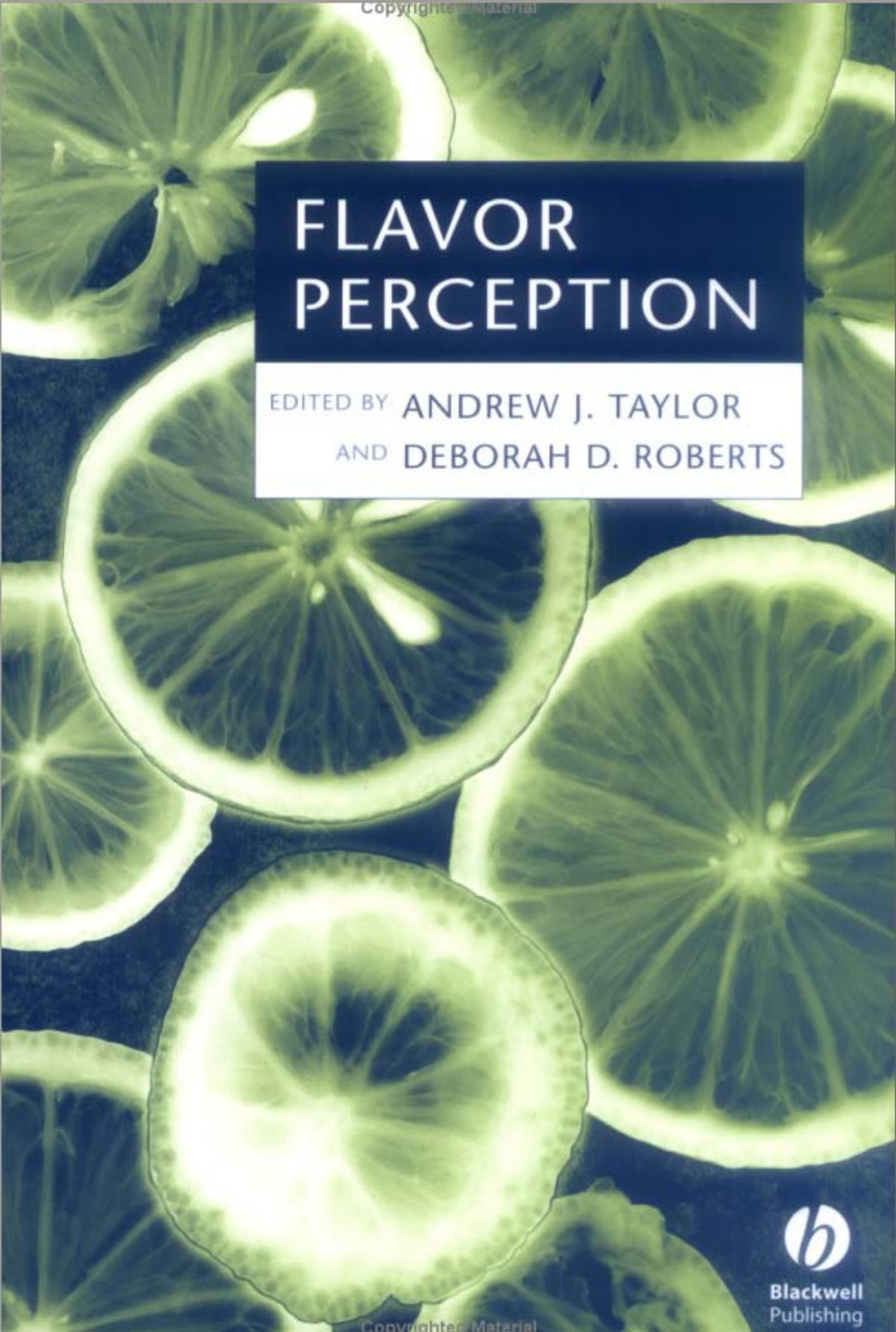

FLAVOR PERCEPTION

EDITED BY ANDREW J. TAYLOR
AND DEBORAH D. ROBERTS

Blackwell Publishing

Flavor Perception

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Preface

Unlike other human senses, the exact mechanisms that lead to our perception of flavour have not yet been elucidated. There are many reasons for our lack of understanding.

First, we now recognise that flavour perception involves a wide range of stimuli (also called modalities). The simple notion that flavour is the result of the well known taste and aroma modalities has been replaced by a realisation that it is a multi-modal phenomenon. Identifying the modalities involved in each food is difficult. Within each modality, there can be many contributing factors and a corresponding array of sensors. Aroma is the most complex, with around 2,500 odorous chemical compounds found in food and around 1000 receptors. Taste is somewhat simpler, with 5 basic components (salt, sour, sweet, acid and umami) and a corresponding number of receptors. Besides taste and aroma, attributes like colour, appearance and sound (e.g. the crispy crunch of some foods) are relatively easy to define. The inner surface of the mouth also contains many receptors that sense stimuli like pain and heat, as well as mouthfeel attributes like creamy, slimy and gritty – but these senses are not as well defined as the taste and aroma receptors.

Second, the chemical compounds and physical structures that activate the flavour sensors change as food is eaten. For instance, the initial crisp nature of a biscuit is replaced by a sticky bolus as the food material becomes hydrated during eating, changing the textural attribute. With wine, the initial aroma sniffed from the glass is replaced by a burst of flavour as the wine is sipped, and that in turn is followed by complex sensations involving terms like fruity, plummy, vanilla, smoky, etc. Measurements of the changes in stimuli with time are therefore essential to an understanding of the relationship between stimuli and perception.

Third, there is a wealth of anecdotal evidence that the individual modalities interact in a complex way and that the overall perception of flavour is not a simple additive effect. Whether these interactions are the result of ‘hard wiring’ of the brain during development or whether they are learned through experience is not entirely clear.

Fourth, the sense of flavour is not as well developed as other senses like speech, music or vision, and we lack a notation (or language) that can adequately describe flavour in terms of the stimuli that create it.

Despite these gaps in our understanding, huge progress has been made in many of the above fields and some examples are listed below.

- Measuring flavour stimuli *in vivo*
- Molecular biology studies of the olfactory and gustatory receptors
- Prediction of olfactory receptor structure and function from primary amino acid sequences
- Neurobiological studies
- Functional MRI
- Multimodal perception

If we are to make further progress in understanding the origins of flavour perception, it is clear that we need to consider the whole process – the release of flavour chemicals in mouth, the transport processes to the receptors, the specificity and characteristics of the receptors, the transduction mechanisms and the subsequent processing of signals locally and at higher centres in the brain. The use of neurobiological techniques and functional MRI can enable us to visualise the effects of flavours on brain function and, coupled with psychological and sensory studies, we can follow each stage of the flavour perception process.

Most of the progress to date has been in deepening our understanding of these individual areas. In order to progress further, approaches integrating these areas will certainly be fruitful. However, a good part of the research related to flavour perception is presented at separate scientific meetings of chemists, biologists, psychologists or physiologists and it is not easy to gain an understanding of the whole field from a single source. This book aims to encapsulate current knowledge in chapters covering the key stages of flavour perception, and to produce a source of information that will be useful to people working in the flavour field, whether they be in academia or in the food/flavour industry.

During the writing of these chapters, we also organised a symposium on the subject, which provided an opportunity for the authors to meet and yielded much useful information which has been incorporated into some of the chapters. We would like to acknowledge the support of the United States Department of Agriculture Cooperative State Research, Education and Extension Service (USDA 2004-35503-14178) and the commercial sponsors (Nestle SA, Firmenich SA, Kraft Foods, International Flavors and Fragrances Inc., Proctor and Gamble, Givaudan Flavors Corporation, and Symrise) for their financial support.

Flavour research is of interest not only on account of its hedonistic implications but because it is one of the factors that limits the acceptance of more

nutritious foods and the enjoyment of food by the ageing population. A basic understanding of the origins of flavour perception would therefore have many uses in the food industry.

AJ Taylor
DD Roberts

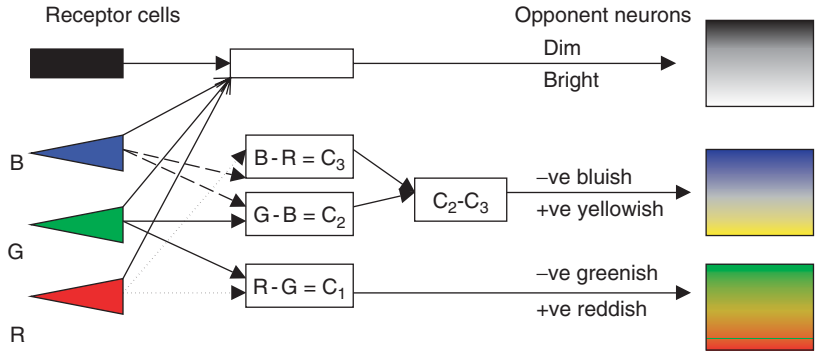

Figure 1.10 Representation of Opponent Colour theory.

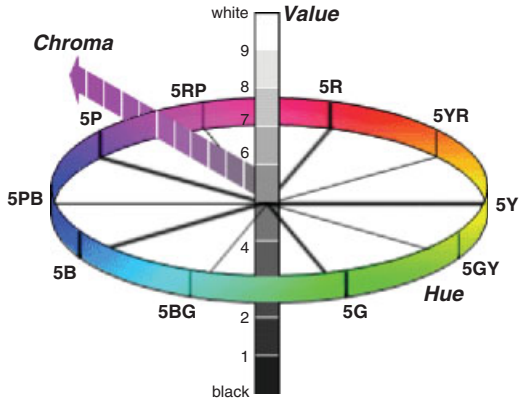

Figure 1.11 Munsell Colour system. (Picture courtesy of HunterLAB.)

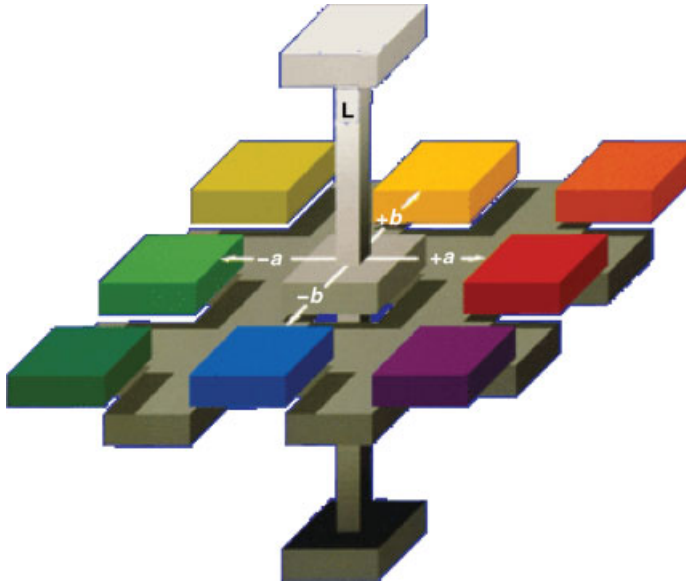

Figure 1.12 HunterLAB colour scale showing the white/black axis (L, vertical), the red/green axis ($+a$ to $-a$) and the yellow/blue axis ($+b$ to $-b$). (Picture courtesy of HunterLAB.)

Flavor Perception
Edited by Andrew J. Taylor, Deborah D. Roberts

1 Measuring proximal stimuli involved in flavour perception

Andrew J. Taylor and Joanne Hort

1.1 Factors influencing flavour perception

Humans eat food both for nutritional benefit and for pleasure. The processes in both cases involve the interaction of food components with specific receptors in the body, which leads to the stimulation of a wide range of sensing systems in humans. While the effects of nutritional stimuli on our behaviour are largely understood (e.g. the effect of carbohydrate consumption and hydrolysis, blood glucose levels and their consequences for attention and appetite), the same cannot be said for flavour stimuli. From our experience, and from formal dictionary definitions of 'flavour', it is clear that several senses (often called modalities) are involved in flavour perception although a complete list of senses/stimuli is rarely given. It is likely that the stimuli involved are highly dependent on the food type. For chocolate, the initial mechanical properties of the solid chocolate and the 'mouthfeel' of the melted chocolate, coupled with the characteristic chocolate taste and aroma, are probably the key modalities involved in flavour perception and in that order of importance. With snack foods, crispness is essential while salt and flavour create the subsequent savoury flavour, whereas with wine it is most likely an equal combination of aroma, taste and mouthfeel. Therefore, we need to identify the key sensory attributes for each food product and not forget that other senses can affect our perception. For example, the discoloration of chocolate due to surface fat crystallisation (so-called bloom) causes little change to the aroma, taste or mouthfeel modalities but reduces enjoyment of the product through a visual stimulus.

1.1.1 Perception of 'taste' and its location

Typical flavour definitions include the taste and aroma modalities, usually with some mention of 'mouthfeel', broadly described as the stimuli that occur in the mouth during eating. These stimuli and their detection are technically described as chemesthesis and are discussed in detail in Chapter 5. There are also definition difficulties due to the imprecise nature of the words that we use to describe food flavour. In English , the word 'taste' means not only the gustatory mechanism found on the tongue for the primary salt, acid, sweet

and bitter modalities but it also describes our overall impression of a food as in the phrase 'that tastes good'. Therefore, although flavour is perceived by receptors in the eye, tongue, nose and mouth lining, the brain interprets the overall sensation as occurring in the mouth. Green (2001) has provided an explanation for this observation by suggesting that the brain 'localises' all the sensory information into the mouth, so we associate that information with the food we consume in the same way we use sight and touch to localise a point on our bodies. Prescott (1999) talks about the 'illusion' of flavour appearing to originate in the mouth and suggests that, since the mouth acts as a gateway to the gut, the sense of flavour determines what foods can enter to keep us safe. Because we localise flavour perception in the mouth, it is not immediately obvious which stimuli are involved in flavour perception and how we combine, vocalise and locate these signals. The aim of this chapter is to study the stimuli involved in flavour perception to set the scene for the following chapters. Subsequent chapters will consider sensing of the stimuli and how the signals produced are processed to provide us with a 'perceptual construct' of that particular flavour.

1.1.2 Multimodal nature of flavour perception

The definitions of flavour listed above are those typically found in scientific literature associated with flavour research but, increasingly, the concepts developed in other scientific disciplines are being applied, leading to a re-assessment of the best definition for flavour. Perception itself has been studied extensively, initially to understand the mechanisms of visual and auditory perception but later to understand how these individual senses combine and interact, leading to the idea of multimodal and crossmodal perception, respectively. In brief, multimodal refers to the fact that several senses (modalities) are involved in flavour perception, and crossmodal indicates that one modality can interact with another to modify the perception. There is now evidence from physiological, neural and functional magnetic resonance imaging (fMRI) studies of brain (see Chapter 7) that leads to a consensus view, namely, flavour perception is no different from other senses and involves both multi- and crossmodal activities. Why has it taken so long to apply this apparently obvious concept to flavour perception? First, it seems that early anatomical studies on neural pathways from the aroma and taste receptors showed that they entered the brain in different places and this was initially interpreted as proof that the two stimuli were independent. A second reason is that early work on multimodal perception proposed that modalities were hierarchical. Vision was considered the most important modality and the hierarchical scheme proposed that vision would always be the dominant sense and would override the input from other modalities. This view is no longer held, as there are many examples that contradict the purely hierarchical idea. A pertinent

example for this book is that a food, however visually appealing, will not be consumed if it emits a foul odour. In this particular case, odour, not vision, is the dominant modality. A third reason for not considering the multimodal approach is that some interactions noted in the literature could be due to physicochemical interactions or artefacts of the sensory analysis methods used, rather than any cognitive, multimodal effect. Here, we define a cognitive effect as any interaction that occurs in signal processing after stimulation of the receptors. For instance, enhancement of aroma by sweeteners (Duran & Costell, 1999) could be due to the presence of sugar at concentrations from 5% to 10% in the samples that might increase aroma release through the well-known physicochemical interaction of solutes (see Taylor, 1998 for review). Thus the observed sensory behaviour could be due to either the physicochemical effect or interaction at the cognitive level. Recent work has shown that low concentrations ($< 10\%$) of solutes like sugar do not affect the *in vivo* release of aromas significantly (unless aromas bind to the solute). In these former cases, the effect is not due to an increased aroma signal being transported to the olfactory receptors (ORs: Breslin, 2001; Davidson *et al*., 1999; Friel *et al*., 2000). Lastly, because of the complexity of flavour, researchers have adopted the classical scientific reductionist approach that seeks to break down the problem into its component parts. This has led to the development of subfields in flavour research such as flavour chemistry, sensory analysis, flavour release and molecular biology of taste and aroma receptors. While this simplifies the systems so they can be investigated, it is imperative that researchers do not lose sight of the whole picture and realise that an understanding of flavour perception requires an integration of all the modalities using a multidisciplinary approach.

Some authors have questioned whether the interactions observed between modalities such as taste and aroma are artefacts of the sensory analysis process itself. In some sensory experiments, panellists may have limited attributes in which to score the samples and there is a notion that panellists may 'dump' any other differences into that (inappropriate) category. For example, when panellists are asked to assess a system containing aroma and sugar for sweetness, they may inadvertently assign a change in aroma perception to the sweetness category. Prescott (1999) has discussed the cause of taste–aroma interactions and has discounted the idea of dumping. He does point out that interactions are seen only if the panellist is presented with the sample in a particular way and if there is already some prior experience of that taste–aroma pairing (so-called congruency). However, the interactions have been noted by many different research groups across the world using a wide range of sensory methods and there is general agreement that the effects observed are real. The general area of crossmodal flavour interactions has been reviewed (Duran & Costell, 1999). Neurobiological studies in animals have demonstrated that there are connections between the taste and aroma systems, and specific neurons that require

a taste and aroma signal before they are activated have been identified (Rolls & Baylis, 1994). All these results are relatively new and, taken together, provide a consensus platform from which our understanding of flavour perception can move forward. Further discussion of interactions between flavour modalities that cause sensory differences can be found in Chapters 8 and 9.

1.1.3 Distal and proximal stimuli

If the notion of multimodal flavour perception is to be studied, we need to be able to identify and measure the stimuli involved, but again we need to bear in mind some principles from other scientific disciplines. The most important is the concept of distal and proximal stimuli, which is demonstrated in Figure 1.1. The distal stimulus is defined as the whole object (in Figure 1.1, a skull), but the perception of that object by vision involves sensing only part of the object, and the image is very small (a few mm) and is expressed upside down on the retina of the eye. Although the eye sees only the front, side or back of the skull at any one time, our familiarity with this object means that we can fill in the missing parts and we imagine that the whole skull is present. For unfamiliar objects, we tend to rotate them to obtain the full distal signal; thereafter, only a partial signal is necessary to recognise the object.

1.2 Aroma

How does the distal/proximal concept apply to the components that make up the sense of flavour? Figure 1.2 outlines the situation for aroma perception. The relationships between distal stimuli, proximal stimuli and aroma perception are shown along with the corresponding compositions and methodologies for measuring the composition at the different stages. Thus the aroma composition of the sample is the distal stimulus, measured by conventional

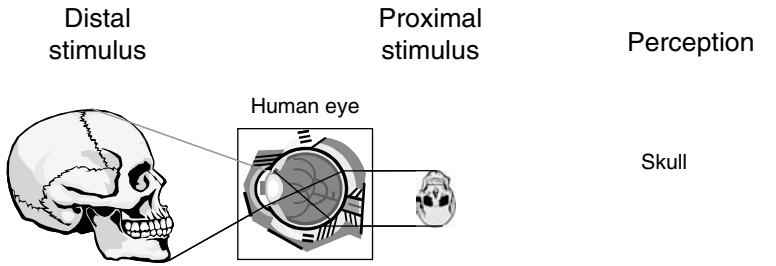

Figure 1.1 Representation of a distal stimulus (a skull), the signal received by the receptor (the proximal stimulus on a small scale – inverted image of the front of a skull) and the perceptual response (recognition of a skull from the information provided).

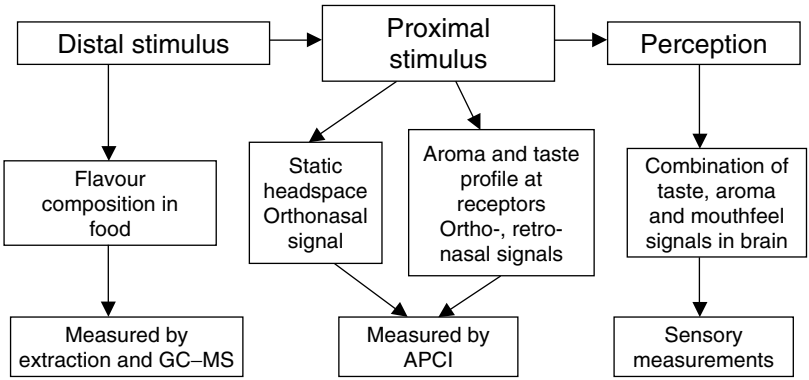

Figure 1.2 Relationship between distal and proximal stimuli (top row), flavour-sampling methods (middle row) and flavour analyses (bottom row).

quantitative extraction and gas chromatography–mass spectrometry (GC–MS). Aroma can be sensed by the orthonasal route (i.e. sniffed through the nostrils), in which case the volatile content in the gas phase above a food (the so-called headspace) is a good indicator of the proximal stimulus that reaches the ORs (see Section 1.2.1). Alternatively, aroma compounds can reach the ORs via the throat during and after the mastication process, the so-called retronasal route. Mastication causes significant changes to the food in terms of surface area, hydration and time in mouth, and these factors in turn affect the mass transfer processes involved in transporting aroma compounds from the food to the gas phase in the mouth and then to the ORs in the nose (van Ruth & Roozen, 2002). Thus the retronasal proximal stimulus may be very different from the distal stimulus of a food. The effects of oral physiology on flavour release are discussed in Chapter 2.

1.2.1 Measurement of orthonasal proximal stimuli

When considering how to measure the orthonasal aroma stimulus, it is necessary to consider the various types of food and our typical ways of consuming them. If we take an example of a packaged food (e.g. roasted peanuts in a sealed bag), the physical conditions inside the bag create an equilibrium headspace, i.e. the aroma compounds are partitioned between the solid peanut phase and the gas phase at an equilibrium (and constant) value. The equilibrium value will depend on temperature but is one of the parameters widely used in aroma analysis of foods as it provides a fundamental value (the air–food partition) that gives an indication of the amount of each volatile compound above a food as well as of the potential flavour release during eating when incorporated in mathematical models (see Linforth, 2002 for a

review of flavour release models). When a bag of peanuts is opened close to the nose, the first stimulus is very close to the equilibrium headspace but there is rapid dilution of that headspace as air is drawn into the packet and the concentrations of individual compounds in the headspace change with dilution. This change in headspace concentration with dilution can be measured using the technique of dynamic headspace dilution where the initial equilibrium between the food and the headspace is disturbed by a flow of gas through the headspace (Taylor, 1998). The technique and the effect of dilution on aroma compounds has been described previously (Marin *et al*., 1999, 2000). The change in headspace composition with time can sometimes be demonstrated with bags of peanuts by opening the packet very close to the nose and immediately taking a deep sniff. Often, a strong sulphur note can be detected but this dissipates almost immediately as the sulphur compound has a very low partition value and the concentration in the headspace is not replenished sufficiently quickly for it to be detected subsequently. Another example of the change of aroma composition with time is provided by the process of wine tasting. Although individual wine tasters adopt slightly different styles of sampling the wine, they all employ the routine of sniffing the 'bouquet' of the wine (i.e. an initial assessment of odour via the orthonasal route) followed by complex mouth movements (some seem to chew the wine), which maximise aroma release and transport the aroma compounds to the OR via the retronasal route. This manipulation of flavour release allows the wine taster to produce a sequence of verbal descriptors, presumably as a result of the sequential delivery of flavour stimuli.

In the case of both peanut and wine aromas, it is clear that the proximal stimuli reaching the ORs are very different from the distal flavour composition of peanuts or wine, and this may be one of the reasons why it has proved difficult to relate flavour perception to flavour composition in the past. Improved devices to mimic orthonasal stimuli are needed; one such device is the gas–liquid interfacial mass-transfer (GLIM) cell proposed by Parker (Parker & Marin, 2000), in which the dynamic release of aroma can be measured as the gas and liquid phases are diluted.

1.2.2 Measurement of retronasal proximal stimuli

The processes involved in delivering the aroma stimuli to the ORs have been reviewed recently (Taylor, 2002). Measuring the stimuli is ideally carried out as close to the receptors as possible. Given ethical considerations and the fact that sampling air at the ORs requires invasive (and potentially painful) methods, it is more convenient to sample air as it exits the nostril. Early work from our laboratory established that release profiles could be obtained by sequential trapping of volatile compounds on Tenax, but the process was very time-consuming (Ingham *et al*., 1995). The development of online mass

spectroscopy (MS) techniques like atmospheric pressure chemical ionisation (APCI) or proton transfer reaction (PTR) to monitor aroma release on a breath-by-breath basis has provided new opportunities for studying aroma release *in vivo*. The techniques have been optimised for sensitivity (typically nanolitres of aroma per litre of air; ppbv) as well as coping with the humidity of exhaled air and the difficulty of ionising a wide range of volatile compounds simultaneously (see Taylor *et al*., 2000 for review of APCI and van Ruth *et al*., 2003 for PTR). Although both APCI and PTR have contributed much to our understanding of aroma release, both techniques have limitations in sensitivity and selectivity. The odour thresholds for some volatile compounds lie in the parts-per-trillion (ppt) range (picolitres of aroma vapour per litre of air), and thus the typical ppbv sensitivity of the MS techniques is inadequate. Some workers have achieved very low sensitivity but mainly with compounds possessing ion masses away from the chemical noise associated with chemical ionisation techniques or with long dwell times that do not allow breath-by-breath analysis. Thus the presence of chemical warfare agents in a smokestack was reported in the low ppt range (Ketkar *et al*., 1991) because the ion mass occurred outside the chemical noise range (giving an excellent signal–noise ratio) and because the ion could be monitored for relatively long periods of time. For aroma compounds with molecular weights (and ion masses) in the 45–200 Da range, ppbv is the typical detection threshold although certain compounds can be followed down to 100 ppt. Secondly, the complex composition of many aromas results in the presence of several compounds with the same molecular weight (isobaric compounds). These cannot be differentiated by the APCI or PTR techniques that only resolve on a unit mass basis. Although some selectivity can be gained by using MS^n (where the original ion is fragmented and the resulting ions analysed using either a tandem MS system or an ion trap instrument), compounds like terpenes create problems as they each have individual aromas but are all found at a few common ion masses.

One of the future drivers for improved real-time MS of aroma release may be developments in MS related to the study of metabolomics. There are many similarities between this area of research and the analysis of flavour compounds. Although there are many definitions of metabolomics, the underpinning idea is the ability to analyse a wide range of compounds of different chemical classes at varying concentrations in a rapid and quantitative manner without creating artefacts or losing labile compounds. Such an analysis has long been the goal of flavour chemists. New developments in MS include the use of alternative ionisation methods. For example, photoionisation (PI) is a soft ionisation that has the potential to ionise molecules in a mixture selectively by using different wavelengths and energies of the light. Another development is the availability of time of flight (TOF) mass analysers that are well suited to the simultaneous analysis of many different ions and also give accurate mass data. Increasingly, hybrid techniques are being proposed such

as the use of PI to ionise molecules, followed by an ion trap to select ion masses and perform fragmentation of the molecule to provide more information on its identity (the MS^n technique), followed by detection in a TOF analyser. The development and manufacture of such machines will be driven by other, more lucrative research fields than flavour analysis, but when such machines become available, there will undoubtedly be a move to adapt them for the analysis of flavours as well as metabolic profiles. The outcome will be improved detection and identification of the proximal flavour stimuli, leading to a better understanding of the concentration change in proximal aroma stimuli with time.

Both APCI and PTR can be used for monitoring the aroma stimuli from model *in vitro* as well as *in vivo* systems. *In vivo* sampling has the advantage that it measures the actual aroma signal in people as they consume food samples. The disadvantages are that person-to-person variation is high due to variability in their mastication behaviour and in their physiology, and both factors cause significant differences in the aroma release profile observed. However, release from an individual is usually consistent (Haring, 1990). Thus if the aim of an experiment is to test the effect of a different food composition on aroma release, it is best to use one or two individuals who release aroma consistently. This will identify the compositional factors influencing aroma release without confusing the issue with the inherent variation in a group of people.

The amounts of volatile compounds released *in vivo* are also small as the food sample is typically limited to about 10 g. One way to overcome the human variation in eating behaviour and the small food sample size is to build a model mouth in which larger samples can be subjected to controlled 'mastication' using the information available on oral physiology (e.g. saliva flow, volume of mouth, air flow). Various devices have been described and all give reproducible results. The question that remains unanswered for many of the model mouths, however, is whether the aroma signal they produce relates to the signal seen *in vivo*. Given the variability of humans, this may seem an impossible task but panellists can be instructed to eat to a certain pattern and individuals with atypical release can be screened out of experiments. There are also some food systems that produce recognisable patterns of aroma release *in vivo* and some validation of model mouths has been attempted. The retronasal aroma simulator (RAS) was tested using APCI to monitor its release pattern for a variety of real foods; these were then compared with human release patterns and a good correlation was found (Deibler *et al*., 2001). The intensity of volatile compounds released from the RAS was much higher due to the larger amount of sample used and the air flow was a key parameter in determining the release profile. Van Ruth has recently reported the same sort of experiment to validate the model mouth developed at Wageningen (van Ruth *et al*., 2002). Rabe has described a model mouth

that uses an automated system of Tenax traps to sequentially collect volatile compounds as they are released from a food matrix (Rabe *et al*., 2002). Validation of the technique with *in vivo* release is currently underway. Piggott's group (Margomenou *et al*., 2000) developed a device to simulate aroma release during consumption of beverages. Factors like volume, dilution with saliva and airflow were taken into consideration and the system tested with whisky. Model mouths can therefore overcome the problems associated with variable mastication/physiology and the low concentrations of volatile compounds released *in vivo*. However, if the data from a model mouth are to be correlated with sensory data, it is essential that the 'proximal stimulus' produced by the model mouth is related to the proximal stimulus measured *in vivo*. Side-by-side comparisons of aroma release *in vivo* and in the model mouth are therefore needed.

Some workers have focused on the release of aroma in mouth and the effect of saliva and mastication on the process. Buettner (Buettner & Schieberle, 2000a, 2000b) has developed methods for measuring aroma release, both in mouth and in model systems, in order to explore the effects of saliva on aromas. More recently, Buettner (2003) has introduced the idea that enzymes in mouth can metabolise some aroma compounds. The significance of the effect is not yet entirely clear as the time taken to cause significant changes may restrict the effect to the perception of aroma persistence, rather than aroma release itself. However, this persistence is part of the proximal stimulus and it may contribute to our perception of complex flavours.

Both *in vivo* and model mouth experiments can provide suitable analyses for the proximal aroma stimulus, but there is still a concern that it does not represent the true aroma profile that reaches the ORs. The reason is that between the nasal gas phase and the ORs lies a layer of mucus containing mucin and odour-binding proteins (OBPs: Paolini *et al*., 1998). The role of these proteins is still not clear but they are thought to assist the transport of aroma compounds to (and from?) the receptors. There are two potential mechanisms for the passage of aroma compounds across the mucous layer: one an active transport mechanism, the other a passive event. Active transport assumes that each aroma compound is bound to a specific OBP that transports it across the mucous layer. The passive idea is that the OBPs act to modify the partition behaviour of aroma compounds in the mucus so that molecules of all polarities can be dissolved in the mucus and are then transported across the mucous layer by the concentration gradient that exists between the gas and mucus phase. There is a report of specific binding of an OBP to an olfactory receptor protein (ORP: Matarazzo *et al*., 2002) but the authors of the paper are cautious in interpreting the result as proof of an active transport system. Further work is needed to examine the nature of the interactions using both physicochemical studies to measure aroma binding to OBP/ORP as well as molecular biology studies to follow OBP/ORP interactions.

1.2.3 Examples of differences in distal and proximal aroma stimuli

Most of the discussion about distal and proximal flavour stimuli so far has the benefit of logic but few data have been presented to prove the hypothesis that there are significant differences between the distal and proximal aroma stimuli. To address this question it is necessary to carry out studies *in vivo*. However, it is clear that the nature of the food matrix will affect aroma release and, to avoid confusion, most experiments have been carried out with aqueous solutions, as the mass transfer of aroma compounds from the aqueous phase to the gas phase is simplest in this system. Buettner developed the exhaled odorant measurement (EXOM) technique of measuring the amount of odorant exhaled after consumption of samples and showed that, after the introduction of a solution of ethyl butyrate into the mouth, the concentration after 5s was about half of the amount found for longer periods. Using the APCI technique in our laboratory, a comparison was made between the concentration of aroma compounds in the equilibrium headspace above a solution and the concentration in mouth and in nose when that solution was consumed (Linforth *et al*., 2002). It is important to record the protocol for consuming samples in this experiment before trying to interpret the data. Panellists were asked to place the aroma solution in mouth, swallow immediately and then breathe out either through the mouth or nose. The exhaled air was sampled into the APCI and the concentrations of aroma compounds measured. The data were expressed as percentages of the equilibrium headspace. Measurement of the exhaled air from the mouth (mouthspace) showed that the differences ranged from 70% (butanol) to 0.17% (α-pinene). This suggested that equilibrium was not achieved for any of the 50 aroma compounds tested and that the amount released in mouth during the short time between the panellist consuming the sample and exhaling varied greatly between compounds. A study of the volatile concentrations in nose (nosespace) after consumption showed a similar wide range but at much lower values (11% of equilibrium headspace (propan-2-ol) to 0.09% (α-pinene)). These data are supported by the work of Roberts (2003) who measured the amount of volatile compound reaching the nose after administration of a 10 ml aliquot of flavoured milk to the mouth, which was swallowed immediately. Only 0.1–0.6% of the volatile compounds was actually measured in nose.

A limited number of experiments have been carried out with real foods but perhaps the best example of the differences between distal and proximal stimuli is provided by work on biscuits containing low- and regular-fat levels (Figure 1.3; Brauss *et al*., 2000). Here, the same amount of flavour (anethole) was applied to the low- and regular-fat biscuit dough but the baking process caused higher losses in the low-fat product. Thus the regular-fat biscuits contained 40 mg/kg of anethole while the low-fat contained 19 mg/kg. However, when the samples were consumed and the nosespace measured,

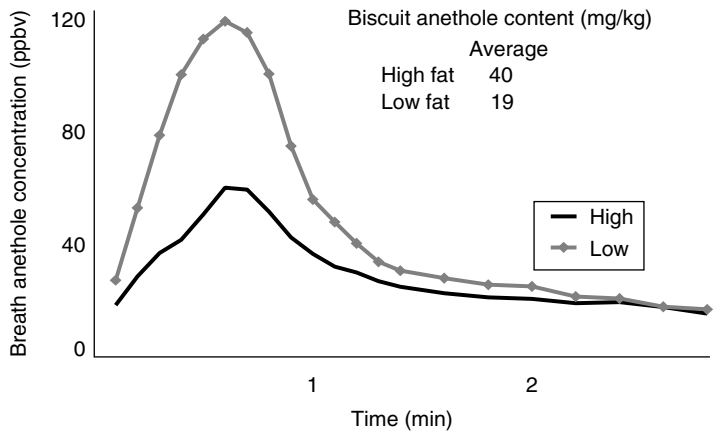

Figure 1.3 Release of anethole from low- and high-fat biscuits manufactured using the same initial amount of anethole. The box shows the anethole content post baking but despite the higher anethole content in the high-fat biscuit, release is greater for the low-fat biscuit containing half the amount of anethole. Here the distal stimulus (the flavour content in the biscuit) does not relate to the perceived sensory intensity but the proximal stimulus (the in-nose signal) does (Brauss *et al*., 2000).

the low-fat biscuits delivered over twice the amount of aroma to the nose than the regular-fat products despite containing only half the anethole content of the regular-fat biscuits.

The results from the solution experiments clearly show that aroma concentrations in nose are significantly lower than the equilibrium values measured *in vitro* and that there is a strong compound effect with a variation of around 100 times in the nosespace data and around 350 times in the mouthspace data. Both the solution work and the biscuit experiments confirm that measuring the proximal stimulus gives more precise information about the stimuli sensed by the ORs compared to the distal stimulus, thus confirming the credibility of the original hypothesis.

1.2.4 Relating proximal aroma stimuli to flavour perception

Given the results above, the next key question is whether proximal stimuli are actually better predictors of flavour perception than distal stimuli. At first, preliminary data from our laboratory seemed to confirm the idea. Herb flavour was incorporated into breadcrumb samples using three different processes. Analysis of the three products (Figure 1.4) demonstrated that the herb content of the samples (the distal stimulus) varied but did not follow the same trend as the perceived flavour intensity, measured using magnitude estimation. When measurement of the maximum concentration in nose during eating of the three samples was taken, the trend related well to the sensory data. This is evidence

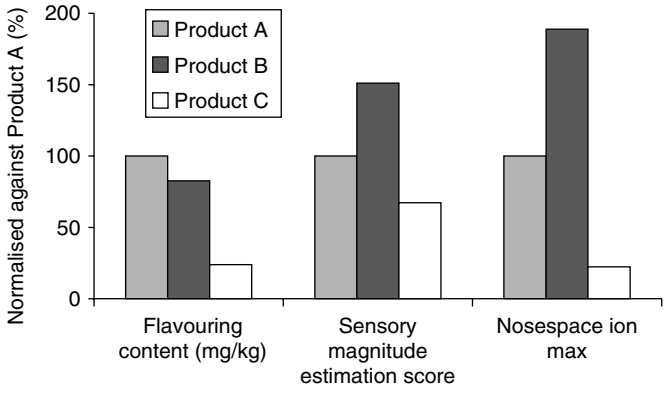

Figure 1.4 Effect of different methods of preparation on the final content of herb flavour, the sensory intensity and the in-nose release in breadcrumb samples. Each sample initially contained the same amount of flavour but losses were different due to the method of preparation. The values are expressed relative to Product A for ease of comparison. The flavour content (the distal stimulus) does not relate well to the sensory intensity but the in-nose concentration of the flavour (the proximal stimulus) shows the same pattern of behaviour as the sensory intensities.

that the proximal stimulus does relate better to sensory perception than the distal stimulus.

Further data was obtained from the same samples by monitoring aroma release with time while simultaneously monitoring perceived aroma intensity using sensory time–intensity analysis. Figure 1.5 shows the time–intensity curve obtained from a panellist with the simultaneous breath-by-breath release. There is good agreement between the curves, within the limitations of the sensory and instrumental methods, again providing evidence that the proximal stimulus relates well to the perceived aroma in this system.

In the above example with herb-flavoured breadcrumb, eating time was fairly short and any adaptation to the herb flavour seemed unlikely. Attention therefore turned to a longer-lasting system, chewing gum, which is known to decrease in flavour perception with time. Typically, panellists report no mint flavour after 3–5 min. With the ability to measure the concentration of mint aroma *in vivo* as the gum was chewed, we carried out an experiment to determine whether adaptation or a decrease in mint aroma delivery was the cause of the decrease in mint flavour perception. Figure 1.6a shows the results for stick gum (sugar incorporated into the gum matrix). Mint aroma delivery from gum was rapid and persisted for up to 20 min with little change from the initial plateau value. Perception of mint, however, decreased much more quickly and it looked like a case of adaptation. The release of sugar from gum was also monitored and the sugar release and mint perception curves were very similar for the stick gum and for dragées (sugar on the

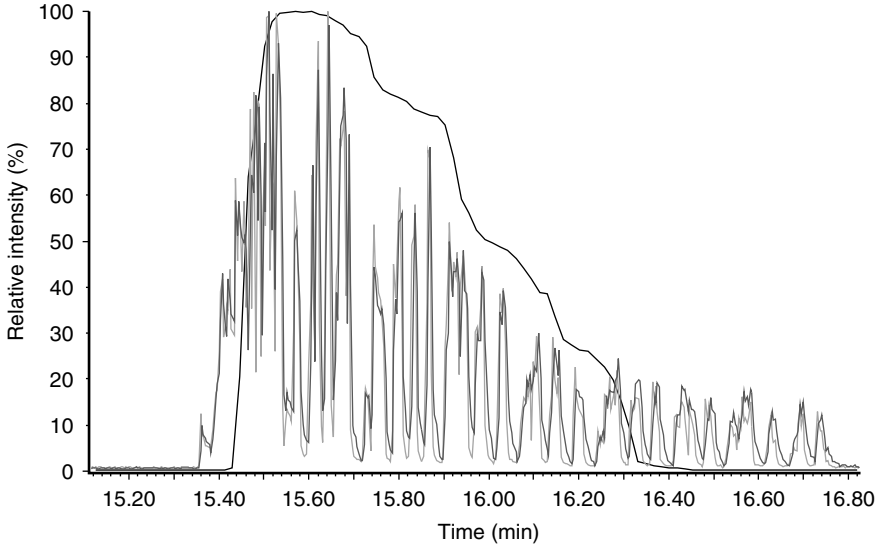

Figure 1.5 Simultaneous measurement of the release of a herb flavour in nose (peaks) with the sensory time–intensity analysis (solid line). Herb flavour was followed by monitoring two ions simultaneously, hence the two traces for the peaks.

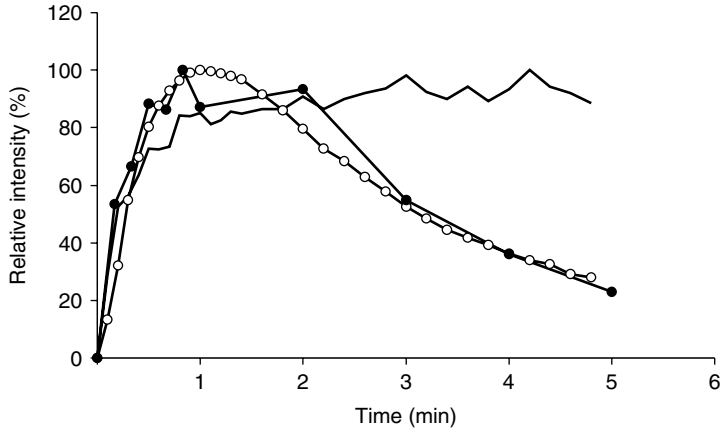

Figure 1.6a The in-mouth sucrose concentration (filled circles), in-nose menthone concentration (no markers) and perceived intensity of mint aroma (time–intensity curve) (open circles) whilst eating a stick-type commercial chewing gum. The sucrose values are the mean of three panellists, while the menthone concentration and perceived mint intensity values are the mean of 11 panellists. Values have been normalised for easy comparison. Release of aroma and sugar measured in nose and in mouth with simultaneous sensory time–intensity assessment of mint flavour from stick chewing gum (sugar incorporated in gum) (Davidson, 2000).

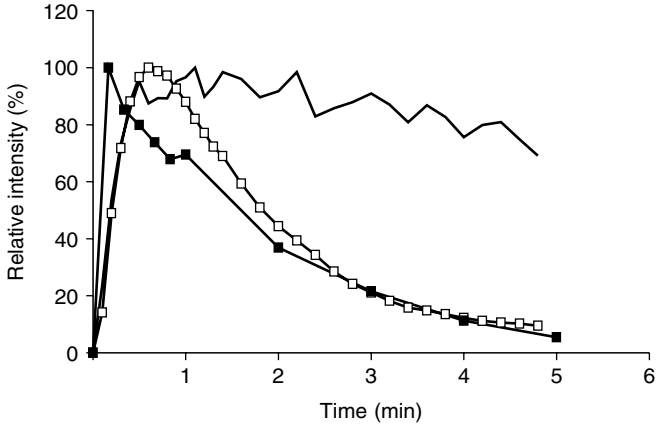

Figure 1.6b The in-mouth sucrose concentration (filled squares), in-nose menthone concentration (no markers) and perceived intensity of mint aroma (time–intensity curve) (open squares) whilst eating a tablet-type (dragée) commercial chewing gum. The sucrose values are the mean of three panellists, while the menthone concentration and perceived mint intensity values are the mean of 11 panellists. Values have been normalised for easy comparison (Davidson, 2000).

outside: Figure 1.6b). This might have been a coincidence but further experiments using a system to deliver solutions of mint or sugar to panellists showed that the perception of mint was sustained by sugar when the mint aroma component was switched off (Hollowood *et al.*, 2002a). Subsequently, this idea of interactions between the various flavour modalities has been further studied and is described in detail in Chapter 9. The outcome was that we now need to develop methods to measure the proximal taste modality.

1.3 Taste

Compared to aroma stimuli, measuring the proximal stimulus of taste compounds seems relatively facile at first. The taste receptors are located on the tongue, which can be easily accessed for direct sampling. However, the process of mastication and dilution with saliva creates a complex mixture of whole food pieces, semi-masticated particles and an unpleasant slurry of saliva/food, which bathes the tongue. It is evident that the system is not mixed to homogeneity and there are big differences in concentrations across the tongue. Added to this is the fact that taste compounds can be present at levels ranging from 100 g/kg (sucrose) to 10 ng/kg (a high-intensity sweetener). The analytical challenge of measuring the change in concentration of such a range of chemicals in a complex biological slurry is huge. At least with aroma

compounds, their volatility releases them into the gas phase, thus removing the problems of analysing compounds in a biological matrix.

However, some systems are amenable to analysis. Beverages are a good example where the initial tastant concentration is decreased due to dilution by the flow of saliva in mouth. This typically flows at 2–5 ml/min depending on the subject and the presence of compounds that stimulate saliva production (e.g. citric acid). Chewing gum is another example of a system where saliva samples (and therefore tastant release) can be easily measured as the food matrix remains in a single piece during eating. Dry foods like biscuits or savoury snack foods absorb saliva initially, making it difficult to collect saliva samples in the early stages of eating. Biscuits are typically hydrated to about 40% moisture content to create a sticky bolus suitable for swallowing, so even at the end of mastication, there is little free saliva available for sampling.

Despite the ease with which samples can be taken from the mouth, the heterogeneity within the mouth and the shape of the mouth make continuous sampling difficult. Various approaches have been adopted to obtain samples from the mouth that represent the proximal taste stimulus. One approach is to use a dental plate fitted with sensors that fits to the roof of the mouth. To measure salt and acid taste stimuli, conductivity sensors have been used (Jack *et al*., 1995) along with miniature pH electrodes (Davidson *et al*., 1998). For food systems that coat the whole mouth (e.g. liquids or semi-solids), the dental plate gives a good representation of tastant release. Dry foods, however, absorb most of the saliva in mouth and there is insufficient aqueous phase in the roof of the mouth to activate the pH and conductivity sensors. In this case, the release of tastants can only be measured once a critical water content has been achieved in the sample. The sensors used are rather nonspecific as the conductivity probe measures all ionic material while the pH electrode only measures hydrogen ion concentration and does not differentiate between malic, lactic or citric acids. Other problems are: each panellist requires a custom-made dental plate; to activate the pH electrode each panellist needs to keep a finger in a solution to form the electrical bridge; and the pH electrode is rather fragile and needs frequent replacement. Figure 1.7 shows the release of tastants using conductivity measurements for mashed potato and potato crisps. It is clear that no sign of salt release from crisps can be seen initially as there is insufficient liquid phase to carry salts to the conductivity detector on the dental plate.

The other main approach for measuring tastant release is to sample the saliva phase on the tongue during eating. Swabbing the tongue at specific time intervals using a pre-weighed cotton bud has been widely used in our laboratory to collect samples (Davidson *et al*., 2000). The technique requires someone to sample the saliva from a particular region on the tongue and, with some types of food, there are problems in deciding whether to sample the saliva or the saliva containing particulate food material. An alternative is to

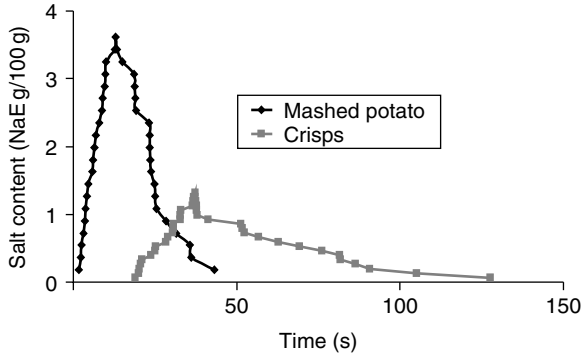

Figure 1.7 Release of salt from samples of mashed potato and potato crisps measured using a conductivity detector in a dental plate. Salt content is expressed as sodium equivalents. Note the delay in conductivity signal from the crisps despite the presence of sodium chloride on the crisp surface.

spit out (expectorate) some saliva at predetermined time intervals and analyse for the tastants. Buettner (Buettner *et al*., 2002) has adopted this method with some success to measure residual volatiles but the method could easily be adapted for tastants. In an attempt to follow tastant release in a continuous manner, a ribbon was pulled across the tongue at a known rate. Regions of the ribbon could be correlated to particular time intervals and analysed for tastant content to build a tastant release curve (Davidson *et al*., 2000). With all these saliva-sampling methods, the weight of saliva sampled needs to be determined to obtain quantitative data from saliva sampling during eating. This requires tedious pre-weighing of cotton buds followed by a second weighing to calculate the weight of saliva sampled. Since many samples are obtained from different panellists, at different times and in replicates, analysis of tastants can become a time-consuming process. Extraction of tastants from the saliva and/or cotton bud is easily achieved by adding methanol and water (50 : 50). Aliquots of the solution can then be analysed for the tastants. Chromatography is a potential analytical method but it is relatively slow, and since the tastants are known, a rapid, direct liquid phase MS technique has been developed to differentiate on the basis of mass in an analogous fashion to the gas phase APCI technique (Taylor & Linforth, 2003). Direct MS of mixtures in the liquid phase is more difficult than mixture analysis in the gas phase due to potential chemical interactions between the ions. Salts especially interfere with the ionisation efficiency and lead to non-quantitative results. A small desalting column to delay the low–molecular weight salts in the mixture so that the larger molecules enter the ionisation source in the absence of salts has been used in our laboratory to overcome some of the drawbacks and produce reliable data. One injection of a 20 μl aliquot can measure five or six tastants

simultaneously within a 30–60-s period. Using the combination of saliva sampling and direct MS analysis, it is possible to measure tastant release *in vivo* as a function of time. Figure 1.8 shows the distribution of sucrose across the tongue when a panellist chewed gum on the right-hand side of the tongue while samples were taken from different regions. The maximum sucrose concentration (11%) was found next to the gum and the sucrose levels were lower elsewhere.

Some preliminary experiments with the swabbing technique for saliva collection (coupled to a direct MS analysis) have shown that the technique is capable of measuring both bulk sweeteners and high-intensity sweeteners well above threshold levels (Taylor & Linforth, 2003). This is shown in Figure 1.9

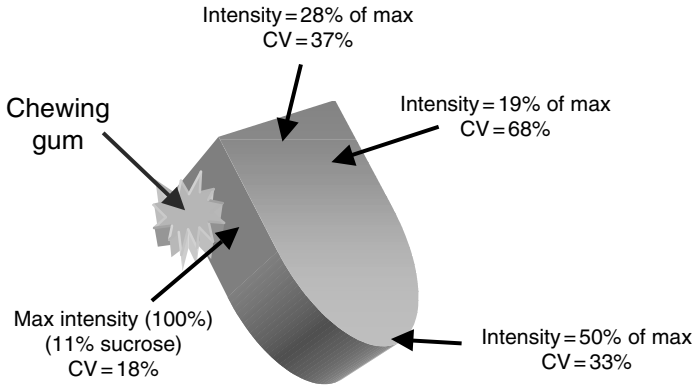

Figure 1.8 Sucrose concentrations at different tongue locations when chewing gum was eaten on the right-hand side of the mouth. Saliva samples were taken by cotton bud swabbing and analysed by direct liquid MS.

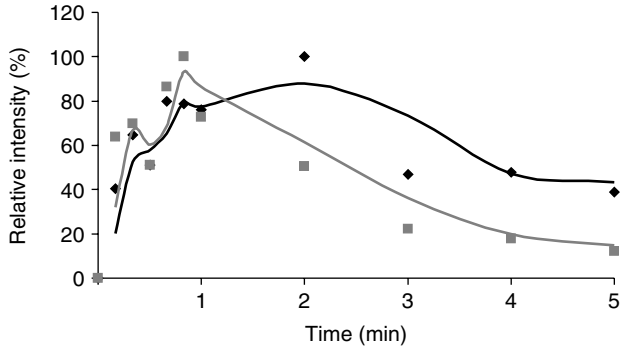

Figure 1.9 Release of sorbitol (diamonds) and aspartame (squares) from chewing gum as measured by cotton bud swabbing and direct liquid MS; lines are the best fit curves to the experimental points.

where the release of sorbitol and aspartame from chewing gum was monitored successfully.

In vivo monitoring is entirely appropriate for food materials but when the taste properties of pharmaceuticals are to be determined, there are ethical as well as safety concerns, and model systems are the choice. Several 'electronic tongues' have been described that can differentiate the basic tastes (Toko, 1998; Winquist *et al*., 2000). Specificity for acid, sweet, bitter, etc. seems to be achieved using either different sensors (typically some sort of electrode) and/or membranes of different permeability over the sensor. These electronic devices can be used to monitor the degree of bitterness of different pharmaceutical preparations. Their role in the wider human sense of taste has been described but the devices do not seem to have been utilised in any major way.

The ability to measure proximal taste stimuli is not perfect but reasonable data can be obtained for all four of the basic tastes as well as other taste-active compounds such as monosodium glutamate and peptides. Thus potential taste–aroma interactions can be studied as discussed in later chapters.

1.4 Texture

Like other senses, texture is an amalgamation of many different signals from receptors located in, or near, the mouth along with an acoustic input for crisp and crunchy foods. Textural stimuli originate from the structure and mechanical properties of the food and the way these break down as food is masticated. For this reason, the relationships between the perceived texture of a food and its mechanical/structural properties have been studied for many years. One of the early examples is the General Foods Texture Profile, developed in the 1960s and defined as 'the organoleptic analysis of the texture complex of a food in terms of its mechanical, geometrical, fat and moisture characteristics, the degree of each present, and the order in which they appear from first bite through complete mastication'. Over 40 years later, the key points of this definition (i.e. texture is composed of different properties and is a dynamic sense) are not disputed although we might wish to add that the components of texture can interact in a crossmodal way to bring the definition in line with current thinking. Although the Texture Profile concept has been known for many years, it has been used more as a semi-quantitative, comparative technique rather than an absolute method for correlating perceived texture to the measurements listed in the definition above. The reasons for this relate to the difficulties in identifying stimuli associated with a particular textural attribute and the lack of interaction terms between different stimuli. The difficulties are neatly expressed by Dobraszcyk and Vincent (1999) as follows:

The fact that few simple mechanical tests have been shown to correlate with sensory perception of texture in foods reinforces the case that sensory assessments of texture involve not only complex interactions between the material properties of the food and its structure but also complex stress states and interactions within the mouth. It is therefore hardly surprising that attempted correlations between sensory assessments and mechanical tests are often inconclusive especially if approached simplistically.

Other possible reasons for the generally poor correlations noted in scientific literature are discussed by Bourne (2002). In the chapter entitled 'Correlations between physical measurements and sensory assessments', he cites inappropriate measurements as a potential cause and uses an analogy with colour perception. Bourne points out that although infrared and ultraviolet wavelengths are part of the electromagnetic spectrum, they do not correlate with perceived colour because our eyes cannot detect these particular wavelengths. In a similar way, not all mechanical measurements of a foodstuff will correlate with its perceived texture because humans lack suitable receptors to detect these properties. Another potential reason is that texture analysers do not operate in the same way as the human mouth, and the differences between measurements from compression machines (which work at constant speed) and the textural stimuli (which result from variable and high-speed compression in the mouth) could explain the lack of correlation.

Lillford (1991) noted that 'physical tests on the intact food structure relate only to the initially perceived textural attributes such as resistance, initial hardness and initial moistness or juiciness'. He proposed a model where size reduction and lubrication of food in the mouth took place as food was chewed and hydrated with saliva. The extent and the rate at which these changes occurred should correlate better with the perceived texture of foods. This topic is discussed in Chapter 2.

Of the many reports in scientific literature, three sensory textural attributes have been chosen to demonstrate the different levels of current understanding. The first is viscosity, a sensory attribute where the importance of the proximal stimulus was recognised some time ago, even if the authors did not quite express their thoughts in terms of multi- and crossmodal interactions. For instance, Sherman (1973) wrote: 'Two questions need to be considered.... First, do we want to characterise the food for quality control purposes and second, do we want information about the consumer's perception of textural properties.' This clearly demonstrates the differences between measuring a 'marker' property that relates to the textural properties of a food sample and the stimuli that cause that textural perception. Sherman applied this thinking to a range of food products and, building on work by Wood (1968), laid the foundations for an understanding of the perception of oral viscosity. The idea was that perceived oral viscosity was related to the conditions under which the

viscous liquid was sheared in mouth rather than to the shear conditions found in rheometers. Using a wide range of food products, the boundaries for in-mouth shear stress and shear rate were presented (Sherman, 1973). In 1992, additional data on the link between perceived viscosity and in-mouth behaviour were proposed (Elejalde & Kokini, 1992), which were very similar to the earlier work of Shama and Sherman (1973). The proposed mechanism is that a sample of viscous food material is squeezed between the tongue and the roof of the mouth causing the sample to flow in an extensional manner. By making some approximations about the way the mouth works (and its dimensions), oral shear parameters could be calculated from conventional rheological measurements performed *in vitro*. Recently, this oral shear stress parameter has been suggested as a key factor influencing the perceived aroma of viscous solutions (Hollowood *et al*., 2002b). Using a fixed amount of odorants and sweeteners, the perceived aroma and sweetness decreased as viscosity was increased – an effect noted by many other workers. With a range of hydrocolloid thickeners, Cook (2003b) went on to show that the decrease in perception was not due to any decrease in the amount of aroma delivered to the ORs (it had been hypothesised that release of aroma during eating might be hindered by increased viscosity). This is compelling evidence of a crossmodal effect where one modality (in this case viscosity) has an overriding effect on another modality (aroma). These crossmodal effects are discussed more fully in Chapters 8 and 9. The reason for mentioning the effect here is to show that the concept of flavour being constructed from different modalities (with crossmodal interactions) is now supported by experimental evidence. The consequences are described in Section 1.6.

The second sensory attribute to be considered is crispness. This attribute has been widely studied with the result that we have a basic understanding of the origin of the proximal stimuli involved. Methods for correlating instrumental and sensory attributes of crispness have been proposed (HenryBressolette *et al*., 1996), although for this attribute, there are undoubtedly inputs from acoustics (see Duizer, 2001 for review) as well as from mechanical receptors in the mouth. There is agreement that the origin of crispness results from 'the sudden drop in load experienced on the jaw adductor (closing) muscles and/or the load sensors in the tooth suspension when a crisp food item breaks between the teeth' (Vincent, 1998). Measuring this load change has been achieved using a texture analyser with a high-speed data collection system to follow the many small breaks as a crisp sample is crushed (Norton *et al*., 1998). From the raw time–force curves, a crispness index was developed by fractal analysis. Put in simple terms, the fractal analysis compares the length of a smooth line drawn through the overall time–force curve with the length of a line that follows every minute fracture event as the food is crushed. These parameters correlated well with sensory analysis of the crispness (Norton *et al*., 1998) and, intuitively, they represent one of the proximal stimuli associated with

crispness. The availability of such measurements opens the door for further experiments to study potential crossmodal interactions of crispness with aroma and taste.

The third textural attribute to be considered is creaminess, another well-recognised and desirable property of foods. Is creaminess a multimodal construct or is it driven mainly by creamy-smelling and creamy-tasting compounds or by the creamy mouthfeel? The information available in scientific literature is clear on some points but less clear on others, and a recent comprehensive review discusses the current status of research (Kilcast & Clegg, 2002). The relationship between the sensory assessment of creamy and instrumental measurements has been widely studied. At its simplest, creaminess has been related to viscosity (Daget *et al*., 1988). More often, more than one instrumental measurement is necessary to obtain a good correlation with the sensory estimation of creaminess (Bourne, 2002), suggesting that there is a multimodal basis for this attribute. An example is given by Kokini *et al*. (1984) that correlated the sensory assessment of creaminess to instrumental measurements related to thickness, softness and slipperiness. The information available on the origins of texture is sometimes confusing and contradictory. Kilcast and Clegg (2002) suggested that the reason for the confusion may lie in the fact that creaminess has been studied in many different systems and its perception may depend on the system used, i.e. there is no universal mechanism for creaminess. The role of the aroma component has not been studied to such a great extent as the rheological and material properties of creamy samples. However, in some systems, perception of creaminess is not affected by the presence of creamy aromas (Kilcast, personal communication). Therefore, in the case of the creaminess attribute, there is still uncertainty about the identity of the crucial proximal stimuli. The evidence is that creaminess involves more than one proximal stimulus (and therefore more than one set of receptors), so it could be considered multimodal. The equations proposed to explain sensory perception of creaminess as a function of instrumental measurements give some measure of the type of interactions that might occur between the modalities. For instance, Kokini (1984) used the following equation:

$$\text{Log creamy} = 0.52 \log \text{ thick} + 1.56 \log \text{ soft} - 0.32 \log \text{ slippery}$$

This suggests that the interaction between the parameters ‘thick’, ‘soft’ and ‘slippery’ is additive/subtractive in terms of the perception of the creamy attribute. This is an area where further work is required to obtain a full understanding of the phenomena involved. Weenen *et al*. (2003) have reported experiments to identify the textural attributes of semi-solid foods such as mayonnaise, custard and sauces. The attributes were then related to instrumental measurements of bulk properties like viscosity, homogeneity and adhesion/

cohesion. Two nontextural measurements were found to relate to the sensory properties, namely temperature and oral irritation. This supports the notion that perceived texture is derived from several different inputs and is not a unimodal percept.

From the review of literature presented above, it is clear that texture is constructed from different stimuli and that it changes with time. Methods have been developed for measuring some of the inputs and the technique of electromyography has proved a simple yet effective technique for following jaw movements and the force applied (Brown *et al*., 1998). In electromyography, electrodes are attached to the skin and the signals produced from the masseter muscles during chewing are recorded using special equipment. From the traces obtained, several parameters can be extracted including the force applied to the food sample as it is eaten. However, food texture involves many other sensory attributes, with the term 'mouthfeel' often used to define the complex sensation associated with events like the melting of chocolate, the changing astringency of wine or the perception of creaminess. Some researchers have measured the lubrication properties of food/saliva mixtures to understand more about the mouthfeel phenomenon (Norton, personal communication).

In these situations, the signals from many different sensors must be undergoing integration to produce the overall textural sensation. In fact, the integration seems to go further and Green (2001) has suggested that we should consider all the receptors in the mouth as one integrated somatosensory system rather than measuring the discrete senses of taste and texture since the two seem to be inextricably linked. Evidence of the integration has been obtained through experiments using fMRI (Cerf-Ducastel *et al*., 2001), which showed that taste stimuli sensed on the tongue and somatosensory stimuli from the mouth were both represented in a common area of the brain, although there were some subtle differences between the two signals. There is a great need to understand how the sensors in the mouth and jaw react to food stimuli and how the subsequent signals are processed in the brain. Since we cannot see inside the mouth during eating and since the methods to measure brain activity (like fMRI) require that subjects remain perfectly still, it is difficult to collect data on the somatosensory output in response to oral stimuli. However, the data presented in this section demonstrate that texture is a component of flavour and, in order to develop a better understanding, we need to measure the different inputs and discover how they interact with each other and with other flavour modalities to form a multimodal construct of flavour.

1.5 Colour and appearance

The description and measurement of food colour and appearance has received considerable attention and has been comprehensively reviewed by Hutchings

(1999). It is widely recognised that appearance attributes, in particular, colour, modify flavour perception to some degree depending on the food product (DuBose *et al*., 1980; Zellner & Kautz, 1990). Brain-imaging techniques have also confirmed different levels of activity in olfactory processing areas of the brain depending on the congruency of colour and aroma presented to an individual (Calvert & Osterbauer, 2002). If the relationship between appearance and flavour perception is to be fully understood, then, as with the other modalities, methods for specifying and measuring colour (and other appearance attributes) need to be representative of the manner in which humans describe and perceive them. This section reviews the development of colour specification models and measurement techniques as they have progressed towards this aim, together with some comments concerning other appearance attributes.

1.5.1 Colour perception

In order to evaluate the colour attributes of an object, the object itself, a light source and a human (the observer) need to be present. Altering the characteristics of any one of these elements will result in different perceptions of the object. Imagine adding a colorant to lemonade, the colour of meat under candlelight compared to fluorescent lighting and finally the response of a colour-blind individual when describing the colours of different fruits.

Light may be absorbed, reflected and/or transmitted by an object. The proximal stimulus for what we see as colour can be regarded as the electromagnetic energy that then radiates from an object. Perception occurs following the impact of the wavelengths of light in the visible spectrum reflected by the object on the human retina (Francis, 1995). The visible spectrum spans wavelengths of light from 390 nm to 760 nm. Essentially, if an object reflects predominantly short wavelengths, it appears blue; in case of long wavelengths it appears red but if it reflects a mixture of long and medium wavelengths, it appears yellow. Several factors affect the ultimate wavelength stimulus reaching the eye, which is why the colour of an object changes under different conditions (Hutchings, 1999). These include the properties of the light source itself: distance, angle, temperature, colour, intensity in addition to aspects such as background colour, viewing angle, size. All such factors need to be controlled for comparative measures of colour.

The actual experience of perceiving colour occurs when the wavelengths of light, converted to a 'code' of electrical signals, are interpreted by the brain. Originally, it was postulated that colour vision was a result of the stimulation of a three-receptor mechanism on the basis that any colour could be made/matched from mixing red, blue and green light. The discovery of three types of cone cells in the retina, each particularly sensitive to wavelengths representing red, blue and green, appeared to support this so-called Trichromatic theory.

Indeed this principle is utilised in the projection of colour in television and monitor displays.

However, it was evident that the theory could not fully explain colour vision. The fact that individuals colour-blind to red cannot see green and those blind to blue cannot see yellow, along with many other observations, led Hering (cited in Goldstein, 1999) to suggest a different mechanism known as the Opponent Colour theory, one now widely accepted. The principle is demonstrated in Figure 1.10.

Neural interconnections between the cone and other retinal cells result in the original three types of signals from the cone cells being eventually reduced to two. For example, combined inputs from the red and green cones are transferred to 'opponent nerve fibres' that are excited by redder wavelengths but inhibited by greener wavelengths. Similarly, combined inputs from 'blue and red' and 'blue and green' cones interact and are transferred to a second type of opponent nerve fibre inhibited by blue wavelengths but excited by yellow wavelengths. The brain finally translates the signal encoded in these opponent nerve fibres and the individual sees 'colour'. At the same time, the lightness of the colour is signalled by a similar opponent mechanism that begins with the stimulation of rod receptor cells and subsequently opponent nerve fibres that respond positively to white light but are inhibited in its absence.

1.5.2 Specifying colour

Individuals do not describe colour in terms of its wavelength. Typically, colour is described in terms of its hue, i.e. whether it is red, green, yellow, etc.; its lightness, i.e. how dark or light it is; and its saturation, i.e. how grey the colour is. In the literature, sensory assessment of food colour tends to be limited to hue although some studies include additional measures of lightness (dark/

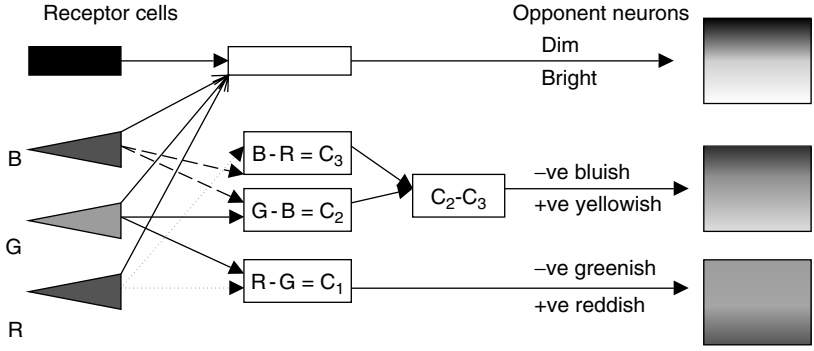

Figure 1.10 Representation of Opponent Colour theory. (Reproduced in colour in the plate section.)

light) and/or brightness (dull/bright) (Lyon, 1988; O'Sullivan *et al*., 2003) and sometimes uniformity of colour, depending on the product. However, such qualitative descriptions do not enable colour to be specified or communicated conveniently – a multitude of food products could easily be described as green or light brown! Humans are effective at detecting colour differences but have a poor colour memory. Consequently, most human colour assessments involve colour matching rather than quantification (Hutchings, 1999).

One of the first modern techniques proposed for a colour notation was the Munsell system. The Munsell notation (see Figure 1.11) first describes colour in terms of its hue – of which there are ten forming the circumference to a sphere; its value – a scale from black to white (lightness); and its chroma – how much a given hue deviates from grey of the same value (Lawless & Heymann, 1998).

This system provided a notation for describing colour analogous to human description of colour and has been used extensively. Most commonly, labelled colour chips are used to match the colour of the object. Such a system obviously requires a human observer and as such is subject to variation. In addition, the colours available are not necessarily representative of those associated with biological systems such as food.

In attempting to develop instrumental methods for colour measurement, a more mathematical approach to colour specification was adopted, which, although complicated, now provides the basis for most colour measurement. It was based on the principle that a colour can be matched by mixing three coloured lights: red, blue and green using physical laws that govern the addition of colours. Essentially, a colour can be specified according to the relative proportion of red (R), blue (B) and green (G) light required to match it. In addition, the intensity of the colour can also be calculated by

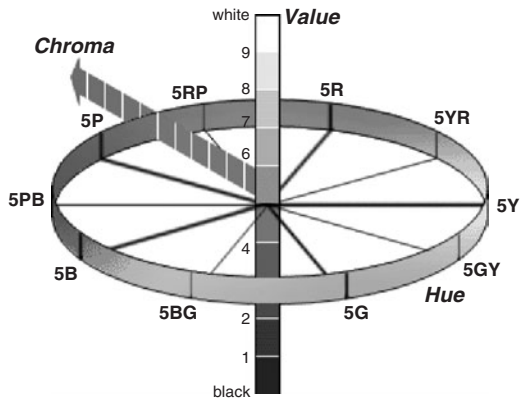

Figure 1.11 Munsell Colour system. (Reproduced in colour in the plate section.) (Picture courtesy of HunterLAB.)

summing the intensity of R, G and B light. Hence, colour could be specified according to the tristimulus coordinates *r*, *g* and *b*. In reality, not all colours could be matched in this way and complications arising due to the occurrence of negative coordinates led to the Commission International d'Eclairage (CIE) adjusting the system to use three abstract primaries *XYZ*. This made all colour coordinates (now *x*, *y* and *z*) positive (see Hutchings, 1999 for a detailed review). This system provides the basis for most other colour notation techniques but in itself lacks the ability to communicate the nature of the colour in a meaningful manner. *Y* relates to 'value' but *X* and *Z* cannot be said to relate to hue or chroma and not surprisingly these coordinates do not correlate well with visual assessments of colour attributes (Lawless & Heymann, 1998).

On the contrary, the subsequently developed LAB system (Lawless & Heymann, 1998) provided a model that reflected the human experience of colour, and thus specified colour in a more logical and communicable way. In practice, measured *XYZ* coordinates are mathematically transformed to new opponent dimensions of yellow/blue, red/green and white/black, analogous to the visual mechanism for colour perception. In the LAB model (see Figure 1.12), *L* (vertical axis) indicates the level of lightness (white/black); the *a*-axis represents red/green ($+a/-a$); and the *b*-axis represents yellow/blue ($+b/-b$). The model is designed to give a perceptually uniform

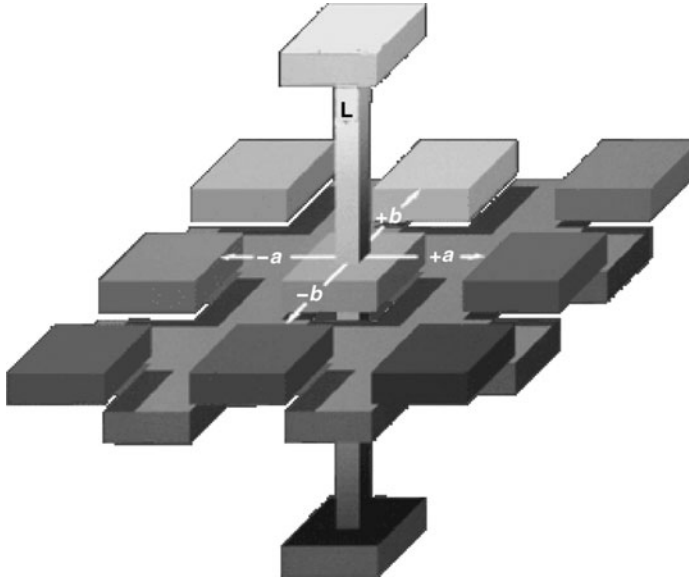

Figure 1.12 HunterLAB colour scale showing the white/black axis (*L*, vertical). (Reproduced in colour in the plate section.) (Picture courtesy of HunterLAB.)

space so that any given change in a value is perceptually equal in any direction.

