

Odour perception in honeybees: coding information in glomerular patterns

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Major advances have been made during the past two years in understanding how honeybees process olfactory input at the level of their first brain structure dealing with odours, the antennal lobe (the insect analogue of the mammalian olfactory bulb). It is now possible to map physiological responses to morphologically identified olfactory glomeruli, allowing for the creation of a functional atlas of the antennal lobe. Furthermore, the measurement of odour-evoked activity patterns has now been combined with studies of appetitive odour learning. The results show that both genetically determined components and learning-related plasticity shape olfactory processing in the antennal lobe.

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Abbreviation

CS conditioned stimulus

Introduction

Research in olfactory systems has received a boost following the molecular characterisation of olfactory receptor genes in vertebrates [1] and recently in insects [2,3]. Our understanding of the neural representation of odours at the level of the olfactory bulb (in vertebrates) or the antennal lobe (in insects) has also improved, particularly as a result of the development of powerful optical recording techniques which allow one to repeatedly measure odour-evoked physiological activity in several glomeruli simultaneously.

In the analysis of olfactory processing, a few model systems are becoming increasingly popular. The first of these is the mouse, which, through the potential for genetic analysis and manipulation [1], has, together with the rat, become an important model for mammalian olfaction. Recently, optical imaging studies have allowed the visualisation of spatial activity patterns for several odours in individual rats [4••]. The second model that has proved useful is the zebrafish; it has only one-tenth of the glomeruli that the mouse has, and its olfactory bulb is also easily accessible for the spatial recording of activity patterns [5,6]. The third model, that of the nematode worm *Caenorhabditis elegans*, is useful because of our excellent comprehension of its genetics and development, because of the ease of identification of individual cells, and also because it is a system that employs strategies very different from those of both insects and vertebrates [7]. Another model is the fruit fly, which is useful

because of its well characterised genetics and the fact that its receptor genes were cloned last year [2,3]. Moths have proved useful for the special case of pheromone and host plant detection, and because of successful electrophysiological single cell recordings. Lastly, the honeybee has been used as a model. This review will focus on this latter species: behavioural paradigms for olfactory learning are well established, and are increasingly used to investigate olfactory coding. Furthermore, recent developments in optical imaging techniques allow odour-evoked activity patterns to be visualised. Together, these approaches have shed important light on the way in which the brain encodes odours.

Why the honeybee?

The 'olfactory code' is a set of transformation rules that lead to a neuronal representation of the olfactory stimulus. This representation is, however, also influenced by the behavioural significance of the stimulus. In order to understand the olfactory code, therefore, it is necessary not only to know the physical properties of a stimulus, but also to characterise its behavioural effects.

The honeybee's strong point as a model system for olfaction research is that odour processing can be studied through tests of learning, with odour as an appetitive stimulus. Several behavioural paradigms are available for the investigation of olfactory learning [8]. These paradigms can be combined with physiological measurements of odour representation [9••]. Furthermore, it is possible to study perceived odour similarity in vast arrays of odours, and thus to obtain a metric of the perceptual space [10].

In honeybees there are 60,000 olfactory receptor cells on the antennae. Their axons project to the antennal lobes, which are subdivided into approximately 160 identified glomeruli, identification being based on the shape and relative position of these glomeruli [11]. The glomeruli are interconnected by about 4,000 local interneurons, and from the glomeruli about 800 projection neurons lead to higher-order brain centres, such as the mushroom bodies and the lateral protocerebrum. Using calcium imaging, it is possible to measure odour-evoked glomerular activity patterns in about 40 of the 160 glomeruli [12–14]. Results from these studies show that each odour elicits a mosaic of activated glomeruli, and that each glomerulus can take part in the mosaic of several odours. Furthermore, responses are graded: a glomerulus may be weakly activated by one odour, and strongly activated by another (or by a higher concentration of the same odour).

