PATTERN RECOGNITION IN THE OLFACTORY SYSTEM OF THE LOCUST: PRIMING, GAIN CONTROL AND CODING ISSUES

Thesis by

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This thesis is dedicated to the memory of my grandfather, Ernesto Moos (1918-2001), whose courage and insight saved his family from the fate of his generation of European Jews, and in doing so gave me the gift of life.

To Eva

To my parents

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Abstract

Object recognition requires both specificity, to ensure that stimuli with distinct behavioral relevance are distinguished, and invariance, to ensure that different instances of the same stimulus are recognized as the same under varied conditions (intensity, pitch, position, …). Psychophysical studies show that an odor can be perceived as identical over significant ranges of concentrations. Whether concentration invariance results, at least in part, from low-level neural phenomena rather than cognitive grouping is so far unknown.

I explore, firstly, the contribution of projection neurons (PNs) in the antennal lobe of the locust, the analog of the vertebrate olfactory bulb, to the recognition of odor identity across concentrations; and secondly, what role spike timing, neuronal identity, and synchronization among neuronal assemblies play in the encoding and decoding of odor information by downstream neurons.

I show the following:

A novel computerized odor delivery system capable of delivering binary mixtures in arbitrary ratios and with arbitrary timecourses selected in real-time.

The locust can recognize odors, and shows innate olfactory preferences.

PNs solve the task of encoding both odorant concentration and odorant identity, independently of concentration, in three ways. First, by multiplexing information in different response dimensions using a code that involves neuronal identity, spike timing (on a timescale slower than previously believed) and synchronization across a neuronal assembly. Second, via a novel phenomenon of experience-dependent plasticity that contributes to PNs' invariance to concentration and sensitizes PNs after exposure to an odor at high concentration, contrary to the adaptation exhibited by receptors. Third, a phenomenon of gain control, whereby excitatory and inhibitory responses balance out massive changes in receptor activity as a function of odorant concentration, maintains the output of PNs within a small dynamic range.

A further mechanism of gain control contributing to keep the activity of early olfactory circuits relatively constant across the wide dynamic range of odorant concentrations in the air is the physical chemistry of odorant reception confers the olfactory system invariance to odorant volatility, a physical property that has hitherto been believed to play a fundamental role in an odorant's effectiveness.

Response patterns sometimes exhibit stable representations over large composition ranges and then abrupt transitions as a function of concentration and mixture composition, suggesting the difference between "same" and "different" odors may be delineated by sharp boundaries in odor space.

Finally, how is the distributed code for odors in PN assemblies decoded? I show that although synchronization among PN assemblies does not augment stimulus information in PN temporal responses, it is necessary for the read-out of odor information by downstream neurons.

In sum, early olfactory circuits appear to employ plasticity, gain control and temporal coding across synchronizing neuronal assemblies to solve the odor recognition problem across multiple concentrations.

Appendices show that the variability of PN responses is correlated across neurons, show how to produce non-cyclic Winnerless Competition (WLC) and a learning rule that causes random networks to self-organize into WLC, present an exact hypothesis test for binomial distributions, improvements to sliding-window cross-correlation and to the K-nearest neighbor classification algorithm, a combinatorial analysis of the connectivity between the locust antennal lobes and mushroom bodies, a didactic exposition of Victor and Purpura's spike cost-based metric and an experiment showing heterogeneity along the length of the locust antenna.

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Outline of the Thesis

There are three threads running throughout the thesis. The first is gain control in the early olfactory system. The second, which is the other side of the same coin, is the generation of invariance to natural variations in the stimuli the olfactory system encounters. The third is a functional analysis of the advantages derived from the coding scheme used by the early olfactory system, using the locust as a model system. Perhaps the three most salient features of the representation of odors in the antennal lobe are that it is dynamic, it exhibits oscillatory synchronization, and it is plastic. This thesis addresses functional roles for the three.

Chapter 1 offers an overview of our understanding of olfaction, ranging from the nature of the computational problem faced and the nature of olfactory stimuli to the nature of the information processing that I believe happens at every stage of the olfactory system. While grounded on a survey of the literature, my goal has been to challenge some conventional views in the field. This review has inherited my advisor's, Gilles Laurent, emphasis on the functional issues in coding and information processing. It aims to be self-contained and accessible to anyone with a minimal background in neurobiology. Wherever applicable, I have included comparisons with other sensory systems, and in particular with other chemical senses, to highlight the coding

and information processing principles at work.

Chapter 2 shows that absorption onto the olfactory mucosa inverts the effects of odorant volatility during evaporation into the atmosphere, making the concentration reaching olfactory receptors largely invariant to an odor's volatility for liquid odorants. I show that this effect provides a mechanism of gain control at the entry point of the olfactory system.

Chapter 3 shows for the first time that the American locust, the model organism employed throughout the rest of this thesis, exhibits olfactory-guided behavior.

Chapter 4 introduces an approach that will be used throughout the thesis: reconstructing the odor stimulus from a series of spike trains, by classifying each spike train as representing the stimulus class whose spike trains are on average most similar to it. Applying this approach for the first time to the olfactory system, I show that the information content of single projection neurons (PNs) in the antennal lobe of the locust is high. Chapter 4 also adds to work by Friedrich and Laurent (2001) by providing a second role for what the dynamic nature of the odor representation buys for the system.

Chapter 5 addresses the role of the second feature of the odor representation in the antennal lobe: oscillatory synchronization. It shows that spikes synchronized to the local field potential do not carry an intrinsically higher informational value, that desynchronized PN assemblies carry as much information as intact ones to an external viewer, but that synchronization of PN assemblies is nevertheless required for the correct readout of odor identity information by downstream neurons.

Chapter 6 presents a novel computer-controlled analog odor delivery system developed to carry out the experiments in Chapters 7 through 9. The system is capable of delivering virtually arbitrary concentrations and binary mixture ratios within two orders of magnitude for a variety of odor components, allows for real-time stimulus choice, shows linear behavior, has high reproducibility and allows for long-term stability in concentration. I show an application of the system showing for the first time that PNs can have abrupt response thresholds.

Chapter 7 refutes three hypotheses in the literature for how odor concentration is encoded in the antennal lobe, and presents an alternative favored by the data: that odor concentration is encoded in the degree of synchronization of PN assemblies to the local field potential, multiplexed with information about odor identity carried in PN slow temporal patterns. I also show that the phenomenon of odor-induced synchronization of PN assemblies is robust across a range of odor concentrations spanning at least two orders of magnitude.

Chapter 8 addresses three questions: 1) How are reports of monotonic increases in antennal lobe odor-evoked activity as a function of odor concentration to be reconciled with non-monotonic accounts of electrophysiological activity of mitral cells or PNs?; 2) How are odor-selective PNs made given the monotonic nature of odor receptors' dose-response functions?; and 3) How does the antennal lobe cope with the large dynamic range of its input? I show that a mechanism for gain control in the antennal lobe, given by the parallel concentration-dependency of excitation and inhibition, can provide the answers to all three.

Chapter 9 shows a novel form of plasticity, which I term priming, contributes to concentration invariance in the antennal lobe of the locust. I also introduce a novel statistical test, which I term the Distance Test, to test whether two series of spike trains are significantly different from each other.

Chapter 10 presents some general conclusions from the thesis.

Appendix 1 contains a presentation explaining the cost-based metric introduced by Victor and Purpura (1997) and used to compare spike trains in Chapters 4, 5, 8 and 9.

Appendix 2 contains a preliminary investigation of the encoding of mixtures of different composition ratios in the antennal lobe of the locust. It shows that the basin of attraction of the dynamic representations of different odors by PNs appear to exhibit abrupt borders in composition space.

Appendix 3 presents a brief investigation of whether olfactory receptors in the locust are homogeneously distributed throughout the antenna, and shows that PN response profiles vary systematically as the position of stimulation in the antenna is changed.

Appendix 4 has a combinatorial analysis of the connectivity between locust antennal lobe projection neurons and mushroom body Kenyon cells that suggests that either Kenyon cells require only few PN inputs to fire, or PN-KC connectivity is not uniform.

Appendix 5 introduces a novel exact hypothesis test to test whether two binomial distributions are significantly different.

Appendix 6 presents a novel classification technique closely related to k-nearest neighbor classification, which I term fractional k-nearest neighbor algorithm, to deal with classes of unequal sizes.

Appendix 7 shows a problem with previous implementations of sliding-window cross-correlograms, and provides a solution.

Appendix 8 shows that Winnerless Competition (WLC), a novel theoretical framework for dynamic attractors developed by Micha Rabinovich et al. (2001), does not require closed loops, contrary to early expectations (Laurent et al., 2001), and that the antisymmetric connection matrix required for WLC can be attained through a local, biologically inspired learning rule.

Appendix 9 shows that the trial-to-trial variability, or 'noise', in PNs' odor responses shows both positive and negative slow correlations across PNs, that these correlations vary for different pairs of PNs, that the correlations are present in some PN pairs in the absence of odor stimulations, and that exposure to an odor can trigger the correlations or turn them off in a stimulus-selective manner.

Collaborators

Chapter 3: Heather Dean and Mary Libby Mosier participated in the data collection and experimental design.

Chapter 4: Michael S. Wehr, Katrina MacLeod and Stijn Cassenaer provided some of the data used.

Chapter 5: Katrina MacLeod collected all of the data and carried out some of the analyses in the paper included at the end of the chapter.

Chapter 6: Yingzhong Tian helped in the construction of the odor-delivery system.

Appendix 2: These experiments were carried out jointly with Michael S. Wehr.

Appendix 3: Gilles Laurent initiated this line of analysis.

Appendix 6: Stijn Cassenaer made the key initial observation that led to the realization of the problem with earlier implementations.

Appendix 7: Valentin Zhigulin verified the two hypotheses raised.

Appendix 9: Michael S. Wehr provided the data used in this appendix.

CHAPTER 1

Olfaction and the Chemical Senses

1.1 Synopsis

This chapter presents an introduction to the thesis. My goal has not been to make this review exhaustive, but rather to place the thesis in a broad functional context, and expand on topics where I felt I had a contribution to make or those where substantial progress has been made recently. The interested reader is pointed to excellent reviews in Laurent (1996, 1997, 1999), Hansson (Ed.) (1999), Wehr (1999), MacLeod (1999) and Laurent et al. (2001).

I purposefully go back and forth between vertebrate and insect model systems, for the evidence suggests that both are strikingly analogous in structure and function, and furthermore, because I am primarily concerned with those common aspects which are fundamental to olfactory computations.

The chapter begins with an exposition of the virtues of the olfactory system as a model system for pattern recognition, followed by a description of the demands put on olfaction by behavior. I then compare the strategy evolved by the olfactory system with that of other chemosensory systems. This section includes a note on potential shortcomings of a widely cited theoretical method to estimate neural information and compute the optimal sparseness of neural representations. I then describe the stimulus space and compare it with that of other sensory systems. That leads to a discussion of the computational problems posed by the stimulus-output requirements. I then argue for the benefits of insects as a model system for olfaction, and provide a brief anatomy of the system.

I then proceed sequentially through the various stages of olfactory processing, from getting odorants to receptors and the nature of olfactory sampling, through olfactory receptors, the antennal lobe and olfactory bulb, the mushroom bodies and pyriform cortex and beyond, with particular emphasis on the processing leading up to and including the insect antennal lobe, the focus of the experimental findings of this thesis. At each stage, I focus on the computational role served by the underlying anatomy and physiology. Of particular interest may be a discussion of non-classical receptive fields

in olfaction and a proposed model for a larger role than has previously been suggested for activity-dependence in the establishment of the connectivity patterns between receptor neurons and glomeruli.

Toward the end, I discuss a little explored aspect of olfaction, that of stimulus variance across space and time, and propose a speculative novel hypothesis for the role of glomerular convergence of receptor neurons expressing the same receptor gene. I end with a discussion of functional differences between the immune and olfactory systems, intra-extra-body counterparts in chemodetection.

1.2 Olfaction as a model system for information processing and pattern recognition

This thesis presents a set of experiments aimed at uncovering a biological solution to the computational problem of information processing for pattern recognition. The choice of olfaction is not coincidental: it is one of the simplest general-use pattern recognition systems in the brain (Hendin et al., 1994) –there are, of course, biological pattern recognition systems outside the brain, and we will explore architectural differences between the olfactory and immune systems in §1.20. This relative simplicity stems from the nature of the olfactory task. In systems such as the visual and auditory systems, where the patterns to be recognized require complex computational processes of reconstruction to convert the raw data from the receptors into a construct that is invariant enough from one exposure to a stimulus to the next so that recognition may take place, recognition cannot occur until several synapses downstream of receptor neurons. To illustrate this, picture the visual system recognizing a given combination of pixels turning on; the probability of that combination repeating itself would be extremely small, since small changes in eye direction, position, angle or illumination would change which photoreceptors are active even if the object observed was exactly the same.

For that reason, visual object recognition operates not on specific combinations of individual pixels, but rather on complex constructs involving several layers of processing leading from pixels to lines on to phase-invariant lines and all the way to faces and other objects. Something similar is true for hearing, a sense used to recognize complex sequences of the single notes that are detected by individual hair cells in the ear. This means that, in both of these systems, it is difficult to address the questions of recognition before achieving a satisfactory understanding of the nature of the representations and how they are developed as signals make their way up the visual and auditory hierarchies. In olfaction, in contrast, the patterns to be detected are not complicated sequences, nor are they complex reconstructions far removed from the raw data gathered by receptors. On the contrary, the chemical signature of an odorant given by what receptor types it binds pretty much defines the pattern to be encoded and recognized. Memory areas are just two synapses downstream of olfactory receptors. This is not meant to belittle the recognition process —we will study at great length some of the difficulties inherent in obtaining invariance to the natural variability in the stimuli—, but rather that the recognition process is not far removed from the periphery and thus may be studied without the need to treat inputs as black boxes or wait until sensory physiologists 'work their way up there.'

1.3 Brief history of the beginnings of research in olfaction

Despite these advantages, research on olfaction has been rather slow in comparison to that on vision and hearing, modalities more crucial to survival of *Homo sapiens* (Vosshall et al., 2000). This cannot be attributed to lack of a good beginning, since Lord Adrian published a neurophysiological investigation of the olfactory tract of fish in 1938 (Adrian and Ludwig, 1938), a full 21 years before Hubel and Wiesel's pioneering paper on receptive fields of single neurons in the cat's striate cortex (Hubel and Wiesel, 1959). Adrian seems to have attracted few early followers, though: when he

published a paper on olfactory discrimination in 1951 (Adrian, 1951), the only reference cited was his own pioneering electrophysiological study of the olfactory bulb and pyriform cortex in the anesthetized hedgehog and the cat (Adrian, 1942). Before that, olfactory research appears to have been limited to anatomical and cytological descriptions (Golgi, 1875; Cajal, 1890; Retzius, 1892, 1897; Gray, 1924), and behavioral investigations. Adrian's ground-breaking studies paved the way for much of olfactory research in the following 60 years. He demonstrated the existence of oscillations in breathing animals and their dependence on odor stimulation (Adrian, 1942). Moreover, remarkably, he demonstrated the existence of spatiotemporal coding in the olfactory bulb, the clustering of these spatio-temporal responses according to chemical similarity of the odorants and the relative invariance of these patterns over a wide range of concentrations (Adrian, 1951). He further hypothesized that these temporal differences might be caused by varying rates of air flow in different regions of the olfactory epithelium or by differences in the solubility of different odorants in water, a prediction that remains untested to this day.

Despite the relative lack of data on olfaction compared to, say, vision[1], research in olfaction has undergone a boom in the last decade[2]. But this sensory system that was among the last to come to the attention of the bulk of the neurophysiological community (Adrian, 1942) has also been around the longest.

1. A search in ISI's SciSearch yields 1,399 papers for the term 'olfactory' for 2000, compared to 10,035 for 'visual.'

2. A search in ISI's SciSearch/ Web of Science yields 242 papers for 'olfaction' for 2000, compared to 167 in 1995 and 17 in 1990, a 14-fold increase in the 10-year period, a percentage gain 50% greater than that of papers with the keyword 'vision', for example, which experienced a 9-fold increase (3005-2139-321) in the same period –a control to ensure the effect was not due to non biological uses of the word 'vision' used the word 'visual' and found the same 9-fold ratio: 10,035/1,166 over the 10-year period.

1.4 Olfaction's place in evolution

For humans, who use vision as their primary window on the world and hearing as their primary mode of communication, olfaction is a rather little used sense, as demonstrated by the relatively normal lives led by so-called *anosmic* people —people who cannot smell. But for much of the animal kingdom, olfaction is the primary means of exploration and communication. Compared to vision, olfaction has the advantage that it works in the dark. Unlike hearing, it does not take moving parts to emanate an odor. It has a longer range than both touch and taste, both contact senses.

More importantly, olfaction evolved sooner than any other sense (Dethier, 1990), 500 million years ago (Hara, 1994). It is thus the most ubiquitous of all senses. There is good reason for this: olfaction requires the least amount of hardware. In its simplest form, it entails nothing more than the process of intra- and inter-cellular communication: a ligand binding a receptor. It thus constitutes a natural extension of such basic protein-protein interactions, if not a precursor of cell-to-cell communication altogether (Hölldobler and Wilson, 1994). It is therefore not surprising to learn that organisms all the way from prokaryotes to mammals, passing through nematodes and insects, all possess olfactory systems.

Biologists were recently surprised with the discovery that, in nematodes as well as in mammals, the family of olfactory receptor genes is large and may encode as many as 1000 seven transmembrane domains proteins (Buck and Axel, 1991; Levy et al., 1991; Ben-Arie et al., 1994; Troemel et al., 1995; Sengupta et al., 1996; Robertson, 1998; Zozulya et al, 2001) –a figure that puts it as using 3% of the mammalian genome and a full 5% of the worm genome (The Genome Sequencing Consortium, 1998). But, viewed in another light, that is still substantially less than the number of receptors used to detect messengers originating in the organism; given that living beings are chemical machines and that they survive by interacting with their surroundings, it is perhaps not surprising that a substantial fraction of the set of genes coding for receptors be devoted to detecting chemical

signals from the outside. We will see later, though (§1.14), that the strategy used by the olfactory system in the selection of receptors is very different from most other processes of chemical detection in the brain.

Possibly by virtue of forming part of the earliest sensory system, cells of the olfactory cortex send and receive information from more brain regions than any other sensory system (Gesteland, 1992). This underscores its importance, but also the potential for future exploration: many of these brain regions are not yet physiologically characterized for odor inputs. In particular, the psychophysical literature has confirmed the popular notion that odor-evoked memories tend to be highly emotional, vivid, specific, rare and relatively old (Herz and Cupchik, 1992, 1995). This leads to the natural question: what is olfaction used for?

1.5 Odor-mediated behavior

Any system is best understood if one knows what its function is. In biology, knowing the function of a system helps one keep in mind what one is trying to explain. The same is true of the olfactory system. This section presents a brief overview of the formidable tasks that are faced by the olfactory system, in the manner of a description of 'features' included, as well as a brief mention of a function commonly presumed present which does not appear to be among olfaction's capabilities.

Firstly, the olfactory system has three essential interrelated roles in feeding behavior: it must be able recognize nutritious foods, it must help recognize poisonous substances, and it must help the animal locate sources of attractive smells. Detection of food odors elicits stereotyped antennal scanning behavior in the cockroach, *Periplaneta americana*, a nocturnal scavenger. This olfactory scanning consists of circular antennal movements at ~1.5–7 Hz, after which both antennae point in the direction of the odor source for 1–2 seconds. The animal then orients toward and approached

the odor source (Chee-Ruiter and Laurent, 1995). We will discuss some of the cues that insects may use in locating an odor source in §1.10.3. The grasshopper, *Melanoplus sanguinipes*, is attracted to the odors of its host plants, wheat, ryegrass, sorghum and alfalfa (Hopkins and Young, 1990; see Hartlieb and Anderson, 1999, for a review on insect odor-mediated behaviors). Dogs are capable of detecting concentrations of certain nitroaromatic compounds as low as 500 parts per trillion (Williams et al., 1998).

Second, olfaction is used for navigation. Salmon, for example, are *anandromous* fish: they grow up in fresh water, migrate to feed in salt water, and then come back to mate in fresh water; the reverse can also happen: eels of the Atlantic Ocean are *catadromous*: they spend their lives in fresh and salt water but breed in the sea. Remarkably, although some straying occurs, most salmon surviving the ocean journey —even though separated from other salmon of their own stock—are able to find their own stream at about the same time as their cohorts, years after departure. Salmon often "test" rivers other than their own, entering the estuary or lower river and then retreating to the sea and moving farther along the coast (Steelquist, 1992). Sockeye salmon use a combination of visual and olfactory cues to return to their natal area (Ueda et al., 1998). Salmon are *imprinted* with the odor of their natal areas during a critical period in development (Morin et al., 1987). Astonishingly, dogs can correctly determine the direction of a trail of 20-minute-old footsteps within 3-5 seconds after encountering it and sniffing only 5 footsteps (Thesen et al., 1993). This remarkable skill has been speculated to employ concentration discrimination (Thesen et al., 1983), although this remains to be demonstrated. Ants encountering a trail leading from a nest to a food source midway cannot tell which direction is which initially, but can do so after walking for a short distance on the trail (Brun, 1914), and may use an odor gradient existing along the trail with the highest concentration near the nest (Bossert and Wilson, 1963, reviewed in Schöne, 1984). Animals may also use temporal and spatial fluctuations in odor intensity to derive information about the direction and distance to an odor source (Murlis et al, 1992; Mafra-Neto and Cardé, 1994; Gomez and Atema, 1996); see §1.10.3.

Feeding and navigation are just two examples of a more general capability: that of recognizing a chemical environment. The same skill can be used by a mother and her calf to recognize each other (Kallquist and Mossing, 1982) and for many other purposes. More importantly, the olfactory system is able to perform such recognition both innately (see Chapter 3; Simpson and White, 1990; Tabuchi et al., 1991; Matsumoto and Mizunami, 2000) and through learning (von Frisch, 1967).

In addition to recognizing a chemical environment, there is evidence that Tiger salamanders (*Ambystoma tigrinum*) generalize a conditioned response to olfactory cues chemically similar to the odor they were trained with (Rusell Mason and Stevens, 1981), suggesting that the olfactory system preserves some notion of chemical similarity between similar compounds. Experiments disrupting neuronal synchronization have also shown that the discrimination among chemically similar odors in bees is more labile to disruption than that among chemically distinct ones (Stopfer et al., 1997).

Animals also exhibit more complex olfactory-mediated behaviors, such as so-called olfactory scene analysis and the learning and identification of abstract relationships. For example, hamsters preferentially remember or value the top scent of a scent over-mark (Johnston and Borade, 1998). What cues do they use to do this? Johnston and Borade (1998) showed that overlap or apparent occlusion are necessary for hamsters to identify the top over-mark, suggesting that these mammals use regions of overlap and the spatial configuration of scents to evaluate over-marks. In a very elegant recent experiment, Giurfa, Srinivasan and colleagues (Giurfa et al., 2001), showed that bees can learn the concept of sameness or difference between two visual or olfactory stimuli and then transfer the learned association, between sameness or difference and a reward, across modalities.

There is behavioral evidence that the olfactory system solves what is known as the blind source separation problem: separating out odors originating at different sources. *Limax maximus*, a mollusk, can discriminate two food odors from separate sources separated by 1 cm (Hopfield and Gelperin, 1989). In humans, delays of 200 to 400 ms between two odors presented monorhinically or

dichorhinically (through one or two nostrils) elicited detection of components (Rouby and Holley, 1995). We will return to this in §1.13.

The capacity to identify components in mixtures of more than three components coming from the same source, in contrast, does not appear to be an ability of humans (Laing et al., 1983; Laing and Francis, 1989; Laska and Hudson, 1992) with or without attention deployed to individual components (Laing and Glemarec, 1992). The same is true of insects, except for some (Smith and Cobey, 1994), but not all (Smith, 1998), binary mixtures. In fact, the presence of one odor can sometimes mask or suppress the perception of a second one (Bell et al., 1987). The olfactory system is therefore not analogous to a gas chromatography or spectroscopy system whereby odors are separated into their components, but rather, odors in mixtures appear to generally blend to form a new odor with few of the characteristics of constituent odors. Whether this is due to the difficulty of the problem or the lack of sufficient adaptive value for such a skill, or both, remains an open question. I suspect there is adaptive value in identifying each blend as a distinct odor, multiplying the information content in the chemical signature of an environment in a combinatorial fashion, even when individual components may be common to many objects or environments. But this is not to say that both abilities, that of assigning a unique identity to a blend and that of identifying the ingredients in a mixture, could not have evolved in concert.

Lastly, the detection of chemical signals has a role in intra-species communications between individuals. That is what we turn our attention to in the next section, after a brief mention of the timescale of odor perception.

1.5.1 How fast is odor perception?

Laing and Macleod (1992) employed psychophysical procedures to determine human recognition

times to three matched intensity levels of the odorants n-butanol, (+)-limonene and propionic acid. A computer controlled the delivery of the odorants from an air dilution olfactometer and measured recognition times. The mean times recorded with the odorants were significantly different and ranged between 680–867 ms. Laing and his colleagues (1994) showed that a time separation of 400 msec between presentation of two odors from separate sources is sufficient to allow significant discrimination of the order in which they were presented. Claims that odors from separate sources presented simultaneously are perceived sequentially (Laing et al., 1994), however, have, I believe, proven unfounded. [3]

1.6 Pheromones, the accessory olfactory system and chemical communication

Once a system evolves the capacity to sense chemical signals from the environment, it is but a small step to evolve a system capable of sending its own chemical signals for detection by other

3. The correlation between order presented and order perceived found by Laing et al. (1994) may be due to the extreme time differences of 400 msec and not true for intermediate values. It is not surprising that presenting an odor almost half a sec before another yields two sequential percepts. Even if the correlation is significant for intermediate values, the odors were presented at different concentrations to generate suppression of one odor by another. That does not seem to be a good condition to test whether two equally perceptible components of a mixture are perceived sequentially. It is perhaps not surprising that a stronger odor is perceived earlier than a faint one, which is the second conclusion of the paper: that the suppressant odor is perceived first. If they wanted to test whether two odors presented at the same time are processed serially, they needed to test whether the order in which they are perceived is significantly different from random (50%-50%). They did not show this, although presumably they have the data. Even if it turned out to be significantly different, it would not be surprising unless the odor concentrations were matched for intensity, or better, for detection latency when presented alone.

individuals. The molecules used for such chemical communication take the name of *pheromones*, from the Greek *pherein,* to transfer, and *hormon,* to excite.

Pheromones have evolved in all animal phyla (Pantages and Dulac, 2000). Fish release an alarm pheromone when disturbed that causes other fish to flee (von Frisch, as cited in Agosta, 1992). The pheromone is carried in large alarm-system cells on the skin of the fish. These cells are fragile, and rupture upon injury, discharging the pheromone into the water. Simply scaring a fish will not discharge these cells, but damage to only a small area of the skin of a single fish is capable of causing fright in an entire school. Honeybees release an alarm pheromone too when disturbed and fan their wings to disperse the signal to their nest mates (Agosta, 1992). Male moths are sexually attracted by bombykol, a pheromone released by female moths. Other pheromones bear messages such as "the queen is in the hive and all is well," "produce more sex hormone," "we are under attack!" and "I am pregnant" (Agosta, 1992). Some species, such as ants and honeybees, use as many as thirty different pheromones to coordinate the activities of their complex communities. It is pheromones that guide ants along their trails, and the importance of chemical signals in the process can be easily demonstrated by crossing an ant's antennae, a procedure which will leave the ant confused and unable to follow the trail in its normal zigzag motion (Agosta, 1992). Bethe (1898, 1900) took an ant from one nest, deodorized it with alcohol and water, and then dipped it in a juice obtained by crushing the bodies of antes of another species. If the ant was placed in its own nest, it was immediately killed, but if placed in the nest of the ants whose odor it bore, it was accepted, although later, when the artificial odor wore off and the ant's own scent became apparent, it was sometimes attacked. Fabre showed that if a female Oak Eggar or Banded Monk moth was placed under a glass, males paid no attention to her, but went straight to a twig on which the female had previously perched at the other side of the room (Moncrieff, 1967, p. 340). He also reported that the smell of a serpent arum flower, which exhales a horrible stench of putrid flesh for two days, attracts hordes of insects, most of which will die engulfed in the capsule after hours of swarming and rolling, unable to resist

the flower's lure, even though they are not ovipositing or feeding, nor are they prisoners (Moncrieff, 1967, pp. 337-338). Other species, such as the *Temnochila chlorodia* beetle, are attracted to the pheromone of their prey. Yet other species imitate pheromones of others to their advantage: bolas spiders mimic moth sex attractant to capture moths (Eberhard et al., as cited in Agosta, 1992). Orchids of the genus *Ophrys* broadcast scents that imitate the sex pheromones of insects resembled by their flowers, hoaxing bees and wasps to attempt copulating with the flowers and impregnating them with pollen in the process (this is successful in part because the male bees emerge earlier than female bees, and the orchids are ready for pollination when there are many males and few females) (Agosta, 1992). Birds are believed to use pheromones too, but evidence for them remains incomplete.

In humans, Darwin showed more than a century ago that an infant with its eyes closed will turn toward its mother, and more recent experiments have shown that infants will display a preference for his or her mother over other mothers. There is also evidence for a chemical attractant that guides human sperm to the egg for fertilization, and about twenty different olfactory receptors have been discovered in sperm tissue (Parmentier et al., 1992, cited in Agosta, 1992). Axillary sweat from women synchronizes other women's menstrual cycles, and axillary sweat from men regularizes women's menstrual cycles. The cause of this striking phenomenon, initially observed in college dormitories by Martha K. McClintock at Harvard University (reviewed in Agosta, 1992), was disputed for a long time, until the chemosensory nature of the cues was pinpointed by an elegant experiment by George Preti and colleagues showing that sweat placed on the lips of women who had never seen or otherwise been in contact with the donors had an effect on the menstrual cycle of the recipient, while controls with no sweat had none (reviewed in Agosta, 1992). Two aromatic compounds also found in musk and civet are produced by bacteria in human sweat of both men and women — although their concentration is higher in males— and constitute the basis for much of today's perfume industry (Agosta, 1992).

Pheromones vary in chemical composition. Some of them are pure substances, while others are complex blends. In the aggregation pheromone system of the desert locust, *Schistocerca gregaria*, six different aromatic compounds that elicit electrophysiological activity in the olfactory epithelium have been identified. Similarly complex odors facilitate individual or group recognition in mammals, e.g., in territorial marking with urine or feces.

In many reptiles and all nonprimate mammals, pheromonal signals, carrying social and sexual information, are processed by the sensory cells in the vomeronasal organ (VNO) of the nose and their central connections in the brain. Together, they receive the name of *accessory olfactory system*. The accessory olfactory system is separate from the main olfactory system and differs from it both in physiology and function. Until recently, it was believed that in some primates, including man, the VNO made a transitory appearance during embryological development but disappeared before birth (Agosta, 1992). More recently, however, an examination of a large number of adult humans showed the VNO, also called Jacobson's organ, present in every one of them (Watson, 2000). The exploration of this most recently discovered human sense, which appears not to influence conscious perception but may well affect behavior subconsciously (Watson, 2000), has only just begun. In a report published this past summer, Savic et al. (2001) used PET to show that women smelling an androgen-like compound activate the hypothalamus, while men, in contrast, activate the hypothalamus when smelling an estrogen-like substance. A role for the hypothalamus in pheromonal processing appears consistent with the subconscious nature of human pheromonal perception (see also Sobel et al., 1999), and may help explain why its effects, such as the synchronization of menstrual cycles described above, appear so surprising to us.

Airborne odorant molecules cannot efficiently enter the dead-end passage containing the VNO. In snakes, the tongue delivers molecules collected from the air and nearby objects to ducts at the entrance to the VNO. In mammals, molecules are transported into the VNO by saliva.

Surgical ablation of the VNO in rodents has been shown to profoundly impair pheromone-induced behaviors such as mating and territorial defense and to perturb associated neuroendocrine responses, such as male testosterone surge and female oestrus cycle (Halpern, 1987; Wysocki, 1989). Pheromone signals ultimately result in activation of centers of the ventromedial hypothalamus involved in reproductive and aggressive responses (reviewed in Wysocki, 1989).

Mammalian VNO neurons use at least three different families of molecular receptors (Pantages and Dulac, 2000; Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997), each composed of 50–100 genes and unrelated to the family of receptors of the main olfactory system. Two of these families are related to each other and to the family of taste receptor genes encoding receptors for bitter tastants; the third is unrelated to the rest. VNO receptors' detection threshold for their corresponding pheromones can be remarkably low, near 10^{-11} M, placing these neurons among the most sensitive chemodetectors in mammals (Leinders-Zufall et al., 2000). VNO neurons show highly selective tuning properties and their tuning curves do not broaden with increasing concentrations of ligand, unlike those of receptor neurons in the main olfactory epithelium (Leinders-Zufall et al., 2000).

Why has such molecular diversity of vomeronasal receptors emerged? The recent analysis of VNO response to pheromonal stimuli directly demonstrates that natural sources of pheromones, such as urine, activate large subsets of sensory neurons (Holy et al., 2000). However, in sharp contrast to the combinatorial mode proposed for olfactory recognition in which specific odorants are recognized by multiple and overlapping populations of olfactory receptor neurons (Buck, 2000), individual pheromonal compounds seem to activate distinct subsets of VNO neurons (Leinders-Zufall et al., 2000). The lack of promiscuity in the VNO neuronal response implies that multiple subpopulations of VNO neurons function as independent chemosensors for many still uncharacterized pheromonal cues (Pantages and Dulac, 2000). Despite the fact that each pheromone activates a distinct subset of

neurons, though, each neuron expresses multiple receptor types (Martini et al., 2001). As we will see below, this is analogous to taste, where multiple receptor types, all binding compounds that are perceived as bitter, are expressed by the same neurons.

Before we concentrate on olfaction for the rest of the thesis, we stop to consider the two remaining chemical senses (§1.7 and §1.8), compare them to olfaction (§1.9) and then consider the nature of the inputs to the olfactory system (§1.10).