However, humans are less sensitive to differences in the green spectrum than they are in the blue and so, in the CIE space, humans can perceive differences between colours with much closer blue notations than equivalent differences in green notations. Different systems try and take such sensitivities into account; e.g. the CIELAB model, representative of 92% of the population with no vision difficulties (Hutchings *et al*., 2002b), is overexpanded in the yellow section whereas the HunterLAB is contracted in the blue region. The LAB-based models are shown to correlate better with human assessments of colour attributes but some researchers have used them to derive further values that appear closer still to human description of colour attributes (Lawless & Heymann, 1998). Thai and Shewfelt (1991) found that L (lightness), C (chroma) and H (hue angle) – all mathematically derived from HunterLAB data, correlated even more closely with human assessments of visual appearance in foods.

1.5.3 Instrumental measurement of colour

To make instrumental measures of colour, the three basic elements, the object, a light source and an observer are still required. However, the observer is no longer the human eye but a colorimeter or spectrophotometer. Colorimeters use photodetectors to convert light reflected from an object through red, green and blue filters to *XYZ* tristimulus values. In a spectrophotometer, light reflected (or transmitted) by the object passes to a photodiode array and the resulting spectral data are then converted to the tristimulus values. Either way, *XYZ* data are obtained, which can then be reported as such or more commonly converted to LAB or other colour model values.

For accurate measurements, colorimeters and spectrophotometers require samples that are opaque, uniform in colour, flat and reflect light diffusely (Hutchings, 1999). Herein lies a problem – for a food sample to exhibit such properties is rare but such instruments are widely used in the food industry with the sample manipulated to enable data to be collected. Adequate measures may be made on some raw materials but assessing the colour of whole foods is certainly more problematic. Consequently, the question arises as to whether measures using such instruments provide data congruent with the perception of a human observer. In addition, the original *XYZ* values are based on the sample being opaque and so subsequent derivations may be disproportionately inaccurate. Add to this the fact that instrumental measures are, as would be expected, made under very controlled and specified conditions but not necessarily those experienced by the human observer, and it is not surprising that data from such instruments do not always correlate with sensory measures.

Emerging is the use of digital technology that allows examination of food colour and other appearance attributes in a manner much closer to the consumer's perspective (Hutchings *et al*., 2002a). The basic principle is to use a calibrated digital camera to capture an image of the object, under appropriately controlled conditions. Information from the pixel-based image can then be quantified and related to the colour, texture, gloss and translucency of the object (Pointer *et al*., 2002). As measures are made on a pixel-by-pixel basis, unwanted background or shadow effects can be eliminated with appropriate software. Promising studies evaluating this technology for food products are emerging in the literature (Kane *et al*., 2003; Lu *et al*., 2000; O'Sullivan *et al*., 2003; Papadakis *et al*., 2000; Pointer *et al*., 2002).

Kane *et al*. (2003) compared the use of colorimetric measures with digital imaging and found both correlated well with sensory measures of cookie colour. O'Sullivan (2003) made similar comparisons whilst assessing pork colour and found superior correlations with human evaluations using the digital approach. Better correlations were attributed to the fact that the area evaluated by the digital image is much larger than that of a colorimeter and is more representative of the stimulus evaluated by human observers. Indeed, this represents one of the key advantages of the technique, together with the fact that a representative image of the object can be retained for later use. The technique offers a promising alternative for measuring appearance attributes in a way that correlates with human perception. Applications include new approaches to shelf life/growing/ripening pattern testing and colour atlas development, as well as the ability to make instrumental appearance evaluations *in situ* (e.g. under supermarket or restaurant conditions; Hutchings, 2002a). Furthermore, coupled with developments in monitor display, it provides the ability to project and manipulate images for appearance attributes/preference evaluations by human assessors (Pointer *et al*., 2002) or identical and stable reference samples for appearance attributes measurement (Kane *et al*., 2003).

Although this section has concentrated on colour, digital technology has the potential to allow a range of appearance attributes to be measured. In general, these attributes, which include size, form, translucency, haze, gloss and uniformity, have received less attention in the literature than colour and, not surprisingly, less is understood about their perception and measurement. The proximal stimulus is the same as for colour but different processing of this stimulus gives rise to the perception of such characteristics. Although instrumental techniques exist for some of these, e.g. nephelometry (haze) and goniophotometry (reflectance/gloss), a lack of understanding has hindered development of techniques that correlate with human assessments. (For further details concerning attributes other than colour, see Hutchings, 1999.)

In summary, the proximal stimulus for appearance attributes, particularly colour, has been known for some time but finding an approach to specify

and measure that stimulus analogous to the mechanisms involved in human perception is challenging. Digital imaging technology appears to have provided a new approach that will enable renewed investigation of the crossmodal relationship between appearance and flavour perception.

1.6 Methods to study and quantify crossmodal interactions

There are many reports of multi- and crossmodal interactions in the literature. The origins of the interactions are now being elucidated and expressed in a way that demonstrates the interaction mathematically. However, most research on the relationship between the stimulus and perception has focused on single modalities and has led to various mathematical expressions that have become known as the Psychophysical Laws. The background to psychophysics has been reviewed and the validity of the different forms of the equations discussed (Hoppe, 1997). The best-known law is Stevens' law (Stevens, 1969), which expresses the relationship between stimulus (S) and perception (P) as a power law function:

$$P = S^n$$

Values for the exponent n are obtained by sensory experiments using a range of flavour concentrations and then plotting log stimulus against log perception to calculate the value of n from the slope of the line obtained. Other psychophysical laws use different scalars to represent the relationship between stimulus and perception. Fechner's law uses a logarithmic scale while Weber's law uses the Just Noticeable Difference (JND) concept. All forms of the law are unimodal and limited in their applications. Factors like adaptation were not originally built into the expressions but have been added more recently (Overbosch, 1986), although their validity still awaits confirmation by experimental data. More pertinent to this discussion, the original psychophysical laws are not well suited to expressing potential interactions between the modalities. These shortcomings have been discussed and the advantages of using a holistic approach have been championed by Booth (1994) for some time. He advocates that much of the data obtained with simplistic model systems cannot be applied when mixtures of modalities are studied, as interactions will inevitably occur and these can only be predicted by experiments with whole systems.

Crossmodal effects are described more fully in Chapters 8 and 9. Here, only a discussion of methodologies is presented. Most studies on crossmodal interactions have focused on the interaction of two modalities such as taste and aroma or viscosity and aroma. Generally, solutions of the modalities have been presented to panellists as they are easy to prepare and flavour release from them is least affected by individual differences in physiology

and mastication patterns. Two approaches have been used. Dalton (2000) hypothesised that a crossmodal interaction between taste and aroma would enhance the perception of one (or both) modalities and result in the detection of a subthreshold stimulus. Thus panellists were asked to compare solutions and report whether they could or could not perceive the stimulus. Other workers have used superthreshold solutions and asked panellists to assess the differences quantitatively. In both cases, panellists have consumed the sample and then given an overall, integrated response to the stimulus. In some food systems, however, the delivery of tastants and odorous compounds changes with time, as demonstrated in the chewing gum example (Figures 1.6a and 1.6b). In this case, the temporal delivery of flavour is important and alternative techniques to deliver the components of flavour to panellists under controlled conditions have been sought. Our solution to the problem has been to pump solutions of flavour components to the mouth, which gives easy control (Cook *et al*., 2003a). A schematic of the technique is shown in Figure 1.13. Total flow into the mouth is around 10 ml/min and composition is changed by switching pumps on and off while maintaining total flow using water as a 'make-up' flow. The technique has been described in detail previously (Cook *et al*., 2003a) and has been named Multichannel Flavour Delivery System (McFlaDS) or Dynataste. By monitoring the proximal taste and aroma stimuli *in vivo*, it has been shown that delivery to the receptors does follow the pattern delivered by the pump.

Using this system, the modalities can be delivered separately and interactions observed. The time span is typically 1–5 min and adaptation may occur

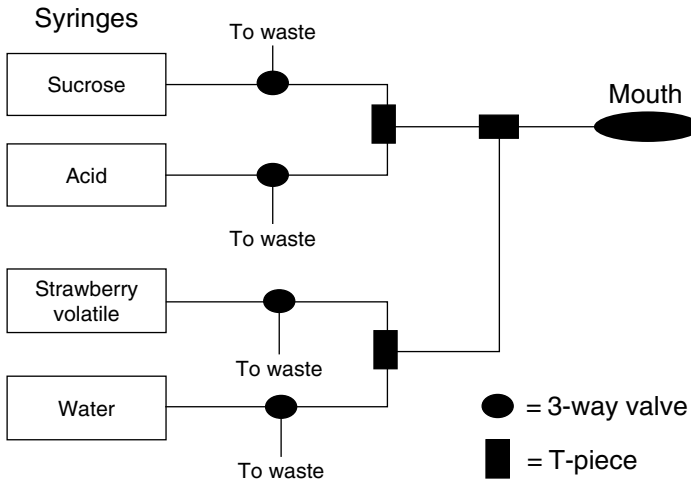

Figure 1.13 Set-up of pumps for measuring crossmodal effects in strawberry flavour.

over this period. Experiments to measure adaptation when a mixture of strawberry aroma and sugar was introduced at a constant level into the mouth showed a decrease in perception of around 15% over 10 min (Cook *et al*., 2003a). Further experiments were conducted where a fruit flavour (orange, banana, strawberry) was given to the panellists with tastants (sugar, acid), and they were allowed to get used to the flavour for a short period (around 30 s); then one of the modalities was withdrawn. Figure 1.14 shows the results for a commercial strawberry flavour and sucrose. The perceived intensity of strawberry flavour was plotted against time and the composition flowing into the mouth is indicated in the figure legend. Two types of behaviour can be observed among the panellists. When sugar was removed, there was always a significant decrease in flavour intensity whereas removal of acid caused a smaller (but still significant) change. Thus panellists reported a dramatic decrease in strawberry flavour perception even though all the usual strawberry volatiles were still being delivered to the nose. Similar results were obtained for other flavours (orange, banana, mint). Even when some panellists were told what was happening, they still reported perceiving the fruit flavour although only sugar was being delivered to their mouths. Measurements of the aroma signal in the nose and the sugar and acid signals on the tongue were made over the time of the experiment and it was shown that all three stimuli decreased rapidly when the respective modality was turned off and increased rapidly when the modality was turned on (Cook *et al*., 2003a). Thus there was no significant reservoir of aroma or sugar/acid that might have been responsible for the sensory behaviour. For the effect to work, panellists have to receive all the modalities initially so they can assess and form an impression of the flavour. After some time to familiarise with the stimuli, withdrawal of a modality creates different effects on the perceived flavour. Counterintuitively, removal of fruit aroma causes little, if any, change. Removal of sugar, however, causes a dramatic decrease in fruit flavour perception. One potential explanation is that once the brain has recognised an object (in this case strawberry flavour), it uses one of the modalities to monitor the perception of that whole object. In the case of strawberry flavour, sweetness is the modality monitored. An analogy can be drawn with face recognition where we use key reference points to identify a person rather than recall every detail of his or her features. When someone changes their appearance in an area that is not a key recognition site (e.g. growing a moustache), we still recognise them and may not notice the moustache unless prompted.

The results obtained using the Dynataste system are open to criticism. One objection is that because panellists are asked to rate only one or two sensory attributes, they may 'dump' any changes they perceive into those categories. As discussed in Section 1.1.2, this is not a universally held view (Prescott, 1999). To determine whether panellists were becoming confused, experiments were conducted in our laboratory with a lemon flavour and sugar, which

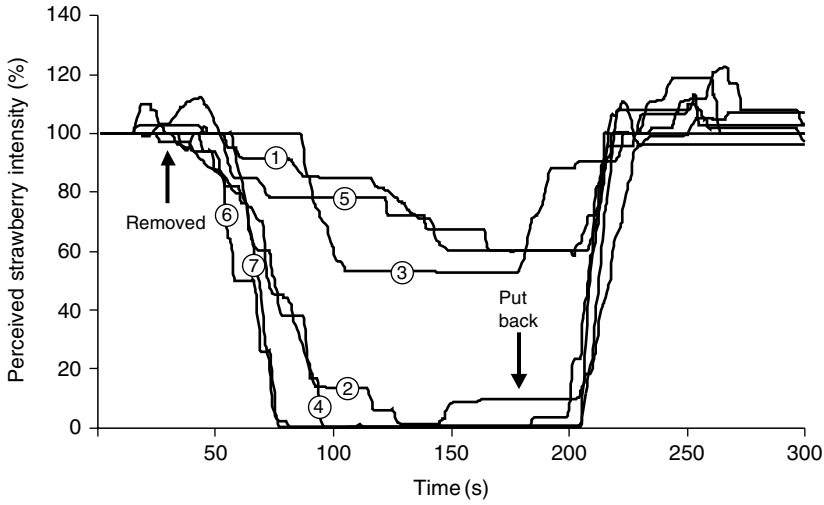

Figure 1.14 Effect of withdrawing and replacing modalities on perceived strawberry flavour. Initially all three components were present (acids, sucrose, strawberry aroma). After 30 s, either one, two or all three of the solutions were replaced by an equivalent flow of water (see *key* below). After 180 s, the mixture was restored to its initial state.
Key: (1) aroma off; (2) sucrose off; (3) acid off; (4) aroma and sucrose off; (5) aroma and acid off; (6) acid and sucrose off; (7) all off.

showed no change in lemon flavour perception when sugar was withdrawn, proving that the panel only registered the effect for congruent flavours. Another objection is that delivering solutions at 10 ml/min over periods of minutes only mimics the eating process for a small number of foods (e.g. chewing gum and hard-boiled confectionery) and that the perception of other foods may be different. However, as a scientific tool for studying crossmodal interactions over time, Dynataste does provide a convenient way of studying the phenomena.

The phenomena reported with the experiments above could be regarded as flavour illusions and analogous to the many illusions reported for vision and selective attention. The influence of colour on the flavour perception of a food has been discussed in Section 1.5. A recent example of a colour–flavour illusion is the aperitif jellies served by the chef Heston Blumenthal at his restaurant, the Fat Duck at Bray. Each jelly consists of two parts, coloured red and orange, respectively and customers are told they are made from beetroot and orange. On eating, customers become confused as the orange part is beetroot-flavoured (being made from yellow beetroot) and the red part is orange-flavoured (being made from blood oranges). Similar effects have been noted when white wine is coloured red and subjected to tasting (Morrot *et al.*, 2001).

From the discussions above, the limitations of traditional psychophysics are such that they cannot be used alone to describe the multi- and crossmodal effects noted for flavour modalities. A more flexible notation is needed, and the mathematical expressions from the textural work in Section 1.4 shows one approach where the intensity of sensory perception can be described as a function of three instrumental measurements (Kokini *et al*., 1984). Another way of presenting the relationships is to use a series of expressions with the format for writing basic computer programmes. The advantage of this approach is that the conditions under which the interactions take place can be defined. As an example, the interactions between viscosity and perceived flavour have been plotted using oral shear stress to give the best correlation. Figure 1.15 shows the relationship for banana flavour with four different hydrocolloids (Cook *et al*., 2003b).

The relationship could be written as:

While [A] > OT and
While (OSS) > log − 0.25 Pa and < log 1.75 Pa, then
If (OSS) ≤ 0.6 then log FP = f[A].− 0.036(OSS) + 1.98 else
If (OSS) > 0.6 then log FP = f[A].− 0.031(OSS) + 2.12

Translating this into words, the first line says that the concentration of aroma [A] has to be above a threshold value (OT). The second line sets the

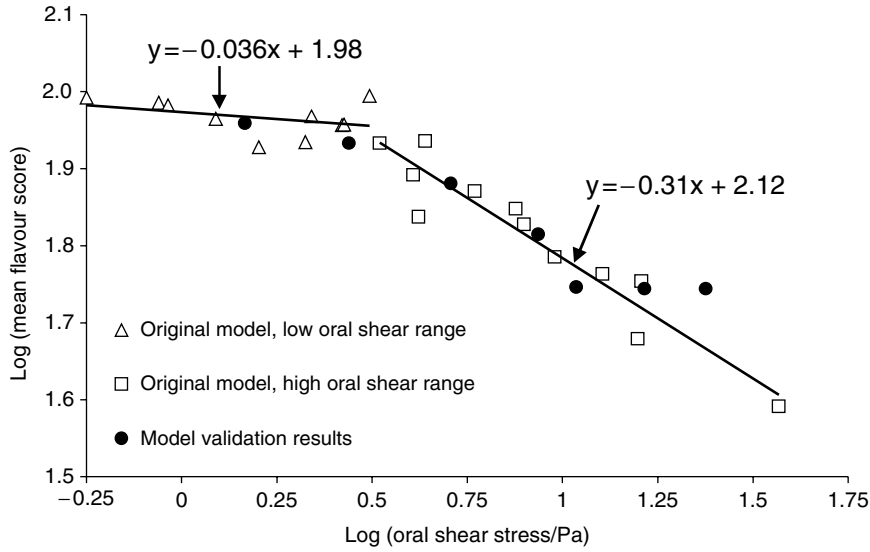

Figure 1.15 Plot of oral shear stress (OSS) against the mean flavour score for banana flavour from four different hydrocolloids (Cook *et al*., 2003b).

limits within which the relationship holds, i.e. the oral shear stress (OSS) has to be within the experimental limits measured. The third line then explains behaviour in the first part of the curve and says that if the log OSS is less than or equal to 0.6, then flavour perception (FP) is a function of aroma concentration [A] multiplied by -0.036 of the OSS plus a constant. The fourth line sets the conditions if the log OSS is above 0.6. The advantage of this format is that it is easy to write and can be used directly for calculation and testing.

1.7 Conclusion

This chapter proposes that identifying and measuring the proximal stimuli associated with flavour may lead to a better understanding of the origins of flavour. Inherent in the proposal is the concept of flavour as a multimodal construct with significant interactions between modalities. The proximal stimuli associated with aroma, taste, texture and colour are discussed, as are methods for measuring them. Progress has been made in developing analyses to measure proximal stimuli, although difficulties still exist. These range from the problems of identifying proximal stimuli to appropriate methods for measuring signals at very low levels as well as signals that change with time as food is masticated and swallowed. Nevertheless, there seems to be a consensus that this approach provides opportunities to gain an in-depth understanding of some flavour systems so that we might express the relationships between modalities and perception on a mathematical basis. This is not only satisfying from the scientific research viewpoint but may help in formulating foods that are more aligned with nutritional goals (less sugar, salt and fat) while maintaining a high-quality flavour.

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2 The role of oral processing in flavour perception

Jon F. Prinz and Rene de Wijk

2.1 Introduction

While all the senses have their role to play during feeding, the special senses, namely vision, taste and olfaction play particularly important roles at various stages. The first task in feeding is to identify and locate food items. At long ranges, vision and smell allow a fruiting tree to be identified, while at closer range these senses together with touch determine the choice of one particular food item over another. Once the food has been transferred to the mouth, taste and texture can be assessed to provide a final check that the ingested food item is suitable to swallow. All the while, information from these multiple sources is processed in the higher centres where it may lead to decisions on the choice of the next food item to be taken. Likewise, the information from the senses may lead to signals sent to areas further down the gastrointestinal (GI) tract, which must prepare to receive the food. Although modern humans seldom need to forage in the forest, these search-and-find skills developed in the cause of human evolution are still in use at the supermarket and at the dinner table to identify the products that will be consumed. Preferences developed in response to scarce resources may explain the general preferences of modern humans for high-fat, sweet, energy-dense foods over foods that are hard to digest and poor in energy, such as leaves and root vegetables. Fortunately, though, our senses allow us to detect the nutrients we need and it seems that our innate food preferences, which include a preference for variety, ensure that most individuals do eat an adequately balanced diet (Lucas, 2004).

The processes that occur in the mouth during mastication alter the physical properties of the food and can dramatically affect the perception of flavour and texture of the food. Details of oral processing have been gathered using a wide variety of techniques, ranging from observations of muscle activity, jaw movement (Heath & Prinz, 1999), particle size distribution (Lucas & Luke, 1983), mixing efficiency (Prinz, 1999), bite mark analysis (Prinz & Lucas, 2001), facial movements, direct observation by videofluorography (Hiiemae & Palmer, 1999; Palmer & Hiiemae, 1997) and ultrasound imaging (Soder & Miller, 2002). Common findings from all these sources are that the following steps are involved in mastication: (1) food is placed onto the anterior one-third of the tongue; (2) the tongue is elevated, compressing the food

against the palate; (3) the tongue is depressed, transferring solid foods to the postcanine teeth; (4) comminution; (5) swallowing; and (6) clearance (Abd-El-Malek, 1955). The main phases of mandibular movement are illustrated in Figure 2.1. In the interval between the food entering the mouth and being swallowed, the food's temperature equilibrates to mouth temperature, it is mixed with saliva allowing the various salivary enzymes to exert their effects, and solids are fractured while liquids and semi-solids may undergo shear thinning.

While the oral processing of solid foods is well understood, the processing of semi-solids lags behind, possibly because those processes are mainly determined by tongue movements that are difficult to monitor non-invasively. However, the amount and type of manipulation to which a food item has been subjected can be inferred by examining the material recovered from the mouth after the main bolus has been swallowed. In solids, the particle size distribution resulting from fragmentation through chewing and the resulting production of new surface area can be quantified (Heath & Prinz, 1999; Liedberg & Owall, 1995; Lucas & Luke, 1983; Prinz & Lucas, 1997). In semi-solids, the addition of a marker to the stimulus allows the degree of mixing to be quantified (Figure 2.2). These data indicate that the mixing of semi-solid food is far from complete when the food is swallowed.

Engelen and van Doorn (2000) asked subjects to describe what they did after placing a semi-solid food in the mouth. Four separate strategies were identified: (1) simple (50%); (2) taster (20%); (3) manipulator (17%); and (4) tonguer (13%). 'Simple' subjects placed the food on the front of the tongue, raised the tip of the tongue to the palate while forming a seal with the sides of the tongue against the teeth, then retracted the tongue and

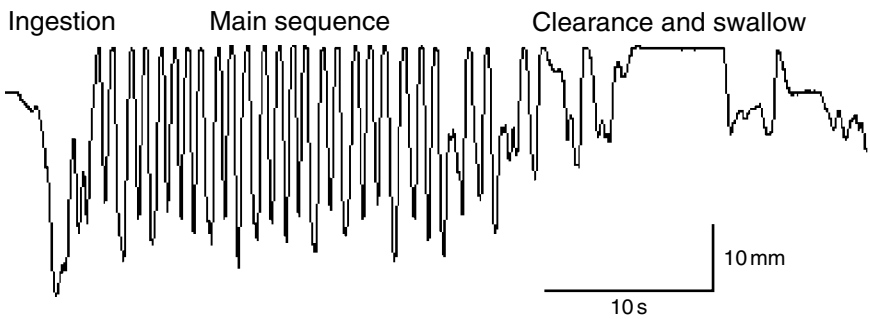


Figure 2.1 Typical chewing sequence showing the vertical displacement of the mid-incisal point from a subject chewing bread. Note the initial (ingestion) phase, where food is transferred from the tongue to the postcanine teeth, the main sequence, and the final clearance and swallowing phases where food is swallowed and the tongue attempts to remove debris from the teeth and oral mucosa. (Data captured using the Sirognathograph system at the Biometrics Laboratory, London Dental Hospital.)

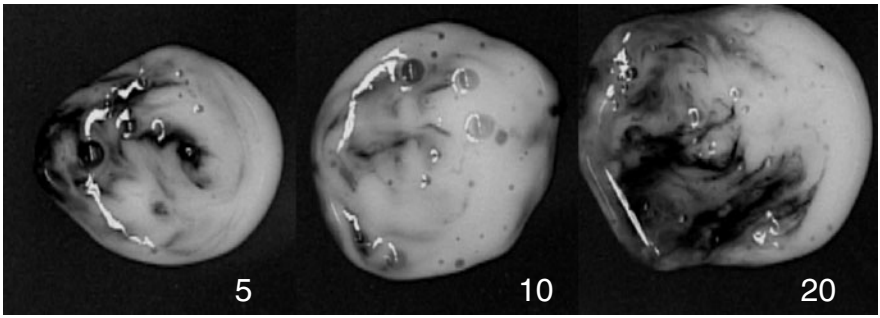


Figure 2.2 Photographs of spat-out custard to which a single 30 μl drop of a black dye has been added. Mixing after oral residence times of 5, 10 and 20 s is shown. For this product, oral residence time is typically less than 10 s.

swallowed the food. 'Tasters' did the same, but made a series of additional short sucking movements before swallowing. 'Tonguers' added back-and-forth and sideways movements of the tongue against the palate, while 'manipulators' described a wide variety of complex behaviours involving tongue, cheeks and jaw movements.

Movements made by the tongue and mandible during eating vary considerably between individuals, but are relatively consistent within subjects. These patterns of behaviour are probably established during development of the deciduous and permanent dentitions between the age of 6 and 18, which suggests that the manipulation of solid and semi-solid foods is a learnt behaviour, unlike the swallowing of liquids, which is innate. Partial or complete loss of dentition in later life can also modify feeding behaviour (Heath & Prinz, 1999).

2.2 Anatomy of the peri-oral structures

Before going further, it is worth reviewing the anatomy of the mouth. Head and neck anatomy is complex, and relevant to feeding are the teeth, salivary glands, cranial nerves, the muscles of facial expression, the muscles of mastication, the supra- and infra-hyoid muscles and the muscles of the pharynx and larynx. Fortunately, it is not necessary to memorise *Gray's Anatomy* (Williams *et al*., 1985) to be able to understand the major structures involved in feeding (Figure 2.3). These are the teeth, tongue, soft palate and pharynx. The soft palate (uvula) acts as a valve that, when raised, seals the nasal cavity off from the mouth. It is the uvula that allows one to drink whilst standing on one's head. In more normal circumstances, though, the uvula hangs down from the roof of the mouth, leaving open a passage from the oral cavity up into the back

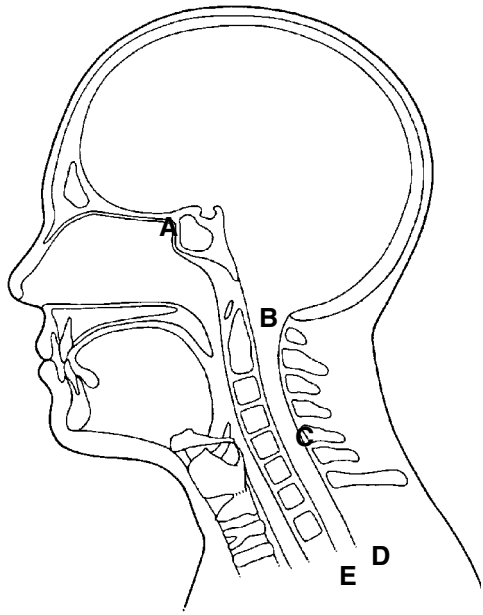


Figure 2.3 Sagittal section of the head. (A) olfactory epithelium, (B) uvula, (C) epiglottis, (D) oesophagus, (E) trachea.

of the nasal cavity through which aroma-laden air may travel, allowing retronasal olfaction to take place (Buettner *et al*., 2001; Burdach & Doty, 1987).

For the ingestion of solid foods, the teeth and the muscles of mastication act in concert as a universal testing machine, sensing load and displacement and allowing the physical properties of the food to be sensed. For liquids and semi-solid foods, mechanoreceptors in the tongue and palate play the role of a rheometer monitoring the forces necessary to deform the bolus, and also monitor the pattern of flow over the tongue.

The pattern of flow over the tongue is determined by the surface profile of the tongue. This varies from region to region and is currently poorly understood. For the purposes of description, the tongue is divided into three parts:

- the anterior one-third is covered with two types of papillae. The fungiform papillae are liberally innervated and contain the taste receptors, while the filliform papillae cover the remainder of this part of the tongue (Figure 2.4a).
- the middle one-third is entirely covered by filliform papillae, although here they are far more keratinised than at the tip of the tongue, and are longer (Figure 2.4b).

- at the boundary between the middle and posterior one-third sit the large circumvallate papillae. It has been proposed that these are predominantly receptors for bitter tastes.

Taste receptors are also present in the palate and the oropharynx. Any movement of the tongue will expand and contract the spaces between the papillae,

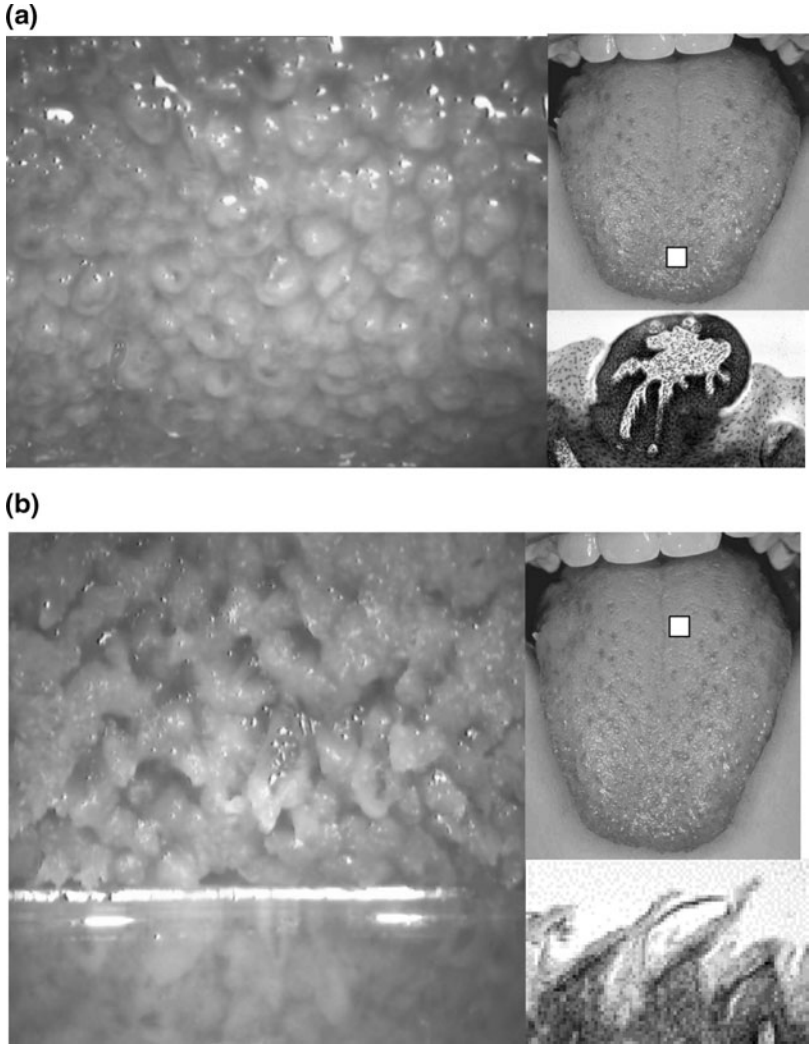


Figure 2.4 Macroscopic and microscopic views of (a) the anterior of the tongue, showing a view of fungiform papillae and (b) the middle one-third, showing filliform papillae.

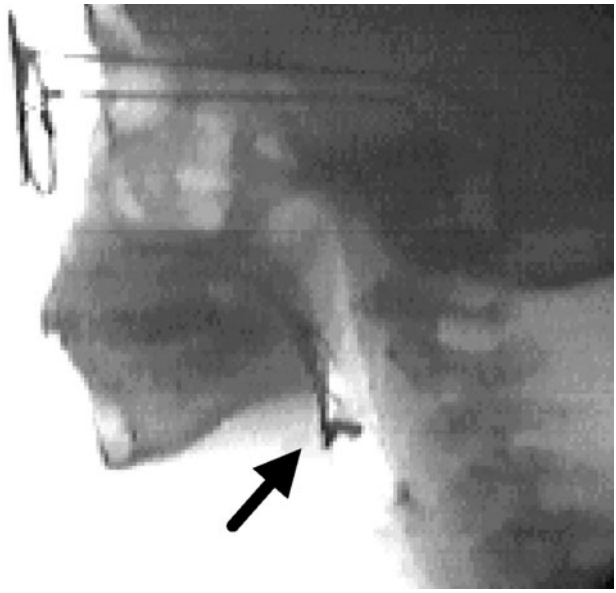


Figure 2.5 Single frame captured from a videofluorographic recording of a subject taken immediately after swallowing. Note the material retained on the pharyngeal wall of the tongue and in the valeculi (arrowed). (From a video supplied by Dr J. Palmer, Johns Hopkins University.)

forcing fluid in and out, and exposing the taste receptors at the base of the papillae to fresh material.

The rougher surface of the tongue in the posterior two-thirds of the tongue causes food to form a thick coating layer, as shown in Figure 2.5.

2.2.1 Saliva

Saliva is derived from three sets of major glands, the parotid, the sublingual and the submandibular (Pedersen *et al*., 2002). In addition, an array of minor glands are located in the oral mucosa of the tongue and cheeks. Parotid saliva is mainly serous, and contains high levels of amylase, proline-rich proteins (PRPs) and bicarbonate ions. The sublingual and minor glands produce a mucin-rich saliva, while the submandibular glands secrete a mixture. In contrast to the parotid, these other glands are continuously active, their main function being to produce secretions that can lubricate the mucosa (Humphrey & Williamson, 2001). Minor glands in the tongue, the von Ebner glands, also secrete the enzyme lipase which breaks down fats in the food. It has recently been suggested that the products of this fat breakdown may be sensed by taste receptors, which, if correct, would give rise to a specific fat taste (Gilbertson, 1998; Mattes, 2001).

Saliva flow rate and composition are modulated by mechanical simulation, taste and expectation. Sour tastes have the greatest effect, increasing flow rates from typical resting values of 0.3–6 ml/min. Other taste compounds have lesser effects and some (such as caffeine) suppress salivary flow. Smells do not appear to have any effect on parotid saliva flow rates, but do have a slight stimulant effect on submandibular saliva flow (Lee & Linden, 1992; Spiers, 1971).

Sour tastes have a dramatic effect not only on the rate of flow of saliva but also on its composition. Parotid saliva in particular responds to increased flow by increasing the concentration of bicarbonate ions, thus enhancing its ability to buffer both dietary acids and the acids formed by anaerobic bacterial metabolism in the dental plaque. This helps to prevent intra-oral pH from dropping below 5.5, the level at which demineralisation of tooth enamel begins to take place. This substantial buffer capacity of saliva rapidly neutralises dietary acids, thereby reducing their sourness.

In starch-based foods, amylase plays an important role in perception. As the starch breaks down, there is a dramatic reduction in viscosity. This leads to the so-called fat surfacing phenomenon, in which the loss of structure in the aqueous phase allows fat droplets to migrate to the surface of the bolus. One would expect that any dilution of the stimulus by saliva would reduce flavour intensity by reducing the effective concentration of flavour compounds. However, since dilution can reduce the viscosity of food and since in sugars reduced viscosity will increase the perceived taste intensity, the opposite can also be the case (Christensen, 1980). For other tastants, though, the flavour intensity is indeed reduced (van Ruth *et al*., 1996). In starch-based semi-solids, the breakdown of the starch by amylase can cause dramatic reduction in viscosity, which allows fat droplets to migrate to the surface, where they can volatilise and form a coating.

There are several other ways in which saliva interacts with both taste and aroma compounds, the most obvious being the interaction of tannins with the PRPs in saliva. PRPs are present in the saliva of most mammals that are exposed to tannins as part of their diet. It has been suggested that the role of PRPs is to sequester any dietary tannins before they can do damage further down the GI tract. In humans, however, it is more likely that PRPs function as a detection mechanism through their effect on friction. Two mechanisms appear to be involved: (1) the tannin-induced precipitation of PRPs that leads to reduced viscosity; and (2) a direct interaction between dietary tannins and the mucosal surfaces that leads to increased friction between the tongue, teeth and palate (Green, 1993; Humphrey & Williamson, 2001; Prinz & Lucas, 1997).

Salivary mucins have also been shown to bind with certain volatile compounds, to the extent that the release of the associated aromas may be blocked completely in subjects with high levels of mucin in their saliva (Bücking,

2000). Tannins have also been shown to interact with salivary amylase, causing a reduction in its ability to break down starch (Kashket & Paolinio, 1988; Zhang & Kashket, 1998). Recent experiments in our laboratory where amylase activity has been modulated *in vivo*, by adding either extra amylase or the amylase inhibitor acarbose, show that starch breakdown by amylase significantly affects both the texture of the food, such as its thickness and 'melting' mouthfeel, and also its flavour. These effects were not seen in control stimuli thickened with carboxymethylcellulose, which is unaffected by amylase (de Wijk *et al*., in press).

2.3 Flavour

Flavour results from the combined input of taste (sensed by receptors on the tongue), smell (sensed in the nose) and irritation (sensed on the mucosal surfaces). However, it is difficult to study each of these aspects of flavour in isolation in real foods, and there is evidence that these sensory inputs are integrated at the central level. Thus, Rolls *et al*. (1999) have demonstrated that single neurons in the orbitofrontal cortex can react to several sensory modalities.

Five basic tastes are usually described: salt, sweet, sour, bitter and umami. Unlike colour vision, where the full gamut of colours is synthesised from just red, green and blue receptors in varying combinations, there appear to be a large number of different types of receptors involved in taste, each responding to one or more of a wide variety of compounds.

If we define taste as originating from receptors on the tongue, it is possible that the classic number of tastes may have to be increased. Recent evidence from animal studies suggests that there are specific lingual receptors for fatty acids, starch and tannins (Gilbertson *et al*., 1998; Mates, 2001; Sclafani, 1991). And it will come as no surprise if additional new receptors are discovered for other nutritionally important compounds, whether beneficial or detrimental (Nelson *et al*., 2001a, 2001b).

Evidence now exists, in rats, of a detector for fatty acids in the tongue. This is not a receptor per se, but makes use of the ability of fatty acids to block potassium channels. The effect is rapid and appears to be highly specific. Other animal evidence suggests that a preference for fat is related to sensitivity to these free fatty acids (Gilbertson *et al*., 1998). Mattes (2001) has shown that in humans, sham feeding – where food is taken into the mouth but not swallowed – increases the concentration of triaglycerols in the blood. The experiments are complex, but the only sensible interpretation is that fats are detected in the mouth, very probably by Gilbertson's (1998) mechanism. Fat substitutes that have the same texture, but which do not contain fatty acids, do not lead to the same changes in blood chemistry.

Fats can also be detected directly by their effect on texture, specifically their ability to reduce intra-oral friction. This is not fat-specific: inorganic oils have been shown to produce the same response in the orbitofrontal cortex in monkeys (Rolls *et al*., 1999).

Recent studies from our laboratory have provided evidence for a third fat detection mechanism. The low thermal conductivity of fat in foodstuffs affects its perceived temperature, so that high-fat products are perceived as being warmer than low-fat ones when served at the same physical temperature. In an experiment where subjects were served 16 samples of commercial mayonnaise varying in fat content from 0% to 82%, there was a very high ($r = 0.99$) correlation of perceived temperature with actual fat content, showing a closer relationship between fat content and temperature than between any of the other attributes assessed in the same experiment (Prinz & de Wijk, 2003).

2.4 Oral processing

How we eat determines how we will perceive the food. An extreme example of this is chocolate, which, at the extremes, can either be chewed vigorously or can be left to melt in the mouth. The experience resulting from these two styles of chocolate eating is clearly different, and similar effects occur with other foods.

The process of feeding, from ingestion to swallowing, is modulated by oral sensations that are in turn affected by the process itself (Alfonso *et al*., 2002). Food begins to be assessed even before it is placed in the mouth, which can result in increased salivation, and a decision is made as to the size of bite to be taken. The first bite is then taken and the food is placed on the tip of the tongue. At this point, the temperature of the food is assessed, both in absolute terms and in terms of its rate of heating or cooling, which in turn allows the fat content of the food to be assessed.

The food is then transferred by the tongue to the posterior teeth and rhythmic mandibular movements start. It is during this so-called 'main sequence' phase that solid foods are fragmented by the teeth, and saliva incorporated into the mass (Alexander, 1998; Prinz & Lucas, 1997). Fragmentation increases the surface area of the food that is exposed to saliva, thus increasing the rate at which taste compounds can dissolve into the saliva and from there be transported to the taste receptors. The increase in surface area and the warming of cold foods also facilitate the release of volatiles, giving rise to enhanced flavour sensations (Engelen *et al*., 2003). Furthermore, aerosols may form as the tongue is removed from the palate, again intensifying flavour release. It is in order to produce aerosols that wine tasters make vigorous tongue movements, and these same movements further enhance the transfer of these aerosols from the mouth to the nose, particularly when tasters hold their

heads down to allow the uvula to drop forwards, fully opening the passage between the mouth and the nose (Zafar *et al*., 2000).

While the tongue is in contact with the palate, the food is subjected to high shear forces, which not only lead to shear thinning of the stimulus but can also result in coalescence of oil droplets (if present), wetting the mucosal surface with oil, increasing the surface area available for volatilisation and allowing more intimate contact between the oil and the taste receptors. All these effects combine to enhance flavour release.

The role of oral movements in perception was investigated in detail by de Wijk *et al*. (2003) who trained subjects to use a variety of feeding styles (see Table 2.1), whilst assessing the stimuli (two custards and two mayonnaises) using a set of attributes to describe flavour, texture and afterfeel sensations. Flavour and texture attributes were assessed 5 s following ingestion, after which the product was swallowed and afterfeel attributes rated. The behaviour modifications affected most of the texture attributes and all of the flavour attributes, which increased in intensity as the movements became more complex (see Figure 2.6). The percentage change in intensity from the still condition, where the food was simply held in the mouth prior to being swallowed, to the normal feeding style was quite dramatic. For the mayonnaises, oily/fatty flavour increased in intensity by 52% and sour flavour by 29%, while for the custards, bitter chemical flavour increased by 81% and vanilla flavour by 67%.

Table 2.1 Subjects were trained to use the following behaviour modifications when assessing semi-solid foods

Modified behaviour	Definition
Still	Stimulus is placed on the anterior one-third of the tongue, held for 5 s and then swallowed
Up	Stimulus is placed on the anterior one-third of the tongue, which is then raised to come in contact with the hard palate, held for 5 s and then swallowed
Up and down	Stimulus is placed on the anterior one-third of the tongue, which is then raised to come in contact with the hard palate, lowered and swallowed after 5 s
Suck	Stimulus is placed on the anterior one-third of the tongue, the posterior two-thirds of the tongue then raised and lowered ten times within 5 s prior to swallowing
Smear	Stimulus is placed on the anterior one-third of the tongue, the tip of the tongue is moved in a figure-of-8 pattern against the hard palate ten times within 5 s prior to swallowing
Normal	Assessment is made 5 s after ingestion, and food is then swallowed in the subject's normal style

Source: de Wijk *et al*. (2003).

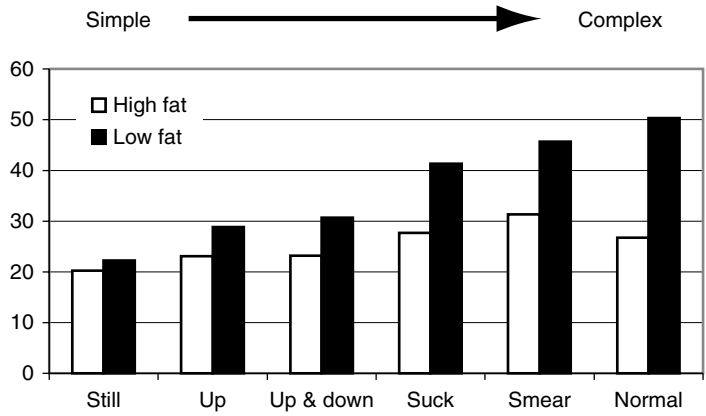


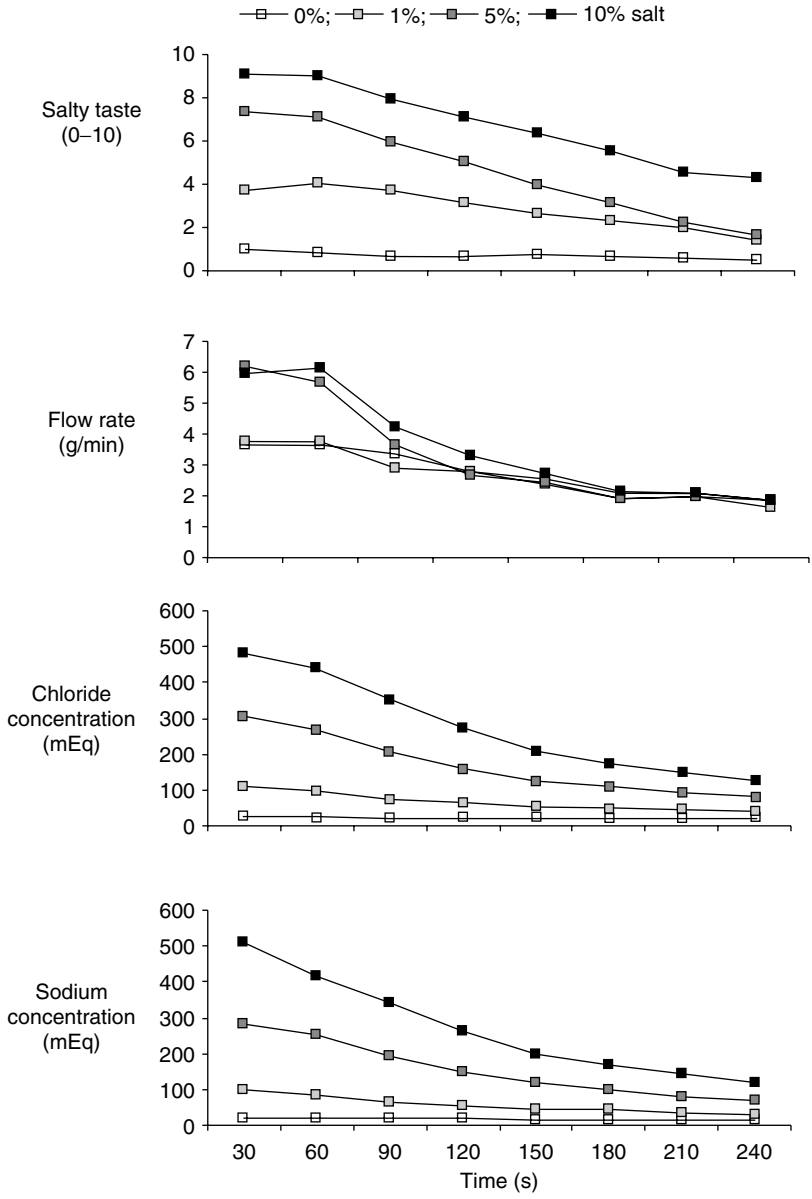
Figure 2.6 Panellist's ratings of bitter chemical flavour for a high- and a low-fat vanilla custard made using the modified behaviours described in Table 2.1. Note how the intensity of, and the ability to discriminate between, the two stimuli increase as oral movements become more complex.

In the same way that the oral behaviours described above can increase the intensity of oral sensations, the intensity of orthonasal olfaction can be increased by sniffing. This increases the volume of air passing over the olfactory epithelium and induces turbulence in the airflow, which facilitates mass transfer (Laing, 1983). As described above, holding the head downwards can further enhance retronasal olfaction by allowing the uvula to come forwards, facilitating the flow of aromas from the mouth to the nose (Matsubara *et al*., 2002; Zafar *et al*., 2000).

The temporal aspects of the release of flavour and the effect on perception are illustrated by experiments carried out by Neyraud *et al*. (2003), in which the concentrations of salt and glucose in saliva were monitored during the mastication of a model salted chewing gum. Subjects recorded the intensities of sweet and salt tastes after chewing gum containing varying amounts of salt. Instead of swallowing the saliva produced during chewing, subjects regularly spat it out, whereupon the concentrations of Na^+, Cl^- and glucose in these spat samples were determined, and conductivity and pH measured. The results (see Figure 2.7) showed a clear relationship between the actual concentration of NaCl and the perceived intensity of salt taste. Interestingly, though, comparing the data for the rated sweetness of the four gums showed taste mixture suppression, i.e. the greater the salt concentration, the lower the perceived sweetness, even though the actual concentrations of glucose in the stimulus and in the saliva were identical.

These effects became even clearer when continuous measurements of intraoral conductivity (a measure of ionic strength, and thus indirectly of salt concentration) were made using electrodes placed in the saliva behind the

lower central and lateral incisors (Davidson *et al*., 1998; Jack *et al*., 1995). While chewing the same four model salted chewing gums, subjects indicated the perceived salt intensity, and simultaneously recorded swallows made. Typical results are shown in Figure 2.8. These data demonstrate the phenomenon of habituation, i.e. the process by which the perceived intensity of a



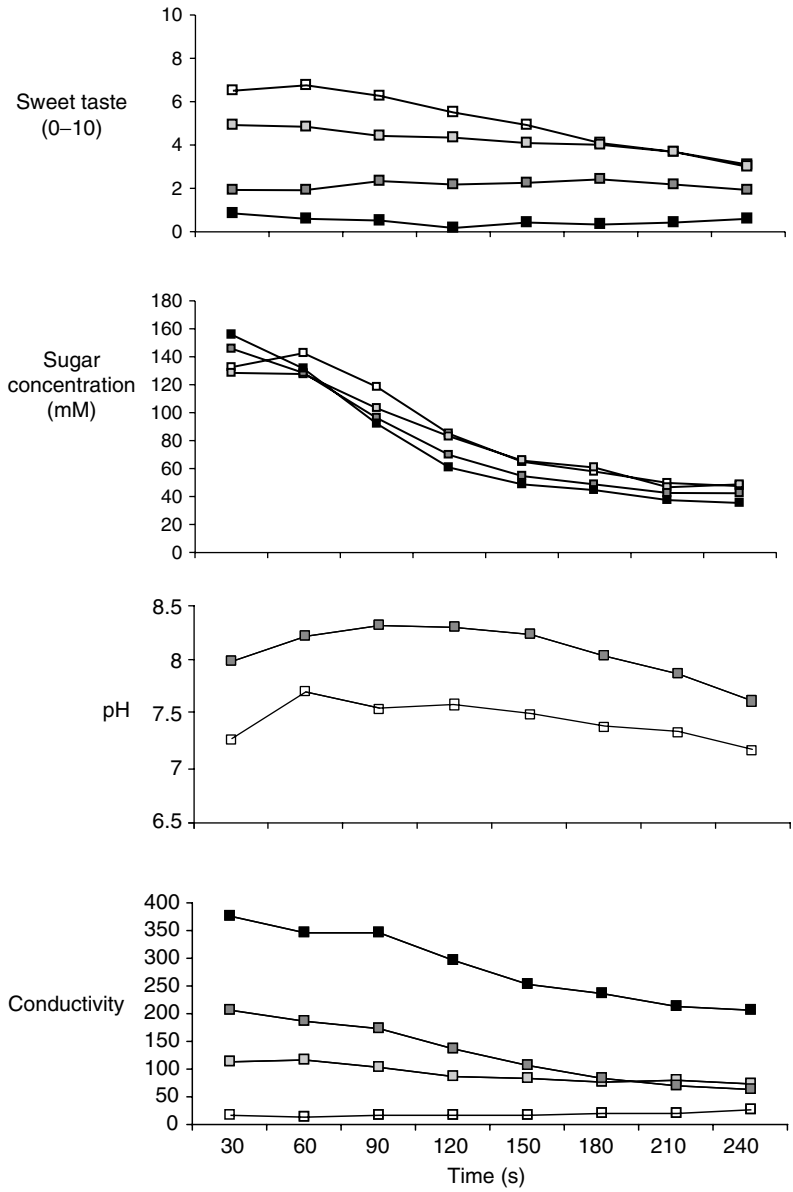


Figure 2.7 The taste, quantity of saliva and composition of saliva sampled at 30s intervals whilst chewing a model salted chewing gum. Values are the means across ten subjects for gums containing 0%, 1%, 5% and 10% salt.

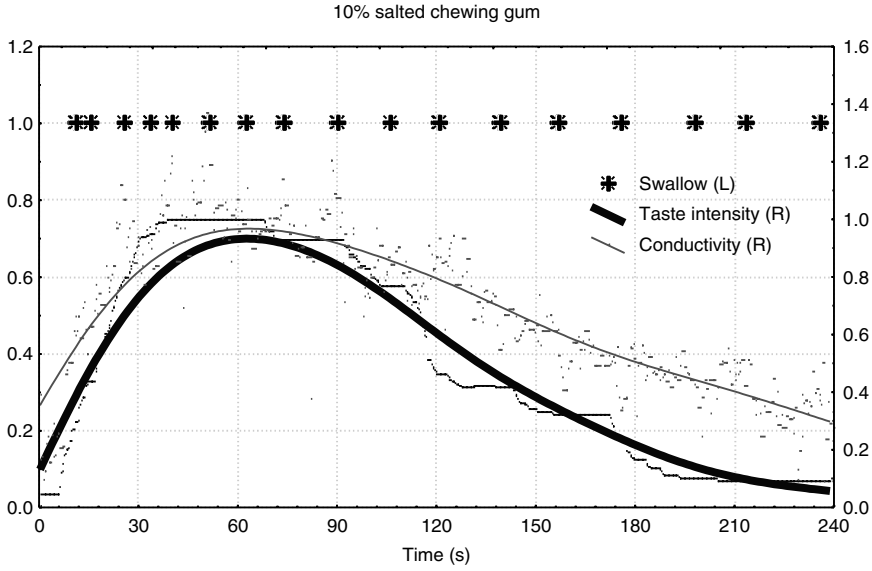


Figure 2.8 Salt taste intensity, conductivity and swallows recorded whilst chewing a 10% NaCl chewing gum. Note that taste intensity declines more rapidly than does conductivity. This is due to habituation.

constant stimulus reduces over time. In Figure 2.8, this is evident in the different rates of decline of perceived intensity and of conductivity (see Chapter 8).

After swallowing, substantial amounts of material are retained in the mouth, either in the form of discrete particles (Heath & Prinz, 1999) or as an oral coating.

This coating tends to form mainly on the posterior two-thirds of the tongue where the surface is roughest (see Figure 2.5) and shear forces are lowest. Particulate debris tends to accumulate in the buccal sulci and in the interdental spaces, regions that are not easily accessible to the tongue during clearance (Heath & Prinz, 1999; Valentová & Pokorny, 1998). In a study (Prinz *et al.*, 2003a) comparing the amounts of the aqueous and fat phases of custard before and after oral processing, it was shown that material recovered from the mouth was deficient in fat compared to the original product suggesting that fats are preferentially retained in the coating (Figure 2.9). This coating continues to release volatile compounds into the air stream and soluble compounds into the saliva, both of which processes contribute significantly to the aftertaste of food.

The movements of the tongue against the palate, and the pressure applied, may play an important role in forming these coatings (assessments of tongue–palate pressure vary from 25 g/cm^2 to 200 g/cm^2 (Takahashi & Nakazawa, 1990)). In normal mastication, different shear rates and pressures can exist simultaneously in different parts of the mouth, and in the same part of the mouth during different phases of the chewing cycle.

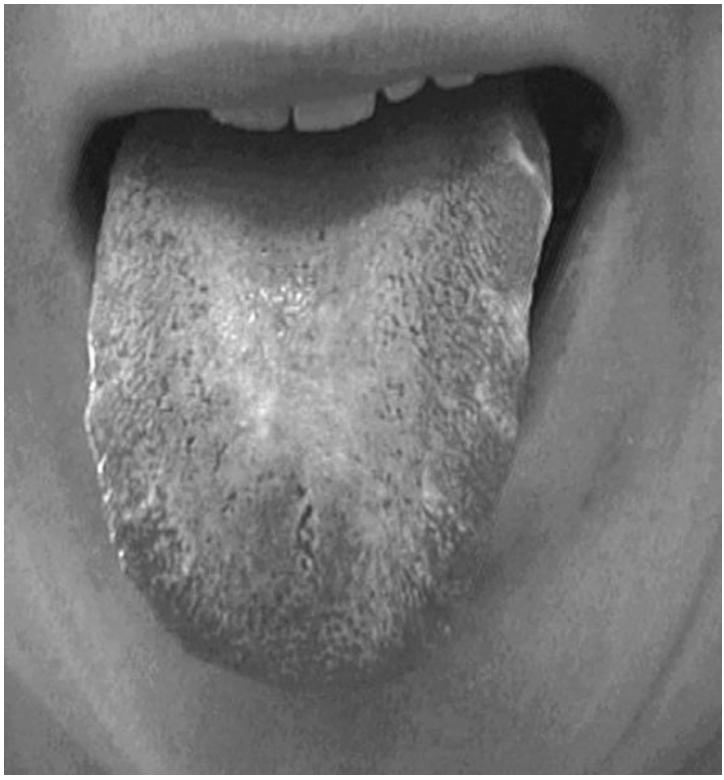


Figure 2.9 Photograph of the tongue taken immediately after the subject has swallowed a spoonful of custard.

2.5 Conclusion

Mastication starts when the food has entered the mouth and ends with swallowing. It is during this process that the palatability of the food is assessed and the food converted into a form suitable for swallowing. The process starts when food is transferred to the mouth and placed on the anterior one-third of the tongue, which then transfers the food to the posterior teeth. During this transfer, the tongue is raised and the food is squeezed against the palate. This forms a coating on the surface of the tongue, which can persist for some time after swallowing (Figure 2.5). Fragmentation of solids and mixing of semi-solids during mastication result in the production of new surface area, and thus in enhanced flavour release. Subsequent oral movements, and heating, amplify these effects.

In addition to the physical effects of mastication, the chemistry of saliva plays an important role in the appreciation of foods. The salivary enzyme

amylase, which breaks down starches, can result in a dramatic change in the viscosity of starch-thickened products such as custards, mayonnaises and sauces within a few seconds of ingestion. Reduced viscosity leads to a redistribution of fat droplets in the bolus resulting in an increased concentration of fat at the surface. This increased concentration of fat in the oral coating increases the availability of fat-soluble flavour compounds. Lipase may also play an important role in the perception of fat. Mucins too play a role, and individual differences in the mucin content of saliva may explain differences in perception (Bücking, 2000).

Even after swallowing, substantial amounts of material are retained in the mouth, which can be recovered by rinsing with water. Analysis to compare the amount of the fat and aqueous phases in the recovered material with the intact product suggests that fats are preferentially retained in the oral coating where they continue to release volatile compounds into the air stream and soluble compounds into the saliva. These aftertastes contribute significantly to the appreciation of the food.

The role of oral processing in the mechanisms of flavour release is becoming increasingly realised and understood. These processes are far from equilibrium, and occur over relatively short timescales. New methods to track changes in taste, texture and aroma as food is processed in the mouth remain a challenge for food scientists, who must remember that what is in the package on the supermarket shelf bears little relationship to the material that enters the stomach. Understanding the mechanisms by which the properties of a food item are altered through oral processing will allow the food industry to develop products that are both more palatable and possibly more healthy to eat.

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Flavor Perception
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3 The cellular basis of flavour perception: taste and aroma

Nancy E. Rawson and Xia Li

3.1 Introduction

The quality of flavour is a delightful amalgam of taste, aroma, texture, chemical irritation and thermal sensation. Understanding the biological bases for these sensations can help develop new approaches to improving the sensory attributes of foods for special dietary needs, as well as improving the quality of the flavour experience for those with impaired ability to detect these stimuli. Beyond this, the neurological systems responsible for these sensations represent perhaps the most diverse, complex and adaptable components of the nervous system. Their study is changing the way we view the nervous system and contributing new insights into basic questions of gene regulation, neuronal plasticity and information coding. This review will focus on the neural systems underlying the detection of taste and odour stimuli, and how they are determined and influenced by genetics, ageing and experience. Aspects of flavour detected via the trigeminal system are discussed in Chapter 5, while issues related to neural processing and encoding of flavour quality within the central nervous system (CNS) are discussed in Chapter 7.

3.2 Taste and flavour

Taste is the sensory system that provides animals with the power to distinguish critical sensory input and check the quality of the food to be ingested, e.g. sweet and amino acid receptors allow recognition of nutritionally rich food sources, while bitter receptors elicit aversive responses to noxious and toxic stimuli (Lindemann, 2001). Humans and other mammals can detect and discriminate between at least five taste qualities: sweet, bitter, sour, salty and umami (glutamate taste). Even newborn human infants can distinguish sweet from bitter and show hedonic expression for sweet tastes but aversive expressions for bitter tastes (Granchrow, 1983; Steiner, 1973; Steiner *et al*., 2001). Recent developments in molecular genetics have provided a much clearer view into the cellular mechanisms underlying the detection of taste stimuli, while advances in imaging and neural recording techniques are yielding new insights into the central processing of taste information and

how that information is integrated with input from other sensory systems to generate the percept of 'flavour'.

3.2.1 Taste buds and taste cells

There has been a widely disseminated yet incorrect 'tongue map' since a twentieth-century textbook showing that each chemoreceptive area of the human tongue specifically responds to each of the qualities of sweet, sour, salty and bitter. In fact, although there are some slight differences in sensitivity across the tongue and palate, all qualities of taste can be elicited from all the regions of the tongue that contain taste buds (Smith, 2001).

On the surface of the tongue (Figure 3.1), there are many taste buds located within taste papillae. The fungiform ('mushroomlike') papillae are pinkish spots located on the front part of the tongue containing one or more taste buds. At the back of the tongue, there are 12 larger taste bud–containing papillae called the circumvallate ('wall-like') papillae. Taste buds are also located in the foliate ('leaflike') papillae, which are on the sides of the rear of the tongue. In humans, the number of taste buds within a papilla varies greatly among individuals (Miller, Jr, 1988; Miller & Bartoshuk, 1991; Miller & Reedy, 1990), and many, particularly the fungiform papillae on the tip of the tongue, may have very few or even none (Brand, personal communication). This variability is not clearly attributable to health status or age (Mavi & Ceyhan, 1999; Miller, Jr, 1988; Miller & Reedy, 1990).

Taste buds are onion-like structures (Figure 3.1), and in rodents, each taste bud contains 50–100 taste cells. Taste cells, like olfactory receptor neurons (ORNs), are continually replaced throughout life, rendering the taste system particularly capable of withstanding the ongoing exposure to stimuli ranging widely in osmotic, ionic, pH, textural and thermal qualities. When tastants (chemicals or proteins) dissolved in saliva contact the taste cells through the taste pores, they interact either with taste receptors on the surfaces of the cells or with ion channels. These interactions cause electrical changes in the taste cells, which trigger them to send chemical signals that ultimately result in impulses along either the chorda tympani nerve (anterior tongue) or glossopharyngeal nerve to the brain (Smith, 2001). Studies of animals and humans show that there is no direct correlation between taste quality and chemical class, particularly for bitter and sweet. For instance, aspartame and sucrose both taste sweet to humans, although their chemical structures are unrelated. The tastants of salty and sour are less diverse and are typically ions.

A single taste cell may be multimodal, with several types of receptors; one type may be more active than the others on that cell. Each taste cell contains more than one type of receptor. Each taste receptor cell (TRC) is connected through a synapse to a sensory nerve ending, which is a peripheral afferent fibre, sending the taste-coding information to the brain. However, a single

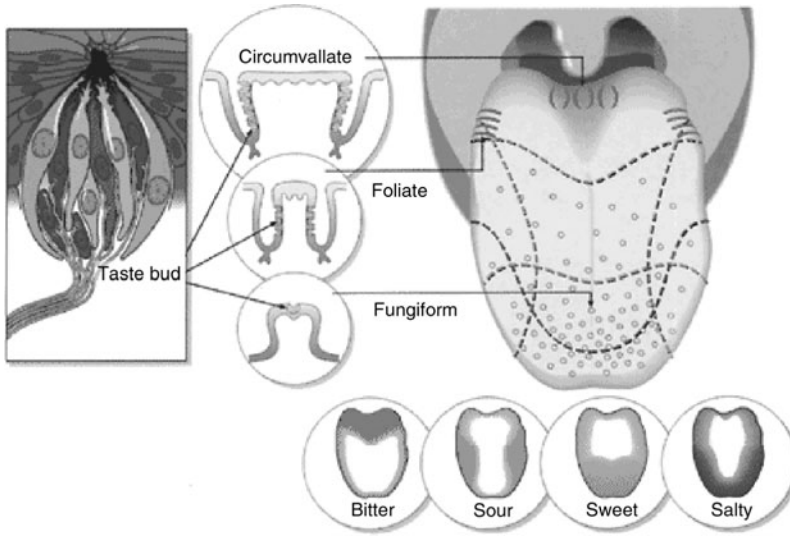


Figure 3.1 Functional anatomy of the human tongue. Diagram of a human tongue, highlighting the taste buds and the regional preferences to sweet, sour, bitter and salty stimuli. While different areas are preferentially responsive to certain taste modalities, there is significant overlap between the regions. (Hoon *et al*., 1999. Copyright 1999. Reprinted with permission from Elsevier, London.)

sensory neuron can be connected to several taste cells in each of several different taste buds. Therefore, the activity of groups of afferent neurons whose cell bodies are within the gustatory cortex is ultimately responsible for coding taste information. Nerve recordings both of the afferent nerves and of the neurons receiving their input in the gustatory cortex are found to respond to multiple taste modalities, but typically are more sensitive to one class of stimuli than another (Smith, 2001).

The taste information relating chemical stimuli to taste perception is encoded by the nervous system. The whole process starts from transduction mechanisms at the receptor and ends in the representation of stimulus attributes by the activity of neurons in the gustatory cortex. Different mechanisms are used by TRCs to transduce chemical stimuli into neural responses, including direct ion-gated channels, paracellular pathways and G protein–coupled second-messenger pathways.

In view of the observations that each neuron responds to many different-tasting stimuli, it is difficult to imagine how the brain can distinguish among various taste qualities. Physiological studies of brain activity patterns have found that unique patterns of activity are generated for a given stimulus type across a large set of neurons. In 1993, Smith *et al*. demonstrated that the same neurons were responsible for taste-quality representation, whether they are viewed as labelled lines or as critical parts of an across-neuron

pattern. Currently available data indicate that similar tastes evoke similar patterns of activity across groups of taste neurons in the gustatory cortex.

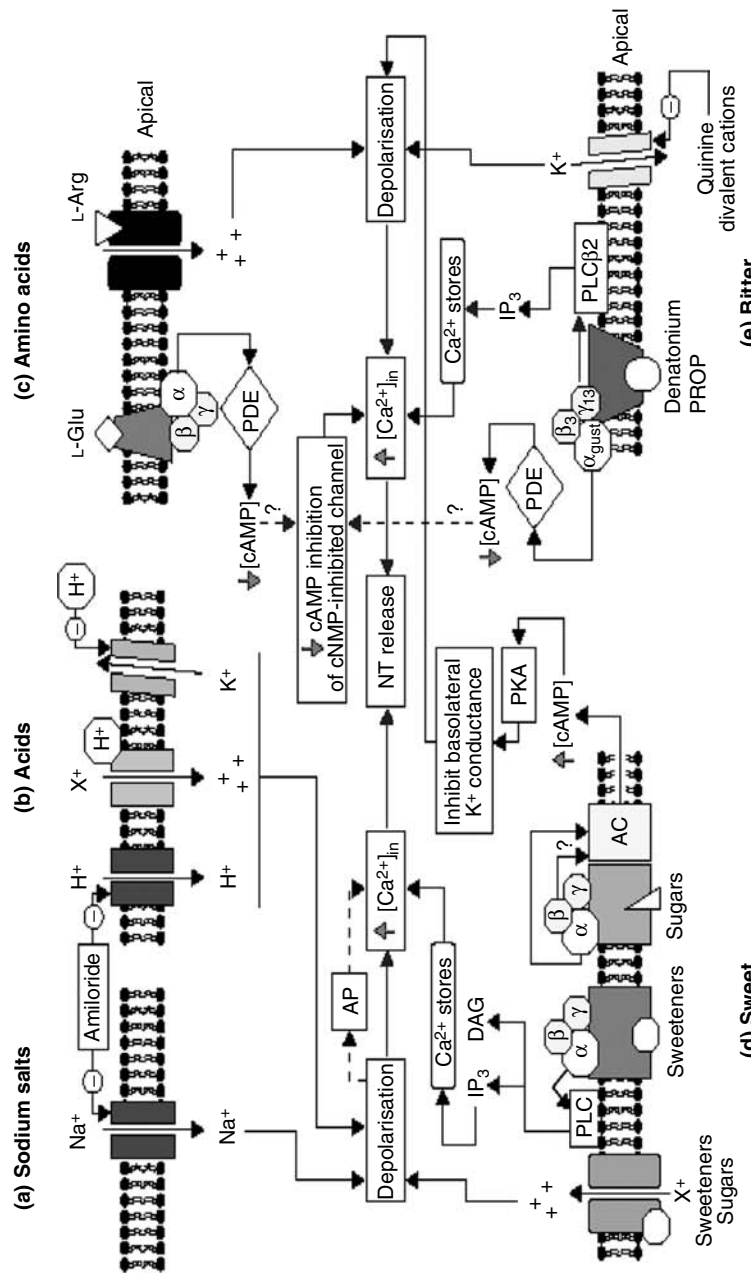
3.2.2 Molecular mechanisms

According to biological and electrophysiological studies, taste cells use a variety of mechanisms to transduce chemical information into cellular signals. The detection of sour and salty tastants is mediated by ion channels. In contrast, sweet, bitter and umami taste transduction pathways involve membrane receptor proteins coupled to intracellular signalling cascades (Gilbertson, 2000; see Figure 3.2). Previous studies show that both peripheral and central gustatory neurons typically respond to more than one kind of stimulus. Although each receptor cell or neuron responds most strongly to one tastant, it usually also generates a response to one or more other stimuli with dissimilar taste qualities (Smith, 2001). This broad tuning has implications for the possible intracellular interactions that might occur among stimuli of different types, and suggests that masking or enhancing effects among stimuli may result from crosstalk within the signalling pathways activated by diverse stimuli.

3.2.3 Salt taste

Salt taste is elicited when sodium ions (Na^+) enter through ion channels on microvilli at the apical end of the taste cells. The sodium ions are accumulated to cause depolarisation, which results in calcium ions (Ca^{2+}) entering the cell. The calcium, in turn, triggers neurotransmitter (NT) release, the identity of which remains a mystery. Nerve endings receive the message and convey a signal to the brain. Taste cells repolarise, or 'reset' themselves in part by opening potassium ion (K^+) channels so that K^+ can exit. Although salt taste is elicited by many ionic tastants, it is mostly relevant to sodium in mammals (Lindemann, 1997, 2001). The only salt that can traverse the channel better

Figure 3.2 Taste is mediated by a diversity of transduction mechanisms. All pathways converge to elicit an increase in intracellular calcium, which triggers neurotransmitter (NT) release. The amiloride-sensitive sodium channel contributes to salt and sour detection (dark blue). Protons released from sour stimuli are also detected via a nonselective cation channel (light blue) and via block of an outward K^+ conductance (light green). Depolarisation results in opening of voltage-gated calcium channels and calcium influx. Glutamate is detected via a G protein–coupled pathway that may include multiple receptor types. In some species, other amino acids can be detected via ligand-gated ion channels, but the presence of this pathway in humans is not established. Multiple pathways exist for detection of sweet stimuli, including both ligand-activated ion channels (ionotropic) and G protein–coupled receptor pathways. Bitter compounds are detected via G protein–coupled receptors activating PLCβ2, leading to release of calcium from intracellular stores. Some bitter compounds and bitter-tasting salts may also act via suppression of K^+ efflux. Symbols and abbreviations: α, β, γ refer to G-protein subunits; AC = adenylyl cyclase; AP = action potential; IP_3 = inositol trisphosphate; NT = neurotransmitter; PDE = phosphodiesterase; PKA = protein kinase A. (Taken from Gilbertson, 2000 with permission.) (See opposite page for figure and text for additional details.)