Linking physiology and behaviour

Ultimately, olfactory coding can only be judged on the basis of behaviour. Honeybees are ideal experimental animals

because they can be trained to respond to an odour with the proboscis extension reflex (PER), which can either be directly observed, or measured physiologically. The occurrence and strength of the reflex is directly related to the learning success. When two odours are presented, of which one is positively reinforced (by pairing it with a sugar-water reward, CS⁺) and the other negatively reinforced (by not rewarding it, CS⁻), the capability of the animal to discriminate two odours can be directly tested: if the animal can discriminate, it will selectively respond to the positively reinforced odour. Using this paradigm, odour pairs can be classified as being similar or distant, and this information can be used in physiological experiments. Odour generalisation can also be used to assess 'perceptual' similarity. For example, Stopfer and co-workers [15] have trained bees to respond to one odour and have then tested for generalisation by presenting the trained odour (CS⁺), a chemically and perceptually similar odour (S) and a distant odour (D) alone and in random order. Generalisation between similar odours is increased when GABA receptors are pharmacologically blocked with picrotoxin, whereas dissimilar odours are not affected by the treatment; this suggests that inhibitory circuits in the brain are important for olfactory fine-tuning [15]. Free-flying bees can also be appetitively trained to an odour. This paradigm has the advantage that a single bee can evaluate up to 50 odours in one experiment, allowing the creation of a similarity matrix between odours [10]. Thus, it is possible to compare similarity in the behavioural responses of bees with the similarity of physiological responses as obtained by optical imaging measurements.

Odour mixtures may be processed as novel perceptual entities ('elementary representation'), or as a combination of their elements ('configural representation'). Several experimental procedures have been applied to distinguish between elementary and configural learning; these experiments show that odour mixtures have mixture-unique properties [16–18]. For example, bees are able to solve a discrimination task where the single odour component is not able to predict the reward, but the mixture is. In these experiments, each odour is part of both a rewarded and a non-rewarded mixture, and bees discriminate (AB⁺) and (CD⁺) from (BC⁻) and (AD⁻), where A, B, C and D are individual odours; the '+' indicates a rewarded stimulus, and the '-' a non-rewarded stimulus [16,19]. The degree of interaction between the odour components in a mixture depends on the odours chosen, and on the number of components. Bees can easily learn to discriminate two components A and B from their mixture AB. However, they have greater difficulties when an additional component is added and they attempt to discriminate AC and BC from ABC [16]. These 'mixture' effects arise both from learning mechanisms and from the way in which odours are represented in the brain. For investigations into the latter aspect, it is particularly interesting that significant mixture effects are observed for some mixtures but not for others. Interaction is less detectable between molecularly dissimilar odorants (e.g. geraniol and 1-hexanol) than between