1.7 The trigeminal system in the olfactory epithelium

No treatise on olfaction would be complete without at least a brief mention of the fact that the olfactory epithelium contains another chemosensory system in the form of *trigeminal nerve receptors*. The fifth cranial or trigeminal nerve (which is the largest cranial nerve and carries the sensory nerves responsible for the face, teeth, mouth, most of the scalp, as well as the motor nerves of the muscles of mastication) provides a second set of nerve endings which are responsible for tactile, pressure, pain and temperature sensations in the areas of the mouth, eyes and nasal cavity. A number of chemical trigeminal stimulants produce effects described as hot, cold, tingling or irritating. For example, 'leavo-menthol' produces the trigeminal feeling of cold at moderate concentrations and 'hot' at high concentrations in the nasal cavity. This type of sensory description is often not just limited to the areas of the nose, mouth and eyes, but also occurs on skin areas not served by the 5th cranial nerve (especially, the genitalia) and thus such stimulants may affect a variety of nerve endings (Leffingwell, 1999). Similarly camphor, which possesses markedly more aroma than menthol, also produces the 'cold' sensation via interaction with trigeminal receptors. Other commonly encountered trigeminal stimulants include the chemicals allyl isothiocyanate (mustard, mustard oil), capsaicin (hot chili powder, mace spray) and diallyl sulfide (onion). The trigeminal sense is relatively

unexplored at present –a search in SciSearch for ‘trigeminal receptor’ yielded but 7 results, and one for trigeminal sense yielded none. We do know that about 70% of all odors are said to stimulate the trigeminal nerve, although, in general, the latter is several times less sensitive than olfactory receptors (Ohloff, 1994).

1.8 Taste

No review of the chemical senses would be complete without a mention of taste, yet another chemical sense that animals are endowed with, and one closely related to olfaction. Taste is the sensory system devoted primarily to a quality check of food to be ingested. Although aided by smell and visual inspection, the final recognition and selection relies on chemoreceptive events in the mouth. There is no life form known that neglects to check its intake using chemoreception (Lindemann, 2001, an excellent review of the topic, from which this section draws heavily). A human baby can already distinguish sweet and bitter and express pleasure for sweet taste but displeasure for bitter taste at only a few days old (Ganchrow et al., 1991). Taste research has seen notable advances in the last few years.

Already in worms, like the nematode *Caenorhabditis elegans*, different cells are involved in olfaction (the detection of airborne molecules) and taste (the detection of soluble attractants and repellants) (Pierce-Shimomura et al., 2001). In the fruitfly *Drosophila melanogaster*, for example, taste sensations are mediated by nerve cells whose sensory dendrites are contained in 'hairs' found on the body surface. Other taste neurons, found on the proboscis (also called the labellum or labial palps), but also the legs, anterior wing margins, and three discrete patches of sensilla in the gustatory tract within the head, express a family of 70 G-protein-coupled receptors (GPCRs) encoded by 62 genes named GR (Clyne et al., 2000; Scott et al., 2001; Dunipace et al., 2001; Robertson, 2001).

A surprising observation is that a subset (four so far) of the gustatory receptor genes examined are expressed in a subset of olfactory receptor neurons in the antennae, the primary olfactory organ in *Drosophila* (Robertson, 2001). These neurons were not previously identified as expressing any of the odorant receptors (Voshall et al, 2000). The neurons expressing one of these genes project axons to a pair of glomeruli in the antennal lobe, rather than to the suboesophageal ganglion (Scott et al., 2001). Thus, they apparently behave as additional odorant receptors (Robertson, 2001).

Vertebrate taste receptor cells are not neurons

In contrast, the taste receptor cells of vertebrates are not neurons, but originate from the epithelial covering of the body (Stone et al., 1995). Vertebrate taste cells are small bipolar cells. To connect to the oral space, they send a thin dendritic process to the epithelial surface. The cells occur either singly or densely packed in taste buds, where up to 100 form a functional unit. Although taste buds also occur abundantly on the body surface and barbels of some fish, all vertebrates have taste buds in the oral epithelium, typically on tongue, palate and pharynx. The marker molecule gustducin, a taste-specific G protein (MacLaughlin et al., 1992), shows additional 'taste cells' in the nasal mucosa (Zancanaro et al., 1999) and in the stomach (Höfer et al., 1996). Each chemoreceptive area of the human tongue responds to each of the qualities of sweet, sour, salty and bitter taste. Only minor differences in subjective thresholds were noted across area (Hänig, 1901; Lindemann, 1999).

A single taste receptor cell expresses many taste receptor genes

Two families of G-coupled transmembrane proteins have recently been identified as mammalian taste receptors (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000). One of

them has two receptors, two of which, T1R2 and T1R3, have been shown, using a heterologous expression system, to combine to function as a sweet receptor, recognizing sweet-tasting molecules as diverse as sucrose, saccharin, dulcin, and acesulfame-K (Nelson et al., 2001). The other family, T2R, codes for 40-80 receptors with different molecular specificities but all expressed in the same group of receptor cells: those responsible for bitter taste detection (Adler et al., 2000; Chandrashekar et al., 2000). Each of the bitter taste receptor cells expresses more than one type of (but not all) T2Rs (Adler et al., 2000). Calcium imaging of taste bud neurons confirmed that only a subset of bitter taste neurons respond to any particular chemical (Caicedo and Roper, 2001). The detection threshold for sugars is roughly 0.1 M, more than five orders of magnitude greater than observed for bitter compounds (Lewcock and Reed, 2001).

The practical consequences of recent efforts to understand the taste receptors are considerable. Based on binding-site structure, advanced techniques of drug design are expected to allow the construction of taste ligands that activate or inhibit a receptor protein, thereby enhancing or inhibiting a specific taste. Thus it might become possible to expand the already huge commercial market for artificial sweeteners into other taste qualities. This would be beneficial in many ways. For example, aged people often have a general decline of taste function (Stevens et al., 1995) and need taste enhancement to once again enjoy their food. And an organic enhancer of sodium taste would be a great help for patients on a low-sodium diet (Lindemann, 2001).

Recordings from the sensory nerve fibers and from the soma of their neurons have consistently revealed that some nerve fibers are specialists, but many are generalists, carrying responses to more than one taste quality (Lundy and Contreras, 1999). A simple 'labeled line' design, where each fiber responds to just one of the qualities, to bitter only or to sour only, is not evident, as many fiber are broadly tuned with respect to taste ligands. These generalist fibers carry responses to salty and sour, to glutamate and sucrose, and so on. Similarly, many taste receptor cells, too, are generalists,

as responses to taste qualities are randomly and independently distributed, varying in intensity across cells (Gilbertson et al., 2001). Given such distributed responses, a part of the information about individual tastants must be buried in the quorum of the receptor cells and the 'across-fiber pattern' of the sensory nerve (Erickson, 2000). All of these properties in the organization of the information processing are strikingly reminiscent of the olfactory system, and are particularly noteworthy given the large differences in the structures used in both systems. We will encounter such an evolutionary convergence once again when we discuss the olfactory systems of evolutionarily distant phyla. The broad tuning of olfactory and taste receptors has been claimed to be optimal: Zhang and Sejnowski (1999, but see [4-5]) maintain that for stimuli space of dimensionality three or higher, more information per neuron (although less information per spike) can be coded by broader tuning and thus lead to maximal resolution using optimal decoding.

The bipolar taste cells have two obviously important specializations: microvilli in contact with the oral cavity and synapses with sensory nerve fibers. Taste receptor proteins are mounted on the microvilli, acting as molecular antennas listening into the chemical environment. On binding taste molecules, taste cells fire action potentials, by means of voltage-gated Na^+, K^+ and Ca^{2+} channels (Avenet and Lindemann, 1987; Roper, 1993; Lindemann, 1996). A local increase in Ca^{2+} concentration is needed for synaptic activation (and hence nerve excitation), and transient rises in the cytosolic Ca^{2+} concentration were observed by fluorescence imaging in taste cells responding to bitter and sweet agents (Akabas et al., 1988), while amino acids triggered either increases or decreases of the Ca^{2+} signal (Zviman et al., 1996; Hayashi et al., 1996). In turn, this process activates synapses and thus causes excitation of the nerve fibers. These carry the signal to the brain stem, where central taste processing begins (Lindemann, 2001).

A number of transmitters have been found within taste buds, but those released by taste cell synapses have been difficult to identify. Noradrenaline and acetylcholine seem to be secreted by nerve

fibers and modulate the responses of taste cells (Herness et al., 1999). Serotonin is thought to act as a paracrine agent between taste cells. Secreted by one cell and modulating the taste response of a neighboring cell, this agent mediates local signal processing within a taste bud (Delay et al., 1997; Herness and Chen, 1997). Glutamate is a strong candidate for a mainstream afferent transmitter secreted by taste cell synapses (Caicedo et al., 2000; Lawton et al., 2000).

Drosophila taste receptor neurons show axonal targeting to stereotypically different regions of the suboesophageal ganglion in larvae and adults, although these brain targets are rather diffuse, pos-

4. There are a number of caveats to note regarding Zhang and Sejnowski's (1999) result. By their use of Fisher information, Zhang and Sejnowski assume that the mean firing rate is a continuous and differentiable function of the encoded stimulus. But this is not necessarily the case. In fact, the responses of retinal ganglion cells, for example, are not properly described by a firing probability that varies continuously with the stimulus. Instead, these neurons elicit discrete firing events that may be the fundamental coding symbols in retinal spike trains (Berry et al., 1997; see also Wehr et al., 1996 for a similar demonstration in the olfactory system). The difficulties of defining a continuous stimulus space are most clearly evident in olfaction. The fact that tuning curves may not be continuous or even well defined, though, renders the method of Zhang and Sejnowski unusable, but does not make the question meaningless: sparseness is an important coding parameter of any representation, independently of whether the stimulus space and the firing rates are continuous or not (see, for example, Pérez-Orive et al., in press).

As Zhang and Sejnowski note, their method applies only to optimal estimation algorithms, for uncorrelated neurons or neurons with weakly correlated noise, and for radially symmetrical tuning curves.

Furthermore, for dimensionality greater than 2 and large tuning widths, Zhang and Sejnowski's result has limited usefulness: it places a lower bound on the error that gets asymptotically close to zero —a lower bound to begin with.

Moreover, their method is only valid for tuning widths that are small relative to the size of the stimulus space. This unstated restriction is most evident in the limit of infinite tuning width: Zhang and Sejnowski's bound for the error is lowest for this case, and yet in reality error rates are at their maximum for that case, for an infinitely wide tuning curve provides zero discriminability. The method is thus unable to estimate optimal tuning widths for stimulus dimensionality greater than 2 (continues in 5)

sibly overlapping, and not nearly as discrete as the glomeruli of the antennal lobe (Robertson, 2001).

1.8.1 Why do vertebrates possess separate gustatory and olfactory systems?

If the difference between taste and olfaction in vertebrates were, as in *C. elegans*, that the former detects soluble molecules and the latter detects airborne ones (Pierce-Shimomura et al., 2001), then how does one explain the fact that fish have both? For this reason, vertebrate olfaction is defined as chemical information transmitted to the central nervous system (CNS) by neurons through cranial nerve I, while chemical information detected by specialized epithelial cells and transmitted to the CNS by cranial nerve VII (facial), IX (glosopharyngeal), or X (vagal) is termed gustation

5. The Cramer-Rao lower bound on the mean squared error of estimation used by Zhang and Sejnowski bounds the error rate *given the amount of information present in the encoding variable chosen*. But nothing guarantees that Zhang and Sejnowski's choice, neuronal firing rates during a time window Tau, are the optimal encoding variable or the one used by the brain. With 1 or few spikes per perceptual event per neuron, mean firing rate may constitute a comparatively poor source of information. Perhaps spike timing, an analog quantity, is a better way to go to optimize estimation.

Zhang and Sejnowski's theoretical results suggest that the accuracy of a 2-D code should be unaffected by the width of the tuning curves. Nevertheless, multiple parallel maps, exhibiting neuronal tuning with different widths, are universal in sensory systems, even when they do not exist at the sensory periphery (Konishi, 1986; Lewis and Maler, 2001). Maps with greater tuning widths have been found to result in equal accuracies of estimation for some parameters, greater accuracies for others and smaller accuracies for others still (Lewis and Maler, 2001).

Finally, it must also be noted that although Zhang and Sejnowski suggested the Fisher information per neuron increases with increasing tuning width for stimulus dimensionalities greater than 2, they showed that Fisher information per spike always decreases with increasing tuning width. If energetic considerations prevail, the latter could be more relevant.

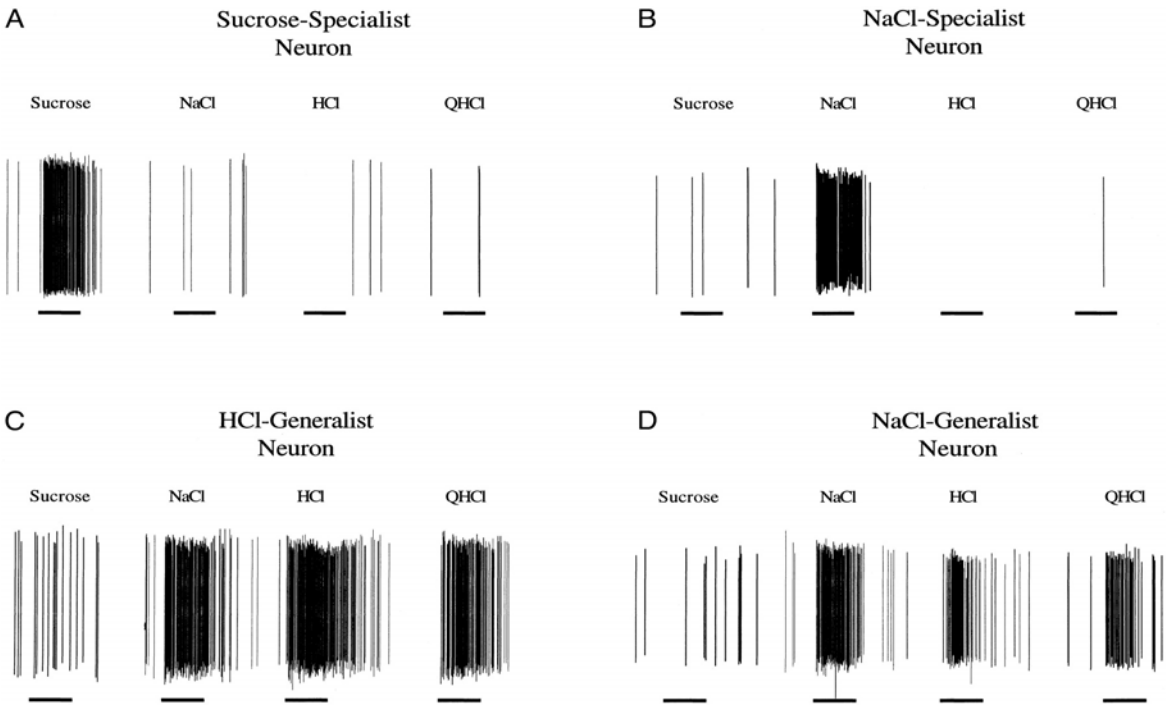


Figure 1.1. Specialist and generalist coding in taste neurons. Each neuron was tested for its sensitivity to 4 chemicals: 0.5 M sucrose, 0.1 M NaCl, 0.01 M HCl, and 0.02 M QHCl. The solid black bar below each spike record represents the duration of stimulus application (15 s) (reproduced from Lundy and Contreras, 1999).

or taste (Hara, 1994). While this makes the definition unambiguous, it leaves unanswered the question of why two separate and apparently nonhomologous systems evolved. If fish were the descendants of land vertebrates, it could be hypothesized that olfaction originally evolved for the detection of volatiles in land animals, and taste evolved for the detection of soluble molecules, and that olfaction was later adapted to detect soluble molecules in fish. But our current understanding of vertebrate evolution maintains that the original vertebrate precursors were aquatic (Encyclopaedia Brittannica Online, 2001).

A second possibility is that gustation evolved to sense ingested molecules, while olfaction evolved to sense the surroundings. There are two problems with this hypothesis. The first is that external taste buds are common in fish: the yellow bullhead (*Ictalurus natalis*), for example, has taste buds

on its entire body surface (Hara, 1994). This problem could be dismissed if this was a secondary adaptation not present in the original vertebrates. The second problem, though, is that even if their locations in the body are different, it is not clear why the same original system could not be co-opted to a different location.

Interestingly, in *Drosophila*, the 33 amino acid signature motif characteristic of the GR gustatory gene family is present but somewhat diverged in 33 of the 60 members of the family of *Drosophila* odorant receptor (DOR) genes. The DOR genes, however, possess additional conserved motifs not present in the GR genes and define a distinct family (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999, 2000). Indeed, the gustatory receptors are an extraordinarily divergent family, of which the odorant receptors are in fact just a single branch among many (Robertson, 2001). This great divergence hints at great antiquity, and indeed five genes have been found that form three lineages within the gustatory family in the nematode *Caenorhabditis elegans* genome, indicating that the superfamily predates the nematode—arthropod divergence (Robertson, 2001). These observations suggest that the putative gustatory and olfactory receptor gene families may have evolved from a common ancestral gene (Scott et al., 2001). Consistent with a common origin, in insects, both types of receptors may be found side-by-side, not localized to different organs as in vertebrates (Schneider, 1963).

In agreement with the large size of the family of taste receptor genes, in fish, taste appears to respond to a wide spectrum of compounds (Kotrschal, 2000). So the size of the stimulus space is unlikely to be the critical distinction between olfaction and taste. In contrast, the difference between them appears to lie in their behavioral outputs. Whereas stimulation of the taste systems alone triggers reflexes, complex, conditional or conditioned behaviors occur only when the olfactory system is intact (Kotrschal, 2000). Thus, our responses to different tastes are to a large degree hardwired — thus the ease with which terms adapted from taste, such as sour and sweet, are co-opted for other

meanings, meaning which are constant across cultural barriers. Descriptions of odors, on the other hand, have a much greater cultural or experience-dependent component, and are therefore impractical as descriptors outside their specific realm.

Finally, olfaction appears to work at low thresholds, designed for remote sensing, while taste appears to operate with higher thresholds and designed mainly for close-distance discrimination (Kotrschal, 2000).

1.8.2 Interactions between olfaction and taste

There are several senses in the mouth. Thermal, touch and pain sensations in the mouth are unambiguous. Olfactory stimuli, however, can have two different origins: orthonasal, when sniffed, or retronasal, to the nasal cavity from the mouth through chewing and swallowing. Our brains interpret these as smell or flavor depending on whether chemosensation is accompanied by touch sensations in the nose, caused by sniffing, or by touch sensations in the mouth, caused by eating or drinking, respectively (Bartoshuk and Beauchamp, 1994). Retronasal olfactory sensations can be localized to the mouth by providing a tactile cue in the mouth: if a tube leading to a chocolate reservoir is placed in the mouth of a subject who then chewed on tasteless odorless gum, the subject perceived the gum to become chocolate when the odorant is turned on (Bartoshuk and Beauchamp, 1994).

1.9 On the nature of the diverse chemical senses

1.9.1 The practicality of labeled lines when the behavioral significance of stimuli is fixed

In essence, VNO neurons, insect neurons that respond to pheromones and project to the macroglomerular complex (MGC), and specialist taste receptors appear to function as labeled lines, each neuron's activity carrying the signal of one particular signal having been detected. Their properties are hard-wired and very different from those of the distributed codes that the main olfactory system employs, and so we will not consider the accessory olfactory system for most of this thesis. This hard-wired character and specificity of receptors is made possible by two factors. First, the specificity and constancy of the signals to be detected has given the pheromonal system, for example, the time to evolve receptor molecules extremely sensitive and exquisitely tuned to the corresponding pheromones. Second, the fixed behavioral meaning of pheromonal signals, as well as the relatively constant biological value of food substances, has allowed taste and the VNO to establish hard-wired connectivity patterns between the periphery and central brain structures. When biology had a limited set of molecules to map onto a set of limited behavioral outcomes, as in the case of pheromonal signals or the five primary tastes, it has favored a labeled line design, with each receptor cell type signaling a distinct behavioral message –not necessarily a unique chemical identity, as witnessed by the large variety of molecules that taste bitter and activate the same group of receptor neurons.

Of course, the simplicity of such a design means that if the meaning of a stimulus changes, evolution must change the system. Indeed, even in closely related species, distinct differences in taste sensory performance may be noted, which seem to match the nutritional 'needs' of a species. Receptor specificity appears to have changed in evolution with the availability of food ingredients (Lindemann, 2001). Such an evolutionary fine-tuning, I argue, may have been necessary due to the relative lack of plasticity of taste compared to olfaction.

1.9.2 The synthetic nature of olfaction?

The main olfactory system, however, cannot afford that luxury: it must be able to recognize *any* odorant that experience may bring upon it, under a variety of conditions and *with any possible behavioral significance*. Much has been said about the former, to the point that this character of olfaction has received a name: its *synthetic* nature, the name given to the presumed need for olfaction to recognize essentially any combination of odorants (Laurent, 1999). This presumed ability awaits experimental demonstration, though (Laurent, 1999); it is unclear to me that we are able to distinguish between the smells of any arbitrary number of different piles of garbage, to use a possible olfactory analog of visual random dot patterns. In fact, electroantennogram recordings paired with gas chromatography (GC), gas chromatography-mass spectroscopy (GC-MS) and a coupled gas chromatograph-electroantennographic detector (GC-EAD) suggest that the olfactory system of the locust responds to only a small fraction of the compounds present in plant volatiles (Njagi and Torto, 1996). The poor ability of human subjects to discriminate among related odor mixtures is even more notorious (Laska and Hudson, 1992). Perhaps it is simply our lack of reliance on olfaction in our daily lives that masks what would otherwise be obvious shortcomings in our ability to discriminate compounds using our noses.

1.9.3 The plastic nature of olfaction

The flexibility of the olfactory system as its hallmark trait, though, has gone comparatively unnoticed. Just as much as the large number of potential odorants, it is precisely the fact that olfactory stimuli have no intrinsic biological meaning (Mombaerts, 1999b) and thus the plastic nature of the association between olfactory stimuli and behavioral significance, as evidenced by the prevalence of olfac-

tory learning (von Frisch, 1967), that makes a labeled line approach, where each neuron may group many signals *with the same behavioral significance* together, like bitter taste neurons do, impractical. How could the system decide which two signals to group together under the same labeled line (which receptors to express in the same neuron, for example) if the significance of each of the two is subject to plasticity, and thus could end up being the same or different depending on experience? In this, the olfactory system shares a generalist task with its visual and auditory counterparts –although in those systems, too, specialist subsystems exist for the detection of specific behaviorally relevant stimuli, such as bug detectors in frogs (Marr, 1970).

We turn our attention to the computational problem faced by the generalist olfactory system in §1.11. Before that, we take a look at the inputs that olfactory systems count with in order to face the formidable challenges posed by their task.

1.10 What constitutes an odorant?

1.10.1 The stimulus space

Odorants are small, generally volatile compounds with molecular weights less than 300 Daltons. The number of different odorant molecules has been guessed to be over 400,000 (Mori and Yoshihara, 1995) —I am aware of no rigorous quantification of this figure. Yet most odors in the natural world are complex blends of these compounds. Each odor, whether monomolecular or a blend, generally evokes a singular percept, leading to an astronomical number of possible smells.

Not all small volatile compounds evoke an odor percept. In order to elicit one, a compound must be able to traverse an aqueous interface (see §1.13) and bind one or more olfactory receptors with a detectable affinity. Thus the existence of odorless small compounds, of which water is perhaps the

most obvious example.

1.10.2 Smells versus images and sounds

Much has been said to the effect that, unlike vision and hearing, olfaction's stimulus space is highly multi-dimensional (Weyerstahl, 1994). What do we mean by the dimensionality of the stimulus space? This is a question more complex than would appear at first sight, so we will build up in complexity gradually. Firstly, a distinction must be drawn between the dimensionality of the stimulus space, independent of the biology, and the dimensionality of the first neural representation of the stimuli. Take color vision, for example: the color of *one isolated monochromatic ray* of light can be uniquely defined by a single number, the frequency of the light. Thus, the dimensionality of the physical color space for *a single ray* is two: frequency and intensity or power. In reality, though, any given point in a visual scene can reflect multiple wavelengths. This means that in reality, the physical stimulus space is much higher dimensional: for each 'pixel', the stimulus space has as many dimensions as quantized levels of light there are in the visible spectrum, since each point in an image is characterized by an intensity for each of those frequencies. To sense color, though, the visual system of humans employs four types of photoreceptors —three cones and one rod—, the activation of each of which is independent from the other due to their different absorption-wavelength functions. This means that only four numbers of information are captured by the visual system about any point in the visual field, thus the input neural representation *of each pixel*[6] in the visual system is four-dimensional. Of these, one is interpreted as luminance or brightness, which leaves our visual system with

6. In reality, the maps of different types of photoreceptors have different tiling densities. Thus, these maps do not have equal spatiotemporal resolutions: the luminance channel's acuity is by far superior to those of the other two (see 6). This is reflected in our behavioral abilities: we have a much lower threshold for detecting lack of focus in luminance than in color (Wandell, 1995).

three dimensions for color[7,8].

In olfaction, in contrast, taking into account a single antenna or nostril, each stimulus is given by a vector in n-dimensional space, where n is at least the number of olfactory receptor types, and possibly the number of receptor neurons if differences in the activation of similar receptor neurons in different locations of the epithelium are preserved downstream of the epithelium (see *Spatial codes in olfaction?*, below). With the number of receptor genes currently estimated at 86 for rats, over 150 for humans (Mombaerts, 1999) and 57 for Drosophila (Vosshall et al, 2000), the number of receptor types is thus substantially larger in olfaction than in vision, and thus olfaction's stimulus space is considerably larger than trichromatic color space. Why, then, does olfaction employ so many more receptors than color vision? Part of the answer lies in the fact that the chemical universe of molecules is nowhere as tidy and linear as the physical universe of electromagnetic radiation: while the color of a monochromatic ray of light can be described by a single number, that of its frequency, describing the chemical structure of a single molecule is more complicated. Furthermore, while the light absorption properties of photoreceptors allow for a complete specification of a visible wavelength by the relative photon absorption levels of two to four (Land, 1977) receptor types with overlapping wavelength-absorption curves, no three receptors are known whose relative affinities for different odorants uniquely specify any odorant.

But is the comparison between the number of molecular photoreceptor types and the number of molecular olfactory receptors a good reflection of the size of the stimulus space for vision and olfac-

7. All four photoreceptor types are employed for a range of brightness levels we call mesoscopic vision. Under bright illumination conditions, only the three cone types contribute to the signal. In scotopic (dark) conditions, only the rods are sensitive enough to respond (Wandell, 1995).

8. The visual representation, of course, changes throughout visual processing. The present description applies to the initial signal in the retina. Downstream, light signals get converted into two color-opponent maps and one luminance map (Wandell, 1995).

tion? I will argue it is not. It is certainly true that olfaction's stimulus space has a higher dimension than *color* space: a color perceived by a human can be completely specified with three numbers, while an odor perceived by one would presumably require ~150. Color space plus luminance is a valid description of visual space for systems that do not form an image, i.e., for the vision associated with the luminance and/or chromatic information in a single point in space, such as that associated with a simple ocellus or ommatidium. But we do not recognize a face by analyzing the color of a single pixel. Rather, visual recognition is the process of identifying a pattern in an image. For each eye, an image is the specification of color and luminance for each location in the retinal surface, or the activities of 5 million cones and 100 million rods (Wandell, 1995). Even if we restrict ourselves to the number of retino-ganglion axons leaving through the optic nerve, there are 1.5 million of those (Wandell, 1995). So the input space of visual cortex is closer to 1.5 million-dimensional than it is to three-dimensional.

The computational nature of any spatial olfactory maps

Olfaction, on the contrary, has many receptor types, but exhibits a very important difference with respect to vision: as far as we know, noses do not form olfactory images (but see §1.19.2). In other words, because of the nature of the transmission of odorants, the spatial distribution of odorant on the olfactory epithelium is not *directly* related to the location of odor sources in the outside world, in contrast with the way in which photons on the retina are directly informative about the location of the objects they come from. As a consequence, the spatial map in the olfactory epithelium does not seem to be preserved in the next layer of olfactory processing. Rather, as we will see in more detail in §1.15.1, a process of remarkable convergence takes place that collects the signals from the all the olfactory receptor neurons expressing the same olfactory receptor type in the same pair of glomeruli in the olfactory bulb (antennal lobe in insects). This means that the dimensionality of olfactory

space is much smaller than that of visual space: if there is no spatial map of receptors, the dimensionality of the output of the olfactory epithelium is only twice the number of olfactory receptor genes (once for each nostril or antenna). In this sense, olfaction appears more similar to hearing than to vision, in that no spatial map of the input is present in the periphery, and any spatial map must be computationally reconstructed from the comparison of the signals arriving in each hemisphere or from a temporal reconstruction of signals collected at different places or different times.

1.10.3 Stimulus dynamics: The nature of odor plumes

So far, we have described olfactory stimuli in any one moment in time. But olfactory systems do not operate on single moments in time, but rather on a time continuum. In most olfactory environments, the simple diffusion of odorant molecules is a negligible means of dispersing odorants (Hopfield, 1991; Murlis et al., 1992). Odors of distant objects are brought to the nose by wind (Hopfield, 1991). Odorant molecules leaving an object follow the path of the air packet into which they evaporate (Hopfield, 1991). This packet already contains odors from upwind objects. The packet will slowly mix with odors from nearby packets, due to microturbulence in the air (Hopfield, 1991). As a result, the odor plume is increasingly mixed with odors from other parts of the environment as time increases. Thus, the stimulus at the nose due to distant objects contains mixtures of odors from many sources, whose relative contributions are constantly changing (Hopfield, 1991). A passive detector placed away from a source experiences intermittent odor pulses lasting from a few milliseconds to more than a second, with interpulse intervals between several 100 ms and minutes (Laurent, 1999). What useful information is there in these temporal fluctuations? Moore and colleagues have shown they carry information on the size, direction and distance of the odor source (Moore et al., 1989; Murlis et al., 1992). The intensity of the odor of a nearby object varies strongly as the wind direction changes. A distant object, in contrast, has an odor plume that is more contorted, broader and weaker, and its

strength will vary less with fluctuations in the wind direction (Hopfield, 1991). The relative timescale of variation also contains information about distance (Hopfield, 1991). Jelle Atema's group at the Boston University Marine Program in Woods Hole, MA, further showed that these fluctuations carry information about upstream obstacles to flow in aquatic environments (Dittmer et al., 1996), and extended these one-sensor results through the identification and statistical analysis of dispersal patterns in a turbulent odor plume using a pair of sensors separated by the 3 cm distance of lobster lateral antennules (Grasso, Basil and Atema, personal communication).

Do animals *use* the information in the temporal fluctuations in olfactory stimuli, though? There is behavioral evidence to suggest they do (reviewed by Murlis et al, 1992): moths flying upwind to a pheromone source will fly faster and straighter upwind, and locate sources more frequently if the plumes are either turbulent or mechanically pulsed than if they are continuous and narrow (Mafra-Neto and Cardé, 1994). Furthermore, lobster chemoreceptor cells show maximum stimulus intensity discrimination when stimulated with odor steps of ~200 milliseconds and showed clear responses at even the shortest pulse durations used (50 msec), demonstrating that they resolve odor peak onsets within the time window corresponding to the 4-5 Hz frequency of olfactory sampling as well as the rapid fluctuation in odor concentration common in natural odor plumes (Gomez and Atema, 1996). Under repetitive stimulation conditions in an aquatic environment, flicker-fusion frequency (that at which two pulses become indistinguishable from one) and synchronization with the stimulus pulse train were concentration dependent: performance rates above 1 Hz became poorer both with increasing pulse amplitude and frequency (Gomez et al., 1999). Flicker fusion frequency was 3 Hz for 100 mmol/l pulses and 2 Hz for 1000 mmol/l pulses. Individual cells showed differences in their stimulus pulse following capabilities (Gomez et al., 1999). These individual differences may form a basis for coding temporal features of an odor plume in an across-fiber pattern (Gomez et al., 1999). Temporal resolution is substantially better in a land animal: cockroach olfactory sensory neurons are able reliably to follow 25 ms pulses of the pure odorant 1-hexanol and 50 ms pulses of the complex

odor blend coconut oil (Lemon and Getz, 1997).

Having examined the nature of the inputs to the olfactory system, what is olfaction's task? What kind of processing shall we expect to turn inputs into outputs usable by behavior? This is what we now turn our attention to.

1.11 Olfaction: The computational problem

Having summarized the range of behaviors that olfaction serves and the nature of the inputs it operates on, we are ready to extract from them the essence of the computational problem at hand, the first level of analysis of any information processing task in Marr's scheme (Marr, 1982). This is important because the nature of the computations that underlie perception depends more on the computational problems that have to be solved than upon the particular hardware in which their solutions are implemented. This becomes particularly obvious when considering the similarities in the principles at work in the olfactory systems of species with hardware as diverse as insects and mammals (§1.12).

First and foremost, olfaction is a process that produces, from raw olfactory receptor activation maps, a description that is useful to the animal. A process may be thought of as a mapping from one representation to another (Marr, 1982). Having examined the nature of the input representation (§1.10), we now ask how that representation must be transformed in order to serve the purposes of odor-mediated behaviors. What is the output of olfaction?

Clearly, the output of olfaction depends on the behaviors it must guide, and thus must vary from species to species (see Marr, 1982). There are likely to be important similarities, though. The first function that the olfactory system needs to perform is to segment the olfactory inputs to separate

individual olfactory sources, or objects (Hopfield, 1991). This is necessary because the olfactory environment is rarely devoid of noise in the form of multiple odor sources, which must be distinguished from one another if a source is to be identified. Hopfield and colleagues have proposed several algorithms by which this can be done (Hopfield, 1991; 1995; 1999; Hendin et al., 1994): by analyzing the fluctuations in concentration common to all odors traveling from the same source, components corresponding to the same source could be grouped together and separated from other sources or noise in the environment. There is indeed behavioral evidence that *Limax maximus*, a mollusk, can discriminate two food odors from separate sources separated by 1 cm but not two odors if they originate at the same location (Hopfield and Gelperin, 1989). Moths can discriminate between a pheromone and an antagonist as long as the sources are 1 mm apart (Fadamiro et al., 1999). In humans, delays of 200 to 400 ms between two odors presented monorhinically or dichorhinically (through one or two nostrils) gave significant increases in the frequency of detection of components, whereas synchronous mixtures favored the perception of a single blended odor (Rouby and Holley, 1995).