(a) Sodium salts
(b) Acids
(c) Amino acids
(d) Sweet
(e) Bitter
Apical
Na+
Amiloride
H+
X+
K+
L-Arg
L-Glu
α
β
γ
PDE
[cAMP]
?
cAMP inhibition of cNMP-inhibited channel
Depolarisation
NT release
[Ca2+]in
Ca2+ stores
IP3
DAG
AP
PLC
AC
PKA
Inhibit basolateral K+ conductance
Sugars
Sweeteners
PLCβ2
β3
γ13
αgust
Denatonium
PROP
Quinine
divalent cations

than Na^+ is lithium, although K^+ and other monovalent ions are somewhat permeable (Heck *et al*., 1984). One mechanism for salt detection is the amiloride-sensitive epithelial sodium channel ENaC. This channel is a hetero-oligomeric complex comprising three homologous subunits, which acts as a salt-taste detector by supplying a specific pathway for a sodium current into taste cells, provided that Na^+ are present in the oral space in sufficient concentration (Kretz *et al*., 1999). While amiloride blocks salt taste in rodents, it is much less effective in humans (Smith, 1995), suggesting the involvement of another unspecified channel or mechanism. Thus, the molecular function of salt taste in humans remains unclear. The anion also plays an important role in detection of salt taste, related to its ability to dissociate from the cation and permeate the extracellular space. The rapid penetration of chloride through the paracellular junctions alters the pericellular ionic composition to facilitate sodium influx (Ye *et al*., 1994). Larger anions such as gluconate are less permeable and therefore result in a less intense salt taste.

3.2.4 Sour taste

Acids taste sour due to the concentration of hydrogen ions (H^+) in solution. In afferent nerve recordings, *in situ* recordings and in isolated taste cells, acidic stimuli elicit action potentials (APs) from taste cells in a dose-dependent manner based on the titratable acidity of the stimulus, rather than its pH (Gilbertson *et al*., 1992; Kinnamon *et al*., 1988). This indicates that perception of sour taste is much like a proton counter. Protons act on a taste cell in three ways: by directly entering the cell; by blocking K^+ channels on the microvilli; and by binding to, and opening channels on, the microvilli that allow other positive ions to enter the cell. The resulting accumulation of positive charges depolarises the cell and leads to NT release. Interestingly, similar to Na^+ salt transduction, members of the degenerin (deg)/ENaC superfamily of ion channels apparently play a role in acid transduction (Benos & Stanton, 1999). Studies by Gilbertson demonstrated that depolarisation of taste cells by H^+ permeation of ENaC contributes to acid taste transduction under conditions of low mucosal Na^+ in hamster (Gilbertson *et al*., 1992, 1993). Moreover, three other members of deg/ENaC family, MDEG1 (mammalian degenerin-1 channel, also called BNaC1 [brain-type Na^+ channel-1]), ASIC (acid-sensing ion channel, also referred to as BNaC2) and DRASIC (dorsal root acid-sensing ion channel) all function as cation channels that are activated by H^+ and are sensitive to the diuretic amiloride (Benos & Stanton, 1999). Therefore, the acid-stimulated activation of these channels would be predicted to lead to depolarisation in cells in which they are present. The role of these diverse mechanisms in human sour taste perception is only beginning to be explored, but involvement of ASIC has been suggested by preliminary studies of human

fungiform papillae in which messenger RNA (mRNA) for several isoforms of ASICs were identified (Huque, personal communication).

3.2.5 Sweet taste

Sweet stimuli, such as sucrose or artificial sweeteners, generate strong hedonic effects in humans and animals. Sweet taste perception is initiated by the interaction of a sweetener with G protein–coupled taste receptors in the apical part of TRCs. When sweet tastants bind to receptors on a taste cell's surface that are coupled to molecules termed guanosine triphosphate(GTP)-binding regulatory proteins ('G proteins'), this triggers separation of the subunits. The subunits activate other cellular processes, including the enzymes that generate second messengers. Interestingly, natural sweet tastants appear to activate a pathway involving cyclic adenosine monophosphate(cAMP)-dependent suppression of a K^+ channel that differs from the one triggered by artificial sweeteners, in which a different second messenger is produced (IP_3) that releases calcium from intracellular stores. However, in both cases, the final step is cell depolarisation and NT release (see Figure 3.2).

There has been great progress in our understanding of sweet taste genetics in the past few years. In 1999, two receptors were identified, *TAS1R1* and *TAS1R2* that were thought to be taste receptors because they were expressed in TRCs and, in the case of *TAS1R1*, because the structural gene was near the saccharin preference (*Sac*) locus (Hoon *et al*., 1999). The *Sac* locus is a region of a chromosome that had been linked to saccharin preference in mice, and the location of *TAS1R1* within this chromosome supported the role of this receptor in the transduction of sweet taste perception. However, as subsequently demonstrated, genetic mapping studies examining sequence variants in *TAS1R1* excluded this gene as a candidate for the *Sac* locus (Li *et al*., 2001). Another member of the T1R family was soon discovered, *TAS1R3*, which is the gene that corresponds to the *Sac* locus (Bachmanov *et al*., 2001; Kitagawa *et al*., 2001; Max *et al*., 2001; Montmayeur *et al*., 2001; Nelson *et al*., 2001; Sainz *et al*., 2001). Support for this comes from experiments with congenic and transgenic mice (Bachmanov *et al*., 2001; Nelson *et al*., 2001) and the results of functional expression of this receptor in heterologous systems (Li *et al*., 2002; Nelson *et al*., 2001, 2002).

Proteins from the T1R family apparently combine ('dimerise') at the surface of the TRC to produce functional receptors. Based on the cellular co-localisation of T1R2 and T1R3, it appears that receptor dimerisation is necessary for sweet compounds to properly interact with the receptor complex (Li *et al*., 2002; Max *et al*., 2001; Montmayeur *et al*., 2001; Nelson *et al*., 2002). Coexpression of mouse and human forms of the receptor suggests that the differences in aspartame sensitivity (but not in sucrose sensitivity)

between mouse and human are due to variants in the T1R2 but not the T1R3 portion of the receptor complex (Nelson *et al*., 2002). Further studies are needed to clarify the transduction pathways for both natural and synthetic sweeteners.

3.2.6 Bitter taste

Bitter taste is generated by tastants such as quinine and 6-n-propyl-2-thiouracil (PROP). Like sweet and umami taste, bitter taste also acts through G protein–coupled receptors and second messengers. Bitter stimuli activate phospholipase C (PLCβ2), resulting in IP_3 production and the release of calcium ions from internal stores. This calcium increase results in depolarisation and NT release. Bitter taste is usually unpleasant. This will effectively warn against the ingestion of potentially toxic or harmful compounds. By mining the human genome sequence database, two research groups identified a multigene family (40–80 members in this family) that codes for the T2R candidate bitter taste receptors (Adler *et al*., 2000; Matsunami *et al*., 2000). The T2Rs have the seven transmembrane domain topology and belong to the G protein–coupled receptor superfamily. The divergence of the T2R sequences may correspond with an ability to interact with the chemically diverse ligands associated with bitter tastes. Although these receptors have been found in human, mouse and rat by sequencing, only a few of them have been characterised functionally. In a heterologous expression system, human T2R4 and mouse T2R8 have been shown to respond to denatonium and PROP; mouse T2R5 (orthologous to rat T2R9) is the bitter receptor for cycloheximide (Adler *et al*., 2000; Chandrashekar *et al*., 2000; Matsunami *et al*., 2000); human T2R16 apparently mediates bitter taste in response to salicin (β-glucopyranosides); and rat T2R9/human T2R10 respond selectively to cycloheximide and strychnine (Bernd & Hofmann, 2002). While these functional expression studies provide powerful tools for structure–activity studies, it remains to be established whether the complete response profile for a given receptor complex is accurately reflected by the heterologous systems.

3.2.7 Umami taste

Umami is a fifth taste quality generated by amino acids such as L-glutamate. It is now known that glutamate binds to G protein–coupled receptors and activates second messengers. In TRCs, L-glutamate has been found to have two different modes of action – ionotropic and metabotropic.

Umami sensation is elicited most potently by glutamate, which is one of the amino acids that make up the proteins in meat, fish and legume. Glutamate also serves as a flavour enhancer in the form of monosodium glutamate (MSG). Genetic studies of umami taste have shown that since glutamate is also an

important nutrient, this may be the reason that some animals have evolved the ability to taste it. Recent progress in the study of the molecular basis of umami taste has demonstrated that among the three members of T1R family, T1R1 dimerises with T1R3 to function as a umami receptor in a heterologous expression system (Li *et al*., 2002; Nelson *et al*., 2002). A second mechanism has been proposed, based on the discovery of an alternatively spliced form of mGluR4 (the first 300 amino acids of the amino (N) terminus are absent) in rat taste buds. This protein, when expressed in a cultured cell line, transduced a response to extracellular glutamate (Chaudhari *et al*., 2000). It remains to be examined how these different pathways work in human taste cells.

In mammals, sweet, umami and bitter-tasting substrates are recognised by G protein–coupled receptors expressed in TRCs that are clustered in taste buds mainly on the lingual epithelium. Activation of TRCs by a stimulus is propagated via afferent neurons and processed in taste centres in the brain. There are controversial views of how these three different taste qualities are perceived and transduced. Two proposed pathways are:

(1) A single TRC could be broadly tuned, and it recognised a multitude of different ligands; hence, an individual cell might express many different receptor types and require numerous signalling molecules that couple to, and transduce the signal from, a given receptor type. Recently, electrophysiological studies revealed that individual TRCs responded to stimuli representing multiple taste qualities (Caicedo *et al*., 2002; Margolskee, 2002; Reed *et al*., 2001).
(2) Individual TRCs might detect only a single taste modality; hence, one would expect that individual cells express only one receptor type. The supportive evidence includes the expression patterns of T1Rs and T2Rs in distinct, non-overlapping sets of TRCs (Adler *et al*., 2000; Nelson *et al*., 2001). This group favours the second pathway. They studied two unrelated families of receptors (T1Rs and T2Rs) that mediate responses to sweet, amino acids and bitter compounds, and two signalling molecules, PLCβ2 and TRPM5 (taste TRP ion channel). They demonstrated that knockouts of PLCβ2 or TRPM5 abolish sweet, amino acids and bitter taste reception but do not impact sour or salty tastes. They have found that a conserved, streamlined signalling cascade is shared by all three receptor types for recognising sweet, umami and bitter-tasting substrates. They concluded that TRCs are not broadly tuned across different taste modalities but mediate only one of these three qualities (Zhang *et al*., 2003). Whether the sour (H^+) and salty (Na^+) stimuli mediated by ion channels are also recognised by TRPM5- or PLCβ2-expressing TRCs, or whether the remaining 50% of cells mediate these taste qualities remains to be elucidated.

3.2.8 Individual variations – polymorphisms in receptors

There are large within-species and between-species variations in sweet taste perception in mammals. While many compounds that taste sweet to humans are palatable to other species, such as rodents and nonhuman primates, even among closely related species, there are large differences in sweet taste perception. For instance, Old World simians can taste aspartame but prosimians and New World simians cannot. The reason for these differences among primates could be due to the sequence polymorphisms in receptors. Sequencing of *TAS1R2* and *TAS1R3* genes has shown that there are some variant sites in nonhuman primates associated with aspartame sensitivity (Li & Reed, unpublished data). In mice, the *TAS1R3* gene is responsible for the *Sac* locus and influences the intake and preference for sweet substances. This gene is allelic in mice. Several groups have identified a missense mutation (I60T) in the mouse, which is the most likely mutation accounting for the phenotypic differences (Bachmanov *et al*., 2001; Kitagawa *et al*., 2001; Max *et al*., 2001; Montmayeur *et al*., 2001; Nelson *et al*., 2001; Sainz *et al*., 2001). The comparable sequence in the human genome *TAS1R3* has been examined to see whether sequence polymorphisms might be associated with individual differences in the sensitivity for sucrose and aspartame among 30 unrelated human subjects. Three missense mutations were found, one of which was common, occurring on 5–10% of chromosomes. Subsequent studies genotyping subjects for this mutation identified 6 heterozygous subjects and 47 homozygous subjects who were compared for their ratings of sweetness intensity and pleasantness. Aqueous solutions of sucrose (0.29 M and 0.58 M) and aspartame (0.0032 M) were reported to be significantly more pleasant for subjects that were heterozygous for the mutant allele than for subjects homozygous for the wild-type allele ($p < 0.05$). Surprisingly, this mutation did not alter sweetness intensity ratings (Reed *et al*., 2001), and detection thresholds were not determined on these subjects. These data are significant as the first demonstration that such genetic variations in a taste receptor could, through altering sweet preference, also influence food preference.

Previous studies show that sensitivity to the bitter compound phenylthiocarbamide (PTC) is a complex inherited trait. Reddy and Rao (1989) have demonstrated that the variability in PTC thresholds is controlled by a major locus with incomplete dominance as well as by a multifactorial component with significant residual heritability. Pedigree segregation analysis supports a model predicting two genetic loci for the inheritance of PTC taste deficiency, which extends the traditional one-locus recessive model by the addition of either another allele or another locus, and in some cases predicts two types of nontasters (Olson *et al*., 1989). The individual variations in PTC taste sensitivity have been associated with a gene on human chromosome 7q that encodes a member of the *TAS2R* bitter taste receptor family. Three coding single-

nucleotide polymorphisms (SNPs) were identified, giving rise to five haplotypes in this gene worldwide (Olson *et al*., 1989). These haplotypes completely account for the inheritance of the classically defined taste insensitivity and for 55–85% of the variance in PTC sensitivity. This gene has a direct influence on PTC taste sensitivity, and sequence variants at different sites interact within the predicted ligand-binding domain of the encoded gene product (Kim *et al*., 2003).

3.3 Perception of aroma

Chapter 4 will provide a detailed view of the peripheral components of odorant detection, including the roles of odorant-binding proteins, olfactory receptors (ORs) and the primary signal transduction pathway for signalling the ligand-binding event to the CNS. Here we will present a neurophysiological perspective of the cellular and systems-level processes that participate in odour detection and initial processing of the incoming signal in the olfactory bulb, where the neural activity pattern responsible for encoding the odorant quality is established. We will also discuss briefly how olfactory detection processes are modulated by cellular and systems-level interactions.

3.3.1 What is the 'signal'?

A change in the electrical activity ('firing rate') of the receptor cell represents the signal within which all data relayed to the CNS about odour binding must be encoded. Mammalian neurons typically exhibit some level of spontaneous activity (Lowe & Gold, 1995). This provides for a multimodal signalling scheme in which the cell may either be depolarised, leading to an increase in AP firing, or hyperpolarised (Sanhueza & Bacigalupo, 1999; Sanhueza *et al*., 2000), leading to suppression of spontaneous activity (Kurahashi *et al*., 1994). This may serve to increase the coding and discriminatory capabilities of the system by improving the signal–noise ratio.

The mechanisms underlying the increase in firing rate have been extensively documented. The triggering event in activation of most mammalian ORNs appears to be the opening of a nonselective cation current in response to elevation of the second-messenger molecules, cAMP or cyclic guanosine monophosphate (cGMP) secondary to activation of the G protein–coupled odorant receptor proteins (Figure 3.3; see also Chapter 4). The amount of cation influx through this channel under normal physiological conditions is not likely to be sufficient to depolarise the cell, but additional mechanisms are activated that amplify the ion flux sufficiently to trigger an AP. In particular, a calcium-activated chloride conductance has been identified in some species that enables efflux of negatively charged chloride to further depolarise the cell

(Kleene, 1993; Kleene & Gesteland, 1991). This amplification mechanism has yet to be identified in human olfactory neurons, but would serve to render the ORN transduction pathway more capable of withstanding changes in the extracellular milieu resulting from altered mucus composition. A variety of voltage-sensitive potassium conductances is then activated in response to depolarisation and serves to allow potassium efflux to repolarise the cell. The molecular nature of these ion channels defines the shape of the AP and the time required for resensitisation. A sodium/calcium exchanger (Noe *et al*., 1997; Reisert & Matthews, 2001) helps to return the concentrations of these ions to their resting state. Many of these channels and transduction elements are modulated by kinases, including calcium–calmodulin-dependent kinase, protein kinase A (PKA) and protein kinase C (PKC) (Boekhoff *et al*., 1994; Gomez *et al*., 2000; Kroner *et al*., 1996; Leinders-Zufall *et al*., 1999; Schleicher *et al*., 1993; Vargas & Lucero, 2002; Wetzel *et al*., 2001). These kinases phosphorylate their target proteins to precisely control the time course of activation and deactivation and resensitisation.

The net effect of this intracellular machinery is that even before the generation of an AP, several levels of signal amplification have already occurred: each odour molecule may activate multiple receptor proteins; each associated G-protein α-subunit may activate multiple adenylase cyclase (AC) molecules; each AC may generate many molecules of cAMP; and each cAMP molecule may gate several cation channels prior to degradation (see Figure 3.3).

Hyperpolarisation and suppression of spontaneous activity may occur through closing of potassium channels (Lischka *et al*., 1999) and/or additional mechanisms. It is not yet known whether odorants can inhibit human ORNs; however, we have found that some cells respond to odorant stimuli with a decrease rather than an increase in intracellular calcium (Rawson *et al*., 1997). This type of response is unusual in that it is not seen in rat ORNs (Restrepo *et al*., 1993; Tareilus *et al*., 1995), but has been reported in catfish (Restrepo & Boyle, 1991). The mechanism for this decrease in calcium is not explained by any model of olfaction yet proposed, and suggests that odour characteristics may be coded somewhat differently in the human olfactory system than in other mammals.

The firing rate may also be modulated to encode information such as intensity or time course in the temporal features of the spike train. The particular profile of ion channels and kinases present in the olfactory neuron determines its sensitivity, adaptation, desensitisation and resensitisation characteristics. Thus, these neurophysiological properties are critical to the understanding of the kinetics of odorant detection. Sequence polymorphisms in any of these proteins could result in dramatic differences in the kinetics of the olfactory experience, but such polymorphisms remain to be explored.

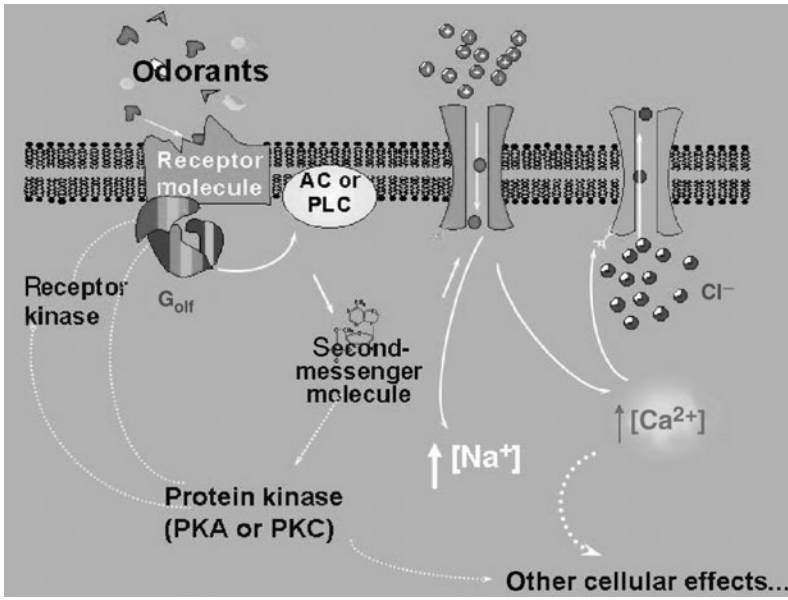


Figure 3.3 Transduction pathways in olfaction. One of the several hundred possible receptor proteins is expressed in a receptor cell, coupled to a signalling cascade involving G-protein activation of second-messenger pathways. Influx of sodium and calcium, coupled with efflux of chloride and activation of voltage-gated ion channels, results in generation of action potentials (APs). The response is rapidly adapting due to phosphorylation at several points in the cascade by various protein kinases (PKs). (Drawing courtesy of G. Gomez.)

3.3.2 How is odorant information encoded?

As described in Chapter 2, when odorants bind to ORs the receptor–ligand complex interacts with GTP-binding regulatory proteins (G proteins) to increase second-messenger levels. Families of genes that apparently encode for G protein–coupled OR proteins have been identified in a broad range of vertebrate and invertebrate species (Krautwurst *et al*., 1998; Reisert & Matthews, 1998; Restrepo & Boyle, 1991; Touhara *et al*., 1999). The similarity of these sequences varies from as low as 40% to as high as 99% both within and between species, with regions of low homology, presumably representing ligand-binding domains, and high homology, particularly within the fourth and sixth intracellular domains (Mombaerts, 1999; see also <http://senselab.med. yale.edu/senselab> for a comprehensive database of ORs). While the majority of these sequences must be considered 'orphan' receptors, with no known ligand, several have now been functionally expressed in heterologous systems (Araneda *et al*., 2000; Kajiya *et al*., 2001; Tareilus *et al*., 1995; Touhara *et al*., 1999). In each of these cases, the receptors appear to be highly selective

in their response profile, and in only one case has evidence been presented that the active ligand is the same in both native and expressed systems (Touhara *et al*., 1999). Sequence polymorphisms have been identified in several of these receptors, but as yet no studies have linked these polymorphisms to individual variations in olfaction. The human OR gene repertoire includes as many as 60% pseudogenes (nonfunctional or non-expressed), many of which have arisen as a result of gene duplication and crossing over (Ben Arie *et al*., 1993; Gilad *et al*., 2000; Glusman *et al*., 2000; Mefford *et al*., 2001; Sharon *et al*., 2000; Trask *et al*., 1998). The profile of pseudogenes and functional genes present in the genome of different populations is currently being investigated to better understand evolution at the genetic level. As these studies progress, it will be fascinating to see how such genetic variations contribute to the individual variations in aroma perception and preference that we all experience!

Currently available data indicate that in vertebrates, individual ORNs express one (Keast & Breslin, 2002) or a few (Rawson *et al*., 2000) OR types per cell. A single OR can recognise different, but structurally related, odorants, and a single odorant can be recognised by different ORs that appear to recognise distinct structural features. The nature of these response patterns may differ across species. For instance, rat ORNs can apparently respond to a variety of odours that exhibit little or no structural or perceptual similarity (Tareilus *et al*., 1995), and tests with a single pure odour may elicit responses from multiple cells. In mice, any single cell might respond to multiple odours, but they are generally rather closely related in structure (Malnic *et al*., 1999; Sato *et al*., 1994). Finally, ORNs from young human subjects are highly selective, and as few as 0.5–1% of neurons tested with a single pure compound will exhibit a physiological response (Rawson *et al*., 1997; unpublished observations). These differences may relate to the particular repertoire of receptor proteins that have remained functional in these different species, but more research is needed to explain these observations.

What is consistent across species is a system in which different odorants are recognised by unique combinations of activated ORs (Malnic *et al*., 1999). This provides the facility for encoding odour quality on the basis of the combined pattern of receptor activation, which, by virtue of the convergence of axons from common OR-expressing receptors onto the same glomeruli (GL) in the bulb, results in a particular pattern of GL activity (see Figure 3.4). Slight changes in odorant structure result in a change in the combination of ORs that recognise the odorant, presumably contributing to the ability to perform fine discriminations in odorant quality.

3.3.3 Odour intensity and quality coding in the olfactory bulb

In addition to the amplification mechanisms employed by the receptor cell, the transmission and detection processes occurring within the olfactory bulb

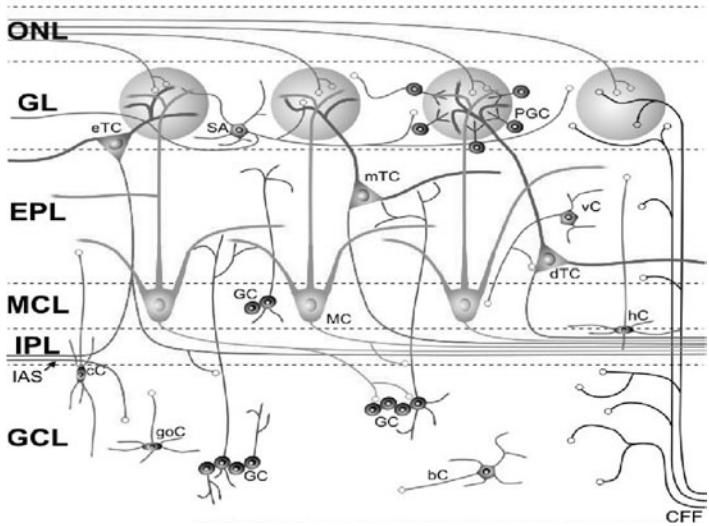


Figure 3.4 The network of input and output relays in the olfactory bulb. Axon terminals enter the olfactory bulb via the olfactory nerve (ONL) to synapse with the dendrites of mitral cells (MCs) within the glomeruli (GL). Modulatory interneurons include external tufted cells (eTC), periglomerular cells (PGC), granule cells and horizontal cells (hC), whose functions are just beginning to be explored. Granule cells are generated throughout life from a population of continually dividing stem cells that migrate from the subventricular zone into the bulb. (Drawing reprinted with permission from Shipley *et al*., 2003.)

are elegantly designed for further amplification and refinement of the incoming odorant detection signal (see Lowe, 2003 for review). Activation of an ORN triggers release of the NT glutamate from the axon terminal within the olfactory bulb (Berkowicz *et al*., 1994). ORN axons are unmyelinated and project in fascicles comprised of bundles of axons encased within nonmyelinating ensheathing cells. Modelling studies suggest that APs can be synchronised within a fascicle. If axons from ORNs expressing different receptor types were present within the fascicle, it would enable presynaptic coordination of the incoming signal as well as signal amplification, although empirical evidence for this is lacking. The glutamate signal is detected via AMPA/kainate receptors residing on the nerve terminals of the olfactory bulb mitral cells (MCs: Aroniadou-Anderjaska *et al*., 1999). These dendrites send excitatory postsynaptic potentials (EPSPs) to the MC soma, and these signals may be modulated by a large complex of lateral interneuron connections. Secondary dendrites may extend horizontally as much as 1 mm from the MC soma, and these are synaptically linked to granule cell dendrites, which contribute to negative feedback and lateral inhibition. Glutamate released from the MC dendrite excites spines on the granule cell processes, which in turn release the inhibitory NT γ-aminobutyric acid (GABA). These interactions may result in

activity-dependent modulation with either facilitation (Lowe, 2002; Margrie *et al*., 2001) or block due to inhibition (Xiong & Chen, 2002). A variety of mechanisms for activity modulation has been described, including phosphorylation of ion channels by kinases (Johnston *et al*., 1999) and altered K^+ channel activity (Christie *et al*., 2001), and additional mechanisms are likely to be discovered with improved technologies for studying bulb circuitry using semi-intact and intact preparations.

Increasing odorant concentration has been reported to raise both spike train frequency and the number of GL activated. Studies of the activity patterns across the olfactory bulb elicited by increasing concentrations of odorant stimuli have provided a picture of odour intensity coding that incorporates both altered axonal output (Duchamp-Viret *et al*., 2000) and, in some cases, activation of additional GL resulting in a new pattern across the bulb (Johnson & Leon, 2000a). For instance, in studies of rat olfactory bulb activation using 2-deoxy-glucose (2-DG) to monitor regions of elevated energy expenditure, rats exposed to pentanoic acid at various concentrations exhibited activity in only one region, and this odour was not perceived qualitatively differently when presented at high or low concentrations. In contrast, the odorant pentanal shows qualitative, concentration-dependent sensory differences: it smells ethereal at low concentrations and quite unpleasant at higher levels. The higher concentrations evoked activity in more GL (Johnson & Leon, 2000a). In the latter case, it has not been established definitively whether the new pattern results from activation of additional receptors by the primary stimulus or from activation of new receptors by contaminants that only reach threshold concentrations at higher stimulus levels. In the case of an aldehyde such as pentanal, rapid degradation to pentanoic acid occurs, so trace levels of this component may reach detection threshold levels as the concentration of the stimulus increases. Due to the chemical instability of many volatile odorants, the extreme difficulty in purification and the great sensitivity of the system, it is likely that many instances of such 'concentration-dependent quality changes' are due to this latter mechanism. It is likely that within the normal range of odorant concentrations, intensity perception is based initially on a higher firing rate frequency in the most sensitive receptors, and at concentrations where those receptors are adapted, on activation of more individual receptor neurons that may express a receptor type with a higher threshold for that particular odorant (Buonviso & Chaput, 1990).

Studies of odorant quality coding in the olfactory bulb have employed a variety of techniques, including detection of 2-DG uptake following exposure of awake, behaving rats to various stimuli (Johnson & Leon, 2000a, 2000b; Johnson *et al*., 1999; Linster *et al*., 2001); imaging using calcium-sensitive dyes in live, surgically prepared subjects (Wachowiak & Cohen, 2001); or imaging of intrinsic signals (Belluscio & Katz, 2001; Luo & Katz, 2001; Meister & Bonhoeffer, 2001; Rubin & Katz, 1999) reflecting neuronal activity

in anaesthetised rats. Recently, magnesium-enhanced functional magnetic resonance imaging (fMRI) has even been successfully used to study odour-induced olfactory bulb activity in the rat (Pautler & Koretsky, 2002). These studies have enabled us to begin to understand the mysteries underlying the encoding of odorant quality, and reveal what some have termed 'modules' (Johnson & Leon, 2000a) of odorant-elicited activity that are consistent across animals and reflect particular structural features of the stimulus. For instance, odorants of a common chemical class such as carboxylic acids of increasing chain length reveal certain modules sensitive to chain length and others to side groups such as a ketone (Johnson & Leon, 2000a, 2000b; Johnson *et al*., 1999). From a synthesis of data from several laboratories and approaches, it is possible to predict the major regions of bulb activity based on the presence of certain chemical features, as well as perceptual similarity based on the bulb activity patterns. This work exploits the existence of stereoisomers that can or cannot be discriminated perceptually. For instance, the stereoisomers of carvone, which can be discriminated, resulted in markedly different GL activity patterns (Belluscio & Katz, 2001; Luo & Katz, 2001; Meister & Bonhoeffer, 2001; Rubin & Katz, 1999). In contrast, stereoisomers of limonene, which were not discriminated by rats in habituation tasks, resulted in activity modules that were not significantly different (Linster *et al*., 2001). Intriguingly, if the 2-DG images were analysed at a finer level, limonene activity patterns would be distinguished, and with reinforcement, rats could be trained to discriminate these compounds (Linster *et al*., 2001). Thus, salience, or relevance to a behavioural outcome, and learning must play a part in determining what level of analysis is to be used by higher cortical centres to discriminate odorant qualities, and one may imagine that relevance or training can result in attending to finer neural activity differences than may be used in the absence of such experience.

In the 'real' world, where single, pure odorants are rarely encountered, the extensive mechanisms for lateral inhibition and dendrodendritic modulation enable a precise activity pattern to be created for a given mixture. Studies of the bulb activity patterns elicited by complex odorants in the mouse olfactory bulb suggest that a simple relationship between the number of odours presented and the number of GL activated does not exist (Schaefer *et al*., 2001, 2002). Thus, a complex odour (like mouse urine) evokes a unique pattern of bulb activity, and does not activate as many GL as might be predicted on the basis of the number of known odorous compounds in mouse urine. Similar studies with flavour compounds are needed, but careful psychophysical studies have revealed that humans are actually quite poor at dissecting the components of odorant mixtures (Jinks & Laing, 1999; Laing *et al*., 2002). This suggests that flavour aromas are, indeed, entities distinct from their components, and that the conversion from signals reflecting activation of individual receptor cells by particular volatile chemicals into

a neural code representing a flavour aroma such as 'coffee' occurs at the level of the olfactory bulb. The MCs relay their activity patterns to higher-order brain regions, including the entorhinal cortex, amygdala and hippocampus, and it is in these areas that a comparison is made of the input signal to stored patterns for identification and recognition of the pattern as a particular quality occurs (see Chapter 7 for further information).

3.4 Flavour perception can be modulated: adaptation, sensitisation and crosstalk

The perceived intensity of an odour or taste decreases upon prolonged exposure. Behaviourally, short-term adaptation (or desensitisation) occurs within seconds to minutes of stimulus exposure, and resensitisation can take several minutes. This short-term phenomenon is likely to relate to both receptor cell processes and perireceptor processes, such as the time required to clear the stimulus from the mucus or saliva. Adaptation processes also occur in the CNS, but are less well understood. It is generally thought that receptor cell adaptation is a short-term phenomenon, while adaptation centrally may last hours, days or weeks. Behaviourally, continuous exposure to an odorant for two weeks at home can increase the detection thresholds for that odorant in a test setting (i.e. reduce sensitivity) even after removal of the odorant from the home (Dalton, 2000; Dalton & Wysocki, 1996). This finding suggests the occurrence of a long-lasting physiological change that affects sensitivity.

Adaptation enables a neural system to adjust its sensitivity and dynamic range as the background level of stimulation changes. In sensory systems, adaptation reduces sensitivity, increasing the threshold and the dynamic range of the behavioural stimulus–response function. This capacity is important to allow the system to respond to changes in stimulus intensity in the presence of differing levels of background stimulation. Adaptation properties of sensory receptor cells also determine the magnitude of the response to the different frequency components present in a stimulus that varies over time, such as the odour plume sensed by an animal as it moves towards the source of the stimulus (Vickers, 2000), or the movement of ingested food through the oral cavity.

The molecular mechanisms that underlie sensory adaptation are not completely understood, but they are likely to take place both in the periphery and in the CNS. In the periphery, there are several processes that contribute to olfactory adaptation. Short-term adaptation appears to be mediated by calcium entry through cyclic nucleotide-gated (CNG) channels (Kurahashi & Menini, 1997; Kurahashi & Shibuya, 1990; Leinders-Zufall *et al.*, 1999). The precise mechanism for Ca^{2+}-mediated adaptation is not known, but it appears that

calmodulin, perhaps via calmodulin-dependent kinase, regulates the activity of the CNG channels (Balasubramanian *et al*., 1995; Granchrow, 1983). A variety of other kinases has also been implicated in OR adaptation, including PKA and PKC (Gomez *et al*., 2000), and these may phosphorylate other components of the detection and transduction cascade. In contrast, long-lasting adaptation appears to be dependent on the production of carbon monoxide (CO) by a pathway involving cGMP (Zufall & Leinders-Zufall, 1997). Thus, it is clear that, even at the level of the OR cell, adaptation is a complex process involving multiple pathways (see Zufall & Leinders-Zufall, 2000 for review).

Even less is known about the cellular adaptation processes occurring in taste. The difficulty in performing single-cell recording from mammalian taste receptors has slowed progress in this area. Evidence has been presented for self-inhibition by sodium of amiloride-sensitive sodium channels (Gilbertson & Zhang, 1998), which may suppress sensitivity in the presence of elevated salt concentrations. Other cellular mechanisms are indicated in adaptation of sweet-sensitive receptor cells, but have not been characterised at a molecular level (Tonosaki & Funakoshi, 1989).

Cross-adaptation can also occur, in which sensitivity to one stimulus is reduced by exposure to a different stimulus (Froloff *et al*., 1998; Keast & Breslin, 2002; Pierce, Jr *et al*., 1995, 1996; Tonosaki, 1992; Tonosaki & Funakoshi, 1989). This phenomenon can occur between chemicals that differ perceptually or structurally, and little is known about the physiological basis, although structurally similar chemicals are presumably detected via the same or overlapping sets of receptor(s). In the taste system, such cross-adaptation is facilitated by the multimodal characteristics of the TRCs. In the olfactory system, crosstalk between intracellular signalling systems may increase the ability of the system to discriminate individual, structurally similar odours. In the lobster olfactory system, two second-messenger pathways within a single cell allow neurons to sharpen odour quality discrimination (Ache & Zhainazarov, 1995), although the mechanism that is employed by this system is quite different than in mammals. This larger range of response variety allows for the coding of a wider range of odours by peripheral ORNs. Although odour responses in the rat olfactory system seem to consist of one type of response (increases in intracellular calcium), the effect of dual pathway activation may be similar. Two pathways that crosstalk may modulate each other and improve contrast enhancement (Ache & Zhainazarov, 1995; Gomez, 2000). For instance, in rodents, activation of phosphatidylinositol 3-kinase by certain odours reduces the sensitivity of the cyclic nucleotide pathway within the same cell (Spehr *et al*., 2002). The presence of this pathway in human ORNs remains to be established. It is also likely that cross-adaptation can result from lateral inhibition or crosstalk within the bulb circuitry. A practical implication of these interactions is the potential for identification of compounds that may

exhibit different sensory qualities, but are capable of suppressing perception of unpleasant or bothersome odours, such as breath or body odour, in a way analagous to the suppression of tinnitus by sound masking (Kemp & George, 1992). However, more studies are needed to characterise the properties of cross-adapting odours to be able to predict what types of chemicals might be effective in this capacity.

While the classic neural phenomenon of sensitisation is rare in odour perception, the ability to detect androstenone can be induced by repeated androstenone exposure in subjects that are initially anosmic to it (Wysocki *et al*., 1989). This effect may be due to reorganisation at the peripheral level, since the electrical response recorded across the olfactory epithelium (electro-olfactogram) is induced in mice treated similarly (Wang *et al*., 1993). Whether repeated stimulation induces changes at the level of receptor expression or affects other components of the detection/transduction pathway remains unknown. Apparent sensitisation may occur in response to training or integration of inputs from multiple sensory modalities (see Chapter 9). These effects are likely to be mediated by network reorganisation or reinforcement within the CNS.

3.5 Flavour perception and ageing

One-third of elderly people report dissatisfaction with their senses of taste or smell, and the actual incidence is probably even higher (Pelchat, 2001). Sensory loss with ageing affects both quality of life and personal safety. The extent, nature and causes for this loss have been the subject of much research, yet we remain largely ignorant of the causes and are entirely unable to either prevent or treat the impairment.

3.5.1 Anatomy and physiology

Several features of these systems make them particularly susceptible to age and disease-associated changes that may lead to functional deficits: exposure to an often harsh external environment; replacement of receptor cells throughout life; dependence on the ability of stimuli to dissolve in, and penetrate through, watery protective layers; dependence on multiple receptor types, all of which contribute to an activity pattern that determines stimulus quality; and dependence on finely balanced ionic exchange between intracellular and extracellular compartments. Sensory loss may be due to changes in the anatomy of the structure (e.g. loss of taste buds or OR cells), changes in the distribution or density of specific receptor proteins, ion channels or signalling molecules (e.g. increases in calcium channels or decreases in second-messenger generating enzymes), or changes in the environment surrounding the receptor cell

(e.g. reduced salivation or altered nasal mucus composition: Doty & Snow, 1988; Doty *et al*., 1991). The effects of denture use that provides a physical barrier to some of the membranes where the receptors reside, along with reduced salivation and compounds released from dental adhesive, certainly account for at least some age-related changes in taste perception. In other cases, the cause may be a consequence of chronic diseases such as diabetes, cancer, kidney disease (Deems *et al*., 1991; Doty *et al*., 1991) or the effects of therapeutic interventions such as medications, radiation or surgery (Cowart *et al*., 1997; Deems *et al*., 1991; Shin *et al*., 1999; Yamagishi *et al*., 1994). In these situations, successful treatment of the disease will often improve chemosensory function, but the time course for recovery can vary considerably.

3.5.2 Taste

Sensitivity and the ability to discriminate taste intensities diminishes with age, but the degree to which this loss is general or quality-specific is unclear. While there is some evidence for differences among tastes with respect to threshold sensitivity, most studies suggest a relatively nonspecific decline in suprathreshold intensity ratings with increasing age (Mojet *et al*., 2003). The context in which the tastant is presented is significant, and studies employing food matrices typically produce different results than those using pure stimuli dissolved in water. Most studies report that salt sensitivity declines slightly with age in humans, and higher concentrations are perceived as more pleasant than they are for younger subjects. For instance, elderly subjects required twice as much salt as younger subjects did in order to detect saltiness in a tomato soup base (Stevens *et al*., 1984). Sour stimuli dissolved in water were rated less intense and were more poorly discriminated by elderly subjects (Nordin *et al*., 2003), but studies using food matrices indicated little age-related change in sour perception (Mojet *et al*., 2003; Stevens & Lawless, 1981). Bitter perception is more sensitive to ageing, but all bitter compounds are not affected equally. For instance, sensitivity to quinine diminishes significantly with age, while sensitivity to the bitter compound urea is not affected (Cowart *et al*., 1994). Again, however, the use of a food matrix minimises the effect of age (Mojet *et al*., 2003). Perception of sweet taste appears least affected by ageing, although certain compounds may appear qualitatively different due to bitter, salty or sour components (e.g. the bitter aftertaste of aspartame may be less noticeable). Only two studies have assessed umami perception as a function of age, and both reported reduced intensity ratings of suprathreshold concentrations (Mojet *et al*., 2003; Schiffman *et al*., 1991), but this difference was not present when the stimuli were tested in a food matrix (Mojet *et al*., 2003). As our understanding of the complex intracellular events occurring in response to taste activation grows,

we can better predict how to tailor flavour ingredients to satisfy the palate of the evergrowing elderly population and to predict the impact of these alterations on other flavour constituents.

3.5.3 Olfaction

The ability to detect, discriminate and identify odours is also sensitive to age-related impairment. Age-related losses in olfaction result from changes at both the anatomical and the molecular level. Anatomical studies in rodents and humans demonstrate non-uniform changes in the density of receptor neurons, immature neurons and proliferating basal cells. As the extent of sensory epithelium declines, olfaction may be altered quantitatively and qualitatively due to selective loss of subsets of receptor types that may be localised zonally (although receptor distribution has not been established in humans). Losses in olfactory sensitivity are likely to be due to changes within the CNS as well as in the periphery. Reduced ability to identify and discriminate odours may be due to changes at the level of the receptor cell. Evidence from our laboratory suggests that with age, more OR cells are broadly tuned, responding to a larger number of different odorants than in younger subjects (Rawson *et al*., 1998). This could account for the poorer discrimination and more rapid adaptation reported in sensory studies (Stevens *et al*., 1989). The decrease in olfactory sensitivity seen with ageing in humans begins sooner in males than in females, and it is possible that oestrogen plays a protective role (Dhong *et al*., 1999), as the rate of decline in post-menopausal females is similar to that of males about ten years earlier (Deems & Doty, 1987; Dhong *et al*., 1999; Doty *et al*., 1984). While some degree of age-related loss in olfactory sensitivity in general has been documented extensively, using both psychophysical and physiological (e.g. fMRI: Suzuki *et al*., 2001) methods, there is considerable overlap between younger and older subjects, and it is possible to retain olfactory function into very old age (Elsner, 2001). There is also evidence that age-associated olfactory loss is not uniform across odours (Ennis & Hornung, 1988; Wysocki & Pelchat, 1993). For instance, in different studies, detection of androstenone (Wysocki & Gilbert, 1989; Wysocki & Pelchat, 1993), butanol (Kimbrell & Furchtgott, 1960) and ethyl vanillin (Pelchat, 2001) was more sensitive to ageing across the lifespan, while the ability to detect phenylethyl alcohol (Pelchat, 2001; Wysocki & Pelchat, 1993), isoamylacetate (Kimbrell & Furchtgott, 1960) and eugenol (Pelchat, 2001) was more robust across age. Insufficient data are available to enable us to determine whether sensitivity by the elderly to certain classes of odours is more likely to be retained than others. Given our current understanding of olfactory cell biology, it seems reasonable to imagine that detection of odorants that are able to activate more types of receptors is more likely to be retained in the face of epithelial injury or loss. The result, however, is that perception of complex odours such

as a food flavour will not only be less intense, but will also be qualitatively different.

3.6 Conclusion

A defining feature of psychophysical data on the chemical senses has always been an unusual degree of variability both within and across individuals. Our current understanding of the neurobiological basis for these sensory systems renders this variability unsurprising. The extensive modulation, plasticity, variable sensitivity to age, health and environmental effects, and sequence polymorphisms in both taste and ORs, leave one to conclude that the 'flavour world' of any given individual at any given moment is likely to be quite unique.

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4 Structural recognition between odorants, olfactory-binding proteins and olfactory receptors – first events in odour coding

Jean-Claude Pernollet and Loïc Briand

4.1 Introduction

All living organisms, including human beings, are able to discriminate between myriad odours, which are airborne compounds. The olfactory repertoire is remarkably diverse, composed of aromatic and aliphatic molecules with varied functional groups and diverse carbon backbones. Functional groups include aldehydes, esters, alcohols, carboxylic acids, ketones, alkenes, amines, imines, thiols, halides, nitriles, sulfides and ethers. Any airborne molecule whose molar mass is sufficiently low and hydrophobicity sufficiently high to allow its volatility in usual conditions is a potential odorant. Consequently, the olfactory system must be able to detect and identify novel odorants whose detection was not planned, in a way analogous to the immune system. Such a property is clearly evidenced by the *de novo* synthesis of artificial perfume compounds, such as methyl dihydro jasmonate.

Many attempts have been made to decipher how odour perception is evoked in the brain in response to odorants, since the sensitivity and quality range of olfactory systems are exceptional. Attempts at correlating the chemical structure of odorants with their odours are indeed as old as synthetic chemistry (Moncrieff, 1949). Until recent years, i.e. until discovery of olfactory receptors (ORs) by Buck and Axel (1991), odorant chemists were compelled to draw 'abiotic' theories in order to establish relationships between the chemical structure and the smell of odorants. For instance, a vibrational theory, based on the vibrational spectrum of the odorant molecule, was developed (Dyson, 1938; Turin, 1996; Wright, 1977). The structure–activity relationships of odorants have largely been investigated (see Chastrette, 1997 and Rossiter, 1996 for reviews). Recently, these have been explored through electronic-topological investigation for some specific odours such as ambergris (Gorbachov & Rossiter, 1999). Implicitly, the more realistic among these theories assumed that the chemical recognition was performed by receptors finely tuned towards chemical functions, and by the relative position of these functions in the odorant molecule. Quantitative structure–activity relationships were also investigated, but these ambitious attempts did not achieve success (Chastrette, 1997). Nevertheless, in the absence of any knowledge of the

mechanisms involved in the biochemical capture of odorants by neurons in the olfactory epithelium, and in ignorance of the subsequent neuronal treatment of the information, these endeavours proved to be of only very limited success (Turin, 2002). They even resulted in pseudobiological theories, based on the comparison with the visual system, in which an odour map is obtained from the combination of at least three fundamental odours; such a theory is indeed so far from reality that it could not be successful. The main explanation for the failure of these attempts lies in the complexity of the biological mechanisms involved in the sense of smell, even at the first molecular steps of odorant capture, i.e. at the pericellular level. Consequently, modelling of odorant detection needs to take into account the molecular mechanisms of odorant uptake by olfactory neurons and the knowledge of the neurological processes in the brain.

This review is focused on the interaction of odorants with proteins involved in olfactory pericellular events with the aim of explaining the stereochemical mechanisms by which odorants are more or less specifically trapped by the olfactory sensory neurons. These mechanisms rest on structural bases that are the first steps of odour coding in the brain and involve a combinatorial system by which odotopic maps are created. Although this chapter will focus on the molecular bases of odorant capture in humans, it will also refer to animal olfaction, which is better known in some aspects, even with some references to insects, since among higher eukaryotes, there is a striking evolutionary convergence towards a conserved organisation of signalling pathways in olfactory systems. The molecular elements involved in odorant uptake are summarised in Figure 4.1. Two kinds of proteins are involved in odorant capture. First, the olfactory-binding proteins (OBPs), also called odorant-binding proteins, are soluble proteins, whose plausible function is to solubilise hydrophobic odorants and carry (and/or protect) them through the aqueous mucus of the olfactory epithelium towards the ORs. As they are soluble, they can be studied by classical physicochemical approaches (spectroscopy, X-ray diffraction, nuclear magnetic resonance (NMR), etc.). Second, there are the insoluble ORs, embedded in the plasma membrane of the olfactory neurons, which activate neuron-signalling pathways upon odorant binding. As they are highly hydrophobic, they require specialist investigation techniques such as functional expression in mammalian cells after gene transfection. Comparison of affinity constants, determined either by biophysical approaches in the case of OBPs or by cellular activation measurements for ORs, provides structural information about the molecular interactions between odorants and olfactory proteins. The knowledge of their 3D structure (either experimentally determined or resulting from computer modelling and molecular docking) gives elements to understand the molecular interactions at the atomic level. These structural data identify functional amino acids within the molecule and allow the design of artificial mutations to generate modified

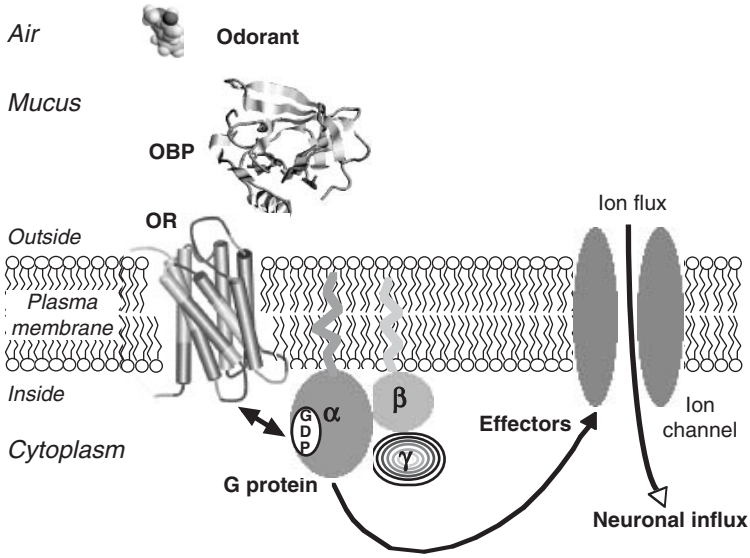

Figure 4.1 Molecular partners of odorant capture. OBP = soluble olfactory-binding protein; OR = olfactory receptor crossing the neuron plasma membrane; heterotrimeric G protein = internal membrane protein, whose α-subunit dissociates upon binding with the internal domain of the OR activated by odorant uptake; the α-subunit activates effectors, which open ion channels, inducing membrane depolarisation, thus resulting in neuronal influx.

proteins through site-directed mutagenesis so that the nature of the odorant-binding sites can be further studied.

A brief survey of signal transduction in the brain emphasises the key role of pericellular events. As a matter of fact, odour coding involves cellular and neuronal mechanisms that are activated as a result of odorant capture. As these topics are thoroughly described in other chapters, they will be briefly considered in this chapter with the aim of understanding how the odour coding is transformed from stereochemical recognition outside olfactory neurons to a topographic map in the brain. The signalling machinery of olfactory neurons, which converts the biochemical signals into nerve impulse through opening of ion channels, will not be detailed. However, the organisation of the olfactory bulb, which transforms the stereochemical detection into an odotopic map and which is maintained in spite of a permanent renewal of the olfactory neuron, is a key feature to be mentioned, since the projection of this globular odotopic map into the higher structures of the brain allows the transfer and the combination of the molecular information detected by the olfactory neurons. It is now agreed that one olfactory neuron only expresses one OR gene (Serizawa *et al*., 2000; Zhao & Firestein, 1999), and that all neurons that express the same OR gene project to the same glomerulus in the olfactory bulb, thus creating an odotopic map. In humans, millions of olfactory neurons project into thousands

of olfactory glomeruli in each olfactory bulb. Their axons form synapses with mitral cells, which in turn form synapses with clusters of neurons at specific sites in multiple olfactory cortical areas, creating another stereotyped sensory map in the olfactory cortex (Zou *et al.*, 2001). So the organisation of the olfactory bulb allows the transformation of the odorant stereochemical detection by ORs into an odotopic map, which is projected in the higher structures of the brain. As described in this review, odour coding results from the combinatorial activation of a set of ORs binding different sites of an odorant. A combinatorial system recognises several thousands of odours based on the binding of a single odorant by several different ORs and, reciprocally, on activation of a given OR by different odorants.

4.2 Anatomy of the olfactory system

In order to understand how odorants reach olfactory neurons, it is necessary to recall some anatomical data concerning the nasal cavity, especially in humans (Farbman, 1992). In vertebrates, the olfactory neurons are the primary sensing cells, which form a neuroepithelium that lines a series of protrusions, called turbinates, in the lateral walls of the nasal cavity in mammals. Turbinates are complex series of folds made of the bony lateral extensions. In macrosmatic animals, such as rodents or dogs, they are more intricate than in many primates, such as humans. In the latter case, the superior and middle turbinates are simple folds, originating from the ethmoid bone, and only the medial part of the superior turbinate exhibits olfactory epithelium, in contrast to macrosmatics whose entire ethmoid turbinates are covered with olfactory epithelium. In humans, most of the olfactory epithelium is found in the olfactory cleft, which is linked to the olfactory bulb of the brain by the cribriform plate (Figure 4.2). In all vertebrates, olfactory neurons are bipolar with a single dendrite going to the central nervous system and a dendrite that terminates on a body surface (i.e. the olfactory epithelium). A thin axon projects from the proximal pole of the cell directly to higher brain regions. From the apical end of the dendritic terminal, narrow appendages extend towards the outside world, forming knoblike swelling from which project some 20–30 very fine cilia that contain ORs and transduction machinery. These cilia lie in the thin layer of mucus covering the tissue, which constitutes protection against drying in terrestrial vertebrates and also prevents this external part of the brain from microbial infection. Secretion of this aqueous mucus originates from supporting cells within the epithelium and from glands associated with the olfactory mucosa, called Bowman's glands.

Human olfactory epithelium, containing sensory neurons, is situated on the cribriform plate and extends for a short distance on the septum, the superior turbinate and, possibly, the upper part of the middle turbinate (Morison

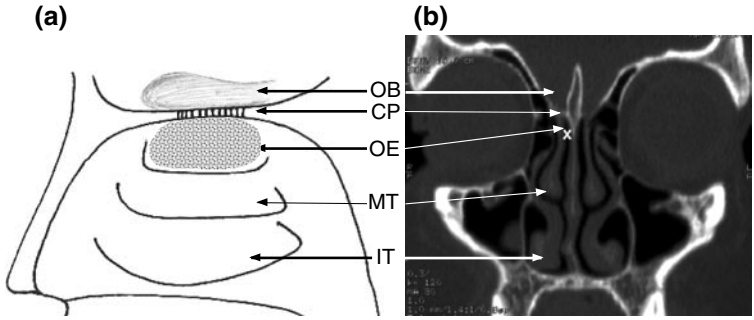

Figure 4.2 The human nasal cavity. (a) Schematic sagittal drawing (adapted from Morison & Moran, 1995). (b) Coronal scan. OB = olfactory bulb; CP = cribriform plate; OE = approximate location of the sensory olfactory epithelium shown as greyed area in (a) and a white cross in (b); MT = middle turbinate; IT = inferior turbinate.

& Moran, 1995). In humans, in each nasal cavity, some six million neurons project through the cribriform plate towards about 8000 glomeruli in each olfactory bulb. Glomeruli are linked to only six mitral cells. In rodents, there are 50 million neurons projecting towards about 2500 glomeruli, each linked to 20 mitral cells. The small area of the olfactory cleft, which can be estimated to be less than 10% of the total area of the nasal cavity in humans, and its inaccessible location (Morison & Moran, 1995) make the sampling of the olfactory epithelium mucus difficult. Access to the neuroepithelium tissue itself is also problematical due to its location next to the brain and the hardness of the facial bones. However, insects use a similar organisation characterised by polarised neurons, which are specialised at one end for chemical detection and at the other for signalling. Specialised structures, called sensilla, maintain olfactory neurons bathed in aqueous lymph. Although involving quite different anatomical structures since insects smell with antennae, comparison of insect and vertebrate olfaction proves to be relevant at the pericellular level because odorant capture undergoes similar biochemical events before neuronal integration. Insects may therefore be used as models, whose behaviour can be experimentally altered through genetic engineering (gene disruption and insertion).

4.3 Olfactory-binding proteins

4.3.1 OBP discovery

In the early 1980s, when searching for ORs using a ligand-binding approach, Pelosi *et al*. (1982) discovered a soluble protein, which was indeed able to bind odorants efficiently. These proteins, first named pyrazine-binding proteins (because they were found with pyrazine derivatives as substrates), could not

be receptors because they were soluble molecules secreted in the mucus. As they were abundant in this secretion (concentration in the millimolar range), putative olfactory roles were suggested such as odorant scavenger, deactivator or transporter. Indeed, airborne odorants are commonly hydrophobic molecules, which have to be rapidly conveyed through the aqueous nasal mucus in order to reach their receptors embedded in the membrane of the olfactory neurons. Pyrazine-binding proteins were then renamed odorant-binding proteins, or alternatively olfactory-binding proteins (OBPs), since they were excellent candidates for playing such a carrier role in the perireceptor events of olfactory processes (Pelosi, 1996, 2001). OBPs were observed to be specifically secreted by Bowman's glands in the olfactory epithelium (Morison & Moran, 1995; Pevsner *et al*., 1988a). These transfer proteins were discovered in both vertebrates and insects and tentatively assigned a common function, in spite of their very different 3D structures. We will mainly focus on vertebrate OBPs, with some comparison with insect OBPs where it is helpful in understanding the functional properties of these olfactory proteins.

4.3.2 General properties of vertebrate OBPs

OBPs have been found in all animal species studied, including cow, pig, rabbit, mouse, rat and, recently, elephant (Briand *et al*., 2000a; Dal Monte *et al*., 1991; Lazar *et al*., 2002; Pes & Pelosi, 1995; Pes *et al*., 1992, 1998; Pevsner *et al*., 1985, 1988a) since the discovery of the first vertebrate OBP isolated from the bovine nasal mucus (Bignetti *et al*., 1985; Pelosi *et al*., 1982). In vertebrates, OBPs are abundant, low–molecular weight (around 20 kDa) highly soluble proteins belonging to the lipocalin superfamily. They are exclusively secreted by the olfactory epithelium in the nasal mucus (Steinbrecht, 1998). Members of the lipocalin superfamily display low sequence similarity, but share a conserved folding pattern made of an eight-stranded antiparallel β-barrel (roughly 10–120 residues) linked by a turn to a short α-helical domain (about 125–140 residues) followed by a short ninth β-strand stuck on the β-barrel (145–150 residues) and ending with a C-terminal tail (Flower, 2000). The β-barrel defines a central apolar cavity, called the calyx, whose role is to bind and transport hydrophobic molecules such as odorants, as observed through X-ray crystallography (Bianchet *et al*., 1996; Spinelli *et al*., 1998; Tegoni *et al*., 1996). Although some rare OBPs exhibit a neutral or slightly basic isoelectric point, the typical nature of OBPs is acidic. These proteins reversibly bind odorants with dissociation constants in the micromolar range (Pelosi, 1994).

4.3.2.1 Structural properties of OBPs

As regards structural properties, up to now, all known vertebrate OBPs belong to the lipocalin superfamily (Tegoni *et al*., 2000). The many OBP sequences

available in the databases, including the putative human OBP (hOBP) genes, have been analysed (Lacazette *et al*., 2000). Lipocalins have low sequence identity, but few characteristic signatures allow their identification: a GxW motif at the N-terminus (position 12 in hOBP-2A), two cysteines in the middle and at the C-terminal end (positions 59 and 151 in hOBP-2A) and a glycine (position 115 in hOBP-2A). A tryptophanyl residue (around position 15) is on the first β-strand and at one end of the β-barrel. One of the conserved cysteine residues, located on the fourth strand of the first β-sheet, forms a disulfide bridge tightening the α-helical C-terminal domain and the β-barrel. Another motif (YxxxYxG), which constitutes the first β-strand of the second β-sheet, is generally conserved in OBPs. Additional details can be found in the review by Tegoni *et al*. (2000).

When comparing OBP sequences (Figure 4.3), one can observe that some other lipocalins exhibit significant similarity with classical OBPs (more than 10% identity): these are Von Ebner gland proteins from saliva (VEGPs) and pheromone-binding proteins (PBPs: boar salivary protein, major urinary proteins, aphrodisin and α_2-microglobulin). Tegoni *et al*. (2000) noticed that distinct subclasses clearly appear for OBPs, VEGPs and pheromone transporters. The percentage of identity among OBPs is low (21–26% on average) with the bovine and porcine OBPs showing a maximal identity (42%), whilst rat OBP-2 exhibits the lowest identity (12–19%) when compared to all other OBPs. Rat OBP-2 is closer to VEGPs (29–39% identity) than to other OBPs, while aphrodisin, which is a pheromonal protein secreted in hamster vaginal secretions, resembles the OBPs, as it is very similar to rat OBP-1F (40% identity) (Briand *et al*., 2000b). Consequently, tissue expression (i.e. in the olfactory epithelium) has to be systematically taken into account in order to classify OBPs.

Porcine OBP can be considered as a typical OBP in contrast to bovine OBP, which exhibits interesting peculiar features discussed later. Porcine OBP is a monomer whose 3D structure is typical of a lipocalin. Two cysteine residues form a disulfide bridge between the C-terminus and the loop joining strands 3 and 4 of the β-barrel (Spinelli *et al*., 1998; Figures 4.3 and 4.4), and a single cavity is observed inside the β-barrel, which does not communicate directly with the external solvent. A few amino acid side chains, which block the access to the solvent, would therefore have to move to make the binding of odorants possible. The cavity is mainly covered with hydrophobic and aromatic side chains.

As regards the quaternary structure of OBPs, some were observed as monomers, such as porcine (Spinelli *et al*., 1998) or rat OBP-3 (Löbel *et al*., 2001), while some others were found as dimers, such as bovine OBP, rat OBP-1F and OBP-2 (Briand *et al*., 2000a; Löbel *et al*., 1998). Nevertheless, in some cases, such as porcine OBP-1, the oligomeric state remains questionable because controversial results provided by different methods have been

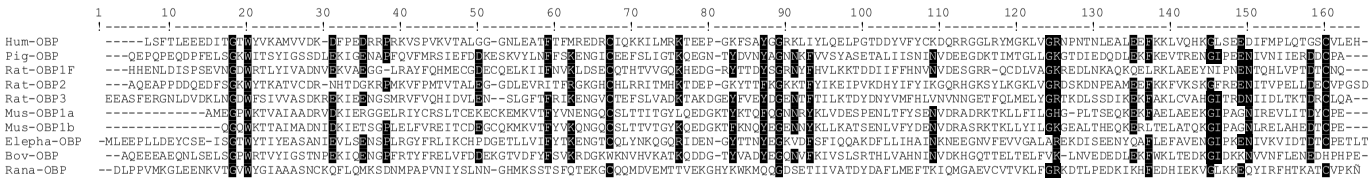

Figure 4.3 Sequence alignment of vertebrate OBPs: hum-OBP, human OBP-2A (Swiss-Prot accession number Q9NY56); pig-OBP, porcine OBP (Swiss-Prot accession number P81245); rat-OBP1F, rat OBP-1F (TrEMBL accession number Q9QYU9); rat-OBP2, rat OBP-2 (TrEMBL accession number Q63613); rat-OBP3, rat OBP-3 (Gene Bank accession number X14552); mus-OBP1a, mouse OBP-1a (TrEMBL accession number P97336); mus-OBP1b, mouse OBP-1b (TrEMBL accession number P97337); elepha-OBP, elephant OBP (TrEMBL accession number Q8HZN2); bov-OBP, bovine OBP (Swiss-Prot accession number P07435); rana-OBP, *Rana pipiens* OBP (Swiss-Prot accession number P06910). Alignment performed with CLUSTAL W. Black background letters indicate the most conserved residues, the line beneath sequences shows the conserved disulfide bridge.

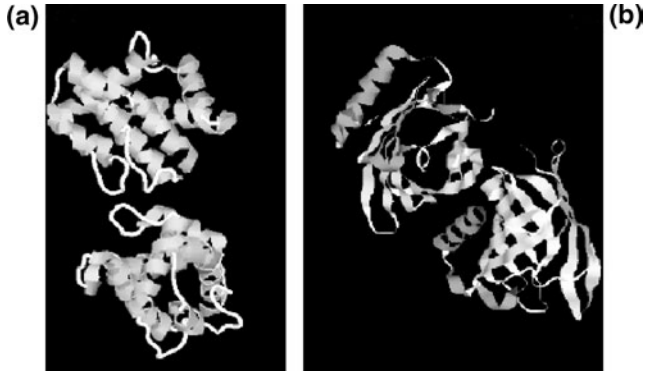

Figure 4.4 Structural comparison of vertebrate (lipocalins) and insect OBPs. Protein backbones are presented with α-helices and β-strands in ribbons and aperiodic structure as a simple line. (a) *Bombyx mori* (silkworm) PBP dimer (computed with the Protein Data Bank accession number 1GMO) made of seven α-helices and no β-strand. (b) Porcine OBP-1 dimer (computed with the Protein Data Bank accession number 1A3Y), forming a typical lipocalin eight-stranded β-barrel, flanked by a single α-helix. (Structures were drawn using RasMac_PPC32_BIT software.)

reported (Burova *et al*., 1999; Spinelli *et al*., 1998). Existence of dimeric structures may create a novel binding cavity at the interface of oligomers.

Like all lipocalins, OBPs exhibit a common disulfide bond pattern, revealing one or two bridges. One of them, immobilising the C-terminal structure formed by an α-helix and an antiparallel β-strand, appears to be more important for the global structure of the molecule than the other disulfide bond when present (Flower *et al*., 2000). Although containing four cysteines, porcine OBP-3 has been reported to exhibit only this single disulfide bond (Scaloni *et al*., 2001). This disulfide bond was observed in hOBP-2A (C59 and C151), which exhibited a free cysteine (C99) (Briand *et al*., 2002a). In the mouse major urinary protein, this bridge (C68–C161) was also observed, whereas C142 remained unbound (Böcskei *et al*., 1992). Although not yet determined, the occurrence of only three cysteines in the rat OBP-2 suggests a similar arrangement. The first disulfide bridge is conserved in all lipocalin sequences identified so far, from bacteria to mammals (Flower, 1996), with the exception of bovine OBP, which diverges significantly from the sequences so far aligned. The absence of the second disulfide bond, which stabilises the C-terminal part of the 3D structure, may favour the occurrence of domain swapping, which was observed in the bovine OBP dimer.

Bovine OBP, which forms a dimer with an elongated shape, was the first OBP whose structure was deciphered through X-ray crystallography (Bianchet *et al*., 1996; Tegoni *et al*., 1996) and was therefore considered as the prototype of OBP, in spite of the absence of the second disulfide bridge. However, the

molecule is not a classical lipocalin, since it exhibited a structural feature called domain swapping (Bennet *et al*., 1995). The β-barrel of each monomer comprises its own strands 1–8, but the ninth strand originates from the other monomer. By this mechanism, the C-terminal part of one of the homodimers rotates and takes the place of the other. In addition to the buried cavity in the middle of the β-barrel, as in monomeric OBPs, a central pocket, composed of residues belonging to the β-barrel domains and to the C-terminal ends, is located at the dimer interface in communication with the solvent.

By comparison of bovine and porcine OBP sequences, Ramoni *et al*. (2002) observed a glycine (position 121) in the hinge linking the β-barrel to the α-helix in the porcine OBP, a residue that is different in bovine OBP. Structural comparison of bovine and porcine OBPs clearly shows that it is located exactly at the position where domain swapping occurs. Ramoni *et al*. (2002) then produced a mutant of bovine OBP in which a glycine residue was inserted after position 121 and, reciprocally, a mutant of porcine OBP in which glycine 121 was deleted. The latter mutation did not result in dimerisation, while mutated bovine OBP became monomeric, suggesting that domain swapping was reversed. They concluded that domain swapping occurs in bovine OBP because the link is shorter and less flexible than in porcine OBP, and that the absence of the disulfide bridge usually conserved in lipocalins may prevent any nonswapped form from being stable.

4.3.2.2 Comparison with insect OBPs

In insects, OBPs have to be classified into three subclasses: pheromone-binding proteins (PBPs), general odorant-binding proteins (GOBPs) and chemosensory proteins (CSPs), corresponding, e.g. to the honeybee antennal specific proteins ASP1, ASP2 and ASP3, respectively (Danty *et al*., 1997, 1998). Neither the sequence nor the 3D structure of insect OBPs is comparable with those of lipocalins. GOBPs and PBPs exhibit some sequence similarity to each other (e.g. around 16% between ASP1 and ASP2: Danty *et al*., 1997, 1999), with a similar disulfide pairing (Briand *et al*., 2001a, 2001b), while CSPs are totally different, both at the sequence and the disulfide bond–pairing levels (Briand *et al*., 2002b). The only known 3D structure of an insect OBP is that of *Bombyx mori* PBP, which was found to be an all-α-protein, comprising six α-helices with a rigid disulfide-linked four-helix scaffold and no β-sheet at all (Damberger *et al*., 2000; Lee *et al*., 2002; Figure 4.4). NMR investigations concerning honeybee OBPs revealed a similar secondary structure disposition (Birlirakis *et al*., 2001; Lescop *et al*., 2001). Nevertheless, insect OBPs, which are as concentrated in the sensillar lymph as vertebrate OBPs, can bind the same odorants as vertebrate OBPs, with affinity constants in the micromolar range. They should therefore play a similar role in olfaction processes.

4.3.3 Experimental approaches to OBP–odorant interactions

To test OBP–odorant-binding properties, i.e. their structure–activity relationships, recombinant proteins and variants, obtained by site-directed mutagenesis (intended alteration of the DNA coding sequence), have to be produced either using bacterial or eukaryotic systems (Briand *et al*., 1999, 2000a; Krieger *et al*., 1992; Löbel *et al*., 1998) in order to obtain amounts sufficient for odorant-binding assays. After purification, these proteins have to be thoroughly characterised (microsequencing, mass spectrometry, peptide mapping, circular dichroism, etc.) to ensure their structural integrity. Early studies used radiolabelled odorants to investigate their binding onto OBPs (Pelosi *et al*., 1982). Nevertheless, this technique, which has been successfully used by Pevsner *et al*. (1985, 1990), remains problematical because of the use of radioactive airborne molecules. One of the most widely used methods is based on the displacement of fluorescent probes by odorants. The fluorescence intensity (i.e. quantum yield) of these probes is highly dependent on the medium polarity: these hydrophobic probes only fluoresce in an apolar environment, so that they perform emission only when they are inside a protein hydrophobic binding pocket, not in aqueous solution. The addition of odorants, which compete with the probe for binding the protein-specific site, induces the release of the probe, and therefore the fluorescence intensity decreases. Dissociation constants (K_{diss}) can then be calculated from a plot of fluorescence intensity against free ligand concentration, obtained using a standard nonlinear regression method. Many probes can be used such as anthroyloxy derivatives (DAUDA, 11-(5-(dimethylamino naphthalene-1-sulfonyl)amino)undecanoic acid) or, more currently, anthracene and naphthalene compounds (e.g. AMA, 1-aminoanthracene; NPN, *N*-phenyl-1-naphthylamine; ANS, 8-anilino-1-naphthalenesulfonic acid). The presence of a tryptophanyl residue located at the bottom of the cavity in all lipocalins may also permit the investigation of interactions using tryptophan intrinsic fluorescence that can be quenched by brominated compounds as observed with insect CSPs (Briand *et al*., 2002b). In addition to fluorescence displacement, isothermal titration calorimetry is a direct physical method for assessing ligand–protein interactions which allows determination of the binding stoichiometry and affinity for unlabelled odorants. This technique has been successfully used for the study of porcine OBP (Burova *et al*., 1999), rat OBP-3 (Löbel *et al*., 2001) and insect OBP (Briand *et al*., 2001a).

The large quantity of recombinant OBPs available also allows the assay of airborne molecules by OBP in conditions close to those of the perireceptor physiology, thus mimicking *in vivo* uptake (Briand *et al*., 2000a). We called this volatile odorant-binding assay (VOBA), aimed to study the uptake of airborne odorants without radiolabelling. Purified recombinant OBP and

a control protein (e.g. bovine α-lactalbumin, a protein that does not bind hydrophobic molecules such as odorants) are dissolved in aqueous buffer to a millimolar concentration comparable to OBP concentration in the mucus (Pelosi, 1994). Protein solutions are placed in a sealed glass chamber containing a pure odorant not diluted in any solvent, with buffer as control. The proteins and the control buffer are then extracted with chloroform and analysed by gas chromatography. This test has also been used to characterise an elephant OBP (Lazar *et al*., 2002). Uptake of airborne odorants in nearly physiological conditions strengthens the role of OBPs as volatile hydrophobic odorant carriers in the mucus of the olfactory epithelium through the aqueous barrier towards the chemosensory cells.

In order to decipher the stereochemical determinants of odorant binding, knowledge of OBP 3D structure is essential. Nowadays, the determination of the 3D structure of vertebrate OBPs can be performed by two approaches: experimental X-ray crystallography or alternatively NMR, which have the advantage of giving direct data on the odorant–protein interactions. Structural predictions can also be used, which is possible since the 3D structures of many lipocalins are already known. The 3D structure predictions can be obtained using the automated comparative protein-modelling server <http://www.expasy.ch/swissmod/SWISS-MODEL.html> after multiple alignments with amino acid sequences of lipocalins of known 3D structures. The prediction procedure involves a thermodynamic refinement based on energy minimisation after a rough model is determined by association of secondary structure motifs originating from the best sequence similarity. Interaction with ligands can then be modelled with *in silico* docking procedures (Spinelli *et al*., 2002).