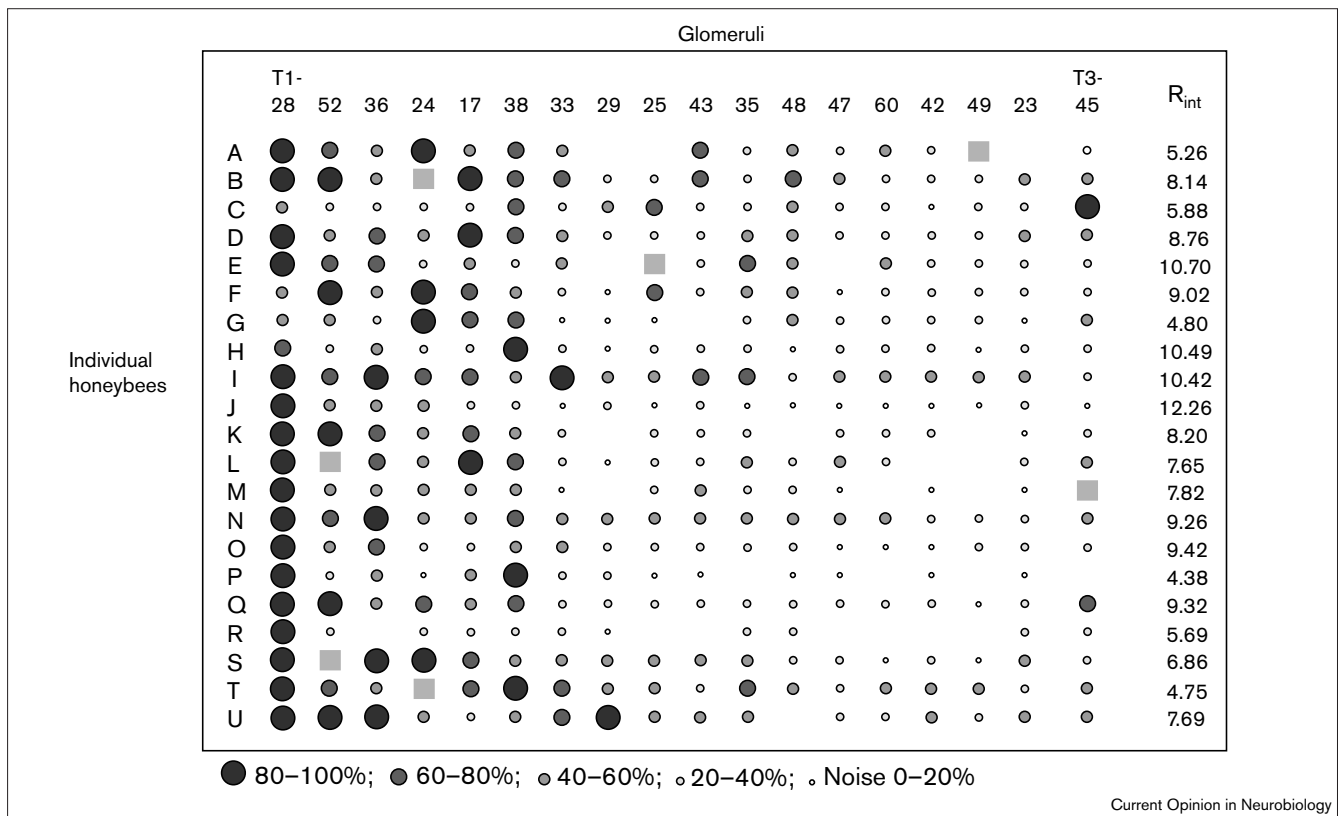
two molecularly similar odorants (e.g. hexanal and 1-hexanol) [17]. In this experiment, bees trained to respond to either hexanol or geraniol responded equally to the trained odour and to a mixture of hexanol and geraniol. However, bees trained to either hexanol or hexanal responded strongly to the trained odour, but much less to a mixture of the two [17]. In 'blocking' experiments, an odour (A⁺) is positively reinforced, and afterwards a mixture with that odour as a component (AB⁺) is reinforced. If blocking is present, the response to odour B alone is reduced in comparison to that of a control group that was only trained to the mixture (AB⁺). Therefore, prior learning of (A⁺) 'blocks' the subsequent learning of component B in the mixture (AB⁺). Though blocking is still controversial in honeybees (it could not be replicated in a thorough analysis [20*]), recent experiments suggest that the occurrence of blocking may depend on the perceived similarity of the mixture components (JS Hosler, BH Smith, personal communication). A prediction from these studies would be that chemically similar odorants have overlapping internal representations. This hypothesis has also been considered in a computational model of the antennal lobe [21] and can be tested using calcium imaging of odour-evoked activity patterns in the antennal lobe of honeybees.

Stereotypy and plasticity of olfactory representation

Activity patterns are genetically determined...