In addition to source separation, the olfactory system needs to eliminate background contaminants for successful recognition, and in the case of simple mixtures of 2-3 known components, it is sometimes able to separate these components, a computational problem in and of itself (Hopfield, 1999).

For each 'olfactory object' (source or component), these are the basic outputs that must be computed by most olfactory systems:

Familiarity: The system must establish whether the odor has been experienced in the past, or whether it is in the presence of a novel odor. Even beyond the problem of specifying an algorithm to arrive at this, this problem is hard to define given the arbitrary nature of what is to be considered sufficiently dissimilar from any previously experienced odor so as to warrant classification as 'novel' (see §1.11.1). Even in the absence of identification, judgment of novelty (vs. familiarity) can aid an

animal by putting it on the alert in the presence of something unusual.

Identification: Related to familiarity, identification consists in arriving at identifying information for an odor for cases in which a percept has been experienced previously. I will show that single projection neurons in the antennal lobe of the locust contain substantial information about odor identity in Chapter 4. Importantly, identification needs to be invariant to concentration (at least to some degree), given that a given odor will seldom be encountered at exactly the same concentration twice. Two ways in which the olfactory system of the locust addresses this problem will form the subject of Chapters 7 and 8. Identification (and consequently encoding) in humans seems to involve at least two different representations: a verbal one, and a nonverbal one stored in the right hemisphere (Ilmberger et al., 2001). Even though identification could in principle be performed concurrently with the assessment of familiarity, the latter appears to precede identification in human sensory systems, as evidenced by the tip-of-the-tongue phenomenon, also called semantic retrieval failure, in which subjects report familiarity with an object before being able to name it or provide other identifying information (Brown, 1991).

Association: Association is intimately related to identification, and in fact probably constitutes the method of identification for neurobiological sensory systems, but is distinct in its scope from it. Identification entails associating one unique identifying character with a percept, such as the name of an odor; association, in contrast, entails associating a host of percepts to the eliciting percept, such as times and places of previous occurrences, visual appearance of the odorant, etc. In reality, identification is probably given by a subset of the associations, which are not instantaneous or simultaneous, so that some associations may take longer than others. Association is key for adaptive behavior, allowing contingencies with predictive value based on previous experience to guide behavior.

Valence: Valence is a special case of association: the association of a percept with positive or neg-

ative reinforcement value. These associations, or value systems, play a key role in shaping behavior, especially in early development when more subtle and complex behavioral plans are absent and behavior is guided by the immediate urges given by positive and negative reinforcement. I will show that particular odors have an innate valence for locusts in Chapter 3.

Intensity: Intensity is a perceptual description, created by the brain, related to the physical concentration of an odorant. The two are not synonymous, though; changing the concentration of an odorant, for example, can lead the percept to change in odor quality and even in valence rather than in intensity (Alcorta, 1991; Ayyub et al., 1990). Intensity judgments are used in evaluating the direction of a trail (Schöne, 1984) and also carry information on the distance to a source (Moore et al., 1989). I will look at the coding of intensity in projection neurons in the antennal lobe of the locust in Chapter 7.

Direction to source: The direction to an odor source is not present directly in the stimulus but rather must be computed by the brain, using, for example, time arrival or concentration differences between both nostrils or olfactory appendages (von Békésy, 1964) or spatio-temporal fluctuations in concentration during successive samplings (Moore et al., 1989). Many olfactory-guided behaviors, including foraging and navigation, require an assessment of the direction to the source of an odor perceived.

Distance to source: Like direction, the distance to an odor source is not carried explicitly by odors and must be computed using the parameters above, plus knowledge of the concentration at the source, when available.

Needless to say, some animals will require specific additional outputs from their olfactory systems. Golden hamsters, for example, will require an output specifying whether a particular scent overmark is on top (Johnston and Borade, 1998).

1.11.1 The computational role of learning in olfaction

Olfaction is an extremely plastic sense, from the very beginning. Rabbit pups, for whom the period of parental care is particularly brief due both to the risks of predation, which forces them into a closed nursery burrow while their mother forages for food, and to the short inter-litter period (26 days), show a preference for food in the diet by their pregnant mothers (Hudson and Distel, 1997). Human fetuses also learn odors from their pregnant mother's diet (Schaal et al., 2000).

The advantage of a learning system is obviously adaptability. Let us consider exactly what needs to happen during learning.

Perhaps the hardest aspect of the pattern recognition problem is the fact that the boundaries around the patterns to be recognized are somewhat arbitrary. In other words, who's to tell the olfactory system that ethyl acetate at a concentration of 10^{-6} should be recognized as the same attractive odor as ethyl acetate as 10^{-5} at low concentration, but that that very odor at a concentration of 10^{-1} should be avoided, and that an intermediate concentration should be recognized as neither of those two? And yet that's exactly what the behavior of unconditioned flies shows toward most odorants (Ayyub et al., 1990; Alcorta, 1991; Acebes and Ferrús, 2001). It must be remembered, then, that the function of olfactory identification is *not* to *reconstruct* the exact nature of the odorant, but rather, to *classify* it as pertaining to the *closest or most likely* class of olfactory memories (using the term to mean odor templates learned or innate), or as a novel odorant altogether. The problem is the same for learned odors: what the process of encoding a novel odor must do is to *imprint* the energy landscape, in the sense of Hopfield's energy function, with a valley leading from the representations of stimuli *similar* to the one being learned to the representation of the stimulus being learned, which will henceforth act as an attractor. Exactly how wide such a valley should be, or how dissimilar an odor

can be before avoiding recognition, is not known. I will address this important topic in Chapter 9, showing that the width of the valley itself is plastic and subject to the influence of experience.

1.12 Convergent evolution? Insects as a model system

The brain is an immensely complex system. To makes things even more difficult, the brain is not a neat modular machine, but rather, each area usually receives feedback from the very brain regions to which it projects, making the isolated study of individual neurons or even brain regions rather limiting (Koch and Laurent, 1999). Luckily, evolution has provided us with a way out —or a way in, so to speak: complex organisms were not created *ab initio*, but rather gradually through a procession of evolutionary steps. Unfortunately, the original ancestors are usually not available to us anymore: every creature alive today has been evolving for the same amount of time: since the beginning of life. Some species, though, appear to have evolved less complexity over evolutionary time than others. And, while biological complexity is very difficult to define (Koch and Laurent, 1999), there are some objective parameters that correlate intuitively with some notion of complexity or at least with our ability to monitor a system's activity. While mapping out the complete connectivity of the nematode *C. elegans* (White et al., 1984) might not get us anywhere close to a complete understanding of its nervous system, it probably brings us closer than not knowing it. And it is certainly harder to obtain such a connectivity map for a brain with billions of neurons than it is for one with 302. Even if you disagreed with the notion that complexity scales in some way with the number of neurons, an experimental reality is that, with the current limitations in our ability to record simultaneously from large numbers of neurons, the number of neurons about whose activity we are ignorant increases with the number of neurons in the brain, giving small brains a practical advantage for the neurophysiologist.

Just how simple a brain do we want to tackle first? Ideally, one that is as simple as possible without losing the computational principles at work in more complex brains. A unicellular organism counts with chemodetection, but clearly no nervous system and no real olfactory system. Perhaps the next obvious candidate would be *Caenorhabditis elegans* , since it can smell and has the advantage that the connectivity of every neuron in its nervous system is known. But the worm has so few cells compared to the number of genes in its genome that it has evolved an entirely different computational strategy, one that expresses up to 20 receptor types in the same receptor neuron (Bargmann and Horvitz, 1991; Colbert and Bargmann, 1995; Troemel et al., 1995, 1999) and thus different from the one-receptor-type-per-neuron doctrine that appears to hold for so-called higher animals (see §1.14). *C. elegans* also differs from higher organisms in many other respects: it has a single neuron expressing each receptor type, and lacks glomeruli, for example. Therefore, even if it may prove a useful model to understand the cellular mechanisms at work in olfactory receptor neurons, the worm is not a satisfactory model system for the vertebrate olfactory system as a whole.

Insects have received a substantial amount of study over the past century or so. Their behavioral repertoire is wide enough to make them more adaptable than most any man-made machine. And yet with a locust brain comprising 360,000 neurons in a volume of 6 mm^3, compared to 100 billion in the 1350 cm^3 of a human brain, it seems like an awfully good place to start. Now, if insect brains were completely unrelated to human brains, they might be easier to comprehend, but that would still not bring us anywhere closer to understanding our brains. While the understanding of insect olfaction would constitute a worthy pursuit in itself, both for intellectual and practical reasons —insects are agricultural pests, disease vectors and are responsible for pollination and for the production of honey—, it is made all the more fascinating by the remarkable parallels between the structure and function of the olfactory systems of insects and vertebrates. It is to this common design that we now turn our attention for a brief overview before we examine each stage of olfactory processing in detail.

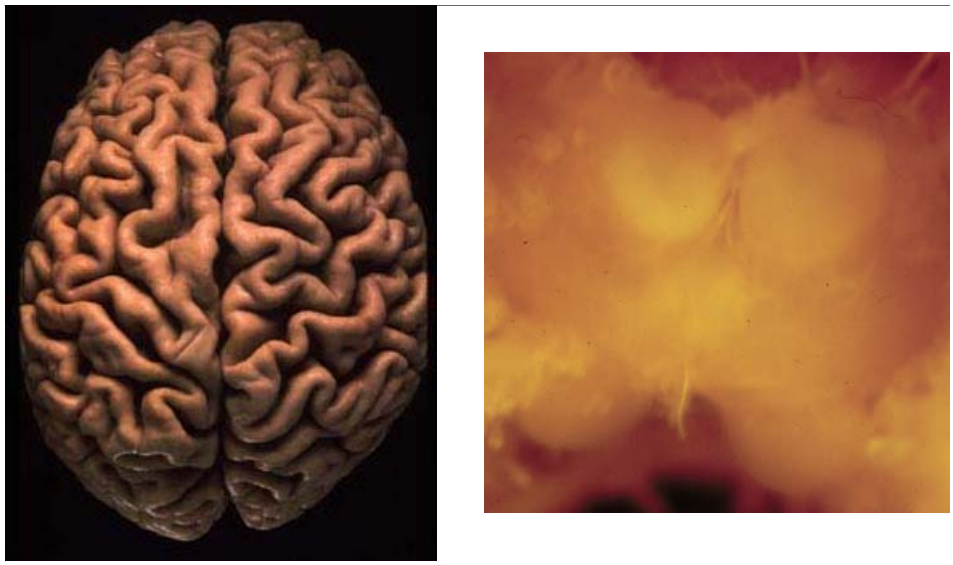


Figure 1.2. A human brain is made of 100 billion neurons and has a volume of 1350 cm^3; a locust brain is composed of 360,000 neurons and is a mere 6 mm^3 (human brain photo courtesy of Virtual Hospital, University of Iowa; locust micrograph by the author).

1.12.1 A brief anatomy of the olfactory system

In both insects and vertebrates, there is massive convergence from receptor neurons to the next processing layer (the vertebrate olfactory bulb [OB] and the insect antennal lobes [AL]) and massive divergence again from there to memory areas. Receptor neurons in both insects and vertebrates are likely to express a single or very few odorant receptor genes (Mombaerts, 1999; Vosshall et al., 2000).

The second processing relays of the olfactory systems of insects, crustaceans, and most vertebrates feature glomeruli, discrete structures of neuropil that have been described as one of the most distinctive structures in the brain (Shipley and Ennis, 1996). Each glomerulus receives projections from receptor neurons expressing the same type of odorant receptor genes (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Vosshall et al., 2000). Even more strikingly, perhaps,

the proteins responsible for glomerular structure, glycoproteins expressed in glial cells, are antigenically very similar and comparable in size in insects and mammals (Krull et al., 1994; Gascuel et al., 1996). These growth inhibitory extracellular matrix molecules (e.g., tenascin and chondroitin sulfate proteoglycans [CSPGs]) have been localized around glomeruli, and it has been suggested that these molecules form a macromolecular wall that restricts axon growth to glomeruli (Gonzalez et al., 1993; Gonzalez and Sliver, 1994; Kafitz and Greer, 1998). Interestingly, these molecules are also responsible for the distinctive patterns of neuropil in rat barrel cortex (whisker somatosensory cortex) (Tolbert, 2000), another structure where the targets of a discrete number of sensory inputs are well separated from each other (see §1.15.1). These second processing relays of both insects and vertebrates have excitatory projection neurons (vertebrate mitral/tufted cells and insect projection neurons [PNs]) and local inhibitory neurons. In addition, mollusks, arthropods and chordates exhibit odor-evoked oscillatory synchronization, whose function has been an enigma for over half a century (Adrian, 1942).

Vertebrate mitral/tufted cells and insect PNs distribute information about odors to several structures in their respective brains. Among those brain targets, two are possibly comparable between phyla (Strausfeld and Hildebrand, 1999). In mammals, the piriform cortex is the main recipient of inputs from the OB and, like the bulb, shows odor-evoked fast oscillations (Ketchum and Haberly, 1993). Mitral/tufted cells also project to other areas of the cortex, including the entorhinal cortex, which sends axons to the hippocampus. Both cortical regions may have parallels in insects. In neopterans, all PNs extend an axon ipsilaterally into a prominent area of the forebrain called the superior lateral protocerebrum and lateral horn (Hornberg et al., 1988). A subset of these PNs provides axon collaterals to the calyces of the mushroom bodies, which are paired, lobed centers consisting of many thousands of intrinsic neurons (Kenyon cells) arranged approximately parallel to each other. Mushroom bodies have been implicated in olfactory learning and memory (Heisenberg, 1998). As in olfactory cortex (Ketchum and Haberly, 1993), neurons postsynaptic to AL PNs, in a region of each

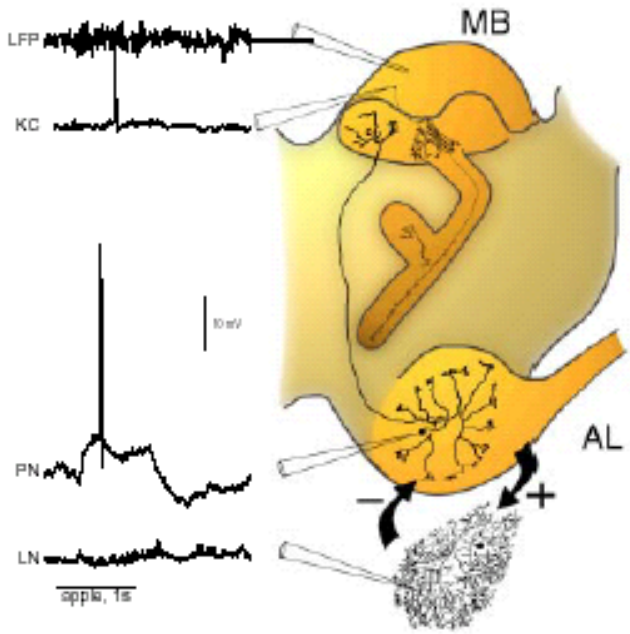


Figure 1.3. The locust olfactory system. Receptor afferents project from the antenna (not shown) to the antennal lobe (AL). The AL consists of projection neurons (PNs) which are spiking, excitatory cells with discrete glomerular arborization patterns, and local neurons (LNs) which are nonspiking, inhibitory cells with global arborization patterns. Both cell types, as well as Kenyon cells (KC), experience odor-evoked membrane potential oscillations as shown in representative intracellular recordings at left in response to apple odor (each cell recorded separately in different animals and aligned to the 1s odor pulse, indicated by solid bar). Projection neurons project to the mushroom body, where odors evoke oscillations in the local field potential (LFP, here lowpass filtered at 50 Hz). Kenyon cells project to the α- and ß-lobes of the mushroom body. Cobalt fills courtesy of G. Laurent. From Wehr (1999).

mushroom body called the calyx, exhibit synchronous activity (Laurent and Naraghi, 1994; Pérez Orive et al., unpublished). Axons from the calyx project to the lobes of the mushroom body, which, like the mammalian hippocampus (Morris et al., 1982), is involved in place-memory functions (Mizunami et al., 1998) and context-specific sensory filtering (Strausfeld and Hildebrand, 1999).

Do these similarities in organization reflect common origins or convergent evolution imposed by the common function of olfactory systems across phyla? The apparent lack of homology between olfac-

tory receptor gene families in insects and mammals, as well as the important differences in olfactory systems of intermediate species, suggest that convergent evolution is responsible (Strausfeld and Hildebrand, 1999). The last word, however, has not been said; molecular studies of the genes involved in the rest of the olfactory system will be of great value in this endeavor.

Figure 1.4. The locust, *Schistocerca americana*.

Having settled on insects as our model system, we need to settle on a particular species for our experiments. My choice of the American locust, *Schistocerca americana*, was largely historical and owed itself to the pioneering studies by Gilles Laurent and his laboratory. The locust is particularly suitable for studies of a generalist olfactory system because of its polyphagous nature (Lee and Bernays, 1988; Bernays and Lee, 1988). There are two distinct advantages of the locust over other insects for its use in electrophysiological investigations: it is a relatively large insect, making surgery easier, and it possesses relatively large neurons, making electrophysiology comparatively easy and allowing us to hold intracellular recordings of single neurons for hours at a time.

Having described the nature of the computations that the olfactory system must perform and the nature of the inputs it counts with for this purpose, and having explained the great similarities between insect and vertebrate systems that allow us to draw on both literatures for the description of the principles at work, the next few sections describe the actual nature of the system, the way we understand it at present.

1.13 Getting odorants to and away from receptors

1.13.1 The active and pulsed nature of olfactory sampling

The olfactory system is not a passive system, and does not rely only on the natural variations in odor concentration. In vertebrates, the sampling of olfactory space is pulsed due to a process known as sniffing (Freeman, 1978; Gray and Skinner, 1988), although dogs can inhale continuously if they are following a trail while running (Steen et al., 1996). In insects, a similar pulsed sampling process can occur due to systematic flicking of the antennae (Mellon, 1997). It has been consistently found that the inhalation and exhalation processes in dogs are complex and are modified by the behavioral task that the dog is performing (reviewed in Kauer and White, 2001), suggesting that the natural statistics of odor plumes are further complicated by the complex sampling mechanisms inherent in sniffing.

1.13.2 The olfactory epithelium is covered by an aqueous mucosa

Before odorant molecules reach olfactory receptors, they must first cross an aqueous interface. In vertebrates, the olfactory epithelium is covered by the nasal mucosa, which is 5–30 micrometers

deep depending on the species and living environment (Menco, 1980). In insects, olfactory receptor neurons (ORNs) are covered by the sensillar lymph, which lies below the cuticular walls of the sensilla.

1.13.3 The rate of air flow through the nose influences olfactory responses differentially for odorants of different sorptions

Different odorants sorb to and cross the mucosa at different rates (Mozell and Jagodowicz, 1973). In the bullfrog, a specific odorant's sorption rate interacts with the rate of airflow across the mucosa to produce varying amplitudes of response in the olfactory nerve (Mozell et al., 1991). This occurs because, when a high-sorption odorant has a low airflow rate, the odorant molecules sorb to the mucosa before moving very far along it. Only a small portion of the epithelium is involved in the response, which is small. When the same odorant flows at a high airflow rate, it spreads across a larger mucosal area before sorbing, so the response is larger. When a low-sorption odorant flows quickly, it moves past the mucosa without sorbing so the epithelial response is small. When the same low-sorption-odorant flows slowly, it has time to sorb across the mucosa and the response is larger (Mozell et al., 1991).

1.13.4 The bilaterality of olfactory sampling

The olfactory system counts with more than just time to look at statistical fluctuations in the distribution of odor plumes: invariably, from insects to mammals, it counts with two spatially separate sensors. Von Békésy (1964) has reported an amazing precision of localization of odor sources by humans. Within an angle of 65 degrees in either direction from the median plane, experienced

experimental subjects could localize an odor source 8 cm away from the nose to within 7-10 degrees. Closing one of the nostrils impaired performance severely (von Békésy, 1964). Using an odor delivery system with tubes connecting into a subject's nostrils, von Békésy (1964) described two processes that could be responsible for such accuracy: a simultaneous process with two sensors and a time interval measurement. For simultaneous measurement, a concentration difference of 5-10% was enough to localize the odor to the side of the nostril that received the higher concentration. In the time interval process, differences of only 0.3 ms in the arrival time of the odor at the right and left nostrils were enough to determine with side the odor came from. Not that the abilities of a single sensor location should be underestimated: even with one nostril plugged, though, a shark can sniff out pieces of food, moving the front part of its body from side to side (Hara, 1971). Bees orient using simultaneous sampling with both antennae or successive sampling depending on the steepness of the odor gradient (Schöne, 1984). Wasps and dung beetles with only one antenna can follow an odor gradient upward (Murr-Danielczick, 1930; Otto, 1951).

1.13.5 Flow rate through each human nostril is differentially regulated and contributes to differential sensitivity to different odorants

In addition to forming separate spatial olfactory images, both nostrils generate chemically distinct filters on the environment, further differentiating the information the brain gets from each. It has long been known that the flow of air is greater into one nostril than into the other because there is a slight turbinate swelling in one (Kayser, 1895; Principato and Ozenberger, 1970; Hasegawa and Kern, 1977). The nostril that takes in more air switches from the left to the right one and back again every few hours (Bojsen-Muller and Fahrenkrug, 1971).

This difference in airflow between the nostrils, combined with the differential dependence on flow rate of responses to odorants of different sorptions described above, causes each nostril to be opti-

mally sensitized to different odorants, so that each nostril conveys a slightly different olfactory image to the brain (Sobel et al., 1999).

1.13.6 The aqueous mucosa provides the olfactory system with invariance to volatility

The olfactory system faces a seemingly formidable challenge in reconstructing the concentrations of olfactory objects in the world, as well as the composition of simple mixtures, given that different substances have different volatilities, and thus the relationship between the concentration found in the gas entering the nose or surrounding an antenna on the one hand, and the concentration at the source on the other, will vary for each substance. We will see in Chapter 2, however, that the system has solved this problem with astounding elegance and simplicity.

1.13.7 The aqueous mucosa concentrates odorants

In land vertebrates, in addition to providing invariance to volatility, the aqueous environment may serve to provide an aqueous environment for the biochemistry of ligand binding. Providing a medium for odorant removal is likely to be another important reason to have an aqueous environment surrounding receptors (Pelosi, 1994). An aqueous layer also serves to concentrate odorants: calculation of partition coefficients using vapor pressures and solubilities shows that the concentrations in grams per liter for all but the most volatile of hydrophobic odorants are actually higher in the aqueous layer than in air by two to four orders of magnitude (Amoore and Buttery, 1978). These cannot be the mucosa's only function, however, since olfactory mucosa are also present in fish (Wehr, 1999). The aqueous mucosa may well serve a protective role for the ORNs, the most

exposed neurons in the entire nervous system: an aqueous layer prevents the receptors from coming in direct contact with potentially toxic substances (Wehr, 1999).

1.13.8 Odorant binding proteins (OBPs)

In insects, most of the sensillar lymph is constituted by a family of odorant-binding proteins (OBPs) and pheromone-binding proteins (PBPs) (Wehr, 1999) secreted by non-neuronal support cells (Kim et al., 1998). OBPs have been found in many species, including numerous vertebrates. In vertebrates, OBPs are members of the lipocalin transport family (Flower, 1996). Lipocalins typically function as carriers of hydrophobic molecules. Invertebrate OBPs, in contrast, constitute a unique family of low molecular weight, chemosensory-specific proteins with six conserved cystein residues (Kim et al., 1998). These show no homology with the vertebrate OBP family (Pelosi and Maida, 1995).

Odorants have been shown to bind directly to these proteins in both mammals and insects (Vogt and Riddiford, 1981; Pelosi et al., 1982; Pevsner et al., 1985; Pevssner et al., 1990; Du and Prestwich, 1995). Vertebrate OBPs bind odorants at the interface of a dimer (Bianchet et al., 1996) while insect OBPs bind ligands as monomers (Sandler et al., 2000). Binding experiments have been performed on bovine and pig OBPs, and have indicated a broad specificity for medium sized hydrophobic compounds, often of green or floral origins (reviewed in Pelosi, 1996).

The *Drosophila* genome contains at least 32 members of this gene family, rivaling the number of odorant receptors in this species (Kim and Smith, 2001). Unlike mammals, whose olfactory cilia are bathed in a common overlying fluid, most arthropods, including insects, have compartmentalized their olfactory neurons into sensilla (Kim et al., 1998). This compartmentalization provides the opportunity to independently regulate the composition of the fluid bathing the olfactory neuron dendrites. Indeed, in *Drosophila*, the identified OBP family members have surprisingly low sequence

similarity and are expressed in different, overlapping zones of chemosensory sensilla (Kim et al., 1998).

One of the *Drosophila* OBPs, *lush*, is required for normal olfactory avoidance behavior responses to a small subset of chemically related odorants (Kim et al., 1998), suggesting that OBPs participate in determining the chemical specificity of olfactory neurons in *Drosophila*. This result illustrates the critical importance of OBPs in olfaction, but it does not address the precise role played by OBPs, since a failure in any of the steps required to get an odorant to receptors would impair behavioral responses to the odorant. Remarkably, expression of a moth pheromone binding protein (not normally expressed in *Drosophila*) under control of the *lush* promoter causes an abnormal repulsion by moth pheromone in transgenic flies (Kim and Smith, 1997), although the concentrations of moth pheromone required are a million times higher than those required by moths (D. P. Smith, personal communication). This result suggests that individual sensilla constitute labeled lines to specific valences, and thus to behavioral reactions to particular odorants.

Moth pheromone-binding protein members of the same family have been shown to bind directly to pheromone with chemical selectivity *in vitro* (Du and Prestwich, 1995) and have been localized to trichodeal sensilla (known to sense pheromones), whereas general OBPs were localized to the generalist basiconic sensilla (Kaissling, 1986; Steinbrecht et al., 1992; Maida et al., 1993; Laue et al., 1994). Moth pheromone-binding OBPs have been found to be expressed in species that are not pheromone responsive too, though, showing that OBPs are not sufficient to confer chemical sensitivity (Zhang et al., 2001). OBPs have also been shown to be expressed in the pheromonal gland of the cabbage armyworm, which has no chemosensory structure (Jacquin-Joly et al., 2001).

What is the function of odorant binding proteins?

In addition to being present in high concentrations in the perireceptor space, OBPs also have a rapid turnover: complete replacement over 48 hours (Vogt et al., 1989). This must represent a large expenditure of energy, which surely is offset by a selective advantage. The facts from the previous section suggest that OBPs may be involved in the solubilization or transport of hydrophobic molecules across aqueous layers. This would be consistent with their influence on chemical selectivity of sensilla, since only hydrophobic odorants that bind an OBP might make it in sufficient concentration to the ORNs and be detected. Since airborne odorants are typically hydrophobic (Wehr, 1999), solubilization to concentrate odorants in the sensillum lymph is indeed very important for odorants to reach receptors. In vertebrates, however, the concentrations of OBPs are too low by an order of magnitude to affect odorant concentrations around receptors (Pelosi, 1994).

A second potential function for OBPs is to provide a means to detect small odorants that might prove difficult for a 7-transmembrane-receptor protein to bind, such as ammonia (NH_3). We know that ammonia is detected by mammals and insects alike (Meijerink et al.,2001). These molecules may prove too small for the pockets of a membrane-associated protein, and it is possible that biology has circumvented this limitation in a manner similar to the way in which other small gaseous molecules are bound in the circulatory system: by using proteins associated with a group, such as the haeme group, that changes conformation in the presence of particular gaseous molecules. On the other hand, quaternary ammonium compounds have been shown to inhibit voltage-activated Shaker K+ channels (Choi et al., 1993), and could be detected that way, and many small gaseous molecules, such as poisonous carbon monoxide, are odorless despite the potential behavioral advantage of detecting it (at least for modern-day humans), suggesting that the olfactory system has not quite circumvented the difficulties associated with detecting small gaseous molecules. More importantly, recent experiments have shown that the ligands for OBPs are larger molecules (Vincent

et al., 2000; Ramoni et al., 2001). 1-octen-3-ol, a typical component of bovine breath and in general of odorous body emanations of humans and animals, has been shown to be the natural ligand for bovine OBP (Ramoni et al., 2001). The recent structural characterization of porcine OBP binding properties (Vincent et al., 2000) has shown that a high degree of hydrophobicity coupled to a molecular mass between 160 and 200 daltons is the main requirement for a ligand to fit the b-barrel cavities of OBP, irrespective of the chemical class, substituents, and molecular structure. Furthermore, biology seems to have circumvented the difficulty of detecting small molecules, such a NaCl, with seven transmembrane (7TM) receptors by employing other membrane-bound receptors, such as the salty taste receptor, a 4TM receptor (Lindemann, 2001).

A third hypothesis (Pelosi, 1994) for the elusive function of OBPs, based on the belief that OBPs' affinities (K_D's of 0.1 to 20 mM) for odorants were poor relative to those of olfactory receptors, suggested that OBPs will bind odorants mostly at high concentration, which in turn suggested that OBPs may act as buffers, keeping odorants' concentration at intermediate values. In this sense, they would act to increase the dynamic range in which changes in odor concentration can be detected, much like the retina acts via multiple mechanisms to keep responses similar across an astoundingly wide range of light intensities. Olfactory receptors' affinities have recently turned out to be of the same order of magnitude, though (Pelosi, 1994). More importantly, experiments done with insect antennal sensilla appear to point in the opposite direction. When the sensillum lymph in *A. polyphemus* was replaced with saline solution, the electrophysiological response to the specific sex pheromones was greatly reduced; normal sensitivity was then restored by the specific purified PBP, but also by bovine serum albumin (Van der Berg and Ziegelgerber, 1991). These results suggest that the function of insect PBP is to concentrate the pheromone, increasing the sensitivity rather than buffering its concentration. In mice, too, the absolute sensitivity of ORNs was much higher in intact epithelium than in experiments using dissociated cells: cells in intact epithelium consistently responded to nanomolar odor concentrations (Ziesmann et al., 2001), suggesting the extracellular

environment contributes to heighten sensitivity rather than to buffer.

Mediating odorant removal constitutes another possible function of OBPs (reviewed in Pelosi, 1994). In vertebrates, the mucosa is constantly being discarded. In insects, however, the sensillum wall prevents this, and thus active mechanisms of degradation must be involved (Pelosi, 1996). The rapid turnover of OBPs suggests that the possibility that they either remain bound to odorant molecules for long periods and thereby mediate odorant removal, or else are permanently modified by binding to odorants and must be replaced after losing functionality.

More recent evidence, however, points to a role of vertebrate OBPs in the VNO rather than the olfactory epithelium (Pelosi, 2001). The elements of evidence towards this view include[9]

– OBPs are structurally similar to pheromone-binding proteins of urine, saliva and vaginal discharge; several subclasses of OBPs have been identified in the same animal species, each best related to a particular group of PBPs;

– OBPs are secreted by glands of the respiratory region of the nasal epithelium; from this area they are translocated to the VNO, but not to the olfactory mucosa; some OBPs are also synthesized in the VNO.

Volatile pheromones are able to activate the enzymatic cascade in the VNO leading to production of cAMP, while a PBP found in the urine of rats binds dissociated VNO membranes and leads to activation of the IP3 signaling cascade (Krieger et al., 1999): this observation would exclude the

9. The finding that olfactory receptors functionally expressed in cells not synthesizing OBPs are still able to respond to odors (Zhao et al., 1998; Wetzel et al., 2001), contrary to early assertions (Pelosi, 2001), does not suggest OBPs do not play a role in the vertebrate olfactory epithelium: on the contrary, recent evidence suggests that intact epithelium is significantly more sensitive to odorants than dissociated receptor neurons (Ziesmann et al., 2001).

requirement of OBPs, at least for one of the two transduction mechanisms active in the neurons of the VNO (Pelosi, 2001).

A final and very interesting role has been recently advanced for bovine OBP (bOBP) by Ramoni et al. (2001), who found that the natural ligand for bOBP is 1-octen-3-ol, a typical component of bovine breath and in general of odorous body emanations of humans and animals, as well as a chemoattractant for mosquitos and many insect species. They thus suggested that bOBP might be used by bovines to remove parts of 1-octen-3-ol from the breath flowing through the nasal cavities and to make them less appealing for several insect species. This would result in a general decrease of the number of insect bites and furthermore might partially protect the animal from parasitosis and infectious diseases carried by these insect vectors.

1.13.9 Olfactory degrading enzymes (ODEs)

Degrading enzymes distinct from OBPs have also been found in olfactory mucosa and sensillar lymph, with very high activity. Olfactory forms of cytochrome P-450, a degrading enzyme of broad specificity first described in the liver, have been found (Dahl, 1988; Ding and Coon, 1988; Nef et al., 1989; Ding et al., 1991), as have enzymes which further detoxify the products of P-450 (Longo et al., 1988; Lazard et al., 1990; Rama-Krishna et al., 1992). These proteins show activity levels in the olfactory system equal to or higher than those in the liver. The functions of these proteins have been speculated to involve protection against toxic odorants as well as removal of odorants to prevent continuous sensory responses. After all, the stimuli for vision and hearing only reach their target organs for as long as they are being emitted from the source, and even gustatory stimuli eventually get ingested, but odorants coming from transient olfactory stimuli would persist in the nasal mucosa or sensillar lymph were it not for an active removal or degradation process. Such a removal process

is particularly important given the importance of dynamic concentration information in guiding olfactory-mediated behavior (see §1.10.3). To investigate the role of ODEs in signal termination, Maida et al. (1995) correlated electrophysiological responses of the moth olfactory epithelium to pheromone with the activity of pheromone-degrading esterase across individuals. While the esterase activity was found to vary over two orders of magnitude, responses to the pheromone retained the same shape and amplitude, ruling out a role for it in signal termination.