4.3.4 Human OBPs

For 20 years the search for hOBPs was unsuccessful. Trials to find hOBPs in the human nasal cavity (Burkhard *et al*., 2001; Lindahl *et al*., 1999, 2001; Maremmani *et al*., 1996; Scalfari *et al*., 1997) failed because sampling procedures were based on washing of the whole nasal cavity. The small area of olfactory cleft (less than 10% of the total area of the nasal cavity in humans) and its inaccessible location (Morison & Moran, 1995) make sampling of the olfactory epithelium mucus difficult (Figure 4.2). In human nasal washing, various proteins were found including tear lipocalin-1 (Burkhard *et al*., 2001; Lindahl *et al*., 1999, 2001; Maremmani *et al*., 1996; Scalfari *et al*., 1997). However, contrary to all other vertebrates so far studied, the presence of OBP-like proteins was not observed in humans. Nevertheless, two putative hOBP genes ($hOBP_{IIa}$ and $hOBP_{IIb}$) localised on chromosome 9q34 have been described (Lacazette *et al*., 2000). The $hOBP_{IIa}$ gene codes for a protein

45.5% homologous to rat OBP-2 and is transcribed in the nasal cavity, in contrast to $hOBP_{IIb}$, which is transcribed in the genitals and codes for a protein that is 43% identical to the human tear lipocalin-1. However, the capability of putative proteins derived from the $hOBP_{IIa}$ gene to bind odorants was still questionable.

We recently evidenced hOBP expression in the mucus covering the olfactory cleft, where the sensory olfactory epithelium is located (Briand *et al*., 2002a). A proteomic analysis of nasal mucus taken under endoscope at different levels of the nasal cavity (inferior turbinate, septum, olfactory cleft) was performed. Putative hOBPs were then observed solely in the mucus of the olfactory cleft, but not detected in the mucus sampled at the surface of the inferior turbinate or the septum. Expression of hOBP appeared limited to the uppermost region of the nasal cavity where odorant molecules are detected by olfactory receptor neurons (ORNs) (Figure 4.2). To demonstrate the odorant-binding activity of this protein, we used a recombinant protein (hOBP-2A), corresponding to the gene $hOBP_{IIa\alpha}$, which was secreted by the yeast *Pichia pastoris*. We obtained hOBP-2A as a well-conformed monomeric lipocalin with its single typical disulfide bond properly formed between C59 and C151 (a conservative feature of all other vertebrate OBPs) and a single expected N-terminus. While the typical nature of OBPs is more acidic, hOBP-2A was found to be neutral (calculated pI 7.8, measured pI 6.5). A ligand-binding study was then performed using fluorescent probes, since no artefactual alteration of the molecule occurred. Recombinant hOBP-2A was found to bind many diverse odorants with dissociation constants in the micromolar range, as found in all known animal OBPs. For instance, seven odorants (2-isobutyl-3-methoxypyrazine (IBMP), α-pinene, eugenol, γ-decalactone, 3,7-dimethyl-2,6-octadienenitrile also called citralva, β-ionone and vanillin) were shown to have different ability to displace fluorescent probes. We found a chemical specificity of this OBP for aldehydes, either aliphatic or aromatic, enhanced by the size of the odorant molecule, as clearly shown with chemical series. Another way of characterising hOBP-2A is by its low affinity for a very potent odorant, IBMP, and a very high affinity for lilial, the odour of the lily of the valley, as well as large aliphatic acids.

4.3.5 Structure of the odorant-binding pocket

The variability of binding thermodynamic parameters corresponding to structurally different odorants suggests that particular constraints and different interactions occur within the binding site. This can be investigated by structural approaches (X-ray crystallography, NMR) and biochemical and biophysical experiments, complemented with modelling and docking computations.

4.3.5.1 Crystallographic observations of complexes of OBPs with odorants

Up to now, only a few odorant–OBP complexes have been submitted to structural analysis. Bianchet *et al*. (1996) observed that two odorant molecules could occupy the β-barrel cavity of bovine OBP. Although Tegoni *et al*. (1996) identified another cavity between the two monomers of the bovine OBP dimer, they suggested, on the basis of porcine OBP data (Vincent *et al*., 2000), that the most likely binding site is inside the β-barrel, since this may be general for all OBPs. The size of the β-barrel pocket was found to be 780 Å^3 (Tegoni *et al*., 1996) for the bovine protein and about 500–550 Å^3 for the porcine OBP (Vincent *et al*., 2000). Using porcine OBP, Vincent *et al*. (2000) tested in solution, and in the crystal, a limited number of odorants with relatively good affinity (affinity constants $> 10^6/\text{M}$) and different chemical groups (aromatic ring, aliphatic chain or polar group). They distinguished two classes of affinity, which appeared to be more or less related to the presence of a relevant portion of linear or moderately branched alkylic chain. Nevertheless, in the crystalline complexes, the ligand orientation inside the cavity proved to be opportunistic with no specific target patches for aromatic or charged group. Interactions between the different odorants and the β-barrel involved most of the residues in the cavity. Except for the two asparagines, which display a polar interaction between the amino acid side chain and the keto-oxygen of benzophenone, all interactions were hydrophobic. The number of these interactions appears to be roughly related to the size of the odorant, but without any correlation with affinity measured in solution. With the limited number of observations so far obtained through crystallography, the interaction with odorants was found to be devoid of specificity. Docking simulations and molecular dynamic experiments have also been performed to examine how the pheromonal ligand occupies the binding pocket of the boar salivary lipocalin, demonstrating a volume increase of the cavity upon steroid pheromone binding and the adaptation of the amino acid side chains to the steroid molecules (Spinelli *et al*., 2002). Biochemical investigations, which dealt with many more OBPs and odorants, gave more subtle information.

4.3.5.2 Biochemical molecular modelling and docking investigation of hOBP

The model of a single binding site per dimer provides the best curve fitting for most fluorescence assays (Briand *et al*., 2000a). Such a stoichiometry is already reported for several OBPs or lipocalins, whatever the method of binding used (Bignetti *et al*., 1988; Burova *et al*., 1999; Löbel *et al*., 1998; Pevsner *et al*., 1986; Ragona *et al*., 2000). The OBP binding site would be localised in the hydrophobic cavity shaped by the β-barrel structure (the calyx) (Ramoni *et al*., 2001; Vincent *et al*., 2000), even in dimeric β-lactoglobulin in

which only one of the two calyces is occupied (Ragona *et al*., 2000). In the case of hOBP-2A, which is a monomer, the binding site certainly lies in the hydrophobic pocket formed by the β-barrel, as attested by the spectral properties of the fluorescence emission: maximal emission wavelengths of dansyl probes decreased while quantum yields increased, revealing a deep burying into an apolar environment (Briand *et al*., 2002a). Since hOBP-2A binds the fluorescent compound 1,8-ANS, it contrasts to rat OBP-2 (Löbel *et al*., 1998). This compound cannot be displaced by odorants, as already observed for bis-ANS (1,1′-bis(4-anilino-5-naphthalene)-sulfonic acid) with rat OBP-3 (Löbel *et al*., 2001).

In the absence of protein crystals for X-ray crystallography experiments, molecular modelling can be applied to vertebrate OBPs since many lipocalin 3D structures have been investigated. For instance, the computed 3D model of hOBP-2A has been obtained by this method (Briand *et al*., 2002a). It revealed that three lysyl residues of the binding pocket (Figure 4.5a) may account for the increased affinity for aldehydes and allowed a better interpretation of the odorant specificity of this OBP.

4.3.6 Potential role of OBPs in odour discrimination

OBPs bind with high efficiency a large number of odorants belonging to different chemical classes. Although no preferential binding was observed with the porcine OBP (Vincent *et al*., 2000), a broad specificity was revealed

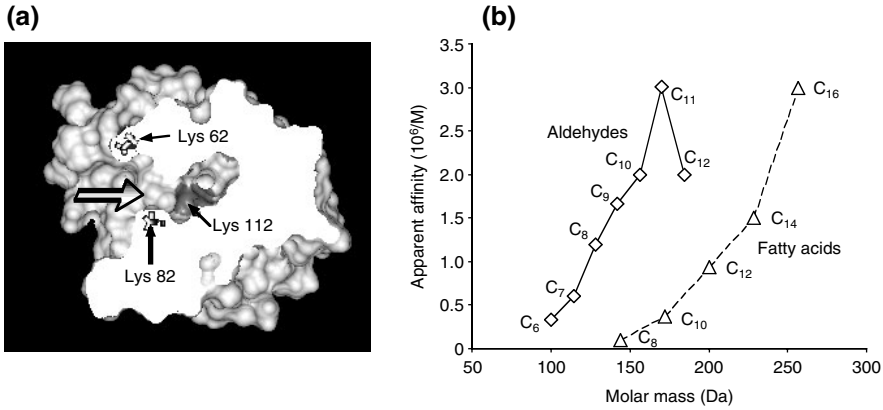

Figure 4.5 Relationships between odorant structure and binding onto human OBP-2A. (a) Slabbed view through the molecular surface and binding pocket of the predicted 3D structure of hOBP-2A. In the binding pocket (arrow), lysine side chains are labelled and surfaces are coloured light grey. (b) Relationships between the size and the affinity of aldehydes and fatty acids. Affinities resulting from fluorescence probe competition experiments by Briand *et al*. (2002a) are reported as $1/K_{diss}$; ◇, series of aliphatic aldehydes; △, series of fatty acids; C_n indicates the number of carbons of aliphatic chains.

by the study of the three rat OBPs, which are specially tuned towards distinct chemical classes of odorants. Rat OBP-1 preferentially binds heterocyclic compounds such as pyrazine derivatives (Briand *et al*., 2000a; Löbel *et al*., 1998) and OBP-2 appears to be more specific for long-chain aliphatic aldehydes and carboxylic acids (Löbel *et al*., 1998), whereas OBP-3 was described to interact strongly with odorants composed of saturated or unsaturated ring structure (Löbel *et al*., 2001). The specificity of hOBP-2A appeared more restricted than that of porcine and rat OBP-1 and OBP-3 (Löbel *et al*., 1998, 2001, 2002; Paolini *et al*., 1999; Scaloni *et al*., 2001) and could therefore contribute to odour discrimination. Rat OBP-2, whose sequence is closest to that of hOBP-2A, binds fatty acids in contrast to other known OBPs, and a similar specificity was expected for hOBP-2A. Although the binding of fatty acids was similar for rat OBP-2 and hOBP-2A, there were some significant differences, suggesting that sequence variations induce structural variations of the binding site (Briand *et al*., 2002a). Among the odorants tested, hOBP-2A interacted preferentially with aldehydes, but not with pyrazines. The secretion of a set of complementary OBPs would therefore allow the solubilisation of a vast array of chemically variable odorants, which are perceived by human beings. Other variants, hOBP-2B and hOBP-2AB, identified in the human genome, have been cloned by Roberto Ramoni (personal communication). They have been expressed and their affinity for various odorants is presently under scrutiny.

To determine the molecular parameters guiding the affinity of odorants for hOBP-2A, the influence of molecular size was investigated using fatty acids as a model (Briand *et al*., 2002a). As illustrated in Figure 4.5b, the fatty acid chain length was correlated to affinity, which regularly increased from octanoic acid ($K_{diss} \sim 2\ \mu M$) to palmitic acid ($K_{diss} = 0.3\ \mu M$), one of the best-tested ligands. To investigate the influence of functional groups, aldehyde derivatives were tested and found to display enhancer effects. The influence of aldehyde derivative size was compared with that of fatty acids. Like acids, aliphatic aldehydes became more efficient as chain length increased up to a maximum at C11. Up to this chain length, the ability of aldehydes to displace the fluorescent probe was equivalent to that of fatty acids with four to five more carbons, indicating that the aldehyde group increases the odorant affinity, which is chiefly caused by hydrophobic interactions. Modelling of the 3D structure of hOBP-2A revealed that lysyl residues of the binding pocket might account for the increased affinity for aldehydes (Figure 4.5a). It can be explained by formation of hydrogen bonds between the aldehyde group and the amine function of a lysyl residue at the rim of the calyx, which could stabilise the docking of the odorants. When the size of an aliphatic aldehyde molecule becomes too large, such interactions are then hampered resulting in reduction of affinity for aliphatic aldehydes containing more than ten carbons, a hypothesis supported by observation that larger fatty acids, compared to

aldehyde derivatives, do not exhibit a decrease in affinity. Nevertheless, hydrophobic interactions are likely to be the main forces driving the binding of odorants onto hOBP-2A, since hydrocarbons such as α-pinene and octane exhibited a significant affinity.

4.3.7 Conclusions about OBP

4.3.7.1 Vertebrate OBP definition

In contrast to insect OBPs, which belong to a well-defined class of proteins whose olfactory role has been proven, vertebrate OBPs are lipocalins, which are hardly differentiated from other lipocalins by structural characteristics, as reported by Tegoni *et al*. (2000). Neither 3D structure nor sequence comparison allows clear-cut definition of OBPs among lipocalins. The tissue expression is therefore a necessary criterion in order to attribute the status of OBP to a lipocalin, provided it does, indeed, bind odorants under physiological conditions. Such a definition is not totally satisfactory, and it is difficult to define a lipocalin protein as an OBP unequivocally. Only the physiological demonstration of the role of a putative OBP will be decisive.

4.3.7.2 Comparison of OBP numbers in vertebrates and in insects and OBP role in odorant discrimination

Different OBP subtypes have been reported to occur simultaneously within the same vertebrate species: three in pig (Dal Monte *et al*., 1991; Scaloni *et al*., 2001), four in mouse (Miyawaki *et al*., 1994; Pes *et al*., 1998; Utsumi *et al*., 1999), three in rat (Briand *et al*., 2000a; Dear *et al*., 1991a, 1991b; Löbel *et al*., 2002; Pevsner *et al*., 1988b), three in rabbit (Garibotti *et al*., 1997) and at least eight in porcupine (Felicioli *et al*., 1993; Ganni *et al*., 1997). This diversity might allow some odorant discrimination. Compared to vertebrates, insects express many more OBPs in the olfactory organs (e.g. in the honeybee antennae) (Danty *et al*., 1998). The knowledge of the fruit fly genome provided a new insight with as many as 30 OBPs found in *Drosophila melanogaster* (Hetmak-Scafe *et al*., 2002), about ten times more than in vertebrates. It is worth noting that the selectivity of insect OBPs (specially the PBPs) is known to be much greater than that of vertebrate lipocalin OBPs (Briand *et al*., 2001a, 2002b; Danty *et al*., 1999), although earlier results concerning lepidopteran PBP (Du & Prestwhich, 1995) are subject to dispute (Campanacci *et al*., 2001).

4.3.7.3 Biological role of OBPs

The essential role of OBPs in eliciting the behavioural response and coding of odour has only been demonstrated in insects. This has clearly been evidenced in fruit fly by gene disruption and reintroduction, which was then associated

with olfactory behaviour (Kim & Smith, 2001; Kim *et al*., 1998). For example, fruit flies normally avoid ethanol, but mutants displayed the opposite behaviour. Analysis of the genome of such a mutant demonstrated that an OBP gene was disrupted. When reintroducing the proper functional OBP gene in this mutant by genetic engineering, Kim *et al*. (1998) obtained flies with restored wild-type behaviour. Wang *et al*. (2001) also showed that the distribution pattern of neural activity, recorded by calcium imaging in the *Drosophila* mushroom body, a neuronal structure equivalent to the vertebrate olfactory bulb, can be altered specifically by genetic manipulation of an OBP. They demonstrated that this OBP alteration was closely associated with a behavioural defect of odour preference and that the OBP was directly involved in odour coding. Fire ants give another example in which natural genetic alterations of OBPs (PBPs) result in social behaviour changes that modify the structure of the colony (Krieger & Ross, 2002).

4.3.7.4 Putative roles of OBPs

In mammals and in insects, ORs are separated from air by a protective layer of hydrophilic secretion, the nasal mucus and sensillar lymph, respectively. Hydrophobic airborne odorants have to cross this aqueous barrier to reach their neuron receptors. OBPs, which have been hypothesised to play such a transporter role, likely appeared during the adaptation to terrestrial life. Considering that it would be practically impossible for a single lipocalin to bind all the different sizes, shapes and chemical functions of chemical compounds, the polymorphism of OBPs supports their carrier role. This role is also supported by their relatively low affinity constant for odorants associated with their high concentration in the olfactory fluids.

Nevertheless, various other physiological roles for OBPs have been proposed besides this function (Pelosi, 1994, 1996, 1998; Pevsner & Snyder, 1990). In addition to a hypothetical scavenger function, which might prevent the saturation of ORs when odorants are highly concentrated, OBPs might be mandatory for the transduction process if ORs merely recognise the OBP–odorant complex instead of the odorant alone. OBPs have also been shown to bind cytotoxic and genotoxic agents (Eckl *et al*., 1995; Marinari *et al*., 1984; Pevsner *et al*., 1990). They might thus play a role in protecting the nasal mucosa and the olfactory neurons, particularly exposed to microbial infection due to airflow and oxidative injuries. Lipocalins, similar to some OBPs, are secreted in biological fluids, such as the tear lipocalin-1, whose biological activity is to protect from microbes. It indeed shows nuclease activity (Yusifov *et al*., 2000) and has also been reported as a cysteine proteinase inhibitor (Redl, 2000). In humans, it is striking to note that OBPs are synthesised at a most vulnerable area where microbes could enter the brain.

4.3.7.5 Lipocalins potentially involved in taste

VEGP is a lipocalin that was found abundantly secreted in saliva by taste buds (Blaker *et al*., 1993). A role similar to OBPs was suggested for this protein that would transfer tastants to the taste receptors (TRs). Nevertheless, as tastants are generally water-soluble molecules, such a role for VEGP is unlikely. Because this protein is exactly the same as tear lipocalin-1 that protects eyes against microbes (Redl, 2000; Yusifov *et al*., 2000), it is highly plausible that VEGP also has a protecting role against microbial infection in the mouth.

4.4 Olfactory receptors

Since OBPs do not have a great discriminatory power, how does the olfactory system manage this sophisticated task? The identification of a large family of ORs allowed considerable progress to be made towards a comprehensive understanding of the molecular basis of olfaction (Buck & Axel, 1991). These developments were supported by novel advanced molecular and physiological techniques, as well as the publication of eukaryotic genomes from various disparate species (e.g. from the worm *Caenorhabditis elegans* to the human genome). They provided a view of molecular coding in the olfactory system that is no doubt incomplete, but nonetheless persuasive. In comparison to OBPs, ORs are membrane proteins, which exhibit a more precise specificity towards odorants. In mammals, about 1000 different, potentially functional ORs are expressed in millions of neurons. Although thousands of putative OR genes have been described in many species, only a very limited number of studies have reported that the proteins encoded by these genes indeed bind odorants: e.g. up to now, a single human receptor, called OR17-40, has been shown to bind a set of odorants, including helional (Wetzel *et al*., 1999).

4.4.1 Discovery of ORs as G protein–coupled receptors

ORs were discovered in the early 1990s by Buck and Axel (1991) who opened the molecular era of olfaction when publishing a multigene family in rat, specifically expressed in olfactory neurons and endowed with membrane receptor properties (see Firestein, 2001 and Mombaerts, 1999a, 1999b for reviews). G protein–coupled receptors (GPCRs) are membrane proteins that are commonly found in many cellular processes involving communication between the cell and its environment, such as hormonal regulation and neurotransmission. Not only do the GPCRs, also called seven-transmembrane (7TM) domain receptors, regulate many physiological processes, but drugs that target these receptors account for more than half of the medicines sold

worldwide. Consequently, ORs were searched for as transmembrane proteins, belonging to the GPCR family, i.e. with sequence features specific for these proteins, which are selectively expressed in the olfactory epithelium. OR discovery was therefore not surprising, but the discovery of as many as 1000 genes for ORs in the mammalian genome was unanticipated. They form not only the largest family of GPCRs but also the largest protein family ever found in the entire genome. It is striking to note that the number of putative functional ORs in humans is approximately 350, which is far smaller than the myriad odours we can perceive, which strongly suggests a combinatorial system for odour coding.

4.4.1.1 General properties of GPCRs

The GPCR family is a very large family of membrane proteins, which share many features in common. They are the first element of a three-step process involving a receptor for external signal reception (input receiver), a transducer (G protein, e.g. transducin in the retina or gustducin in taste sensing) and an effector enzyme (e.g. phospholipase C, PLC and cyclic GMP phosphodiesterase also called adenylyl cyclase, AC). Among membrane-bound receptors, GPCRs are certainly the most diverse. They are capable of transducing messages as different as photons, small organic molecules such as odorants, pheromones, tastants, carbohydrates, nucleotides, nucleosides, peptides and lipids, and also macromolecules such as proteins. Many studies led to an indirect comprehensive general structure of GPCRs, which has been recently confirmed by the determination of the crystal structure of one of them, the bovine rhodopsin (Palczewski *et al*., 2000). They all have 7TM domains, with an external domain (also called ectodomain) outside the cell, corresponding to the N-terminal end of the protein, and an internal cytoplasmic C-terminal domain (also called endodomain). The seven membrane–spanning sequences are characterised by highly hydrophobic amino acids, and are linked by six loops, three outside the cell and three in the cytoplasm. The 7TM domains form a seven-α-helix bundle, which composes a central core whose global structure is common to all GPCRs. In many cases, a pocket formed by the membrane-spanning segments is involved in ligand (also called agonist) binding, although it is also observed that the ectodomain (and the external loops in some cases) may be involved in this function.

This diversity allows GPCRs to bind an amazing number of ligands of very different types. Upon binding an agonist molecule, the transmembrane bundle and the internal loops undergo conformational changes (modification of the relative orientation of TM3 and TM6, alteration of the structure of the second and third internal loops), which may differ from one GPCR to another, unmasking G protein–binding sites located in the cytoplasmic C-terminus and internal loops. This allows interaction with G proteins, which, in turn, induce a series of intracellular reactions leading to important metabolic

alterations. The interaction with G proteins gave the name GPCR to these membrane proteins. Coupling with G proteins makes transduction mechanisms more intricate, since many different G proteins, coupled with various transduction pathways, have been observed to occur with different GPCRs. We will restrict our description to those involved in chemical senses.

As regards GPCR structural features, two cysteine residues (one in external loop 1 and the other in external loop 2), which are conserved in most GPCRs, likely form a disulfide bridge, which is probably important for the stabilisation of ORs. Some GPCRs have been found to form either homo- or heterodimers not only with a structurally different GPCR but also with membrane-bound proteins having one transmembrane domain such as nina-A, odr-4 or receptor activity–modifying protein (RAMP), the latter being involved in their plasma membrane targeting, function and pharmacology. This is also the case of TRs that recognise sugars and amino acids. Sweet responses require coexpression of taste receptor 1, member 2 (T1R2) and taste receptor 1, member 3 (T1R3) receptor proteins, whereas umami (amino acid) responses require coexpression of the T1R1 and T1R3 receptor proteins (Nelson *et al*., 2001, 2002). Whether this dimerisation affects trafficking or whether it is primarily structural remains to be determined. Such a mechanism has not yet been reported for ORs or has never been investigated specifically.

4.4.1.2 GPCR classification

Although all these receptors have in common a central core domain, there are at least three families of GPCRs showing no sequence similarity (see Bockaert & Pin, 1999 and Pierce *et al*., 2002 for reviews). Aside from sequence variations, GPCRs differ in the length and function of their N-terminal extracellular domain, their C-terminal intracellular domain and their external and internal loops (Figure 4.6). GPCRs can be classified into three main families (1, 2 and 3), easily recognisable when comparing their amino acid sequences, which generally share over 25% identity in the transmembrane region and a distinctive set of highly conserved residues and motives. Receptors from different families share no sequence similarity. Family 1 is the largest one that contains most GPCRs including ORs and can be divided into three subfamilies. Subfamily 1a, which includes rhodopsin, contains GPCRs for small ligands such as odorants, adrenalin, catecholamines, adenosine, adenosine triphosphate (ATP), opiates and enkephalins. Their ectodomain is very small (20–30 amino acids) and their binding site is located within the 7TMs. Subfamily 1b contains receptors for peptides, cytokines, thrombin, whose binding site includes the short N-terminus and extracellular loops. Subfamily 1c contains GPCRs for glycoprotein hormones (lutropin, thyrotropin, follitropin). A large extracellular domain (300–400 amino acids) characterises this subfamily. The binding site of subfamily 1c is mostly the

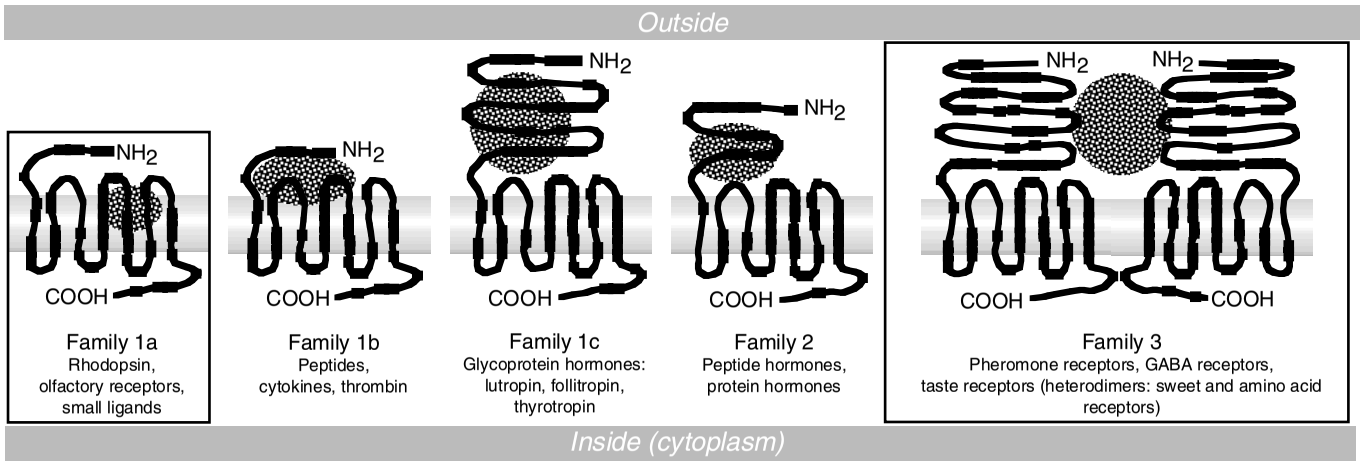

Figure 4.6 Schematic G protein–coupled receptor classification. The ligand position is approximately shown by a spotted oval; family 1a and family 3 (boxed figures) comprise receptors involved in chemical senses, olfactory and gustatory, respectively. (Adapted from Bockaert & Pin, 1999 and Montmayeur & Matsunami, 2002.)

ectodomain but also involves at least contacts with extracellular loops 1 and 3. This ectodomain undergoes several glycosylations that are involved in the receptor activation (Vu-Hai *et al*., 2000). Like subfamily 1c, family 2 GPCRs have a quite large ectodomain, but they do not share any sequence homology. Members of this family are not so numerous (about 25 proteins) and their ligands include the gastrointestinal peptide hormones (glucagons, vasoactive intestinal peptide and secretin), and also calcitonin, corticotrophin-releasing hormone and parathyroid hormone. Family 3 also contains few members, including metabotropic glutamate receptors (mGluRs), the GABA receptor involved in neurotransmission and the calcium-sensing receptor, as well as some TRs (Montmayeur & Matsunami, 2002). It also comprises a group of putative pheromone receptors coupled to the G protein G_{α_0} (termed VRs and G_{α_0}-VN) (reviewed in Bargmann, 1997). In addition to these three families, Bockaert and Pin (1999) identified two others; family 4 comprises other pheromone receptors (VNs) associated with G_{α_i} (Dulac & Axel, 1995), whereas family 5 includes receptors involved in embryonic development.

A large variety of molecular mechanisms are involved in the activation of the core domain by diverse ligands. Among family 1 (GPCRs activated by small ligands; subfamily 1a), the ligand binds in a cavity formed by TM3–TM6. In the case of rhodopsin, the light-activated receptor, retinal (photon target), is covalently linked in this cavity, and its light-induced change in conformation activates the receptor. As discussed further, ORs have a similar binding site, opposite to family 3 receptors, which comprise taste and pheromone receptors, endowed with a very large ectodomain involved in ligand binding.

4.4.1.3 G proteins as transducers

More than 30 years ago, Rodbell *et al*. (1971) imagined that a guanine-nucleotide regulatory protein functionally connected glucagon receptors with AC (effector), generating the second-messenger cyclic AMP (cAMP). This was the discovery of G proteins, later shown to be heterotrimeric systems, comprising α-, β- and γ-subunits: the α-subunit is responsible for guanosine triphosphate (GTP) binding and hydrolysis (the feature that gave the name of G proteins to this family), whereas the β- and γ-subunits are associated in a tightly linked complex. Each of these subunits is known to be a member of a gene family; to date 16 α, 5 β and 12 γ proteins have been cloned (Pierce *et al*., 2002). G proteins are generally referred to by their α-subunits (e.g. the G_s heterotrimeric complex contains G_{α_s}). Four distinct α-subunit subfamilies are recognised: G_s proteins induce stimulation of AC; G_i proteins induce inhibition of AC; G_q proteins couple to the activation of PLC; and G_{12} protein couples to the activation of rho guanine-nucleotide exchange factors. However, due to the combinatorial complexity of αβγ heterotrimers, the specific subunit composition of the G proteins that function in specific pathways is

poorly understood. As regards chemical senses and *in vitro* experimental approaches reported here, the following G proteins have to be cited (Figure 4.7): G_{α_s} and $G_{\alpha_{olf}}$, which increase AC activity (Ma *et al*., 2000; Zheng *et al*., 2001); G_{α_0} and G_{α_i}, which decrease AC activity; $G_{\alpha_{gust}}$, also called gustducin, involved in bitter and sweet taste, which increases phosphodiesterase activity (McLaughlin *et al*., 1992); and $G_{\alpha_{15,16}}$, which increases PLC activity (Pitcher *et al*., 1998).

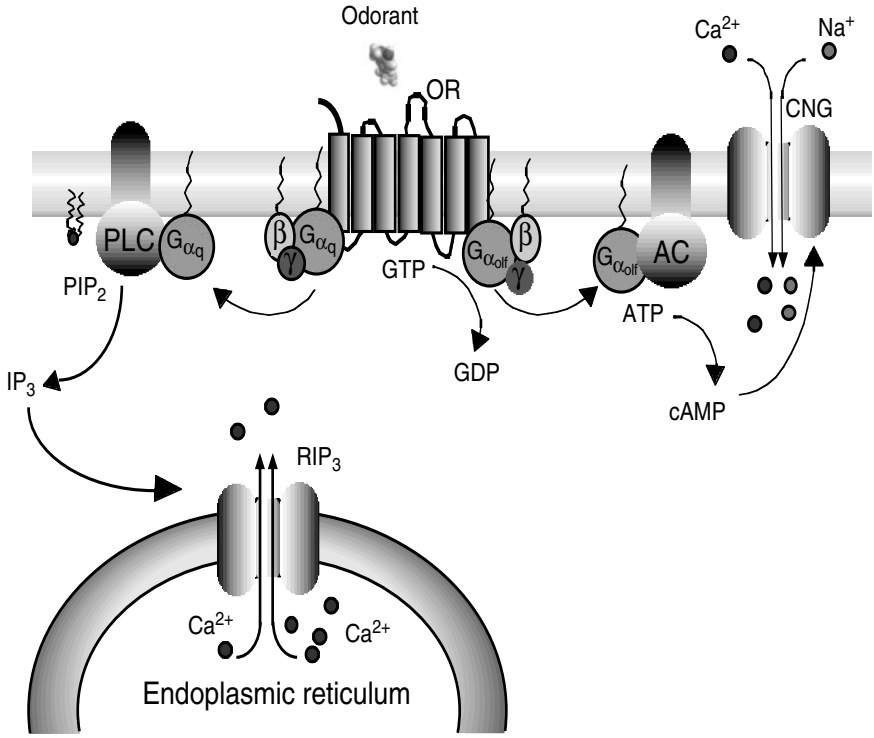

Figure 4.7 Olfactory transduction cascade. Within the cilia of olfactory neurons, a cascade of enzymatic activities transduces odorant binding onto an OR into a calcium influx, which leads to an electrical neuronal signal that can be transmitted to the brain. G proteins are composed of three distinct subunits called α, β and γ. G_{olf} is the olfactory G_α, which, in inactive state, binds guanosine diphosphate (GDP). Upon OR activation, G_{olf} binds GTP and activates the enzyme called adenylyl cyclase (AC) that catalyses the formation of 3′,5′-cyclic adenosine-5′-mono-phosphate (cAMP) from adenosine triphosphate (ATP). A cyclic nucleotide-gated (CNG) channel is then opened by cAMP, allowing cation entrance into the cytoplasm. G_{olf} hydrolyses guanosine triphosphate (GTP) into GDP and goes back to inactive state. Alternatively, an inositol-(1,4,5)-triphosphate (IP_3) pathway has been described in olfactory neurons. When G_q α-subunit is activated, it stimulates the enzyme called phospholipase C (PLC) that cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) in the cell membrane to release IP_3. IP_3 diffuses into the cytosol and binds to IP_3 receptors (RIP_3) located in the endoplasmic reticulum membrane which permit subsequent Ca^{2+} ion release into the cytoplasm. Arrows indicate stimulatory pathways.

4.4.2 Peculiar properties of ORs

Although vertebrate ORs exhibit typical features of GPCRs, they share certain specific characteristics, such as an unusually long second extracellular loop, (which is much conserved among ORs), an extra pair of conserved cysteines in that loop and other short sequences (Zhao & Firestein, 1999). Figure 4.8a illustrates a typical OR structure with the rat r1C6 receptor. Within the family of ORs, there is a range of similarity, from less than 40% to over 90% identity, as discussed further. ORs lack a definable N-terminal signal peptide, and the short ectodomain contains a single putative N-glycosylation site. Consequently, ORs are small GPCRs made of only 300–350 amino acids. In addition to the N-terminal N-glycosylation site, another consensus one is observed in the first external loop (Zozulya *et al*., 2001). There are several potential phosphorylation sites in the relatively short C-terminal endodomain, which may contribute to the desensitisation process. The second extracellular loop is made of 38 amino acids, with three highly conserved cysteines, and is considerably longer than that of most other GPCRs, while the third cytoplasmic loop, which contains only 15–17 residues, is comparatively short. The absence of sizeable external domain of ORs is a considerable advantage for gene cloning, since the membrane-spanning domain of GPCRs (i.e. the 7TMs and the six loops) is known to be devoid of introns, those 1 kb nucleotide sequences that are removed from the pre-mRNAs (messenger RNAs) before their translation by the ribosome. This uncommon feature makes experimental molecular approaches of ORs far easier.

Mombaerts (1999b) and Zhao and Firestein (1999) have reviewed the sequence peculiarities that distinguish ORs from other GPCRs and allow GPCRs to be classified as ORs, with some degeneracy (Buck & Axel, 1991; Pilpel & Lancet, 1999; Probst *et al*., 1992). A recent article on the human OR repertoire reports the identification, physical cloning and comparative sequence alignment of the 347 putative human full-length functional OR genes (Zozulya *et al*., 2001). The first cytoplasmic loop IL1 contains a consensus motif LHTPMY, the end of the third transmembrane helix (TM3) and the beginning of the loop IL2 exhibit the sequence MAYDRYVAIC, while SY ends TM5. In addition, the consensus sequence KAFSTCXSH is found at the N-terminal part of TM6 and, finally, PMLDPF is observed in TM7. The most conserved region is therefore in the second intracellular and extracellular loops, as well as within TM2, TM6 and TM7. Opposite to these constant regions, it is worth noting the existence of hypervariable sequences within TM3, TM4 and TM5 (Pilpel & Lancet, 1999), which likely participate in the odorant-binding site (see Figure 4.8b).

4.4.2.1 The second external loop of ORs

The second external loop of ORs exhibits two peculiar features: an unusually large size and frequently a distinctive sequence pattern. It is therefore tempting

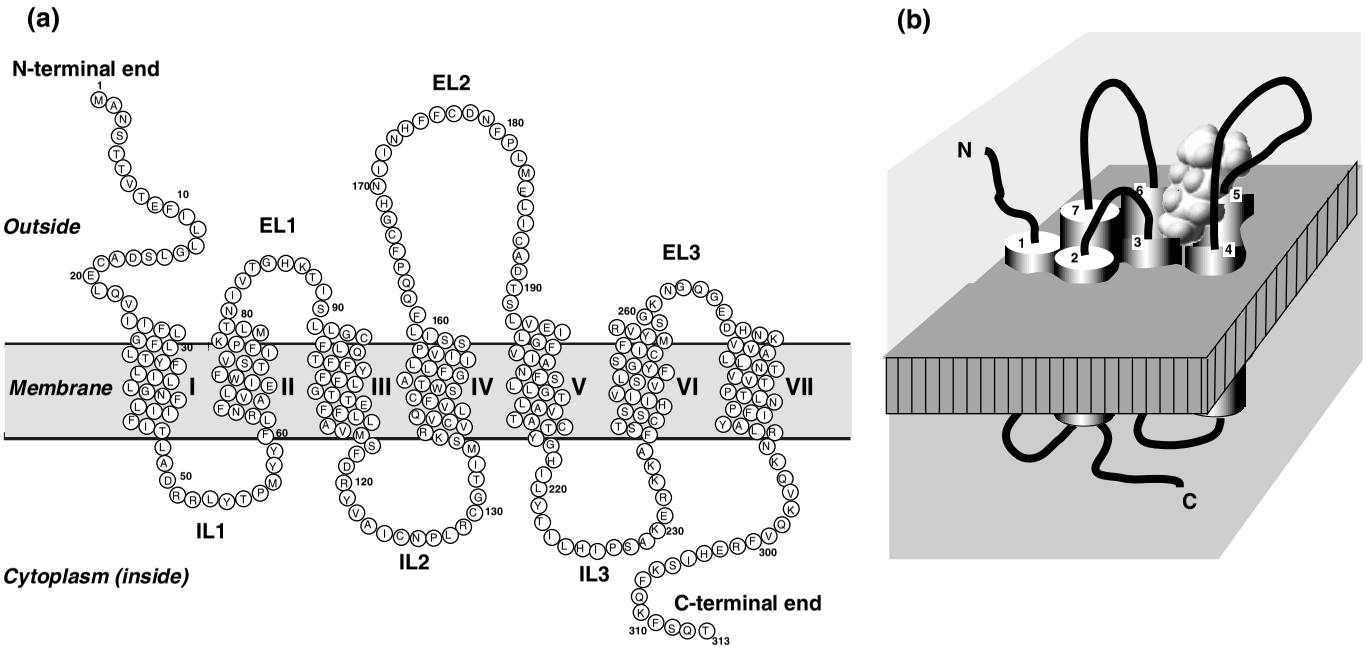

Figure 4.8 Olfactory receptor structure. (a) Arrangement of sequence domains with reference to the plasma membrane using the rat receptor 1C6 as a model; transmembrane-spanning α-helices, shown as cylinders, are numbered with roman figures; EL stands for external loops and IL for internal loops. (b) Schematic OR α-helix organisation, hypervariable transmembrane segments (3, 4, 5 and 6) are indicated by hatched top; menthol is schematically located at the binding crevasse entrance.

to postulate a role in odorant capture for this loop. The first idea is that it could be a binding site for a common molecular partner, e.g. OBPs, as discussed further. The hypothesis that the relatively long second extracellular loop may play a role in guiding axons of olfactory neurons (Singer *et al*., 1995) has not been supported by strong evidence so far.

Another hypothesis has recently been put forward by Wang *et al*. (2003), with some preliminary experimental arguments. These authors first noted that volatile compounds that smell strongly are also good ligands for metal ion coordination complexes. For instance, humans are extremely sensitive to amines and thiols but not to alcohols. The former compounds are good ligands for metal ions as well as for ORs. To explain this observation, Wang *et al*. (2003) hypothesised that ORs are metalloproteins with the metal ion located in their binding sites. They then looked for possible metal ion–binding sites in the genome sequences of ORs, and revealed a consensus sequence in the second external loop. They found the sequence HXXC[DE], where X is a hydrophobic residue, in nearly three out of every four ORs. They then synthesised a pentapeptide that contains this putative binding site and found that it not only has high affinity for Cu(II) and Zn(II) ions but that it also undergoes a dramatic transition to an α-helical structure upon metal ion binding. Based on these observations, they proposed a 'shuttlecock' mechanism for the possible structural change in ORs upon odorant binding. This mechanism involves membrane penetration of the second external loop after residue charge neutralisation by metal ion binding, penetration that modifies the second internal loop and therefore the possibility to interact with G proteins. This tricky and interesting hypothesis has now to be submitted to scrutiny using site-directed mutagenesis and functional analysis.

4.4.3 Odorant recognition by ORs: structural relationships between OR and odorant

The discovery of GPCR-like genes specifically expressed in the olfactory epithelium does not experimentally satisfy the ability of putative ORs to mediate an odorant-induced response. *In vitro* and *in vivo* functional analysis is the principal approach that can be used. In order to investigate the structural characteristics of odorant–OR interactions, protein modelling and *in silico* docking are the only techniques that can currently provide pertinent information, in complement to protein engineering.

4.4.3.1 Experimental studies of odorant–OR interactions

Because ORs are not water soluble, they cannot be studied by many of the classical techniques used to elucidate structure–function relationships (spectroscopy, X-ray diffraction, NMR, etc.). Instead, the structure–function relationships of water-insoluble ORs need to be investigated by functional

expression in eukaryotic cultured cells after gene transfection in cells that usually do not express these proteins. This requires efficient heterologous expression systems able to target ORs towards the plasma membrane where they naturally function. The clonal expression of ORs in natural conditions, i.e. the expression of a single allele among all the OR genes in a given olfactory neuron (Serizawa *et al*., 2000), has also been used to perform *in vivo* studies either in rat (Zhao *et al*., 1998) or in the worm *Caenorhabditis elegans* (Milani *et al*., 2002). Comparison of affinity constants, determined by indirect fluorescence assays revealing activation of the G-protein transduction machinery, provides structural information about the molecular interactions between odorants and ORs. Nowadays, researchers have adopted a pharmacological approach that uses a large and diverse pool of odorant compounds to characterise the molecular receptive field of an OR (Araneda *et al*., 2000; Schmiedeberg *et al*., 2003).

The comparison of odorant affinities for related natural proteins and the prediction of their 3D structures, resulting from computer modelling and molecular docking, identify the key amino acids involved in odorant binding and therefore suggest mutations with which binding can be studied further. In order to engineer ORs, mutations have to be performed by site-directed mutagenesis resulting in modified recombinant proteins used to explore the odorant-binding site, which must reach their membrane destination. Alternatively, the comparison of natural variants of a given OR, e.g. originating in different but close animal species, may provide very useful information concerning structure–activity relationships.

As regards GPCR 3D structure, it is difficult to express and extract large amounts of protein, and then obtain good crystals for X-ray diffraction analysis. The chances of obtaining a 3D structure for OR by these means are unlikely in the short term. Currently, only protein modelling can be helpful in studying the odorant–receptor interactions at atomic scale. Nevertheless, the determination of the crystal structure of bovine rhodopsin in 2000 was a landmark in the study of GPCRs (Palczewski *et al*., 2000). Bovine rhodopsin is the prototypical GPCR and also the first and only GPCR for which detailed 3D-structural information has been obtained, thanks to its abundance and quasi-crystalline state in the retina membrane. A recent review reports the latest structural information on the activation of this model protein (Albert & Yeagle, 2002). However, the key question is how well this structural information can be extrapolated to ORs. Bissantz *et al*. (2003) have recently shown that models of a range of GPCRs based on the rhodopsin structure can be successfully used to identify both antagonists and agonists by virtual screening of compound libraries. So it seems that such approaches could be usefully exploited in searches for OR ligands, the more so as bovine rhodopsin belongs to the same GPCR subfamily as ORs (i.e. devoid of large ectodomain).

4.4.3.2 OR functional studies reveal broad odorant selectivity

Only a few ORs have been expressed in heterologous cell systems. Difficulties in membrane targeting were overcome by fusing a signal peptide to the OR amino terminal (e.g. that of rhodopsin), which has enabled low levels of membrane expression in mammalian cells (e.g. HEK293, human embryonic kidney cell line or COS-7, African green monkey kidney cell line). In addition to the OR-modified gene, the complementary DNA (cDNA) of a G_{α} protein, which may couple with the OR and transduce the signal to a chosen transduction pathway, must be co-transfected. For instance, $G_{\alpha_{15}}$ and $G_{\alpha_{16}}$ can couple receptors, which are normally inducers of the cAMP pathway through $G_{\alpha_{olf}}$, with the phosphatidyl bisphosphate pathway (IP_3, inositol triphosphate). Target enzyme activation resulting from odorant binding with the heterologous OR is then able to open ion channels that induce intracellular calcium release. Calcium increase is measured by fluorescence intensity of a probe that has been injected into the cell (e.g. Fura-2 or Fluo-4). This technique is called calcium imaging (Figure 4.9). Some success has been achieved in oocytes with ORs from fish (Speca *et al*., 1999) and from humans (Wetzel *et al*., 1999). But in general these approaches remain difficult and reliability is still insufficient. Nevertheless, efficient systems for expressing and assaying ORs are being developed with permanent cell lines, which will allow automated odorant screening (Schmiedeberg *et al*., 2003).

As regards *in vitro* experiments using cell cultures, a successful attempt was reported by Krautwurst *et al*. (1998) using HEK293 cells and a vast repertoire of mouse ORs. They generated an expression library of chimeric receptors that contained a large and diverse repertoire of mouse OR sequences from the beginning of TM2 to the end of TM7, which were flanked by the sequence from the N-terminal to TM2 and the C-terminal sequence of a mouse OR named mOR4. The odorant-induced response was measured by calcium imaging in an artificial reporter system that detects OR activation at the single cell level using appropriate $G_{\alpha_{15,16}}$. By testing 80 chimeric receptors against 26 odorants, three receptors were identified as responding to the odorants carvone, (−)citronellal and limonene, respectively. The authors further showed that the response profile of a receptor was conserved between the receptor chimera and the full-length receptor. This system was also used by Kajiya *et al*. (2001) for the mouse OR named mOR23 and by Gaillard *et al*. (2002) for the mouse OR named mOR912-93. This system proved reliable, since it provided the same ligand specificity for mORI7 and mOR23 as found in the *in vivo* system involving olfactory neurons functioning in their own tissue environment. Alternatively, a cAMP assay can be used to monitor heterologously expressed activated ORs (Kajiya *et al*., 2001).

In this alternative approach, Zhao *et al*. (1998) have taken a strategy of using a homologous system, i.e. olfactory neurons in the olfactory epithelium, for functional expression of ORs. They used a recombinant adenovirus to drive

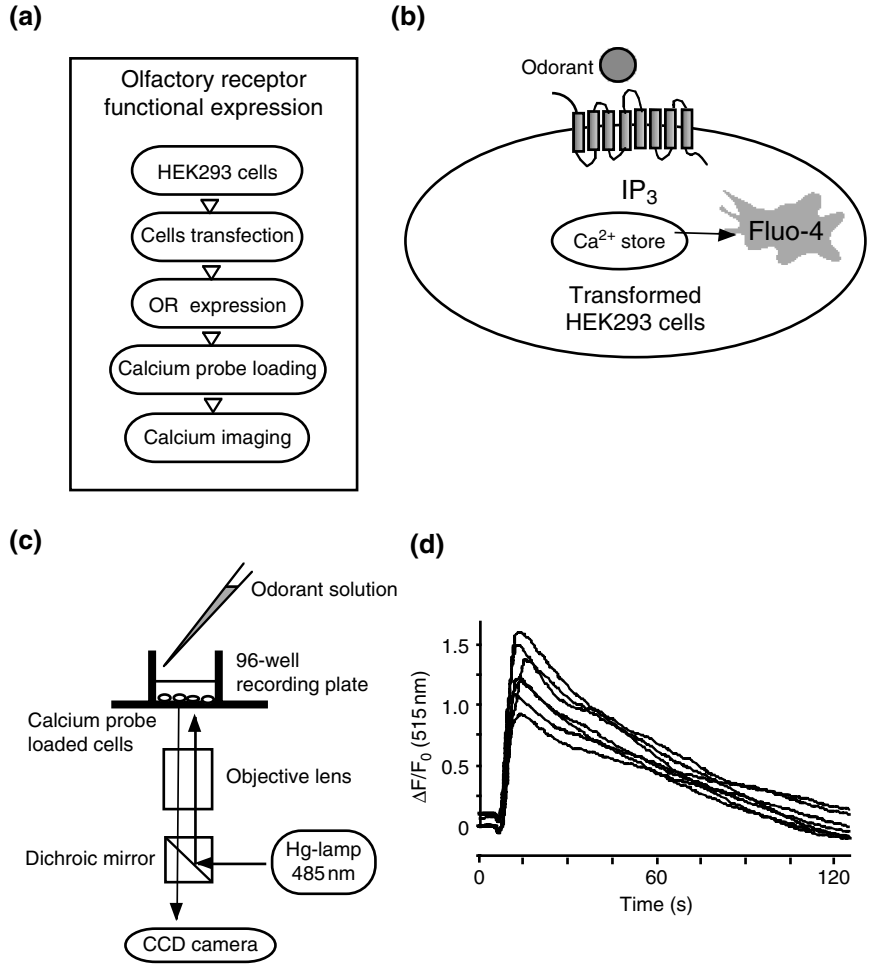

Figure 4.9 Olfactory receptor functional expression in transfected mammalian cells observed by calcium imaging. (a) Schema describing the functional expression of an OR in mammalian cells. HEK293 cells are genetically modified (transfected) with a given OR DNA, in order to express it in its plasma membrane. After calcium probe loading, modified cell activation by a specific odorant is monitored by calcium imaging. (b) Odorant stimulation elicits receptor-mediated signalling (IP_3 pathway) leading to internal Ca^{2+} store influx. In the cytoplasmic compartment, calcium probe (Fluo-4) chelates Ca^{2+} cations, leading to an increase in Fluo-4 fluorescence intensity. (c) Schematic drawing of calcium imaging set up to record odorant-induced calcium increase in adherent transfected HEK293 cells under inverted light microscope. Odorant solutions (10 μM concentration in solution) were manually applied to the 96-well recording plate using a pipette. (d) Time-course response of seven single cells to odorants reflects changes in Fluo-4 fluorescence intensity measured at 515 nm.

expression of a particular receptor gene in an increased number of sensory neurons in the rat olfactory epithelium. Electrophysiological recording showed that increased expression of a single gene led to greater sensitivity to a small subset of odorants. They wished to determine if related odorants might be recognised by the rat I7 receptor. They verified that rORI7, which was originally cloned by Buck and Axel (1991), was indeed overexpressed in transformed cells. The response profile of the I7 receptor appeared relatively specific for C7–C10 saturated aliphatic aldehydes. Among a 74-odorant panel, including both aromatic and short-chain aliphatic compounds with various functional groups, they found that, in addition to octanal, the most active odorant, there were significantly increased responses to heptanal, nonanal and decanal, but not with other compounds. The results indicated that a member of the olfactory multigene family identified by Buck and Axel (1991) does indeed encode an OR that mediates a physiological response to a specific set of odorants. These were the first results to demonstrate that ORs display specificity, but not to a very narrow extent.

The same approach was successfully performed for mOR23 to list the odorants that provoke the response of individual olfactory neurons responding to lyral from which the receptor genes were functionally cloned (Touhara *et al*., 1999). The procedure combined calcium imaging and single-cell reverse transcription–polymerase chain reaction (RT–PCR: Malnic *et al*., 1999). The ligand specificity of mORI7 and mOR23 means that ORs recognised various odorants that possessed some chemical similarity, consistent with a mode of ligand recognition of other GPCRs. This procedure led to a breakthrough in linking an odorant with its receptor(s). It is based on the mutually exclusive and monoallelic expression of ORs, which means that an olfactory neuron expresses a single OR (Nef *et al*., 1992; Ressler *et al*., 1993; Serizawa *et al*., 2000; Strotmann *et al*., 1994; Vassar *et al*., 1993). It proceeds in two steps: the response profiles of isolated, individual olfactory neurons to a panel of odorants is examined by calcium imaging, and the OR that is solely expressed in each neuron is then identified by single-cell RT–PCR. In the study carried out by Malnic *et al*. (1999), 13 ORs from 14 olfactory neurons were identified as responding to some of 17 aliphatic odorants. More details concerning these experiments aimed at deciphering odorant–OR pairing can be found in a review by Touhara (2002).

The results showed that an OR from an individual olfactory neuron recognises multiple odorants and that a single odorant is recognised by multiple receptors in different neurons – a landmark clearly set forth by Malnic *et al*. (1999) who were the first to decipher the combinatorial receptor code for odours. These results are in total agreement with those obtained by electrophysiological measurements using isolated rat neurons (Duchamp-Viret *et al*.,

1999) and calcium imaging performed on mouse intact olfactory epithelium (Ma & Shepherd, 2000). In addition, different odorants are recognised by different OR combinations. This capability of ORs to bind several odorants is well adapted for encoding the vast odour space. Araneda *et al*. (2000) showed that there is a high specificity for certain molecular features, and high tolerance for others, which enables the olfactory system to be both highly discriminating and to recognise several myriad odorants. Moreover, since each of the ORs recognising a common odorant shows different affinities to the odorant, the repertoire of the receptors activated by an odorant changes as a function of concentrations (Kajiya *et al*., 2001). Homologous ORs have been shown to bind overlapping sets of odorants with different specificity and affinity, demonstrating that a change in the concentration of an odorant results in a change in its receptor code. These results may fairly explain our experience that the quality of some odorants is perceived differently at different concentrations. The evaluation of affinity constants of odorants for diverse ORs is less documented than for OBPs. The threshold of detection in reconstituted systems can be found to be comparable to physiological odorant concentrations ($10^{-6} - 10^{-8}$ M) (Gaillard *et al*., 2002; Krautwurst *et al*., 1998), although most studies used between mM and μM odorant concentrations to obtain responding cells (Kajiya *et al*., 2001; Wetzel *et al*., 1999). Waiting for more information, one would expect dissociation constants to be in the μM–nM range.

As regards odour-intensity coding, it has been shown that more olfactory neurons, i.e. more ORs, are activated at higher concentrations of odorants (Ma & Shepherd, 2000). Recently, Hamana *et al*. (2003) investigated the sensitivity-dependent odour coding by mouse ORs. They isolated 2740 mouse olfactory neurons and classified them in terms of their sensitivities and tuning specificities to a chiral pair of odorants, *S*(+)-carvone (caraway-like odour) and *R*(−)-carvone (spearmint-like odour). Using a calcium-imaging approach, they found that the majority of ORs, which were activated at the lowest stimulus concentration, represented the principal odour qualities characteristic of each enantiomer. The number of chiral-non-discriminating ORs increased by 3.7 times that of *R*(−)-carvone-sensitive ORs when the stimulus concentration was augmented tenfold. They observed that more than 80% of the responsive ORs exhibited overlapping sensitivities between the enantiomers. This study suggests that the information processing of odours involves the selective weighting of the signals from the most sensitive ORs, and that principal odour qualities are encoded by the most sensitive ORs, whereas lower-importance odour qualities are encoded by less sensitive ORs. Generalisation of these results needs, however, further experiments with several diverse odorants.

4.4.4 Odorant-binding site in the receptor transmembrane bundle

In the absence of a significant ectodomain, the OR binding site for odorants has to be located either in external loops or in the pocket formed by the α-helix bundle of the transmembrane domain. As already mentioned, analysis of external loop sequences does not show large variability. Most interesting is that there is a region of hypervariability, where the OR sequences show particularly strong divergence, located in the third, fourth and fifth transmembrane regions. The more variable transmembrane segments 3, 4, and 5 were then assigned to the odorant-binding region, the more so as the inferred variable helical faces are largely positioned towards the interior of the receptor transmembrane bundle. A limited set of hypervariable residues, which point to the barrel interior and are more extracellularly disposed, may constitute the odorant complementarity–determining regions. Reviewing these OR properties, hypotheses were proposed for the molecular mechanisms underlying the OR functions and a global model built in order to locate the known functional mutations in TM3–TM6 (Shepherd *et al*., 1996; Singer & Shepherd, 1994; Singer *et al*., 1995, 1996). The accumulation of hundreds of OR sequences allowed Pilpel and Lancet (1999) to improve this model. In the 3D model of rhodopsin, these three α-helical barrels face each other and form a pocket about one-third of the way into the membrane (Palczewski *et al*., 2000). A similar arrangement is therefore thought to occur in all GPCRs belonging to subfamily 1a (Pilpel & Lancet, 1999). Based on studies from other GPCRs of this subfamily, such as adrenergic receptors (Kobilka *et al*., 1988), and in common with the binding site of retinal in rhodopsin (Palczewski *et al*., 2000), this pocket is the probable binding site for ligands. The variability among hundreds of OR sequences, based on natural variations observed in vertebrates in the region homologous to the rhodopsin binding pocket, has been used to roughly decipher the odorant-binding site, and provides the first molecular basis for understanding the range, diversity and large number of odorants that can be detected and discriminated (Pilpel & Lancet, 1999; Shepherd *et al*., 1996). More recently, Liu *et al*. (2003) applied an automatic process to a database of mammalian OR genes in order to map the sequence motifs that may correlate with function, especially those involved in ligand binding. Figure 4.8b summarises the data presently available.

The recent availability of detailed models of other GPCRs, based on the knowledge of rhodopsin 3D structure (Palczewski *et al*., 2000), permitted analysis of the OR amino acid variability patterns in a more precise structural context owing to OR 3D-structure prediction at the atomic level (Singer, 2000; Vaidehi *et al*., 2002). These authors studied a receptor known as I7 as the mouse and rat orthologues of this OR showed a differential response with one being more sensitive to *n*-octanal (Zhao *et al*., 1998) and the other to *n*-heptanal (Krautwurst *et al*., 1998). The functional expression of a rat recep-

tor, rORI7, and its counterpart in mouse, mORI7, was performed by Krautwurst *et al*. (1998). The experiments showed that rORI7 responded to *n*-octanal at a lower concentration than to *n*-heptanal, whereas mORI7 did the reverse. Remarkably, this difference in ligand specificity could apparently be attributed to a single residue change. Among the 15 amino acids that are different in the two genes, only three are located between TM2 and TM7, with a single residue in transmembrane domain TM5 (K90E in the first extracellular loop, V206I in TM5 and F290L in TM7). The substitution of valine for isoleucine at position 206, through site-directed mutagenesis, caused rORI7 to recognise *n*-heptanal preferentially, and the substitution of isoleucine with valine led mORI7 to recognise *n*-octanal preferentially. Interestingly, the nature of these changes, valine versus isoleucine and *n*-octanal versus *n*-heptanal, is consistent with compensatory alterations in the structures of odorant and receptor, as shown by Singer (2000) who modelled the ORI7.

Nonetheless, other authors reported recently that both mORI7 and rORI7 are activated by both *n*-octanal and *n*-heptanal (Bozza *et al*., 2002; Gaillard *et al*., 2002). Using novel 3D-structure prediction and molecular-docking algorithms developed and successfully tested with other GPCRs, Vaidehi *et al*. (2002) predicted independent structures for both rORI7 and mORI7, which proved to be very close. The calculated binding energies for *n*-octanal and *n*-heptanal in rORI7 and mORI7 indicated that little or no preference exists for *n*-octanal over *n*-heptanal for these receptors, as predicted by the model of Singer (2000) and in agreement with the experiments of Bozza *et al*. (2002). However, one cannot exclude that these minor differences might lead to a difference in the activation of G-protein second-messenger pathway. In the model of Vaidehi *et al*. (2002), TM3, TM4 and TM6 form the binding cavity, but not TM5. Lys164 forms a hydrogen bond and is critical in recognising the aldehyde functional group for both *n*-octanal and *n*-heptanal, in disagreement with previous modelling of rORI7 by Singer (2000). Other residues important in binding are Phe109, Cys114, Cys117 and Ile255. They found that none of the sequence differences between rORI7 and mORI7 are located near the binding pocket, supporting the similarity of binding profiles found by Bozza *et al*. (2002).

This anecdote illustrates that by now GPCR prediction has to be taken with caution and that functional experiments also still lack reliability. Nevertheless, the prediction of OR functional sites provides suggestions for site-directed mutagenesis experiments to alter odorant specificity and this promises to be a potential development with the further improvement of 3D-structure prediction and molecular docking. Recently, the structure of another mouse OR, mORS25, identified by Malnic *et al*. (1999), was modelled through well-established molecular dynamics methods. *In silico* docking studies were conducted with 24 odorants (Floriano *et al*., 2000) and then compared with odorant specificity obtained by single-cell RT–PCR. This predicted OR

structure revealed a likely odorant-binding site composed of residues from TM3 to TM7. Floriano *et al*. (2000) also independently predicted the two compounds (hexanol and heptanol) that experimentally best activate mORS25. These results confirm that ORs exhibit broad specificities and this combined approach significantly enhanced the number of assigned odorants for the many members of the OR family.

4.4.5 Conclusions on the odorant–OR structural relationships and odotope definition

The predicted 3D structures and binding sites allow the design of mutagenesis experiments to validate and improve the structure–function prediction methods. However, to date, only few site-directed mutation experiments have been reported (Krautwurst *et al*., 1998), although this approach has largely been used to investigate structure–activity relationships of many other GPCRs. As these structures are validated, they can be used as targets for the design of new receptor-selective antagonists or agonists for ORs. Such results provide the impetus for developing a pharmacology of ORs that would produce activity matrices of large numbers of receptors tested against equally large chemical libraries.

The RT–PCR cloning approach to identify retrospectively the exclusive OR gene that neurons express permitted the functional characterisation of ORs that specifically bind a particular odorant. This strategy allowed the identification of many ORs, which could all potentially recognise a single odorant. Surprisingly, a broad specificity was found for all ORs so far studied. Their molecular receptive ranges were found to be highly tolerant in some aspects, but highly specific in other areas, therefore establishing a complex combination of ORs, which bind a specific odorant. The direct experimental evidence for this combinatorial code model was provided by recent reconstitution studies in which structurally related ORs recognised overlapping sets of odorants with distinct ligand specificity and affinity (Kajiya *et al*., 2001).

The location of the binding site is now well established as being in a pocket formed by the transmembrane helices 3–7, opened to the outside of the cell. Docking experiments using the whole molecule have failed to find any other location for the odorant-binding site (Floriano *et al*., 2000; Vaidehi *et al*., 2002). However, direct evidence needs to be provided to confirm this highly plausible model. In addition to the odorant-binding pocket, another structural feature that seems to be important for OR functioning is the second external loop, which is specific to ORs.

In summary, each odorant is encoded by a unique set of ORs at a given concentration. The olfactory system determines the odour quality by identification of the combination of activated ORs, which may therefore vary as a function of odorant concentration. There are some parallels between odorant

binding to ORs and the immune system, which may prove useful. Structural relationships between antibodies and antigens are now very well understood. The immune system expresses proteins that exactly fit the 3D structure of antigenic determinants called epitopes. Fine-tuning is performed between the hypervariable part of the antibody, called the paratope, and one epitope of the antigen. A given antigen usually bears several epitopes, each of them being recognised by an antibody (called monoclonal), so that a set of several antibodies (called polyclonal) specifically binds to the antigen at different sites. Although the molecular processing used to generate several hundred ORs is quite different from that used to create hundreds of millions of antibodies, we can define the odotope as the region of an odorant molecule that is specifically recognised by one OR. This does not imply that a single OR must bind a given odotope. It is plausible that different ORs can bind a given odotope, but with variable affinity. One odorant may bind several receptors through different odotopes, resulting in a combinatorial activation of a limited set of receptors that is specific for an odorant. Reciprocally, one receptor may bind different odorants through a similar odotope. The odour coding results then from the combination of information originating from activation of different receptors, which is transmitted to the olfactory bulb. Like the immune complex, the olfactory system is built on an open logic system conditioned by the impossibility to predict, a priori, what molecules it might encounter. This apparently fuzzy system allows maintaining an indeterminate but nonetheless precise sensory array. However, it raises difficulty in the classification of odours (Rossiter, 1996).

4.4.6 OR classification and genome comparison

Our purpose here is not to describe the olfactory genomes (i.e. the repertoire of OR genes) in great detail, but only to emphasise some features that may be helpful to understand how the human olfactory system has evolved and is able to detect and recognise tens of thousands of odours. We will deal with the classification of ORs, their nomenclature, the comparison of OR genomes among vertebrates and also invertebrates (nematodes and insects) and the diversity of human olfactory genomes.

4.4.6.1 Classification of the OR gene family

When comparing sequences, ORs can be divided into subfamilies such that the members of a given subfamily share significant sequence conservation (Buck & Axel, 1991). The criterion of 60% sequence identity has been used to classify ORs into different subfamilies (Glusman *et al*., 2000; Zhang & Firestein, 2002). The range of divergence is considerable, with some ORs in different subfamilies sharing less than 20% identity, whereas ORs in the same subfamily may differ by only a few amino acids. Genomic Southern blot

analysis was formerly used to classify ORs, profiting from the absence of introns in the short 1 kb coding region of the OR. Nowadays, sequence comparisons are widely used and allowed a clear-cut classification (Glusman *et al*., 2000). A major subdivision was proposed by analysis of the genome of the amphibian *Xenopus*, which is endowed with a gene repertoire encoding both 'fish-like' receptors and 'mammalian-like' receptors (Freitag *et al*., 1995). Class I comprises 'fish-like' ORs, whereas 'mammalian-like' ORs are gathered in Class II. The recently cloned ORs from the ancient vertebrate lamprey represent another distinct class (Berghard & Dryer, 1998).

The human OR genes, like other mammals, contain some Class I 'fish-like' sequences (Zozulya *et al*., 2001). Rouquier *et al*. (1998, 2000) showed that 72% of human OR genes are pseudogenes, i.e. genes that cannot be expressed as protein. Generally, the genes for human Class I ORs have fewer pseudogenes (52%) than those in Class II (77%) (Glusman *et al*., 2001). A recent article on the human OR repertoire reports the identification, physical cloning and comparative sequence alignment of the 347 putative human full-length OR genes (Zozulya *et al*., 2001). Analysis of the human genome draft sequences has revealed a more complete portrait of the OR gene repertoire in humans than was available previously: Crasto *et al*. (2001) reported 368 full-length and potentially active human OR sequences and Zozulya *et al*. (2001) reported 347 genes. As regards the chromosomal location of human OR genes, Rouquier *et al*. (2000) determined that they are distributed over all chromosomes except chromosomes 20 and Y. Most of the OR genes are gathered in two main clusters on chromosome 11. Next in order of frequency are those on chromosomes 1, 9, 6 and 14, whereas chromosomes 10, 22 and X each carry only one OR gene. None of the 347 genes encoding putative functional ORs identified by Zozulya *et al*. (2001) were found on chromosomes 2, 4, 18 or 21.

4.4.6.2 OR nomenclature

Developing a systematic classification and nomenclature for this huge gene superfamily proved to be a very difficult task, since the average percentage identity for OR is only 27% (Zozulya *et al*., 2001). At present, several nomenclatures are being used. The one proposed by Glusman *et al*. (2001) is based on sequence identity percentage as illustrated in the Human Olfactory Receptor Data Exploratorium website <http://bioinformatics. weizmann.ac.il/HORDE> (Safran *et al*., 2003). Another nomenclature for functional human OR candidates has been proposed at Senomyx, Inc. (La Jolla, CA, USA) by Zozulya *et al*. (2001) reflecting both phylogenetic clustering and chromosomal localisation. The sequences of all the available OR genes are accessible on the web at the Olfactory Receptor Database <http://senselab. med.yale. edu/senselab/ORDB/default.asp>, with a classification simply based on the chronological order of sequence publication (Crasto *et al*., 2002). More details

can be found in recent reviews concerning human OR genes (Crasto *et al*., 2001; Mombaerts, 2001).

4.4.6.3 Variability of ORs within the human species

Allelic variants of human OR genes presumably occur very frequently as first reported for a limited number of subjects (Menashe *et al*., 2002). Such a study is not an easy task because the high sequence similarity of some OR genes makes it difficult to distinguish between alleles and genes. Menashe *et al*. (2002) investigated the population differences in patterns of single nucleotide polymorphisms (SNPs) for a 400 kb OR gene cluster on human chromosome 17. Samples came from four different ethnogeographical origins and allowed 74 SNPs to be identified. These analyses revealed substantial differences in nucleotide diversity and haplotype distribution among the different human populations. For instance, three functional SNPs were observed to segregate at different frequencies in the various ethnogeographical groups. In a very recent paper, Menashe *et al*. (2003) investigated whether the most recent OR gene disruptions might still segregate with the intact form by genotyping 51 candidate genes in 189 ethnically diverse humans. They revealed an unprecedented prevalence of segregating pseudogenes, identifying one of the most pronounced cases of functional population diversity in the human genome. This suggests that different evolutionary pressures may have shaped the chemosensory repertoire in different human populations. These original data suggest that OR genes may have evolved to create different functional repertoires in distinct human populations. The existence of OR alleles in the human population offers the exciting opportunity to correlate olfactory function, determined by psychophysical assays, directly with specific OR genes.

4.4.6.4 Comparison of the OR number between man and animal and between vertebrates and insects

In mammals, the OR gene repertoire has been estimated to consist of 500–1000 genes, making the OR gene family the largest in the genome yet identified, which comprises 2–3% of the entire coding genome. In rodents (rat, mouse), as many as 1000 OR genes per haploid genome are observed (Nef *et al*., 1992; Ressler *et al*., 1993; Rouquier *et al*., 2000; Strotman *et al*., 1994), while in the dog, the size of the family was estimated to be more than 400 genes (Parmentier *et al*., 1992). The OR gene superfamily of the mouse has been recently analysed, thanks to the nearly complete genome established by Celera (Zhang & Firestein, 2002). As many as 1296 mouse OR genes were found, which were distributed on all chromosomes except 12 and Y, like human OR genes with only about 20% pseudogenes. A large number of apparently functional 'fish-like' Class I OR genes in the mouse genome suggest that they may have important roles in mammalian olfaction. As reported by Glusman *et al*. (2001), Class I ORs, previously considered

a relic in terrestrial vertebrates, constitute as much as 10% of the human repertoire, in which they exhibit a lower pseudogene fraction, suggesting also a functional significance. Fish and amphibians are less well endowed, with about 100 ORs. In catfish, the OR gene family was first estimated to contain less than 100 genes (Ngai *et al*., 1993). In zebrafish, the size of the OR gene family was estimated to be in a similar range (Barth *et al*., 1996; Weth *et al*., 1996). The significantly smaller number of receptors in fish may reflect the smaller repertoire of aquatic odorants.

Receptor gene families have also been described in two invertebrate species, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. In the latter species, the number of ORs was established to be about 62 (Vosshall *et al*., 2000), far fewer than in vertebrates. Analysis of the recently deciphered *Anopheles gambiae* genome showed the existence of a similar number, 79 candidate ORs (Hill *et al*., 2002). In *Caenorhabditis elegans*, about 500 functional OR genes were counted (Ngai *et al*., 1993).

4.4.6.5 Human OR genome compared to macrosmatic animals

Comparisons of the OR gene sequences of humans and other species have been carried out to identify orthologues (i.e. related genes whose mutations are randomly scattered all along the sequence) and map out the evolutionary patterns of the development of ORs. For instance, Lane *et al*. (2001) identified seven functional orthologues between OR genes of the mouse and human P2 clusters. Eighteen mouse and eight human ORs were identified, and the orthologues showed greater than 80% identity. The smaller size of the P2 gene cluster in humans supports the conclusions of Rouquier *et al*. (2000) that the range and acuity of the human OR array is diminished compared to rodents. Nevertheless, the recent genome analysis of the mouse OR genes (Zhang & Firestein, 2002) clearly revealed that human ORs cover a similar odour space as the mouse ORs, suggesting that the human olfactory system has retained the ability to recognise a broad spectrum of chemicals even though humans have lost nearly two-thirds of the OR genes as compared to mice. Rouquier *et al*. (1998, 2000) showed that more than two-thirds of human OR genes are pseudogenes, and that a significant reduction of potentially active OR genes is found in prosimian primates (the Old World apes). Lower primates (the New World monkeys) possess less pseudogenes, whereas such inactive genes are more rarely observed in rodents or zebrafish. Similar results were recently obtained by comparing human ORs to their orthologues in apes (Gilad *et al*., 2003). When primates stood up, as they gained a more acute vision, they concomitantly lost olfactory potential. Such an evolutionary process is also observed for the functional vomeronasal organ (VNO), which is not present in the Old World apes and humans (Trotier *et al*., 2000). In other mammals, some pheromones are detected primarily by this specialised anatomical structure, so called for its proximity to the vomer bone located at

the base of the nasal septum (Døving & Trotier, 1998; Keverne, 1999). VNO neurons are connected to the accessory olfactory bulb and to the brain centres involved in neuroendocrine regulation.