Odours evoke across-glomeruli activity patterns in the antennal lobe of the bee. These patterns can be visualized using calcium imaging: the brain is stained with a dye that increases its fluorescence when the neurons are active. The stained bee is stimulated with an odour, and the spatial pattern of neural activity can be measured as increased fluorescence with a CCD (charge-coupled device) camera [12,13]. Using a computerized morphological atlas of antennal lobe glomeruli [22], it was possible to map the identity of glomerular units onto the physiological recordings done with calcium imaging. Therefore, the morphological identification of glomeruli was accomplished independently of the physiological recordings. This is important, because if the activity patterns were compared between individuals and found to be equal solely on the basis of these patterns, that would create a vicious circle. With this procedure, it was possible to compare the response profiles of individual glomeruli between specimens (Figure 1). In this experiment, two main issues were addressed: first, whether odour representation is conserved within the species; and second, whether the activity pattern elicited by an odour is sufficient to predict the stimulus odour. Statistical analysis was carried out on 18 of the 160 glomeruli of the bee (11%). More precisely, a discriminant analysis was used to test whether odour representations of each given odour form a coherent 'cloud' in the multidimensional space where axes are defined by each of the identified glomeruli. In 86% of cases the odour could be identified correctly from the glomerular activity [23**]. Thus, odour representation is

Figure 1



Calcium responses to 1-hexanol in 18 identified glomeruli (columns) of 21 individual honeybees (rows A–U). T1 and T3 denote the antennal nerve tract innervating the glomerulus, and the number is a unique identifier. Larger circles indicate stronger responses (as indicated by the key below the figure). Gray boxes indicate glomeruli that could not be identified in that animal. Animals differed in their overall response intensities, which were calculated as an integral of the calcium response. The maximum response values are given as R_{int} in the right

column. In order to compare the response patterns between individuals, each row is normalised to its maximal response (R_{int}). Within the T1 glomeruli, those with the strongest odour responses are shown to the left; glomeruli with the weakest responses to the right. Note that glomerulus T1-28 is the strongest in 17 out of 21 patterns. Of the glomeruli responding strongly to 1-hexanol in some animals, glomeruli T1-36, T1-17 and T1-33 are direct spatial neighbours to T1-28 glomeruli; T1-24, T1-52 and T1-38 are not. Adapted from [23**].

highly conserved within the species *Apis mellifera*, and the functional organisation of the antennal lobe appears to be genetically pre-determined. (See also [24*], where a functional atlas is published for those glomeruli and odours mapped so far.)

The best-studied non-honeybee case in which physiological responses can be dissected into cellular components and mapped to a group of anatomically identified glomeruli is the macroglomerular complex of the heliotine moth *Heliothis virescens*. Here, four subcompartments respond to four pheromone components. Using electrophysiological recordings of receptor neurons followed by neuron tracing, the physiological input has been mapped for each of the four compartments [25]; similarly, the projection neuron responses have been recorded and mapped onto identified glomeruli [25,26]. The interesting finding here is that both the input and output of each glomerulus have the same odour response specificities. Thus, glomerular computation appears to be more closely related

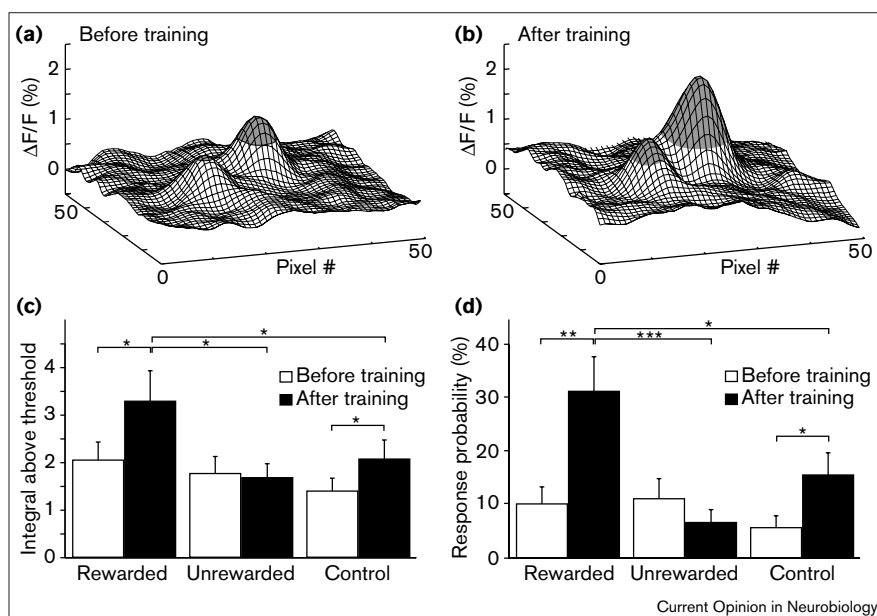
to temporal properties or signal-to-noise improvement than to chemical discrimination. However, this is not necessarily a universal picture: in noctuid moths, input and output of individual glomeruli seem to differ [27].