1.14 A broad array of generalist sensors: Olfactory receptors

1.14.1 Olfactory receptor (OR) genes

Vertebrate olfactory receptor genes make up a huge family

Until recently, the molecular transducers responsible for odor detection were unknown. Although much remains yet unknown, the last decade has seen a revolution in our understanding of olfactory receptor (OR) genes spearheaded by Richard Axel's laboratory at Columbia University and a small army of postdoctoral fellows in his lab, now in their own laboratories around the country.

This explosion derived from the initial isolation of OR genes from the rat (Buck and Axel, 1991) using an experimental design based on three assumptions (Mombaerts, 1999). First, ORs were likely G-protein coupled receptors, and these are generally seven-transmembrane (7TM) proteins. This first and most critical assumption was based on biochemical evidence that had implicated G proteins in olfactory signal transduction (Pace et al., 1985; Sklar et al., 1986; Jones and Reed, 1989). The 7TM superfamily includes rhodopsin, dopaminergic, adrenergic, muscarinic and other receptor types (Dohlman et al., 1991). Second, ORs are likely members of a multigene family of a considerable size, because of the immense variety of chemicals that can be discriminated by the

olfactory system. Third, ORs are likely expressed selectively in olfactory receptor neurons (ORNs). Buck and Axel designed a series of degenerate PCR primers based on conserved regions of 7TM proteins, and amplified a multigene family from cDNA of olfactory epithelium. They then demonstrated by Northern blot analysis that members of this family are expressed only in the olfactory epithelium of the rat. Stuart Firestein and his colleagues then used an adenovirus vector system to overexpress a putative mammalian odorant receptor in the rat olfactory epithelium and measured elevated physiological responses to octanal and some related odorants (Zhao et al., 1998).

Screening rat genomic libraries suggested that the OR gene family has 500-1000 genes (Buck, 1992), making it the largest family in the mammalian genome (Mombaerts, 1999). Sequence divergence in this family is highest in transmembrane domains 3 to 5, which are believed to be involved in ligand binding in other 7TM proteins (Kobilka, 1992), suggesting, as expected, that different receptor genes bind with odorants of widely varying chemical structure. A given OR gene will typically cross-hybridize with a few others (Mombaerts, 1999). These sets of similar genes are called subfamilies. Because OR genes are intronless, genomic DNA has been successfully used to create primers to clone OR genes in multiple vertebrate species (Mombaerts, 1999). The genes form clusters throughout the genome, just like all gene superfamilies (Mombaerts, 1999).

There is a high frequency of pseudogenes in the human OR repertoire

A recently reported enigma is the high frequency of pseudogenes in the human OR repertoire (Mombaerts, 1999c): Because of frameshifts, non-sense mutations, and deletions, between 38 and 76% of the 500 to 750 OR-like sequences do not appear to encode full-length polypeptides (Mombaerts, 1999b). By contrast, no pseudogenes have been reported among 200 OR sequences in mouse and rat (Mombaerts, 1999b), and in other vertebrate species, OR pseudogenes are also

scarce. This raises the interesting issue of whether the pseudogenes contribute to perceptual diversity in the human population, with individuals having different pseudogenes (Mombaerts, 1999b). The massive degeneration of the human OR repertoire may be related to our inferior sense of smell relative to other species. Perhaps less selective pressure was exerted on the OR repertoire during the evolution of *Homo sapiens*, who apparently came to rely more on the visual and auditory senses (Mombaerts, 1999b). Analogously, the relative lack of pseudogenes in other species pinpoints the importance olfaction and the huge diversity of receptor genes has in those species.

Insect olfactory receptor genes appear not to be homologous to their vertebrate counterparts

Difference cloning, along with searches in the recently completed *Drosophila* genome, yielded a family of 57 7TM genes which are expressed in the third segment of each of its antennae (Clyne et al., 1999b; Gao and Chess, 1999; Vosshall et al., 1999; Adams et al., 2000; Rubin et al., 2000). Two recent experiments showed that this family indeed codes for functional odorant receptors. Stortkuhl and Kettler overexpressed the *Or43a* gene in the fly antenna and tested for an increase in odor response *in vivo*. *Or43a* is normally expressed in circa 15 olfactory receptor neurons (ORNs) of the antenna, but Stortkuhl and Kettler were able to drive its expression in a high fraction of the approximately 1,200 antennal neurons by using the GAL4/UAS system. They then found a concomitant elevation in antennal response to a subset of odors, as measured by electroantennograms (EAGs), which are extracellular recordings of the receptor potentials of populations of neurons. Stortkuhl and Kettler found that overexpression of the *Or43a* gene conferred increased response to cyclohexanol, cyclohexanone, benzaldehyde, and benzyl alcohol, each of which contains a six-member carbon ring with a single attached polar group. Responses to several other tested odorants, including some others containing six-member rings, were unaffected. In a second experiment, Vetzel and col-

leagues (2001) showed that heterologous expression of the *Or43a* gene in oocyte cells conferred oocytes responsiveness to cyclohexanol, cyclohexanone, benzaldehyde, and benzyl alcohol.

Interestingly, the family shows no homology with the vertebrate family of OR genes. The insect family as a whole is extremely divergent and exhibits from 17% to 26% amino acid identity. However, each of the genes shares short common motifs in fixed positions that define these sequences as highly divergent members of a gene family. As in vertebrates, analysis of the sequence of all 57 receptors reveals the existence of discrete subfamilies whose members exhibit significantly higher sequence identity, ranging from 40% to 60% The *Drosophila* OR genes are widely dispersed in the genome and most exist as single genes that distribute on each of the *Drosophila* chromosomes, although a few are in clusters of two or three genes (Vosshall et al., 2000). Given the high level of divergence shown by the OR gene families, however, ancient similarities may be hard to come by, and the last word in terms of arthropod and vertebrate olfactory receptor homology may be yet to come.

1.14.2 Olfactory receptor neurons (ORNs)

The number of ORNs substantially exceeds the number of ORs

The ‘nose’ of an insect is the third segment of each of its antennae, which bears olfactory sensilla housing olfactory receptor neurons (ORNs) that supply axons to discrete islets of neuropil called olfactory glomeruli (Strausfeld and Hildebrand, 1999). A locust antenna has 50,000 olfactory receptor neurons (Leitch and Laurent, 1996). In humans, each of the two nostrils is about 2.5 square centimeters containing in total approximately 50 million primary sensory receptor cells (Lefingwell, 1999). In dogs, the number is close to one billion. This number, tens of thousands to hundreds of thousands of times larger than that of inner hair cells, for example, surely serves a purpose. We will

explore this in §1.19.

Each ORN expresses a single OR gene

The olfactory system seems to have gone to great lengths to ensure that each ORN expresses a single OR gene. Two mechanisms are used to this end.

First, each ORN transcribes a single OR gene, both in mammals (Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994; Malnic et al., 1999) and in insects (Vosshall et al., 2000).

Second, allelic inactivation of odorant receptor genes ensures that only a single allele of an OR gene is expressed in each ORN (Chess et al 94). Whether the paternal or maternal allele is inactivated is chosen independently, apparently at random, in each ORN (Serizawa et al, 2000; Ebrahimi et al., 2000).

We will explore a novel hypothesis for the reason for this apparent selective pressure for ORNs representing a single chemical signature in §1.19.

Selectivity for odotopes grants ORs high specificity for some features and broad tolerance for others

Odorant receptors are thought to work by forming a pocket which binds epitopes in odorant molecules (odotopes). Thus, they provide a signature of 3-D structure of the odorant molecules, and are capable of discriminating enantiomers (Kraft and Frater, 2001). The response of a given OR type across different molecular odorants has been found to be highly specific for some molecular features and highly tolerant for others (Fujimura et al, 1991; Araneda et al., 2000; Wetzel et al., 2001).

This combination of wide and narrowly tuned detectors sensitive to features common to several molecules allows the olfactory system to be able to perform fine discrimination of thousands of odors (Araneda et al., 2000).

Bilateral symmetry in the early olfactory system

Wes and Bargmann (2001) recently showed that *C. elegans* odor discrimination requires bilateral asymmetric diversity in olfactory neurons. The same is true of taste neurons in *C. elegans* (Pierce-Shimomura et al., 2001). In insects and mammals, though, asymmetry in the olfactory bulb may be limited to fine structure. Experiments using optical imaging to assay the olfactory bulb's responses to odorants in mice, patterns of activated glomeruli were bilaterally symmetric and consistent in different individual mice, but the precise number, position, and intensity of activated glomeruli in the two bulbs of the same individual and between individuals varied considerably (Belluscio and Katz, 2001). In the honey bee, calcium imaging showed bilateral symmetry in the activation of the antennal lobes (Galizia et al., 1998). This symmetry held true for all odors tested, irrespective of their role as pheromones or as environmental odors, or whether they were pure substances or complex blends.

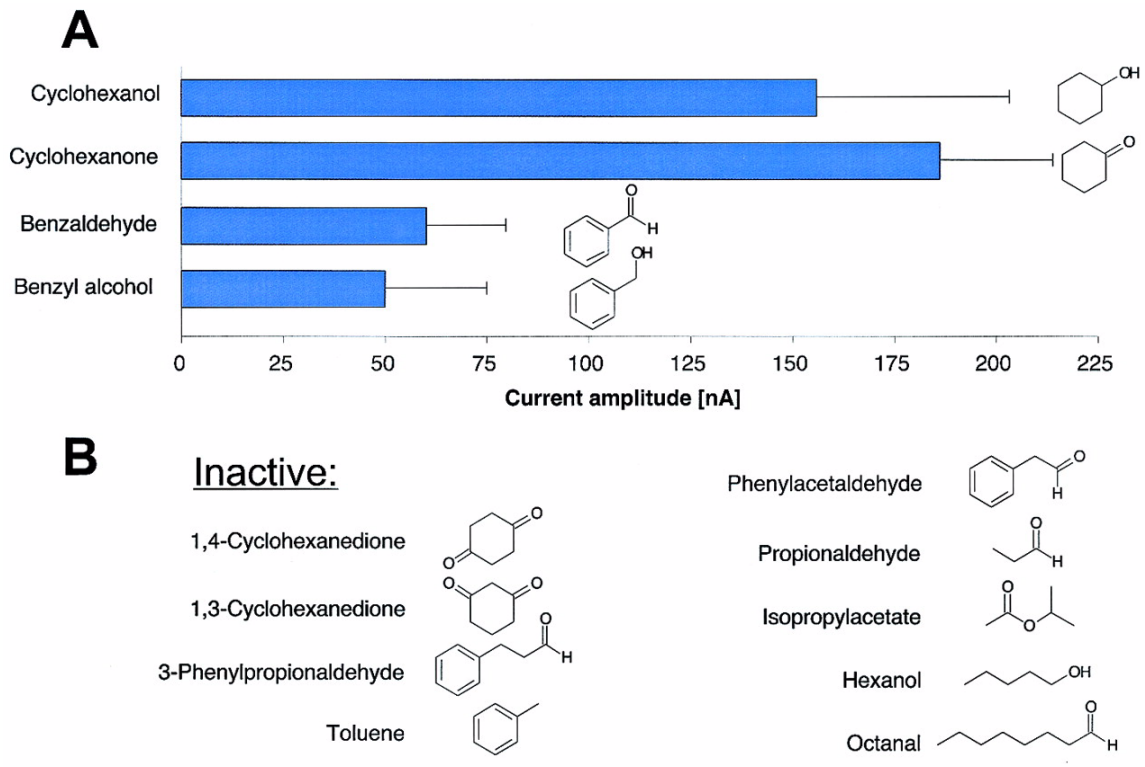


Figure 1.5. (A) Diagram showing the structure and the activity of the compounds tested on oocytes injected with Drosophila OR43a. The stimulatory action of agonists is presented as the peak amplitude of the induced currents (mean +- SE). The odor concentration was 1 mM. Only cyclohexanol, cyclohexanone, benzyl alcohol, and benzaldehyde were active as agonists at the Or43a. (B) Structures of compounds that were inactive at millimolar concentration at the OR43a (from Wetzel et al., 2001).

A topographic map of OR gene expression: Each OR is expressed apparently randomly within one zone of the olfactory epithelium

Both in mammals (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994) and in the fly (Vosshall et al., 1999;Vosshall et al., 2000; de Bruyne et al., 2001), each receptor gene is expressed in an apparently random subset of a zone within the olfactory epithelium, and, conversely, each receptor neuron expresses only one or a small fraction of the receptor genes.

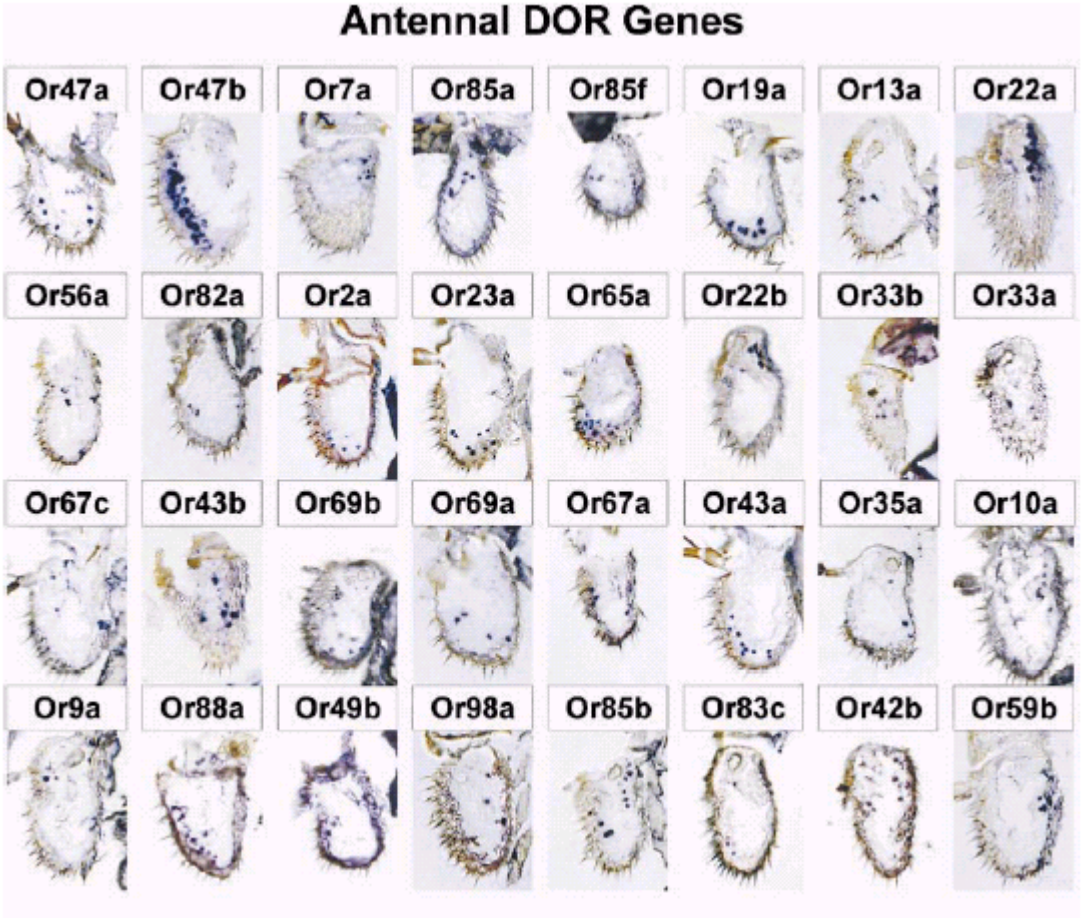


Figure 1.6. Each odorant receptor is expressed in a subset of odorant receptor neurons in the fly (from Vosshall et al., 2000).

Individual ORNs can show either excitatory or inhibitory responses to odors

ORNs exhibit multiple modes of response dynamics: an individual neuron can show either excitatory or inhibitory responses, and can exhibit different modes of termination kinetics, when stimulated with different odors (de Bruyne et al., 2001). In *Drosophila*, there are 16 ORN classes combined in ste-

reotyped configurations within seven functional types of basiconic sensilla. One sensillum type contains four ORNs and the others contain two neurons, combined according to a strict pairing rule (de Bruyne et al., 2001).

A case for non-classical receptive fields in olfaction

As had previously been shown for the lobster (Ache, 1994), Breer and colleagues have shown that different odorants activate different second messenger signaling pathways in the rat: one using cAMP and the other using IP3 and diacylglycerol (Breer and Boekhoff, 1991; Schandar et al., 1998). Brunet et al. (1996) showed, however, that targeted disruption of both alleles of the mouse olfactory cyclic nucleotide-gated cation channel eliminates extracellular electrophysiological responses to odorants that activate *either* of the two pathways. Despite the contention that these results are difficult to reconcile (Schandar et al., 1998), an interpretation is readily available if one considers the nature of non-classical receptive fields (those in which a stimulus causes a response detectable only in the presence of other stimuli), that have now been well demonstrated in vision. Taken together, these results suggest that IP3-activating odorants cause only suppressive or modulatory responses in ORNs, so their effect is not noticeable unless the cAMP pathway is also activated. Indeed, some odorants have been shown to suppress the inward current in newt olfactory receptor cells, by a mechanism that is distinct from inhibition and adaptation (Kurahashi et al., 1994). Suppression may sharpen the odorant specificities of single cells (Kurahashi et al., 1994). Given that most natural odorants are complex blends likely to contain components activating both pathways, an inhibitory or modulatory response may well be as important as an excitatory one in facilitation discrimination between odors.

Primary odors?

In vision, the concept of primary colors —the minimum set of colors necessary to create the percept of any color— has proved extremely useful, both from a theoretical standpoint, because knowing that vision depends on 3 to 4 receptor types is crucial to understanding color vision, and from a practical standpoint, because it has allowed the development of television using only 3 color signals (RGB) and of printers using only 4 ink colors (CMYK). In olfaction, in contrast, the concept has proved less fruitful, if only because the number of olfactory receptor types is so much greater than of photoreceptors. Nevertheless, attempts have been made to find primary colors via genetics, by finding inheritable specific anosmias —an increased olfactory threshold for specific compounds (Amoore, 1977). This is a tricky business, though: some anosmias could be due to regulator genes rather than receptor genes, and many receptor types could go unnoticed due to redundancy. In any case, we are still in the infancy of the mapping of the receptive fields, or set of agonists, for each OR.

How many odorant molecules are needed for behavioral detection? How many ORNs must be activated?

Like the visual system, the olfactory system both is remarkably sensitive and is able to operate over a huge dynamic range, possibly the entire available physical range. For the skunk odorant, for example, only about 40 receptor cells in the human nose need be stimulated by no more than nine molecules each to give a detectable odor sensation (chemoreception, Encyclopaedia Brittannica Online, 2001). Some insects have been reported to be capable of detecting single molecules (Kaissling and Thorson, 1980). At the other end of the scale, animals often discriminate odors at the physical maximum of concentration, given by saturated vapor —inside a flower for a bee or against a potential mate's behind for a dog, for example (Laurent, 1999).

Temporal resolution of olfactory receptor neurons

Adaptation and disadaptation rates determine the temporal response properties of sensory receptor cells. In olfaction, temporal filter properties of receptor cells are as yet poorly understood. In the lobster, antennular chemoreceptor cells recover from a 5–s adapting pulse after time intervals ranging from 1 to 60 s. After complete adaptation by the adapting pulse, individual cells recovered at different rates. After 1 s, a third of the cells respond with a mean response of 3 spikes/cell, representing approximately 20% recovery. Mean full recovery is within 25 s, with a time constant of 14 s, independent of stimulus concentration (Gomez and Atema, 1996b). A study in frog ORNs, on the contrary, found that the duration of adaptation increased with increasing concentration of the adapting pulse (Reisert and Matthews, 2000). The duration of adaptation has been found to increase with increasing durations of exposure (Getchell and Shepherd, 1978; Strausfeld and Kaissling, 1986).

The bandwidth of olfaction

In a provoking recent review, Gilles Laurent (2000) challenged readers to imagine reading an article with their noses: although possible in principle (one might learn to assign odors or concentrations to words or letters), the rate at which information could be conveyed appears to be low. Olfaction seems to be poor at following many or rapidly varying signals, he claimed, and is as such a low-bandwidth sense. Given humans' lack of reliance of olfaction, though, it might be more appropriate, in order to estimate olfaction's bandwidth, to think of a task that olfactory-guided animals might be adept at. Dogs, for example, are able to rapidly follow a track using odor cues: they are thus 'reading' the direction in which to go (an analog time-varying quantity requiring many bits for its digital representation) at every step of the way (Thesen et al., 1993). Dogs' ability to track on the fly is all

the more impressive when judged by the frequency with which my co-pilot fails to read a (visual) map in time to make the right turn in an unfamiliar environment.

Due to the difficulties involved in generating predictable and controllable natural odor stimuli, the stimuli used in the great majority of experiments on olfaction, unfortunately, including the ones described in this thesis, lack temporal structure: they usually consist of single pulses provided with relatively constant intensity from a nearby source. A step in the right direction has recently been made by two studies that used more natural odor sources that generate temporally varying plumes (Vickers et al., 2001; Stopfer and Laurent, unpublished).

It is true that sniffing usually occurs on a relatively slow time scale (Laurent, 1999), but it is not clear that olfaction is impervious to temporal fluctuations on a timescale faster than that of sniffing — vision is of course sensitive to temporal fluctuations faster than the frequency of eye blinks. Even though at least some insects produce stereotypic antennal movements in response to odor stimulation (Chee-Ruiter and Laurent, 1995), their olfactory systems clearly do not require those movements in order to respond to odors (Laurent and Naraghi, 1994; Laurent and Davidowitz, 1994; Laurent, Wehr and Davidowitz, 1996; Wehr and Laurent, 1996; Stopfer et al., 1997; MacLeod et al., 1998; this thesis). Lemon and Getz's data (1997) suggests that the olfactory system of the cockroach is indeed capable of updating its representations in response to stimulus changes every few tens of seconds, a timescale comparable to that of the visual system (Meister and Berry, 1999). Whether these rapid variations are present in natural odor plumes and whether they are behaviorally relevant remains to be ascertained. Electroantennogram (EAG) recordings in the presence of natural odor plumes in the field have already shown that insect receptors are capable of responding to temporal structure in the plumes that provides relevant information on distance to the source up to a frequency of at least 5 Hz (Murlis et al., 2000)[10]. The 5 Hz figure must be considered a minimum, given that the EAG is a very coarse measure of receptor activation and that responses at a

finer timescale of individual receptor neurons might be averaged away in it. Thus, it is important to remember that even if odor identity does not vary very rapidly in the field (even this is questionable for a bee rapidly flying from one flower to a neighboring one), this environmental constancy does not apply to the rapid fluctuations in concentration given by the spatiotemporal structure of odor plumes, and it may well be precisely these that occupy the bulk of the olfactory system's bandwidth. In summary, a characterization of the bandwidth of olfaction must await until we understand the nature of the information that the olfactory system recovers from natural olfactory stimuli.

Fetal ORNs respond to odors with no selectivity

Rat olfactory receptor neurons begin to differentiate from stem cells on day E10 of embryonic life in the rat. By day E16, the receptor epithelium is well populated and receptor neurons respond to odors. However, they were not selective. Each cell responded to nearly all of the substances in the stimulus set. The first synaptic connections between receptors and mitral cells are established on day E18. The olfactory marker protein appears first in the receptors on the same day. By day E21, single unit responses changed dramatically: the cells became selective, responding to about half of the substances in the set used (Gesteland et al., 1982).

The epithelial map of expression and patterning of genes encoding ORs can develop in mice lacking olfactory bulbs, suggesting retrograde influences of the bulb on the epithelium are not required (Sullivan et al., 1995). ORNs degenerate within 5-14 days of neuronal age in a bulbectomized adult rat, suggesting the olfactory bulb is necessary for prolonged survival of ORNs, as is the case in other

10. This timescale would allow a temporal representation of 4 cycles/stimulus in projection neurons in the antennal lobe of the locust (see below). The period in between bursts of detectable odor 'packets' is highly variable (Murlis et al., 2000), however, and longer interburst periods would allow longer temporal representations.

sensory systems (Schwob et al., 1992).

1.15 Noise reduction, analog to digital conversion and decorrelation: The convergence to the insect antennal lobe and the vertebrate olfactory bulb

1.15.1 Glomeruli: Converting a spatial code into an identity code

In insects, the antennal lobes (ALs), the structures immediately downstream of ORNs, exhibit an array of structurally and functionally identifiable glomeruli (Rospars and Hildebrand, 1992; Vickers et al. 1998; Galizia et al., 1998) from which classes of projection neurons (PNs) send axons to distributed nuclei in the forebrain or protocerebrum (Homberg et al., 1988). In vertebrates, the analogous structure is termed the olfactory bulb, and also exhibits glomeruli.

Glomeruli are compartments of neuropil constrained by glial cells to a location in the bulb or antennal lobe. Glial cells accomplish this by expressing tenascin-like molecules on their cell surface during the period of glomerulus formation. These molecules repel growing neurites of many AL neurons *in vitro* (see Hildebrand et al., 1997 for a review), and may constrain neuropil growth within glomeruli.

Similar molecules are involved in the formation of *barrels* in the rat primary somatosensory cortex, where inputs from each whisker are compartmentalized into units called barrels, which are the flat equivalent of glomeruli in the cortical surface.

By virtue of this compartmentalization, glomeruli convert a spatial code, embedded in the position of afferents, into an identity code, which carries information in the identity of the neurons activated. This would only be meaningful if there was indeed a spatial code in the afferents to the antennal

lobes and olfactory bulb. In 1994, Vassar and colleagues found that there is. The key to it lies in the projection pattern of olfactory receptor neurons to the antennal lobes and olfactory bulbs.

1.15.2 The convergence of like olfactory receptor neurons: Noise reduction?

ORNs expressing the same OR project to the same glomerulus

Using in situ hybridization with five different receptor probes, Vassar et al. (1994) demonstrated that axons from neurons expressing a given receptor converge on at most a few glomeruli within the olfactory bulb of the rat. Moreover, they found that the position of specific glomeruli is bilaterally symmetrical, and constant in different individuals. Each glomerulus receives converging inputs from about 3000 ipsilateral neurons (Meisami, 1979, 1989).

In Drosophila, ORNs, which are located within sensory hairs, send projections to one of 43 glomeruli within each antennal lobe of the brain (Laissue et al., 1999; Stocker, 1994). Drosophila ORNs expressing the same receptor project to the same one or two glomeruli in both the ipsilateral and contralateral antennal lobe (Vosshall et al., 2000). The sorting zone (SZ) region of the antennal nerve of the moth *Manduca sexta* comprises a glia-rich domain just outside the antennal lobe of the brain. During development, ingrowing olfactory receptor neuron (ORN) axons abruptly change their trajectories upon encountering this domain, lose association with their neighbors, and exit in large fascicles of axons destined for particular glomeruli.

Glomeruli have indeed been shown to be functional units in the encoding of odors in the input to the olfactory bulb and antennal lobe: 2-deoxyglucose, voltage-sensitive dyes and calcium imaging studies have all shown it is common to see a glomerulus respond in its entirety in response to an odor (Kauer and Cinelli, 1993; Galizia and Menzel, 2001).

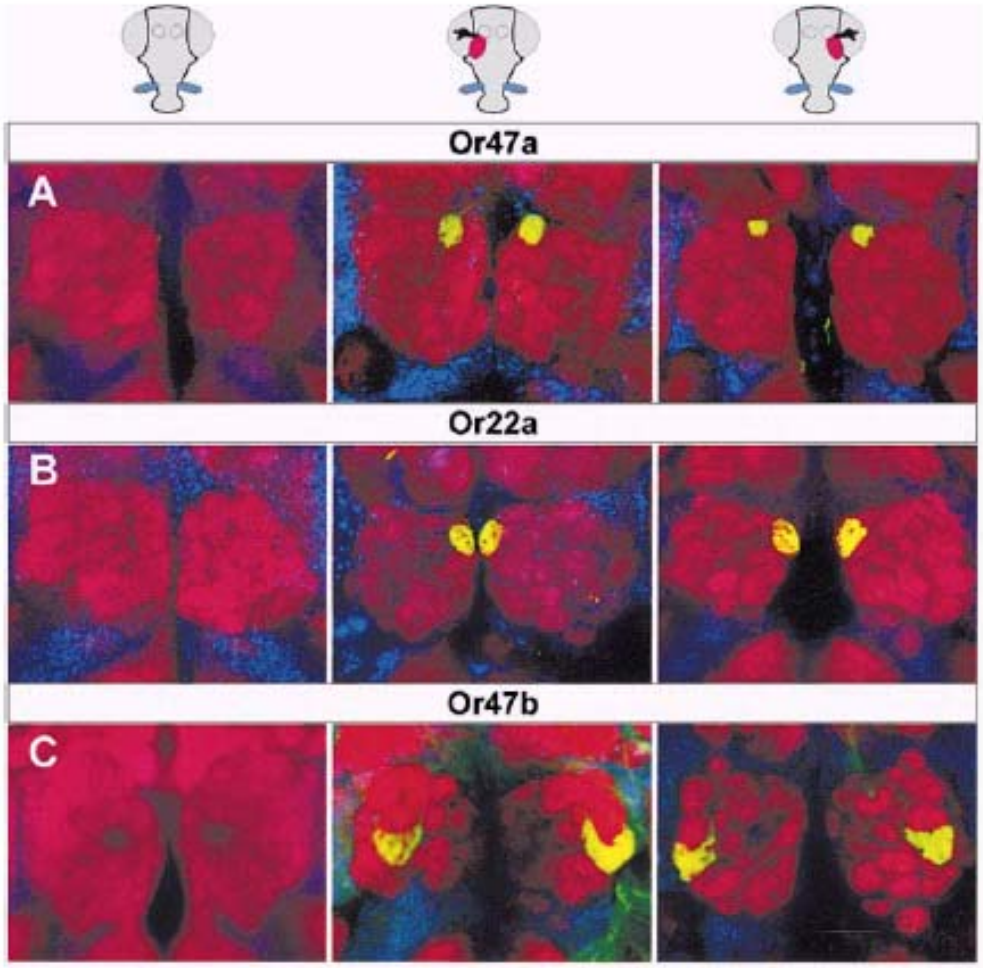


Figure 1.7. The set of receptor neurons expressing any given OR projects both ipsi- and contralaterally to 1-2 pair(s) of bilaterally symmetrical glomeruli in the fly *Drosophila melanogaster*. Cells expressing GFP under the control of different OR promoters project to different glomeruli. Bilateral deafferentiation resulted in complete loss of ORN staining in the antennal lobe (left). Unilateral deafferentiation of left (center) or right antenna (right) show labeling in both antennal lobes (from Vosshall et al., 2000).

The common wisdom is that convergence of like receptors to the same glomeruli achieves an increase of the signal to noise ratio through the averaging away of uncorrelated noise. We will discuss this further in §1.19.2.

Regarding summation of signals across afferents, we do know that the *Drosophila* mutant *gigas,* which establishes more synapses than normal, is attracted to concentrations of ethyl acetate to

which sibling controls are indifferent (Acebes and Ferrus, 2001). In addition, the intensity of responses is augmented at both attractive and repulsive odorant concentrations with respect to that of controls.

Are all inputs to a glomerulus equal? There is evidence to suggest they are not. In the mouse, input to a single glomerulus shows a dynamic range much greater than that reported for single neurons (Wachowiak and Cohen, 2001).

How do ORNs know where to project? A larger role for activity-dependence than previously suspected

The development of the complex connectivity pattern between ORNs and glomeruli is a flourishing field of study (see Mombaerts, 2001 for a recent review). Below, I summarize some exciting recent developments and put forth a novel proposal for an expanded role of activity dependence in shaping the connectivity pattern between epithelium and olfactory bulb.

Briefly, how ORNs know where to project is currently unknown. It has been argued that odorant receptor proteins are involved in the process of axon guidance to the bulb (Mombaerts et al., 1996; Wang et al., 1998; Mombaerts, 2001). The evidence for this is fourfold: 1. OR mRNA is present in ORN axons (Vassar et al., 1994), although data on OR protein expression in the axons is lacking; 2) mutations and deletions of the coding region of an OR gene lead to disruptions in the pattern of projection of the corresponding ORN to the bulb (Wang et al., 1998); 3) swapping one OR gene for another leads the ORNs to project to a third glomerulus that is neither the donor or the target (Mombaerts et al., 1996; Wang et al., 1998); 4) the projection patterns of ORNs and the odor response patterns of glomeruli are largely conserved across different individuals of the same species (Vassar et al., 1994; Galizia et al., 1998; Galizia and Menzel, 2001).

More recently, the developmental pattern has been shown to be more heterogeneous across the OR ensemble. In a study that shows the magnificent power of molecular biology used intelligently, Zheng et al. (2000) showed that, in mice rendered practically anosmic (Brunet et al., 1996; Parent et al., 1998) by a mutation in a cyclic nucleotide-gated channel, OCNC1, the projection pattern of ORNs expressing the P2 OR remain comparable to those in the wildtype, but that of ORNs expressing the M72 OR becomes more diffuse, terminating in additional glomeruli. When additional crosses were used to generate mice with both OCNC1-positive and OCNC1-negative ORNs expressing M72 in an OCNC1-negative background for ORNs expressing the rest of the ORs, axons from channel-positive and channel-negative neurons expressing the same receptor terminated in distinct glomeruli in the bulb. Importantly, OCNC1-negative axons converged mostly to glomeruli rather than projecting diffusely, and these glomeruli were close to the targets in the wild-type. Zheng and colleagues concluded that glomerulization per se and axonal pathfinding to a restricted area of the bulb are not dependent on OCNC1, and that neural activity subsequently refines the connectiviy pattern. In a follow-up study, Potter and colleagues showed that M72-expressing ORN axons occupy a large surface area of the bulb postnatally and coalesce into a single protoglomerulus only later in development, at a reproducible stage (Potter et al., 2001).