4.4.7 Other possible functions for ORs

In addition to their role in odorant binding and olfactory signal transduction, ORs have been shown to have other functions. They are thought to play a role in axon guidance to the correct glomeruli during the development and regeneration of olfactory neurons, in agreement with the observation that olfactory neurons expressing the same ORs project their axons to the same set of glomeruli in the olfactory bulb (Mombaerts *et al*., 1996; Ressler *et al*., 1994; Singer *et al*., 1995; Vassar *et al*., 1994; Wang *et al*., 1998). This hypothesis was experimentally tested by gene knockout and replacement, which showed that the OR is required for convergence of olfactory neuron axons (Mombaerts *et al*., 1996; Wang *et al*., 1998). In addition, using OR minigenes that coexpress histochemical markers, Vassalli *et al*. (2002) have recently shown that the determinants in the sensory neurons required to generate the stereotyped olfactory bulb map are the same as those needed for appropriate expression of the OR.

About 10% of mammalian ORs are transcribed in testes, and have been detected on mature spermatozoa (Asai *et al*., 1996; Parmentier *et al*., 1992; Walensky *et al*., 1995, 1998). ORs may then be involved in chemotaxis of sperm during fertilisation. Most testis ORs are also expressed in olfactory epithelium, but some receptors seem to be only expressed in testes (Vanderhaeghen *et al*., 1993). Expression of OR genes in the male germline may result from loose regulation of transcription, but, as most testicular ORs are not pseudogenes, it can be proposed that these genes fulfil a specific biological role. Such roles could be in regulating sperm maturation, sperm motility (chemokinesis) or sperm attraction to oocytes (chemotaxis). Branscomb *et al*. (2000) tried to elucidate whether testis-expressed ORs play roles in sperm chemotaxis or are ordinary nasal ORs that are expressed gratuitously in testes. They analysed the evolution of these ORs, since, under the sperm chemotaxis hypothesis, testis ORs should be subject to intense sexual selection. Their results suggested that some ORs might perform internal non-olfactory functions in testes, but other hypotheses could not be definitely discarded. Since several ORs were observed to be expressed predominantly, even exclusively, in human spermatogenic cells, and since these ORs are located in the spermatozoa flagellar midpiece, several authors have investigated the function of ORs that reside in spermatozoa (Parmentier *et al*., 1992; Spehr *et al*., 2003; Vanderhaeghen *et al*., 1993). Spehr *et al*. (2003) cloned and functionally expressed a previously undescribed human testicular OR named hOR17-4. Using calcium imaging, they established the molecular receptive fields for this OR, both native and expressed in HEK cells. They showed

that aromatic aldehydes, notably bourgeonal, lilial and florazone, were powerful agonists for hOR17-4 (both recombinant and native). Bourgeonal was also independently proved to be a strong chemoattractant for spermatozoa. In contrast, undecanal was observed to be a potent OR antagonist to hOR17-4. These results indicate that at least one OR, but plausibly many others, play a role in human sperm chemotaxis and in the fertilisation process.

In addition, it was recently observed that polymorphic genes of the human major histocompatibility complex (important in determining resistance to parasites and avoidance of inbreeding) were linked to testis-OR genes, which might not only participate in olfaction-guided mate choice but also in selection processes within the testis (Ziegler *et al*., 2002). These data suggest that testes-expressed polymorphic human leucocyte antigen (HLA) and OR are functionally connected in order to serve the selection of spermatozoa, enabling them to distinguish 'self' from 'non-self'. Finally, it has been proposed that some ORs may play still unknown roles during early development as observed in the olfactory system (Leibovici *et al*., 1996; Nef *et al*., 1992, 1996), and even in the heart (Drutel *et al*., 1995).

4.4.8 Concluding remarks about ORs

Mechanisms of odorant binding have been studied in cellular expression systems, in conditions far from natural. For instance, odorants are applied in solution, not as airborne molecules. Molecular and cellular biology data are combined with molecular modelling of ligands and receptors, which gives evidence of interactions between the odorants and a binding pocket in the receptor. However, direct observations supporting the very plausible hypothesis that the binding pocket resembles that of other GPCRs are necessary to validate this model, e.g. through site-directed mutagenesis or affinity labelling in order to identify the residues specific to the binding pockets of different ORs with different odorant specificity. It is, however, remarkable that the diverse approaches summarised here have yielded largely convergent results, which have been validated by data mining of the human and other genomes. In hardly more than ten years, we have changed from the conceptual idea of the odour receptor (without physical reality) to the molecular description of actual proteins. Moreover, these proteins belong to one of the most studied families (GPCRs) for which very powerful pharmacology approaches are being developed. Another remark about OR ligands is that whereas only a very few number of studies identify odorants that play the agonist role for a very limited number of ORs, only one (Spehr *et al*., 2003) has very recently dealt with antagonists, in the case of testis ORs. This discovery is very important, since it shows that some odorants may inhibit the binding of others, which is a key to understand how some odours can be masked by others.

Moreover, we have no idea of the quantitative aspects of OR expression: we know neither how many copies are expressed within a neuron nor how many neurons are currently expressing the same OR. The next step in OR studies should produce evidence for the regulatory mechanisms involved in gene expression. This important question is related to alteration of the OR genome expression in the course of development all along the lifespan of the organism: Are all the OR genes expressed simultaneously, or are there some stages when some are specifically expressed? For instance, one may speculate that 'fish-like' Class I ORs, which are devoted to aquatic life and binding of hydrosoluble odorants, would be more strongly expressed during fetal life and be silenced after birth in mammals. Changes in preferences in the course of adolescence or in old age might also be related to alterations in OR expression. It would also be very interesting to study the OR pattern expression as a function of physiological and environmental conditions, in order to better understand, e.g. why certain odours, which are pleasant before eating, become unpleasant when satiety is reached. This information will need to be integrated with studies of odour maps and neuronal circuits in the olfactory pathway before understanding the brain mechanisms underlying odour perception. The relationships between the conscious odour perception and the actual capture of odorants are an exciting challenge which has to take together all the many factors occurring in the coding processes that alter the initial signal coming from olfactory neurons.

4.5 Biochemical mechanisms involved in odorant capture

Although an understanding of the structural and binding features of the main molecules involved in odorant capture (namely, OBP and OR) is becoming clear, the interactions between OBP and OR, as well as the relationships between OR and other proteins, which lead to a neural signal, are not yet well understood.

4.5.1 Interactions of OBP with OR

The crucial biological role of OBPs, which has been proven in insects, has not yet been demonstrated in vertebrates. A plausible hypothesis is that, in the absence of an odorant, the OBP cannot interact with the OR, and that an odorant-induced conformational change might trigger the association of the odorant–OBP complex onto the receptor, before transfer of the odorous molecule from the lipocalin pocket to the receptor binding site. The sequence conservation of the peculiar second extracellular loop of ORs and the OBP-specific consensus sequence are good reasons to propose such a mechanism. Thus, OBPs could not only dissolve hydrophobic odorants but also transport and deliver them specifically to the ORs. Without this specificity, odorants

might be transported to other nonreceptor sites, like the membrane lipids, reducing their detection. This theory that ORs are only able to recognise the OBP–odorant complex suggests that OBPs, after complexing their ligands, would interact with ORs through specific binding. Receptors for lipocalins have indeed been described (see Flower, 2000 for review), e.g. the human tear lipocalin-1 receptor (Wojnar *et al*., 2001). Although this receptor does not belong to the GPCR family, some GPCRs are well known to interact with proteins as agonists (see Bockaert & Pin, 1999 for review).

Initial investigations concerning interactions of ORs with OBPs were reported by Boudjelal *et al*. (1996) in an attempt to unveil the mechanism of odorant capture. They showed that radiolabelled bovine OBP was able to specifically bind isolated membranes from bovine nasal mucosa and reveal a single population of binding sites. OBP binding was abolished upon proteolytic treatments of these membranes and inhibited by lipocalins, which are similar with OBP and bind similar ligands (rat and mouse major urinary proteins). Nevertheless, this putative OBP receptor was not restricted to olfactory tissues. More recently, Matarazzo *et al*. (2002) studied the interaction of radiolabelled porcine OBP with two human ORs that were stably expressed in mammalian COS cells in culture as fusion proteins with green fluorescent protein (GFP), a fluorescent reporter protein. They observed that only one of the two ORs was able to exhibit specific and saturable binding for porcine OBP with a dissociation constant around 10 nM in the absence of an odorant. However, they did not test any odorant on the OR–OBP association. Like Boudjelal *et al*. (1996), they found that the radiolabelled OBP bound mainly olfactory tissues, but was also able to interact with other tissues. Using fluorescence spectroscopy, we also observed that hOBP-2A is co-localised with the human recombinant receptor OR17-40 at the surface of the cell membrane (Figure 4.10). Nevertheless, these rare results are not convincing enough to infer a reasonable molecular mechanism of odorant capture. They have to be confirmed by more rigorous data, obtained with proteins originating from the same species, and verified in the presence and absence of odorants known to be captured by the ORs studied as well as being delivered in physiological conditions, i.e. as airborne molecules.

However, odorant response of ORs, expressed in heterologous cultured cells and measured by calcium imaging, has been demonstrated in the absence of added OBPs, which does not exclude a better affinity of the receptor for the OBP–odorant complex. The model of bacterial chemotaxis (Koshland, 1981) suggests that OBPs, like the equivalent bacterial periplasmic protein, would undergo a significant conformational change upon odorant complexation before being able to interact with the receptor. Such a transconformation has not been observed for porcine OBP (Paolini *et al*., 1999; Vincent *et al*., 2000), but recent studies on rat OBP-1F show a limited conformational change (Nespoulous *et al*., 2004).

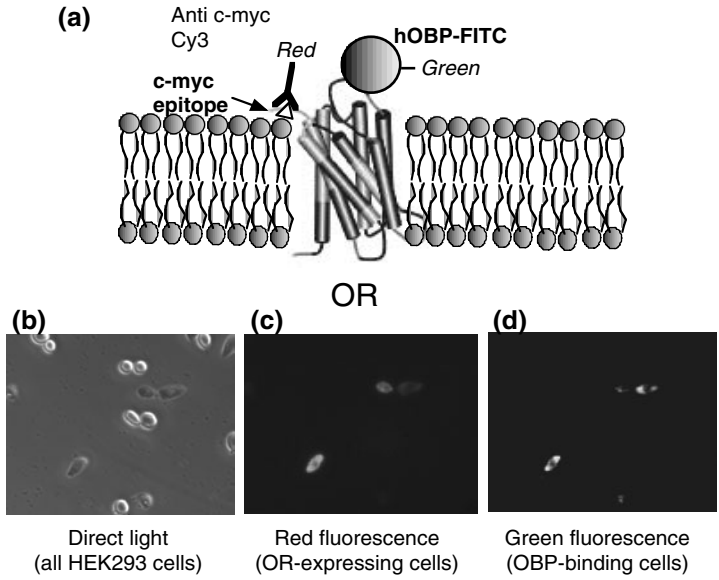

Figure 4.10 Fluorescence microscopy observation of OBP–OR interaction. (a) Schema describing the OBP–OR interaction detection method. HEK293 cells are transfected with a cloned OR gene tagged with c-myc epitope; cells transiently expressing the OR gene are visualised with an antibody raised against the c-myc epitope conjugated with a red fluorescent dye (Cy3); hOBP-2A is revealed using chemical labelling with a green fluorescent group (FITC); HEK293 cells are incubated for 30 min at 37°C with Cy3-conjugated anti-c-myc antibody and FITC-hOBP-2A derivative. Due to 50% transfection efficiency, all the cells do not express OR but all cells expressing OR bind OBP, as revealed by co-localisation. (b) Transfected HEK293 cells observed under bright field. (c) Cells expressing the OR protein, visualised through red fluorescence. (d) Cells interacting with hOBP-2A, visualised through green fluorescence.

A final thought on OBP–OR interaction concerns the expression of an hOBP transcript in testes (Lacazette *et al*., 2000). It corresponds to hOBP-2A, which exhibits preferences for cyclic aldehydes (Briand *et al*., 2002a), and hOR17-4 that binds these aldehydes, which is also specifically expressed in sperm (Spehr *et al*., 2003). This expression of a 'pair' of OBP receptor and OR for the same class of molecule adds weight to the notion of functional interaction between hOR17-4 and hOBP-2A.

4.5.2 Signal transduction in olfactory neurons and neural impulse formation

Following odorant binding by OR, a cascade of events begins that transforms the chemical recognition into a neural signal, leading to a change in the membrane potential of the olfactory neuron (see Firestein, 2001 for review).

4.5.2.1 Receptor activation and downstream signalling

This process is being studied for some GPCRs and will only be briefly summarised in this chapter (Figure 4.7). Deciphering this signalling pathway was noted by changes in the different components of the pathway, which resulted in loss of transduction. Agonist activation of the receptors induces conformational changes, which are, up to now, poorly understood. Conformational changes seem to involve, at minimum, rearrangements of membrane helices 6 and 3 (Ballesteros *et al*., 2001; Farahbakhsh *et al*., 1995). Once the receptor is activated by its agonist, i.e. an odorant molecule, it can interact with the heterotrimeric G protein, and serves as a guanine-nucleotide exchange factor to promote guanosine diphosphate (GDP) dissociation, and GTP binding and activation. In the current model, the activated heterotrimer dissociates into an α-subunit and a $\beta\gamma$-dimer, both of which have an independent capacity to regulate separate effectors. Hydrolysis of GTP to GDP leads to reassociation of the heterotrimer and termination of the activation cycle (Ross & Wilkie, 2000). In the case of ORs, the odorant-bound OR activates a specific G protein named G_{olf}, which in turn activates an AC, named AC III, as an effector. This enzyme converts the intracellular ATP into cAMP, an intracellular signalling molecule that binds to the intracellular face of a cyclic nucleotide-gated (CNG) ion channel embedded in the plasma membrane of the olfactory neuron, enabling it to conduct cations such as Na^+ and Ca^{2+} (Firestein *et al*., 1991). When the CNG channels open, the olfactory neuron is depolarised, so that the cell reaches threshold and generates an action potential which is propagated along the axon, reaching neurons in the olfactory bulb. This second-messenger process provides amplification and integration of odorant binding to ORs. A single odorant molecule is thought to be sufficient to produce a measurable electrical event in an olfactory neuron, and correlated opening of just a few channels could induce action-potential generation (Lynch & Barry, 1989; Menini *et al*., 1995). Other effectors have been shown to be involved in OR signalling, responsible for other pathways. As reviewed by Paysan and Breer (2001), some potent odorants were found not to induce the cAMP pathway, but to elicit an increase of IP_3 through activation of a PLC as an effector, involving a G protein other than G_{olf} (Schandar *et al*., 1998). Additional events may also occur to increase the excitatory response magnitude. Integration of these events and their physiology has been thoroughly reviewed (Rawson & Gomez, 2002). This simplified view is complemented by the information in Chapter 3.

4.5.2.2 OR desensitisation

A last crucial point needs to be underlined, namely desensitisation, which is of prime importance for deactivation of olfactory signals. Of course, desensitisation processes may occur at different steps of the signalling cascade and could be different. Such mechanisms operate at both the level of the receptor as well as downstream, e.g. at the level of G proteins. We will only

deal with those that directly involve OR molecules. Other regulations include a variety of events, described in other chapters, which comprise, e.g. a recently discovered protein named regulator of G-protein signalling (RGS) that apparently acts on the AC to decrease its activity (Sinnarajah *et al*., 2001).

Desensitisation means that signalling dampens rapidly, even in the presence of continuing agonist stimulation. At the receptor level, several desensitisation mechanisms may occur (see Pierce *et al*., 2002 for review). Rapid dampening of receptor function is usually controlled by receptor phosphorylation, which is mediated by second-messenger kinases, or by a distinct family of G protein–coupled receptor kinases (GRKs) (Pitcher *et al*., 1998). Kinases that phosphorylate activated receptors send them into a desensitised state (Dawson *et al*., 1993; Schleicher *et al*., 1993). As regards second-messenger kinase regulation, phosphorylation of the endodomain and intracytoplasmic loops directly uncouples receptors from their respective G proteins, and thereby works as classical negative-feedback regulatory loops.

Opposite is the GRK–β-arrestin system, another very general mechanism for regulating GPCR activity (Krupnick & Benovic, 1998; Pitcher *et al*., 1998). This mediates ligand-specific desensitisation, since only the ligand-activated conformation of the receptor is phosphorylated by GRKs. GRK phosphorylation induces binding of another protein, called arrestin, to the receptor, which sterically inhibits further interactions between the receptor and the G protein. Usually, arrestin binding leads to the redistribution of the receptor away from the cell surface in clathrin-coated pits. This process is called endocytosis, alternatively internalisation. Receptor phosphorylation is then reversed by phosphatases that are localised in those intracellular vesicles to which the receptors traffic after stimulation and desensitisation (Krueger *et al*., 1997; Pitcher *et al*., 1995). Receptor phosphorylation generally operates over short periods of time (seconds to minutes). This mechanism has indeed been observed for ORs (Peppel *et al*., 1997; Walker *et al*., 1999). The regulation and control of these mechanisms are not so well known in the case of ORs, compared to other processes involving G proteins, and still need much more investigation.

4.5.3 Beyond the olfactory neuron

As reviewed by Zhao and Firestein (1999) and Crasto *et al*. (2001), in most vertebrates, only one OR gene is expressed in any given ORN (Chess *et al*., 1994; Lancet, 1986; Malnic *et al*., 1999; Vanderhaegen *et al*., 1993), i.e. ‘one neuron, one receptor’ (Axel, 1995). Even very homologous ORs are expressed in different neurons (Kubick *et al*., 1997; Leibovici *et al*., 1996). In the mouse, OR gene expression was shown to be derived exclusively from one of two alleles (Chess *et al*., 1994; Mombaerts *et al*., 1996). To study the mutually exclusive expression of OR genes, Serizawa *et al*. (2000) generated transgenic mice that carried the murine OR gene *MOR28*. Expression of the transgene

and the endogenous *MOR28* was differently visualised by using two different markers, β-galactosidase and GFP, respectively. This approach allowed the authors to demonstrate the mutually exclusive expression of OR transgenes. Nevertheless, the mechanisms underlying exclusion of the other alleles and other OR genes are not yet understood. Lane *et al*. (2001) failed to reveal conserved sequence motifs that might be related to transcription factor binding sites or promoter regions.

Not only is the expression of ORs clonal but also all neurons expressing the same receptor converge to the same olfactory glomerulus. In the olfactory epithelium of the mouse, there are about 5 000 000 olfactory sensory neurons (Resler *et al*., 1993), each of which expresses only one of about 1000 different OR genes (Malnic *et al*., 1999). Neurons expressing the same OR are scattered throughout one of the four olfactory epithelium zones that have corresponding zones in the olfactory bulb (Resler *et al*., 1993; Vassar *et al*., 1993). In the olfactory bulb, their axons form synapses with bulb mitral cells and tufted relay neurons in only a few of the 2000 glomeruli of the bulb (Mombaerts *et al*., 1996; Resler *et al*., 1994; Vassar *et al*., 1994), resulting in a stereotyped spatial map in which inputs from different ORs are targeted to different glomeruli and bulb neurons. Consistent with patterns of odour-induced activity (Buck, 1996; Hildebrandt & Shepherd, 1997; Mori *et al*., 1999), an odorant receptor code is represented by a dispersed ensemble of neurons and in the bulb by a specific combination of glomeruli. Using genetic tracing, Zou *et al*. (2001) showed that a stereotyped sensory map is further created in the olfactory cortex in which signals from a particular OR are targeted to specific clusters of neurons. They prepared transgenic mice that coexpressed a transneuronal protein tracer with only one of the different ORs. The tracer travelled from nasal neurons expressing that OR to the olfactory bulb and then to the olfactory cortex, allowing imaging of neurons that receive input from a particular OR. They demonstrated that inputs from different ORs overlap spatially and could be combined in single neurons, potentially allowing for an integration of the components of an odorant combinatorial receptor code. In addition, signals from the same OR are targeted to multiple olfactory cortical areas, permitting the parallel processing of inputs from a single OR before delivery to the neocortex and limbic system.

4.6 Conclusion

4.6.1 Complexity of stereochemical odorant recognition and subsequent odour coding

From the discussion above, it is clear that a receptor can interact with several different odorants but with different affinities (Malnic *et al*., 1999). Conse-

quently, odour coding involves sets of ORNs, each expressing a single OR, with varying degrees of activation in response to a given odour or odour mixture. Both odorant binding onto receptor and signal processing are therefore complex and fuzzy processes. In addition, taste and trigeminal neural signals are combined with odour signals in the brain to generate a global feeling that can hardly be analysed and whose components cannot be distinguished from each other. For instance, an odorant such as menthol is simultaneously recognised by two different kinds of receptors, an OR, which induces the odour perception, and an ion channel receptor, which mediates the cold feeling through trigeminal mediation (McKenny *et al*., 2002; Peier *et al*., 2002).

Up to now, nearly all ORs are orphan receptors, whose ligands are unknown. Only a very low number of ORs (less than 1%) have been assigned odorants in humans as well as in animals, since only an elite handful of ORs have been expressed in functional form. Such information is necessary to understand how odorants (and mixtures) are perceived by humans as a result of the concomitant activation of a limited set of ORs. Considerable effort is being invested using high throughput technology to identify those ORs that are of interest to the food industry (Schmiedeberg *et al*., 2003). This will be very helpful to understand how different odotopes of a given odorant bind different ORs. In addition, it will provide a key to decipher specific anosmias and their link to individual genomes. Considering this topic, it is worth noticing that, in mouse, one OR gene cluster matches a known locus mediating a specific anosmia, indicating that the anosmia may be directly due to the loss of ORs (Zhang & Firestein, 2002). As emphasised by Sanchez-Montañés and Pearce (2002), olfactory neurons form a population that possesses unspecific receptive fields. These authors showed that overlapping sensory neuron tunings that respond to common chemical compounds have better estimation performance than perfectly specific tunings. This type of organisation seems to be particularly suited to identify myriad odorants with a minimum number of OR genes.

Although presented as an alternative to the vibrational theory (Turin, 2002), the odotope theory is based on ligand–protein interactions, directly observed through structural biology. Such a molecular mechanism has been supported by thousands of examples in all domains of biology, e.g. the epitope–paratope interactions of antibody–antigen recognition, or the even more widely documented substrate–enzyme interactions. Numerous membrane–receptor interactions have also been observed, including GPCRs. This is notably detailed in the case of agonist–antagonist pharmacology, which involves GPCRs in more than half of medical applications. Nevertheless, in the case of ORs, which do not exhibit large soluble ectodomains, the direct observation of odorants interacting with the transmembrane helices is not easily achievable with current techniques. However, functional studies associated with genomic

comparison, based on natural mutations, and site-directed mutagenesis have clearly proven that odorants are recognised through a stereochemical-guided interaction with receptors embedded in the neuron plasma membrane. All these results, taken together with neurophysiology experiments, totally dismiss the vibrational theory.

4.6.2 Taste and VNO receptors compared to ORs

However, chemical senses not only involve ORs, but also TRs. In Chapter 3, TRs have been described but, here, the receptors that capture pheromones in the accessory olfactory system, located in the VNO linked to the accessory olfactory bulb, will be discussed. As reviewed by Firestein (2001), two additional families of GPCRs, unrelated to the family of ORs, have been identified in the mouse VNO which are not coupled to the same type of G proteins (Jia & Halpern, 1996). V1Rs are of the same general type of GPCR as the ORs (short external ectodomain), whereas V2Rs, which belong to GPCR family 3, have a long extracellular N-terminal region. However, in humans, the accessory olfactory system, which involves the VNO, is vestigial (Trotier *et al*., 2000) and all putative members of the VR family are pseudogenes, with only one exception whose role is unknown (Rodriguez *et al*., 2000).

Humans can detect and discriminate between at least five taste qualities: sweet, sour, salty, bitter and umami. Umami means delicious, and corresponds to glutamate taste. This is a vestigial chemical sense devoted to L-amino acid identification, which is of prime importance for animal survival. Sour and salty are perceived by ion channels (see Chapter 3) compared to the three other taste qualities whose receptors are found in taste buds on the tongue. In a recent review, Montmayeur and Matsunami (2002) describe the latest identification and characterisation of mammalian receptors for bitter, sweet and umami taste stimuli. Like ORs, these receptors are also GPCRs, but exhibit some peculiarities.

Twenty-four human bitter receptor genes, named T2Rs, have been described (Bufe *et al*., 2002), showing a weak amino acid identity (Adler *et al*., 2000; Matsunami *et al*., 2000), in agreement with a capability to interact with the chemically diverse molecules associated with bitter tastes. They look like ORs that contain a short N-terminal ectodomain, so it is predictable, although not yet proven, that the residues important for tastant-binding specificities are located in the transmembrane domains and possibly also in the extracellular loops. Several dozens of putative T2R genes have been observed in humans and mice. As far as we know, T2Rs are connected to a special G protein named gustducin, which can be activated by bitter compounds (Adler *et al*., 2000; Margloskee, 2002; Ruiz-Avila *et al*., 2001; Wong *et al*., 1996). Although it has not been definitely demonstrated, it seems plausible that a single taste bud cell can express multiple T2R genes, which could explain the lack of perceptual

differentiation between different bitter compounds (Adler *et al*., 2000). To study receptor–ligand interactions for T2Rs, an expression system akin to those used to characterise ORs allowed the screening of numerous taste stimuli (Bufe *et al*., 2002; Chandrashekar *et al*., 2000). Among others, they identified cycloheximide as a ligand for mT2R5, which was also shown to activate gustducin when bound with this tastant.

Sweet and umami receptors proved to be quite different, belonging to GPCR family 3. Both are heterodimers made of two 7TM receptors with an enormous external domain suspected to be the tastant-binding site, by analogy with mGluRs (Kunishima *et al*., 2000). Sweet and umami receptors share one subunit in common, T1R3, while the other was found to be specific for each taste quality, T1R2 for sweet taste and T1R1 for umami (L-amino acids) taste, since results, obtained in the mouse, suggest that T1R1/3 heterodimers may function as umami receptors (Li *et al*., 2002; Nelson *et al*., 2001, 2002; Figure 4.6). In contrast to bitter receptors, no T1R was shown to mediate signalling through gustducin (Nelson *et al*., 2001). Both mono- and disaccharides, and sweeteners were shown to activate T1R2/3 (Li *et al*., 2002). T1R2 and T1R3 are probably both critical to form the sugar and sweetener binding site(s) of the receptor complex.

Recently, Ma *et al*. (2003) found that the mouse septal organ, a distinct chemosensory organ in the mammalian nose, made of olfactory neuroepithelium located bilaterally on the nasal septum, was able to capture and transduce olfactory signals. Its sensory neurons express olfactory-specific G protein and AC type III, as does the main olfactory epithelium. The septal organ resembles the main olfactory epithelium in odorant response properties and projection to the main olfactory bulb. This is a novel trail to fully understand how chemicals are perceived in mammals.

4.6.3 Comparison of human and animal olfactory systems

Whereas several hundreds of different ORs participate in the capture and detection of odorants, only a small number of OBPs are expressed in vertebrates, which are widely tuned towards different chemical classes of odorants (Briand *et al*., 2000a, 2002a; Löbel *et al*., 2002). Olfactory discrimination in insects is also a two-step process, but with ten times more OBPs (of a different protein type) and only about twice that number of ORs. However, the number of combinations between OBPs and ORs leads to a similar complexity. Insect OBPs (notably those involved in sexual pheromone detection) would exhibit a greater specificity than vertebrate OBPs, in such a manner that they more actively participate in odorant–pheromone discrimination. As regards aquatic vertebrates, the number of ORs is about ten times less abundant than terrestrial species but the role of OBPs is not so clear due to the fact that odorants are already in an aqueous medium. Nonetheless, they may participate in

hydrophilic molecule capture, as in bacterial chemotaxis (Koshland, 1981). The olfactory system of the nematode *Caenorhabditis elegans* appears to be more primitive, allowing this worm to respond to chemosensory cues from the environment in a limited manner (Prasad & Reed, 1999). Eisthen (2002) has discussed the observation that olfactory systems in diverse animals are very similar. Either they have evolved from a common ancestor or resulted from a convergent evolution. Up to now, no definitive answer can be drawn, since it seems that olfactory system features that are similar across very disparate animal species may reflect both responses to similar constraints and adaptations to similar tasks.

Comparison of humans with animals on an olfactory point of view raises the question of their relative aptitude to distinguish odorants. The answer to this question is not easy, since no clear comparison can experimentally be performed. Obviously, it is clear that, as primates acquired a vertical standing position, they lost a large part of their functional OR genes, which became inefficient pseudogenes (Rouquier *et al.*, 2000). However, the human ORs cover a similar odour space as the mouse ORs, so humans are not yet proven to be microsmatic beings, in contrast to what is frequently claimed.

4.6.4 Inferences from the recent knowledge about olfaction pericellular events and future progresses

4.6.4.1 Odotope mixture cannot be distinguished from odorant mixture

The combinatorial system that performs the recognition of several thousands of odours relies on a fuzzy principle, in which a single odorant binds several different ORs and, reciprocally, a given OR is activated by different odorants. Odour coding therefore results from the combinatorial activation of a set of ORs binding different sites of an odorant. A consequence of this functional organisation is that odorants or odorant mixtures cannot be distinguished from each other by the olfactory system. As an odorant mixture is nothing but an odotope combination, it is necessarily perceived as an odorant with numerous odotopes.

It is highly probable that affinity constants of diverse odotopes for various ORs are quite different from each other. In these conditions, all the ORs, which are potentially activated by the odotopes of a given odorant, have different affinities, so that at low concentration only some of them are activated. Consequently, the resulting odotopic map, and therefore odorant perception, changes as a function of odorant concentration (Kajiya *et al.*, 2001). And because the odorant concentration that reaches the olfactory epithelium changes as a function of time, the quality of a single odorant may be differently perceived in the course of time, which would explain a common observation (Figure 4.11).

Great steps have been achieved in the understanding of odour sensing, but the delivery dynamics of the airborne molecules to the olfactory epithelium

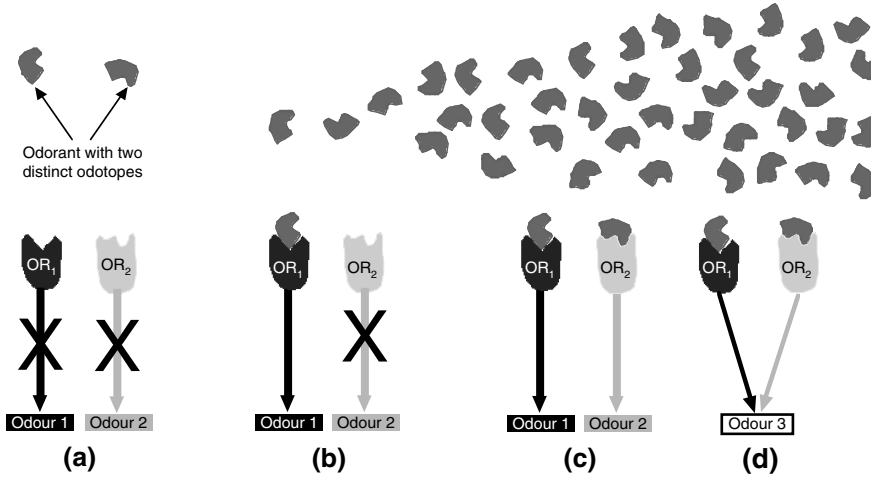

Figure 4.11 Hypothetical explanation of odour change as a function of odorant concentration. (a) A single odorant has two distinct odotopes, which can independently bind two distinct ORs, OR_1 and OR_2 coding for 'odour 1' and 'odour 2', respectively. (b) Supposing that the binding affinity is greater for OR_1 than for OR_2, then, at low odorant concentration, only OR_1 is activated, generating the smell 'odour 1'; at higher odorant concentration, OR_2 also binds the odorant so that either (c) a second distinct smell is perceived or, alternatively, (d) the combinatorial signal generated in the olfactory bulb gives rise to another smell 'odour 3'. Crosses on arrows indicate neuronal pathway deactivation.

still needs to be deciphered. Together with the dynamics of the odorant capture by the association of OBPs and ORs, this is a crucial question that has to be solved, in addition to the understanding of olfactory neuron desensitisation. This question is related to the molecular mechanisms regulating odour perception, which can occur either at the odorant capture level (e.g. odour masking by antagonist binding onto ORs or OBPs) or further during neural signal coding and treatment.

4.6.4.2 Human individual variability

Genomic studies are revealing important genetic diversity among humans for odorant perception, putting forward the urgent necessity to compare human genomes and sensory phenotypes (Menashe *et al*., 2002, 2003). It is worth noticing that Menashe *et al*. (2003) extrapolated the number of segregating OR pseudogenes in the entire human genome to be at least 60, a number in rough agreement with the reported count of different modes of specific anosmias. It seems therefore plausible that OR gene mutations or disruptions might be involved in specific anosmias, whereas OBP gene alteration could be responsible for a general decrease of olfactory aptitude. Such increases in detection thresholds, called hyposmias, are very frequent in the human

population. They affect subjects who cannot smell any randomly chosen odorant at a concentration that normal individuals can detect. Odorant solubilisation by OBPs would therefore be involved in odour detection threshold. Abraham *et al*. (2002) have already modelled the relationships between odorant solubilisation by OBPs and detection thresholds. However, the proposed model is oversimplified because it does not take into account the occurrence of several OBPs having diverse affinity for odorants. Once the affinity constants of the various hOBPs have been more thoroughly characterised for a large set of odorants, an extended model will likely prove more realistic. The binding of diverse odorants to a single hOBP demonstrated that they bound with different affinities, e.g. a low affinity for some very potent odorants, such as eugenol, and a high affinity for aldehydes and also large fatty acids (Briand *et al*., 2000a). Therefore, OBP specificity could contribute to odour discrimination, as also suggested for rat OBP-1, OBP-2 and OBP-3, which were shown to have complementary properties in binding odorants (Löbel *et al*., 2002). Through this mechanism, OBPs could modulate the discriminating role of ORs.

The difficulty of classifying odorants and odours (Rossiter, 1996) certainly finds its origin in the diversity of individual OR repertoires, which are likely to be unique for each person (Menashe *et al*., 2003). Such a diversity would be responsible for a mandatory subjectivity in odour description, since each subject would perceive odorants with their unique OR repertoire. As the functional OR gene repertoire in humans is confined to about 350 genes, it seems possible to define the most frequent alleles of each of these genes in various populations. Rapid approaches, such as DNA chip technology, could be used to associate specific hyposmias to certain OR genes and alleles, at least in some cases. This would not only bring comprehensive knowledge about the interindividual variability of odorant responsiveness, with biological and medical involvements, but also be useful to the fragrance and food industry, once some OR genes have been shown to occur differently in identified populations. The link between various human genotypes and both qualitative and quantitative perception will certainly be a goal for agroindustry, in order to optimise food products in different areas of the world. The assignment of a set of odorants (or odotopes) to each OR is thus a high throughput challenge, which is being taken up.

4.6.5 Olfactory biosensors

Another issue concerning the proteins that capture odorants lies in potential applications based on their use as novel biocaptors as part of a biosensor. Although acellular expression of membrane proteins such as ORs is still currently a very difficult technical challenge, which will take some time to resolve, OBPs offer a very good opportunity to build odorant biosensors

based on field-effect transistor technology. OBPs, which are naturally adapted to bind odorants, can be chosen as templates to design and build specific biocaptors. Indeed, the lipocalin scaffold has been already used to engineer biocaptors (Schlehuber *et al*., 2000; Skerra, 2000). Sensory biotechnology could be the first among many practical applications with commercial potential that will be derived from the molecular and cellular understanding of the olfactory system, which has been acquired over the past decade (Gilbert & Firestein, 2002; Schlehuber & Skerra, 2001).

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Flavor Perception
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5 Oral chemesthesis: an integral component of flavour

Barry G. Green

5.1 Overview

Chemesthesis, the chemical sensibility of the skin and mucous membranes, was long considered a minor and unidimensional modality that had little to do with flavour. Interest and research in oral chemesthesis has grown substantially over the past two decades, spurred mostly by its recognised importance for perception of pepper and mint flavour, and reinforced by evidence that it often contributes to the 'taste' of salts, acids and bitter stimuli. In addition, clear evidence has been found that principally chemesthetic stimuli interact with principally gustatory and olfactory stimuli during flavour perception. Whereas recent studies of receptor biology have begun to uncover the peripheral basis of chemesthetic sensitivity and its relationship to temperature and pain, advances in understanding the perceptual processes and neurophysiological mechanisms of chemesthesis have been less dramatic. What follows is an overview of progress on both of these fronts. Emphasis is placed on the multimodal nature of chemesthesis, including its interactions with temperature, touch, taste and smell, and how these interactions make it essential that chemesthesis be studied as an integral component of flavour.

5.2 Introduction

In addition to their taste, smell and texture, many foods, beverages and oral products have sensory properties that arise from a less well-known chemical sensibility. The burn of hot peppers and mustards, the tingle and prickling of carbonation and the sharp coolness of peppermint are all qualities of chemesthesis. The term *chemesthesis* is relatively new, having been coined in 1990 (Green *et al*., 1990) to replace an older terminology that inadequately characterised the physiological basis of these types of sensations. The 'common chemical sense', as described by Parker (1912) 78 years earlier, was believed to be a mucocutaneous chemosensory system 'as distinct and well defined as smell or taste' (p. 221). We now know that the chemical sensitivity of the mucous membranes does not derive from a separate, specialised sensory system, but rather from chemically sensitive neurons of the

classically defined somatosensory systems, particularly temperature (warmth and cold) and pain (Treede, 1995). For example, peppermint evokes coolness because the menthol it contains stimulates cold fibres, and chilli pepper evokes burning because the capsaicin it contains chemically stimulates heat-sensitive pain fibres. Accordingly, chemesthesis can be sensed wherever temperature and pain are sensed, and thus is a property of all types of skin. What enables the mucous membranes to be particularly sensitive is the absence of a substantial stratum corneum, which serves as a barrier to penetration of chemicals in hairy and glabrous (smooth) skin. The less effective barrier properties of the mucosa make it possible for otherwise innocuous chemical stimuli such as salts, mild acids and alcohol to provoke surprisingly strong sensory irritation (Bryant, 2000; Bryant & Moore, 1995; Simon, 1992; Wang *et al*., 1993). As a consequence, oral chemesthetic stimuli comprise a remarkably wide range of chemical compounds, including many, such as salts and acids, which have traditionally been considered 'pure' taste stimuli. It will be argued here that a vanishingly small number of conventionally defined taste stimuli excite only the taste system, and that chemesthesis is therefore a much more common component of the flavour of foods and beverages than generally recognised.

5.3 The neurophysiological basis of oral chemesthesis

Before considering the sensory and perceptual aspects of chemesthesis in greater detail, it is useful to review what is known about its neurophysiological basis. In the oral cavity, chemesthesis is mediated by three different nerves: the trigeminal (cranial nerve V), the glossopharyngeal (cranial nerve IX) and the vagus (cranial nerve X). The trigeminal nerve has an extensive innervation field that includes the anterior oral cavity and tongue, the nasal cavity, the face and parts of the scalp. Because this nerve is the exclusive mediator of chemical irritation in the nose and serves such a large area of the mouth, chemosensory scientists have often referred to chemesthetic (and somesthetic) sensitivity as 'trigeminal sensitivity'. But this designation is too limiting even for nasal chemesthesis, as inhaled irritants pass into the nasal and oral pharynx, where they stimulate the vagus nerve. Chemesthetic stimuli that are ingested inevitably reach the posterior tongue and oral pharynx during mastication and swallowing, where they stimulate the glossopharyngeal and vagus nerves.

Whereas the anatomy and physiology of trigeminal innervation of the tongue and palate has been studied fairly extensively (Dixon, 1961; Farbman & Hellekant, 1978; Harada *et al*., 1987; Sostman & Simon, 1991; Suemune *et al*., 1992; Wang *et al*., 1993), less is known about the general sensory function of nerves IX and X. However, both the glossopharyngeal nerve (Bradley *et al*.,

1986; Sweazey & Bradley, 1989;Yamada, 1966) and vagus nerve (Hauser-Kronberger *et al*., 1997) are known to contain somatosensory neurons, which give them the potential to respond to chemesthetic stimuli; and psychophysical studies have in fact shown that the sensitivity to chemical irritation is at least as high in the throat as it is in the mouth (Breslin *et al*., 2001; Rentmeister-Bryant & Green, 1997). More will be said about these studies later in the context of spatial differences in chemesthetic perception.

By far, the most detailed understanding of the sensory receptors responsible for chemesthesis has come from the study of capsaicin, the pungent principle in chilli pepper. A member of the vanilloid family, capsaicin was first identified as a stimulus for pain fibres (nociceptors) over four decades ago (Jancso, 1960; Jancso *et al*., 1961). Interest in capsaicin stemmed not only from its potent presence in hot peppers, but also from its ability to desensitise afferent fibres, principally c-polymodal nociceptors (Buck & Burks, 1986; Carpenter & Lynn, 1980; Kenins, 1983; Szolcsanyi, 1977b, 1985; Wall & Fitzgerald, 1981). The cellular mechanism of desensitisation is still not fully understood, although it seems likely that an influx of calcium through a nonspecific cation channel plays a significant role (Bhave *et al*., 2002; Docherty *et al*., 1996; Mohapatra *et al*., 2003). Better understood is the molecular receptor responsible for gating this channel. The vanilloid receptor 1 (VR1), which is sensitive not only to capsaicin and other vanilloids but also to heat and low pH (Caterina *et al*., 1999; Jordt, 2003; Liu & Simon, 2001; Tominaga & Julius, 2000), was the first member of what is now recognised as a family of so-called transient receptor potential (TRP) ion channels (Gunthorpe *et al*., 2002). Moreover, psychophysical studies of cross-desensitisation by capsaicin, many of which will be discussed later, have shown that neurons possessing VR1 mediate the chemesthetic qualities of numerous other compounds as well. The multimodal sensitivity of VR1 helps explain why capsaicin induces a sensation of burning heat, and why, as will be discussed later, the intensity of this sensation can be strongly modulated by temperature. VR1 has recently been found throughout the oral and lingual mucosa, including in fungiform and circumvallate papillae where they lie in close proximity to – though not on – taste receptor cells (Ishida *et al*., 2002; Kido *et al*., 2003; Kusakabe *et al*., 1998).

Other members of the TRP family are also important for oral chemesthesis. One of these, TRPM8, was recently found to respond to menthol (McKemy *et al*., 2002; Peier *et al*., 2002) and is thought to mediate sensitivity to cooling over temperatures from about 10°C to 25°C. TRPM8 has been assumed to be expressed by cold fibres because it is sensitive to moderate cooling as well as to noxious cold. This assumption does not take into account the recent finding that temperatures as mild as 28°C can cause sensations of burning and stinging (Green & Pope, 2003), nor the older evidence that cold-sensitive nociceptors can have thresholds as high as 30°C (Georgopoulos, 1976; LaMotte &

Thalhammer, 1982). Given that menthol evokes sensations of burning and stinging as well as cold (Cliff & Green, 1996; Green, 1992b), it is premature to conclude that TRPM8 encodes cold and not burning and stinging. It is possible that another TRP receptor will be found that responds to still more moderate cooling (i.e. $> 25^{\circ}C$) as well as to menthol and other cooling agents.

Psychophysical evidence indicates that the receptor responsible for menthol's irritancy is also involved in perception of nicotine: desensitisation of the tongue tip to menthol reduces the sensitivity to nicotine (Dessirier *et al*., 2001a), which causes stinging but not coolness. On the other hand, studies have shown that desensitisation to nicotine fails to cross-desensitise the irritancy of capsaicin or piperine (Dessirier *et al*., 1997, 1998). This pattern of results implies that menthol's irritancy depends upon stimulation of nicotinic acetylcholine receptors that are expressed in a small subset of capsaicin-sensitive nociceptive neurons, a possibility supported by electrophysiological evidence that some individual trigeminal ganglion neurons possess both nicotinic and vanilloid receptors (Liu & Simon, 1996). More recently, Viana *et al*. (2002) reported that some cold-sensitive neurons that respond to menthol are also sensitive to capsaicin. Theoretically, such neurons would be capable of responding to menthol, capsaicin, cold, heat and low pH, and thus would be more likely to be polymodal nociceptors (Bessou & Perl, 1969; Torebjork, 1974) than cold fibres.

Another common oral chemesthetic stimulus whose transduction mechanism has begun to be understood is carbonation. It had been hypothesised in the past that the sensation of carbonation arose from mechanical stimulation produced by CO_2 bubbles (Yau & McDaniel, 1990), but research has shown that CO_2 stimulates nociceptors after it goes into aqueous solution and is converted to carbonic acid by carbonic anhydrase (Dessirier *et al*., 2000a; Komai & Bryant, 1993). Whether it stimulates nociceptors via the same cellular mechanism (e.g. VR1) as other weakly acidic chemicals is unclear, but the tendency for cold to enhance rather than inhibit perceived carbonation (Green, 1992a) is inconsistent with mediation by a heat-sensitive receptor. What is clear is that on the tongue, sensory irritation from both CO_2 (Dessirier *et al*., 2001b) and citric acid (Gilmore & Green, 1993) has been reported to be reduced following exposure to capsaicin. But because capsaicin can disable whole neurons as well as specific receptors (Holzer, 1991), cross-desensitisation does not rule out the possibility that weak acidity is mediated by more than one transduction mechanism on a common set of neurons.

Thus, even though the full variety of sensory receptors serving chemesthesis remains to be discovered, several different types are already known to exist which, when expressed alone and in different combinations on sensory neurons, have the potential to produce a variety of different chemesthetic sensations.

5.4 Psychophysical characteristics

5.4.1 Sensation quality

Implicit in the early notion that the common chemical sense was an undifferentiated system of chemically sensitive nerve endings was the idea that it could detect the presence and amount of sensory irritants, but could provide no information about their quality (Silver, 1987; Silver *et al*., 1985; Walker *et al*., 1979). The foregoing evidence that a variety of sensory receptors and neurons serve chemesthesis belies this old view. With the recent demonstration that a class of chemicals called alkylamides can stimulate mechanically sensitive trigeminal neurons as well as thermally sensitive ones (Bryant & Mezine, 1999), we must conclude that chemesthetic stimuli are capable of stimulating all elements of the classically defined somesthetic senses: temperature, pain and touch. As shown in Figure 5.1, subjects reported a curious 'buzzing' sensation from an extract that contained an alkylamide (in this case spilanthol) that was consistent with stimulation of low-threshold mechanoreceptors of the sort that are sensitive to vibration.

The ability of some chemicals to stimulate mechanoreceptors underlines the fact that the qualitative richness of chemesthesis rivals, if not surpasses, that of

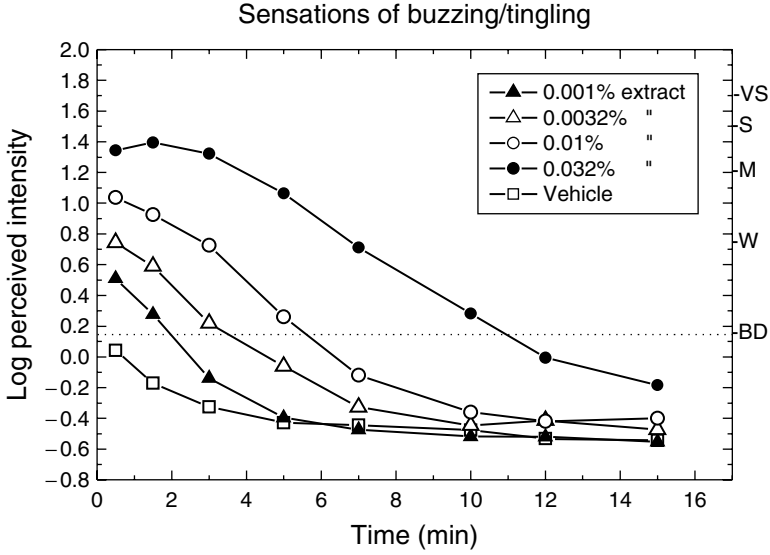

Figure 5.1 The perceived intensity of sensations of buzzing and tingling produced by a single exposure to jambu extract, which contains the alkylamide spilanthol. The parameter was the concentration of the extract in aqueous solution. Sensation intensity was rated on the labelled magnitude scale (LMS); the right *y*-axis displays intensity in terms of semantic descriptors of the LMS: BD = barely detectable; W = weak; M = moderate; S = strong; VS = very strong. (Green *et al*., 1993.)

taste. 'Hot' spices such as red and black pepper, ginger and cinnamon fall along different qualitative coordinates in multidimensional scaling tasks (Cliff & Heymann, 1992), and common oral chemical irritants have been shown to yield 'flavour profiles' (Green, 1991a) characterised by qualities such as burning, stinging, tingling and numbness. It is by virtue of this perceptual diversity that irritants contribute distinctive qualities to some of the most widely consumed and universally liked flavours: along with the coolness of menthol and the heat of peppers there is the burn of cinnamon from cinnamic aldehyde, the tartness and sting of lemon and vinegar from citric and acetic acid, the sting of radish and spicy mustards from isothiocyanates and so on. Though these flavours might be recognisable without their pungent dimensions, excluding these qualities the sensory impact of each would be greatly lessened. They would, in a word, be bland.

5.4.2 Spatial factors in sensitivity and sensation quality

In addition to these purely qualitative differences, the ubiquity of somesthetic innervation in the oral and nasal cavities introduces the perceived site of stimulation as a potentially salient feature of chemesthesis. Perhaps the most obvious example is the unmistakable nasopharyngeal burn of horseradish and mustard, which is categorically different from the lingual and oropharyngeal burn of peppers. Spatial differences of this sort most likely result from a combination of physicochemical and neuronal factors, e.g. differences in stimulus diffusion, rates of stimulus absorption, and innervation type, density and depth. The relative importance of these factors undoubtedly depends upon the stimulus. In the case of horseradish and mustard oil, their high volatility compared to vanilloids allows them to disperse into the nasal pharynx and nose. Equally important, of course, the epithelium of the pharynx is apparently well innervated with receptors that can be stimulated by isothiocyanates. The tongue must contain at least some of the same receptors, because mustard oil applied to the tongue in rats is an effective trigeminal stimulus (Carstens *et al*., 1998). On the other hand, the much stronger irritation in the throat than on the tongue from the nonsteroidal anti-inflammatory drug ibuprofen (Breslin *et al*., 2001), which is not at all volatile, suggests that the throat, which is innervated by cranial nerve X and to a lesser extent cranial nerve IX, possesses a certain type of receptor that does not exist in significant numbers, or perhaps at all, in the anterior tongue.

Capsaicin represents yet another type of spatial variation that may reflect the combined effects of receptor density and either stimulus absorption or depth of innervation. When capsaicin is swallowed, it too produces a stronger burn in the throat than in the front of the mouth (Rentmeister-Bryant & Green, 1997); but capsaicin is of course an excellent stimulus on the tongue as well, where its effectiveness corresponds with the density of trigeminal innervation. Burning sensations are strongest on the tongue tip and anteriolateral edges

(Green, 1988; Green & Schullery, 2003; Lawless & Stevens, 1988), where an abundance of trigeminal nerve endings innervate the fungiform papillae and outnumber chorda tympani fibres (Farbman & Hellekant, 1978; Whitehead *et al*., 1985). In contrast, in the circumvallate papillae the majority of peptidergic nerve endings, which are likely to be nociceptors and therefore sensitive to capsaicin, reside deep in the epithelium near the base of the papillae (Kusakabe *et al*., 1998).

Figure 5.2 illustrates the marked difference in capsaicin's pungency on the front and back of the tongue. Menthol (not shown) exhibits the same spatial pattern with respect to its irritancy (Green & Schullery, 2003). However, the coolness of menthol (Figure 5.3) is significantly stronger on circumvallate papillae than on the tongue tip. The spatial distribution of cold-sensitive neurons in the tongue is apparently different from the spatial distribution of nociceptors. Data on the sensitivity to physical cooling support this hypothesis, inasmuch as cold perception (in contrast to warming) is fairly uniform throughout the tongue and mouth (Green, 1984; Green & Gelhard, 1987). A further prediction that can be made from the data of Figure 5.3 is that if TRPM8 is expressed in neurons that encode cooling rather than burning or

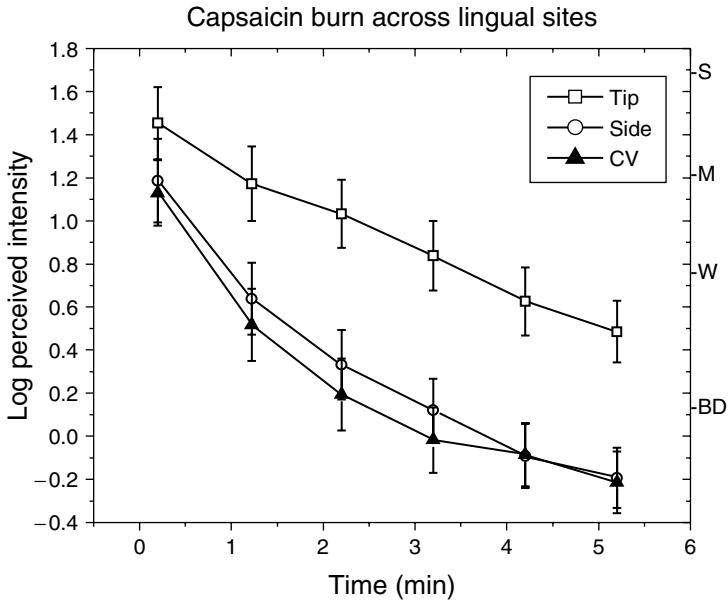

Figure 5.2 The perceived intensity of burning sensations as a function of time after exposure to capsaicin applied to three different areas of the tongue via saturated cotton swabs: Tip = anterior edge/fungiform papillae; Side = posterior–lateral edge/foliate papillae; CV = posterior tongue/circumvallate papillae. Vertical bars represent standard errors of the mean (SEMs). (Adapted from Green & Schullery, 2003.)

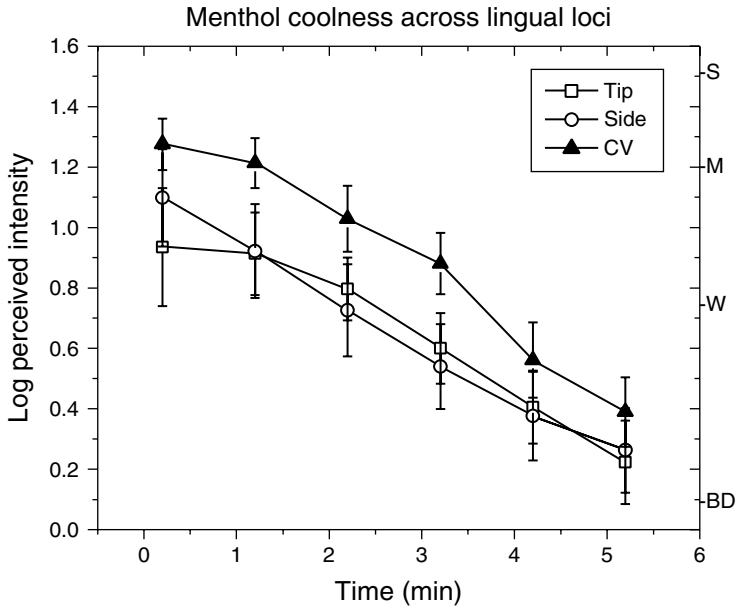

Figure 5.3 The perceived intensity of coolness as a function of time after exposure to menthol applied to three different areas of the tongue via saturated cotton swabs: Tip = anterior edge/fungiform papillae; Side = posterior–lateral edge/foliate papillae; CV = posterior tongue/circumvallate papillae. Vertical bars represent (SEMs). (Adapted from Green & Schullery, 2003.)

stinging, these receptors should be more abundant in cranial nerve IX than in cranial nerve V, or perhaps be localised on nerve endings that rise closer to the mucosal surface in circumvallate papillae than in fungiform papillae.

5.5 The roles of sensitisation and desensitisation in chemesthetic perception

In most sensory systems, constant or repeated stimulation causes a progressive decline in sensation intensity and/or threshold sensitivity (sensory adaptation). This is rarely the case for chemesthetic stimuli. Instead, continuous or recurrent stimulation often leads either to a progressive growth in irritation ('sensitisation') or to a decrease in sensitivity that usually requires a lull in stimulation to develop ('desensitisation'). As alluded to in the discussion of chemesthetic receptors, desensitisation provides a means for determining whether particular sensations are mediated by common sensory neurons. The irritant best known for inducing both sensitisation and desensitisation is of course capsaicin, but piperine, zingerone, eugenol, mustard oil and menthol

can all produce similar effects. In general, shorter interstimulus intervals (ISIs) are conducive to sensitisation (Green, 1998) and longer ISIs are conducive to desensitisation (Green, 1989, 1991c; Green & Rentmeister-Bryant, 1998); a pause of 10–15 min between exposures to capsaicin maximises desensitisation (Green, 1993). Thus, contrary to what happens with most other sensory stimuli, frequent exposure increases the sensory impact of many chemesthetic stimuli, whereas waiting several minutes between stimuli (i.e. to avoid adaptation) invites desensitisation. Because continuing excitation can offset or delay desensitisation, it has been hypothesised that desensitisation and excitation (or sensitisation) derive from opponent cellular processes (Green, 1993). Even after strong desensitisation has been established, continuing exposure causes sensation to build again to nearly the same level as before desensitisation (Green, 1998; Green & Rentmeister-Bryant, 1998). This phenomenon, called Stimulus-Induced Recovery (SIR), has been observed in trigeminal ganglion neurons (Dessirier *et al*., 2000b) as well as psychophysically on the tongue, which indicates that it has a peripheral rather than a central (i.e. central nervous system, CNS) source. Repeated stimulation may cause stimuli to build up in the epithelium and/or sensitise the receptors themselves. Whatever its cause, SIR explains why eating a hot and spicy appetiser does not prevent one from perceiving the chemical heat of the main course. Even if desensitisation occurs it would be likely to reduce the heat of only the first few bites, after which the burn would build as consumption continues. A temporal delay in growth of the burn might easily go unnoticed during casual dining.

On the other hand, studies of SIR have also provided clues about how a perceptual tolerance for chilli pepper might develop over time. First, an important feature of SIR is that it is significant only when subsequent stimuli are at least as strong as the desensitising stimulus (Green & Rentmeister-Bryant, 1998). Eating foods that contain higher amounts of capsaicin can, therefore, reduce the perceived heat of foods that contain lesser amounts of capsaicin. This is consistent with the idea that the cellular mechanisms of excitation and desensitisation are opponent processes (Green, 1993), such that desensitisation can only be reversed when capsaicin is reintroduced in quantities at least as high as that which caused desensitisation in the first place. We have also found that repeated exposure to the same capsaicin concentration on a daily basis slowly reduces the magnitude of SIR, such that recovery becomes progressively less complete. This trend is shown in Figure 5.4, which contains data from an experiment in which we applied 330 μM capsaicin to the tip of the tongue daily, excluding weekends, for three weeks. Whereas SIR was virtually complete across three blocks of ten exposures to capsaicin on day 1, there was a significant trend toward less and less recovery over days. For example, on day 2 the intensity of the first stimulus in block 1 was reduced by almost half (47%) compared to day 1, but this difference diminished by the last stimulus of block 1 (due to SIR) to only 8.8%. By day 15, these reductions had increased

to 72.5% for the first stimulus and 31.9% for the last stimulus. It can be inferred from these results that desensitisation involves at least two separate processes: one that acts over minutes, which can be reversed by re-exposure to capsaicin (SIR), and another that acts over longer periods of time (on the order of days and weeks), which cannot be reversed by re-exposure. The longer-term process may involve a progressive degeneration of unmyelinated terminals of capsaicin-sensitive neurons, as evidenced by neurohistological studies on the effects of capsaicin to hairy skin (Reilly *et al*., 1997; Simone *et al*., 1998). If so, individuals who frequently consume large amounts of chilli pepper or other spices that contain capsaicin may actually have fewer sensory neurons carrying the VR1 receptors in the mouth and throat than do less avid chilli pepper consumers. This loss need not be permanent, however, as intraepithelial nerve endings regenerate over a period of weeks if capsaicin is not reintroduced (Reilly *et al*., 1997; Simone *et al*., 1998). Thus, only those consumers who frequently eat large amounts of capsaicin are liable to have significant and prolonged degeneration of the subset of pain fibres that are sensitive to capsaicin. Interestingly, loss of capsaicin-sensitive neurons seems to have little perceptual effect beyond reductions in sensitivity to chemical irritants. Changes in sensitivity to heat pain are minor and there are no consistent changes to milder temperatures or to pain produced by mechanical stimulation (Simone & Ochoa, 1991).

From a research standpoint, the temporal characteristics of capsaicin and similar stimuli impose very severe limitations on experimental design. The

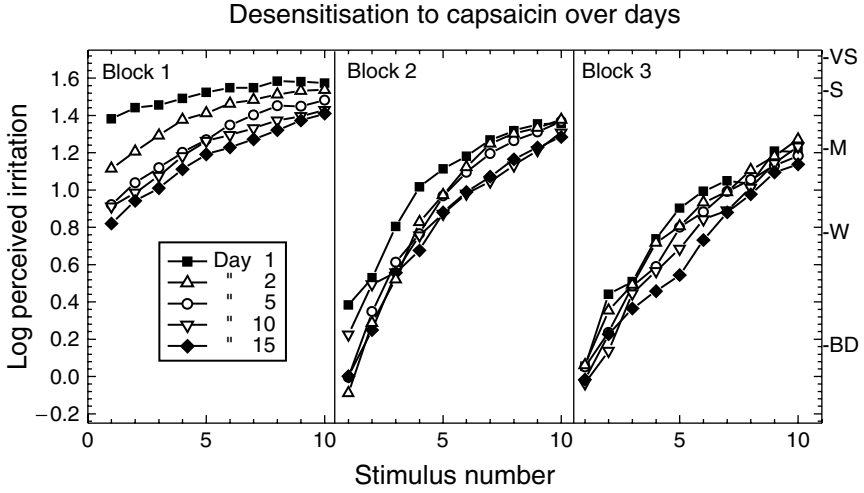

Figure 5.4 Perceived intensity of burning sensations from capsaicin as a function of number of applications in three blocks of trials over 15 testing sessions. There was a 15-min hiatus in stimulation between successive blocks. Testing was conducted on consecutive days, with weekends off. Vertical bars represent SEMs.

preceding data make it clear that stimuli delivered to the same site within the same experimental session, or even 24 h or more later (Karrer & Bartoshuk, 1991), cannot be assumed to be perceptually or physiologically independent. To make matters worse, in addition to the well-known individual differences in sensitivity to stimuli like capsaicin and menthol, the magnitudes of sensitisation and desensitisation also vary from person to person (Cliff & Green, 1996; Prescott, 1999). Depending upon the goals of a particular experiment, it may be necessary to use more than one stimulus concentration to make certain that all subjects experience similar levels of sensation, and to adjust and control the ISI to hold sensation as constant as possible across trials.

5.6 Temperature and chemesthesis

Because chemesthetic stimuli very often stimulate thermally sensitive neurons, temperature can drastically influence chemosensory irritation by either increasing or decreasing its intensity. In general, cooling suppresses chemical irritation and heating enhances it (Green, 1986, 1991b; Sizer & Harris, 1985). Once again, these effects are best understood for capsaicin. Because VR1 is sensitive to heat, heating the receptor while it is being chemically stimulated leads to an enhanced perceptual response (Ringkamp *et al*., 2001; Szolcsanyi, 1977a). On the other hand, cooling was recently found to directly inhibit capsaicin-induced calcium currents in trigeminal ganglion neurons (Babes *et al*., 2002). As might be expected, the chemical cooling effect of menthol is stronger at cooler than at warmer temperatures (Green, 1985), presumably because the effects of chemical and thermal stimulation on TRPM8 (assuming that it is indeed a 'cold' receptor) are additive. For both menthol and capsaicin, chemical stimulation can be viewed as sensitising thermally sensitive neurons to respond at temperatures that would otherwise be below threshold (Hensel & Zotterman, 1951; Szolcsanyi, 1977a). The result is that peppermint makes inhaled room temperature air feel noticeably colder, and capsaicin causes skin or oral temperatures that are just a few degrees above normal to feel burning hot. Cooling can also have an attenuating effect on pain, and thus on the nociceptive qualities of capsaicin and other sensory irritants, via inhibitory mechanisms in the CNS (Wahren *et al*., 1989). The latter effects can occur even if cooling takes place away from the immediate area of stimulation. A cardinal rule of chemesthetic research is therefore to minimise temperature change in the oral cavity during stimulation and throughout the response period. A common mistake is to deliver test solutions and water rinses at ambient temperature (20–22°C), or to allow subjects to breathe through the mouth after capsaicin or menthol exposure. Retracting the tongue after stimulus application curtails evaporation and helps keep the tongue near normal oral temperature. From a practical standpoint, the temperature sensitivity of chemesthetic 'heat'

also offers an obvious (short-term) solution when the burn becomes uncomfortably strong. Drinking any cool liquid will immediately quell the burn, though, unless the capsaicin itself is removed (e.g. by rinsing with a lipid-containing substance like milk), the burn will return as the oral cavity reheats to its normal temperature (Green, 1986).

5.7 Interactions with touch

Although to date only alkylamides are known to stimulate mechanoreceptors, touch can nonetheless have a significant effect on chemesthetic perception. Like cooling, mechanical stimulation has the potential to inhibit pain, and hence sensory irritation, via a central neural process (Melzack & Wall, 1965). Even subtle mechanical stimulation, like that produced by sipping and expectorating a liquid, appears sufficient to attenuate capsaicin burn significantly (Green, 1986). One can only assume that more exaggerated oral movements, such as 'smacking' the tongue and lips during tasting, or the act of chewing, would have even stronger effects. A recent study showed that merely touching a thermal stimulator to the skin, causing only a modest sensation of contact, could attenuate sensations of burning and stinging that normally accompany cooling (Green & Pope, 2003). The latter result raises the possibility that the pungency of menthol might similarly be dulled by oral mechanical stimulation during ingestion, mastication and swallowing.

5.8 Interactions with taste and smell

A primary emphasis in psychophysical research on oral chemesthesis has been its potential for interactions with taste. The majority of this work has focused on possible inhibitory (or masking) effects on taste (e.g. Cowart, 1987, 1998; Lawless & Stevens, 1984; Prescott & Stevenson, 1995a, 1995b; Prescott *et al*., 1993; Simons *et al*., 2002; Stevens & Lawless, 1986). Despite this emphasis, it appears that the only consistent effect is a small reduction in sweetness. A recent study in rats indicates that capsaicin may produce this effect, and perhaps also weaker effects on the tastes of monosodium glutamate (MSG: 'umami') and quinine, by disrupting taste transduction (Simons *et al*., 2003). Studies of the effect of capsaicin burn on retronasal odour perception have also uncovered only small effects on perception (Green, 1996; Prescott & Stevenson, 1995a), which would necessarily have central rather than peripheral origins. These relatively small and often inconsistent effects on flavour perception are surprising in light of the commonly reported experience of being unable to 'taste' strongly spiced foods. The reason for this discrepancy is most likely that only when burning sensations become too strong during a spicy

meal, we are unable to appreciate flavours, and experimenters generally avoid giving volunteer subjects concentrations of capsaicin high enough to cause intense pain. It is possible that at such concentrations a central (masking) effect adds to the peripheral suppression of taste. At the very least, intensely painful burning sensations would draw attention away from the benign and, at that moment, relatively unimportant flavour of the food.

Carbonation, though generally thought of as producing a milder sensory irritation than capsaicin, has also been studied as a modifier of taste (Cometto-Muniz *et al*., 1987; Cowart, 1998; Yau & McDaniel, 1990, 1992). Rather than masking tastes, carbonation tends to add to overall taste intensity (Cowart, 1998) as well as to specific taste qualities. Sweetness again stands out as the quality most often altered by CO_2, though sourness and saltiness may also be enhanced. It seems probable that these effects arise from biochemical reactions at the receptor (resulting, perhaps, from conversion of CO_2 to carbonic acid) rather than from central interactions via stimulation of nerves V, IX or X (Cowart, 1998).

In addition to potential excitatory effects of chemesthesis on taste, researchers have investigated the possibility that capsaicin desensitisation can impair taste perception. The first work on this topic in humans was not, however, directed toward desensitisation of taste per se; rather, it explored whether the pungent qualities of acids and salts could be desensitised, and thus be mediated by capsaicin-sensitive neurons. Gilmore and Green (1993) answered this question in the affirmative for the irritancy of sodium chloride and citric acid. However, sourness and saltiness were also somewhat lower after capsaicin, which the authors attributed to the inability of subjects to judge the chemesthetic and gustatory parts of the sensation independently. Karrer and Bartoshuk (1995) later found that the bitterness of quinine and 6-n-propyl-2-thiouracil (PROP) and the sourness of citric acid were all reduced following capsaicin. Like Gilmore and Green (1993), they concluded that most, but perhaps not all, of the depression of taste was attributable to reductions in irritancy. However, a follow-up experiment indicated that subjects perceived PROP and quinine to have only a weak ‘tactile’ component. The possibility that bitterness itself could be desensitised by capsaicin was bolstered by an earlier study in rats that found evidence of higher rejection thresholds for bitter substances in capsaicin-treated animals (Silver *et al*., 1985).

Recently, we made the serendipitous discovery that when applied locally to the tongue capsaicin tastes bitter. The first systematic study of the intensity and incidence of this bitterness showed that about 50% of individuals reported it, and that the sensation was stronger when capsaicin was applied to the circumvallate rather than the fungiform papillae (Green & Schullery, 2003). Figure 5.5a shows that capsaicin’s bitterness was rated more than twice as strong in the circumvallate region than in the fungiform region of the tongue, and Figure 5.5b shows that menthol evokes bitterness in a similar way. The

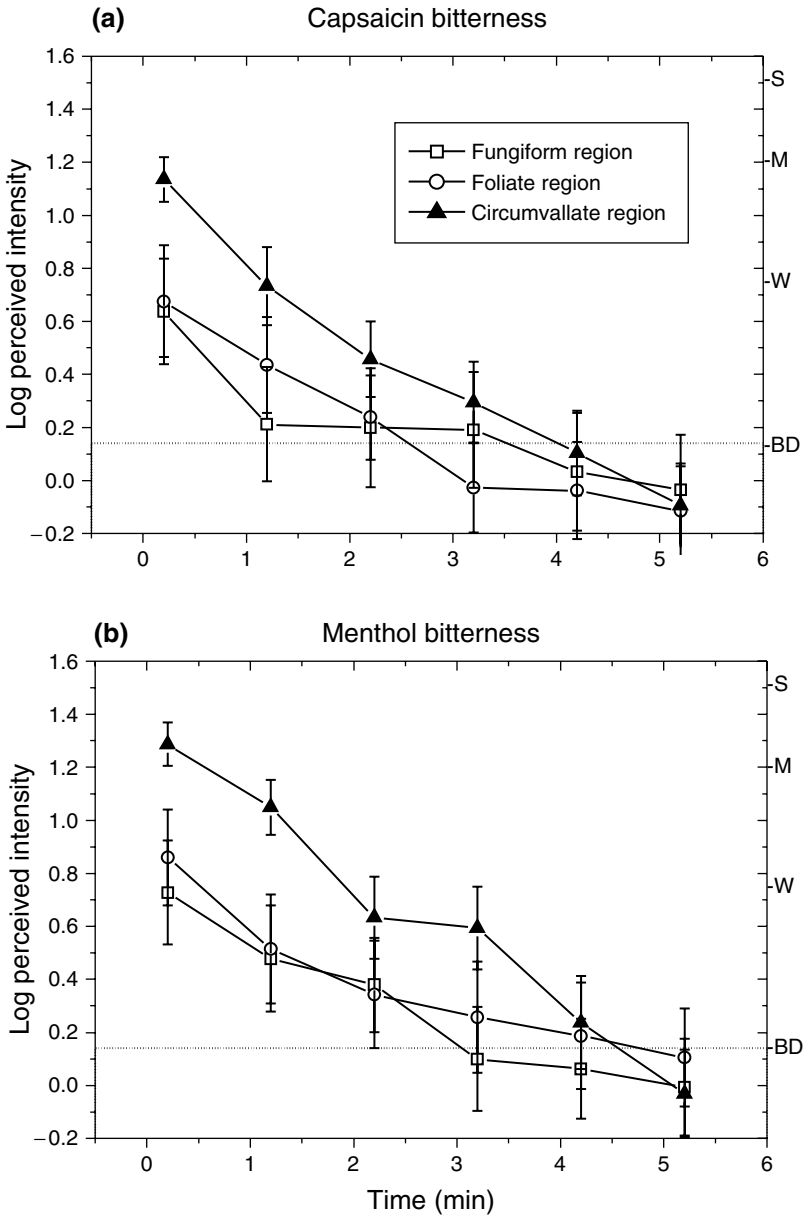

Figure 5.5 Perceived intensity of bitterness reported for (a) capsaicin and (b) menthol on three areas of the tongue. Subjects also rated taste sensations of sweetness, sourness and saltiness; none were reported significantly above BD. Vertical bars represent SEMs. (Adapted from Green & Schullery, 2003.)

discovery that capsaicin could stimulate bitterness was surprising in view of its prominence as a prototypical chemesthetic and chemical pain stimulus. However, the fact that it does so may help explain why pre-exposure to capsaicin reduces the bitterness of traditional gustatory stimuli like quinine and PROP. One possible explanation for capsaicin's bitterness is that subsets of taste cells that possess 'bitter receptors' also possess VR1. Unfortunately, there is no evidence at present that VR1 is associated with taste cells in either the front or back of the tongue. Though VR1 has been found in association with taste papillae (Ishida *et al*., 2002; Kido *et al*., 2003), it has been assumed that its presence reflects the dense innervation of taste papillae (as opposed to taste *cells*) by nociceptors. Furthermore, expression of VR1 by taste cells would not explain menthol's bitterness. Using the same logic, taste cells would have to possess both VR1 and TRPM8 to account for the data of Figure 5.5. Alternatively, capsaicin and menthol bitterness may mean that one or more of the burgeoning class of bitter taste receptors (T2Rs: Bufe *et al*., 2002; Chandrashekar *et al*., 2000) is sensitive to these two chemicals and possibly other chemesthetic agents (e.g. nicotine). No studies have been published to date that have tested T2Rs for sensitivity to nontraditional bitter tastants.