...and still there is plasticity

On top of the genetically determined response properties of glomeruli, there is an experience-dependent component. When animals were studied which learned a particular set of odours, odour representation in the antennal lobe of honeybees was found to be plastic [9**]. In particular, three odours were included in a fully balanced conditioning study. One odour was paired with a sugar reward (CS⁺), one was delivered to the antennae but not paired with a reward (CS⁻), and one was tested before and after the training period, but not presented to the animal during the training session (this control odour was used to test whether the learned changes were specific for CS⁺ and CS⁻, or whether they generalised to a different odour). The representations of the three odours were measured in the

Figure 2

Appetitive learning leads to an increased response in the antennal lobe. **(a)** Surface plot representing the activation pattern in response to an odour. Activity is measured as relative change of fluorescence ($\Delta F/F$). To clearly extract active glomeruli, a threshold is introduced at the top 25% of the signal range before training, and activity values above the threshold are shown in dark gray. **(b)** Response to the same odour in the same animal, but after that odour has been paired with a sugar reward five times. Note the increased area above threshold as compared to that in (a). **(c)** Statistical analysis of the results averaged over 20 animals: the response significantly increases for the rewarded odour, but not for the non-rewarded odour. The response to a control odour, which was not presented during the training phase, is also increased; this shows a generalisation effect from the trained to a non-trained odour. **(d)** Appetitive learning leads to the honeybee extending the proboscis in expectation of the sugar reward. In this experiment, proboscis extension was electrophysiologically monitored via the extensor muscle, and the same experiment was performed as in (c). Whereas the



unrewarded odour does not lead to any change in response, pairing with sugar-water leads to an increased response probability, and a control odour also leads

to an increased response. Thus, the behavioural data mimic response strength in the antennal lobe. From [9**].

antennal lobe before and after the experimental animals' training session. The learned odour significantly increased its response strength, as did the generalisation effect. The CS⁻ odour, however, showed no change in its elicited response (see Figure 2). The correlation between patterns elicited by the CS⁻ and the CS⁺ odours decreased as a consequence of learning.

Therefore, superimposed upon the genetically predefined odour representation, there is a plastic component reflecting the animal's associative experience. It is unclear whether the increased response leads to a lower odour detection threshold, and thus, possibly, to earlier odour detection in the field, and/or whether it decreases detection errors (which would be suggested by a decreased correlation between the response patterns). These alternatives are yet to be tested.

These results indicate the presence of an olfactory memory trace that exists alongside a similar such trace in the mushroom body – a notion suggested by several studies [8] and recently confirmed by pharmacological analysis [28]. It has yet to be determined whether the memory trace in the antennal lobe is a primary process of memory formation or whether it is established under the control of the mushroom bodies.

How is the olfactory code organised?