In a related truly beautiful and groundbreaking recent study, Zhao and Reed (2001) exploited the same phenomenon used by Zheng et al., X inactivation, to generate a mosaic mouse, half of whose cells expressed a wild-type copy of OCNC1 and half of which expressed a mutant, inactivated copy together with a reporter gene. X inactivation is a natural phenomenon through which one of the two chromosomes in any female gets inactivated for transcription. The mosaic mouse allowed Zhao and Reed to study the effects of odorant-induced activity on competition, by setting up a situation in which cells which differ only in whether they exhibit odor-induced activity or not compete with each other for innervation of glomeruli. In male mice hemizygous OCNC1-deficient mice, whose neurons do not compete with OCNC1-wild-type neurons, the epithelium and olfactory bulb was found to be

morphologically normal. The normality in the face of a lack in odor-induced activity prompted Zhao and Reed to predict that the epithelium of heterozygous females would consist of a mosaic of wild-type and mutant ORNs. To their surprise, however, they found that the epithelium of adults consisted apparently entirely of wild-type neurons. In contrast, the epithelium of neonatal animals consisted of both mutant and wild-type neurons. Zhao and Reed hypothesized that homozygous OCNC1-deficient females would result in a noncompetitive situation and show a mosaic of mutant and wild-type neurons, and found that, indeed, roughly equal numbers of patches of mutant and wild-type neurons are visible. The clusters were not mutually exclusive, but were intermingled in the epithelium and in each glomerulus of the bulb.

Even more interestingly, unilateral naris occlusion shortly after birth led to a recovery of the mutant population, such that at 40 days to 4 months afterwards the epithelium had both mutant and wild-type neurons, much like the hemizygous male's. The unoccluded hemisphere —ORNs project ipsilaterally to the bulb— showed a depletion of mutant neurons. Zhao and Reed concluded that ORNs compete with each other for scarce resources potentially provided by target downstream neurons, and that odor-mediated neural activity gives a neuron an advantage in that competition. When all neurons are subject to the same lack of neural activity, no type is preferentially impaired. When one group of ORNs has a selective disruption of neural activity, that group of neurons is disadvantaged in the competition.

Why did Zhao and Reed see a depletion in mutant neurons with reduced odorant-induced activity while Zheng et al. did not? The difference may lie in the fact that Zhao and Reed's neurons faced competition from odorant-induced-activity-positive neurons expressing *each* of the 1000 OR's in the mouse repertoire, while Zheng et al.'s faced competition only from odorant-induced-activity-positive neurons expressing the M72 OR. This means that Zheng et al's OCNC1-deficient neurons were not at a relative disadvantage with respect to other neurons in any glomeruli but the site of projection of

wild-type M72. This may explain both why the channel-negative neurons survived and why they projected to a different glomerulus than the wildtype.

There is a further apparent contradiction between Zhao and Reed's findings and Zheng et al's. Zhao and Reed report that without competition, male hemizygous OCNC1-deficient mice have normal epithelium and bulb. Zheng et al., on the other hand, report that M72-expressing ORNs have abnormal projection patterns in an OCNC-1 deficient background. I presume that given that Zhao and Reed's mice had all ORNs equally labeled, though, it would be hard for them to notice an additional glomerulus in the projection pattern of a particular OR type. Alternatively, if they can indeed see M72's projection pattern to be normal, M72 may be protected by a lack of its ligand in their laboratory's olfactory environment, a ligand that may be present in Zheng et al's laboratory. This could be tested by analyzing whether M72's projection pattern is affected in Zhao and Reed's female mice (whose OCNC-1-negative neurons do face competition, but only in the face of olfactory stimulation).

Zhao and Reed's findings warrant a major reevaluation of the olfactory development literature far beyond their paper's claims. The demonstration, firstly, that ORN axons compete for survival, secondly, that this survival is activity dependent, and thirdly, that activity plays a permissive role only in a competitive situation, as shown by the fact that OCNC1-negative neurons are unaffected in olfactory-deprived hemispheres, suggests a larger role for activity-dependence in the establishment of the projection pattern of ORNs to the bulb. Importantly, it provides an alternative explanation for the previous finding that deletion or substitution of an OR gene affects the corresponding ORNs' projection pattern (Mombaerts et al., 1996; Wang et al., 1998): rather than owing to the lack of the OR gene products in the axons, the abnormalities could be due to the corresponding alteration in odor-induced activity of the neurons. This possibility had been downplayed after the discovery that practically anosmic mice developed normal projection patterns for P2-expressing ORNs (Belluscio et al., 1998; Lin et al., 2000). These mice, however, faced a uniform down-regulation of activity in all

ORNs, though, and thus would presumably not have been affected by the activity-dependence demonstrated by Zhao and Reed, that surfaces only upon competition with ORNs exhibiting normal activity. A small remnant of odorant-induced activity, as indeed observed in the so-called “anosmic” mutants, particularly among newborns (Belluscio et al., 1998; Zhao and Reed, 2001), could be enough to bring the competiiton-based mechanisms observed by Zhao and Reed into play. Indeed, the small remnant of activity left in OCNC-1-negative neurons in hemizygous males was enough to prevent the odorant activity-dependent phenotype of OCNC-1 +/- heterozygous females. Furthermore, ORN activity or lack thereof was not assayed by single-cell recordings, but only indirectly via electro-olfactograms (EOG). The generality of the conclusions stemming from the analysis of the projections of P2-expressing ORNs is further put in doubt by the discovery that the development of ORN projection patterns is heterogeneous for different ORs (Zheng et al., 2000; Potter et al., 2001), and that inferences about the lack of a requirement for odor-induced activity have been generally based on analysis of one or two ORs (e.g., Lin et al., 2000).

Zhao and Reed’s paper allows a further important conclusion to be drawn. Because olfactory deprivation led to the elimination of any competitive advantage of neurons with a wild-type OCNC1 allele, constitutive activity, such as the wave-like activity found in the retina that guides visual development (Stellwagen and Shatz, 2002), cannot be responsible for the development of ORN axons —discarding one of three modes of OCNC1 action proposed by Zheng et al. (2000). At least two reasons come to mind to explain this difference between the visual and olfactory systems. First, since neighbor relations in the olfactory epithelium do not correlate with functional proximity, as opposed to the visual, auditory and somatosensory epithelium, wave-like spontaneous bursts of activity propagating through gap-junctions cannot generate a meaningful wiring pattern in which similar inputs wire together. Second, unlike the mammalian visual system, which remains in darkness during development due to eyelid closure and the poverty of the womb as a visual environment, olfactory responsiveness has been demonstrated at early embryonic stages in the rat (Gesteland et al., 1982),

rabbit (Hudson and Distel, 1997) and human (Schaal et al., 2000). Natural stimuli are thus available to guide olfactory development since early on.

An activity-dependent competitive scenario for the establishment of ORN-glomerular projection patterns is attractive from several standpoints. Firstly, it would obviate the need for a large set of axon guidance molecules to interact with the set of 1000 OR proteins which have herefore been postulated as axon guidance cues. With the set of ORs already constituting 1 in every 30 genes in the mouse and human genomes, the need for an additional set of receptors for them might prove embarrasingly expensive for our as-of-recent smaller-than-expected genome (Venter et al., 2001; Lander et al., 2001). Secondly, activity-dependent competition is the norm in the development of other senses, and has been demonstrated particularly thoroughly in the visual system. The demonstration that the same mechanisms are at work in the olfactory system would prove satisfyingly parsimonious. Thirdly, simple mathematical models of activity-dependent unsupervised learning have been shown to maximize the difference between the responses of different mitral cells in a model of adult neurogenesis of granule cells (Cecchi et al., 2001). The same class of models (see below) could be used to demonstrate how simple competitive rules could lead to ORNs expressing different ORs survive differentially in different glomeruli starting out with broad projection patterns that are pruned via differential survival, as seen experimentally (Potter et al., 2001). Finally, as noted by Zhao and Reed (2001), an activity-dependent projection scheme would prove evolutionarily helpful, since a mutation in an OR gene, which caused a change in its odor-mediated activity pattern, would automatically and simultaneously eliminate the corresponding ORNs' projection to their old target and lead them to assemble into a novel and unique glomerulus.

Towards an activity-dependent model for the generation of the ORN-glomerular mapping

How would activity regulate the formation of the projection map seen experimentally and ensure that ORNs expressing different ORs end up projecting to different glomeruli? I propose a model with a) initially broad projections of ORNs across the bulb (observed by Potter et al., 2001), b) slight initial inhomogeneities across different areas of the bulb in connection numbers or strength of ORNs expressing any given OR type, such as could be generated by a gradient of neurotrophins, c) winner-take-all competition between ORNs expressing different receptor genes such that only ORNs with similar patterns of activity survive in the projection to any one glomerulus, as observed empirically by Zhao and Reed (2001), and thus each glomerulus eventually receives input only from ORNs expressing one OR type, and d) winner-take-all competition between glomeruli for ORNs expressing any given OR type, such that ORNs expressing any given OR type will project to only one glomerulus (or one in each of *n* separate maps, such as observed for the medial and lateral bulb). The latter competition, which need be OR-specific, may be implemented via granule-cell-mediated inhibition, whose specificity could derive from STDP in the synapses between granule cells: the winning glomerulus, due to its larger synaptic efficacy, would result in earlier firing than the rest and would thus precede spikes in granule cells, but all other M-T cells would fire later, leading to strengthening of the inhibitory synapses to them, eventually leading to the retraction of the corresponding ORN projections, due to a decreased ability to make the postsynaptic cell fire.

Importantly, the initial heterogeneities in connection strengths or numbers do not need to be different for ORNs expressing each OR type: a spatially distributed projection map is ensured nevertheless because once ORNs for one OR type take over one glomerulus, the winner-take-all competitive mechanism between ORNs with different activity patterns will have pruned the synapses of ORNs expressing other ORs and thus forcing to project to another glomerulus. Alternatively, the initial gradient could be a double opposing gradient, such that different relative affinities of

each receptor for each of the two guiding molecules would lead to a different spot of maximal attraction (Ofer Mazor, personal communication). This model requires that most every receptor type bind to some degree to each of the guiding molecules, something that would only be expected if there are conserved regions across all receptor types.

The consequence of this set of assumptions is a diagonalization of the OR-glomerulus matrix, as described for adult neurogenesis of granule cells (Cecchi et al., 2001), via the following sequence of events: 1) all ORNs being with broad projection patterns across much of the bulb, 2) ORNs with the strongest odor-evoked activity win over competition for the glomerulus to which they project most strongly due to initial inhomogeneities, while a winner-take-all competitive mechanism between glomeruli mediated by lateral inhibition prunes out projections of those ORNs to all other glomeruli, 3) the process in (2) is repeated for ORNs expressing the next strongest driven OR, with the constraint that a glomerulus that has already been dominated by ORNs expressing a different OR are not available for further colonization, and so on until all ORNs expressing a given OR project to a unique glomerulus.

The model makes predictions that can be tested experimentally. First, it predicts that ORNs expressing different ORs with the same odorant specificities will project to the same glomeruli. Second, it predicts that odor exposure during early development will guide the order in which ORNs expressing each OR type will take over given glomeruli. Thus, changing such early exposure through the introduction of exogenous odorants *in utero* should lead to a change in the order in which the projection patterns of ORNs expressing individual ORs crystallize into a unique glomerulus. Such changes in the temporal sequence of development for ORNs expressing each OR may or may not lead to a change in the final mapping, depending on the specificity of the initial bias present in genetic chemical gradients for different ORs.

The final word is not said, and the picture is likely to include a combination of genetic cues and activ-

ity-dependent modifications. It is too early to say the degree to which activity-dependent competitive mechanisms shape olfactory development. It is also too late to say they don't.

Multiglomerular projection patterns of locust ORNs

In contrast to mammals and flies, all single locust ORNs stained have been found to project to several (~2-6) glomeruli in the antennal lobe (Hansson et al., 1996). The total number of glomeruli in *Schistocerca gregaria* has been found to be over 1000 (Hansson et al., 1996), substantially more than in the fly or bee brains. Combined with the fact that locust projection neurons arborize in several glomeruli as opposed to a single one for most other species studied (see §1.15.3), this suggests that a single glomerulus in other species may be analogous to a group of several glomeruli in the locust. Whether glomeruli innervated by any one PN are isofunctional or not remains to be tested.

1.15.3 The anatomy of the vertebrate olfactory bulb and insect antennal lobe

The insect antennal lobe is composed of two main classes of neurons: spiking excitatory output neurons called projection neurons (PNs) and inhibitory local neurons (LNs). In the locust, PNs number about 830, fire action potentials and arborize in several glomeruli. In other insect species as well as in mammals, PNs arborize in a single glomerulus. Locust LNs number 300 or so and are non-spiking, althoug this is not generally the case in other species. PNs respond to a subset of odors; LNs appear to be tuned more broadly —less sparsely— both in time and in odor space.

The existence of two major tracts between the primary and secondary olfactory processing areas is a common neural organization of olfactory systems, both in vertebrates and insects. In the honey-

bee, functional subdivisions of PNs have been described (Mueller et al., 2001): morphologically distinct subpopulations of PNs projecting to different regions of the mushroom bodies were seen to have different coding properties. lACT neurons appeared to code odors by spike rate differences, have broader response profiles to different odors, and convey the information rapidly. Neurons in the mACT tract coded odors by latency differences, had more specific response profiles, and conveyed the information with a delay. Thus more general information about the olfactory stimulus might reach the mushroom bodies first via lACT neurons, and then mACT neurons add more specific odor information.

In vertebrates, the architecture is functionally very similar, although somewhat more complex. The projection neurons are mitral and tufted cells. These neurons are similar in many ways, so they are often lumped together as mitral/tufted cells. In mammals, both send their primary dendrite to a single glomerulus. In amphibians, fish and reptiles, mitral cells can receive input from several glomeruli through multiple apical dendrites (Herrick, 1931). Both cell types are excitatory and project to the piriform cortex via the lateral olfactory tract. In addition to mitral and tufted cells, periglomerular cells also receive input from glomeruli, including ORNs, mitral/tufted cells. Their axons, which ramify in other glomeruli, are GABAergic but have been shown to have an excitatory action on each other and on mitral/tufted cells. Such GABA-mediated excitation has been described elsewhere (Cherubini et al., 1991; Michelson and Wong, 1991), and is believed to come about through an inverted chloride potential across postsynaptic cell membranes. The inhibitory cells in the bulb are termed granule cells. They make dendrodendritic synapse with other granule cells and with the basal dendrites of mitral/tufted cells, which can extend laterally for up to 1 mm in rats. Finally, there is a little known small population of short axon cells (Scott et al., 1987).

Recently, a precise intrabulbar connection of tufted cells was shown to link glomeruli in the medial and lateral sides of the bulb that receive input from ORNs expressing the same OR type (Lodovichi

et al., 2001). As this projection terminates in the granule cell layer, which provides inhibitory input to the external tufted cells, this finding reveals that a set of mutually inhibitory connections link isofunctional glomeruli in the medial and lateral maps of olfactory receptors.

It has been argued that the olfactory bulb is analogous to primary sensory cortices in other senses (Johnson et al., 2000), while piriform cortex is analogous to associations cortices (see below).

1.15.4 Odors are represented by overlapping assemblies of PNs

Because individual odorant receptors respond to a variety of odorants, and potentially also because individual PNs sample from several glomeruli, PNs respond to a variety of odorants (Laurent and Davidowitz, 1994; Wehr and Laurent, 1996; Laurent et al., 1996; this thesis). Thus, odors are said to be coded in a combinatorial or distributed code, with the identity of an odor at any time encoded in the set of active PNs.

1.15.5 Oscillatory inhibition: Analog to digital conversion

A striking characteristic of neurons in the insect antennal lobe and the vertebrate olfactory bulb is their tendency to respond to odor stimulation with oscillations in their membrane potential and their firing rates. Since their original discovery in the hedgehog (Adrian, 1942, 1950), these oscillations have been found in the cells themselves or in the local field potential of many vertebrate species: fish (including rainbow trout, salmon, char), amphibians (frog, toad), reptiles (iguana, caiman, snakes and turtle), birds (including albatross, duck, vulture, pigeon and others), and mammals (rat, rabbit, cat, dog, monkey and human)[11].

In insects, the oscillations were first described in the locust (Laurent and Dawidowitz, 1994) and have since been shown in the honeybee (Stopfer et al., 1997), moth (Wu et al., 1995), wasp and cockroach (Stopfer et al., 1999). Oscillations have also been found in the olfactory system of molluscs (Gelperin and Tank, 1990).

Oscillatory synchronization is not exclusive to the olfactory system. It has been described in the visual cortex of cat and monkey (Eckhorn et al., 1988; Gray et al., 1989, 1990, 1992; Engel et al., 1990; Livingstone, 1996), motor cortex (Murthy and Fetz, 1992, 1996, 1996b), and hippocampus (Whittington et al., 1995).

In the locust, this oscillatory synchronization involves different PNs in different epoques of the response, and is also odor dependent (Laurent and Davidowitz, 1994; Laurent et al., 1996). The oscillations require fast (20 Hz) periodic GABA inhibition by local neurons in the antennal lobe (MacLeod and Laurent, 1996), which do not fire action potentials but do produce Calcium-like spikelets. When the oscillatory synchronization was disrupted by injecting a GABA antagonist, picrotoxin, in the antennal lobe, the ability of bees to discriminate between chemically similar odorants was impaired (Stopfer et al., 1997). The ability to discriminate between chemically unrelated odorants was not impaired.

By dividing responses in discrete cycles of the oscillations, the system may be effectively digitizing the signals, converting analog time into a digital series of vectors, where each element represents one PN.

When I embarked on the investigations described in this thesis, the functional role of oscillatory synchronization was unknown. Chapter 5 is devoted to a series of explorations that begin to address this issue.

11. From 33 references, the originals of which are cited in Wehr (1999), pp.37-38.

1.15.6 Temporal patterning of responses: Decorrelation

Projection neurons in the antennal lobe and mitral cells in the olfactory bulb respond to odors with temporally complex patterns of excitation and inhibition (Kauer, 1974; Laurent and Davidowitz, 1994). For some neurons, the response patterns can even change from one oscillation cycle to the next (Wehr and Laurent, 1996); most neurons, however, show a high correlation in the timescale of hundreds of milliseconds in their response properties (see Chapter 4). These changes mean that, over the entire PN assembly, the set of PNs that fires changes as the response dynamics unfold.

What these response dynamics, which occur in response to static (non changing) stimuli, buy for the animal remains unclear. Friedrich and Laurent (2001) have shown that in fish, the responses for chemically similar odorants get decorrelated as the response evolves in time. What is most remarkable about their results, though, is that while the dynamics in the antennal lobe amplified initial differences between the representations of chemically similar odorants, the variance across trials decreased slightly. In other words, unless the stimuli were *exactly* identical across multiple trials, we can assume that the dynamics suppressed the differences between stimuli that were very close together, or that had acquired a common functional meaning, while amplifying differences between stimuli that were farther apart, or that had different functional meanings. How the system manages to retain this sensitivity to small differences in stimulus space without simultaneously increasing intertrial variabiilty remains a mystery. The response sparseness, on the other hand, remained constant in time. Functionally, the system may be remapping chemical odorant space into a representation space that is more regularly populated, a process that can ease odor classification by increasing the distance between closest neighbors in stimulus space. A caveat of these experiments, though, is that only three trials were presented for each odor/cell pair, so intertrial variability may have been hard to assess, and could in any case not be representative of steady-state

responses, since the first few trials of any odor presentation are known to evoke responses different from later ones in the locust (Stopfer and Laurent, 1997). A second caveat is that the neurons were not recorded simultaneously but rather over many animals.

1.16 Sparsifying, digital decoding, intermodal association and learning: The divergence to the insect mushroom bodies and the vertebrate piriform cortex

1.16.1 The insect mushroom bodies

PNs in insects project to Kenyon cells (KCs) in the mushroom bodies, so called because of their shape, and continue on to synapse on the lateral protocerebral lobe. Kenyon cells project on to α- and ß-lobe neurons, which feed back onto Kenyon cells, forming a loop. Very little is known about how information processing continues beyond the mushroom bodies.

Kenyon cells show a long after-spike-hyperpolarization period and exhibit sparse response patterns to odors, both in time and in odor space: they respond with one or few spikes and only to one or few odors, although there appears to be some heterogeneity across the population (Pérez-Orive et al., 2002). KCs can exhibit high reliability across multiple presentation of the same stimulus (Pérez-Orive et al., 2002).

Kenyon cells exhibit non-linearities which are consistent with a role in coincidence detection across PN inputs (Pérez-Orive et al., 2002). Furthermore, their membrane potential oscillates at a fixed offset to PN membrane voltage oscillations, and are systematically inhibited during part of each oscillation cycle. Thus, it is conceivable that their spikes happen in response to synchronized spikes only, and that the KC assembly provides a readout of which PN combinations are active and synchronized at any given cycle of the oscillations.

An intriguing observation is that Kenyon cells of adult Drosophila exhibit synchronous oscillation of intracellular calcium concentration, with a mean period of approximately 4 min (Rosay et al., 2001).

The mushroom bodies have been implicated in learning and memory both through lesions and genetic studies. Chemical ablation of the mushroom bodies leads to total loss of olfactory learning (de Belle and Heisenberg, 1994). More subtle manipulations have shown a requirement for mushroom body signaling during memory retrieval, but not during acquisition or consolidation (McGuire et al., 2001; Dubnau et al., 2001). The mushroom bodies have also been implicated in the perception of odor attractiveness, but not aversiveness (Want et al., 2001). In other words, blocking the output of Kenyon cells does not interfere with memory acquisition, which suggests that memories are encoded upstream of KCs, possibly in ORN-PN and/or PN-KC synapses.

The mushroom bodies also receive multimodal inputs, including visual and tactile inputs in addition to olfactory ones (Strausfeld and Li, 1999; Li and Strausfeld, 1999).

1.16.2 The mammalian piriform cortex

As opposed to other senses, which project to cortex through the thalamus, mammalian mitral/tufted cells project directly to pyramidal cells in piriform cortex. Piriform cortex, also called paleocortex, is a three-layered structured simiar to reptilian cortex, in contrast to the six-layered neocortex of all other primary sensory areas. Layer 1 consists of pyramidal apical dendrites, afferent mitral cell fibers and intrinsic cortico-cortical collaterals. Layer 2 contains pyramidal cell bodies, and layer 3 has basal dendrites of pyramidal cells, deep pyramidal cell bodies, and multipolar neurons. The set of cortico-cortical collaterals, or association pathway, has been likened to the recurrent feedback chatacteristic of artificial neural network models. Deep within the piriform cortex lies the endopiriform nucleus, a large group of multipolar cells.

Piriform and endopiriform efferents target cortical rather than nuclear structures. Extensive projections from the endopiriform nucleus extend to most basal forebrain areas including the piriform cortex, entorhinal cortex, insular cortex, orbital cortex, and all cortical amygdaloid areas. The perirhinal cortex, olfactory tubercle, and most subdivisions of the hippocampal formation receive light projections. Projections are highly distributed spatially within all target areas. Efferent axons from the endopiriform nucleus are unmyelinated and give rise to boutons along their entire course rather than arborizing locally. Efferents from the endopiriform nucleus lack the precise laminar order of those from the piriform cortex, and provide a heavy caudal to rostral pathway that is lacking in the cortex (Behan and Haberly, 1999).

Pyramidal cells in piriform cortex project widely to many areas of cortex, including areas involved in high-order function (Johnson et al., 2000). This has lead Johnson et al. (2000) to compare piriform cortex with association cortices in other sensory modalities, rather than with primary cortices. Within piriform cortex, pyramidal cells' arbors are highly distributed with no regularly arranged patchy concentrations like those associated with the columnar organization in other primary sensory areas (i.e., where periodically arranged sets of cells have common response properties, inputs, and outputs) (Johnson et al., 2000). A lack of columnar organization is also indicated by a marked disparity in the intrinsic projection patterns of neighboring injected cells (Johnson et al., 2000).

1.17 Beyond

Beyond piriform cortex, our knowledge is only sketchy.

Neuronal responses in rat orbitofrontal cortex are more likely to reflect associations between simultaneously trained odors than between odors that predict similar responses (Alvarez and

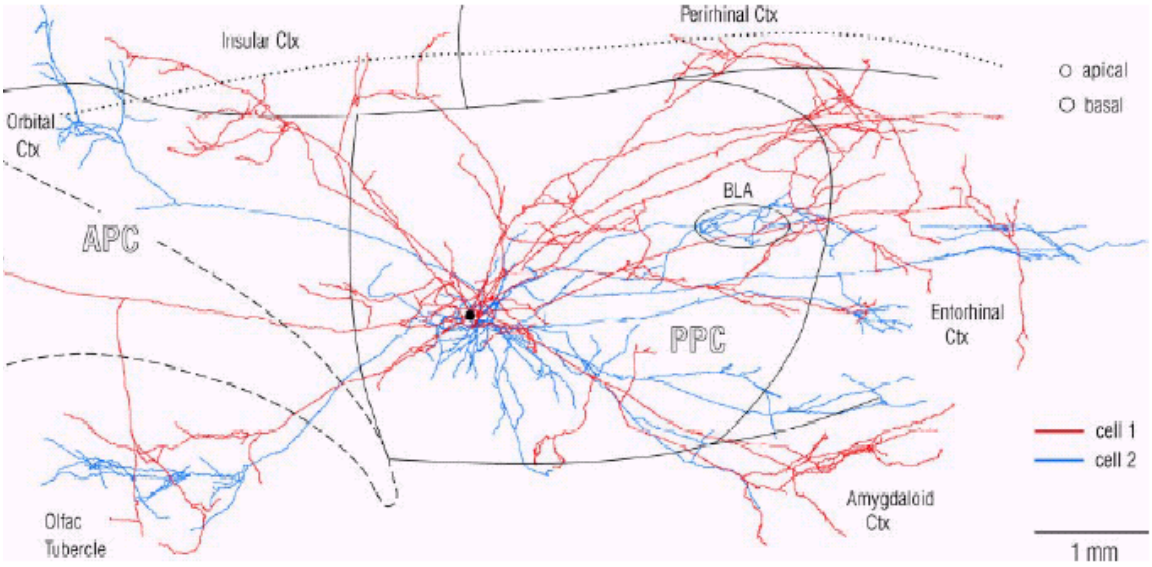


Figure 1.8. Pyramidal cells in piriform cortex project widely over large regions of cortex. Association (cortico-cortical) axons from a pair of neighboring superficial pyramidal cells in posterior piriform cortex. Note the minimal overlap of the two axonal arbors outside the ;1 mm diameter local collateral region that surrounds SP somata. The arborizations from the second cell (*blue*) in the orbital cortex (*top left*) and basolateral amygdala (*BLA, oval*) are deep to piriform cortex. The *black spot* indicates the position of the cell bodies. The *circles* at *top right* denote typical diameters of pyramidal cell dendritic trees at the depths where they are contacted by association fibers (proximal apical dendrites in layer Ib and basal dendrites in layer III). The borders of piriform cortex and the insular-perirhinal border are indicated by *solid lines*; the *dashed line* outlines the lateral olfactory tract; the *dotted line* is the rhinal sulcus. From Johnson et al.,

Eichenbaum, 2001).

The parahippocampus has been involved in the discrimination of the odor of individuals in hamsters, which use scent-marking (Petrulis et al., 2000).

Single-unit activity of 15% of the neurons recorded in the human amygdala was positively correlated with perceived odor unpleasantness (Buchanan et al., 2001).

Finally, hemispheric asymmetries have been found in olfactory recognition of artificial, but not natural, odors (Ilmberger et al., 2001).

1.18 Neuromodulators and learning

The field of olfactory learning and neuromodulators is outside the scope of this introduction, but I will point out two outstanding series of studies (see also reviews by Hasselmo and Bower, 1993; Hasselmo, 1995).

An octopaminergic neuron mediates unconditioned stimuli in the bee brain

In a beautiful study, Martin Hammer showed that, in the honeybee, an identified octopaminergic neuron mediates the effect of the unconditioned stimulus (US) in learning, and showed that you could replace the US by injection of octopamine (Hammer, 1993; Hammer and Menzel, 1995).

Oxytocin mediates attachment in the mammalian brain

Oxytocin is a neural mediator of attachment, both to sexual partners (Shapiro et al., 1991; Winslow et al., 1993; Williams et al., 1994; Insel et al., 1997) and for infant-mother relations (Nelson and Panksepp, 1998).

Oxytocin induces recognition acting on the olfactory bulb

Oxytocin induces preservation of social recognition and the induction of maternal behavior in rats by activating receptors in the olfactory bulb (Yu et al., 1996, 1996b; Dluzen et al., 2000).

1.19 Olfaction: A sense of variance

As we saw in §1.10.3, even though most of olfactory research has gone into unraveling how the olfactory system encodes the chemical identity of odors, it is perhaps in the encoding and decoding of spatiotemporal patterns of odor identity and intensity that most of the complexities of olfaction lie. Many odor-driven behaviors, such as the search for a mate in moths, depend on the analysis of chemically simple but physically complex signals that allow orientation as well as detection (Laurent, 1999).

1.19.1 Evaluating variance takes time…

If the olfactory system extracts information out of fluctuations in the absolute and relative intensities of different odorants, it must compare intensities over time and/or space. Do responses of projection neurons in the antennal lobe of the locust correlate better to differences in odor intensity than to absolute concentrations? This important question remains unanswered, although the means to answer it are now at our disposal.

1.19.2 …or space and convergence: Olfactory images and spatial codes in olfaction?

Alternatively, fluctuations could be evaluated spatially, as the differences between activation of identical receptors in different locations of the epithelium. Let us examine this possibility, hitherto largely unexplored. The olfactory epithelium of a dog has a surface area larger than that of the retina (Adrian, 1951). Furthermore, unlike in the visual, auditory and somatosensory systems, the spatial coordinates of olfactory receptors do not correlate with their physiological properties. Also differently

from vision, hearing and touch, the projection patterns of olfactory receptors do not preserve neighbor relationships between source and target. It has been argued that this may reflect the fact that olfaction is not a spatial sense (Zheng et al., 2001). If olfaction was a non-spatial sense, though, why have such a large sensory surface? The conventional explanation suggests that this redundancy, coupled with the convergence of olfactory receptor neurons (ORNs) expressing the same receptor genes onto the same glomeruli in the olfactory bulb of vertebrates or the antennal lobe of insects, is there to sum over similar inputs and reduce noise (Laurent, 1999). Yet there is no a priori reason why a large organ should be more sensitive to smells than a small one (Adrian, 1951). While it is certainly plausible that summing across receptors is indeed what convergence in glomeruli accomplishes, the lack of evidence for it does not warrant the widespread assumption that this is the case. In the visual system, convergence of many rods onto a single retinoganglion cell is effective because a) the physics of light transmission ensures that different photoreceptors will receive different inputs corresponding to different points in the visual field, and b) noise in the photoreceptors is indeed the limiting factor for light detection (Meister and Berry, 1999). There is no compelling evidence to my knowledge, however, that olfactory receptor neurons are both sufficiently noisy and sufficiently uncorrelated that such a summation would indeed help much. Individual ORNs, in fact, appear to have basal firing rates more than five times lower than photoreceptors (compare Lemon and Getz, 1997, with Meister and Berry, 1999). It is noteworthy that in the auditory system, there are only about 15,000 hair cells in each ear (SFN, 1994), only 3500 of which are inner hair cells, less than 1/10,000th of the number of odorant receptor cells in humans and less than 1/200,000th of that number in dogs. Hecht, Schlar and Perrine demonstrated in the 40's that human subjects can reliably detect single photons (Boroditsky, 1999; Wandell, 1995), thus showing that light can also be detected reliably without the benefit of summation across receptor neurons. In other words, biology does not require a large number of identical cells in order to obtain a trustworthy signal: sensory neurons are in general reliable detectors. Furthermore, even if ORNs turn out to be affected by sub-

stantial uncorrelated noise, it is unclear that this could not be averaged away without convergence of like receptors onto the same targets. Odor responses of Kenyon cells, for example, are more reliable than those of their PN inputs, even if each PN has a different 'tuning curve.'

But a large epithelial surface will certainly provide a screen on which the pattern of excitation can be mapped in greater detail (Adrian, 1951). In fact, the nose has a complex structure that seems designed to ensure that the stimulus is distributed inhomogeneously over the epithelium (Adrian, 1951). Rather than *summing* over inputs to *reduce* variance (Laurent, 1999), convergence may perhaps be *estimating variance* across the olfactory epithelium, extracting information from an olfactory *image*.

Is there any plausible biophysical implementation of the computation of the variance in the activity of inputs? Indeed, stochastic resonance has been shown to increase neuronal firing rates as a function of noise levels, i.e., as a function of the variance in the signal (Douglass et al., 1993; Gammaitoni et al., 1998). Although in the past these mechanisms have been proposed to use noise to amplify signals, they could potentially be exploited to measure noise *per se*.

This new hypothesis is consistent with both the one cell – one gene expressed finding and the allelic exclusion exhibited by olfactory receptor genes. If the responses of individual ORNs were homogenous across the epithelium, there would be little incentive to force the expression of a single allele – indeed, cells in the VNO express multiple receptors (Martini et al., 2001): the discrimination of odors can be done regardless of whether the 'bases' of the vectorial representation of odors represent pure odotopes or a more complex chemical signature—; if, on the contrary, activation was diverse across the epithelium and the olfactory system extracted information from the analysis of *differences* across ORNs, then there would be an advantage to having the detectors be homogeneous: otherwise, differences in the spatial pattern of activation of one receptor would be multiplexed and confused with differences in the spatial pattern of activation of another receptor expressed in the same

cells, differences that need not always be aligned. If proved correct, then, this hypothesis could serve to explain the evolutionary reasons behind (i) the one cell – one gene expression pattern, (ii) allelic exclusion, (iii) the nonlocalized distribution of ORNs expressing the same receptor gene, (iv) the large surface of the olfactory epithelium and (v) convergence of cells expressing the same receptor type onto the same glomeruli.