In light of the evidence that even capsaicin is capable of stimulating taste, there is perhaps no oral chemesthetic stimulus that cannot be 'tasted' as well as 'felt'. Likewise, because salts and acids evoke burning and stinging at moderate to high concentrations, only stimuli that taste sweet or bitter should be considered as 'pure' taste stimuli throughout the normal physiological range. But there is evidence that some bitter substances can stimulate the trigeminal nerve in rats (Liu & Simon, 1998), and a recent study in my laboratory revealed that out of four bitter tastants (quinine, magnesium chloride, PROP and urea) we tested, only one (quinine) failed to induce at least mild chemesthetic sensations at concentrations that produced just weak to moderate bitterness on the tongue tip (Green & Hayes, 2003). This result narrows the range of potentially pure gustatory stimuli even further. Indeed, exclusively sweet-tasting chemicals may be the only class of stimuli that can confidently be considered specific tastants.

5.9 Individual differences

The sensitivity to sensory stimuli varies from person to person in every sensory system, but the degree of individual variation in chemesthesis is particularly large, and thus particularly important to measure and understand. Certainly, the finding that only about half of the individuals we tested reported capsaicin to have a bitter taste stands as an example of how pronounced such variation can be. But by far the most obvious example of individual differences is the sensitivity to, and tolerance of, chilli pepper, and the preference

for hot and spicy foods that appears to follow these two factors. Such differences have been attributed to everything from lifelong experience to genetics to personality (Karrer & Bartoshuk, 1991; Lawless *et al*., 1985; Prescott & Stevenson, 1995b; Rozin & Schiller, 1980; Rozin *et al*., 1982; Stevenson & Prescott, 1994). While each of these factors may play a role in liking and preference for chemical heat, personality probably has less to do with sensitivity than with factors such as reactivity (i.e. the emotional response to painful heat) and the willingness to try spicy foods. Nonetheless, personality may well affect sensitivity indirectly by limiting an individual's exposure to capsaicin and other spices. That mere exposure can alter the perceived intensity of capsaicin's burn (at least over a period of days and weeks) is evident in Figure 5.4, and a role for genetic variation has been demonstrated via a correlation between perception of capsaicin's burn on the tongue and sensitivity to PROP (Karrer & Bartoshuk, 1991). The marked individual differences in bitterness from capsaicin and menthol almost certainly result from genetic differences in the number and/or types of bitter receptors – whether T2Rs or others – expressed by gustatory neurons. Similarly, variations in expression of VR1 and TRPM8 may well underlie differences in the nociceptive and thermal qualities of capsaicin (and other vanilloids) and menthol (and other cooling agents). Future research in chemesthesis will therefore benefit greatly from understanding the genetics of receptor distribution. It will be just as important though to conduct parallel psychophysical studies that enable the perceptual sensitivity and responsiveness associated with these receptors to be fully characterised and understood. In these and other studies of oral chemesthesis, it will also be important to include measurements of all perceptually significant modalities of stimulation (i.e. taste and retronasal olfaction), not just chemesthetic ones. Only via multimodal studies of this kind will we eventually gain an understanding of how chemesthesis contributes to the perception of overall flavour, and the ways in which this contribution varies from person to person.

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Flavor Perception
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6 Flavour perception and the learning of food preferences

Anthony A. Blake

6.1 Introduction

Everyone has their own likes and dislikes for specific foods and flavours: sometimes the liking can be so strong that it can be described as a craving, while equally we can also dislike foods to the extent that we are disgusted by seeing others eat them. All parents at some time have experienced the frustration of getting their children to eat a new food for the first time. 'How can you say you don't like it when you haven't even tried it?' is a question that must have been asked more than once in every family in every country in every generation. The ways in which we develop our personal preferences for food and especially its flavour is a subject that until recently has been only partially answered, and the main reason for this is that the questions which need to be discussed and the issues they raise spread into so many scientific disciplines, such that at present there are few who can provide all the answers. However, in recent years there has been a confluence of scientific techniques, ideas and research results that is starting to change the whole understanding of what flavour is and how we come to have preferences for certain foods while excluding others. This chapter attempts to review the relevant issues related to this new understanding and will of necessity touch on aspects of food and flavour science, nutrition, neonatal development, neurology, experimental psychology and the social sciences. Thus, the author can claim a personal expertise in only a small part of the literature being reviewed and trust that those more expert in each of the other disciplines will excuse any errors or naïvety that appears in the text. The defence is that only by taking a multidisciplinary approach to this subject is it possible to advance it to a proper understanding of issues that certainly affect us all, which are important to much of the pleasure we have in eating and which, in the future, will very likely have a major influence on our approach to good nutrition and dietary health. (For a further discussion of this view, see Schrödinger, 1944.) This chapter is primarily aimed at food scientists for whom many of the issues being discussed will probably be quite new.

In the last ten years, there have been several important developments within quite disparate disciplines that are especially relevant to the subject of flavour perception and learning, and it is worth briefly reviewing what these are in

very general terms before proceeding to show the collective importance they have to our new understanding of flavour.

6.2 Flavour as an example of molecular communication

Our senses of taste and smell are specific examples of chemical message signalling – the earliest form of communication in living systems. All the cells within the body send messages between and within themselves. In the cells of the nervous system and brain, information is transmitted as electrical impulses, but a much older means of conveying information plays a key role as well, which relies on interactions between small signalling molecules and receptor molecules built into the structure of the cells themselves. Many of these signal transduction processes rely on a particular class of protein molecules found in cell walls, the seven-transmembrane G-protein receptors, given this name because they loop from the inside to the outside of cell walls in seven steps; and through conformational changes, which take place when the signalling molecules are bound, are able to transfer information from the outside of a cell to its inside. Nature uses this technique over and over again in living systems. From a spermatozoa trying to find and fertilise an ovum, an insect trying to find a mate, a blood platelet knowing when to become sticky and help to form a blood clot to a person sniffing his glass of wine and enjoying its bouquet, in each case very similar biochemistry is used to transmit relevant information to make the process work. Sending any message is an essentially dynamic process, where a time dimension is necessary for the decoding of the information; as you read this page, the words make sense only because you look at them in a specific sequence within time.

For many years the preoccupation of flavour scientists was to identify and establish the structure of the molecules that exist within our food and are responsible for giving it taste and odour. The molecules that give taste are water soluble and usually nonvolatile, while the molecules responsible for smell or odour are often more hydrophobic and are by definition volatile, since these are the molecules transferred from the food to the nose in the breath during exhalation. Until very recently, the analysis of these molecules concentrated on their structure and the quantities in which they are present in food, i.e. the analysis considered a single point in time. But in very recent years, a few flavour scientists have become increasingly interested in the dynamic processes of how these molecules are transferred from food to the nasal epithelium, from which information about their nature and concentration is passed on to the brain.

To continue the analogy with reading, until recently the objective had been to discover the words within the message, but now attention is focused on establishing the sequence of the words, and hence the time course of the

message itself. A key breakthrough was made at the University of Nottingham in England when a technique was developed to quantitatively monitor flavour molecules released to the breath while eating, using real-time analysis (Linforth *et al*., 1998). For the first time, the dynamics of flavour release during eating could be studied, and hence the relationships between the presence of volatile flavour molecules in the nose and the conscious perception of flavour could be determined. The importance of this work to our present understanding of what flavours are and how we learn to like them will be further developed in this chapter, but first we will introduce two other quite separate fields of research that are relevant. One relates to our knowledge of the brain and how it grows, and the other to how the human brain processes the information arriving from the different senses, specifically those that are relevant to flavour perception.

6.2.1 The human brain

An understanding of how the human brain performs its functions and a proper explanation of the phenomenon we know as consciousness has been described as one of the greatest unsolved problems of science. Many authors have written on this topic at a popular level, yet coming from very diverse backgrounds including mathematics (Penrose, 1994), psychology (Sacks, 1985), (Pinker, 1998), physics (Cotterill, 1998), neurology (Damasio, 1999; Ramachandran & Blakeslee, 1999), neuropharmacology (Greenfield, 1997, 2000a, 2000b) and scientific journalism (Carter, 1998, 2002; McCrone, 1999; Scientific American, 1999). Much of our knowledge of brain function has come from correlations made between damage to specific areas of the brain caused by trauma or stroke and the subsequent impairment of function. Experimental work has also been carried out on the living brain during surgery in order to establish the function of brain regions by direct stimulation and its consequence (Libet *et al*., 1964).

In recent years, non-invasive techniques have become available, which allow direct observation of the brains of conscious, healthy individuals. Magnetoencephalography (MEG) is a development of the old technique of electroencephalography (EEG), but much more sensitive and precise. It monitors the activity of brain cells by detecting slight changes in the magnetic field within the brain caused by changes in their electrical activity. The technique is fast and spatially precise but works best on the outer surface regions of the brain and is not very good at looking deep within it.

Nuclear magnetic resonance imaging (NMRI) and the more recent functional magnetic resonance imaging (fMRI) are two techniques that use radio waves to detect subtle changes in the behaviour of certain atomic nuclei within the brain when it is positioned in a powerful magnetic field; the precise interactions between the atomic nuclei and the radio waves depend on their

surroundings, including what type of cell and tissue they are in. Both methods are able to scan all regions of the brain but differ in that while the earlier NMRI allows a detailed picture to be created of the internal structure of an organ such as the brain, the newer fMRI goes further in that it can also give information about metabolic function within the target organ (Wiesmann *et al*., 2004). Although it is much slower than MEG, this procedure is able to identify those regions of the brain that are most active and are using up oxygen during the performance of some specific mental task such as tasting or smelling a food (Anderson *et al*., 2003; Rolls, 1997). It is now routine to 'look' inside an individual's head and watch the brain working as it performs some mental exercise, and where the information that arrives from the different senses is processed (Calvert, 2001; Posner, 1994). The technology continues to be developed in terms of both sensitivity and speed of response but is already established as a routine tool for studying the brain.

Another quite different imaging technique is positron emission tomography (PET), which also detects specific regions of brain activity but has the disadvantage of requiring the injection of a radioactive material into the subject; so, while safe, cannot be regarded as a non-invasive procedure. It may seem that these essentially medical diagnostic techniques are a long way from the day-to-day enjoyment of food, but they are becoming increasingly relevant to understanding the processes within the brain, which organise and make sense of the signals arriving from the outside world.

The notion that the brain has discrete areas that are wholly and solely dedicated to processing each different sensory channel has been dramatically revised. The general consensus of opinion is now that the brain operates in a truly holistic way in that all parts of it have the potential to be connected to each other, and this interconnectivity is essentially determined by experience. It is known that the brain has remarkable plasticity in the way it develops and restructures itself according to the sensory inputs it receives, and this is particularly true for the developing brains of infants and children. There is a stunning example reported recently (Cleaver & Derbyshire, 2002) of a seven-year-old girl who could speak two languages even though half of her brain that normally engaged in speech had been removed when she was three years old. The human brain only approaches maturity at about the age of 16 and is particularly able to modify its structure until that time, but even in adults it can show degrees of plasticity as is seen when patients recover from a stroke or brain injury.

Not only is the human brain highly adaptable in the way it can structure the connections between its neurons and change the way it functions, it is now obvious that the parts of the brain which process the different sensory inputs have a much greater degree of interconnection than had been previously thought. It is estimated that at birth the human brain has about 10^{11} neurons, which is about the same as in an adult brain. What then changes dramatically

as the child grows and the brain develops is the extent and nature of the dendritic interconnections between the individual neurons (King, 2003). It has been estimated that any one neuron can be linked in this way with several thousand other neurons, meaning more than 10^{14} connections within an adult brain (roughly equivalent in computer terms to a 10-terabyte hard disk!).

Food-eating experiences and their associated flavours will be as important to the structuring of a child's brain as are all the other sensations it receives while it matures; yet this aspect of brain development has hardly been considered by the food scientists. One of the key questions is how information, received not only through our external senses of vision, touch, taste, smell and sound but also internally generated as thoughts, feelings and emotions, is integrated within the brain for any particular eating experience, since it is during this process that we literally mould our brain structure and form our memories and expectations for food enjoyment in the future. Put simplistically, our brains can be considered as computers, which continually restructure their hardware and software depending on the tasks they have done in the past and are now being asked to do. The way the brain handles different but simultaneous sensory signals is a very active topic for the experimental psychologists and neurologists as will now be discussed.

6.2.2 Multisensory perception

Six years ago, fMRI (Calvert *et al*., 1997) showed that when scans were made of the brains of people engaged in silent lip-reading, the hearing areas of the brain were activated as well as the visual areas even though there was no sound being heard. In other words, there were sufficient connections between the vision and auditory region of the brain such that the two functioned together. This was a clear case of what is now known as multisensory perception, a phenomenon already well known to experimental psychologists and one which is very much involved in our new understanding of what flavours actually are and how we learn to like them.

The way in which the brain integrates the information it receives via the different senses to give us our conscious impression of the world around us is now being very actively studied. Almost 30 years ago, it was found that the lip movements of a person speaking can change the audible perception of sounds (McGurk & MacDonald, 1976). Since then, the way that one sensory input can modify the perception of another sense has been demonstrated many times, and it is now realised that perhaps all of our senses are modulated by the information simultaneously received by the others (Driver & Spence, 2000). Although up until now most of the research done by the experimental psychologists has been on vision, hearing and touch, it appears that taste and smell are also strongly interactive.

There appears to be a number of multisensory convergence sites in the brain that are specialised in processing information arriving from different senses (Calvert, 2001; Calvert *et al*., 1998). The orbitofrontal cortex, a small region in the front of the brain behind the eyes, had already been identified as a place where vision, smell and taste signals converged (Rolls & Baylis, 1994). We will go on to discuss these three recent developments in scientific understanding and their relevance to food flavour, but let us first restate the key points made in this introduction:

(1) Much of the information about the food we have in our mouth is transmitted to our nose in our breath, and this time-dependent or dynamic process can now be accurately monitored.
(2) Our brain develops rapidly after birth and this is primarily the way its neurons form the network of dendritic connections between themselves. The nature of this interconnectivity is very much determined by sensory experiences and not least by those associated with eating and drinking.
(3) The brain is a holistic processor of information and although we have brain regions that are largely devoted to any one specific sensory input such as taste or olfaction, there is a constant communication between these brain regions, which changes the conscious perceptions we experience.
(4) Our flavour experiences and memories of them are multisensory and associative.

6.3 What flavour is and how we learn to like it

It is important to spend a little time defining the word ‘flavour’. It is a simple word that we all use in everyday language, but we usually use it incorrectly or at best vaguely (Gibson, 1966). We taste our food to see if we like the flavour, we talk of wines with the taste of oak, raspberries or spice, and of course we all know where we experience the flavour of our food – in our mouth. The reality is that much of our information about food derives from our sense of smell detected high up in our nose; it is estimated that as much as 80% of the information about our food depends on olfaction (Murphy *et al*., 1977). The olfactive epithelium is in fact a direct extension of the brain and is the only part of it that protrudes from the skull directly into the outside world. Even though we are not conscious of its location (behind the eyes and midway between the ears), it is from the receptor cells in this organ that signals are sent to the brain giving information about the changing pattern of volatile molecules being released from food during eating. The olfactive receptor cells are continually renewed during our lifetime and are among the fastest growing cells in our body.

All individuals differ in their olfactive sensitivities and there are well-reported cases of genetically linked anosmias; one of the best known is the differing ability to smell the volatile steroid androstenone (Wysocki *et al*., 1989). Repeated exposure to an olfactive stimulant can also induce enhanced sensitivity to that odorant so that an individual's ability to smell and distinguish different volatile compounds can be trained. It has also been reported (Dalton *et al*., 2002) that such induction of sensitivity is observed to a much greater extent with women of reproductive age than is the case with men. This supports the many anecdotal reports that women show a much higher sensitivity to environmental odours than men; such heightened sensitivity would probably have major advantages for mother–infant bonding and for the recognition of safe food sources. We may sniff our food before putting it in our mouth as a preliminary and cautious step to checking its quality, but it is during eating, drinking and swallowing that the volatile components of the food are transported in the breath to the olfactive receptors, and this is what provides most of the information about our food, which we consciously perceive as flavour.

The fact that we cannot 'taste' our food when we have a cold (another example of the inaccurate way we use these words) means that the volatile molecules cannot get up into the nose and we lose the olfactive signals. Of course, even with a blocked nose we can in fact still 'taste' our food because our sense of taste is truly located in our mouth but limited to just five informational components – sweet, sour, bitter, salty and umami. Our perception of taste is very important to the overall flavour as will become increasingly apparent, but it is the olfactive signal that gives the brain the information that allows it to discriminate and recognise what it is that we are eating.

Up until the 1990s, several research groups had studied flavour release from food in the mouth and this work is well reviewed (Overbosch *et al*., 1991). However, it was not possible at that time to study the fine details because the analytical instruments available were too slow to record dynamic changes or too insensitive to measure the quantities of flavour molecules being released from food. As already mentioned in the introduction, the breakthrough came a few years ago at the University of Nottingham in England (Linforth *et al*., 1998) when an atmospheric pressure chemical ionisation mass spectrometer (APCI-MS) was modified to allow it to make real-time analysis of flavour molecules in the breath of a volunteer while eating. Work using this new dynamic analysis of flavour release could be linked to sensory perception. For the first time the actual release of flavour molecules from food could be related to the conscious impression they created, and through this came the understanding of how the nose and brain transduce the information and give us a conscious impression of it. Most importantly, it was realised that our perception of odour strength of a volatile molecule did not depend on the absolute concentration of it in the breath but rather that it responded to changes in its

concentration (Linforth *et al*., 2000) – a discovery that was to lead to a paradigm shift in understanding how to add flavouring materials more effectively to food; rapid bursts of flavour are more important to perception than a steady rate of release.

However, flavour does not rely solely on taste and olfaction; the mouth also sends information via the trigeminal nerve, one of the main nerves leading from the face to the brain. It is through this that we know whether our food is hot or cold, whether it is spicy (as with chilli or pepper) or cooling (as with menthol), and what texture it has. Our mouths are incredibly good at assessing the textural characteristics of food via our sense of touch, but it is only in recent years that we have come to understand that texture also plays a key role in what we recognise to be flavour.

Using this new ability to measure flavour release, the researchers at Nottingham went on to study how food texture could affect it and hence change its perception. It is well known that above a limiting viscosity the perceived intensity of sweetness and flavour of a thickened drink is reduced as the viscosity of the drink increases (Baines & Morris, 1989). The Nottingham group confirmed this finding (Hollowood *et al*., 2000), but contrary to expectation, found that the viscosity had no measurable effect on the actual concentration of flavour volatiles that were released to the nose. Irrespective of viscosity, the concentrations of volatile flavour molecules in the breath during consumption of the drinks were identical within the experimental errors. Clearly, the change in viscosity was affecting the perception of flavour intensity through a mechanism that did not directly involve olfaction. These results could not be fully understood when they were presented at an American Chemical Society (ACS) meeting in March 2000 (Hollowood *et al*., 2002). At this same meeting, Dr Paul Breslin from the Monell Chemical Senses Center in Philadelphia showed that the sense of taste could directly affect olfactive perception. In this now classical paper, the authors (Dalton *et al*., 2000) showed that the presence of subliminal sweetness in the mouths of tasters was able to modify the threshold level for their olfactive detection of benzaldehyde (see Figure 6.1).

In other words, a taste signal was shown to be directly modifying an odour signal whereas in the Nottingham experiments it was the texture of the drinks that was affecting odour perception. Whether this latter result was directly through the tactile receptors or because of reduced sweetness perception is still not completely resolved, but since that time we realise that in both sets of experiments what was being described were effects of crossmodal perception.

A further important finding came out of the Nottingham work, which concerned the intensity rating of flavour in chewing gum. According to volunteer tasters, the perceived peppermint intensity of a gum was found to reduce to insignificant levels after about 5 min of chewing even though the measured intensity of menthol and menthone on the breath was found to be as

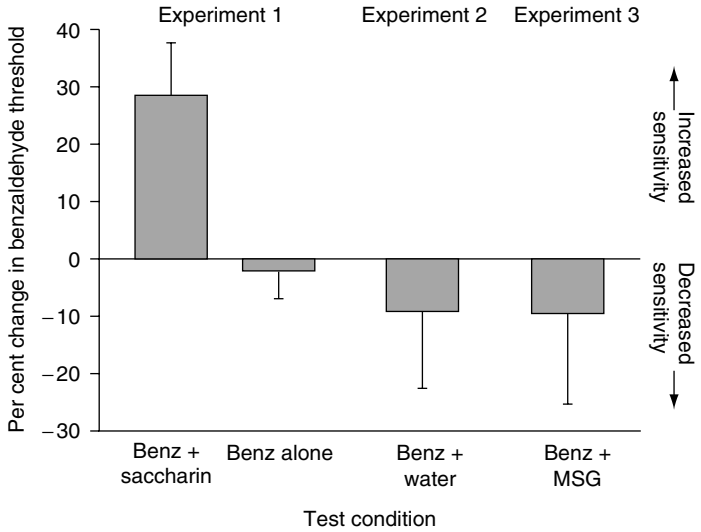

Figure 6.1 Shows the change in the threshold level for benzaldehyde odour when different tastants are present in the mouth. (Reproduced with permission from *Nature Neuroscience*.)

high as it had been at the beginning of the test (Davidson *et al*., 1999). This research further showed that the perceived intensity of the peppermint flavour did, nevertheless, correlate well with the sweetness of the gum measured by an analysis of sucrose present in the saliva; however, there is nothing inherently sweet about peppermint, the plant is not sweet so our association of its aroma with sweetness is entirely learned and is dependent on the way we use its flavour in our food. The fact that we have learned to associate peppermint aroma with sweetness probably starts from the time we first brush our teeth with sweet, peppermint-flavoured toothpaste. The way in which we learn to associate specific olfactive stimuli with a particular taste is an example of multisensory learning and clearly plays a very important role in our judgement of flavour acceptability. It is only with uncooked food that our accepted standards of normality are decided by nature. We expect a lemon to be sour and a mango to be sweet, but many volatile flavour molecules are only paired with specific tastes through the cuisines and cooking techniques that create them. Whether we relate the flavour of tea or coffee to sweetness or bitterness is entirely consequent on how we have trained ourselves to accept these drinks. Nor is perception of flavour determined only by olfaction, taste and touch; recent work shows that vision is also involved. Food scientists have long known how much colour can influence our appreciation of food, which is one reason why taste panels are often conducted under lighting conditions to reduce colour bias; however, it seems that colour is also an important factor in flavour learning. A recent report discusses how a group of experienced wine

tasters were asked to describe the quality and characteristics of a selection of white wines, which they did expertly; when the same wines were again presented but now coloured red by the addition of a nonvolatile food dye, the experts were quite unable to correctly identify the same wines and could only describe them in terms of quite inappropriate red wine descriptors (Morot *et al.*, 2001).

The fact that we have great difficulty in recognising flavours that are presented in association with inappropriate colours is not just a party trick; it is because our brains have been trained and moulded by experience and consequently have great difficulty in processing mismatched stimuli. Flavour is therefore a synthesis of all the associated sensory signals received during eating and drinking, including the internally generated emotions associated with the occasion. Gibson (1966) argues that a better word for the composite sensation we call flavour would be palatability. Whatever term is used, it describes what is arguably the most multisensory experience we have, and it should now be obvious why it is so important for the experimental psychologists who are studying multisensory perception to be talking to the flavour scientists who are studying taste and olfaction. It should also be acknowledged, however, that almost 100 years ago the psychologist E. B. Titchener (1909) wrote of the 'curiously unitary character' of the senses when eating a peach:

> Think, for instance, of the flavour of a ripe peach. The ethereal odour may be ruled out by holding the nose. The taste components – sweet, bitter, sour – may be identified by special direction of the attention on them. The touch components – the softness and stringiness of the pulp, the puckery feeling of the sour – may be singled out in the same way. Nevertheless, all these factors blend together so intimately that it is hard to give up one's belief in a peculiar and unanalysable peach flavour.

He was remarkably close to the modern description of a multisensory experience which is learned through our eating experiences wherein sensory signals that cause neurons to fire together on a repeated basis permanently change their neural connectivity, thus moulding the way the brain will process future sensory inputs. Put very simply, when we eat a particular food on a regular basis, our brain will ultimately combine all the signals received from the different senses during each of our eating experiences and create the memory we recognise as the 'flavour' of that food. The acceptability of this food is therefore a fundamentally personal thing and will depend very much on how familiar it is to us and our previous histories of eating it. If we eat a food and its flavour is even slightly different from what we expect, then our brain will tell us to pause and consider what it is that we have in our mouth; if the flavour is associated with a previously bad eating experience or is totally different from anything eaten before, then almost certainly our reaction will be to spit it out. Flavour is Nature's way of letting food communicate with us in order that

we know what it is that we have in our mouth and can decide whether or not we continue to eat it.

Flavour has been described as a psychological construct of the brain by Prescott (1999) and he makes the point that although olfactory signals are detected in the nose, the illusion of olfactory quality of food appears to originate in the mouth. Prescott also proposes that this olfactory illusion (of it being in the mouth) is important because of the high survival value of correctly identifying safe food. He says that 'since the mouth acts as a gateway to the gut, our chemical senses can be seen as part of a defence system to protect our internal environment' (Prescott, 1999, 2003). Some years earlier Gibson (1966) made the point that the sensory qualities within flavours were unlikely to be independent, and that there was more and more evidence showing how we learn to associate the different sensory aspects of flavour and integrate them into a total memory experience. According to Frank and Byram (1988), certain food odours such as strawberry and vanilla enhance sweetness. These authors suggested that perceptual similarity between a taste and an odour might arise through a history of association of these qualities in foods.

There is also evidence that the most pronounced multisensory interactions occur when the sensory stimuli are presented close to their threshold levels, exactly the situation reported in the Monell work. A region of the brain known as the superior colliculus shows little activity when dim lights or quiet sounds are presented in isolation, but its neurons fire at a dramatically increased rate when the two stimuli are presented simultaneously (Stein *et al*., 2003). Such crossmodal reinforcement of sensory signals close to their threshold levels would clearly have had enormous evolutionary advantages, since the integration of signals from all the senses would allow the recognition of opportunity or danger below the limit of detection of any one sensory channel. However, such integration is not always positive and there are also examples of one sensory input suppressing another (Stein & Meredith, 1993), which in certain circumstances could also have been advantageous, e.g. in focusing concentration on a specific task.

Without going too deeply into all the work which has now been reported on the way the brain can integrate the different sensory inputs it receives, it is clear that from now on we have to think about the perception of flavour as a daily multisensory experience. This new understanding of flavour is less than five years old, and at present there are very few food scientists who think in these terms. Inevitably, we are also brought to a conclusion that since the multimodal perception of flavour is learned, how this comes about will play a determining role in what foods we enjoy eating in later life. Let us now discuss the moments in our lives when we are first exposed to new eating sensations and what it is about them that will influence us to remember them as delicious or disgusting.

6.3.1 Learning to like flavour

Americans in general like the flavour of wintergreen, which is used in many foods and drinks in the USA, particularly in sweet confectionery and most notably in root beer. Europeans as a rule do not like these products and associate the smell of wintergreen with liniment, skin creams and other medications, but certainly not food. Clearly, this is not a phenomenon that depends on genetic differences since both populations derive essentially from the same gene pool. This is a clear example where nurture rules over nature, and the flavours liked by a group of people are consequent on their collective experiences as children. There are many similar cases; e.g. the Dutch enjoy liquorice confections, which is also true for many other countries, but in the Netherlands *dropjes* are often salty, which seems to be a preference reserved for them and some Scandinavians. The English (and most of their colonial cousins) put mint sauce on their roast lamb while much of the rest of the world thinks that this is a rather strange thing to do. My English daughter who was born in Switzerland also puts mint sauce on her lamb but does not particularly like the flavour of British chocolate. Having thought about her preferences, it is now clear to me that her favourite foods at meal-times are entirely based on those cooked by her mother, whereas her choice of leisure foods has come from those she has experienced with her childhood friends; the relevant question is when did she start to form her preferences.

It is apparent from much recent work that we are able to experience flavour far, far earlier than has generally been considered to be the case; already at 11 weeks after conception (six months before birth) the human fetus has a functioning olfactory epithelium (Doty, 1992). During pregnancy, the fetus continually swallows amniotic fluid, which contains flavour molecules transferred from the mother's blood, and there is no doubt that by the time of its birth the baby has already experienced many of the flavours that derive from the mother's diet. Results presented at the European Symposium on Olfaction and Cognition held in 1999 in Lyon, France show that three-day-old babies are more attracted to the odour of their own amniotic fluid than to that of an unrelated fetus (Schaal *et al*., 1998, 2002). Scientists at Monell have also shown that pregnant mothers who consumed carrot juice in their last three months of pregnancy gave birth to babies who responded more positively to carrot-flavoured cereal in comparison with control babies whose mothers had drunk water in place of the juice before they gave birth (Mennella *et al*., 2001; see Figure 6.2). Newborn babies have also been shown to have preferences for the foods relating to their mothers' diets (Schaal *et al*., 2000).

After the baby is born, a whole new world of sensations opens for it. It has for some time been able to hear, and recognises its mother's voice (De Casper & Fifer, 1980; De Casper & Spence, 1986). Now it is exposed to light and

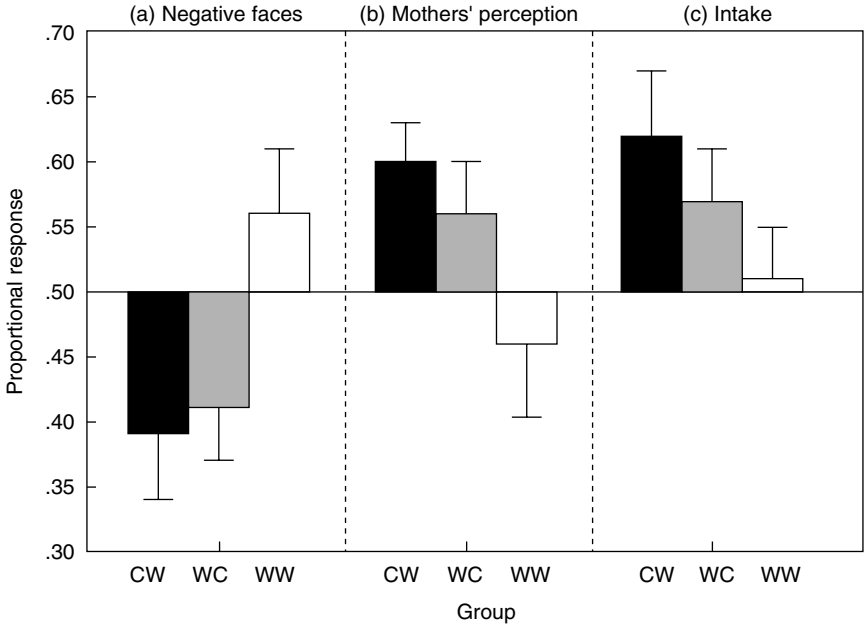

Figure 6.2 The infants' relative acceptance of the carrot-flavored cereal as indicated by display of negative facial expressions (left panel), mothers' ratings of their infants' enjoyment of the cereals (middle panel), and intake (right panel). Proportional responses were calculated by dividing each infant's response to the carrot-flavored cereal by his or her response to the carrot cereal plus plain cereal (carrot/[carrot + plain]). For example, scores above 0.50 indicate increased display of negative facial expressions, maternal ratings of infants' enjoyment, or increased intake when feeding the carrot-flavored cereal relative to the plain cereal. Mothers of infants in the CW group drank carrot juice during pregnancy and water during lactation; those in the WC group did the opposite; whereas those in the control group (WW) drank water during both exposure periods. (Mennella *et al*., 2001. Reproduced with permission from the authors and the American Pediatric Association.)

develops sight; it also experiences touch and tactile stimulation from others, especially its mother, in an entirely new way. Of particular relevance to this discussion is the fact that it starts to drink and experience the sensations of feeding, the flavours that come with the milk and all the other sensory and emotional aspects of the feeding process: nourishment, security, contact, warmth and attention. Sullivan and Taborsky-Barba (1991) have shown that odours combined with 30 s of massage of one-day-old babies condition them positively for that particular odour.

Already at a very young age a baby can recognise the smell of its own mother's milk and is aware of changes in its flavour; when nursing mothers eat either garlic (Mennella & Beauchamp, 1991) or vanilla (Mennella & Beauchamp, 1996), in both cases there is a change in the sucking rate of the baby.

Breastfeeding gives the benefit that the baby's diet is not monotonous since the foods eaten by the mother will affect the flavour of the milk and provide variety. It has been shown that mothers who consume specific flavours during breastfeeding will enhance acceptance of those flavours in their child's diet at the time of weaning (Mennella *et al*., 2001). Breastfed infants are also reported to be more willing to accept a novel vegetable on first presentation than are formula-fed infants (Sullivan & Birch, 1994). It also appears that there is an odour characteristic of human milk that makes it particularly appealing to babies (Marlier & Schaal, 1999).

The Monell scientists have also shown that early infant feeding from a bottle rather than breastfeeding and the type of formula feed can have a marked effect on food choice several years later (Mennella & Beauchamp, 2002). Children who had been bottle-fed with three different types of compound feed during their first year were subsequently compared for eating behaviour at the age of four to five years. The feeds given in the early months of life differed in that one was derived from milk, one was based on soya and another was based on protein hydrolysates; the last was described by the researchers as being unpalatable to adults with a bitter, sour taste. The children, who were now about four years older than when they were first given the formula feed, showed clear differences in their preferences for differently tasting apple juices, which were either presented as such, or soured by the addition of lemon juice or given a bitter taste by the addition of naringin, the bitter chemical in grapefruit. Milk-fed children preferred the untreated apple juice, soya-fed children preferred the bitter apple juice, while the hydrolysate-fed children preferred the acidified juice. Children were also offered the original three formula feeds. Those who had been fed hydrolysate or soya in infancy were most likely to judge the flavour of hydrolysate formula as pleasant when compared with those originally fed milk. Those fed hydrolysate also judged the odour of hydrolysate as more pleasant than those fed milk. A further interesting observation was that the mothers of infants given hydrolysate or soya feeds were more likely to rank broccoli as one of their children's favourite foods in comparison with those fed on the milk-based feed. Introducing children to a range of flavours at the time of weaning has also been shown to improve their subsequent acceptance of novel foods (Gerrish & Mennella, 2001). It is obvious from these studies that exposure of very young children to differently flavoured foods, whether at the breast or the bottle, is already forming patterns of acceptability that will certainly last into childhood and probably into adult life; yet, this fact seems to be largely ignored in books on child rearing and is essentially unknown to mothers-to-be.

A group of workers at the Department of Human Development and Family Studies at Pennsylvania State University have shown another interesting consequence of bottle-feeding on later food consumption (Fisher *et al*., 2000). In this case, comparisons were made between toddlers who had either

been breastfed or bottle-fed earlier in life. One of the main conclusions of this work was that breastfeeding in the first year showed benefits on food intake and feeding style, which persisted into the toddler phase. However, an interesting result of this work was the conclusion that these benefits were indirectly derived from the mother–child relationship that developed during the phase of breastfeeding; mothers who breastfed their children could not see how much milk the babies consumed, and developed a relaxed attitude to the fact that they were giving them enough to eat; mothers who bottle-fed their children could see how much they were giving and their concern about how well the children were eating persisted beyond the bottle-feeding stage. In a perverse way, the children put under pressure to eat more in fact ate less than the breastfed children with more relaxed mothers. It is frequently found that children coerced to eat one food in preference to another or to eat more of a particular food develop eating preferences that are quite contrary to what is wanted by the parent. As children reach the age of about two, they start to develop their sense of independence and their wish to control their own environment and those around them – the period in their lives described as the 'terrible twos'. The children express themselves both vocally and physically and now show strong preferences or dislikes for foods. They increasingly show distrust for any new food or flavour, and this is described as food neophobia. Several psychologists have studied this subject; in particular, the publications of Birch and Marlin (1982), Pliner (1994), Pliner and Hobden (1992), Pliner *et al.* (1993) and Rozin and Schulkin (1990) cover it in detail. We will return to it when we discuss food preferences in adults.

There is considerable evidence that young children left to their instincts are quite capable of selecting an adequate and nutritious diet. In a classic study, which would be virtually impossible to repeat today, 15 infants from 6 to 11 months of age (presumably in an orphanage) were allowed to self-select their diets over a period of six years (Davis, 1939). Each day there were three or four meal occasions when the children were offered the range of 34 different foods shown in Table 6.1. The supervisory staff who were present at the meal had strict instructions not to influence in any way the infants' choice of food but only to give practical help with feeding when required. It should perhaps be added that most mothers today would be delighted if their children only ate the foods described here (no sweets, no hot dogs, no ice-cream!).

Not surprisingly some of the food combinations selected were unusual. One example given is a breakfast consisting of a pint of orange juice and a bowl of minced liver, followed by a supper of eggs (mentioned in the text but not listed in the table), bananas and milk. However, all the children thrived on their self-chosen diets and instinctively met energy and nutritional needs without guidance. They were regularly checked for health and were judged above average in this respect. In the early stages of the experiment, all the children tried virtually all of the foods presented, often several times, but after a few

Table 6.1 Foods available to children during the self-selection trials of Dr Clara Davis

(1)	Water	(18)	Potatoes
(2)	Sweet milk	(19)	Lettuce
(3)	Sour (lactic) milk	(20)	Oatmeal
(4)	Sea salt	(21)	Wheat
(5)	Apples	(22)	Cornmeal
(6)	Bananas	(23)	Barley
(7)	Orange juice	(24)	Ry-krisp
(8)	Fresh pineapple	(25)	Beef
(9)	Peaches	(26)	Lamb
(10)	Tomatoes	(27)	Bone marrow
(11)	Beets	(28)	Bone jelly
(12)	Carrots	(29)	Chicken
(13)	Peas	(30)	Sweetbreads
(14)	Turnips	(31)	Brains
(15)	Cauliflower	(32)	Liver
(16)	Cabbage	(33)	Kidneys
(17)	Spinach	(34)	Fish (haddock)

weeks they settled for the few they preferred, although these were different from child to child. Sometimes patterns of choice changed, particularly after illness when it was noted that children ate increased amounts of raw beef, carrots and beets. No comments were made about the table manners or social behaviour of the children at mealtimes!

There has been a great deal of research on why animals, particularly rats, choose to eat one food rather than another. Several scientists have shown that flavours associated with post-ingestive nutritional benefits come to be preferred (see Sclafani, 2001 and Myers & Sclafani, 2003 for recent papers), while those associated with foods that cause sickness and especially gastro-intestinal illness are subsequently rejected. In this review, however, the author has largely avoided making reference to animal studies for several very simple reasons: firstly, humans are exposed to a far greater variety of flavours than other animals by virtue of the fact that they largely create their food and its associated flavours by cooking; secondly, humans are imaginative and innovative and seek novelty and variety in their experiences including their choice of foods in ways that animals do not; and finally, they communicate through language and can influence each other about different foods and their acceptability. Many conclusions from animal experiments will of course be relevant to flavour learning in humans: e.g. where nutrient deprivation or hunger is concerned, the foods that correct these physiological needs will

condition positively for the associated flavour; conversely, foods eaten to satiation will result in a temporary reduction in the liking of that food, and in the extreme case, the flavours of foods that result in gastrointestinal illness will lead to their future rejection (Kalat, 1985). However, even in the case of satiety, it has long been recognised that how humans learn to initiate and terminate meals is not only physiological but also depends on emotional and sociological factors (Hawkins, 1977). It is the author's opinion that the social occasion and the feelings of status and well-being associated with eating different foods are the most important factors in determining the attractiveness of their associated flavours, especially in those societies where hunger is not an issue. Particularly in early infancy, the bonding between mother and child will play a uniquely important role, influencing future food preferences of the child far more than is generally assumed to be the case. Birch (1998) wrote:

> While children do not need to learn to like sweet or salty foods, and will readily accept even novel sweets, there is substantial evidence that children's preferences for the majority of other foods are strongly influenced by learning and experience, especially via the impact of associative learning.

This is central to the learning of evaluative responses (Martin & Levey, 1978). It has been reported that the sight of a mother eating a particular food can positively influence a child to also try that food (Harper & Sanders, 1975). If we consider the wide range of foods eaten by all human groups on earth, one must ask the question whether any edible material that provides nourishment with no ill effects can be regarded as inherently disgusting, since if presented at a sufficiently early age with positive reinforcement from the childcarer, it would become an accepted part of the diet. The eating of insects is a good example because, while in most European and North American cultures this is regarded as disgusting, it is nevertheless perfectly acceptable in parts of South America, Australia and Asia. In the Nuba mountains of Sudan, the local tribespeople produce a fermented food called okah, which is used as a food flavouring very much in the way soya sauce is used in other parts of the world; indeed this condiment is aged and allowed to mature in just the same way as the better qualities of soya sauce are (Dirar, 1993). Okah is probably a valuable source of minerals and proteins for the tribespeople who consume it, but the starting material of cow's urine will probably preclude it becoming a serious competitor to soya sauce in Asian restaurants!

The influence of parent–child relationships and observational learning by the child from siblings and other adults (see Dunn & Munn, 1985 and Lazarus, 1991 for reviews) appear to have been only rarely investigated in the case of eating habits. This may, however, prove to be one of the most important factors for food acceptance and hence flavour learning in the young child. One-year-old children respond to signals of fear or disgust from their mothers in as brief a period as

30 min but the effects of these social signals are retained well beyond the occasion of the signals (Hornik *et al*., 1987). A mother's expression of disgust at something a young child is attempting to eat may well provide a powerful and negative association for the future acceptability of that item as a food.

A fascinating study at the Monell Chemical Senses Center has looked at the liking young children have for the smell of alcoholic beverages (Mennella & Garcia, 2000). The children studied fell into two quite clear-cut groups: those who reacted positively to the smell of drinks such as beer and those quite negatively. The most interesting finding was that in the families of the children who liked the smell, alcohol had a neutral role in family life; in the families of the children who reacted negatively to the smell of beer, there was evidence of alcohol abuse or dependency on it in the family group. It is assumed that the children had never themselves experienced alcohol but were remembering its smell in the context of unpleasant family situations.

The association of a smell with a stressful situation has also been investigated by the scientists at Monell. Five-year-old children were given a puzzle to solve in a room that was perfumed with a specific odour; the only slightly unfair aspect was that the puzzle was designed so that it could never be resolved (Epple & Herz, 2001). After this frustrating experience, the children were given a second puzzle, which was relatively easy to complete, either in an unperfumed room, in a room perfumed with a completely different smell or in a room with the same smell as the first. The children doing the second test in the last room performed significantly more poorly in their ability to solve the puzzle than either of the other two groups exposed to a different smell or no smell at all. This was not an eating experience, but clearly children can form associations between a smell and an emotional experience and vice versa, and it is hard to escape the relevance this could have to the process of forming flavour preferences and the situational or emotional environment of the meal.

For very young children, the relationship with the mother or childcarer will play a key role in making the meal occasion a happy or stressful situation, and this will be an important factor in forming positive or negative food preferences. Although there appears to be only little that is published (Mitchell, 1968), the particular event where a child is recovering from an illness provides an interesting learning situation. There will usually be extra concern and attention given by the carer and it is often the case that particular foods are offered as being easy to eat or especially tempting to the patient. From personal experience and discussion with many colleagues and friends from different nationalities and backgrounds, this seems to be a common pattern and frequently these comfort foods are those that an adult will return to when feeling unwell. In my case it is the beef broth that my grandmother was convinced a sick child needed to get better.

There does not appear to be a great deal of work published about how family meal occasions can influence food and flavour preferences. A generation or

two ago, most children were raised in larger groups where grandparents, aunts and uncles played an important part in family life and the family meal was a real social occasion. One of the most obvious trends in modern industrialised societies is the decrease in the size of the family unit. The influence this change has had on the eating habits of young children does not appear to have been studied in depth, but it is tempting to speculate that reducing the family meal to a trivial occasion where the child does not interact with adults will inevitably have important consequences on the flavour preferences it will develop as it grows up.

Much work has been done on what is needed to get a small child to try and then come to like a new food. Familiarity and repeated exposure appear to be the key requirements to overcoming neophobia (Birch *et al*., 1998; Sullivan & Birch, 1994), and peer pressure appears to play a role even in preschool life (Birch, 1980). However, it is not sufficient to simply see the food being consumed by others; it has to be actually tried by the child on several occasions (Birch, 1987, 1990) and will then be gradually accepted. Some work has related the nutritional value of the food to the flavour, and there is evidence that flavour acceptance is positively associated with energy-rich foods (Birch, 1990), especially those containing fat (Johnson *et al*., 1991). However, it appears that many parents fail to get their children to like new foods because their strategies usually involve either coercion, 'Mummy will get really cross if you don't even try it', or punishment, 'you'll sit there until you finish all those nice green beans', and often bribery, 'if you eat up your broccoli, you can have some ice cream afterwards'. Several researchers have shown that all these strategies fail and most likely because the stress of the situation and the parent's displeasure simply become associated with the food and equally importantly its flavour.

The effects of presenting foods as rewards has also been studied in three-to-five-year-old children (Birch *et al*., 1980). The food was either presented as a reward for specific behaviour, paired with extra attention from an adult or simply presented in a nonsocial context (left to be generally available or offered at lunchtime). The foods given as a reward or paired with attention got enhanced preferences. However, the idea of enhancing preference for a food by offering it as a reward need not always work. The same group (Birch *et al*., 1984) showed that when children were allowed to play only if they consumed a flavoured drink, this led to a decreased acceptance of the flavour, i.e. play was the reward but consuming the drink was the condition. Indeed the offering of one food as a reward for eating another less popular choice simply gives positive reinforcement for the reward food and negative feelings about the target food (Newman & Taylor, 1992). A better strategy for encouraging the consumption of vegetables might be: 'If you don't finish all your ice cream, you won't be able to have some of this lovely broccoli.'

In most developed countries, there is more and more concern about the poor diets of many people, especially children – not poor in terms of available food but poor in terms of dietary balance, i.e. a low consumption of fruit, vegetables and those foods containing dietary fibre, and an excessive consumption of sweet, fatty and high-calorie products. Several groups of nutritionists and psychologists have tried to find ways of correcting this imbalance in children during their early school years. For several years, the Department of Psychology at the University of Wales, Bangor, has investigated techniques for changing the eating habits of children in primary schools (Horne *et al.*, 1995; Tapper *et al.*, 2003). In a series of trials, this group have studied a variety of interventionist campaigns aimed at influencing children's attitudes to eating more fruit and vegetables. Typically, during the course of the trial, the participants are introduced to daily video cartoons showing good and bad characters. The heroes are the Food Dudes who extol the virtues of eating fruit and vegetables while the Junk Punks seek to rid the world of these foods and destroy the vital force necessary for human life. At the morning break and at lunchtime, the children are provided with packs of fruit or vegetables. Those children who normally had a home-provided packed lunch were also given additional fruit or vegetables. During the course of a campaign, typically 16 days, the children who ate target quantities of fruit or vegetables were given rewards in the form of stickers, pencils or erasers featuring the 'heroes'. In a typical trial, fruit consumption was reported to rise from 36% to 79% and was maintained at 61% thereafter; vegetable consumption rose from 44% to 66% and then was maintained at 59%. These results show that it is possible to modify the eating preferences of children, but the way of doing so must be part of a planned and positively focused strategy. Nevertheless, the author's opinion is that such interventionist tactics are attempts to correct a situation that should never have taken place. There is no escaping the conclusion that as parents we need to think about training our children to like the correct foods and their flavours far sooner and in a much more fundamental way than has been previously thought necessary.

6.3.2 Flavour learning in adults

Much of what has been discussed so far focuses on how young humans are exposed to flavours during the early years of their lives when the range of flavours they are exposed to and their memories of these experiences are largely determined by those who take care of them. Adults are in principle free to make their own decisions about their diets, but in fact this freedom is limited, not only because they are constrained by their own patterns of flavour acceptability formed during their childhood but also by the eating habits of the society in which they live.

There are features of human food selection that are very difficult to understand in comparison with the behaviour of animals. We have already discussed the fact that as small children become mobile they develop patterns of food neophobia, and as with other omnivorous animals, this is explained in terms of the evolutionary benefits from only eating foods proven to be safe and by avoiding anything new which might be harmful (Birch & Marlin, 1982; Pliner, 1994; Pliner & Hobden, 1992; Pliner *et al*., 1993; Rozin & Schulkin, 1990). Many animals that have a choice of potential foodstuffs in fact restrict their diet to only a handful of these; the Californian sea otter has a selection of more than 20 foods available to it, but each pup is taught by its mother to select only three or four of these, which it then sticks to for the rest of its life (Monterey, 2003).

It is not difficult to understand why neophobia in humans should exist. What is much more difficult to understand is the fact that human beings both individually and collectively do not stick to eating the same foods throughout their lives. Even with young children, we see patterns of food experimentation that are hard to understand. For example, they eat and apparently enjoy confectionery products with levels of sourness that are totally unacceptable to their parents. It is reported that Charles Darwin commented on his own children's preferences for rhubarb, unripe gooseberries and Holz apples, which he found disgusting (Liem & Mennella, 2003). In a recent study carried out at Monell, it is reported that over one-third of five-to-nine-year-old children show preference for highly acidic confections and that this correlates with lower levels of neophobia (Liem & Mennella, 2003). What is puzzling is that children should show this behaviour at all since it is hard to explain it in terms of any real benefit other than gratification of behaving differently from older people and of seeking novelty.

For adults it is equally hard to see why a society with an adequate and nutritious diet would spontaneously change its food and flavour preferences from generation to generation. Population groups have their own cuisine styles, which have been described as culturally transmitted food practices (Rozin, 1973). It has also been argued that flavours (herbs, spices, sauces, etc.) are added to basic foodstuffs to satisfy the human desire for variety within what would otherwise be a monotonous diet dependent on a limited range of staple ingredients. It has also been said that an important function of flavouring is to label foods as familiar and thus safe (Rozin, 1977), whereas neophobia encourages the conservatism to stay within this range of proven foodstuffs. In spite of this, the history of food acceptance patterns in many cultures does not fit with this logic. In eighteenth-century England, the weekly diet included a high consumption of salt fish but today this is only seen for sale in speciality food shops. In 1920 there were more than 200 shops preparing and selling tripe in Manchester and now there are essentially none (Mason, 2002). I recently had a conversation with a taxi driver in New Jersey who had first-generation

Italian parents, and because he had trained as a butcher he liked to dress his own tripe although, as he ruefully explained, he was the only person in the house who liked it and he could not get his kids to try it! Yet, who would have thought 20 years ago that Americans would now be eating raw fish and that sushi bars are to be found there in most small townships?

Such shifts in food preferences are not new although in the last century they have probably happened faster because of our global awareness. The flavours of tomatoes, chillis, vanilla, potatoes and maize were unknown outside the Americas until the fifteenth century but became totally assimilated into the other cuisines of the world. In all developed countries of the world food preferences have shifted significantly. One well-researched study by Cwiertka (1999) looks at changes in eating habits in Japan over the last hundred years. In her thesis she writes:

> Dietary changes in modern Japan went hand in hand with the changing lifestyles, tastes and budgets of the population, and were not merely determined by the introduction of new technologies or the availability of new foodstuffs. The emergence and growth of the urban population of white-collar workers employed in banks and offices, referred to as the 'new middle class' as opposed to the 'old middle class' formed by the merchants and craftsmen, was crucial for the cultural and social development of twentieth-century Japan. This new class played an important role in disseminating Western traits and propagating their adoption. By the 1960s the new family created by, and in the framework of, this class had become culturally dominant in Japan. The impact of the cultural ideal of domesticity on the attitude to family meals was great, and in the long term contributed to the variety as well as the quality of food prepared in Japanese homes.

These shifts in eating habits are more to do with fashion and status than nutrition, and although it has been said that our food preferences are defined by sociocultural rules rather than being influenced by physiological need (Rozin, 1982), this does not explain why we do not stick to the tried and proven foods we know, which would be the expected behaviour for a truly neophobic animal. There has been a limited amount of work on the reactions of people fed a monotonous but nutritionally adequate diet; 20-year-old volunteers on such a regime reported a dramatic increase in their food cravings, which diminished once they returned to a varied diet whereas older people in their seventies did not show this (Pelchat, 1997; Pelchat & Schaefer, 2000). Other studies also report an age-related decline in food cravings (Basdevant *et al*., 1993; Hill *et al*., 1991). One is left with the conclusion that in spite of our inherent neophobia, which is known to differ from individual to individual (Pliner & Hobden, 1992), we show a unique behaviour as human beings in that we need to try new foods and flavours and that this characteristic declines as we get older. Ironically, olfactive discrimination also decreases with age (Schiffman & Pasternak, 1979) so that elderly people, in spite of a reduced incidence of food cravings, are nevertheless more willing to

try novel foods (Pelchat, 2000). Some very recent work has used fMRI to study the brains of people who experience food craving and there are some very preliminary suggestions that the brain regions which are active during this are also those involved with drug addiction (Pelchat, 2002; Pelchat & Ragland, 2003).

Many of the flavours we find most attractive are first experienced and become accepted in childhood. Vanilla is the most popular flavour tonality worldwide and this is thought to be because of its widespread use in baby-food formulations. This is a globally popular flavour but many flavours uniquely belong to specific societies where children's early exposure to the flavours is the key to their acceptance. A classic example is the liking for autolysed yeast. In some countries waste yeast from breweries is processed into a characteristically sticky, savoury brown paste, which is particularly rich in B vitamins, and sold as a nutritious spread (Marmite® in the UK, Vegemite® in Australia and New Zealand, Cenovis® in Switzerland). Many children in these countries are introduced to these products as a thin filling in sandwiches or on toast, which then remain popular at all age levels. However, to people brought up outside these countries their flavour is generally regarded as quite disgusting. The fact that we frequently like flavours that have unpleasant features is not, however, unusual and is another intriguing and puzzling aspect of human beings and how they form their flavour preferences. Rozin (1977) describes as a uniquely human characteristic the fact that we develop strong likings for foods and drinks that are unpleasant when first tasted. Indeed the most popular beverages in the world, tea, coffee and beer, all have this feature, and there has been much written on the curiously popular appeal of chilli (Rozin, 1990). It is generally accepted that at birth we have only one intrinsic and innate flavour preference, namely for things that are sweet. So what are the reasons people learn to like flavours that according to all the accepted rules should be rejected?

The drinking of coffee is an interesting subject of study, not only because of its wide popularity even though it has intrinsically unpleasant flavour characteristics but also because it is served in so many different versions; an Italian ristretto in Milan is a very different drink from a café latté served in New York. Several groups have investigated whether the stimulant action of caffeine could explain our liking for coffee (Rogers *et al*., 2003; Yeomans *et al*., 2000). These workers were able to show that when flavours were paired with drinks containing caffeine, some people showed enhanced preferences for these flavours in comparison with drinks that did not contain the stimulant. However, this only occurred for people who were already consumers of coffee and had been deprived of caffeine prior to the trials. People who were not dependent on caffeine showed no enhanced liking for flavours paired with it, so a post-ingestive boost from caffeine would not explain why people learn to like the flavour of coffee before they become caffeine-dependent. This therefore leaves completely open the question of why we persist in drinking

coffee when our first experiences of it are unpleasant. One possible explanation which does not appear to have been investigated is that as children we learn to associate coffee, and in particular its powerful and characteristic smell, with positive emotional situations that are essentially adult. Thus the smell of freshly brewed coffee at the start of a new day when breakfast is being prepared, the experience of adults enjoying coffee together in a social situation, e.g. at the end of a meal, might be markers for contentment and maturity (see also Rozin, 1982). Has the smell of coffee and the association of its flavour as something intrinsically adult made the eventual acceptance of it as a drink a rite of passage from childhood to maturity?

From the preceding discussion it must be clear that there is still much that we need to learn about the peculiarities of why we like the flavours that we do and the extent to which these preferences are tied in with our culinary traditions. It is a peculiar fact that many of the flavour molecules to which we are most sensitive, i.e. the ones to which we have the lowest threshold levels, are only generated by cooking and are not found as such in nature. It is hard to explain how we have evolved exquisite sensitivity to molecules that did not exist before man used fire and started to cook meat. In spite of these gaps in our understanding, there has nevertheless been a lot of work done on how we can modify and train our liking for foods and their associated flavours. Just as with children, the key issue to acquiring a liking for new flavours is that they are experienced over several eating or drinking occasions; even if they are disliked and rejected on a first exposure, familiarity can ultimately lead to liking. The University of Florida has studied how to teach children to accept the flavour of grapefruit (Capaldi, 2001). In this case, by sweetening the juice, children who did not like its flavour could be persuaded to drink it; after 30 daily tasting sessions of sweetened juice, the children came to like the grapefruit flavour even when unsweetened. In our own laboratory, we have selected people with specific dislikes for certain fruits (rhubarb, durian), and with less than ten exposures shifted this to acceptance and even liking for the flavours. In a further trial, we exposed a panel of people to an olfactive stimulus they had never before experienced, but in association with different tastants. What we wanted to see was whether we could train an acceptance of a previously unknown odour in conjunction with either a sweet or a salty taste (Blake & Cayeux, 2003). The particular odour we chose was that of perilla (shiso) oil, which is used to flavour confectionery products and pickles in Japan but is largely unknown in Europe. In tasting sessions held over ten weeks, we were able to significantly increase the acceptability of this flavour in a soup but only for people who had received repeated exposure to perilla flavour in a salty situation; those exposed to the odour in a sweet situation showed no enhanced acceptability. Thus, it was the context of the odour and taste together that was learned and not the olfactive component alone.

As adults we experience several changes in life which may directly affect our food choices and each of these will, to a greater or lesser degree, modify our flavour preferences. Some of these are self-chosen but others are determined by social pressures. Typical examples of the former are changes in diet for health reasons where there has been a dramatic shift in many countries toward low-fat foods. When people change to these foods, they initially dislike the flavour but gradually come to accept it. Immigration or relocation to a new country is a situation where social pressures usually force a change in eating habits. It is reported that in such situations there is a shift in the acceptability of odours in general, which gradually moves toward those of the host country (Hudson & Distel, 1999). At an even more personal level, when tea or coffee drinkers decide to no longer add sugar to their drink, they will at first dislike it but very quickly readjust and will soon find sweetened versions unpalatable. One particular situation that has been studied in some detail is the way marriage requires a shift in food acceptability for the partners and the emergence of a joint spousal food system (Bove *et al*., 2003). Seen in the context of flavour learning and how the brain gradually integrates taste and olfactive signals, we can more easily understand the ways in which we behave in these situations.

An area of interest that will need much more work is the effect which ageing has on food acceptability and preferences in view of the growth in the number of people in this category. It is known that our olfactive abilities decrease with age (Hummel, 1999) and older people frequently complain that food does not taste the same as it did when they were young. The fact that flavour is now seen as a multisensory experience may allow us to compensate for a declining ability in one sensory channel by enhancing or modifying the others.

It is clear that within the food and flavour industries, this new realisation that our perception of flavour is multisensory and learned through experience will raise many issues and questions that will need to be answered. One issue that is especially pertinent is the extent to and way in which consumer panels are used for conducting market research. Many food and beverage products are researched for acceptability or preference on the basis of a single exposure to a consumer and outside the context of either the final product (packaging, presentation) or the eating situation (location, company, ambience). In so far that flavour acceptance changes with repeated exposure and familiarity, and that the enjoyment of a food depends so strongly on the emotional and situational experience surrounding previous eating experiences, it is difficult to see how such an approach can work. Perhaps even more questionable is the widespread use of ‘trained expert panels’ in consumer research. The more trained such tasters are, the more different they will be from the average consumer and the less relevant will be their judgements. The remarkably high failure rate of new product launches is perhaps evidence that we need a new approach that will take into account the actual changes in brain structure

and the psychological issues which are intrinsic to flavour learning. There is already an exploration of how some of the latest techniques in brain imaging can be used to literally get inside the head of the consumer and understand at a more fundamental level what it is that makes foods delicious or disgusting. In the next ten years we are likely to see a dramatic reassessment of the techniques and equipment that will be needed for consumer research.

6.4 Conclusion

In recent years, there has been rapid progress in the understanding of how the human brain develops and grows. It is realised that the fine detail of the connectivity between the neurons depends very much on the sensory information received by the brain, particularly in early childhood. This plasticity of the brain allows human beings to be uniquely adaptable to their environment, but this takes time and that is why the human infant takes so long to reach adulthood.

Although we have specific sensory inputs of sight, sound, touch, taste and smell, the brain processes the information it receives in a multisensory way because the brain regions that deal with each sensory channel are interactive. The precise detail and extent of this interaction is largely determined by personal history; thus, every brain is different because no two people can ever have identical experiences. Eating is one of the most multisensory experiences we have, although olfaction provides most of the information about what is in our mouths, the other senses strongly influence and determine our final conscious perception of flavour. The associations between flavour experiences and feelings of contentment or well-being strongly determine which flavours become desirable or disliked. This process starts even before birth when the foods eaten by the mother influence the olfactive environment of the growing fetus via the amniotic fluid in which it lives. After birth the mother–child relationship influences flavour preferences not only directly via the food given but also through contact with the childcarer and the emotional content of the eating situation.

As children develop their independence, they show a reluctance to try new foods and this neophobia is strongly influenced by their previous eating experiences; in general, babies exposed to as wide a range of flavours as possible before and after birth are more willing to try new foods. Repeated exposure to a novel flavour usually leads to its acceptance if it is associated with a positive emotional situation, but attempts to introduce new foods to children fail if coercion is used or the eating experience is stressful. The decline in the importance of the family meal in modern societies is a trend that needs to be considered in terms of the adverse consequences it may be having on children's eating habits.

Taking the wide range of foods eaten by all people on earth, it is hard to believe that any flavour is intrinsically disgusting, and those that are judged to be so are simply the ones never introduced in early childhood or that were taught to be avoided. Although humans are considered neophobic, they show behaviour that is inconsistent with other omnivores. The need to try new foods and the shifts in accepted fashions for them occur in all societies and particularly when food is plentiful. Yet, this is a trait that is difficult to understand and may be driven by a basic human desire for sensory stimulation, novelty and change. Why many popular foods are those that are unpleasant on first exposure is a particularly unique human characteristic for which there is no adequate explanation.

The new understanding of flavour as a multisensory and learned experience will have many consequences for the food and beverage industries. Not least will be the need for a fundamental reconsideration of the way in which consumer research is used to assess the acceptability of new products and the flavours they have.

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7 Functional magnetic resonance imaging of human olfaction

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7.1 Introduction

Functional magnetic resonance imaging (fMRI) enables us to visualise neuronal activation of the brain to a given stimulus, be it a simple stimulation paradigm or a complex task. More precisely, fMRI non-invasively detects the regional haemodynamic response of the brain. It can be used to locate regional activation and determine the spatial or temporal organisation of distributed processing centres. In the past years, fMRI studies have greatly advanced our understanding of human brain function. Most sensory studies, however, were focused on the visual, sensorimotor or auditory systems. Detailed knowledge about how and where the human brain encodes smells, odour-associated memories and odour-related emotions is still limited. In the past, information about the neural correlates of olfaction was derived almost exclusively from experimental animals, particularly from rodents and to a limited extent from monkeys or human lesion studies. Although the data from these studies advanced our understanding of brain regions involved in cerebral processing of odours, it is through functional imaging that the window is beginning to open more widely with the chance to reveal finer details of brain function during olfactory processing in humans. So far, only a small number of studies have been published in the area of functional imaging on olfaction, but these few studies have already demonstrated the obvious potential to advance our knowledge of the neural correlates of odour processing in humans.

7.2 The methodological basis of functional magnetic resonance imaging

7.2.1 Magnetic resonance imaging

The magnetic properties of hydrogen nuclei ('water protons') in the human body are the basis for MRI. The MRI scanner creates a strong permanent magnetic field (B_0). Magnetic field strengths between 0.5 and 1.5 tesla are typically used in clinical studies, although higher magnetic fields are already approved for humans. When a person is placed within the scanner, the spin orientation (or magnetic 'dipole moment') of the water protons in his or her

body aligns with this static magnetic field. During imaging, some of the protons are excited by a radio frequency (RF) pulse. This means that energy is transmitted to promote some of the protons from a lower energy state, in which protons are aligned to the static magnetic field, to a higher energy (anti-aligned) state. Depending on their magnetic properties, the protons will realign to the static magnetic field within milliseconds to seconds. This process has been termed relaxation. Because the changes of magnetisation during relaxation can induce small electric currents in a conducting loop, the process of relaxation can be measured using an adequate 'antenna', i.e. the MRI receiver ('coil'). There are different ways of excitation (characterised by certain manipulations of the magnetic field). In addition, different parameters of relaxation can be measured defining the image contrast (e.g. T1 relaxation, T2 relaxation or $T2^*$ relaxation). The 'MRI sequence' determines the specific way of excitation and which relaxation parameters are measured to create an image. Brain tissues and fluids differ with respect to their intrinsic relaxation times (T1, T2, $T2^*$). Usually, the information of consecutively acquired images with different image contrasts is combined to characterise the anatomical structure of the object under study. (For further details on MRI, see McRobbie *et al*., 2003.)

MRI is safe and non-invasive, and allows excellent visualisation of soft tissues such as the brain. Data acquisition of a typical set of anatomical images usually takes minutes. However, using MRI sequences such as single-shot echo planar imaging (EPI), data acquisition of single images can be performed in less than 100 ms (Mansfield, 1984).

The sensitivity of MRI, i.e. adequate anatomical resolution and image contrast, depends mainly on the signal–noise ratio (SNR). SNR is influenced by the magnetic properties of the probe to be studied, magnetic field strength of the scanner, dimension of a voxel, sensitivity of the detection coil and total acquisition time. Strategies to increase SNR include using scanners with higher field strengths, reducing the anatomic resolution (increasing voxel size) or temporal averaging (increasing data acquisition time).

7.2.2 Functional magnetic resonance imaging

The goal of fMRI is to detect activation of circumscribed brain regions induced by a specific stimulus or cognitive task. In principle, during an fMRI session multiple measures (images) of the functional state of the brain are acquired, while the brain of the person to be studied changes from a resting state to receiving sensory stimulation or performing various tasks. These images can then be compared. Statistical analysis might reveal a correlation of regional activation to distinct stimuli or tasks. The underlying physiological process is a change in synaptic activity in circumscribed brain regions, which then leads to an increase or decrease of neuronal spike rate (Duncan & Stumpf,

1991; Duncan *et al*., 1987). This process cannot be visualised directly in MRI. Instead, fMRI utilises the physiological principle that regional changes in synaptic activity lead to characteristic changes in cerebral blood flow (CBF) in the respective brain region. Increased synaptic activity results in a higher local metabolic rate (Magistretti & Pellerin, 1996), and the response to a local increase in metabolic rate is increased delivery of blood to the activated region (Matthiesen *et al*., 1998). This phenomenon is known as neurovascular coupling.

Three different strategies to visualise CBF using MRI are used in neurological research (see Moseley & Glover, 1995 for review).

7.2.2.1 Exogenous contrast agent injection

The early fMRI studies were based on defining changes in local blood volume with increased activation (Belliveau *et al*., 1991). Paramagnetic contrast agents alter the relaxation properties of blood water and of the tissue water that surrounds blood vessels. Consecutive images are acquired following intravenous injection of a bolus of such a contrast agent. The degree of signal alteration on the MRI images correlates with cerebral perfusion. By applying tracer kinetic analysis to the concentration–time curve, maps of CBF and cerebral blood volume (CBV) can be calculated. This strategy has its limitations for fMRI because each time CBF is measured a separate injection of contrast agent is required. Possible future availability of long-lived circulating contrast agents such as monocrystalline iron oxide nanoparticles (MIONs: Palmer *et al*., 1999) could change these limitations, even when multiple observations need to be made.

7.2.2.2 Arterial spin labelling

The method of arterial spin labelling (ASL) has been described as early as 1992 but has not been used widely for fMRI (Kwong *et al*., 1992). Although this approach has the advantage that the CBF can be measured quantitatively, it suffers from rather poor SNR. Proximal to the slice of the brain to be studied, protons in the arteries that supply the brain are labelled using a special RF pulse, i.e. their steady-state value of longitudinal magnetisation is modified. Directly after labelling, the slice of interest is imaged. The recovery rate of longitudinal magnetisation depends on arterial inflow of these labelled protons and correlates with cerebral perfusion. During functional activation the CBF increases, and will thus deliver increasing numbers of labelled protons into an imaged slice (Detre *et al*., 1994; Kim *et al*., 1997; Wong *et al*., 1995).

7.2.2.3 Blood oxygen level–dependent fMRI

Deoxygenated haemoglobin differs in its magnetic properties from oxygenated haemoglobin (Pauling & Coryell, 1936). A change in the proportion of deoxygenated versus oxygenated haemoglobin therefore leads to changes in the local

distortions of a magnetic field applied. Thulborn *et al*. (1982) demonstrated that the T2-relaxation rate of blood varies exponentially with the proportion of deoxygenated haemoglobin in a fashion precise enough to allow determination of blood oxygenation directly from the line width of the water proton MR signal of blood. The effect was shown to increase with applied magnetic field strength, as predicted for a phenomenon based on differences in magnetic susceptibility between blood cells and the surrounding medium (or tissue). However, it was Ogawa *et al*. (1990) who described the first true blood oxygen level–dependent (BOLD) contrast imaging experiment with a report that gradient echo MR images of a cat brain showed signal loss around blood vessels when the animal was made hypoxic. The experiments of Thulborn (characterising T2 changes) and Ogawa (characterising $T2^*$ changes) define distinct phenomena, although they are related. Both T2 and $T2^*$ changes contribute to BOLD contrast. While in the technique of ASL fMRI, only the rate of cerebral perfusion is quantified, in BOLD fMRI, the rate of cerebral perfusion and the degree of oxygenation both contribute to the signal (Ogawa *et al*., 1990, 1992). In temporal order, three components of the BOLD signal can be discerned. The first component is increased oxygen consumption, which is a consequence of increased metabolic demands and which initially *decreases* the intravascular ratio of oxygenated–deoxygenated haemoglobin. The second component is a rapid increase in regional CBF (rCBF) as a consequence of dilation of small arteries (arterioles) in the respective region of the brain. Now significantly more oxygenated haemoglobin molecules enter the local compartment per time and the net effect is an *increase* in the ratio of oxygenated–deoxygenated haemoglobin. A few seconds later, venous vessels in the affected brain region also dilate. This leads to a pooling of deoxygenated haemoglobin in the tissue. Consequently, the ratio of oxygenated–deoxygenated haemoglobin *decreases* again. Shortly thereafter, rCBF and oxygenation ratios return to normal levels.