Molecular response profiles

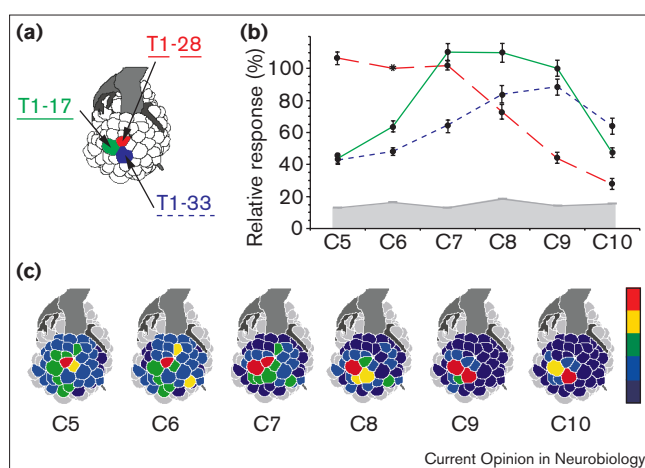
Relating olfactory responses to individual glomeruli, and averaging the results across animals, allows the measurement

of molecular response profiles of olfactory glomeruli. When testing aliphatic alcohols (both primary and secondary), ketones, aldehydes and alkanes with carbon chains varying in length from C5 to C10, one general property is apparent: whenever a glomerulus responds strongly to a chemical, it will also respond to a 'neighbouring' chemical with a carbon chain length of +1 or -1 [29**]. This fuzziness in glomerular response properties also applies to the functional group; for example, glomeruli preferentially responding to a particular range of alcohols will generally also respond to the corresponding ketones and aldehydes (alkanes generally elicit only weak responses in the investigated glomeruli, and do not fit into this description). However, the relative response magnitude is glomerulus-dependent; in other words, whereas one glomerulus has stronger selectivity for alcohols, another may be preferentially activated by ketones. Therefore, the olfactory system must compare activity in several glomeruli in order to identify an odour (cross-glomeruli code).

Broad response profiles to carbon chain length are reflected in honeybee behaviour: when the ability to distinguish between members of homologous series of aliphatic substances is tested, a significant negative correlation is found between discrimination performance and structural similarity of odorants in terms of differences in carbon chain length [10].

Spatial arrangement of olfactory glomeruli

Glomeruli with similar response profiles are often direct neighbours. Figure 3 shows responses in honeybee

Figure 3

Representation of aliphatic alcohols. **(a)** Schematic view of the honeybee antennal lobe, with the three glomeruli T1-28, T1-17 and T1-33 indicated. These three glomeruli are direct neighbours. **(b)** Responses of the T1-28, T1-17 and T1-33 glomeruli to a series of alcohols varying in carbon-chain length from C5 (1-pentanol) to C10 (1-decanol). Note that T1-28 responds most strongly to short-chain alcohols, T1-17 to intermediate chain lengths, and T1-33 to longer chain lengths. Each point represents the average of 14–21 individuals (error bars shown). Responses are shown relative to the response to hexanol in glomerulus T1-28 (asterisk). The shaded area indicates noise levels. **(c)** Spatial activity patterns elicited by the same six alcohols as those in (b). Red indicates strong activity and dark blue indicates low activity. Glomeruli that could not be measured are shown in gray. Note the continuous shift in activity between T1-28, T1-17 and T1-33 as the carbon-chain length increases. Also note that the response patterns are not limited to these neighbouring glomeruli. Adapted from [29**].

glomeruli T1-28, T1-17 and T1-33 to a homologous series of alcohols [29**]. These three glomeruli have overlapping response profiles, differing mainly in the carbon chain length of the most effective stimulus: T1-28 is most responsive to short chains, T1-17 to intermediate chains, and T1-33 to longer chain alcohols. Is there a functional reason for them being neighbours?