How could one test such a hypothesis? It would predict that differential stimulation of two ORNs (or groups of ORNs) expressing the same receptor type would excite some mitral cells (or insect PNs) in the glomerulus the ORNs innervate more than identical stimulation of both. In contrast, the traditional summing hypothesis predicts that stimulation of additional receptors will always lead to additional excitation of all mitral cells or PNs in the corresponding glomerulus, and thus stimulation with a strong stimulus to both groups of ORNs would lead to more excitation of than stimulation of one group of ORNs with a high concentration and stimulation of the other group with a low concentration. The test would need to be careful to stimulate only one receptor type to ensure that stimulating an additional site affects only the sum and difference of excitation of receptors of the same type, and does not introduce a confound of additional receptor types stimulated. This is particularly important given the uneven distribution of receptor types across the epithelium. Second, the test should measure the activity of PNs or MCs innervating the glomerulus targeted by the receptor type stimulated, to measure the direct action of convergence onto the glomerulus, and not indirect effects due to bulb or AL dynamics. Lastly, the test should be exhaustive, since the coexistence of summation and variance estimation may occur in different mitral cells or PNs.

While this hypothesis is entirely speculative at this point, its possibility should serve to focus efforts to demonstrate the role of glomerular convergence rather than assume it to be the simplest alternative.

1.20 Architectural differences with the immune system

It is of considerable interest to compare the olfactory system with biology's other major chemosensory system, the immune system, to find similarities and differences in the strategies they evolved, speculate on the functional reasons behind them, and draw on our knowledge of one to make predictions for the other.

1.20.1 Output requirements and specificity of response

The output requirement of the immune system is very different from that of the olfactory one: while olfaction needs only to recognize chemical signals, but not act on the signals themselves, the immune system has to mount an attack on them. The immune system is much more ambitious, just like the Star Wars Missile Defense system is more ambitious than a simple attack identification system. The immune system's need to provide a target-specific attack mechanism requires selectively amplifying molecules (antibodies) and cells that bind the targeted intruding molecule and kill intruding and infected cells.

This difference in output requirements dictates a difference in sensing strategies. The broad specificity of olfactory receptors allows the olfactory system to be sensitive to a huge variety of stimuli with a much smaller set of receptors. For the immune system, instead, broad specificity is not a desirable property. If the system goes awry and specificities become too broad, autoimmune disease ensues. Killing only the intended target, and not benign self cells, using broad specificities would require having the killing dependent on the binding of a particular *combination* of antibodies or T-cells. Such a combinatorial detection capability could be hard to implement given the physical constraints of getting a large number of different T-cells (or even antibodies) to bind the same, potentially small, intruding molecule.

1.20.2 Different mechanisms to generate diversity of specificities

A second, and related, difference between both systems lies in the mechanisms of diversity generation. The complexity of the odorant receptor repertoire is estimated in mouse and rat at 2000 genes, or about 3 percent of the genome, surpassing that of the immunoglobulin and T cell receptor genes combined (Mombaerts, 1999b). While the immune system has developed a sophisticated combinatorial mechanism to generate diversity, this does not appear to be the case with chemosensation, which uses a fixed (and large) repertoire of genes instead. Why? In particular, once one system appeared in evolutionary history, why was it not co-opted for a second function, since biology has shown to co-opt genes with much greater ease than that with which it evolves new ones? I believe the answers can be found by two converging lines of analysis.

The tradeoff between specificity and a small receptor set

First, could either of the mechanisms of diversity generation work in the other system? Because antibodies and T-cells cannot afford broad specificities (see above), a much larger diversity of molecules is needed to recognize as large a set of antigens. Furthermore, while a low-affinity receptor might provide enough signal for accurate odor detection and recognition, the immune system's task requires much more than simple detection and recognition. It is not enough to detect the presence of a foreign antigen; each and every antigen-bearing molecule or cell that binds the receptor of an activated B-cell needs to be attacked. The immune system's effectiveness in doing this depends directly on antibodies' affinity for an antigen, and an ellaborate set of processes involving amplification of activated cells, somatic hypermutation and the germinal center have evolved to maximize antibodies' affinity for the antigens that activate a B-cell. Thus, every second cell generated during

the process of hypermutation has a different receptor (Janeway and Travers, 1994). Clearly, it would be immensely costly in genome size, if not impossible, to achieve such a large repertoire of high affinity receptors with a fixed set of genes. Specificity of response calls for a more ellaborate system of diversity generation.

Could immunoglobulins work as odor detectors? Clearly, they could be expressed on the surface of olfactory receptor neurons; several members of the immunoglobulin superfamily are membrane-associated. Whether antibodies can evolve to bind small volatile molecules is an open question, but they are known to bind small molecules. Would the large repertoire of specificities of the immune system work in the olfactory system? Possibly not: each odorant receptor gene acts as a labeled line to the brain, not only to glomeruli in the mammalian olfactory bulb and insect antennal lobes, but also beyond into the insect protocerebrum (Wong et al., 2002; Marin et al., 2002), and it is unclear that the benefits of increased dimensionality of the olfactory representation offset the costs at increasingly large number of such labeled lines. Indeed, a large fraction of the members of the human odor receptor family are pseudogenes, suggesting that, at least in a species with little reliance on olfactory-mediated behavior as humans, there is not much selective pressure for an increased set of odor receptors. A gene family evolved from immunoglobulins could have developed broader specificities, though; MHC molecules are one such family. The critical question, then, is which came first: olfactory receptor genes or immunoglobulins. ORs came first, the reason for the evolution of immunoglobulins is apparent. If, on the other hand, immunoglobulins preceded ORs, then perhaps that indicates a fundamental inability of immunoglobulins to evolve affinity for small organic molecules or to develop broad-tuned response profiles.

The evolutionary history of the immune and olfactory systems

Let us take a brief look at the history of both systems. How old is the immune system? Although even primitive creatures such as sponges have means of distinguishing self components from non-self components, cellular immunity does not appear until worms or starfish, none of the complement system appears until arthropods, and immunoglobulins and lymphocytes do not appear but in vertebrates (Steiner, 1996; Encyclopaedia Brittannica Online, 2001) —although immunoglobulin-like domains have been found with functions different from antigen recognition in *C. elegans* and *Drosophila* (Steiner, 1996). Analysis of genetic diversity suggests the divergence from the ancestral immunoglobulin took place some 200,000,000 years ago. Based on paleontological evidence, this is about the same as the time at which amphibians are thought to have diverged from the main vertebrate line. It is not until one reaches the level of the terrestrial vertebrates—amphibians, reptiles, birds, and mammals—that a complete immune system with thymus, spleen, bone marrow, and lymph nodes becomes evident and that both IgM and IgG antibodies are made. Antibodies of the IgA class are only found in birds and mammals, and IgE antibodies are confined to mammals.

Olfactory receptors, instead, appear to be much older, and a complete olfactory system is present in insects as well as vertebrates, with simpler membrane-bound chemodetection systems present in worms, yeast and even bacteria (see §1.4). The evidence is thus consistent with the fact that immunoglobulins evolved much later than odorant receptors to fulfill a function that odorant receptors could not have performed themselves.

Hereditary response profiles?

Note that the differences in the diversity-generation mechanisms dictates a secondary difference: while odor-response profiles are largely hereditary, immune response profiles have both a genetic

and random components. Whether this difference was a driving force for the evolutionary differences or a consequence is debatable: it is advantageous to both systems to preserve specificities selected for in the past, as it is advantageous to both systems to have a diverse set of specificities in a population.

1.20.3 Monospecificity is common to lymphocytes and olfactory receptor cells

As opposed to taste receptor cells, which can express several receptor genes in the same cells (Adler et al., 2000; Chandrashekar et al., 2000; see §1.8), olfactory receptor neurons and lymphocytes exhibit monospecificity: each cell expresses a single type of receptor out of the diverse array in the population of cells. In the immune system, the advantages for this are clear: clonal selection can thus selectively amplify responses to one antigen without simultaneously upregulating responses to another. In the olfactory system, the reasons are as of yet less clear (but see §1.19.2 for a hypothesis). Perhaps the lessons from the immune system can help. We know that new olfactory receptor cells, as well as new granule cells in the olfactory bulb, which exhibits monospecificy of glomeruli as well, are born throughout life. Further, persistent exposure to an odorant can selectively upregulate responses to it both at the behavioral (Rabin, 1988; Wysocki et al., 1989; Laska and Hudson, 1991; Nevitt et al., 1994; Dittman et al., 1997; Moller et al., 1998; Hudson and Distel, 1998) and peripheral neural (Wang et al., 1993; Yee and Wysocki, 2001) levels, even causing the detectability of odorants for which a person was originally anosmic. Finally, although the question was asked in the context of development rather than plasticity, there is recent evidence that odorant-induced activity upregulates survival of olfactory receptor neurons (Zhao and Reed, 2001). It is thus not unreasonable to predict that some sort of clonal selection may be at work in the olfactory system, selectively enhancing the representation of common odorants by upregulating the survival of cells that respond to them.

CHAPTER 2

The olfactory system is invariant to odorant volatility

2.1 Abstract

It is sometimes held that volatility is a primary determinant of our ability to smell any given compound. Sometimes, corrections are even made for volatility when comparing olfactory responses to odorants at any given concentration in solution (Brockerhoff and Grant, 1999). These corrections do not significantly reduce the dynamic range of the amplitude of olfactory responses, though. I show here that if the odorant source is a solution whose composition is similar to that of the mucosa covering olfactory receptors, and in equilibrium, the system is invariant to the volatility of the odorant, i.e., two compounds present in a solution in equal activities will reach the olfactory mucosa in equal activities even if one is significantly more volatile than the other. This is due to the fact that the solubilization process is the inverse of the evaporation process if the activity coefficients of the odorants are similar for the source and the mucosa, and their strong dependencies on volatility therefore cancel each other out. Furthermore, even when the compositions are not similar, odorant activity at the mucosa is not an explicit function of the volatility, and depends only on the activity coefficients of the odorant at the source and the olfactory mucosa —the gas phase acts merely as a carrier. I extend the analysis to solid odor sources, for which the same concept holds. Interestingly, the same holds not only for noses whose receptors are immersed in solution, but also for artificial noses for which odorants are detected by sorption into a polymer phase.

2.2 Introduction

The olfactory system faces a seemingly formidable challenge in reconstructing the concentrations of olfactory objects, as well as the composition of simple mixtures, given that different substances have vastly different volatilities[1], and thus the relationship between the concentration found in the gas entering the nose or surrounding an antenna on the one hand, and the concentration at the source on the other, will vary for each substance. In addition, the olfactory system faces another daunting challenge, that of being sensitive to a huge dynamic range: all the way from single or a few molecules (Kaissling and Thorson, 1980) to saturation (inside a flower for a bee, for example).

If two odorants of different volatilities are present in aqueous solution (called *source* hereafter) in equal concentrations, what will their concentrations in the mucosa covering odor receptors be after evaporating from the source and reaching a nose? I show here that the aqueous mucosa solves the problem of invariance to volatility and reduces the dynamic range the rest of the olfactory system must cope with, by factoring out the volatility of the odorant. The reasoning is that the gaseous phase acts merely as a carrier medium between a solid or liquid source and a liquid target, the olfactory mucosa. If the activities coefficients of the odorants in the mucosa and in the source solution are similar enough, the process of evaporation into the carrier gas is the inverse of the process of solubilization from the carrier gas into the mucosa, and the effects of the two tend to cancel each other out. This means that two odorants will dissolve in an aqueous medium such as the olfactory mucosa in concentrations equal to their concentrations at the source[2], even if the two have different volatilities, provided that they both are dilute enough and have similar enough activity coefficients in the source and in the mucosa. Thus, for physiologists interested in the concentration reaching olfactory receptors, the most relevant concentration, contrary to previous belief, is that at the source

1. Throughout, the terms volatility and vapor pressure are used interchangeably, and specify vapor pressure in the pure state.

2. Excluding active effects such as that of any odorant binding proteins with affinity for the odorants in question, which would change an odorant's activity coefficient in the mucosa.

solution, and not that in the air carrier.

2.3 The olfactory epithelium is covered by an aqueous mucosa

Before odorant molecules reach olfactory receptors, they must first cross an aqueous interface. In vertebrates, the olfactory epithelium is covered by the nasal mucosa, which is 5–30 micrometers deep depending on the species and living environment (Menco, 1980). In insects, olfactory receptor neurons (ORNs) are covered by the sensillar lymph, which lies below the cuticular walls of the sensilla (Pelosi and Maida, 1995). The functions of this mucosa have remained a puzzle. In vertebrates, it has been proposed that they serve a protective function for the olfactory receptor neurons (Pelosi, 2001). In insects, though, where the cuticular walls of the sensilla provide more of a protective layer than the mucosa itself, its presence is puzzling.

2.4 In equilibrium, the concentration of an odorant in the mucosa is given by the fraction of its partial pressure over the vapor pressure of the pure odorant

The concentration of an odorant in an aqueous phase in equilibrium with the vapor phase[1] is governed by the partition coefficient between the vapor and the solution, which dictates that the concentration in the aqueous phase is proportional to the ratio of the partial pressure of the vapor over the vapor pressure of the odorant:

$$a_{mucosa} = g_{mucosa}\, x_{mucosa} = p / p^* , \qquad \text{(Equation 1)}$$

where *a* is the odorant activity, x is the equilibrium mole fraction of the odorant, g is the odorant activity coefficient in the mucosa, p is the partial pressure of the odorant in the airspace above the

1. Equilibrium between phases is assumed throughout the discussion.

epithelium, and p* is the vapor pressure of the pure odorant (Doleman et al., 1998). If the activity coefficients, which account for the specific solvation interactions between the sorbent phase and the odorant molecules, are similar for odorants within a homologous series, then the concentration of any member of the homologous odorant series in the mucosa will be primarily determined by the fraction of the pressure at the nose over the vapor pressure of the pure odorant, as opposed to being determined primarily by the absolute concentration of the odorant in the vapor phase (Moulton and Eayrs, 1960; Amoore and Butter, 1978; Doleman et al., 1998). Indeed, the olfactory detection thresholds for homologous series of alkanes and alcohols are to a first approximation a constant fraction of the vapor pressure of the odorant both in humans (Mullins, 1955; Cometto-Muniz and Cain, 1990; Doleman et al., 1998) and rats (Moulton and Eayrs, 1960). This relationship has also been reported for electrophysiological thresholds in the olfactory mucosa of the frog (Ottoson, 1958) and the trigeminal nerve of the rat (Silver et al., 1986).

What determines, then, the fraction of the vapor pressure of an odorant in the gas phase? The process of evaporation of an odorant from a solution to air is the inverse of the sorption of that odorant into the solution:

$$p_{source} = g_{source} \cdot x_{source} \cdot p^* \ , \qquad \text{(Equation 2)}$$

which is just a rearrangement of equation 1, with the subindices changed from mucosa to source.

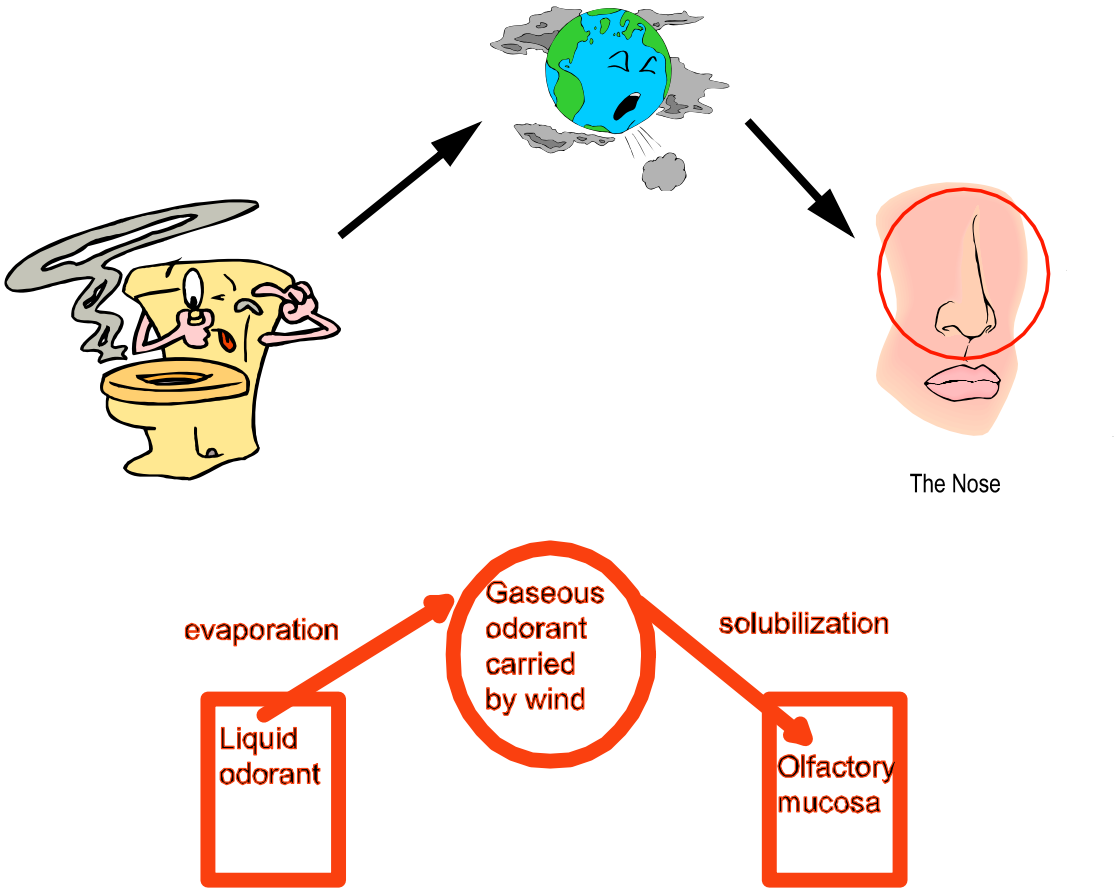
2.5 Transport of odorant molecules from source to nose

In most olfactory environments, the simple diffusion of odorant molecules is a negligible means of dispersing odorants (Murlis and Jones, 1982; Murlis et al., 1992). Odors of distant objects are brought to the nose by wind, following the path of the air packet into which they evaporate (Murlis and Jones, 1982). This means that, while the frequency of the packets decreases with distance from the source (Murlis et al., 2000), the absolute concentration in each packet varies less (Murlis et al.,

2000), and even after dilution into surrounding air, the relative concentrations of different components will remain constant. The partial pressure of an odorant above the mucosa will then be

$$p_{mucosa} = d \cdot p_{source} \quad , \qquad \text{(Equation 3)}$$

where d stands for a dilution factor $(0<d<=1)$[1].



Solubilization is the inverse process of evaporation, making the net process of odor transport independent of volatility.

1. This dilution factor accounts for the failure of the vapor to reach saturation before being swept by an air current, dilution by mixture with air during travel from odor source to the nose, and failure of the odor to reach saturation in the mucosa if the odor is present only transiently. Because this dilution is to a first approximation homogeneous for all odorants (Murlis et al., 1992), it does not affect their relative concentrations.

2.6 Invariance to volatility

The above considerations have an interesting consequence. It is sometimes said that more volatile odorants will reach odor receptors in higher concentrations and thus are more effective olfactory stimulants (e.g., Brockerhoff and Grant, 1999, but see Mullins, 1955). In reality, however, the volatility (p^*) appears in the *numerator* of equation 2 determining the concentration of an odorant in the vapor phase, but it appears in the *denominator* in equation 1 determining the concentration of an odorant in the mucosa. Thus, in the relationship between the concentration in the liquid source and that in the mucosa, the volatility appears in both the numerator and the denominator, and therefore cancels out. Combining equations 1 and 3, then

$$a_{mucosa} = g_{mucosa} \cdot x_{mucosa} = p_{mucosa} / p^* = d \cdot p_{source} / p^* \qquad \text{(Equation 4)}$$

Combining equations 2 and 4:

$$x_{mucosa} = d \cdot (g_{source} / g_{mucosa}) \cdot x_{source} \qquad \text{(Equation 5)}$$

The presence of an aqueous mucosa over the epithelium has, then, the important consequence that the concentration encountered by the olfactory system is independent of the volatility of the different components[1,2].

A second consequence of the presence of the mucosa and its invariance to volatility is a dramatic compression of the dynamic range of concentrations faced by the olfactory system, compared to what it would be if detection happened directly in the gaseous phase. For a system already facing a dynamic range of many dB's, such a compression could play a fundamental role in the ability of animals to respond to concentrations ranging from a few molecules of a distant source to the saturated

1. Of course, a substance with *zero* volatility will never reach the mucosa, so this discussion holds for non-zero volatilities.

2. It is possible in principle that an implicit dependence on volatility is contained in the dependence on the activity coefficient ratios for non-ideal solutions.

vapor pressure faced by, say, an insect inside a flower.

For dilute odorants (and the olfactory system is sensitive to very low concentrations (Kaissling and Thorson, 1980; Devos et al., 1990)), the relationship in equation 5 is even simpler.

2.7 Raoult's law

When the components in a solution are chemically similar, the mixture is called an ideal solution and the vapor pressure of each component can be approximated by Raoult's law:

$$p_A = x_A \cdot p^*_A \qquad \text{(Equation 6)}$$

or rearranging:

$$p_A / p^*_A = x_A \quad , \qquad \text{(Equation 7)}$$

where p_A stands for the vapor pressure of component A, p^*_A is the vapor pressure of A as a pure liquid, and x_A stands for the mole fraction of A in the liquid (see Atkins, 1989).

When the components are dissimilar, strong deviations from Raoult's law can occur. Even then, though, the law becomes increasingly accurate as x_A approaches unity. In other words, the law is a good approximation for the solvent so long as the solution is dilute.

2.8 Henry's law

In real non-ideal solutions, the solute does not follow Raoult's law. The English chemist William Henry realized that the vapor pressure of the solute in a dilute solution is proportional to the mole fraction, even if the proportionality constant is not the vapor pressure of the pure liquid (Atkins, 1989). This is a simple consequence of the fact that any curve can be approximated with arbitrary

accuracy by a straight line in the limit of its length going to zero (see, for example, Feynman, 1965); Henry's empirical discovery was that the range of validity of this approximation was sufficiently broad for what came to be called *ideal dilute solutions* so that it was useful.

2.9 The concentration in the mucosa for ideal solutions

For ideal solutions, the odor-dependent activity coefficients are unity, and thus, activities are equal to concentrations in the solution. Equation 5 simplifies and becomes independent of the odor and linear in the concentration at the source. The relative concentrations of components of a mixture in the epithelium then equal the relative concentrations in the original liquid odorant:

$$x_{mucosa} = d \cdot x_{source} \qquad \text{(Equation 8)}^{1}$$

or, for ideal dilute solutions, per Henry's law,

$$x_{mucosa} = d \cdot k \cdot x_{source} = k' \cdot x_{source} \qquad \text{(Equation 9)}$$

with k and k' constants for any given set of mixture components.

2.10 Solid-state odor sources

The independence of the concentration of odorants in the mucosa with respect to volatility holds for solid odor sources as well as liquid ones. The process of solubilization in the mucosa is of course

1. Slight differences between the relative concentrations of two odorants at the source and those at the epithelium may remain due to failure to reach equilibrium and differential kinetics of absorption of components – especially at high flow rates through the nose—, differential diffusion of components, temperature differences between source and mucosa and differential contamination due to different presence of components in the background. Diffusion is a negligible component of odorant kinetics, though (Murlis et al., 1992).

the same whether the source was a solid or a liquid. What is then the vapor pressure created by a solid in equilibrium with its surroundings? At the temperature of fusion (T_{fusion}), solid and liquid phases are in equilibrium with each other, which means their chemical potentials are equal to each other. Since the pressure of the vapor in equilibrium with a phase is dependent on the chemical potential of the phase, the pressure of the vapor created by sublimation of a solid (p^{subl}) is equal to that created by vaporization of a liquid (p^{vap}) at T_{fusion}:

$$p^{subl}_{Tfusion} = p^{vap}_{Tfusion} \qquad \text{(Equation 10)}$$

But, p^{subl} and p^{vap} are both (different) functions of the temperature (Atkins, 1989):

$$p^{subl} = p^{subl}_{T_{fusion}} e - C_{subl} \quad , \qquad \text{(Equation 11)}$$

where p^{subl}_0 is p^{subl} at $T=T_0$ and

$$C_{subl} = \frac{\Delta H_{subl}}{R}\left(\frac{1}{T} - \frac{1}{T_{fusion}}\right) \quad , \qquad \text{(Equation 12)}^1$$

where ΔH_{subl} is the enthalpy of sublimation and R is the gas constant. Likewise,

$$p^{vap} = p^{vap}_{Tfusion} \cdot e^{-c}_{vap} \quad , \qquad \text{(Equation 13)}$$

where p^{vap}_0 is p^{vap} at $T=T_0$ and

1. Assuming ΔH_{vap} and ΔH_{subl} to be independent of temperature in the range T_{fusion} - $T_{ambient}$.

$$C_{vap} = \frac{\Delta H_{vap}}{R}\left(\frac{1}{T} - \frac{1}{T_{fusion}}\right) \quad , \qquad \text{(Equation 14)}$$

where ΔH_{vap} is the enthalpy of vaporization. Combining equations 10-14 to obtain p^{subl} as a function of p^{vap}, we arrive at

$$p^{subl} = p^{vap} \cdot e^{\frac{-(\Delta H_{subl} - \Delta H_{vap})}{R}(\frac{1}{T} - \frac{1}{T_{fusion}})} \qquad \text{(Equation 15)}$$

Thus, the pressure of a vapor in equilibrium with a solid odorant is proportional to its vapor pressure in liquid form, with the proportionality constant dependent on the temperature, the fusion temperature and the difference between the enthalpy of sublimation and that of vaporization. So, just like in equation 2 for liquid odorants, the vapor pressure is in the numerator of this equation, and thus the concentration of odor in the mucosa resulting from a solid source is independent of its volatility:

$$x_{mucosa} = \frac{d}{\gamma_{mu}\cos a} e^{\frac{-(\Delta H_{subl} - \Delta H_{vap})}{R}(\frac{1}{T} - \frac{1}{T_{fusion}})} \qquad \text{(Equation 16)}$$

2.11 Discussion

In summary, the problem of reconstructing an odorant's composition at the source in the face of varying volatility of components turns out to be solved by the very physics of the process by which an odorant reaches the olfactory epithelium of land animals. The olfactory mucosa, exploiting the fact that most behaviorally relevant odor sources are solid or liquid rather than gaseous[1], achieves

invariance to the volatility of the odorant by virtue of inverting the process of evaporation of an odor from its source to the atmosphere. This allows the mucosa to act as a gain control mechanism to maintain the concentration of different odorants in the mucosa at more similar levels than would be the case were odor receptors detecting odorant concentration in the vapor phase. In doing so, the mucosa dramatically compresses the dynamic range faced by olfactory receptors, facilitating their task of recognition across many orders of magnitude of concentration.

A similar trend holds not only for olfactory thresholds but also for sorption into a carbon black-polymer sensor: the mean response intensity of electronic nose detectors is essentially constant for members of an homologous series if their activities are held constant (see Doleman et al., 1998). This suggests the mucosa does not need to be aqueous to create invariance to volatility: solubilization or adsorption into other media will have a similar effect. The critical step to achieve invariance to volatility is to make the odor receptors responsive not to the absolute concentration in the vapor, as mass spectroscopy or flame ionization detection is, but rather to the thermodynamic activity of the odorant. In addition, the more similar the composition of the mucosa to that of the odor source, the more similar the activity coefficients for different odorants will be for both phases, and the less the relative concentrations in the mucosa will differ from those at the source.

2.12 Thermodynamic equlibria versus particle counters

More generally, gas quantification processes can be separated into two classes: those counting par-

1. Even predicting the toxicity of a gas is better served by identifying the concentration of the toxin in aqueous solution, or better, its activity, than by measuring the gas' pressure in the vapor phase (see Ferguson, 1939). This does not apply to all gas measurement applications, though. To measure the amounts of different gases in the atmosphere that could absorb sunlight, for example, the relevant concentration is the absolute concentration in the vapor phase. Neither does it apply to all gas detectors: mass spectrometers, for example, measure absolute concentration in the gas phase.

ticles, such as nuclear, mass, photonic or electric processes of detection, including geiger counters, mass spectroscopy and flame ionization detection, and those relying on a thermodynamic equilibrium, such as biological noses and carbon-black polymer sensors. The former detect absolute concentration in the gas phase, whereas the latter detect activities. In evaluating a molecule's biological effect, such as in measuring toxicities, it is the activity that is more relevant. Furthermore, the invariance to volatility that characterizes the latter detector class, but not the former, makes the responses of thermodynamic detectors more correlated with a mixture's concentration in the original condensed phase than that of particle counters.

2.13 Predictions and empirical support

The analysis presented here suggests the prediction that, if olfactory detection thresholds for different odorants are predominantly driven by the concentration of odorant that reaches the mucosa (Doleman et al., 1998), then thresholds based on concentration of the odorant in solution should be significantly more similar for different odorants than thresholds based on concentration in air. A survey of the literature shows that this is indeed the case. While the human thresholds in g/l in air observed for n-butanol, pyridine and isovaleric acid vary over four orders of magnitude (four studies compiled by Laffort, 1963, cited in Amoore and Buttery, 1978), the thresholds in g/l in water are comparatively constant, varying by a factor of two or less (Amoore and Buttery, 1978) (Table 1).

A second prediction is that, despite decades of attempts to correlate olfactory thresholds with volatilies (Ottoson, 1958; Moulton and Eayrs, 1960; Brockerhoff and Grant, 1999), olfactory thresholds, measured in concentration at the source, should be independent of volatility. In other words, two ideal compounds of different volatilities but equal in all other regards will accumulate in the mucosa in concentrations proportional to those at the source and independent of their volatilities. This prediction is harder to test, because it is difficult to vary volatility without varying other physicochemical

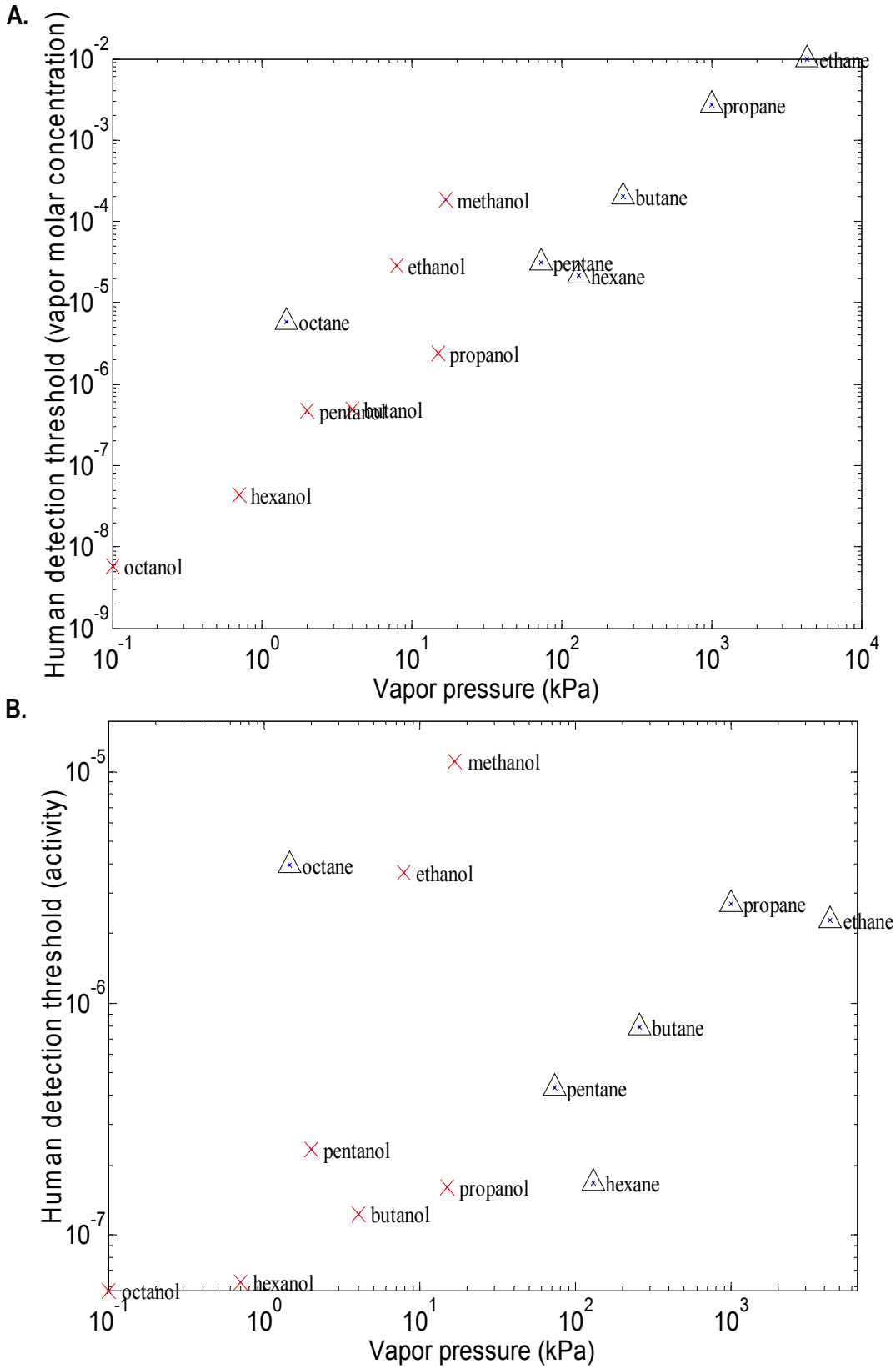


Figure 2.2. Human detection thresholds for 1-alcohols (red crosses) and n-alkanes (black triangles) are more tightly correlated with vapor pressures when measured in concentrations in the vapor (A)

than in thermodynamic activities (B). Activities were calculated as the ratio of the partial pressure in the vapor at threshold over the vapor pressure of the pure substances. Olfactory thresholds from Devos et al., 1990. Vapor pressures from the CRC Handbook (1999) and http://chemfinder.cambridgesoft.com/.

properties of the odorant that are bound to affect olfactory thresholds. One way to do that might be to change the composition of the carrier stream adding an insoluble gas to air in concentrations large enough to change the affinity of odorants for the vapor phase. This would have the effect of changing an odorant's vapor pressure, without a concomitant change in the physicochemical properties of odorants or their affinity for the sorbed phase. The prediction would be that odor detection thresholds (measured in activity, which can be approximated for ideal solutions by concentration of the source solution) would be unaffected (provided that the activity coefficients of the odorant are similar in the source and the mucosa).