The time course of the BOLD signal has been defined best for the primary visual cortex, where the timing of the stimulus can be controlled precisely (Ernst & Hennig, 1994). In the primary visual cortex, there is an initial small decrease in signal intensity ('early dip') that evolves over the first second following a stimulus. There is a progressive increase in signal intensity over the next 2–4 s. For a simple stimulus that does not cause physiological habituation, the signal change is maintained at a relatively constant level for the period of stimulation (Bandettini *et al*., 1997). After the stimulus stops, the BOLD signal decreases over a further few seconds to a level below the initial baseline (the 'undershoot'), from which it recovers slowly over a further few seconds. Overall, even for a brief stimulus, the time from onset to final return of the signal intensity to baseline may be 12–18 s (Figure 7.1). In comparison to the ASL technique, BOLD fMRI offers superior temporal resolution and higher SNR. Therefore, the BOLD technique has become the standard

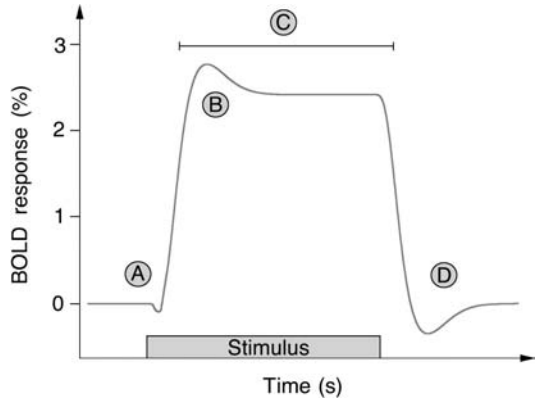

Figure 7.1 fMRI BOLD signal response. Schematic representation of the common features of the fMRI BOLD signal response to a period of neuronal stimulation. Immediately after electrical activity commences, there may be a brief period of approximately 0.5–1 s during which the MRI signal decreases slightly below baseline (A). This subtle effect, which has been termed 'early dip', is not seen in all experiments. Subsequently, a more robust 'positive BOLD response' is observed (C). Typically, the MRI signal rises above noise baseline 1–2 s after neural activity has begun, peaks at about 5–8 s (B, overshoot) and reaches a plateau if stimulation is maintained. Upon cessation of the stimulus, there is a return of the BOLD response to baseline, often accompanied by a 'post-stimulus undershoot' (D). Eventually, the response returns to baseline. At 1.5 tesla, the positive BOLD response typically amounts to 2–3% signal change in cortical areas.

technique in fMRI over the last years. The drawback is that the sequences used in BOLD fMRI are prone to susceptibility artefacts and geometrical distortion.

Two different strategies to provoke a haemodynamic response are used in fMRI. The most time-efficient approach for comparing brain responses to different tasks during the imaging experiment is the 'block' design (Bandettini *et al*., 1993; Friston *et al*., 1999). This design uses relatively long alternating periods (e.g. 30 s), during each of which a discrete cognitive state is maintained. In the simplest form, there may only be two such states, a 'rest' and an 'active' state (although 'rest' is defined only with respect to the specific activity being considered). These are alternated through the experiment in order to ensure that signal variations from patient movement, small changes in scanner sensitivity or attention have a similar impact on the signal responses associated with each of the different states. The other approach is the single-event fMRI (Buckner, 1996). In this design, activation data are acquired serially after discrete stimuli or responses. By averaging data acquired after many such discrete events, the time course of the haemodynamic response can be defined. This is a potentially powerful approach, as it allows considerable flexibility for determining, e.g. responses to novel aperiodically presented stimuli. In most paradigms, block designs lead to a more pronounced haemodynamic response with a prolonged plateau phase of the BOLD response, thus increasing the statistical power of the data. However, cognitive and 'rest'

states are difficult to control and many types of stimuli (particularly olfactory) may show rapid habituation (Bowtell *et al*., 2001; Poellinger *et al*., 2001).

Different functional brain imaging methods are usually compared and contrasted in terms of both their temporal and spatial resolution. Positron emission tomography (PET) and fMRI provide information on the increases in blood flow accompanying neuronal activation with relatively high spatial resolution (approximately 1–10 mm), but both have a temporal resolution limited by the rate of the much slower haemodynamic changes that accompany neuronal depolarisation. However, advantages of fMRI compared to PET are that firstly, it is highly accessible and less costly than PET imaging. Secondly, because there is no radioactive exposure, subjects can be scanned repetitively. Thirdly, for acquisition of entire brain volumes, the temporal resolution can typically be kept as low as 4 s. Because of this, series of hundreds of such images can be obtained from the same individual within a typical imaging session during which various activation paradigms are enacted.

The major disadvantage of fMRI studies is that the ventral frontal and temporal brain areas and the brainstem are often compromised by artefacts. The underlying bone and air produce magnetic field inhomogeneities, which can result in signal dropouts or geometric distortions. Especially affected are the $T2^*$-weighted EPI ($T2^*$-EPI) sequences used in BOLD fMRI. This can make evaluation of these regions difficult, or sometimes even impossible. Several strategies to reduce these artefacts have been proposed (Chen & Wyrwicz, 1999; Glover & Law, 2001; Li *et al*., 2002; Wilson *et al*., 2002; Young *et al*., 1988). However, areas such as the orbitofrontal cortex (OFC) have often been found to be unmeasurable in many fMRI studies.

7.3 fMRI and perception of odorous compounds

Over the last years a considerable number of imaging studies have addressed the question: 'To what degree is the processing of odours related to topographical organisation in the human brain?' Using fMRI, specific regions involved in the processing of olfactory information have been demonstrated. It has to be considered, however, that odorous signals in man may be processed by two different systems – the olfactory and the trigeminal (Kobal & Hummal, 1988; Kobal *et al*., 1992). While some substances act only on olfactory nerves (unimodal odorants), others stimulate both olfactory and trigeminal nerves (bimodal odorants: Doty *et al*., 1978; Kobal, 2003). To date, imaging data suggest that the perception of odorants is mediated by a set of core regions that are different for unimodal and bimodal odorants (Bowtell *et al*., 2001; Yousem *et al*., 1997). In addition, the piriform cortex, which is the most prominent part of the primary olfactory cortex (POC), is suggested by some authors to become activated by olfactory stimuli and

habituate rapidly to odour stimuli (Bowtell *et al*., 2001; Poellinger *et al*., 2001; Sobel *et al*., 2000), while others find 'that sniffing, whether an odorant is present or absent, induces activation primarily in the piriform cortex' and 'a smell, regardless of sniffing' does not (Sobel *et al*., 1998a). Table 7.1 summarises fMRI studies that focus on the perception of odorants. Along with a discussion of the results of fMRI experiments, the basic anatomy of the olfactory system will be outlined.

7.3.1 Anatomy and organisation of the olfactory system

Conceptually, the human olfactory system can be divided into three parts: (1) olfactory receptors (ORs), olfactory nerves and olfactory bulb; (2) POC (anterior olfactory nucleus, olfactory tubercle, piriform cortex, amygdaloid, periamygdaloid region, entorhinal cortex); and (3) secondary olfactory regions (including hippocampus, ventral striatum and pallidum, hypothalamus, thalamus, OFC and agranular insular cortex and cingulate gyrus). Since the olfactory epithelium with its olfactory neurons as well as the olfactory nerves and the olfactory bulb is not accessible by any imaging technique in humans at this point in time, only the cortical olfactory areas are discussed in this chapter.

Table 7.1 fMRI studies of olfactory perception in humans

Odorants used	Activated areas	References
U	Bilateral orbitofrontal cortex R > L, cerebellum	(Yousem *et al*., 1997)
B	Bilateral orbitofrontal, cingulate, temporal and occipital cortices, cerebellum	
U	Bilateral frontal and perisylvian cortices R > L, anterior cingulate cortex	(Yousem *et al*., 1999b)
B	Bilateral orbitofrontal, entorhinal and anterior cingulate cortices	(Levy *et al*., 1997)
U	Bilateral orbitofrontal and piriform cortices R > L, peri-insular cortex	(Sobel *et al*., 1998a)
U and B	Anterior olfactory nucleus and tubercle, orbitofrontal, inferior frontal, peri-insular and anterior cingulate cortices	(Sobel *et al*., 2000)
B	Bilateral frontal cortices, cingulate cortex, orbitofrontal cortex, amygdala, insula	(Wiesmann *et al*., 2001)
U	Bilateral piriform, orbitofrontal cortices, hippocampus, amygdala, insula, cingulate cortex, cerebellum	(Cerf-Ducastel *et al*., 2001)
U	Orbitofrontal cortex R > L, bilateral piriform cortex, amygdala, entorhinal cortex, anterior hippocampus, anterior insula, frontal operculum, basal ganglia	(Poellinger *et al*., 2001)
U	Bilateral piriform cortex, amygdala, orbitofrontal cortex	(Gottfried *et al*., 2002a)

7.3.1.1 Olfactory receptors, olfactory nerves and olfactory bulb

The OR neurons (ORNs) in the nasal cavity constitute the first neurons in the olfactory system. We know that, in humans, cAMP-gated ion channels activated by G_{olf} protein–coupled receptor proteins are responsible for odour-induced activity of OR cells (Thürauf *et al*., 1996). OR cells only express one or maybe two of these relatively unspecific receptors. In addition, all neurons expressing the same protein send their axons to the same two glomeruli in the olfactory bulb. In vertebrates, one olfactory stimulus, e.g. vanilla, does not activate one specific OR. Instead, a large number of receptors are activated, although the intensity of activation differs within all of them. A different olfactory stimulus will activate a different set of ORs, of which some may have been activated by the first stimulus, while others may not. Again, however, there is a characteristic intensity pattern of the activated receptors. Hence, quality coding seems to be related to the neuronal analysis of the topographical distribution of the receptor proteins (Mombaerts, 1999b; see Axel, 1995 for a review).

The axons from the ORNs group together into small bundles to form the olfactory nerves, or fila olfactoria. They run upwards through the foramina of the cribriform plate of the ethmoid, entering the anterior cranial fossa. On the way from epithelium to olfactory bulb the axons regroup to form more homogeneous bundles (Daston *et al*., 1990).

The olfactory nerves terminate at the ipsilateral olfactory bulb. In the olfactory bulb, the axons synapse with the mitral/tufted cells, which represent the secondary neurons in the olfactory system. The two olfactory bulbs, which have a volume of 45–55 mm^3 in young adults (Bhatnagar *et al*., 1987), lie in a bony groove formed by the cribriform plate. Although our current understanding of the synaptic organisation of the olfactory bulb depends entirely on observations made in animals, it is very likely that the human bulb has the same basic organisation. (For a detailed review, see Price, 1990.)

In sensory systems, individual neurons are frequently arranged into functional units, e.g. columns or barrels in the cerebral cortex. The rat olfactory bulb contains about 2000 functional units called glomeruli, which are responsible for the transduction and encoding of odour information. Recent studies suggest that glomeruli in the olfactory bulb are similar to other synaptic structures in the central nervous system (Mombaerts, 1999a; Shepherd & Greer, 1998). In the glomeruli, the olfactory nerve axons synapse with the mitral/tufted cells, which represent the secondary neurons in the olfactory system. Glomeruli have an assumed spheroidal diameter of $\sim$150 μm ($\sim$0.002 μl).

Until recently, it seemed impossible to localise the activation of such single functional units using fMRI. Although Yang *et al*. (1998) were able to map odour-elicited olfactory responses at the laminar level in the rat olfactory bulb,

Kida *et al*. (2002) were the first to report clear *in vivo* evidence of repeated and reproducible localisations from individual glomeruli in the rat olfactory bulb with a high-resolution fMRI at 7 tesla and using an in-plane resolution of 0.11×0.11 mm with a slice thickness of 0.125 mm (spatial resolution ~ 0.0015 μl). Their data were consistent with traditional topographic methods, electrophysiology, optical imaging and previous fMRI data to support the hypothesis that olfactory information is processed as a mosaic pattern of activated glomeruli (Xu *et al*., 2000). Olfactory stimuli activate characteristic patterns of glomeruli, which are topographically independent of the concentration of the odorant. Instead, different concentrations are assessed and discriminated by variable magnitudes of activation. Because of anatomical as well as technological restraints, high-resolution fMRI studies of the human olfactory bulb are not yet in sight.

7.3.1.2 Olfactory tract and primary olfactory cortex

Olfactory tract. The axons of the mitral cells of the olfactory bulb – the secondary olfactory neurons – leave the olfactory bulb in the lateral olfactory tract, which runs along the olfactory sulcus to reach the posterior part of the orbital surface of the forebrain. Unlike in several non-mammalian species, there is no medial olfactory tract in mammals, including primates (Price, 1990). At the rostral part of the anterior perforated substance, the lateral olfactory tract divides into its three roots, or striae. This area is called the olfactory trigone. The medial olfactory stria curves upward to the septal region. The lateral olfactory stria curves laterally and leads to the medial surface of the temporal lobe. Delineated by the medial and lateral striae is the anterior perforated substance. The intermediate olfactory stria continues onto the anterior perforated substance, ending at the olfactory tubercle.

POC. All areas receiving a direct projection from the lateral olfactory tract constitute the POC. The POC consists of anterior olfactory nucleus, olfactory tubercle, piriform cortex, anterior cortical nucleus of the amygdala, periamygdaloid cortex and a small anteromedial part of the entorhinal cortex. As a group it also comprises most of the paleocortex, which is somewhat thinner and structurally less complex than the most extensive neocortex (Jacob, 2002; Figure 7.2).

Anterior olfactory nucleus. The anterior olfactory nucleus consists of several subdivisions. At its caudal end, the lateral olfactory tract fuses with the orbital cortex. Just caudal to this level there are several small groups of cells, which belong to the anterior olfactory nucleus. In primates, including humans, other cells of the anterior olfactory nucleus are distributed from the olfactory trigone along the olfactory tract into the deepest part of the olfactory bulb itself (Price, 1990).

Olfactory tubercle. From the olfactory trigone, the intermediate olfactory stria continues onto the anterior perforated substance. On top of the anterior

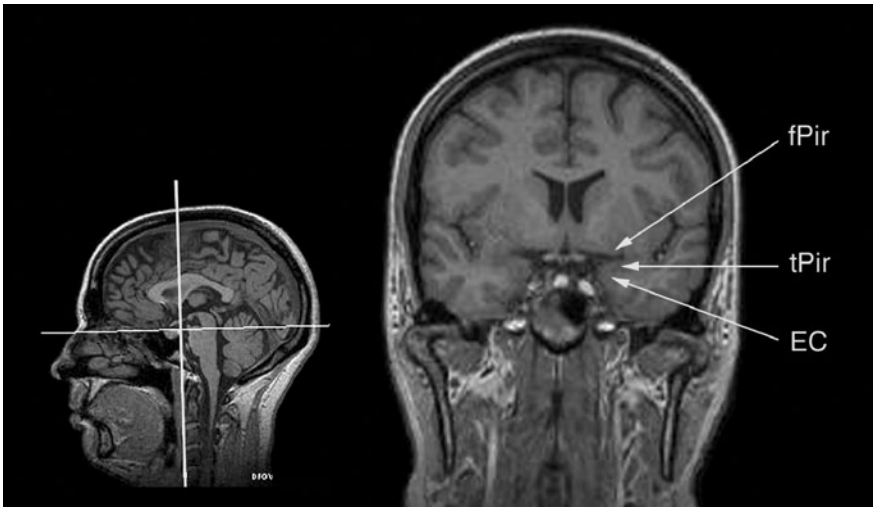

Figure 7.2 Primary olfactory cortex. Coronal section of the POC. T1-weighted MR image. fPir = frontal piriform cortex; tPir = temporal piriform cortex; EC = entorhinal cortex.

perforated substance, there is a layer of grey matter, which is called the olfactory tubercle. In rodents and carnivores, the olfactory tubercle is a prominent structure. In the monkey, the tubercle is very thin but still readily recognisable. In humans, the olfactory tubercle is so poorly developed that, without a method of labelling, it is difficult to distinguish the tubercle with certainty.

Piriform cortex. The piriform cortex is the largest olfactory cortical area in most mammals. It was previously referred to as the 'prepiriform' cortex. The piriform cortex is situated along the lateral olfactory tract on the caudolateral part of the orbital cortex, near the junction of the frontal and temporal lobes, and continues onto the dorsomedial aspect of the temporal lobe.

Anterior cortical nucleus of the amygdala. Only one of the nuclei of the amygdala, the anterior cortical nucleus, receives secondary olfactory projections. The anterior cortical nucleus is a relatively small structure in the dorsomedial part of the amygdala.

Periamygdaloid cortex. The periamygdaloid cortex is a paleocortical region on the medial surface of the amygdala. It belongs to the uncus of the temporal lobe, and has been described as very heterogeneous (Price, 1990).

Olfactory entorhinal cortex. In humans, the entorhinal cortex (Brodmann area 28) is situated in the ambient and parahippocampal gyri of the temporal lobe. In rodents and carnivores, a large fraction of the entorhinal cortex receives fibres directly from the olfactory bulb. In primates, only a relatively small 'olfactory' part of this cortex receives a direct olfactory projection. In

humans, the extent of the olfactory bulb projection to the entorhinal cortex is unclear, but an anteromedial region has been suggested to correspond to the olfactory entorhinal cortex of the monkey (Price, 1990).

Connections within the POC. In rodents and carnivores, it has been shown that there is an extensive system of associational connections within the areas of the POC. These fibres originate in all of the olfactory areas except the olfactory tubercle. Many of the associational fibres also extend into cortical regions beyond the area that receives fibres from the olfactory bulb, including portions of the entorhinal, perirhinal and insular cortex, and the medial amygdaloid nucleus (Price, 1990). Data on these projections in either man or monkeys are scarce.

Contralateral connections. The projection of the olfactory bulb itself is entirely unilateral. However, fibre bundles originating from those cells of the anterior olfactory nucleus, which are located in the olfactory bulb, run with the lateral olfactory tract to cross in the anterior commissure and reach the contralateral olfactory bulb and cortex. Similar commissural fibres also originate more caudally in the anterior part of the piriform cortex (Price, 1990).

Centrifugal projections to the olfactory bulb. Many of the olfactory cortical areas, including the anterior olfactory nucleus, piriform cortex and periamygdaloid cortex, send fibres back to the olfactory bulb. The projection of the anterior olfactory nucleus is bilateral. There is also a substantial projection from the nucleus of the horizontal limb of the diagonal band to the superficial layers of the olfactory bulb (Price, 1987).

So far, a clear transformation of the topographic map of the bulb onto the olfactory cortex has not been demonstrated. Small areas of the olfactory bulb project to virtually the entire olfactory cortex, and small areas of the cortex receive afferents from virtually the entire olfactory bulb (Haberly & Price, 1977).

7.3.2 fMRI studies of the primary olfactory cortex

In some fMRI studies dealing with the perceptions of odorous compounds, activation of the POC could be demonstrated (Cerf-Ducastel & Murphy, 2001; Gottfried *et al*., 2002a; Kettenmann *et al*., 1999; Poellinger *et al*., 2001; Sobel *et al*., 1998a, 2000; Figure 7.3). However, in other experiments, higher-order olfactory areas showed strong activation, whereas the POC appeared silent or showed only inconsistent activation (Kettenmann *et al*., 2000; O'Doherty *et al*., 2000; Wiesmann *et al*., 2001; Yousem *et al*., 1999a, 1999b). Despite the fact that the POC is rather small, this finding was still surprising, given the fact that other primary cortices like the primary visual or primary auditory cortex show a consistent and strong activation in fMRI studies. It is generally supposed that secondary regions stand in a hierarchical arrangement relative to primary regions, and that initial sensory processing takes place within the

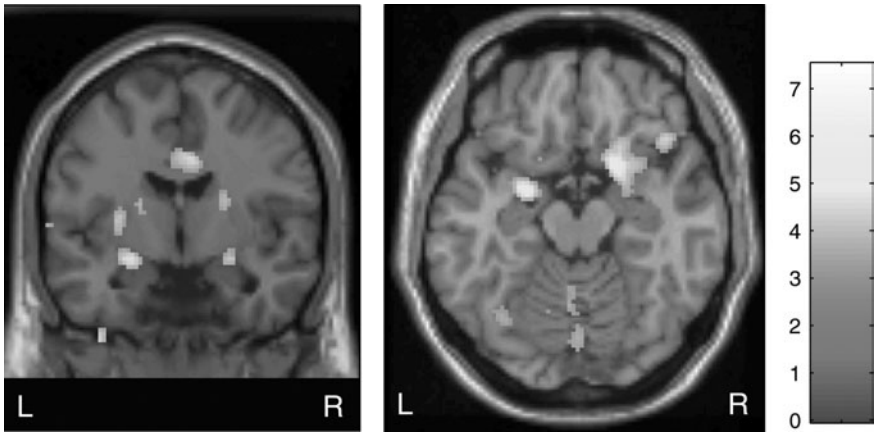

Figure 7.3 fMRI activation of primary olfactory regions. Activation maps showing significant increases in the BOLD contrast signal following olfactory stimulation using eugenol. Single-subject analysis using the SPM99b software (Wellcome Department of Cognitive Neurology, London, UK). Coronal slice (left) shows bilateral activations of the piriform cortices, cingulate cortices and basal ganglia ($p < 0.001$). Axial slice (right) shows bilateral activations of the piriform and posterior OFCs, and activation of the right lateral OFC. Also observed is cerebellar activation.

primary area. If this assumption were correct, one would expect to find POC activity very consistently. Technical limitations (susceptibility artefacts) as well as physiological effects of habituation (Bowtell *et al*., 2001; Poellinger *et al*., 2001; Sobel *et al*., 2000) may contribute to the inconsistent POC activation observed:

(a) *Image artefacts*: the POC is situated in the ventral frontal and temporal brain. As discussed above, in fMRI studies these areas are often compromised by signal dropouts or geometric distortions due to magnetic field inhomogeneities. Many olfactory fMRI studies have been performed using techniques that precisely map higher-order olfactory areas at the cortical surface, but show severe signal loss in the area of the POC.
(b) *Habituation*: desensitisation to odour is a common experience in everyday life and well-documented in the literature (Hummel & Kobal, 1999). When you enter a room you might sense the presence of an odour immediately. With time, however, you notice it less and less, until eventually you may not notice it at all. ORs, olfactory bulb and POC all contribute to this phenomenon. Adaptation of the OR requires several seconds of a constant stimulus (see Dalton, 2000 for review). With prolonged stimulation, an initial phasic component can be discerned followed by a lower-amplitude tonic component. Repeated short odorant pulses also produce adaptation of the OR. Interstimulus intervals of 60 s

are sufficient to allow recovery of receptor adaptation (Kobal, 1981). Within the olfactory bulb, the mitral/tufted cells – secondary olfactory neurons – show habituation modulated by central structures including the POC (Shipley & Ennis, 1996). In an fMRI study of the rat olfactory bulb, odorant stimulation for 4.8 min did not affect any decrease in activation, whereas after longer exposure (27.6 min) habituation occurred (Yang *et al*., 1998). The piriform cortex is the most distinctive part of the POC. Compared to the olfactory bulb, the piriform cortex shows a much faster habituation. With repeated short stimuli as well as with prolonged stimulation of odours, the piriform cortex neurons habituate rapidly and completely (Wilson, 1998). In an fMRI study by Poellinger *et al*. (2001), the question of habituation was addressed. Consistent POC activation was demonstrated when a short stimulus (9 s) was used. After a long stimulus (60 s), the temporal pattern of the BOLD response signal from the POC showed a short, phasic increase, followed by a prolonged decrease below baseline. On the contrary, in higher-order olfactory regions, namely the OFC, a sustained signal increase was observed using the long stimulus. Consequently, Gottfried *et al*. (2002a) were able to show a consistent activation of the posterior part of the piriform cortex to odour stimulation using an event-related fMRI design. These findings have to be taken into consideration for fMRI data analysis. In fMRI analysis, voxels are termed activated if their signal curve is temporally correlated with an expected BOLD response function. Usually the BOLD response function is expected to be similar to the stimulus. If the haemodynamic response is modified by habituation, the curve of the measured signal intensity will not match the stimulus. As a consequence, activation of the POC can be shown if either short odour stimuli are used or the POC is analysed using a modified expected BOLD response function (Sobel, 2000). The majority of olfactory fMRI studies do not fulfil these criteria.

7.3.2.1 Secondary olfactory regions

From the POC, information is transmitted to several other parts of the brain, including the OFC, hippocampus, ventral striatum and pallidum, medial and lateral hypothalamus, medial thalamus, and agranular insular cortex. These areas have been referred to as secondary olfactory cortex. Most of the data about these connections, however, are derived from studies on rodents or carnivores. Projections to and among these areas are complex. Most of these areas are not specific for the processing of olfactory stimuli but show activation by other sensory inputs as well.

Of the secondary olfactory cortex, the hippocampus and anterior insula have been shown to demonstrate a BOLD response function similar to that of piriform cortex, indicative of rapid habituation, whereas the OFC demonstrated a different haemodynamic response with persistent activation

after long stimuli (Poellinger *et al*., 2001). Among secondary olfactory regions, the OFC and amygdala have been studied most extensively.

7.3.2.2 Orbitofrontal cortex

The OFC forms the ventral surface of the frontal lobe (Figure 7.4). OFC activation is a consistent finding in fMRI studies of human olfaction (Figure 7.5). There is converging evidence that specialised areas within the OFC are engaged depending on the specific task of olfactory processing and that there is some functional lateralisation.

In the monkey, two separate regions with rather specific functions in olfactory processing have been identified within the OFC: an area in the lateral posterior orbital cortex (LPOC), in which cells respond rather selectively to specific odours; and a region in the central posterior orbital cortex (CPOC), in which neurons respond much less selectively. Lesion studies in monkeys indicated that the LPOC is involved in the identification or discrimination of specific odours (Price, 1990; Takagi, 1984). These areas approximately correspond to the lateral orbital gyri and posterior orbital gyri in man.

Analysis of patients with OFC lesions indicate a right OFC dominance for certain types of olfactory processing (Jones-Gotman & Zatorre, 1993; Zatorre *et al*., 1991). Correspondingly, both PET and fMRI studies of olfactory

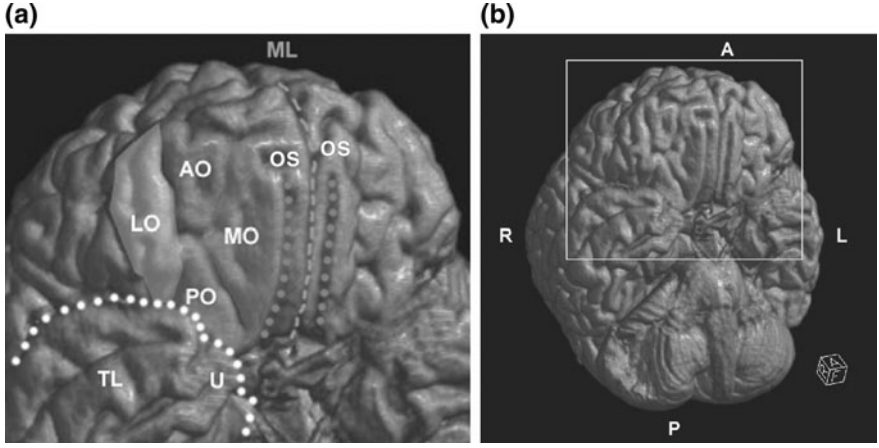

Figure 7.4 Orbitofrontal cortex anatomy. 3D-reconstructed MR image of a human brain specimen. Orbitofrontal region (Figure 7.4a) as viewed from below (Figure 7.4b). The orbital surface is marked by the triradiate or H-shaped orbital sulcus, which constitutes the principal landmark for the subdivision of the orbital gyri. Olfactory bulbs and tracts have been removed. AO = anterior orbital gyrus; LO = lateral orbital gyrus; MO = medial orbital gyrus; PO = posterior orbital gyrus; OS = orbital sulcus; ML = interhemispheric fissure (midline); TL = temporal lobe; U = uncus of the temporal lobe; A = anterior; R = right; L = left; P = posterior.

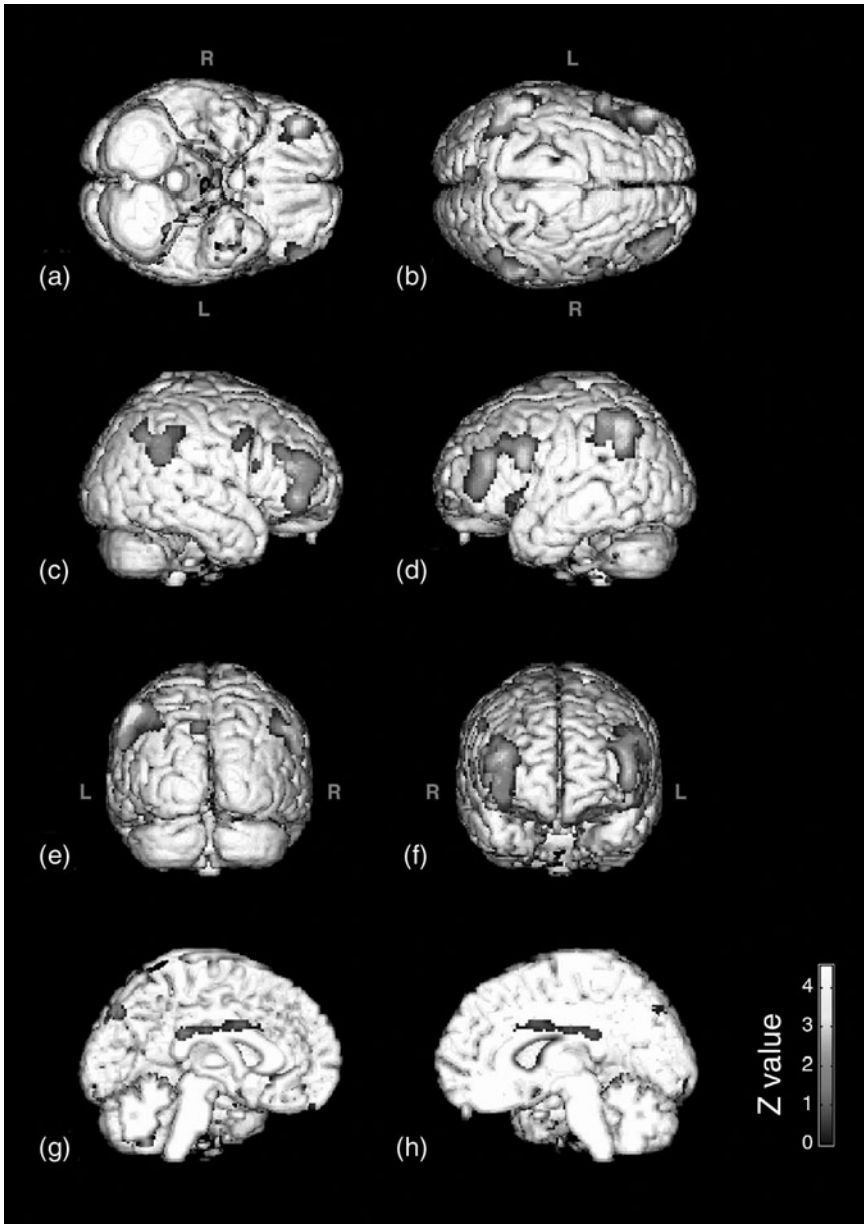

Figure 7.5 fMRI activation of secondary olfactory regions. Activation maps showing significant increases in the BOLD contrast signal ($p < 0.01$) following olfactory stimulation using eugenol. Data correspond to a group analysis of six subjects using SPM96b (Wellcome Department of Cognitive Neurology, London, UK). Results are spatially normalised to the stereotactic space of Talaraich and Tournoux and displayed on a 3D

(*Continued overleaf*)

stimulation report more frequent (or stronger) right OFC activations than left OFC activations (Dade, 1998; Francis *et al*., 1999; Royet *et al*., 1999; Small *et al*., 1997; Sobel *et al*., 1998a; Yousem *et al*., 1997; Zatorre *et al*., 1992). Based on PET and fMRI data, it has been postulated that the right central OFC may play a critical role in recognition memory, while the left lateral OFC region may play a more central role in hedonic judgements (Zald & Pardo, 2000).

Another fMRI study demonstrated that regions of the OFC are related to olfactory sensory-specific satiety (O'Doherty *et al*., 2000). The activation of some regions in the OFC produced by an odour of food eaten to satiety decreased, whereas there was no similar decrease for the odour of food not eaten in the meal. In addition, a study by Francis *et al*. (1999) showed that parts of the OFC that are concerned with representing the positively affective aspects of somatosensory stimuli are different from the areas activated by taste and smell.

7.3.2.3 The role of the amygdala in hedonic processing of odours

A few imaging and lesion studies have begun to explore the functional anatomy of hedonic and semantic processing of odours, i.e. the induced emotional construct and the qualitative meaning of an odour. For example, the odour of vanillin can have the qualitative meaning of sweet sugar biscuits (semantic) with a positive emotional construct (hedonic) of something that is always eaten at a relaxed and pleasant teatime.

Figure 7.5 (*Continued*) reference brain (SPM template). The reference brain is shown in view from caudal (a), above (b), right lateral (c), left lateral (d), occipital (e), frontal (f), left medial (g), right medial (h) sections. Orientations are indicated (L = left; R = right).

Chemosensory stimuli were presented by means of a special constant-flow olfactometer that presents odours to both nostrils separately without altering mechanical or thermal conditions at the stimulated mucosa. Odour stimuli consisted of 800 ms suprathreshold monorhinal pulses of eugenol that were embedded into the constant-flow air stream with controlled temperature (36.5°C) and humidity (80% relative humidity). Olfactory stimuli were presented to the left nostril only with a concentration of approximately 3 ppm. No attempt was made to synchronise stimulus presentation with the breathing of the subjects. Instead, subjects were trained to breathe through the mouth (velopharyngeal closure) to avoid respiratory airflow in the nose. fMRI was performed on a 1.5 tesla MR scanner (Siemens Vision, Erlangen, Germany) using gradient echo, echo planar T2*-weighted images with blood oxygenation level–dependent (BOLD) contrast. Functional image volumes were collected continuously every 6 s in one run. Periods of rest and olfactory stimulation alternated. Activation periods lasted for 48 s (eight volumes being collected in this time). Rest periods lasted for 42 s (seven volumes being collected in this time). Four periods of olfactory stimulation and five periods of rest were analysed. During the periods of olfactory stimulation, one odour stimulus was applied at the beginning of an activation period. Three more odour stimuli were applied with an interval of 12 s. In total, four odour stimuli were given within each period of olfactory stimulation. Experimental details have been described elsewhere (Wiesmann, 2001).

Following olfactory stimulation, bilateral activations were observed in the supramarginal, middle frontal, inferior frontal and lateral orbitofrontal gyri. Smaller clusters of activation were found in the cingulate gyrus, the right precentral gyrus and the left precuneus.

The amygdala is proposed to play an important role in affective responses in general, and in olfactory hedonics in particular. Of all the senses, olfaction possesses the most intimate relation with the amygdala. The anterior cortical nucleus of the amygdala is considered to be a part of the POC. In addition, associational fibres from other parts of the POC extend into the medial amygdaloid nucleus (Price, 1990). Seizures or electrical stimulation of the human amygdala frequently produce unpleasant olfactory hallucinations, but rarely produce pleasant hallucinations (Andy, 1967; Andy *et al*., 1975; Chitanondh, 1966). Temporary changes in judgements of odour pleasantness have been reported after reversible electrolytic lesions of the right amygdala (Hughes & Andy, 1979). Following temporal lobe resection including excision of the amygdala, an increased variability in the rating of odour pleasantness was found (Duerden *et al*., 1990).

The neuroimaging literature on the amygdala's response to the hedonic value of sensory stimuli converges with this clinical evidence. There is a preferential activation of the amygdala during exposure to aversive tastes, unpleasant visual stimuli and aversive odours (Irwin *et al*., 1996; Lane *et al*., 1997; Taylor *et al*., 1998; Zald & Pardo, 1997; Zald *et al*., 1998). Apart from the amygdala, activation following aversive odours was also found in the left OFC (Zald & Pardo, 1997). It has been speculated that, over evolution, the amygdala has taken on a more negative valence-specific role in sensory processing (Zald & Pardo, 2000). In a clinical study, aversive odour stimuli lead to bilateral activation of the amygdala in social phobics as well as in healthy controls. Following the presentation of neutral faces, only the social phobics demonstrated amygdala activation (Birbaumer *et al*., 1998). These data suggest that the amygdala is active in human phobics when they are exposed to potentially fear-relevant stimuli.

Interestingly, in a recent study by Anderson *et al*. (2003), the role of the amygdala for the processing of aversive stimuli has been questioned. Anderson *et al*. compared the effects of olfactory stimuli that varied in arousal and pleasantness. They claimed that arousal and pleasantness are processed independently, by different brain regions. The amygdala responded strongly to the emotional arousal elicited by high-arousal odours, but did not differentiate between the pleasantness and unpleasantness of the odours. Conversely in the same study, a region in the right medial OFC responded preferentially to pleasant stimuli regardless of arousal, whereas a region in the left lateral OFC responded preferentially to unpleasant stimuli. The latter finding was confirmed in a second fMRI study by Gottfried *et al*. (2002b). These results were partly supported by other imaging techniques. In these studies, the left OFC was also found to be activated in response to emotional visual, auditory and gustatory stimulation (Royet *et al*., 2000; Zald & Pardo, 2000). Apart from these results, the study by Gottfried *et al*. showed that an anterior part of the piriform cortex is engaged by the hedonic value of odorous stimuli.

In conclusion, the amygdala has often been portrayed as the centre of fear and loathing in the brain. However, in future studies investigating the role of the amygdala, the effects of arousal will have to be carefully controlled. There is the possibility that hedonic processes engage orbitofrontal areas and parts of the piriform cortex rather than (or in addition to) the amygdala. Findings doubting the role of the amygdala in the processing of emotions and hedonic portion of odour perception need to be replicated in carefully designed and controlled studies.

7.3.2.4 Other brain regions

Neuroimaging studies also indicate that olfactory responses often occur outside traditionally defined olfactory areas, e.g. in the cingulate (Figure 7.5) or the cerebellum.

7.3.2.5 Cingulate

Although there are connections between the cingulate gyrus and frontal areas involved in olfaction (Barbas *et al*., 1999; Vogt & Pandya, 1987), the cingulate gyrus has not typically been considered part of the olfactory system. The cingulate gyrus is involved in the processing of information of various kinds. More specifically, the anterior cingulate is frequently involved in tasks requiring attention to sensory features in the environment (Devinsky *et al*., 1995). In olfactory studies, activations have been reported in anterior as well as posterior parts of the cingulum (Fulbright *et al*., 1998; Kettenmann *et al*., 1999, 2000; Levy *et al*., 1997; Yousem *et al*., 1997). Interestingly, the cingulate gyrus has also been reported to be of critical importance in the processing of painful sensations (see Peyron *et al*., 2000 for review). Thus, one might speculate that emotions induced by either odours or pain relate to the similarity in the pattern of brain activation seen after odorous or nociceptive stimulation.

7.3.2.6 Cerebellum

In several studies, cerebellar activation following olfactory stimulation has been reported (Cerf-Ducastel & Murphy, 2001; Sobel *et al*., 1998b; Wiesmann *et al*., 2001; Yousem *et al*., 1997). Yet, the functional significance of these findings remains unclear. Sobel *et al*. (1998b) compared the effects of smelling versus sniffing on cerebellar activation and hypothesised that the cerebellum maintains a feedback mechanism that regulates sniff volume in relation to odour concentration. Further studies are needed to elucidate the role of the cerebellum in olfaction.

Whereas the studies mentioned so far focused on the aspects of odour perception, there is also research on other processes related to olfaction, namely olfactory memory, interaction between olfaction and other sensory modalities, and the transduction of pheromone-like compounds.

7.3.2.7 Imagination of odours and memory

In lesion studies, deficits in odour memory have been found in patients with temporal as well as frontal lesions (Jones-Gotman & Zatorre, 1993). Very few imaging studies have as yet been conducted on odour memory. Levy *et al*. (1999) used fMRI to measure odour memory–induced brain activation. They concluded that odours can be imagined and similar brain regions are activated by both imagined and actual odours. However, in their study, imagination of odours elicited quantitatively less brain activation than actual smells of corresponding odours. In another fMRI study, Kettenmann *et al*. (2000) compared the imagination of an odorant with actual olfactory stimulation. They drew almost the same conclusions even though the areas activated in the two studies were different from each other. Analysis revealed surprising similarity in cortical activation during stimulation and imagination/recall of the odorant. The areas activated in this experiment were mainly secondary/tertiary cortical areas related to association, i.e. the supramarginal gyrus, medial frontal gyrus, inferior frontal gyrus, the transition of the orbitofrontal gyrus, the insular gyrus and the cingulate gyrus. In this study, the intensity of activation during the two conditions was similar except for the medial frontal gyrus and the cingulate gyrus, which were more activated during the imagination/recall task. Using another imaging technique, Dade *et al*. (2001) studied olfactory working memory. They found that olfactory working memory and face working memory engaged similar brain regions, namely the dorsolateral and ventrolateral frontal cortex and the inferior parietal cortex. Visual working memory, but not olfactory working memory, additionally engaged the left superior parietal cortex.

7.4 Interaction between olfaction and other sensory modalities

Another important area in the perception of odours is the highly integrative interaction between olfaction and other sensory modalities. As already mentioned above, Francis *et al*. (1999) investigated the processing of somatosensory stimuli that feel pleasant (soft velvet touch to the hand) and compared them with wood, presumably an affectively neutral touch. To separate the affective aspects of the tactile stimuli from modality-specific aspects, two positively affective stimuli in other sensory modalities, taste and smell, were also investigated. It was shown that pleasant touch to the hand produced a stronger activation of the OFC than touch considered affectively neutral. In contrast, the affectively neutral, but more intense touch produced more activation in the primary somatosensory cortex than the pleasant stimulus. The authors concluded that parts of the OFC are concerned with the representation of positive affective stimuli. The comparison of these data with the results obtained from gustatory and olfactory stimulation showed that

the orbitofrontal areas activated during pleasant touch are different from the orbitofrontal area activated by taste and smell. This finding was extended by another study of the same group where satiety-related olfactory activation of the human OFC was investigated (O'Doherty *et al*., 2000). The paper describes the response of the OFC to odours when food was eaten to satiety. Activation of regions in the human OFC was related to sensory-specific satiety. In particular, activation of the OFC decreased to the odour of a food eaten to satiety (banana), but not to another odour of a food that had not been eaten at all (vanilla). The particular aspect of the sensory stimuli that had the strongest effect in this experiment was the pleasantness of the taste or smell of the food. Eating food to satiety did not alter the intensity of the corresponding odour. The authors concluded that when food is eaten to satiety, its reward value and its pleasantness decreases. This experiment identified orbitofrontal areas that specifically signal olfactory information, but only under certain circumstances, namely when the individual is hungry. This finding is in line with results from electrophysiological studies in monkeys (Rolls *et al*., 1996). It would be highly interesting to study patients with eating disturbances using this experimental paradigm.

7.4.1 Transduction of pheromone-like compounds in humans

A separate and intriguing issue in olfactory processing is pheromone transduction in humans, which has been highly questioned because the human vomeronasal organ lacks the neural elements that are capable of transmitting such signals to the brain (Trotier *et al*., 2000). Although the data are still limited, neuroimaging studies including PET and fMRI have shown that putative human pheromones activate core regions of the olfactory system and that there is a sex-differentiated response (Savic *et al*., 2001). Even an undetected airborne chemical was shown to induce activation in the brain – although not in the piriform cortex (Sobel *et al*., 1999).

7.5 Conclusion

In conclusion, functional imaging data so far support a model of hierarchical organisation of olfactory processing (Royet *et al*., 2001; Savic *et al*., 2001; Schab, 1991). From the ORs and olfactory bulb, olfactory information is projected to the POC, which is located at the posterior part of the orbital cortex and the medial aspect of the temporal lobe. The piriform cortex is the most prominent part of the POC in man. Neuroimaging, neurophysiological and neuroanatomical data reported in the literature suggest that this area is at least minimally engaged during all olfactory tasks. A variety of secondary regions have been shown to receive projections from the POC, among which the right

OFC seems to be engaged in most olfactory processing. Thus, core areas within the olfactory system may play a mandatory initial role. However, the involvement of secondary regions seems to vary with specific task demands, e.g. whether odour processing is related to recognition or emotional response. Finally, a third level of organisation appears to involve brain areas that fall outside of the typically defined olfactory system which become engaged during specific types of processing. Examples of these tertiary areas are activation of the cingulate cortex as a multimodal sensory processing area or involvement of the middle frontal gyrus in tasks requiring olfactory memory processing. In summary, odour processing seems to comprise a serial processing of information from primary to secondary and tertiary cortices, and also a parallel, distributed processing engaging a complex and distributed network of brain regions whose pattern of activation varies depending on the specific requirements of the task. fMRI offers a non-invasive means to study the functional topography of olfactory processing in man, and has the potential to further advance our understanding of the complex mechanisms of odour perception in man.

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Flavor Perception
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8 Flavor interactions at the sensory level

Russell S. J. Keast, Pamela H. Dalton and Paul A. S. Breslin

8.1 Introduction

The positive expectations of consuming an apple begin when we visualize it and handle it, but the majority of sensory information comes when we take the first bite. The perceived flavor of the apple is derived when three independent sensory systems are activated, taste, olfaction and oronasal somatosensations (irritation, tactile, thermal), making flavor a multimodal experience. Figure 8.1 is a schematic diagram of the various sensory/physical inputs and influences on flavor perception. Chemicals within a food and the physical properties of the food activate a wide variety of receptor mechanisms within each sensory system that convert the chemical or mechanical message to electrical signals which are transduced via afferent nerve fibers to specific regions of the brain. The brain decodes the electrical signals in both specialized regions and regions common to the different modalities. What we perceive as the flavor of the food/beverage is the result of complex stimulus–response interactions between a food matrix and human sensory, perceptual and cognitive processes. Thus, to understand flavor, we need to examine the relevant psychophysical literature that investigates interactions among sensory modalities. What follows is an overview of properties of sensory modalities, interactions within and between modalities and the influence these factors have on flavor.

8.2 The psychophysical curve: physical intensity vs. perceived intensity

There are orderly relationships between regular variations in the physical world, such as variations in concentration, and our perceptions of these changes as a function of how the stimuli affect our sensory systems. The physical and psychological parameters can be plotted as a concentration–intensity function with the physical parameter (chemical concentration, energy decibels, photon flux) on the *x*-axis (abscissa) against perceived intensity on the *y*-axis (ordinate). The final shape of the plot is the psychophysical intensity curve for the stimulus and subject or sample population, and illustrates how perceived intensity grows with increasing physical intensity. The shape of this function will vary both as a function of the particular stimulus employed and the observer under study.

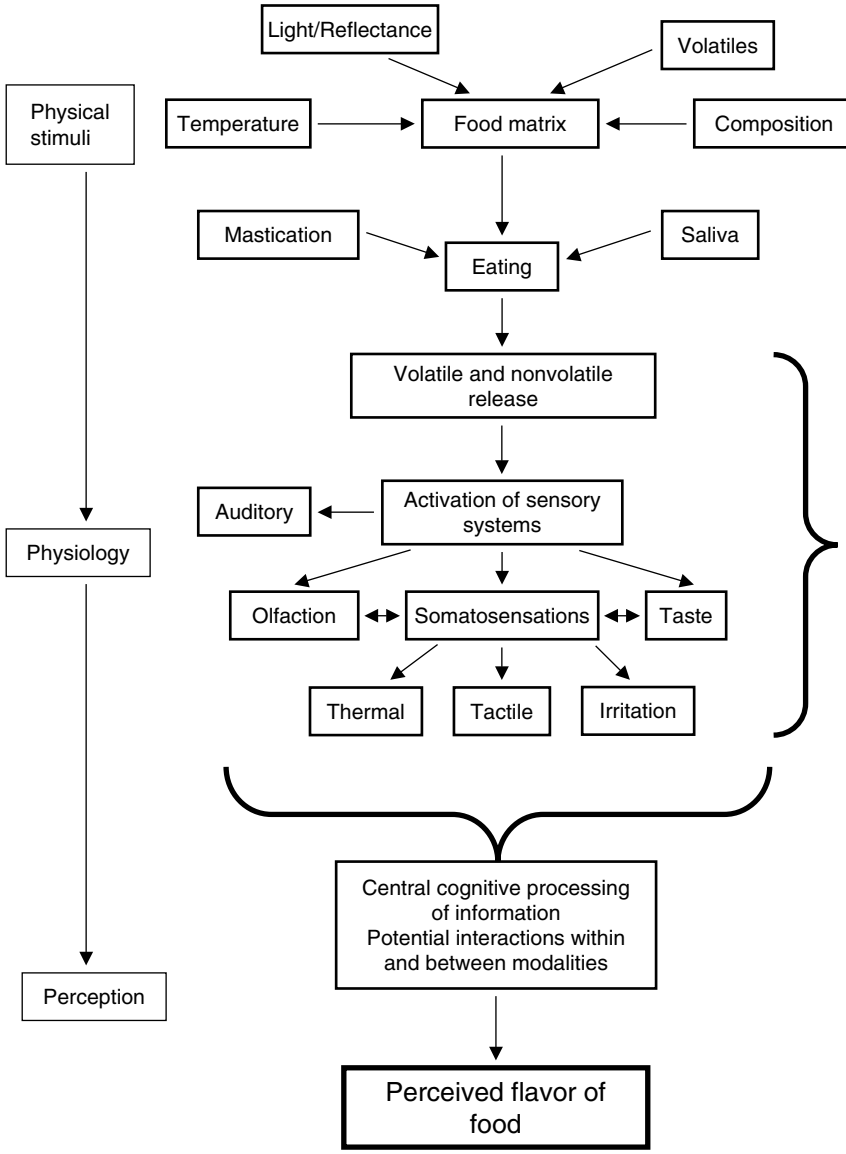

Figure 8.1 Flow diagram outlining various stages and factors that influence flavor perception.

Human sensory receptors for environmental cues vary in structure, and therefore in their psychophysical intensity functions. For example, the basilar membrane in the cochlea of the ear responds indirectly to changes in pressure (sound waves) at the tympanic membrane (eardrum). Different frequencies of

sound waves (Hz) produce maximal activity at different positions along the basilar membrane. Thus, at a frequency of 10 kHz and signal level of 20 dB, a weak vibration will occur in a spot on the basilar membrane and only a few hair cells and their fibers may respond. As the decibel level increases at 10 kHz, hair cells that were unresponsive at 20 dB and are ideally tuned to other frequencies (2–14 kHz) are recruited. This type of recruitment grows exponentially as the decibel level increases across an 80 dB range (auditory psychophysical curve A in Figure 8.2). The somatosensory system, like the auditory system, has a capacity for perceived intensity to continue to grow with physical intensity up to the point of physical damage to the receptor system. In contrast, the gustatory and olfactory systems have a much more limited response range to increases in chemical concentration.

A compound's concentration–response function is usually nonlinear (Figure 8.3). As the physical concentration of a compound increases, the perceived intensity elicited by that compound also increases but at varying rates. The curve can take on a sigmoidal shape: at very low concentrations of sapid compound the taste intensity can grow in exponential fashion; at

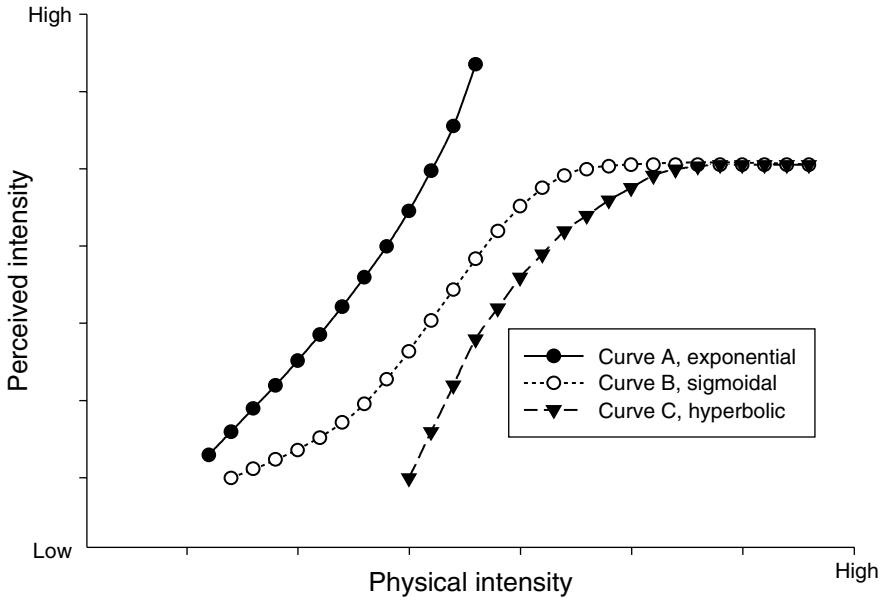

Figure 8.2 Three examples of psychophysical curves. Curve A is a theoretical psychophysical curve from the auditory system. Curves B and C are theoretical psychophysical curves from the taste system. The auditory function exponentially increases with increasing dB. The taste functions show an asymptote at maximum taste intensity.The *y*-axis represents perceived intensity and the *x*-axis represents physical intensity. (Reprinted from Keast & Breslin, 2003, with permission from Elsevier.)

medium concentration the perceived intensity can increase in linear fashion; at higher concentrations the perceived intensity may plateau, resulting in three specific regions of a typical psychophysical concentration–intensity function. Stevens' power law (Stevens, 1969) can be applied:

$$I = kC^n$$

where I is the perceived intensity, k is a constant related to the flavor-eliciting compound, C is the concentration of the compound and n is the exponential variable associated with the shape of the curve. The three regions correspond to (1) expansive, (2) linear and (3) compressive (Bartoshuk, 1975). The expansive region should result in hyperadditivity of intensity when low concentrations of compounds are added together. The linear region is in the middle of the psychophysical function and should result in intensity additivity when two concentrations within this range are added together. The compressive region is found in the upper portion of the psychophysical function; when two concentrations from this region are added together, we expect to see suppression of the expected intensity. When coordinates are plotted in log–log, the exponent n from Stevens' power law represents the shape of the line for each phase. For the expansive phase the exponent is greater than 1, for the linear phase the exponent is equal to 1 and for the compressive phase the exponent is less than 1. Thus, there are portions of the intensity curve that are expansive and others that are compressive. The shape of this function can often lead to the spurious conclusion that two stimuli in mixture enhance or suppress one another. Based on a popular understanding in the literature, sensory enhancement means that the mixture of two stimuli results in a sensation that is greater than their sum, such that $1+1>2$; similarly, the term additivity means $1+1=2$ and suppression means $1+1<2$. This simple understanding of enhancement or suppression fails to consider the shape of the psychophysical intensity function.

Keast *et al*. (2003) assessed synergy between two compounds by evaluating the actual intensity of the mixed components, compared to self-addition (i.e. a weak intensity of a secondary compound is added to the primary compound and the resulting intensity is compared to a weak intensity of the primary compound added to itself). Figure 8.4 illustrates the types of sensory interactions that can occur to a psychophysical function of a compound (E) when mixed with another compound (D or F). When a fixed concentration of compound D (activating the same or different sensory modality) is mixed with compound E, the psychophysical function of E is shifted to the left and the slope increased, which illustrates that compound D has an enhancing effect on the intensity of E. The linear part of the psychophysical function of E could also be left-shifted without affecting the slope (D1: Figure 8.4 bottom). The asymptote may also be affected (D′); in this situation the maximum attainable

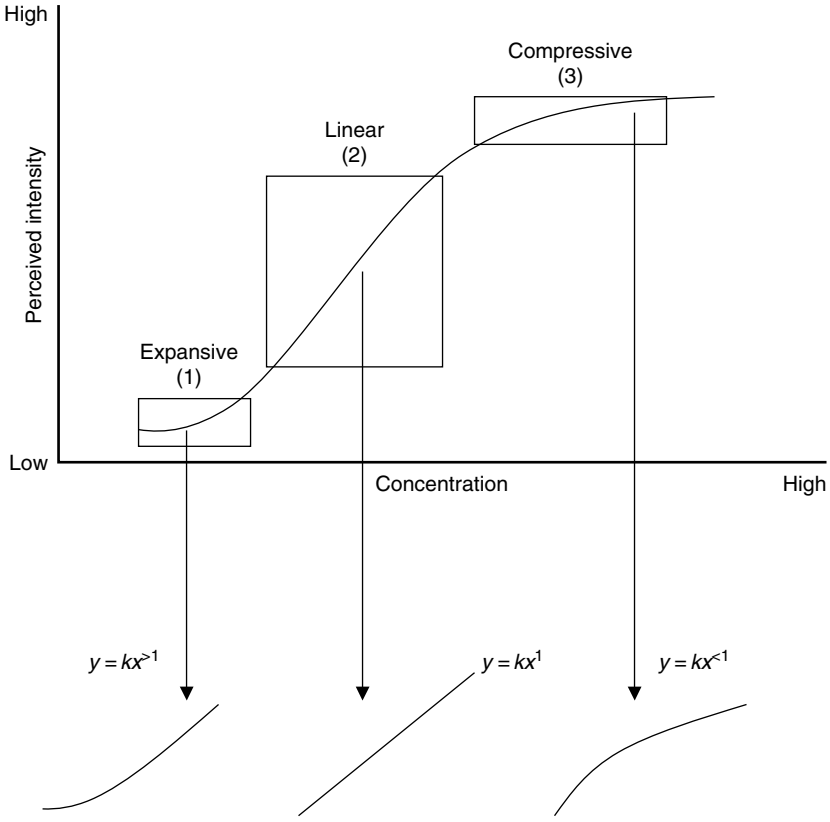

Figure 8.3 Theoretical psychophysical concentration–intensity function for flavor compounds. Concentration of the flavor compound is plotted along the *x*-axis and perceived intensity is plotted along the *y*-axis. (Reprinted from Keast & Breslin, 2003, with permission from Elsevier.)

intensity elicited by E is also increased. These scenarios show that compound D enhanced the perceived intensity of compound E at a given concentration. Figure 8.4 also shows the effects on the intensity of compound E when a fixed concentration of compound F is added. Compound E's psychophysical function is right-shifted by F and the slope decreased, revealing that the addition of F suppresses the intensity of E. In parallel to D1, the psychophysical function can be shifted to the right without any change in slope (F1: Figure 8.4 bottom). The asymptote may be affected, as shown by F′; in this situation F has blocked the maximum achievable intensity of E, as would be expected with a noncompetitive antagonist. Enhancement (left-shift of curve; curve D) interactions may occur within a modality, such as when the psychophysical curve of the umami taste from monosodium glutamate (MSG) is shifted to the left and the

slope increased when certain 5′-ribonucleotides are added (enhancement of umami taste). Alternatively, these interactions can happen between modalities, such as when the psychophysical curve of the sweetness from sucrose is

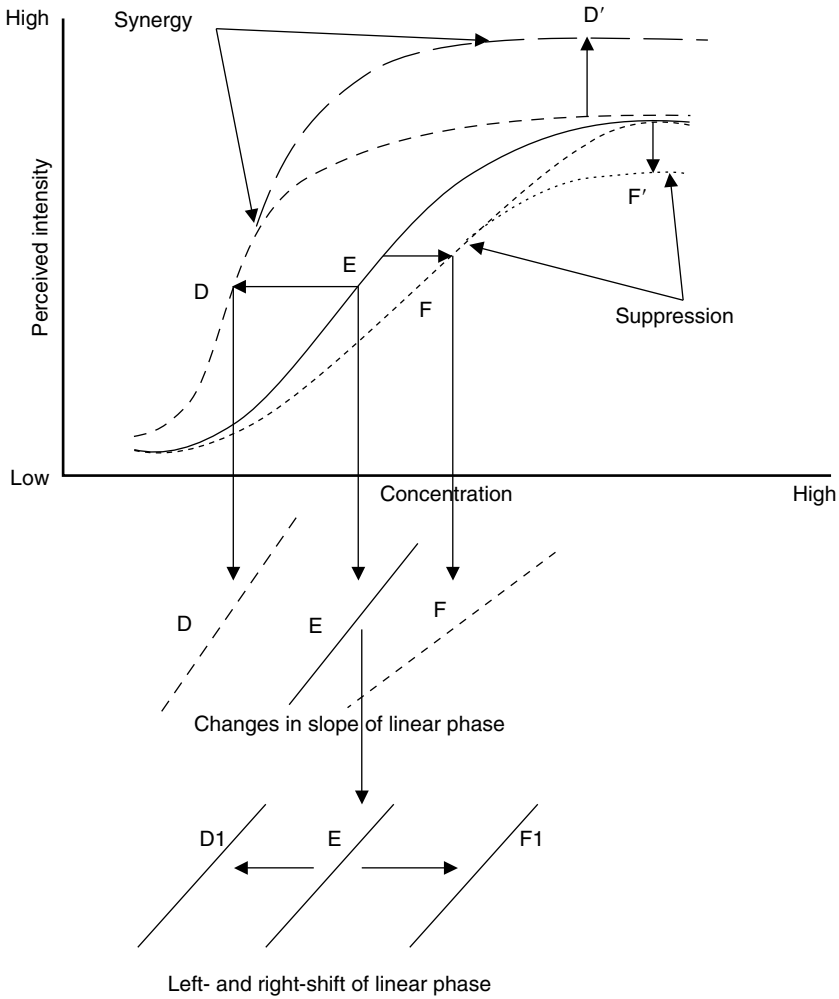

Figure 8.4 Effect of multiple-flavor compounds on a psychophysical curve. Changes in slope or maximum height of psychophysical curves show inhibition or synergy. Curve *E* represents the concentration response of a sapid compound plot against increasing concentrations of the compound. To the left of curve *E*, curve *D* shows an increase in slope with an additive. This suggests that synergy is occurring between the sapid compound and the additive. Curve *D′* shows that the maximum intensity is elevated above what the sapid compound can stimulate alone. To the right of curve *E*, curve *F* shows a decrease of slope when mixed with an additive. This suggests that masking or inhibition is occurring. Curve *F′* shows suppression of maximum achievable intensity. (Reprinted from Keast & Breslin, 2003, with permission from Elsevier.)

left-shifted when vanilla odor is added (see Section 8.7.3). Suppression (right-shift of curve; curve F) interactions may also occur within and between modalities, and suppression is more common than enhancement when flavor-eliciting compounds are mixed. As a general heuristic, when two or more flavor-eliciting stimuli (above threshold) are mixed together, the intensity is less than the sum of the individual intensities (mixture suppression: Pangborn, 1960; Keast & Breslin, 2003).

To understand complex flavors and the sensory interactions at work within them, the first step is to investigate single-compound interactions and build knowledge from there. Psychophysicists have been methodically investigating interactions among flavor compounds for over 100 years, but still relatively little is known about sensory interactions. To assemble a psychophysical function for one stimulus requires a small amount of time and effort; to assemble a complete psychophysical function for a binary combination of flavor stimuli would be a much more substantial effort; to empirically measure functions for the hundreds or thousands of flavor-eliciting compounds in foods is obviously impractical. Thus, a more practical approach has been to use prototypical stimuli that represent a quality or modality (e.g. sucrose (sweet) mixed with capsaicin (hot/irritating)) and to generalize stimuli that elicit similar qualities. Our ability to generalize from prototypical stimuli, however, is often limited.

8.3 Attributes of sensory modalities

There are four sensory attributes that all complex flavors possess: quality, intensity, temporal and spatial patterns.

8.3.1 Quality

The attribute 'quality' is a descriptor to categorize sensations that stimuli elicit (see Table 8.1 for quality descriptors). The quality of a stimulus sensation is its single most important defining feature. The following are examples of the importance of particular qualities in flavors: we could not substitute sucrose (sweet) for iso-α-acids (bitter) in beer without rendering the beer a different beverage; or replace a banana's aroma with a blue cheese aroma and still identify the fruit as a banana; or replace the tingle of CO_2 in soda with the burn of capsaicin without changing the identity of the beverage.

8.3.2 Intensity

The attribute 'intensity' is a measure of the magnitude of sensation(s) elicited at a set concentration of a flavor stimulus at a given time. The perceived

Table 8.1 Examples of quality descriptors from the sensory modalities primarily responsible for flavor. The lists for odor and somatosensory qualities are merely examples of the many adjectives available to describe quality.

Taste	Smell	Somatosensation
Sweet	Minty	Tingling
Sour	Woody	Stinging
Salty	Resinous	Burning
Bitter	Burned	Cool
Umami	Nutty	Rough
	Moldy	Smooth
	Sulfurous	Astringent
	Rancid	Sharp

intensity of a stimulus may be plotted against its concentration to produce a psychophysical function (Figure 8.2; see Section 8.2). Many flavor compounds may have accelerating (intensity increases faster than concentration) or decelerating (intensity increases slower than concentration) concentration ranges depending on the region of the psychophysical curve that is being utilized (Figure 8.3). Therefore, doubling the concentration of a flavor compound will not necessarily double the perceived intensity. There are many reasons why nonlinearities exist in intensity functions, including physical, chemical, physiological and psychological factors (see Section 8.5).

8.3.3 Temporal pattern

The 'temporal' pattern of a stimulus is defined as the time course of the intensity of sensation of that stimulus (McBride, 1976). Figure 8.5 shows time–intensity graphs of two irritating stimuli (CO_2 and capsaicin) that were matched for overall perceived intensity, but as the graph shows, the intensity of compound CO_2 decays rapidly, while compound capsaicin lingers in the oral cavity with a duration of intensity over 1 min. Notice that these compounds do not achieve the same peak height, despite having been matched for overall intensity. Understanding the temporal profile of compound intensity is a critical concept in food science, as different temporal properties of compounds cause flavor problems in products. For example, one of the reasons high-intensity sweeteners have had difficulty replacing sucrose in foods is their extended time–intensity profiles that render them clearly unsucrose-like.

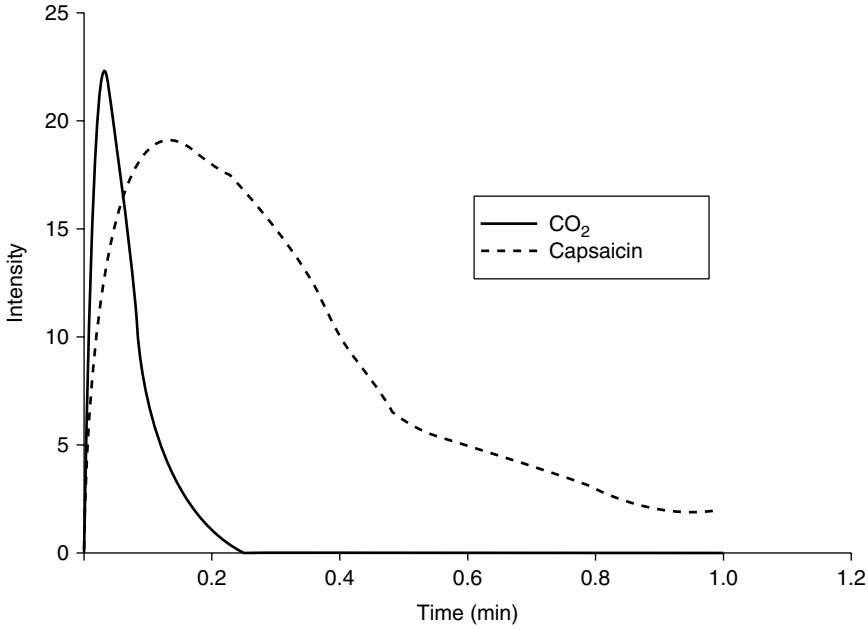

Figure 8.5 Time–intensity curves of two irritating stimuli matched for peak intensity. The *y*-axis is a measure of intensity using the labeled magnitude scale (Green *et al*., 1993), the *x*-axis plots time in minutes. The graph shows that capsaicin and CO_2 elicit the same peak intensity, but the time course of their sensations is different with capsaicin irritation lasting longer than CO_2 irritation.

8.3.4 Spatial pattern

The 'spatial' topography refers to the localization of stimuli in sensory coordinates (e.g. left vs. right or front vs. back: McBride, 1976). Perceptible localization of sensations requires two steps: a physiological map of the receptors' layout and the psychological ability to access this 'map' and identify the receptors activated by stimuli. A compound may only stimulate a certain receptor at a specific location, e.g. iso-α-acids (primary bitter components of beer) elicit bitterness at the back of the tongue and throat region but leave the anterior portion of the tongue relatively unaffected, whereas quinine (tonic water) elicits bitterness from the side to the back and front of the tongue. This is most likely due to differences in the patterns of receptor distribution. Receptors responding to iso-α-acids may be located primarily in the posterior lingual and pharyngeal region, while receptors for quinine may be more distributed within the taste buds of oral cavity.

The different sensory modalities vary widely in their ability to convey spatial information: the tactile sense (touch) is very accurate at localizing point of contact; the sense of taste is moderately accurate (Delwiche *et al*.,

2000); and olfactory stimuli cannot be localized/lateralized to the left or right nares without accompanying irritating input (Kobal *et al*., 1989).

8.4 Adaptation

Adaptation can be defined as a decrease in perceived intensity or detection threshold for a stimulus under conditions of continuous/repetitive exposure to that stimulus. Presumably, sensory systems evolved to react to change, and when the local environment is held constant, the system recalibrates or re-zeroes itself. Adaptation is a primary feature of all the senses including taste, olfaction, tactile and thermal stimulation (irritation often sensitizes as well as desensitizes: see *Nasal irritation* under Section 8.6.3.1). For example, when you enter a room with a specific odor, after a short period of time it will diminish; or if you go swimming in a cool mountain lake, the initial shock of the perceived coolness subsides as the thermal senses adapt. Adaptation processes can potentially influence flavor perception in both subtle and substantial ways, as adaptation of one sensory system (e.g. olfaction) may affect the overall flavor of a food. For example, if a worker at a strawberry candy factory ate one of the strawberry candies being manufactured, he or she might find it difficult to identify the olfactory characteristics of the candy due to the ambient strawberry odor in the factory and resulting adaptation. Thus, the candy would taste sweet with little strawberry character.

8.5 Four levels of flavor interactions

Four levels of interaction must be taken into account when analyzing flavor: (1) chemical interactions occurring in the food matrix that may directly affect flavor perception; (2) mechanical/structural interactions of the food and mastication with the release of compounds; (3) peripheral physiological interactions; and (4) cognitive interactions among tastes, odors and somatosensations perceived together.

8.5.1 Physiochemical interactions

Physiochemical interactions can change flavor intensity or even generate new flavors. They occur in a simple aqueous solution: an acid in combination with a base will result in the formation of a salt; weak attractive forces, such as hydrogen or hydrophobic bonding, will result in altered structures; precipitation of the compounds will render them weaker or tasteless. The addition/formation of salt in a complex aqueous matrix will increase volatile release. The chemical composition of a food matrix will influence perceived flavor; whether the

food is primarily lipid, protein, carbohydrate or aqueous will affect release of flavor-active compounds from the matrix. (For a comprehensive review of flavor release from food matrices see van Ruth & Roozen, 2002.)

8.5.2 Mechanical/structural interactions

The structure of the food and the efficiency of mastication will affect how food is broken down in the mouth (see also Chapter 2). Whether the food is hard, viscous, cohesive, adhesive, lumpy determines how the physical structure collapses and subsequently releases flavor-active compounds that stimulate tastes, smells and the skin senses (Agrawal *et al*., 1997). The efficiency of mastication determines the speed of food breakdown and the extent of release (Heath & Prinz, 2002).

The temperature of the mouth also determines flavor release. For example, the temperature of ice cream ($< 0^{\circ}$C) ensures that olfactory compounds are not volatile, but when placed in the oral cavity ($\sim 30^{\circ}$C), the frozen structure begins to melt and volatile compounds activate the olfactory epithelium via the retronasal passage (Heath & Prinz, 2002). Nonvolatile compounds (such as sugars and fats) are solubilized and available for receptors in the oral cavity. In addition, the temperature of the ice cream activates cold-sensitive nociceptor fibers in the somatosensory system. The melting process is dynamic and flavor-active compounds are continually released as the matrix is warmed in the mouth.

8.5.3 Oral and nasal peripheral interactions

When two compounds are mixed, there is potential for one compound to interfere with receptor cells or transduction mechanisms associated with the other compound. For example, this type of peripheral interaction occurs between sodium salts and certain bitter compounds. Sodium salts suppress the bitterness of selected compounds (Bartoshuk & Seibyl, 1982; Breslin & Beauchamp, 1995; Keast & Breslin, 2002a, 2002b; Schifferstein, 1994a). This suppression is a peripheral oral effect (at the cellular/epithelial level) rather than a cognitive effect (central process). To demonstrate the peripheral effect, Kroeze and Bartoshuk (1985) applied a bitter stimulus to one side of the tongue and a sodium salt to the other side of the tongue (split tongue methodology). The stimuli were applied independently and simultaneously. The intensity of bitterness was only reduced when the stimuli were applied to the tongue in mixture together, compared to independent simultaneous application of the two stimuli on different sides of the tongue. This peripheral interaction between sapid compounds could occur at a number of sites on or in the taste receptor (TR) cell (Keast *et al*., 2001). Gillan (1982), when investigating sucrose–NaCl interactions, performed a similar split tongue experiment and found evidence for both peripheral and cognitive interactions.