Inter-glomerular inhibition is most likely to occur between glomeruli with similar response profiles in order to sharpen their somewhat fuzzy response profiles. If glomeruli with similar responses were generally direct neighbours, it could be organised as a lateral inhibition mechanism. Indeed, lateral inhibition has been found between mitral cells in rabbit olfactory bulbs [30–32] where neighbouring mitral cells have similar response profiles. However, inter-glomerular inhibition must not be limited to direct neighbours for the following reasons. First, from the point of view of an interneuron, all glomeruli are approximately equidistant: the antennal lobe is spherical, with all glomeruli covering the outside of the sphere. Local interneurons — those neurons responsible for inter-glomerular computation — innervate several glomeruli; however, their neurites do not travel from one glomerulus to the neighbour, but rather from one glomerulus to the central neuropil and from there to other

glomeruli [33,34]. Second, not all glomeruli with similar response profiles are direct neighbours [29**]. Furthermore, the usefulness of lateral inhibition in an olfactory system has been challenged [35].

Alternatively, neighbourhood relationship could be merely a consequence of developmental constraints. In mammals, the receptor gene has an instructive role for the glomerular target recognition of receptor neuron axons [36]. Furthermore, axons from receptor neurons expressing similar receptor proteins terminate in adjacent glomeruli in the olfactory bulb [37]. Assuming that receptor proteins with highly homologous genes often have similar molecular response profiles, this finding would explain the neighbourhood relationships observed, and the spatial arrangement of olfactory glomeruli would be a more developmentally dominated than a functionally dictated organisation.

The two alternatives are not mutually exclusive. For example, the developmental constraints may have been a sort of pre-adaptation for efficient mutual inhibition.

Conclusions

Progress has been achieved in understanding olfactory coding in the bee through the development of techniques for mapping physiological activity onto morphologically identified glomeruli, using animals that are able to learn appetitive responses to an odour stimulus.

What is still missing is a better understanding of the separate steps involved in olfactory processing in the antennal lobe. Indeed, the major advantage of the studies reviewed here is, at the same time, their major drawback: as a result of the staining protocol, all antennal lobe cells are stained. Therefore, the measured activity is the integral activity of the entire glomerulus, and the relative contributions from receptor cells, local interneurons and projection neurons are unknown. The selective measurement of the spatial activity patterns in these neuron populations, hopefully under conditions in which their spiking behaviour can be monitored simultaneously, is an important goal for the future.

Part of the olfactory information may lie in the timing of action potentials [38*]. For example, when olfactory processing is disturbed by applying the GABA-antagonist picrotoxin (which also affects the temporal pattern of antennal lobe neuron firing, but may also affect the spatial representation of odours), similar odours are no longer distinguished by honeybees [15]. Many more experiments are needed to understand the relationship between temporal and spatial odour representation [15]. One difficulty lies in the paucity of electrophysiologically recorded cells whose innervated glomeruli have been identified, and which can therefore be used to correlate the observed spatial and temporal activity patterns. The atlas of the antennal lobe will help to establish, for each glomerulus, its olfactory response profile and its temporal response patterns both for the afferent input and the projection neurons. It will

then also be possible to evaluate more clearly which neural parameters change during olfactory learning.

Update

Following the completion of this review, King and co-workers [39*] have recorded the projection neurons from an identified glomerulus in female moths of *Manduca sexta* and shown that this glomerulus selectively responds to linalool, a common plant-produced odour. These findings support the idea that each glomerulus has a characteristic, limited molecular receptive range.

References and recommended reading

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In this study the authors related odour-evoked spatial activity patterns in the antennal lobe to the underlying morphological structure. This approach paves the way for creating a functional atlas of the antennal lobe (see also [24*], where this atlas is published for those glomeruli and odours mapped so far).
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 - at URL: <http://www.neurobiologie.fu-berlin.de/honeybeeALatlas>.

This site is constantly updated. Glomerular response patterns to a variety of odours are shown, together with examples of optical imaging recordings and three-dimensional reconstructions of the honeybee antennal lobe.
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Responses to five series of homologous aliphatic hydrocarbons are measured in honeybees. The results show that responses are fuzzy with respect to carbon-chain length and functional group, implying that only a pattern of glomerular activation is capable of accurately coding any of the tested odours. Furthermore, the arrangement of glomerular responses is not random with respect to their functional response: often neighbouring glomeruli have similar response profiles.
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