TABLE 1. Comparison between aqueous and gaseous olfactory thresholds (adapted from Amoore and Buttery, 1978).

Compound	Water threshold (g/l)*	Air threshold (g/l)$^{+}$
n-Butanol	6.5×10^{-3}	4.3×10^{-5}
		1.0×10^{-6}
		3.2×10^{-6}
		3.7×10^{-5}
Pyridine	4.2×10^{-3}	4.1×10^{-5}
		3.2×10^{-5}
		3.7×10^{-6}
		3.9×10^{-8}
		7.4×10^{-7}
		1.6×10^{-7}
Isovaleric acid	1.2×10^{-4}	7.6×10^{-9}
	*: Amoore and Buttery, 1978.	+: From data compiled by Laffort (1963), where the originals are cited.

It is easier to show that olfactory thresholds are indeed less correlated with volatility when measured as concentration in solution than when measured as concentration in the vapor phase. Indeed, the correlation between a compound's volatility and its olfactory threshold measured as concentration in solution varies widely for different homologous series (for acids, Spearman rank correlation r=0.1, p>0.8, Fig. 2.3a; for aldehydes, r=0.7, p=0.19, Fig. 2.3b). Unfortunately, for decades, olfactory researchers have been biased toward measuring thresholds as concentrations in the vapor phase, partly because that obviates the need for specifying a solvent, which might be different for different odorants. Thus, thresholds measured in solution are scarce in the literature compared to the wealth of thresholds measured in the vapor (see Devos et al., 1990, for a compilation). Fortunately, we can use the ratio of thresholds in the vapor phase over the vapor pressure of the compound to calculate activities, since the activities in the vapor phase and in solution are the same in equilibrium. In man, thresholds measured as activities for 1-alcohols and n-alkanes have a much weaker dependency on volatility than that reported for thresholds measured as concentration in the vapor (Spearman rank correlation r=0.998, $p<10^{-4}$ for concentrations in vapor; r=0.035, p>0.1 for activities; see Fig. 2.2; compare Mullins, 1950 with Doleman et al., 1998, respectively)[1]. Detection thresholds, measured in activities, for the same homologous series (n-aliphatic alcohols) also show stronger dependencies on the species in which they are measured than on the volatility of the substance (Fig. 2.4). Finally, "correcting" for differences in volatility among olfactory stimuli (Brockerhoff and Grant, 1999) does not significantly reduce the variance in EAG responses induced by different odorants in a cone-

1. Performing the correlations separately for each series did not yield a better correlation for activities and vapor pressures of n-alkanes (r=0.03, p>0.9, down from 0.94, p<0.005 for concentrations in the vapor), but yielded a barely significant correlation for 1-alcohols (r=0.8, p<0.05, down from r=0.96, $p<4x10^{-4}$ for the vapor concentrations). Interestingly, this correlation was positive, not negative, meaning that, if anything, more volatile substances are less effective odorants.

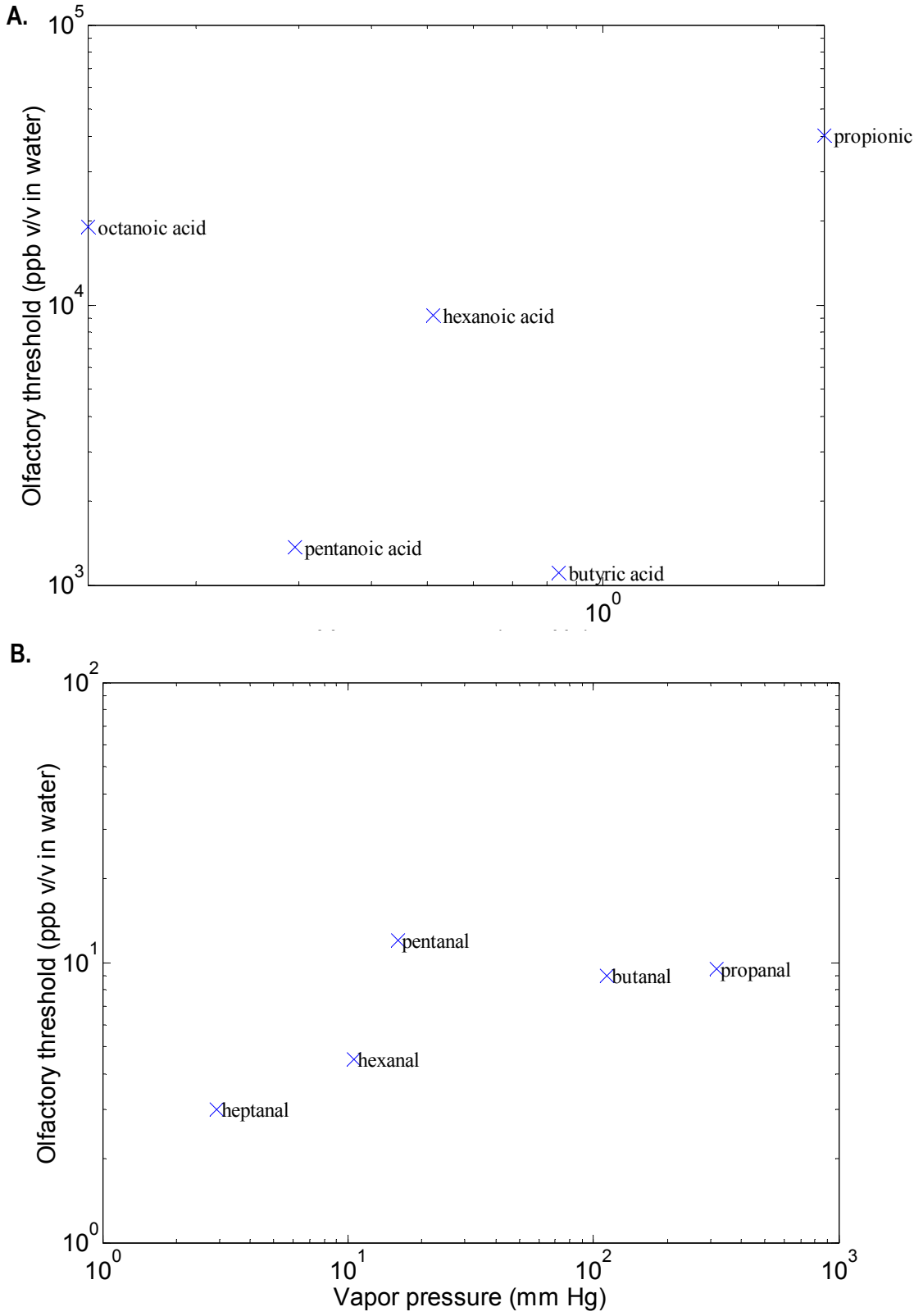


Figure 2.3. Human olfactory thresholds measured as concentrations in solution, as a function of volatility for a homologous series of (A) acids and (B) aldehydes.

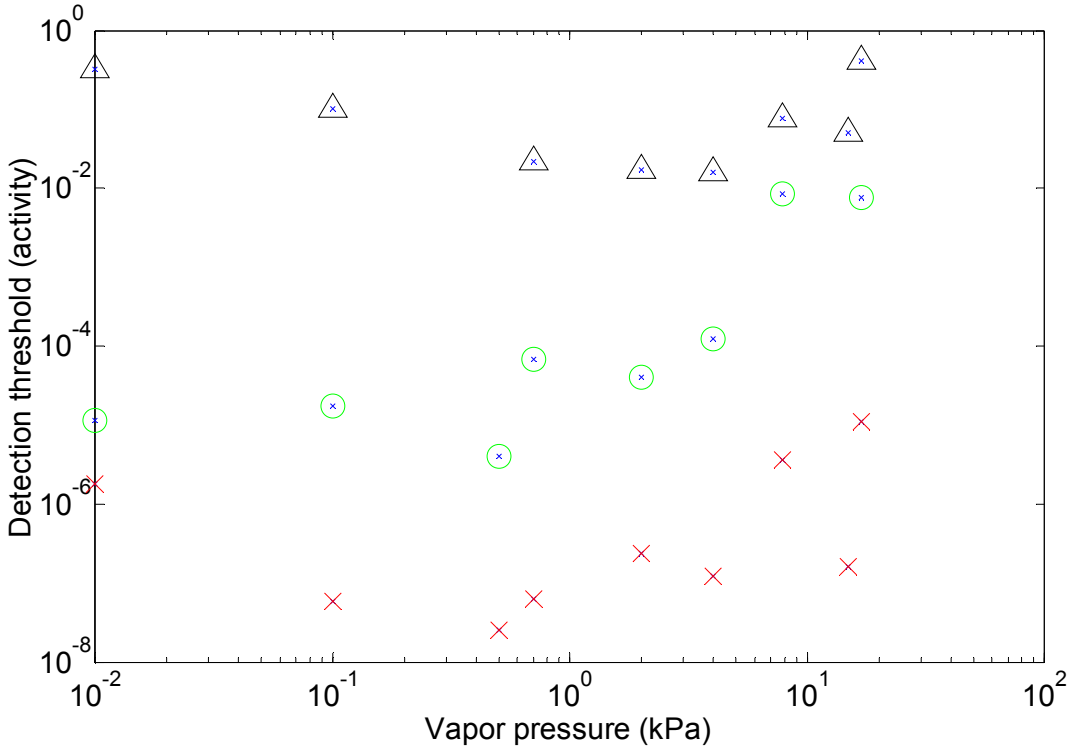


Figure 2.4. Relation between detection threshold, expressed as thermodynamic activities, for l-alcohols in humans (red crosses; Devos et al., 1990), rat (green circles; Moulton and Eayrs, 1960) and blowfly (black triangles; Dethier and Yost, 1951).

worm. Together, these observations suggest that olfactory detection thresholds are not primarily driven by volatility, if they depend on it at all.

Of course, detection thresholds depend on the affinity of the highest-affinity receptor for the compound in question, as well as, in some cases, on specific odor-binding proteins (Kim, Repp and Smith, 1998). The responses of the top-responding receptors, however, appear to be comparable for many molecules in homologous series, even when the identity of the top-responding receptors changes for different elements of each series (Fujimura et al., 1991). Furthermore, it is likely that olfaction operates under nonequilibrium conditions for at least some odorants. Olfactory stimulation is transient and intermittent, due both to the turbulent nature of odor plumes (Murlis et al., 1992) and to periodic sampling given by sniffing (Freeman, 1978; Gray and Skinner, 1988) in vertebrates or by

antennal movements in arthropods (Mellon, 1997). A complete characterization of the determinants of olfactory thresholds must await an *in vivo* characterization of each of the hundreds of receptor types, in its natural habitat of the mucosa and under conditions of intermittent stimulation.

Acknowledgements

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CHAPTER 3

Hedonic valence of odors in the locust, Schistocerca americana

3.1 Introduction

Behavior is the starting point for any neurophysiological investigation, for it is the function of nervous systems that neurophysiology seeks to explain, and this we call behavior. Thus, before Mark Konishi, and the long series of outstanding scientists he has trained, started work on the neurophysiology of auditory localization in the owl, he first demonstrated that the nocturnal birds were capable of precisely locating a target using sound alone (Konishi, 1973). Analogously, the exploration of the fly's visual system has been guided by the pioneering studies of German ethologists showing what an insect's visual system is capable of (see Wolf and Heisenberg, 1990, for example). More recently, the demonstration that bees are able to learn to discriminate the category of symmetrical images from that of asymmetrical ones (Giurfa et al., 1996) and that of sequentially presented pairs of 'same' objects from that of 'different' objects, even across modalities (Giurfa et al., 2001) will surely pave the way for a neurophysiological investigation of how the bee brain achieves that.

In olfaction, research has been hampered by humans' relative lack of reliance on that sense. Thus, it is only arduously that we can start to piece apart, even at Marr's algorithmic level (Marr, 1982), complex olfactory behavior, such as how dogs are able to determine the direction of a trail (Steen, 1990) or how hamsters detect which over-mark is on top of another (Johnston and Bhorade, 1998). Most research has thus concentrated on the comparatively simple olfactory tasks of odor detection and discrimination.

The rest of this thesis deals with a neurophysiological investigation of the olfactory system of the American locust, *Schistocerca americana*. The first and foremost question when investigating how locusts recognize odors, then, is *whether* locusts indeed recognize odors. This question is made especially important in the light of a report that palpation, which involves contact chemoreceptors rather than olfactory receptors, is involved in all instances of food selection by *Schistocerca americana,* both for acceptance and rejection (Chapman and Sword, 1993). In the past, however,

and despite the abundance of physiological investigations on its olfactory system, this question had remained unanswered, due to the locust's lack of motility (one would almost say its apathy) and the consequent difficulties associated with measuring observable behavior. This chapter deals with the first successful attempt to demonstrate olfactory-guided behavior in *Schistocerca americana.* We set out to test for the presence of innate preferences for or against odors. In later work, Heather Dean and Brian Smith showed that locusts can also be trained to respond to an odor to which they have no innate preference (unpublished observations).

3.2 Failed beginnings

Our initial attempts consisted in placing droplets of an odorant in one corner of a box, placing a locust in the center and measuring the tendency of the locust to go to that corner as compared to the other corners. This experiment failed to reveal a preference or avoidance for any odor tested.

A second approach consisted in placing locusts (both males and females) on sand at the downstream end of a wind tunnel we constructed for this purpose, having odorized air flow along the direction of the tunnel, and observing the position of the locusts as time went by. This too, failed, for the locusts remained mostly static and clung to the walls.

The beginnings of success came using an apparatus constructed by Christine Chee-Ruiter for work with cockroaches (Chee-Ruiter and Laurent, 1995). The apparatus consists of a large cylinder with eight small openings on one plane, around the perimeter of the circle. The openings are large enough for an insect to go through, and each leads to a small compartment that has a smaller hole at the outside end. Through this smaller hole, odorized air can be made to flow into the large cylinder. There is a small hole at the bottom of the large cylinder, to serve as an exhaust. Finally, a cover allows the insects to be restricted to the short section of the cylinder which contains the plane of the openings after they have been introduced in the apparatus. This apparatus serves as a multi-

ple-choice test-bed for odor preferences, allowing an insect to escape through the opening which smells best to it. Preliminary results using locusts in it showed some odor preferences of little significance. We wondered whether the large number of openings made it hard for the locusts to identify the direction from which an odor was coming, and sought to simplify the task.

3.3 Schistocerca gregaria

At this point, frustrated by our poor luck getting *S. americana* to demonstrate odor-induced behavior, we resorted to a cousin, *S. gregaria*, which inhabits Africa. *S. gregaria* exists in two states: solitary and gregarious, depending on the amount of aggregating pheromone segregated by cohorts into the medium. *S. americana*, instead, exists in a single state that appears visually similar to *S. gregaria*'s solitary phase. Odor-mediated behavior of *S. gregaria* has been demonstrated before (Loher, 1958; Haskell et al., 1962), and we decided to use the African locust as a positive control for the apparatus. We obtained male specimens of *S. gregaria* from Dr. Ahmed Hassanali at the ICIPE, in Kenya. In our hands, *S. gregaria* exhibited significantly more locomotion than their American counterparts in our colony. With these locusts, we were able to refine a protocol to demonstrate olfactory preferences in the locust, and then were able to apply the same protocol to *S. americana* and show that they, too, had innate preferences for odors.

3.4 Naive locusts show preferences for cherry and grass odors

The design that finally served our purpose had several improvements over our previous attempts. Most importantly, the task was simplified to a two-alternative forced choice by placing locusts —several in preliminary experiments, one at a time in later experiments— at the bottom of a vertical Y-maze, at the top of which we placed a light source. Locusts are attracted by the heat and

the light of the bulb, and move upwards. Upward movement was further facilitated by the placement of mosquito netting along the inside surface of the Y-maze. Locusts like to cling on to the netting (somewhat like they cling to grass) and tend to move more when they can grab onto it. A locust can choose, then, between one of two symmetrical arms to climb into. Each arm had a hole at the top through which air could be made to flow in, and there was an exhaust at the bottom. One of the arms had air going into it, while the other had an equal flow rate of odorized air. Experiments were counterbalanced by switching which arm received odorized air after every experiment. Following Simpson's (1990) demonstration of the importance of food deprivation in observing odor preferences, our locusts were starved for at least 2 hours, usually several hours, before each experiment. An experiment then consisted of placing an adult locust in the maze while odor flowed into one arm and air into the other, and recording which arm the locust went into first. If the locust went into neither arm within 5 minutes, the experiment was aborted and not included in the statistics, since we were not interested in the probability that a locust would move, but rather the probability that a locust which did move did so toward the odorized air significantly more frequently than toward the non-odorized control.

For odors that appeared to cause repulsion using the above setup (i.e., through a preference of locusts *not* to enter the odorized chamber), we confirmed repulsion by using a different setup which more directly tested repulsion: we placed the Y-maze upsidedown and compared the tendency of locusts to exit the odorized arm with their tendency to exit the control arm with pure air[1], by placing 3 locusts on each arm of the inverted Y-maze and recording which branch the locusts

1. A simultaneous test of attraction and repulsion can be implemented by using a modification of the elevator maze used by Tully and Quinn (1985), where the insect is placed between two arms, one of which leads toward an odor source and the other leads away from it. Heather Dean and I built such a maze, which was used in experiments by Natalia Caporale (unpublished results).

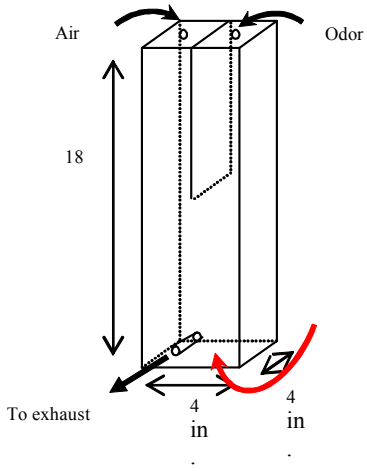

Figure 3.1. The Y-maze. The odor and air input as well as the exhaust are indicated by black arrows. The location of the air and odor tubes was alternated between experiments. The red arrow indicates the place in which the animal was introduced into the device (diagram courtesy of Natalia Caporale).

were more likely to exit within 5 minutes.

In preliminary experiments, 6 locusts were placed at the base of the Y-maze and each trial was scored by counting the number of locusts to climb each branch during the course of 5 minutes. These experiments unveiled a significant preference of *Schistocerca gregaria* for wheat grass ($p<0.008$, binomial test, n=8 experiments).

In later experiments, we restricted each trial to a single locust and changed the locust on every trial, to ensure that we were sampling decisions by every locust and that each of them was an independent choice. The results with single locusts were found to be similar to those using several locusts. In these experiments, *Schistocerca americana* showed attraction towards fresh crushed wheat grass ($p<0.04$, binomial one-sided test, n=8) and cherry extract (Lorann oils) ($p<0.006$, n=27), a significant avoidance of pentanol ($p<0.001$, n=14), and no significant effect by apple, hexanol, octanol, citral, strawberry, lavender, cineole, spearmint, parsley, cilantro, apple-blossom and geran-

iol (p>0.05, n=21 to 53 experiments for each odor, Table 1).

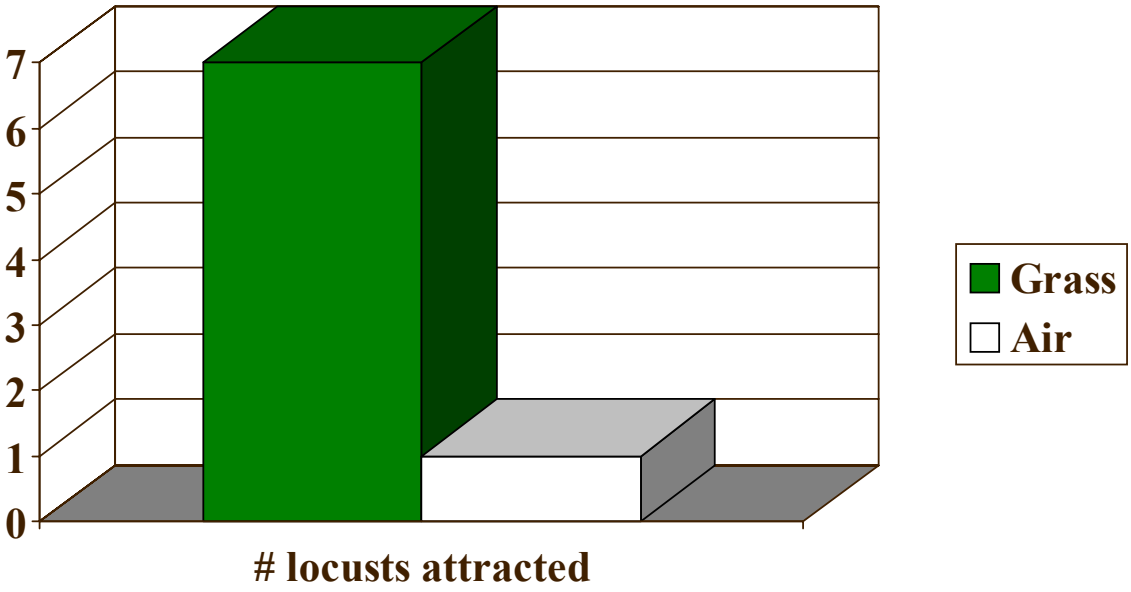

Figure 3.2. The odor of crushed wheat grass attracts *Schistocerca gregaria*.

TABLE 1. Olfactory preferences of *Schistocerca americana*

Odor	Chose odor	Chose air	No response	p-value
grass	38	23	16	0.04
cherry	16	4	7	0.006
apple	23	13	11	0.066
strawberry	14	17	12	0.76
lavender	10	12	8	0.42
cineole	23	13	17	0.06
citral	13	16	27	0.356
geraniol	5	4	19	0.5
spearmint	11	7	10	0.24
parsley	8	6	14	0.395
cilantro	8	6	14	0.395
apple-blossom	10	12	13	0.416
octanol	6	7	8	0.5
hexanol[a]	14	16	8	0.428
pentanol[a]	13	1	0	0.0009

a. **Hexanol and pentanol were tested using the inverted Y-maze.**

3.4 Conclusions

In summary, we have demonstrated that the American locust, *S. americana*, does indeed smell, as evidenced by olfactory preferences. Furthermore, we have showed that at least one of these preferences (cherry) and one of these deterrences (pentanol) are innate, since the animals had not been exposed to the odorants previous to testing. This paves the way for continued studies to explain the physiological basis that allows locusts to perform discrimination between odors and that grants some odorants a positive or negative valence.

Acknowlegements

This research was funded by a grant from the GAAC to A.B. and Wolfgang Stein, a Sloan Fellowship to A.B., and an NIH grant to G.L.. Section 2 of this chapter is the result of joint work between Ari Hershowitz, Yingzhong Tian and Mary Mosier, in chronological order, and myself. Section 3 is fruit of a collaboration with Wolfgang Stein. Section 4 is the result of joint work by Heather Dean and myself; Heather finally succeeded where others had failed in the past. The results in Section 4 were later corroborated and expanded by Natalia Caporale.

CHAPTER 4

The information content of neurons engaged in population temporal coding in early olfactory circuits

4.1 Abstract

It has long been believed that the information content of single neurons in the first olfactory processing nucleus is very low, both because of the overlapping nature of the ensemble representation of odors and because of their low signal to noise ratio, which has been estimated to be as low as 10^{-4} (Freeman, 1990). Here, I take the point of view of the organism and applied to olfaction for the first time an algorithm to identify the odor presented by observing spike trains of projection neurons in the antennal lobe of the locust. The information in one spike train of a single neuron sufficed to identify a stimulus among several presented on up to 95% of all trials. I characterize the timescale of optimal information extraction for two different neural codes: while information in single neurons is robust to a variety of readout temporal scales, including a rate code, the information rate across assemblies of neurons is significantly greater when taking temporal response patterns into account. PN assemblies are shown to be most informative when decoded with a time constant on the order of several hundred milliseconds. This is shown to be due to the burstiness of PN spike trains: The timescale at which PN response patterns are found to vary is on average significantly longer than that previously reported (Wehr and Laurent, 1996). I characterize the information present in assemblies of increasing numbers of neurons. Finally, I characterize the reliability and sparseness of the representation as a function of the timescale of the code with which it is read out.

4.2 Introduction

Perhaps the foremost task of the early olfactory system is to convey enough information about olfactory stimuli to be able to discriminate between odors with different behavioral relevance. In the olfactory bulb of vertebrates and the antennal lobe of insects, it has long been believed that information is coded by distributed assemblies of neurons. In addition, these neurons have been thought to be very noisy, with signal-to-noise ratios close to 10^{-4} (Freeman, 1990). The information in single neurons has thus been considered insufficient to identify odors. The present work quantifies such information present in single projection neurons (PNs) in the antennal lobe of the locust.

4.3 Results

Single PNs allowed correct odor identification in 81% of trials on average when a single concentration of each odor was presented (chance = 50%) (n=12 PNs) and above 95% for several PNs exposed to three odors (chance = 33%) (Fig. 4.1). When multiple concentrations of each odor were presented, the task of the recognition of odor identity was naturally made more difficult. Single PNs nevertheless exhibited enough information to recognize odor identity correctly in up to 50% of all trials on average (chance = 26%) (n=46 PNs) (Fig. 4.2).

To find the duration at which any further information in spike trains becomes redundant with earlier information, I varied the length of the spike trains used for classification systematically. The information content of spike trains saturated at windows of 0.5–1 sec (n=13 PNs, Fig. 4.4). The mean informational content of spike trains for the discrimination of odor quality peaked at odor onset and suffered a fall 500 msec after odor onset (n=13 PNs, Fig. 4.5).

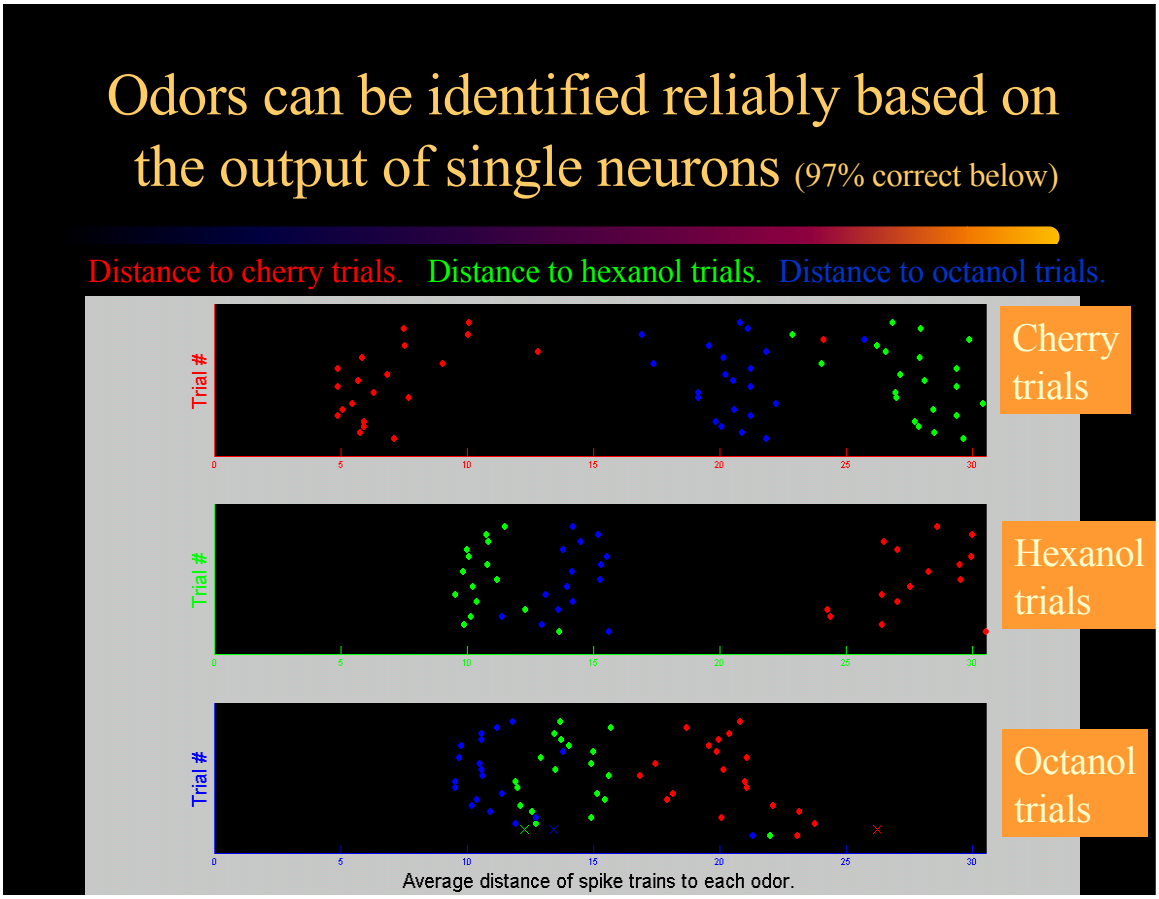

Figure 4.1. Information in single PNs can reliably identify the odor presented. Each plot shows all trials for one odor. Each line shows one trial; the first trial of the corresponding series is displayed at the bottom of each plot. The axis for each point represents the mean distance between a spike train and all (other) spike trains for a given odor (class). Trials whose distance to their own class is smaller than that for other classes are correctly classified; others are shown as crosses —only the second octanol trial is misclassified, as hexanol.

Although for individual PNs temporal information could yield significantly better classification than spike counts alone (Fig. 4.5), on average across all PNs tested, information in single neurons was barely greater using temporal information than using spike counts alone (Fig. 4.2). Furthermore, firing rate variations over longer timescales was less variable across trials and concentrations, and therefore classification based on spike count was better than that based on T values on the order of the length of an oscillation cycle (50 ms) (Fig. 4.2).

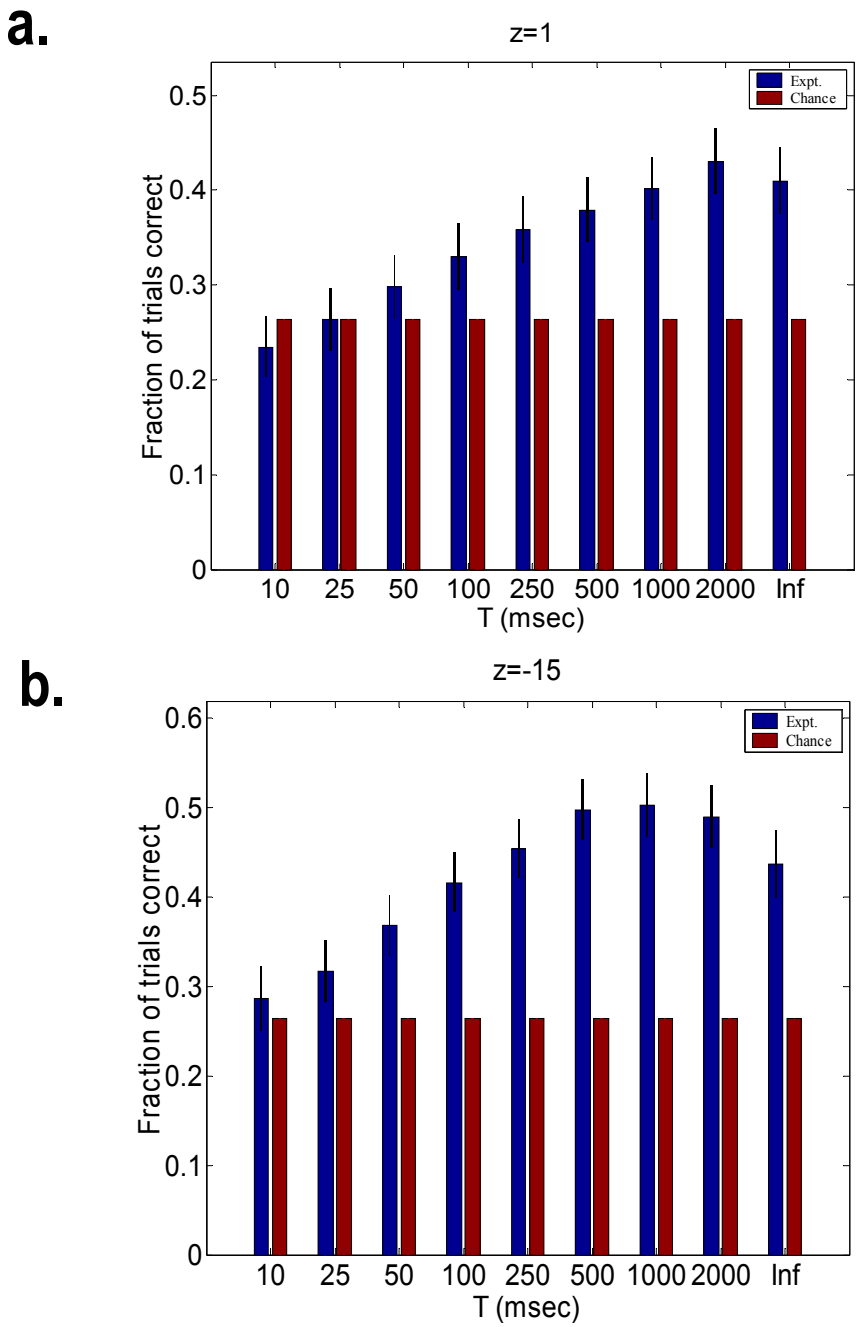

Figure 4.2. Fraction of trials for which the odor was correctly classified as a function of the timescale at which the spike trains of single PNs were decoded. Each trial is assigned to the class with the lowest arithmetic mean distance to it (a) or to the class with the lowest geometric mean distance to it, with exponent=-15 for the averaging (b) (blue bars, n=47 PNs). The former classifies spike trains to the class closest on average over all trials for that odor; the latter amounts to classification into the class with the closest trials altogether. Each odor was presented for 1 sec at various concentrations from 2% to 100% of saturated vapor pressure. For a complete explanation of T, see methods. Red bars denote chance levels of classification.