8.5.4 Central cognitive interactions

Flavor-active compounds stimulate receptors of the sensory systems, and afferent signals are sent to the processing regions of the brain where the signal is decoded and sensations perceived. As a general heuristic, when two or more suprathreshold stimuli of the same modality are mixed together, the intensity is less than the sum of the individual (unmixed) intensities. This phenomenon is known as mixture suppression (Pangborn, 1960) and has been extensively reported in taste interaction research (Frank *et al*., 1993; Kroeze, 1982, 1990; Lawless, 1979b). Kroeze and Bartoshuk (1985) effectively demonstrated this by using the split tongue methodology. Kroeze found suppression of individual qualities, such as sweet and bitter, when the compounds were mixed, regardless of whether the compounds were applied independently to either side of the tongue or together as a mixture. This showed that suppression had a central cognitive rather than just a peripheral oral effect. This conclusion is possible because the two lateral halves of the tongue are neurologically independent until the ascending neurons interact in the brain (Tucker & Smith, 1969).

We must also consider subjective cognitive influences relating to emotions and preferences. In effect, we cannot remove the emotional attachment we have to certain flavors. The distinctive aroma of freshly baked apple pie may bring back happy memories of grandmother's kitchen or of long hours on the factory floor if you work for a pie manufacturing company. Such cognitive processes can have halo effects on rating intensities (Lawless & Clark, 1992; Schifferstein & Verlegh, 1996). For example, the apple pie may be fruitier and sweeter for the person who associates it with early childhood memories. The key features of consuming a food are not only the nutrient value the food may contain but also the pleasure we receive while eating/drinking.

8.6 Intramodal interactions

Human physiology involved with flavor detection systems is complex. To gain an understanding of each component sensory system, research has concentrated on interactions within individual sensory systems. What follows is a brief review of intramodal interactions within the taste, olfaction and somatosensory systems.

8.6.1 Taste

It is widely accepted that there are five major categories of taste quality: sweet, sour, salty, bitter and umami (savory). There may also be subcategories of taste, but there is no consensus on this issue. Taste is the essential foundation

upon which flavor can be constructed and is elicited when saliva-soluble compounds stimulate taste cells (specialized epithelial cells) in the oropharynx and laryngeal areas. The majority of taste receptor (TR) cells are organized into rosette-like structures called taste buds, which are embedded in folds or lingual bumps called papillae. These papillae are located on the tongue; fungiform papillae occur at the anterior of the tongue, vallate papillae occupy the posterior sides of the tongue and circumvallate papillae are located at the posterior dorsal surface of the tongue. In addition, there are large numbers of taste buds on the soft palate and pharynx in humans, but these do not occur in papillae. Taste buds contain taste cells/TR mechanisms responsible for detecting compounds that elicit taste qualities (sweet, sour, salty, bitter, umami). All taste qualities can be experienced at all sites in the oral cavity that contain taste buds; thus, the often-cited theory of a tongue map is incorrect. For example, the tongue map states that the tip of the tongue is sensitive to sweet, the back of the tongue is sensitive to bitter. To convince yourself that areas of the tongue respond to all qualities, dip the tip of your tongue into solutions of tonic water or strong coffee to assess bitterness, honey to assess sweetness, salt water to assess saltiness, lemon juice to assess sourness and consommé to assess umaminess. The nose may be pinched closed while tasting to eliminate olfactory input. As you will find, all five qualities may be elicited from the tip of the tongue. It is important to note that the same statement may not hold true for all representative member compounds of a quality. For example, although bitterness can clearly be tasted from the tip of the tongue in strong coffee, the same may not be said for the bitterness in beers such as Guinness, which comes from iso-α-acids.

The chemoreceptive transduction begins when chemicals in ingested foods/drinks/oral products activate specialized receptor proteins at the apical ends of taste cells (exposed to the external environment in the mouth). The chemical signal carried by the stimulus is converted to an electrical impulse that is sent via afferent nerve to the nucleus of the solitary tract and ultimately to cortical regions of the brain (see Lindemann, 2001).

8.6.1.1 Single-quality interactions

There are thousands of chemically distinct nonvolatile compounds that elicit taste. In order for the taste system to detect a wide range of nutritive and toxic compounds, it must have an adequate receptor system. It appears that receptors may be broadly tuned for the detection of chemically diverse structures. For example, the only discovered putative sweet TR (a dimer of two G protein–coupled receptors T1R2 and T1R3) is activated not only by sugars but also by dipeptides, diterpenes and cyclamates (Li *et al*., 2002). There is a family of approximately 25 bitter TRs (T2Rs) and thousands of structurally diverse bitter-tasting compounds, which indicates that the bitter TRs are broadly tuned (Chandrashekar *et al*., 2000).

This raises the question: If compounds, such as the sweeteners, activate the same sweet taste pathway, how do we perceptually distinguish among them? If sweet taste were the same for all compounds (i.e. the taste quality of all sweeteners is identical), then the replacement of sucrose with a high-intensity sweetener would be universally accepted; yet no intensive sweeteners perfectly mimic the taste of sucrose. Theoretically, quality and spatial topography of compounds sharing the same receptor will be similar, but separate binding sites on the receptor may affect the intensity and temporal pattern. There may be different affinities of compounds to the receptor that cause perceptual differentiation among the sweeteners. In addition, compounds sharing the same receptor may be distinguished by their activation of other sensory modalities (e.g. menthol elicits a taste, an odor and several somatosensations). Thus, a sweetener such as saccharin will stimulate bitterness as well as sweet taste in many subjects.

8.6.1.2 Multiple-quality interactions

When one compound that tastes bitter is mixed with another compound that tastes sweet, the resulting mixture is often a combination of the two tastes (a bitter–sweet solution). This is not the case in all sensory systems. The perception of mixtures of taste compounds can often be analyzed by introspection into its component tastes, but not always. A number of studies have investigated the perceptual outcome when two taste-eliciting compounds are mixed, and a review of such interactions was recently published (Keast & Breslin, 2003). As a general rule in humans, binary taste interactions follow the predictions of the different phases of a sigmoidal-shaped psychophysical function (Figure 8.3). At low intensity/concentration, taste enhancement of the components was often reported, at moderate intensity/concentration there was a mix of enhancement, suppression and linear interactions, and at high intensity/concentration, taste suppression of the components was most common. This is a cognitive model of mixture interactions and does not address the many potential peripheral physiological interactions that may occur (e.g. sodium suppression of bitterness, 5′-ribonucleotide and MSG synergy of umami taste).

8.6.2 Odor

Given the small number of taste qualities, it is not surprising that the immense diversity of flavors we associate with foods is primarily derived from olfactory input via the volatile compounds that are released in the oral cavity when food or liquids are chewed and swallowed. These volatile compounds are known to activate a large family of seven transmembrane G protein–coupled receptors (up to 350 in humans: Young *et al.*, 2002), which are expressed in olfactory neuronal cilia (Buck, 1992). Olfactory receptors (ORs) are located high in the nasal cavity, and odorants must travel through an aqueous mucous layer that

encases the cilia of the olfactory neurons. There are two routes of activation in ORs: orthonasal, when the subject actively sniffs food; and retronasal, where the aroma from the ingested food travels via the back of the mouth/throat.

Specific receptors can be activated by a variety of ligands (broadly tuned) (Mombaerts, 1999a, 1999b), and the quality of odor may be coded by spatial pattern recognition of signals sent via activated ORs (Kauer, 1987). ORs vary in their receptiveness to odorants over a range of concentrations. Therefore, the concentration of an odorant can alter the perceived quality of an odorant (Kajiya *et al*., 2001; Laing *et al*., 2003; Wetzel *et al*., 1999). The chemical signal carried by the odorant is converted to an electrical signal that is sent to the first processing area, the olfactory bulb, where the signal can be integrated/modulated by other signals. From there, the signals are sent both directly and indirectly to the primary olfactory cortex and other cortical areas where the odor perception arises and ultimately can be integrated with other sensory modalities (Rolls, 2001).

8.6.2.1 Odor interactions

Mixture suppression/masking of suprathreshold aromas is a common phenomenon when odors are mixed (Cometto-Muniz *et al*., 2003; Koster, 1969; Laing & Willcox, 1983; Laing *et al*., 1984). As a general rule, odor interactions will follow a sigmoidal psychophysical curve (Figure 8.3; Cometto-Muniz *et al*., 2003; Gregson, 1986; Lawless, 1997; Patterson *et al*., 1993), with enhancement at threshold/very low intensity, additivity at low/moderate intensity and suppression at higher intensity. Lawless (1997) reviews several mathematical relationships that have been proposed to describe intensity interactions in odor mixtures.

When an odor mixture contains more than three odors, identification of the components is often difficult (Jinks & Laing, 1999, 2001; Laing & Francis, 1989; Livermore & Laing, 1998a, 1998b), yet it is very rare, outside a laboratory setting, that perceived aroma is from a single compound. When we perceive an aroma from a food/beverage, it is a combination of hundreds of volatiles, some of which may be more important to the overall aroma than others. Odor mixtures tend to blend to form homogenous percepts in which the quality of the component aromas may bear no resemblance to the quality of the aroma of the product. For example, the vast majority of the 850 identified volatiles that are present in coffee aroma have no distinguishable coffee qualities, e.g. 2-methyl butanoate has a blue cheese quality, 1-phenylpropanedione has a floral/herbaceous quality (Flament, 2002).

8.6.3 Somatosensations are components of flavor

Textural and irritant sensations are perceptual components of flavor. These oronasal (upper airway) sensations are conveyed to the central processing areas

of the brain primarily via polymodal nociceptors of the trigeminal nerve (fifth cranial nerve), but also via the vagus (tenth cranial nerve), glossopharyngeus (ninth cranial nerve) and chorda tympani (seventh cranial nerve).

8.6.3.1 Chemesthesis: irritation

Green and Lawless (1991) proposed the word 'chemesthesis' to describe a sensation that arises when a chemical stimulates free endings of nerve fibers. The free endings of individual nerve fibers innervating both the oral and nasal mucosa have specialized sensory receptors that respond to noxious heat (Caterina & Julius, 2001; Caterina *et al*., 1997) and noxious cold (McKemy *et al*., 2002), both of which evoke thermal and pain sensations. The oronasal nerve fibers are not independent sensory systems, but a component of the pain and temperature fibers that occur throughout the skin. In addition, there are primary sensory fibers (Aβ fibers) that detect innocuous stimuli and do not contribute to pain (Julius & Basbaum, 2001; mechanoreceptors, which are involved with texture detection, are also part of the pain system). The detection mechanisms of the irritation system are required to identify a diverse range of stimuli. For example, the heat/pain sensation initiated by the receptor VR1 is activated by the chemical capsaicin (component of chili peppers) and is also a molecular thermometer activated at $\sim 43^{\circ}$C (Caterina *et al*., 1997). The cold/pain sensation initiated by the receptor CMR1 is activated by the chemical menthol and is also a molecular thermometer activated at $\sim 26^{\circ}$C (McKemy *et al*., 2002). A common feature of oronasal chemical irritation is the long latency of sensation relative to that of taste or smell, due to the time taken for the chemicals to diffuse through tight junctions or the epithelium to engage receptors on the nerve fibers. The disparity in temporal onset between the odor/taste and chemesthetic sensations adds complexity to the perception of flavor.

There are a number of chemicals that are capable of activating irritant sensations and different adjectives to describe the sensations: the burn of chili pepper, the warmth of ethanol, the tingle of CO_2, the pungency of wasabi. However, the terminology associated with oronasal irritant sensations lacks the qualitative breadth of olfactory sensations and may be influenced by the taste and smell co-elicited with the irritation. For example, the perceived burn from chili pepper or pungency from wasabi may be the same irritant sensation, but when eaten they appear perceptually distinguishable. This may be due to the interactions of irritation with the taste and odor components rather than the quality of irritation per se.

Oral irritation. Capsaicin (chemical heat) and menthol (chemical cool: menthol also elicits taste, odor and nasal irritation) are the most widely studied oral irritants and both exhibit chemical desensitization (reduced intensity) and sensitization (enhanced intensity) when subsequently exposed to themselves (Cliff & Green, 1994; Dessirier *et al*., 2001; Green, 1989; Karrer &

Bartoshuk, 1991). That both enhancement and reduction of irritant intensity can occur on subsequent stimuli exposures demonstrates the complex temporal nature of irritation, which is primarily due to the time lag of the subsequent exposures (Dessirier *et al*., 2001). The time lag required for sensitization or desensitization may differ among compounds (Prescott & Stevenson, 1996), and there are also compound-specific differences in whether an irritant exhibits sensitization or desensitization across trials (Carstens *et al*., 2002). Menthol and capsaicin can cross-sensitize or cross-desensitize each other, but the effect is also dependent on the time of stimulus application (Cliff & Green, 1996; Green & McAuliffe, 2000). Further complicating the physiology of irritation is the observation that sensitization and desensitization may not occur for all chemical irritants as demonstrated by ethanol, which failed to sensitize (Green, 1990).

Nasal irritation. Most volatile compounds that elicit odor have the potential to also elicit nasal irritation at higher concentrations; however, the psychophysical function relating perceived irritation to concentration is far steeper than the psychophysical function for odor sensation. In addition, there appears to be less variability in nasal irritation thresholds than in nasal odor thresholds for the same compounds (Cometto-Muniz *et al*., 1998). Similar to oral irritation, nasal irritation can exhibit either sensitization or desensitization following repetitive exposure, with the direction of the effect being largely dependent on the time course of stimulation; sensitization tends to occur early in the course of exposure or with brief, punctuate exposures, while desensitization occurs following longer periods of stimulation (Cometto-Muniz & Cain, 1995). Although nasal irritants can exhibit cross-adaptation in which the perception of an irritant such as menthol is decreased by the prior presentation of ammonia, there are many examples where such cross-adaptation does not occur; moreover, there are no demonstrated examples of cross-sensitization in the nasal cavity, although such might exist. Recent modeling work has suggested that nasal irritation thresholds can be predicted by a quantitative structure–activity relationship based on a solvation model incorporating a number of physicochemical properties of the stimulus (Abraham, 1993; Abraham *et al*., 1998).

8.7 Texture

'Texture is a sensory and functional manifestation of the structural, mechanical and surface properties of foods detected through the senses of vision, hearing, touch and kinesthetics' (Szcesniak, 2002; see Rosenthal, 2002 for a review). The importance of texture to flavor should not be underestimated. The thought of eating entirely pureed foods would not only modify the pleasure of eating but also cause problems identifying the foods you eat. In a

study where subjects were asked to identify pureed foods, only 40% of foods were correctly identified (Schiffman, 1977).

8.7.1 Visual texture

Before we physically contact a food, our eyes tell us much about the object: shape, color, size and surface characteristics. Walking around the fruit and vegetable department of a supermarket will reveal many examples of visual texture; the limpness of celery or the wilting of spinach are both examples of how sight can prepare or predispose a consumer to the quality (and flavor) of a product. Visual texture is primarily influenced by the surface characteristics of foods; if the potato chip looks soggy or the asparagus stalk has developed wrinkles, the expectation of the perceived texture, flavor and liking of the food is affected. By examining the movement of liquids, we can assess the viscosity of a drink – a thick shake will appear more viscous than a glass of apple juice. In addition, if the color of a product is incongruent with the flavor (e.g. a blue-colored coffee), the identification and perceived intensity of the attributes of that product will be affected (Christensen, 1983; DuBose *et al*., 1980). Our vision provides expectations of texture and flavor even before we have touched or eaten the food, largely due to associations between appearance and flavor.

8.7.2 Auditory texture

Distinctive sounds are emitted when our teeth break potato chips, or when we open a bottle of beer and hear the pressure release signaling a lively carbonated beverage. If the sounds from such examples are not crisp/lively, the expectations of freshness and liking of the product will be diminished.

8.7.3 Tactile texture

We have gathered much information on the possible oral tactile texture prior to the food entering the oral cavity. Oral texture is the perception that arises when food interacts with teeth, saliva and tactile receptors in the oral cavity. The oral mucosa supports many nerve endings beneath the epithelium, which are sensitive to touch, pressure and vibration (mechanoreceptors), and the oral muscles, tendons and joints are innervated and relay information on jaw position to central processing areas (see Gillespie & Walker, 2001 for a review). Teeth are also highly sensitive to pressure and tension. The first bite and manipulations of the food are the most important in assessment of texture (Agrawal *et al*., 1997). When you bite into a fresh apple, the first few jaw movements collapse the food structure and provide information about the quality and flavor of the apple. As the process of mastication continues, the texture of the food changes as particle size of the food is reduced and a bolus

suitable for swallowing formed with the addition of saliva. Hyde and Witherly (1993) suggest that the change in texture during eating is responsible for the enjoyment of certain foods. It is also worth noting that the process of mastication varies among people and this affects the food breakdown and subsequent texture and flavor of the food (Brown *et al*., 1996). While texture descriptors such as hardness may be conceptually understandable and relatively easy to measure on a category scale for hardness (from Philadelphia cream cheese (soft) to rock candy (hard): Szcesniak, 1963), other descriptors such as juiciness are very complex and involve multiple perceptual phenomena (Szcesniak & Ilker, 1988).

8.8 Interactions between modalities

8.8.1 Interactions of orosensory chemesthesis, tactile sensations and taste

The oral peripheral mechanisms of taste, tactile sensations and chemesthesis are closely associated in the mouth. The association is not only at an oral peripheral level but also in the central nervous system with the possibility for interactions in the first taste relay (nucleus of the solitary tract: South & Ritter, 1986; Saxon and Hopkins, 1998; Whitehead, 1990) through to cortical regions of the brain (Plata-Salaman *et al*., 1993; Yamamoto *et al*., 1981). Green (2002) states that 'so ubiquitous is the entanglement of gustatory with somesthesis that the concept of taste as an independent sensory system may no longer be useful'.

An example of such interactions is the influence tactile stimulation has over taste and thermal sensations. The perceptual phenomenon of 'taste capture' refers to localization of taste sensations to areas of nearby tactile stimulation rather than areas where taste buds are located (Green, 2002; Todrank & Bartoshuk, 1991). 'Taste referral' occurs when the perceived taste sensations of one object are perceived in an adjacent flavorless object (Delwiche *et al*., 2000). Closely related to taste referral is thermal referral, where sensations of temperature are localized to areas of nearby tactile stimulation (Green, 1977). Touch determines localization of taste and thermal stimulation. It may also determine localization of odors to the mouth (see Section 8.8.2).

The physical temperature of a solution can also influence chemical taste intensity, although the evidence regarding magnitude and direction of the change in taste intensity as temperature changes is often contradictory (Bartoshuk *et al*., 1982; Calvino, 1986; Green, 1986; Green & Frankmann, 1987; McBurney *et al*., 1973; Moskowitz, 1973). Green demonstrated that the temperature of the tongue was the determining factor in taste–temperature interactions and speculated that lack of control of tongue temperature might be responsible for much of the variation in published studies (Green &

Frankmann, 1987). Alteration in stimuli temperature may produce thermal taste; warming the tip of the tongue elicited perceived sweetness, while cooling the tip of the tongue evoked sourness and saltiness (Cruz & Green, 2000). At the back of the tongue, cooling evoked sourness and bitterness, while warming failed to produce a sensation. This could mean that specific TRs/taste cells are molecular thermometers or there is convergence of taste-afferent signal with thermal-afferent signal downstream of receptor activations.

8.8.2 Interactions of odor and somatosensations

Compounds that stimulate olfactory neurons may also stimulate nasal irritation at higher concentrations. Many common odors have the capacity to elicit both an odor quality and irritation, e.g. benzaldehyde (cherry/almond odor), iso-amyl acetate (banana odor). Both nasal irritants and odors can interact with perceived intensity of each other (generally suppressive) and the effect can be either central cognitive (Belousova *et al*., 1983; Cain, 1990; Cain & Murphy, 1980) or nasal peripheral interactions (Bouvet *et al*., 1987).

Interestingly, despite the strong contribution of retronasal olfactory cues to the overall perception of flavor, flavor sensation is almost always localized to the oral cavity and not the nose (Murphy & Cain, 1980). This illusion may be mediated through the oral somatosensory stimulation that almost always accompanies actual taste stimulation, but does not necessarily accompany odor unless the olfactory stimulus has both olfactory and chemesthetic properties. Thus, the term 'taste-referred olfaction' coined by Murphy and Cain (1980) may be more appropriate as 'tactile-captured olfaction' as it is likely that tactile input during eating is responsible for oral perception of aroma.

8.8.3 Interactions of odor and taste

It is unlikely that there is a common physiology at the periphery linking the taste and olfactory systems, thus any interactions between the two modalities are likely to be a result of central cognitive processing (see also Chapter 9). There are many papers written regarding odor–taste interactions at suprathreshold levels and the effects these independent sensory systems have on each other (Bonnans & Nobel, 1993; Cliff & Nobel, 1990; Frank & Byram, 1988; Frank *et al*., 1989, 1993; Prescott, 1999; Schifferstein & Verlegh, 1996; Stampanoni, 1995; Stevenson *et al*., 1995, 1998; van der Klaauw & Frank, 1996). In summary, suprathreshold odors can enhance tastes (strawberry aroma enhances sweet taste: Frank & Byram, 1988), and tastes can enhance odors (intensity ratings of ethyl butyrate increase as the concentration of sucrose increases: Hornung & Enns, 1994), but the effects are likely due to congruency of the pairing (e.g. peanut butter odor does not increase sweetness: Schifferstein & Verlegh, 1996; Frank & Byram, 1988) or 'learned synesthesia', where one

modality can evoke qualities of another modality after frequent co-occurrence (Prescott, 1999; Stevenson *et al*., 1998). The phenomenon of enhancement of odor or taste intensity by each other may also be due to 'dumping' effects (Clark & Lawless, 1994; Lawless & Clark, 1992). In essence, lack of appropriate scales results in subjects 'dumping' intensity into a similar scale, e.g strawberry odor appears to enhance sweetness because there is no scale to rate fruitiness (Frank, 2002; Frank *et al*., 1993).

The congruency of odor–taste pairings appears critical for interactions at subthreshold levels as well. Studies that have investigated the ability of a subthreshold tastant (e.g. sucrose) to lower the threshold for an odor compound (e.g. benzaldehyde) have found that crossmodal integration occurs for odor–taste combinations that are culturally appropriate (Dalton *et al*., 2000) or that can be learned (Belanger *et al*., 2002). This observation affirms the notion that many enhancement effects to odor–taste combinations may reflect associations based on prior experience (Rolls, 1997).

8.9 Sources of error in sensory research

Human psychophysical studies have investigated mixture interactions as early as 1894 (Kiesow, 1894), and much of the literature since then has been contradictory, at least in parts.

8.9.1 Individual variation

Perception of taste, smell and somatosensation varies between people (Burdach *et al*., 1985; Delwiche *et al*., 2001; Koelega, 1994; Shusterman, 2002; Yokomukai *et al*., 1993). One person may find a 50 ppm iso-α-acid solution extremely bitter, while a second person barely notices the bitterness. Thus, differences in sample populations between experiments may affect results. Even with an individual, the perceived intensity of a compound may vary according to the time of day or choice of food/beverage prior to testing (Faurion, 1987). There will be variation in perception from myriad sources, including sensory adaptation, background noise in the nervous system as well as a variety of peripheral receptors being activated during the course of eating.

8.9.2 Experimental protocol

The experimental design has a direct influence over the results. The method of stimulus delivery has an impact: flowing tastants over the anterior tongue or exposing the whole mouth to the taste compounds will alter the psychophysical function (Bartoshuk, 1977). The psychophysical function of compounds may be altered depending on the method used. For example,

delivering a concentration series in ascending order versus random order can alter the shape of the concentration–intensity function. Also the number of subjects and their training can influence final results. Kamen *et al*. (1961) employed close to 1000 subjects in a simple half-replicate design with single-sample methodology, while Beebe-Center *et al*. (1959) had only two subjects, but utilized more powerful paired comparison and direct matching protocol.

8.9.3 Choice of flavor-active compound

The choice of flavor-active compound can cause large variation in experimental outcome. Both urea and quinine are perceived as bitter but presumably activate different taste transduction pathways (Keast & Breslin, 2002a; Lawless, 1979a; McBurney *et al*., 1972; Yokomukai *et al*., 1993). The observation that compound X affects the bitter taste of quinine does not predict that X will affect the bitter taste elicited by urea (Breslin & Beauchamp, 1995). Further, concentrations of compounds required to elicit isointense sensations vary; 0.4 mM quinine can elicit a strong bitterness but 0.4 mM urea is usually tasteless.

8.9.4 Psychophysical function of a compound

Different experimenters use different single concentrations of a given compound, and as previously stated, the position of this concentration along the expansive, linear or compressive phase of its psychophysical function will influence results.

8.9.5 Method of rating

There are three main scaling techniques used to measure the intensity of taste samples: visual-analog, magnitude estimation (Meilgaard *et al*., 1991) and the labeled magnitude scales (Green *et al*., 1993, 1996). Depending on the method utilized, the scale may expand or compress different portions of the psychophysical curve. In addition, the experimental context and number of scales and qualities simultaneously used can have an impact on final data (Frank *et al*., 1993; Schifferstein, 1994a, 1994b; Schifferstein & Frijters, 1992; Stillman, 1993).

8.10 Practical implications for flavor

Tastes, odors and somatosensations are stimulated throughout the oronasal region as the food bolus is manipulated during eating. The release of

flavor-active compounds from a food is a dynamic process related to the food composition and structure as well as mastication efficiency. The sensory systems are also dynamic in nature as they adapt to stimulation and recover from adaptation. We accept the dynamic aspects of flavor when we eat foods without conscious thought about the flavor profile. However, we notice when the flavor of a favorite product changes. For example, if the sucrose in a soft drink were replaced with a non-nutritive sweetener, invariably consumers would notice the difference. The reason for the difference is not sweetness intensity per se as the sweetness can be matched for intensity, but that the sweeteners have different time–intensity profiles and are possibly localized to different regions of the oral cavity. The sweetness of the non-nutritive sweetener lingers longer than the sweetness of sucrose and may originate from the posterior tongue creating noticeable differences between the two compounds. In addition, the odor associated with the beverage also appears to linger with the sweetness. In this situation, the flavor length has been extended and not just the sweet profile. When consumers' expectations are altered, the new product may be rejected or alternatively, consumers may take time to adjust to the new flavor profile that was created when the non-nutritive sweetener replaced sucrose.

There are many potential sites within the sensory systems for interactions between or within modalities. There could be peripheral interactions occurring between compounds at a receptor site, there could be interactions between cells/nerve fibers that send signals to higher cortical areas and there could be interactions in the cortical regions of the brain. At any of these stages, the interactions could result in a change to the final perceived flavor of a food. The changes in flavor that occur when formulations are altered can be difficult to predict; the foolproof way is to collect empirical data showing whether there is a change in flavor or not.

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Flavor Perception
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9 Psychological processes in flavour perception

John Prescott

9.1 Flavour as sensory integration

Eating and drinking provide a constant stream of sensory information – gustatory, olfactory, visual, tactile – that informs about the identity, and thus suitability, of what we are about to ingest. Yet, except in those limited circumstances where the sensory properties of foods or beverages are evaluated individually (e.g. descriptive analysis of wines or foods by trained panels or specialist judges), the major concern for most of us, most of the time, is whether or not the flavour is acceptable. Determining the relative contributions of tastes, odours and tactile qualities to overall flavour is not only not a priority, consumers are typically even unaware of the degree of importance that the sense of smell plays in flavour perception (reflected in the interchangeable use of the words *taste* and *flavour*, at least in the English language – see Rozin, 1982).

Some combinations of sensory inputs, e.g. taste and tactile sensations in the mouth, are so well integrated that we cannot begin to disentangle them (Rozin, 1982). By contrast, we can, on reflection, deconstruct our experience of a beverage such as orange juice into the individual sensory properties of 'orange-ness', sourness and sweetness. Nevertheless, our initial response is to *orange flavour* – an effortless combining of all of these qualities into a single percept. Thus, what we ultimately perceive during consumption – as opposed to the sensations we experience – are *flavours*, in which the physiological inputs are 'functionally united when anatomically separated' (Gibson, 1966, p. 137).

Gibson (1966) has argued that the primary purpose of perception is to seek out objects in our environment, particularly those that are biologically important. As such, the physiological origin of sensations is less important than the fact that these sensations can be used in object identification. Because of its adaptive significance, flavour perception is perhaps the most prominent example of this notion. Likes and dislikes naturally spring from the integrated perception of flavour, since we are responding to substances that we have learned to recognise as foods and that are therefore important to survival. Initial 'gut' responses to foods are almost always hedonic, and this naturally precedes accepting or rejecting the food. Thus, what we perceive when we sit down to dinner are, thankfully, integrated perceptions – a steak and a glass of red wine – rather than a collection of distinct sensory signals.

Such integration of information from physiologically distinct sensory modalities appears to be a general property of the mammalian nervous system (Gibson, 1966; Marks, 1991; Stein & Meredith, 1993). Its purpose may be to enhance the detection of, and reduce ambiguity associated with, stimuli, particularly in those cases where a single sensory modality fails to supply all the necessary information about the stimulus. Integration across sensory modalities is reflected in the presence of multimodal neurons that receive converging sensory information, as well as by the presence of areas of heteromodal cortex, within which inputs from different sensory systems are represented. Multimodal neurons may respond specifically to combinations of different sensory inputs, or sensory-specific neurons may be responsive to modulation by other sensory modalities (Calvert *et al*., 1998; Meredith & Stein, 1983; Stein & Meredith, 1993).

In the processing of olfactory and gustatory stimuli, multimodal neurons in the orbitofrontal cortex of the monkey have been shown to respond specifically to qualities that occur together in flavours, e.g. the sweetness of glucose combined with fruit odours, rather than to incongruous combinations such as saline and these same odours. It is thought that these neurons develop from unimodal neurons that originally responded to olfactory information, through learning of appropriate combinations of signals during repeated coexposure of particular tastes with odours (Rolls, 1997; Rolls & Bayliss, 1994). Distinct patterns of neural activity reflecting flavour perception have also been demonstrated in humans. Using positron emission tomography (PET) – a technique for measuring cerebral blood flow that reflects regional activity of neurons in processing information – to examine flavour perception, Small *et al*. (1997) found evidence for cortical processing of flavours as unique sensory events. Patterns of neural activity in primary gustatory, and secondary gustatory and olfactory cortices during simultaneous presentation of odours and tastes that occur together in flavours were found to be distinct from patterns produced by independent presentations of identical stimuli.

9.2 Qualitative and psychophysical evidence for odour–taste integration

Psychophysical and behavioural parallels to neural integration across sensory modalities have been repeatedly demonstrated in animal studies. For example, Stein *et al*. (1988) showed substantial enhancement of behavioural responses to visual stimuli by auditory tones in cats. In humans, odour–taste integration is evident in two linked phenomena: the first of these is the common observation that some odours are described using taste properties such as sweet or sour; the second is the ability of these odours to enhance those tastes when combined with the tastant in solution. This chapter will focus largely on these two phenomena and their implications for understanding flavour perception.

The reason for this emphasis is that investigations of these phenomena have provided insights into the origins of the integration of odours and tastes as flavours, as well as providing a model through which to examine the psychological processes that are involved in the formation of flavour perceptions.

9.2.1 Taste properties of odours

Some odours, when sniffed, elicit descriptions of qualities that are more usually associated with basic taste qualities (Burdach *et al*., 1984). For example, in Dravnieks' (1985) descriptive analysis of odour characteristics, 65% of his assessors gave 'sweetness' as an appropriate descriptor for the odour of vanillin, while 33% described the odour of hexanoic acid as being sour. These descriptions appear to have many of the qualities of synesthaesia, in which a stimulus in one sensory modality reliably elicits a consistent corresponding stimulus in another modality (Martino & Marks, 2000; Stevenson *et al*., 1998). For example, musical notes have been reported to reliably invoke sensations of shapes in some individuals (Cytowic, 2002). While in other modalities, synesthaesia is a relatively uncommon event, the possession of taste properties by odours is almost universal, particularly in the case of commonly consumed foods, e.g. the sweet smell of honey and the sour smell of vinegar. In fact, for some odours, taste qualities may represent the most consistent description used. Stevenson and Boakes (2003) reported data showing that, over repeat testing, ratings of taste descriptors for odours (e.g. *sweetness* of banana odour) were at least as reliable as ratings of the primary quality of the odour (i.e. *banana*). Such universality may reflect the functional significance of this phenomenon.

9.2.2 How do taste-related odour qualities develop?

Frank and Byram (1988) proposed that odours take on taste qualities through frequent co-occurrence with particular tastes. Many food-related odours are seldom, if ever, experienced in the mouth without an accompanying taste. Given that combinations of specific tastes with specific odours are relatively invariant for most foods and beverages (at least within cultures), this is a plausible explanation. We are unlikely to consume combinations such as vanilla odour and saltiness or chicken odour and sweetness very often, whereas if we exchange these tastes and odours, the combinations are much more familiar. In fact, this consistency in odour–taste combinations provides much of the basis of culture-specific flavour principles (Rozin & Rozin, 1981). By contrast, some culture-specific odour–taste pairings may result in some odours having different sniffed taste properties, depending on the cuisine in which they are experienced. Such examples might include pumpkin odour, which is associated with sweetness in the USA (pumpkin pie) but with savoury qualities

in many other countries, since it is primarily consumed as a vegetable as part of a main course. Consistent with this, it has been reported that French and Vietnamese vary in their judgements of odour–taste harmony, i.e. the extent to which odour and taste are seen as congruent (Nguyen *et al*., 2002).

This associative explanation for the origins of odour 'taste' properties was examined in a series of experiments (Stevenson *et al*., 1995, 1998) in which relatively novel odours (lychee, water chestnut) were repeatedly paired with either sweet (sucrose) or sour (citric acid) tastes in solution. The odours used in each case were rated as low in smelled sweetness and sourness when sniffed prior to pairing with the sweet or sour tastes. The method used to provide the coexposure of the odour and taste was a series of mock discrimination tasks in which subjects were asked to pick the odd sample out of three identical odour–taste pairs. This method of covertly providing repeat exposures, together with the use of interspersed dummy pairs – other odours paired with water – ensured that the purpose of the experiments was not explicit. These studies were able to consistently demonstrate that these novel odours were rated significantly higher in smelled sweetness or sourness, depending on the taste with which they were paired, when sniffed following the exposure phase. Figure 9.1 shows these effects, as well as the fact that odours associated with one quality (e.g. sweetness) also decreased in the extent to which they possessed the other quality (e.g. smelled sourness). Stevenson *et al*. (1998) have argued that the outcome of this associative process represents an example of learned synesthaesia.

9.2.3 How 'real' are smelled taste qualities?

To what extent does the attribution of taste qualities to odours reflect some sort of perceptual reality? Specifically, are these changes in ratings really changes in the perceived quality of the odour – as opposed to the development of a metaphor ('sweet like sugar') – or even a change in hedonic value? This possibility needs to be considered since we use terms such as 'sweet', 'bitter' and 'sour' as taste qualities but also, amongst other things, to describe the hedonic properties of facial expressions, emotional states or personality traits. In particular, the term sweetness is indelibly linked to food/beverage qualities that are liked. It is possible, then, that ratings of increased sweetness in odours are merely a synonym for the increased liking for flavours, which is known to occur following repeated exposure with sweetness (Zellner *et al*., 1983).

In fact, there is good evidence that such learning produces an odour whose qualities, and not merely hedonic values, have changed. In several studies of the same type (Stevenson *et al*., 1995, 1998), increases in the smelled sweetness or sourness of novel odours occurred with no appreciable change in ratings of liking. Moreover, in these studies, whether or not the subjects

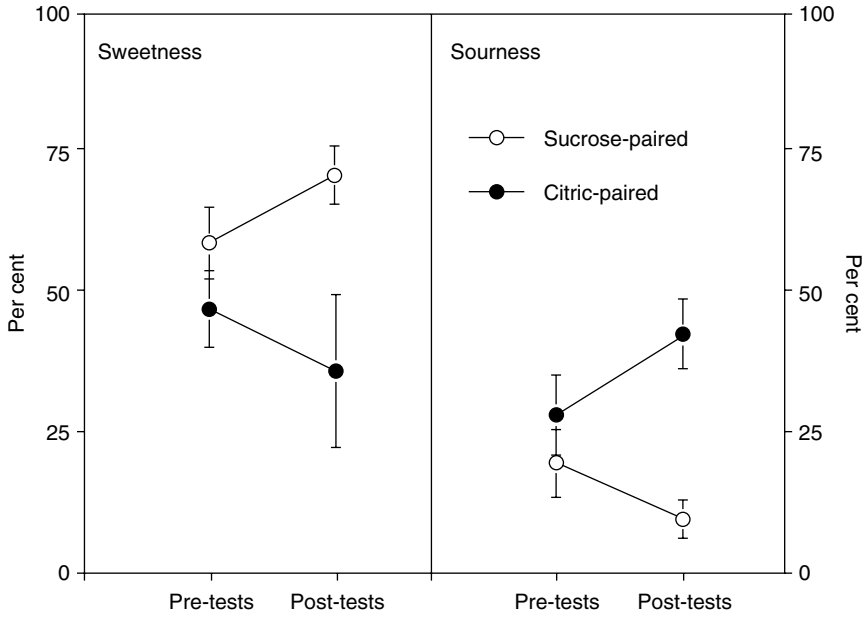

Figure 9.1 Mean pre- and post-exposure ratings of the smelled sweetness or sourness of novel odours that had been repeatedly paired with either sucrose or citric acid in solution. When paired with the sweet taste, the odour became sweeter, and less sour-smelling when sniffed. The converse pattern is seen with odours paired with sourness. (From Stevenson *et al.*, 1995.)

were explicitly aware of the specific odour–taste pairing during exposure did not influence the degree of change in the smelled properties of the odour. This argues against the results being interpreted either as a metaphor for liking or originating from expectations that certain odours are *supposed* to smell sweet or sour because of a specific experimental pairing. As will be discussed in detail, these odours are also able to predictably influence the sensory properties of odour–taste mixtures in which they are incorporated, suggesting a certain degree of perceptual reality. Studies by Stevenson (2001a, 2001b) showing that odour qualities are influenced by pairing with other odours also support the view that associative pairing can alter the quality of an odour. Thus, mushroom odour repeatedly paired with cherry odour begins to smell more cherry-like (and vice versa). It is difficult to see how either changes in liking or the development of a metaphor can explain these changes in odour quality. One important implication of these associative conditioning studies is, therefore, that odour qualities are, to some extent, malleable, whether through association with another odour or a taste.

9.2.4 Influence of smelled taste qualities on the perception of tastes

Further evidence for the integration of the components of flavours lies in studies showing the lack of psychophysical independence of odours and tastes, when combined in mixtures. Many, if not all, odours that are judged to have significant taste qualities when sniffed are also able to influence judgements of taste intensity, especially when added to tastants in solution. The most studied example of this effect is the ability of food odours such as strawberry or vanillin to enhance sweetness when added to solutions of a tastant like sucrose (Bingham *et al*., 1990; Clark & Lawless, 1994; Cliff & Noble, 1990; Frank & Byram, 1988; Frank *et al*., 1989, 1993; Prescott, 1999; Stevenson *et al*., 1999). These increases in taste intensity are not mediated by chemical interactions between the odorant and tastant, since pinching the nose abolishes the effect. The odours are also typically determined to be tasteless when experienced alone in solution. Moreover, rather than resulting from either a general sensory summation or being related to the chemical compounds used, the effects are specific to the taste and odour *qualities*. Frank and Byram (1988) showed that strawberry, but not peanut butter, odour enhanced the sweetness of sucrose; conversely, saltiness was not enhanced by strawberry odour. This effect is shown in Figure 9.2 in which the mean judged sweetness for each of three food odours in solution with sucrose is rated as sweeter than sucrose alone.

More recently, these findings have been extended by studies showing that odours added to tastants can also suppress taste intensity. Prescott (1999) found that odours judged to be low in smelled sweetness (peanut butter,

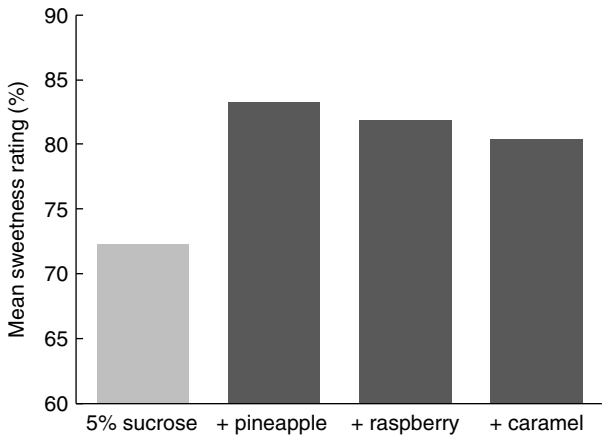

Figure 9.2 The effect of adding the sweet-smelling odours pineapple, raspberry and caramel to 5% sucrose in solution. In each case, the sweetness is rated as higher than the solution of sucrose alone (Prescott, unpublished data).

oolong tea) suppressed sweetness when added to sucrose in solution, in contrast to raspberry odour, which enhanced it. Stevenson *et al*. (1999) showed that sweet-smelling caramel odour not only enhanced the sweetness of sucrose in solution but also suppressed the sourness of a citric acid solution. Importantly, this latter effect appears to conform to the pattern of interactions seen with binary taste mixtures, in that the addition of sucrose would similarly suppress the sourness of citric acid. Such findings provide further evidence for the perceptual reality of odour–taste properties.

The ability of odours possessing smelled taste qualities to influence tastes has recently been demonstrated in paradigms in which neither the identification of quality nor judgement of intensity is required. Once again, these studies point to a perceptual reality for combining odour–taste qualities and tastes. Dalton *et al*. (2000) assessed orthonasal (i.e. sniffed) detection thresholds for the odorant benzaldehyde, which has a cherry/almond quality, while subjects held a sweet taste (saccharin) in the mouth (see also Chapter 8). Sensitivity for the odour was significantly increased (reduced thresholds) compared with benzaldehyde alone or in combination with either water or a nonsweet taste (MSG). The most plausible interpretation of these findings is that the smelled sweetness of benzaldehyde and tasted sweetness of saccharin were being integrated at subthreshold levels. Using a somewhat different experimental protocol in which both the odorant and taste were presented together in solution, Delwiche and Heffelfinger (2003) replicated these findings, although surprisingly, the same effect on sensitivity was apparent both with a sweet odour–taste pair (pineapple/aspartame) and a pair in which the tastant was MSG, a savoury (umami) quality.

If such threshold effects do represent integration of qualities common to odour and taste, then increased sensitivity for the tastant should also be apparent. Djordjevic *et al*. (2003) recently reported that the detection accuracy of sucrose at around threshold level was improved with the addition of an orthonasally presented sweet-smelling odour (strawberry) as compared to a nonsweet odour (ham). Data from our laboratory (Johnstone & Prescott, unpublished) show much the same result, using a priming procedure in which the odour preceded the taste presentation. Following determination of individual thresholds for sucrose in solution, subjects undertook a detection task consisting of presentation of two solutions successively, one containing sucrose. The subject's task was to determine which solution contained the sucrose, and each trial was preceded by presentation of an odour in a sniff bottle. One group of subjects received an odour previously judged to be sweet-smelling – caramel – while two other groups received either a nonsweet-smelling odour (peanut butter) or no odour (distilled water). Figure 9.3 shows that the sweet-smelling odour produced the greatest change in detectability (here presented as log d, a measure of sensitivity) relative to no odour. The measure of response bias (i.e. the tendency to respond in a particular way,

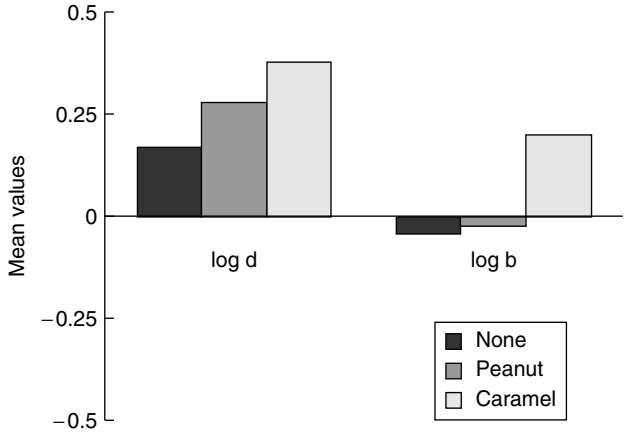

Figure 9.3 Measures of discriminability (log d) and response bias (log b) for the detection of a sweet taste following presentation of either a sweet-smelling odour (caramel), a nonsweet-smelling odour (peanut butter) or no odour. Both measures increased following the sweet-smelling odour (Johnstone & Prescott, unpublished data).

also known as the criterion or log b) was also greatest for the sweet-smelling odour. However, log d is an independent measure of sensitivity, indicating that the priming effect on sensitivity was not an artefact. Interestingly, the non-sweet odour also produced changes (although smaller) in detectability of sucrose suggesting, like Delwiche and Heffelfinger's (2003) threshold data, that there may be aspects of odour–taste integration for which the odour–taste pair does not necessarily need to share the same 'taste' quality.

A study of reaction times to sweet and sour tastes has shown similar priming effects at suprathreshold levels (White & Prescott, 2001). Simultaneous orthonasal presentation of odours that shared the same taste quality with the tastant (sweet-smelling cherry odour/sucrose; sour-smelling grapefruit odour/citric acid) facilitated the speed of naming of the taste, relative to incongruent pairs (cherry odour/citric acid; grapefruit odour/sucrose), or neutral/control pairs (either butanol or no odour plus either sucrose or citric acid). Some of these effects are illustrated in Figure 9.4.

9.3 Smelled taste qualities and taste modification as indicators of flavour formation

Orthonasal (sniffed) taste qualities and retronasal (tasted) taste modification by odours appear to be different aspects of the same perceptual phenomenon. Stevenson *et al*. (1999) directly tested this hypothesis by examining the

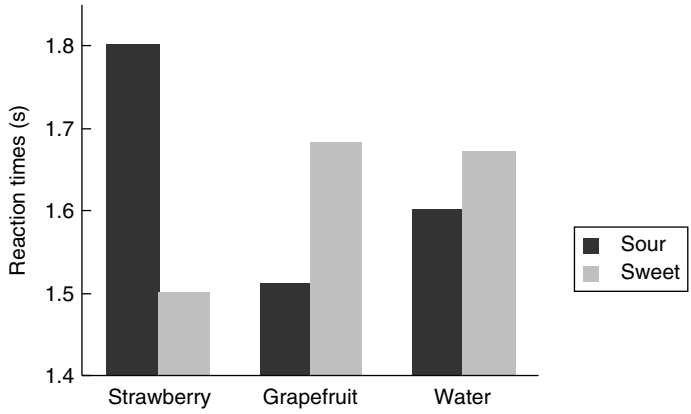

Figure 9.4 Reaction times to name a taste (sour; sweet) presented simultaneously with an odour (strawberry; grapefruit) or no odour (water). Reaction times are significantly lower when the odour and taste are perceptually similar, i.e. they share a perceptual quality such as sweetness (strawberry/sweetness) or sourness (grapefruit/sourness). (From White & Prescott, 2001.)

relationship between ratings of smelled sweetness and sweetness enhancement. In this study, subjects were asked to rate the smelled sweetness of a variety of odorants (including both food-related odours such as lychee, caramel and mango, and nonfood odours) as well as their tasted sweetness as flavours in 10% sucrose. Smelled sweetness was found to be a significant predictor ($r = 0.67$) of the extent to which the odour modified the tasted sweetness of the sucrose solution. That smelled taste qualities are the best predictors of taste enhancement was subsequently confirmed by Nguyen *et al*. (2002).

Like the acquisition of taste qualities by odours, the ability of odours to influence tastes has also been shown to be a function of associative learning. Prescott (1999) demonstrated that odours that initially had either no impact on sucrose sweetness (water chestnut) or actually suppressed sweetness (peanut butter) both enhanced sweetness following an exposure phase of repeated pairings with sucrose in solution. Similarly, odours repeatedly paired with either sucrose or citric acid acquired the ability to facilitate the speed of naming of the associated taste, relative to the unassociated taste, when presented simultaneously with the taste (White & Prescott, 2002). Subthreshold integration of odours and tastes, as reflected in reduced odour thresholds when combined with a taste, has been shown to occur following specific odour–taste pairings, but not with odours and tastes not previously paired (Breslin *et al*., 2003).

What significance do these phenomena have for our understanding of flavour perception? Frank *et al*. (1991, 1993; van der Klaauw & Frank, 1996) proposed that enhancement of a sweet taste by an odour such as strawberry occurs because these qualities have become perceptually similar.

As a consequence of their prior association, the boundaries of odour and taste are perceptually blurred, and it may therefore be unclear to an assessor where the odour qualities end and those of the taste begin. It is suggested that, effectively, the odours and tastes may be encoded as dimensions of the same compound stimulus – the flavour – in which qualities such as sweetness (whether associated with a taste or an odour) are perceptually combined. In this view of odour–taste interactions, the co-encoding of the odour and taste as a flavour in memory determines the subsequent interactions of the elements of the flavour (Frank *et al*., 1991; McBurney, 1986; Schifferstein & Verlegh, 1996).

According to Stevenson *et al*. (1995), when a flavour's odour component is subsequently experienced, the taste component is also evoked. Thus, for example, sniffing caramel odour activates memorial representations of caramel flavour, which includes a significant sweet component. This results either in perceptions of smelled taste properties such as sweetness or, in the case of a mixture, a perceptual combination with the physically present taste in solution. In other words, perceptions are being constructed from a combination of both 'real' tastes and taste properties of the odour that are encoded in memory. Stevenson and Boakes (2003) have recently elaborated on this explanation by proposing a configural account of how odours come to take on the properties of tastes (or even the qualities of other odours). They argue that experiencing novel odours involves comparison to memories of previously encountered odours. If the initial experience of the odour is that it is paired with either a taste or another odour, a configural (unitary) stimulus is encoded in memory. Subsequently, sniffing the odour alone will evoke the most similar odour memory, i.e. the odour–taste combination. It is suggested that it is this memory that constitutes the odour experience. Such explanations are consistent with data showing that memorial representations of chemosensory qualities can combine with physically present stimuli to produce mixtures that show very similar interactions to those of identical combinations of physically present qualities. These 'mental mixtures' have been demonstrated with combinations of different tastes (Stevenson & Prescott, 1997), odours (Algom & Cain, 1991) as well as odour–taste mixtures (Algom *et al*., 1993).

Similar models of odour–taste integration are to be found in the animal learning literature. For example, Rescorla (1980; Rescorla & Durlach, 1981) describes within-event learning in which the simultaneous presentation of distinct qualities (either taste–taste or taste–odour) can lead to the encoding of a compound stimulus to which the individual elements come to be perceived as similar (as assessed by the ability of the elements to elicit behaviours conditioned to the compound stimulus). He notes that the presentation of each element may also activate the whole compound stimulus. This clearly has parallels with the ability of a sniffed odour to activate a previously encoded odour–taste compound in humans.

9.3.1 The role of spatial and temporal factors in odour–taste integration

Rescorla (1980) demonstrated that simultaneous, rather than successive, presentation of the stimulus elements results in more effective learning of the compound stimulus in rats. Two aspects of this finding are also pertinent to human flavour perception. Firstly, Rescorla (1980, p. 216) suggested that simultaneous presentation 'discourages the formation of individual representations', which may act to inhibit learning. Stevenson and Case (2003) similarly showed that exposure to novel odours prior to pairing those odours with tastes (sucrose, citric acid) inhibited learning of an odour–taste association, i.e. the odours did not become significantly sweeter or more sour following coexposure with the taste. Secondly, spatial and/or temporal contiguity of the odour and taste may also be a crucial determinant of integration in humans. Central to this process may be facilitation of learning by the well-known olfactory location illusion, in which retronasal perception of odours (i.e. via the mouth as part of flavours) is universally interpreted as originating in the mouth, rather than the nose (as a sniffed odour would be). This is likely to be mediated by concurrent taste or tactile stimulation (or both). While these two olfactory routes differ physiologically perhaps only in efficiency of delivery of odours to the olfactory epithelium (Pierce & Halpern, 1996; Voirol & Daget, 1986), Rozin (1982) has suggested that retronasal olfaction, which occurs during food consumption, can be seen as functionally distinct from orthonasal olfaction in terms of its cognitive impact. The importance of the olfactory component of flavours lies in the fact that, in association with tastes and other sensory properties, they uniquely identify foods located in the mouth. It is therefore understandable that a bias exists towards perceiving a food's sensory qualities as a unique flavour belonging to the food, rather than as a set of separate sensory stimuli (Gibson, 1966). If this is the case, the location illusion mediates the integration of odours and tastes as flavours.

While the apparently close spatial proximity of the odour and taste may have cognitive significance, there is evidence to suggest that Rescorla's findings (1980) regarding the importance of simultaneous presentation apply to human flavour perception as well. The location illusion appears to depend on *both* the spatial and temporal contiguity of the discrete sensory inputs. Von Bekesy (1964) emphasised the importance of temporal factors by showing that the perceived location of an odour (mouth vs. nose) and the extent to which an odour and taste were perceived as one sensation or two could be manipulated by varying the time delay between the presentation of the odour and taste. With a time delay of zero (simultaneous presentation), the apparent locus of the odour was the back of the mouth and the odour–taste mixture was perceived as a single entity. When the odour preceded the taste, the sensation was perceived as originating in the nose. Consistent with this, Sakai *et al.* (2001) demonstrated that odour-induced taste enhancement can occur whether

the odour is presented orthonasally or retronasally, provided the odour and taste are presented simultaneously. More generally, while there are instances of either spatial or temporal co-occurrence being important in integration across sensory modalities, integration can be facilitated when both types of co-occurrence are present (Calvert *et al*., 1998; Driver & Spence, 2000) as in the case of odour–taste mixtures.

9.4 Cognitive processes in the development of flavour perception

Even though an odour's sniffed 'taste' qualities and its ability to enhance that taste in solution are highly correlated (Stevenson *et al*., 1999), demonstrating that a sweet-smelling odour, for example, can enhance the sweetness of sucrose in solution appears to operate under some constraints. Stevenson *et al*. (1995, 1998) were able to produce sweeter-smelling odours through repeated pairing with sucrose. However, these odours subsequently failed to enhance the sweetness of a sucrose solution. In fact, whether or not an odour enhances taste, and hence the degree to which the two modalities could be said to be integrated, appears to be dependent on what the subject is asked to judge. Thus, Frank *et al*. (1993) found that while strawberry odour enhanced the sweetness of sucrose in solution when the subjects were asked to judge only sweetness, the enhancement disappeared when subjects rated the sourness and fruitiness of these mixtures as well. In addition, Frank *et al*. (1991, 1993) found suppression of the sweetness of the strawberry–sucrose mixtures when the subjects rated total intensity of the mixture and then partitioned their responses into sweet, salty, sour, bitter and/or other tastes. Interestingly, these effects were also noted (Frank *et al*., 1993) for some taste mixtures in which the elements are often judged as similar (e.g. sour/bitter taste mixtures), but not others with dissimilar components (e.g. sweet/bitter mixtures). Similar effects have been reported by Clark and Lawless (1994) who found significantly less sweetness enhancement when subjects rated, in addition to sweetnees, the strawberry-flavour strength of strawberry–sucrose mixtures or the vanilla-flavour strength of vanilla–sucrose mixtures, than when they rated sweetness alone.

9.4.1 Taste modification by odours: a rating effect?

One interpretation of the dependence of enhancement effects on the number of rating scales used has been to see them as examples of 'halo-dumping' (Clark & Lawless, 1994; Lawless & Clark, 1992). This occurs when a response alternative is not provided for a salient attribute and subjects 'dump' the values for the attribute onto other rating scales that are provided. In the case of odour–taste mixtures, salience reflects perceptual similarity. Hence, when subjects are unable to express sensory qualities such as *fruitiness* in their rating of a

fruit odour–taste mixture (e.g. by being asked to rate sweetness only), they 'dump' these odour qualities onto the *sweetness* rating, thereby producing enhancement. Under conditions where multiple, appropriate scales are provided, enhancement is not seen since, it is argued, the subject is able to rate all qualities. In other words, enhancement is a function of the number of scales used influencing the subject's rating strategy (Clark & Lawless, 1994).

If odour-induced taste enhancement results merely from the way in which subjects use rating scales, it clearly undermines the idea that this phenomenon reflects the integration of odours and tastes into flavours. However, there are data that cast doubt on this interpretation of taste enhancement. Although ratings do have an impact on the ability of odours to influence tastes, it also seems to be the case that such effects are also a function of the odour itself. Stevenson *et al*. (1999) demonstrated that, while keeping the number of rating scales constant, some sweet-smelling odours (e.g. lychee, caramel) enhanced sweetness in solution, and others that were rated very low in smelled sweetness were able to suppress the sweetness of sucrose and, in one case, the same sweet-smelling odour (caramel) not only enhanced sucrose sweetness but also suppressed the sourness of citric acid. In addition, Prescott (1999) demonstrated that following repeated pairing with a sucrose solution, an odour that initially suppressed sweetness could be changed into one that enhanced sweetness, again without manipulation of rating scales. These latter findings argue strongly that a 'taste' property can effectively become part of an odour's overall quality. Nguyen *et al*. (2002) have shown that dumping as a function of number of rating scales can occur with odour–taste mixtures, but that it appears to be more a generalised tendency to integrate, rather than a response to the perceptual similarity of odours and tastes in the mixture. By manipulating congruency (perceptual similarity) of odour–taste pairs as well as number of rating scales, they were able to demonstrate that enhancement involved another mechanism beyond, or at least in addition to, dumping.

9.4.2 Taste modification by odours as a function of perceptual strategy

In attempting to explain the rating scale effects, Frank *et al*. (1993; Frank, 2003; van der Klaauw & Frank, 1996) emphasised the role of the task demands, without necessarily implying that taste enhancement merely reflects rating strategies. They suggested that, given perceptual similarity between an odour and taste, the conceptual 'boundaries' the subject sets for a given complex stimulus will reflect the task requirements. In the case of an odour–taste mixture in which the elements share a similar quality, combining those elements is essentially optional. Rather than 'dumping' one quality onto the scale associated with a similar quality in another sensory modality, this explanation invokes the notion that integration of perceptually similar dimen-

sions is determined by the attentional focus demanded in the task. These effects of instructional sets are analogous to those seen in studies of cross-modal integration of vision and hearing. Melara *et al*. (1992), for example, showed that focusing on the overall similarity of visual or auditory stimulus pairs, representing different stimulus dimensions versus focusing on their component dimensions, could influence whether the pairs were treated as interacting or separable dimensions (Garner, 1974).

An interpretation of taste enhancement effects as a perceptual, rather than a response, process would suggest that the apparent influence of the number of rating scales on odour–taste interactions (i.e. asking subjects to rate different number of scales) results from the impact of these scales on how the odour and taste are perceived. In keeping with this view, van der Klaauw and Frank (1996) were able to eliminate taste enhancement by directing subjects' attention to the appropriate attributes in a taste–odour mixture, even when they were only required to rate sweetness. In other words, rating all appropriate attributes of a mixture is not a prerequisite to eliminate taste enhancement. Similarly, Clark and Lawless (1994) noted that prior knowledge of those attributes that are to be rated should also abolish any halo-dumping effects even if these are rated in succession, since the subject realises that there is no need to 'dump' qualities. Thus, rating scale effects may be more appropriately interpreted in terms of the subject's attention to the sensory properties rather than a response to rating scales per se. The existence of rating effects can therefore be seen as an important example of the extent to which taste modification by odours is determined by cognitive processes.

9.4.3 Analysis and synthesis in the perception of flavour

Unlike the effects of mixing chemicals to produce a specific new compound, the mixing of sensory stimuli may therefore be partly determined by the adoption of different cognitive/perceptual strategies. Two common modes of perception have been found to be useful descriptions of how interactions occur *within* sensory modalities. The blending of odours to form entirely new odours is a commonplace occurrence in flavour chemistry and perfumery, and hence is referred to as *synthetic* interaction (analogous to the blending of light wavelengths). However, even this process depends on the number of odours blended, since the components of binary odours often remain distinct (Laing & Willcox, 1983). By contrast, the mixing of tastes is typically seen as an *analytic* process, because individual taste qualities do not fuse to form new qualities and, like simultaneous auditory tones, can be distinguished from one another in mixtures. As noted above, however, when the elements of some taste mixtures are perceptually similar, such as sour and bitter, the influence of the task demands on the responses can be observed (Frank *et al*., 1993).

McBurney (1986) has suggested a further category of interaction, namely, *fusion* – the notion of sensations combined to form a single perception, rather than combining synthetically to form a new sensation. He argues that this applies to perception of flavours that remain analysable even when perceived as a whole. This is illustrated in Figure 9.5, which shows that both analysis and synthesis can be alternative responses to food flavour. An implication of this notion of fusion is that whether an odour–taste mixture is perceived analytically or synthetically can be determined by the responses required of the subject. Multiple ratings of appropriate attributes force an analytical approach, whereas a single rating of a sensory quality that can apply to both the odour and taste (e.g. the tasted sweetness of sucrose and the smelled sweetness of strawberry) encourages synthesis of the common quality from both sensory modalities. The components of these flavours may not be treated separately when judged in terms of sweetness or other single characteristics (including *overall intensity*). When instructions require separation of the components, however, this can be done – the components of a flavour are evaluated individually, and sweetness enhancement is eliminated. van der Klaauw and Frank (1996, p. 26) also adopt this view, suggesting that 'providing appropriate response alternatives will encourage observers to separate the component attributes of a complex stimulus, whereas they are more likely to integrate dimensions when their response alternatives are limited'. In other words, rating requirements lead to different perceptual approaches (analytical or synthetic), which, in turn, influence the degree of perceptual integration that occurs.

The congruence (Murphy & Cain, 1980; Schifferstein & Verlegh, 1996) or perceptual similarity (van der Klaauw & Frank, 1996) of the odour and taste components influences this process. Schifferstein and Verlegh (1996) suggest that evaluating taste or odour intensities is primarily analytical, except when the stimulus components are perceptually similar, forming a congruent, synthetic whole. Consistent with this, Frank *et al*. (1991) found that the degree of enhancement produced by an odour for a particular taste was significantly correlated with ratings of the perceived similarity of the odorant and tastant. Whether an odour–taste combination is seen as congruent depends upon familiarity, or experience with the components as a combination, i.e. it is a product of prior association (see Section 9.2). Presumably all common odour–taste pairs would be rated highly in terms of stimulus similarity.

9.4.4 Investigating cognitive processes in flavour perception

The concept of fusion suggests strongly that flavour perception is highly dependent on both past experience with specific odour–taste combinations (the origin of congruence) and cognitive factors that influence whether the flavour elements are combined or not. However, to date, there have been few

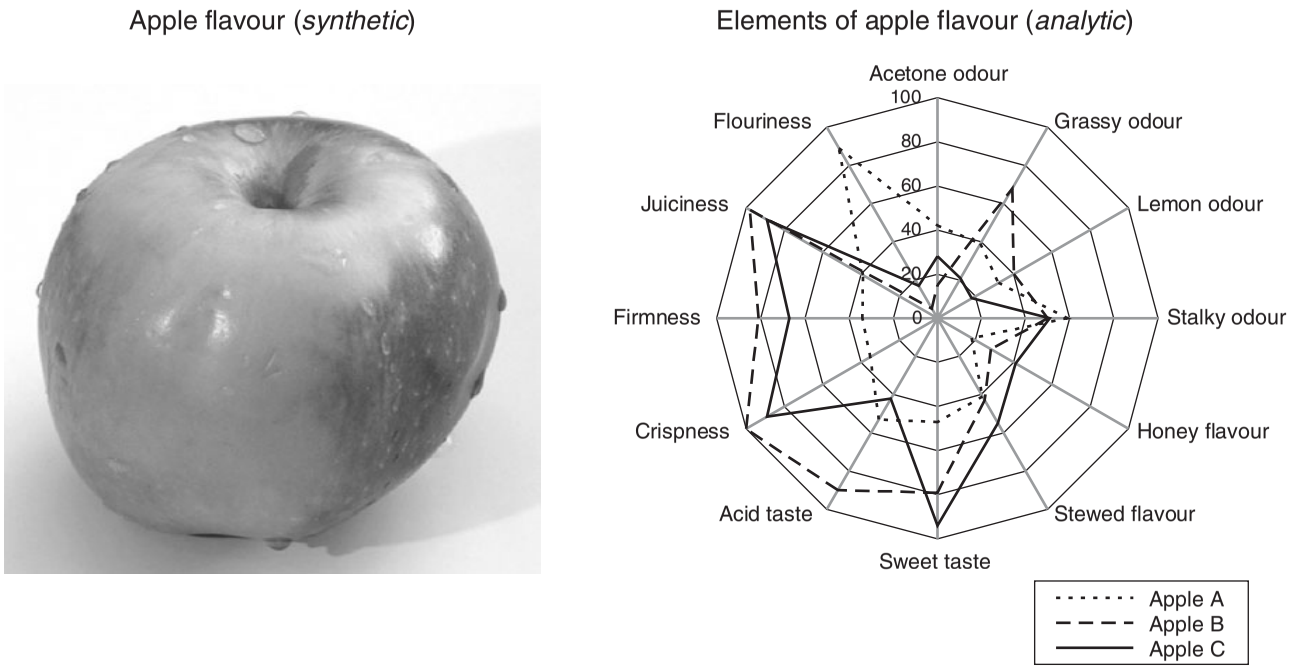

Figure 9.5 Synthetic and analytic views of a flavour. In each case, the sensory signals are identical, but the perception differs – the whole flavour of the apple versus a collection of sensory qualities on which different apples may vary. (Spider web graph courtesy of Shane Walker and Hort Research, New Zealand.)

studies investigating the role of cognitive factors in flavour perception. Since psychophysical responses (e.g. ratings) are used to infer perceptual processes, testing the interpretation that odour-induced taste modification reflects perceptual/cognitive (as opposed to response) processes is not straightforward. One approach to this question is to manipulate the perceptual strategy used (analytic vs. synthetic) while maintaining a constant number of rating scales. Although they did not deliberately attempt to investigate this issue, Bingham *et al*. (1990) showed that untrained panellists judged solutions of the odorant maltol plus sucrose as sweeter than a solution of sucrose alone. In contrast, a trained descriptive analysis panel found no such enhancement. The important point here is that a key aspect of the training of such panels is for them to adopt an analytical approach to the sensory properties of complex stimuli such as foods or beverages.

If it is the case that odour–taste interactions can be influenced by the extent to which an analytical or synthetic perceptual approach is taken during rating, this suggests the possibility that the extent to which the odours and tastes become integrated (as shown increased perceptual similarity), as well as any influence of odours on those tastes in solution, might similarly be determined by the way in which the components of the flavour are associated during their initial joint exposure. An exposure strategy that emphasises the distinctiveness of the elements in the odour–taste mixture (an analytical perceptual strategy) should inhibit increases in the taste properties of the odour, and the subsequent ability of the odour to influence tastes in solution. In contrast, treating the elements as a synthetic whole is likely to encourage the blurring of the perceptual boundaries, fostering subsequent odour–taste interactions.

Some studies have specifically trained subjects to distinguish the individual odour and taste components of flavours in order to determine if such prior training influences the ability of novel odours to take on taste qualities following coexposure (Stevenson, 2001a, 2001b; Stevenson & Case, 2003). Stevenson and Case (2003) examined changes in smelled sweetness and sourness of novel odours paired with sucrose and citric acid, respectively, in trained and nontrained subjects. The trained group were first given explanations of the relative contributions of odours and tastes in flavours, and taught to distinguish the odour and taste components in mixtures. In contrast to the results of Bingham *et al*. (1990), however, such training had no impact on the extent to which the odours took on taste qualities following subsequent conditioning with a new set of odours.

While Stevenson and Case (2003) argued that the integration of odour and taste during their joint exposure is ‘cognitively impenetrable’, it is probable that their failure to find an effect of training reflects the sensitivity of odour–taste integration to the nature of their joint exposure. It is unlikely that the ‘trained’ group used by Stevenson and Case (2003) responded to the new odour set to which they were exposed in an analytical manner. Specifically,

the exposure procedure, in which both trained and untrained subjects were asked to rate their *liking* for the new odour/citric acid and odour/sucrose mixtures, is likely to have encouraged synthesis of these elements. Hedonic responses tend to reflect a global or synthetic response to a food, beverage or flavour, which is regarded as generally incompatible with an analytical approach.

Another approach to assessing the impact of perceptual strategies on interactions within flavours has been to predispose subjects to adopt different strategies *during* joint exposure of odours and tastes. Prescott (1999; Prescott *et al*., in press) reported studies in which groups of subjects were forced to adopt strategies that encouraged either synthesis or analysis of the odour and taste components of flavours during their repeated coexposure. Subjects received multiple exposures to sucrose combined in mixtures with relatively novel odours that were low in smelled sweetness or, as a control, the odours and sucrose solutions separately. Two groups that received mixtures made intensity ratings that promoted either synthesis or analysis of the individual elements in the mixtures. The synthetic approach was to require subjects to attend only to overall flavour intensity of the mixtures during exposure. In the analytical group, subjects were informed that the solutions were made up of sucrose and an odour and their task was to attend to, and separately rate, the components of the mixture. The earlier study (Prescott, 1999) showed only that a synthetic approach produced increases in the smelled sweetness of low-familiarity odours, but without any alteration in the ability of that odour to enhance sweetness in solution. In later studies in which the analytic and synthetic strategies were better differentiated (Prescott *et al*., in press), the odours became sweeter-smelling irrespective of group – and interestingly after only a single coexposure with sucrose. However, as predicted, only adopting a synthetic strategy during the exposure phase produced odours that subsequently enhanced sweetness in solution. These data reinforce the view that the perceptual approach taken – whether the odour and taste are perceived as a single entity or multiple elements – is an important determinant of the effects discussed in this chapter and hence of how flavours are 'constructed' and perceived.

9.5 Implications and future directions

These alternative cognitive strategies, here demonstrated experimentally, map very well onto real distinctions that exist between consumer and 'expert' perception of food and beverage flavours (see Figure 9.5). Without motivation to do otherwise, consumers will naturally integrate sensory information, producing a response (typically hedonic) to the whole food or beverage. Analytical panellists and other experts (e.g. wine judges) are not only trained to view

complex flavours as a set of individual elements, but the tasks typically required of them also involve assessment of multiple sensory attributes, each assumed to be independent. As has been discussed in this chapter, many such attributes are not independent, even though they may be derived from distinct physiological sources. The data on the role of cognitive processes in flavour perception, presented here and elsewhere, raise the question of whether it is even possible for a trained panel to perceive a whole flavour.

These data also have implications for the measurement of sensory qualities. Clearly, the questions asked will determine how perceptually similar flavour qualities are combined or separated, with consequences for the intensity of sensory qualities. This raises the question of what qualities *should* be measured in evaluations of food/beverage flavours. Thus, in a complex mixture such as a sweet food, which part is the 'real sweetness'? Clearly, it depends on whether it is important to know about the sweetness of the flavour, or the sweetness associated with the odour or produced by the taste. However, the distinction between odour sweetness and taste sweetness may be artificial if one is evaluating flavours, since the sweetness of the flavour components may be represented cognitively, and even perhaps neurally, as equivalent. They may be functionally equivalent, as well. As noted earlier, odour sweetness can suppress tasted sourness, just as a sweetener such as sucrose does (Stevenson *et al*., 1999). Frank *et al*. (1993) suggest that the question might be an empirical one, especially for consumers. That is, what form of sweetness best predicts overall responses to foods? In this regard, overall flavour sweetness may be the most ecologically valid measure.

Although not considered in this chapter, whether a flavour is viewed analytically or synthetically may not only determine the psychophysical interactions that occur between the elements but may also influence the hedonic properties of the overall flavour. Synthetic perceptions may facilitate hedonic responses, whereas analytic perceptions are usually seen as antithetical to making hedonic judgements. This issue has yet to be explored in detail, although preliminary data from our laboratory suggest that odours coexposed with sucrose during a synthetic strategy increase in smelled sweetness *and* in liking, while those exposed under an analytical strategy show either decreased, or no change in, liking. There is reason to believe that the psychophysical and hedonic changes are independent, but this needs to be examined with other taste qualities besides sweetness to be certain.

9.6 Conclusion

Recent data on odour–taste mixtures have provided compelling evidence that sensory integration as a result of prior association is responsible for the formation of distinct flavour perceptions. The evidence that this integrative

process, and thus flavour perception, is determined by individual (and perhaps even cultural) learning, memory and other cognitive processes should not be surprising. This is true in all other spheres of perceptual experience. Moreover, that there exists a functional flavour perceptual system is not a new idea. Brillat-Savarin (1825, p. 41) noted in his gastronomical meditations that he was 'tempted to believe that smell and taste are in fact but a single sense, whose laboratory is the mouth and whose chimney is the nose'. The recent psychophysical data on odour–taste interactions are now providing the empirical and theoretical basis for this prescient observation.

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