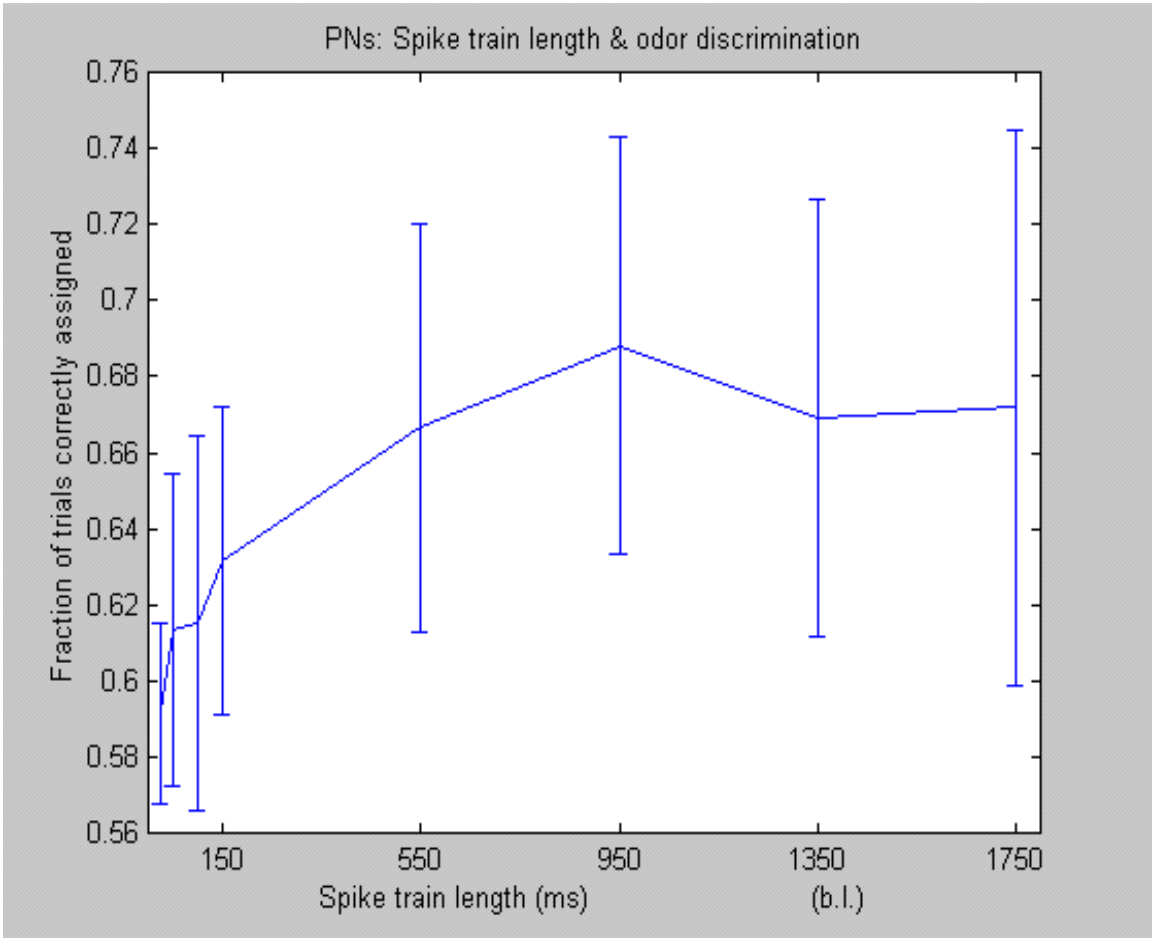

Figure 4.3. Odor discrimination as a function of the length of the spike trains used for classification. The latency was kept at the one yielding best classification in the range starting from 1 second before the odor onset to 1 second after the odor onset. Discrimination was performed for a single concentration of different odors.

To test whether this was the consequence of using single cells for classification, I classified the spike trains of 19 PNs simultaneously recorded by Stijn Cassenaer in response to one concentration of 16 odorants. Classification was performed using two different neural codes that employ different ways to pool across neurons (see Reich et al., 2001). The first, which I call population code in keep-

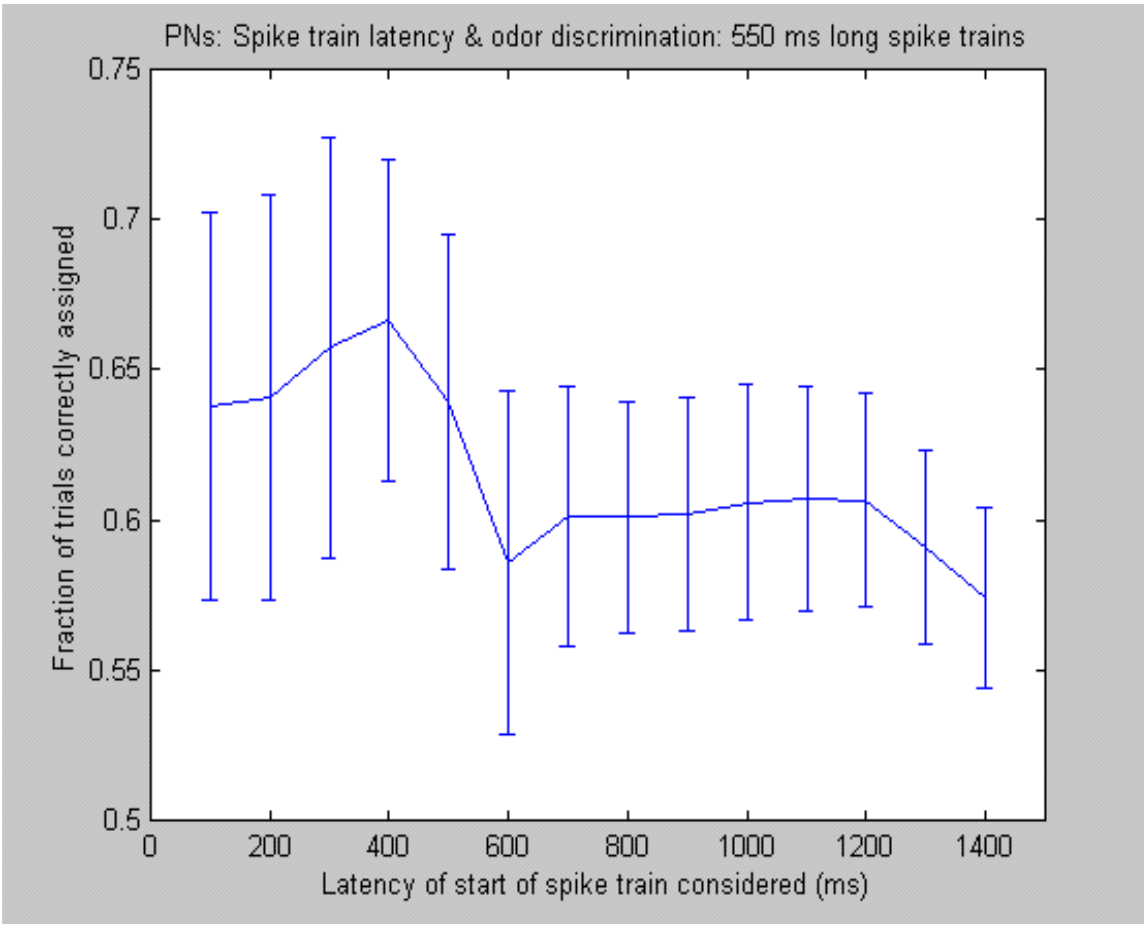

Figure 4.4. Odor discrimination as a function of the latency of the start of the spike train considered. Discrimination was performed for a single concentration of different odors.

ing with Reich et al.'s nomenclature, simulates an integrate-and-fire downstream decoder by treating spikes from all neurons equally regardless of neuronal identity. One spike train was computed that aggregates the spikes across all PNs, and classification was performed based on distances between these aggregate spike trains. Using this algorithm, classification was significantly better using temporal information than using spike counts alone (Fig. 4.6), but was hardly better than using just a single cell. The second algorithm, called labeled line code, does not throw away information about neuronal identity of each spike. All neurons receive equal weight in the classification decision:

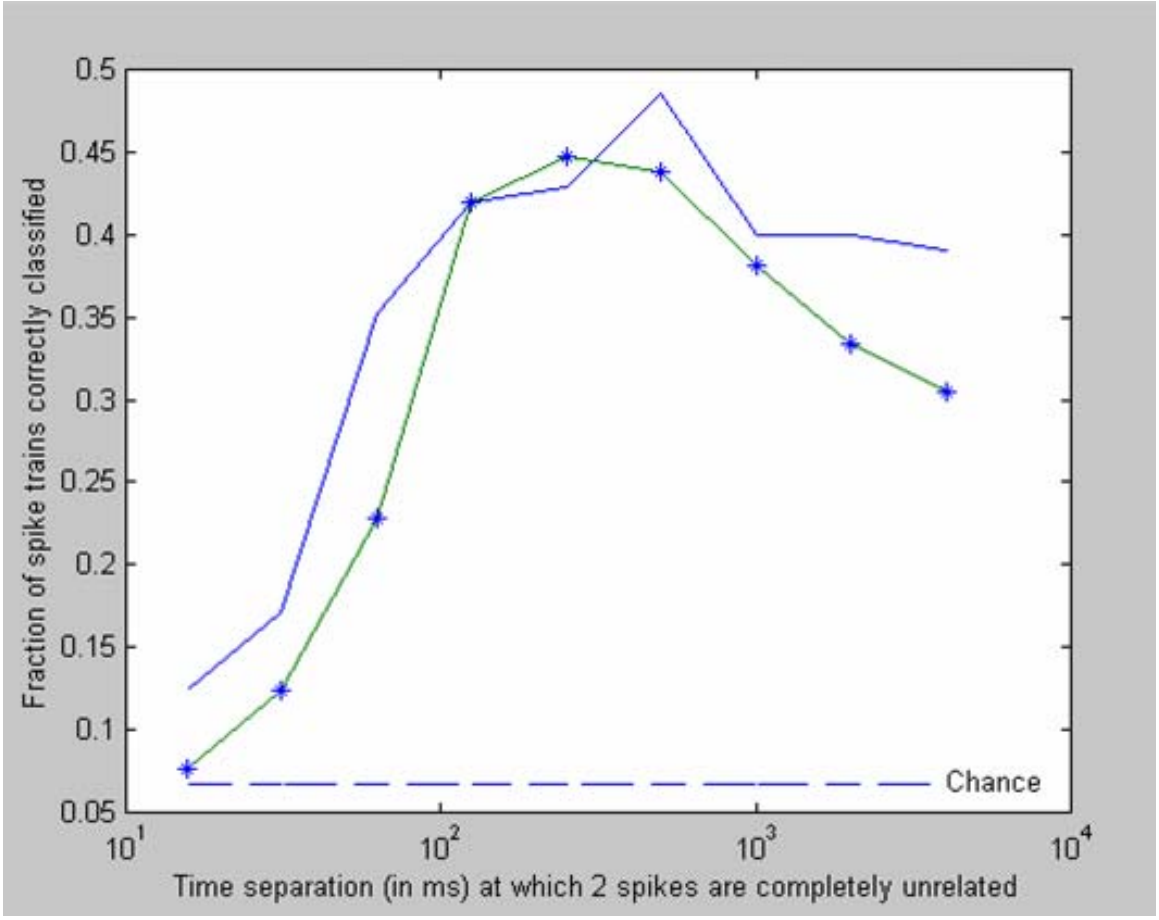

Figure 4.5. A single PN exhibits better classification using temporal information than using spike count alone (T tending to Infinity) when classification is to the closest class performing linear averaging across all trials (green) and geometric averaging with exponent = -15 (blue) (see Methods; see Fig. 4.2 for mean classification-across 19 PNs).

the distance used for classification is the sum of the distances for individual cells. Because each PN carries different stimulus information, classification using the labeled line code was significantly better than that using the population code (Fig. 4.7). Classification using the labeled line code yielded 98% correct classification among the 16 odors presented, and presented almost perfect classification even when only using spike counts (Fig. 4.7).

To test whether this timescale independence of the labeled line code was due to a saturation in information due to the high ratio of # of neurons/ # of odors, I calculated classification rates as a

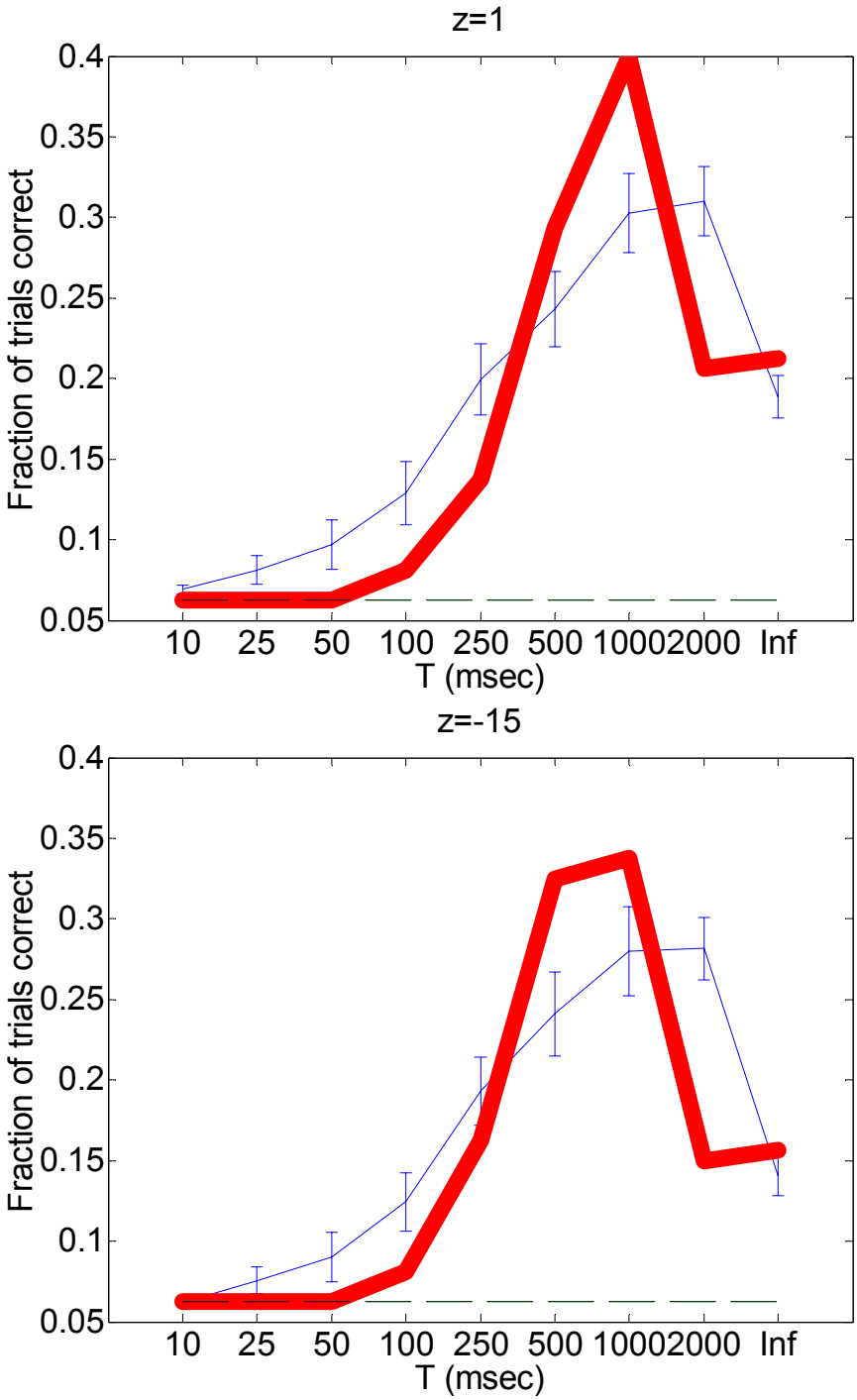

Figure 4.6. Odor discrimination by an assembly of 19 simultaneously recorded PNs among 16 odors using the population code (red, see text) and using single PNs (blue; means and S.E.M.), as a function of the timescale T of decoding (see Methods), for linear averaging across trials (z=1, above) and geometric averaging (z=-15, below). For each trial, 7.5 sec beginning with the onset of a 3 sec long odor pulse were used for discrimination. Chance levels are indicated by the black dashed line.

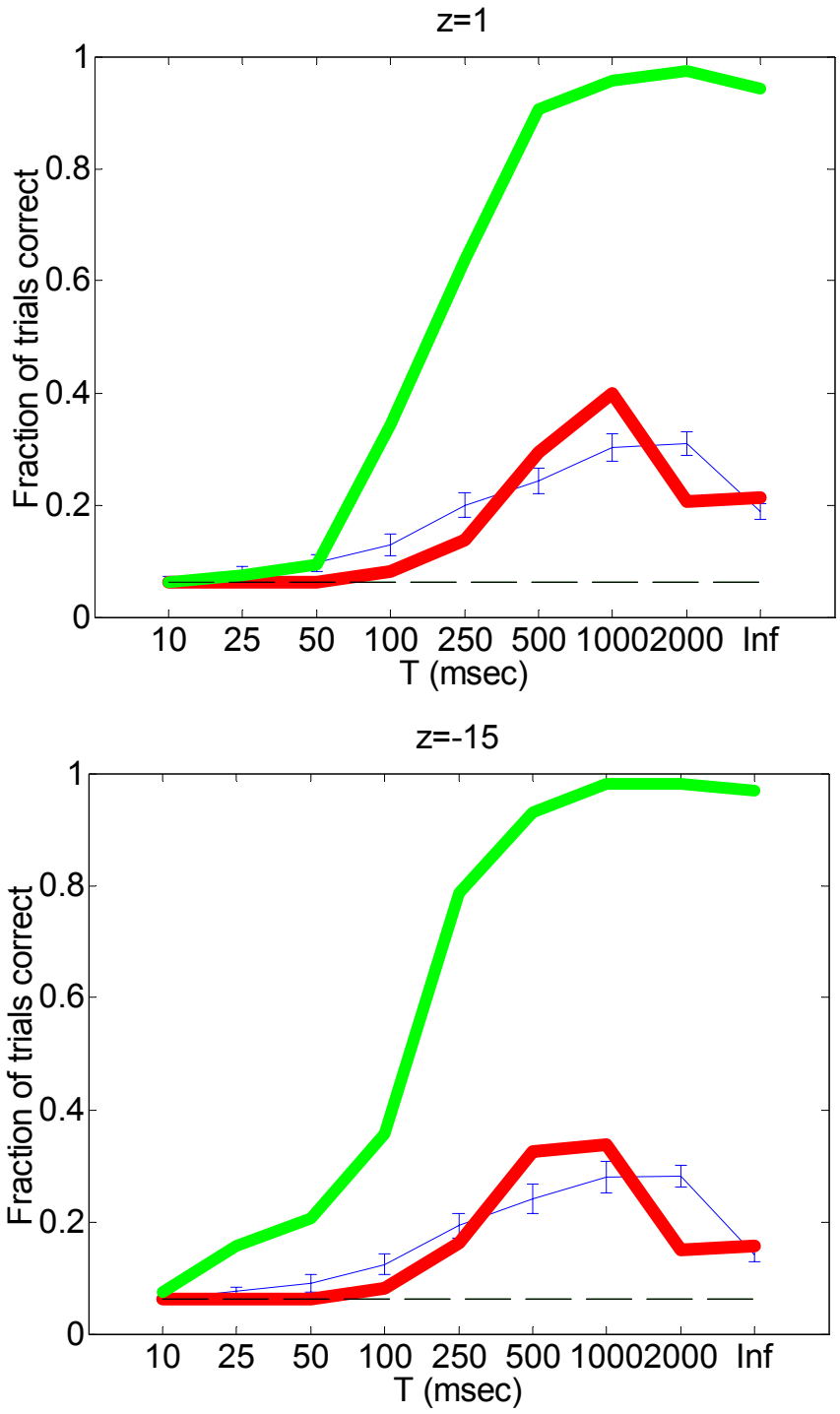

Figure 4.7. Odor discrimination by an assembly of 19 simultaneously recorded PNs among 16 odors using the labeled line code (green, see text), the population code (red) and single cells (blue), as a function of the timescale T of decoding (see Methods), for linear averaging across trials (z=1, above) and geometric averaging (z=-15, below). For each trial, 7.5 sec beginning with the onset of a 3 sec long odor pulse were used for discrimination. Chance classification levels are indicated by the black dashed line.

function of the number of neurons used in decoding for each decoding timescale used (Fig. 4.8). For each number of neurons, 40 randomly chosen subsets of the 19 neurons were used (unless the maximum number of combinations was less than 40, in which case all combinations were used). The same information is plotted in a different format in Fig. 4.9, which shows that indeed, for smaller PN assemblies for which the information content is not saturated, temporal information improves classification accuracy. The transmitted information per cell is approximately constant at 0.3 bits/ neuron after the first neuron and up to within 0.5 bits of the total stimulus information available in our experiments (Fig. 4.18). Assuming this linearity holds throughout the entire antennal lobe when the stimulus set is sufficiently large, this yields a total bandwidth of 250 bits for the locust antennal lobe (830 neurons x 0.3 bits/neuron), which would allow the discrimination of 10^75 odors.

Classification rates, however, were maximal for timescales much larger than the timescale of the oscillations which were previously hypothesized to form the basis for a temporal code based on the fact that some PNs exhibit different firing probabilities for successive cycles (Wehr and Laurent, 1996). This suggested that PN responses might exhibit significant correlations on timescales smaller than 1 second. To test that, I calculated the matrix of conditional probabilities

P(x,y) = p(# of spikes in cycle N=y | # of spikes in cycle N-1=x)

over all trials of all concentrations of all odors presented to 46 PNs (data collected by the author; Fig. 4.10) and over all trials of all odors at one concentration presented to 12 PNs (data collected by Katrina MacLeod; Fig. 4.11). These matrices showed a strong correlation between the number of spikes in successive non-overlapping 50 msec windows. This correlation was present both following odor presentations ($r=0.67$, $p<<10^{-6}$, Spearman ranksum correlation test, 3 sec period following odor stimulation, Figs. 4.10-4.11) and during the 1 sec period preceding odor presentation ($r=0.64$, $p<<10^{-6}$, Spearman ranksum correlation test, Fig. 4.12). The effect is very signficant: over all trials

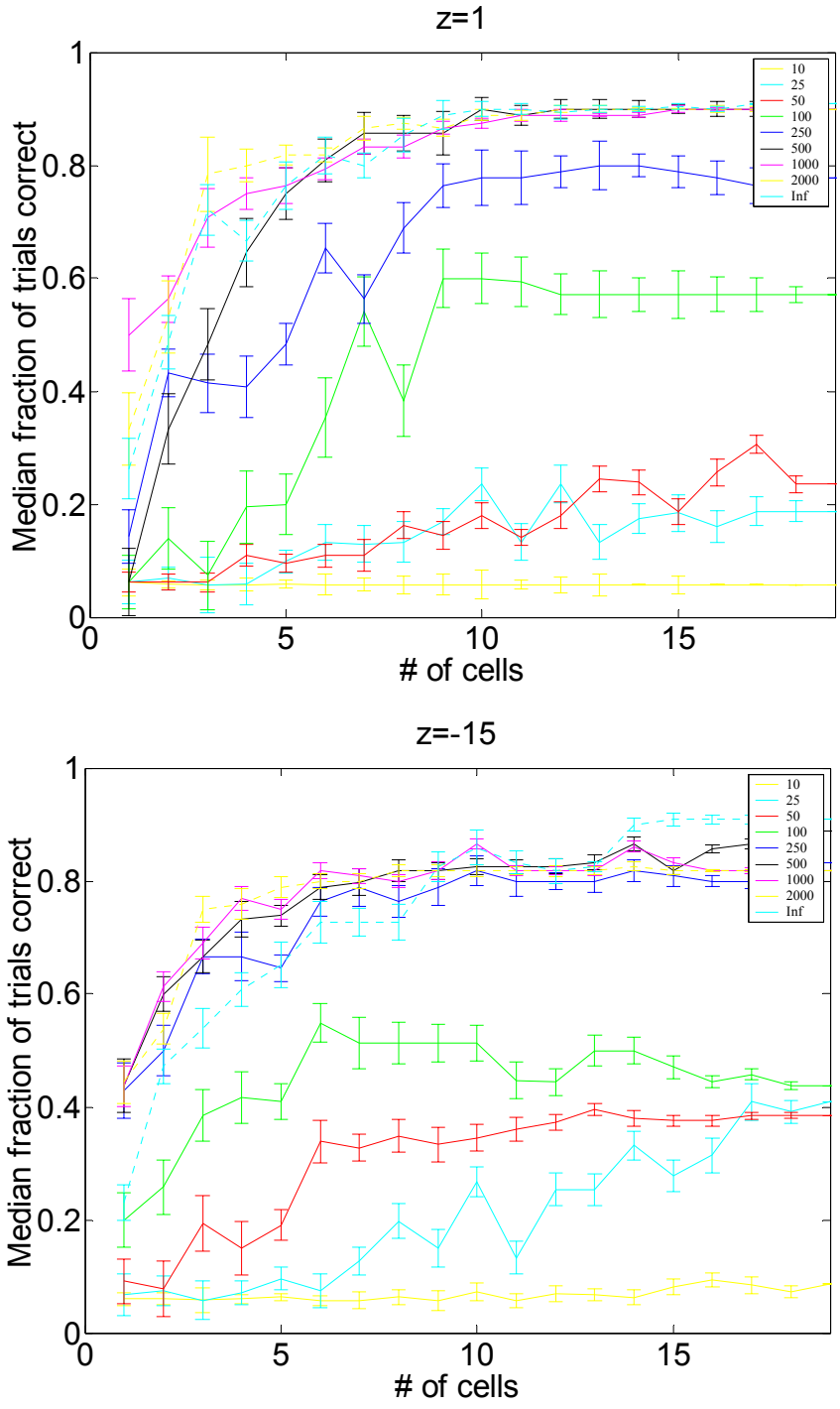

Figure 4.8. Odor discrimination for the labeled line code as a function of the size of the cell assembly used for decoding. Each line represents a different timescale used for decoding (T values, inset).

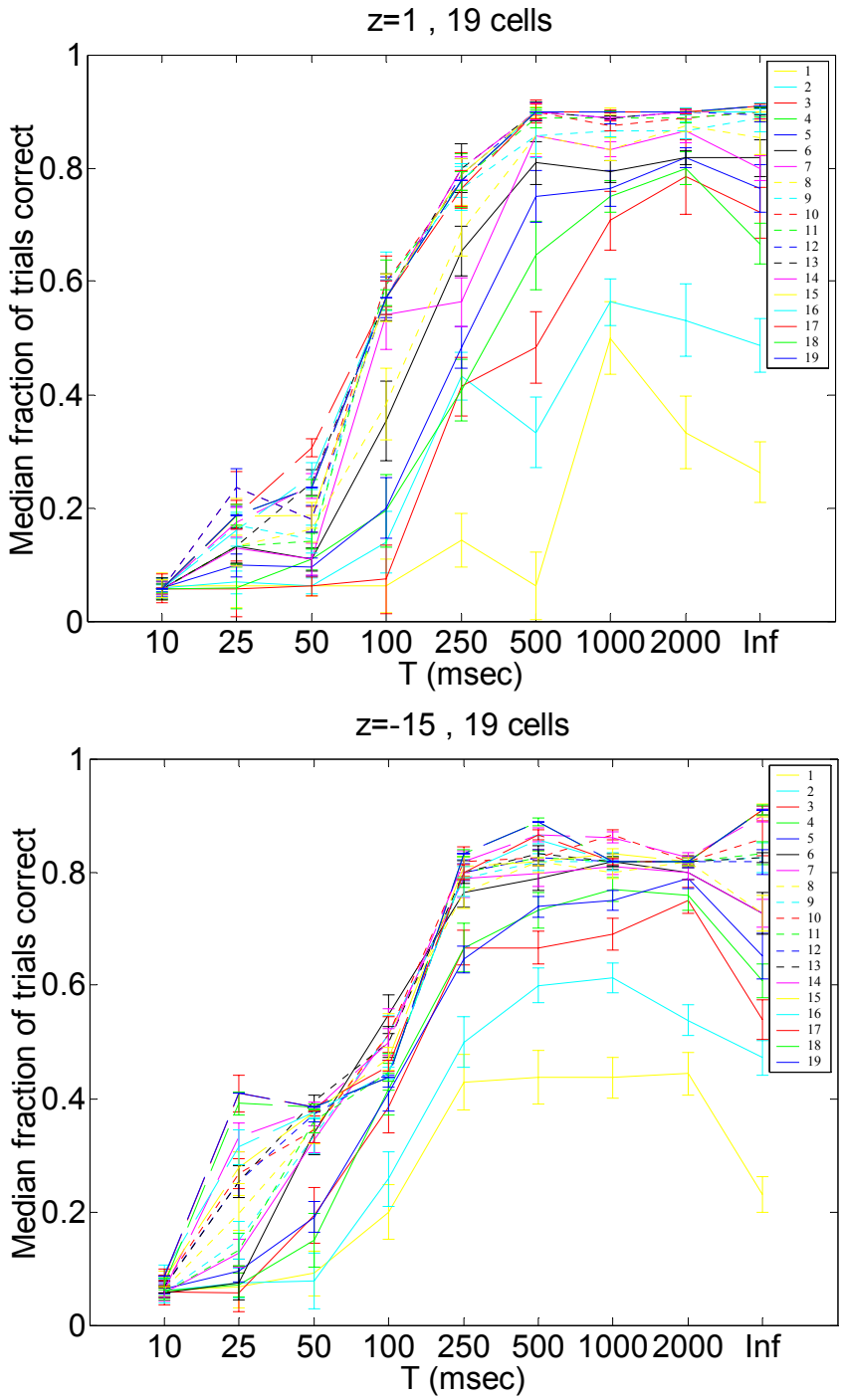

Figure 4.9. Odor discrimination for the labeled line code as a function of the timescale used for decoding (T). Each line represents a different size of the cell assembly used for decoding (key, inset).

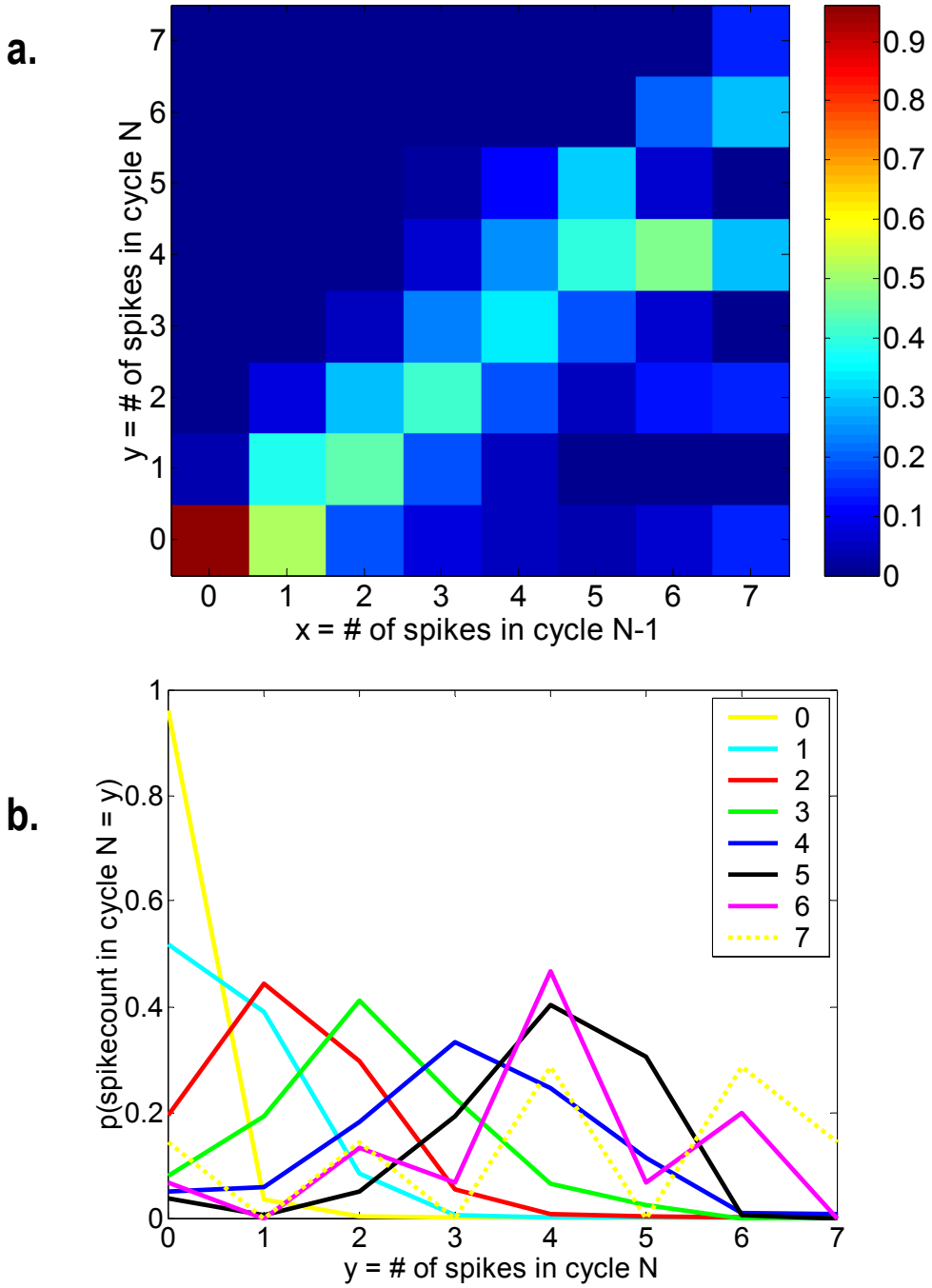

Figure 4.10. PNs are bursty: The probability of firing of a PN in any given cycle is significantly enhanced if the PN has fired in previous cycles, and the number of spikes in successive cycles is significantly correlated. a) P(x,y) = p(# of spikes in cycle N=x | # of spikes in cycle N-1=y). Probabilities calculated over several concentrations of more than 100 PN-odor pairs of 46 PNs. **b)** The same data plotted as line plots of the probability distributions of the # of spikes in cycle N-1. Each curve represents a different # of spikes in cycle N (see legend). Note the shift rightward in the curves as the # of spikes in cycle N increases.

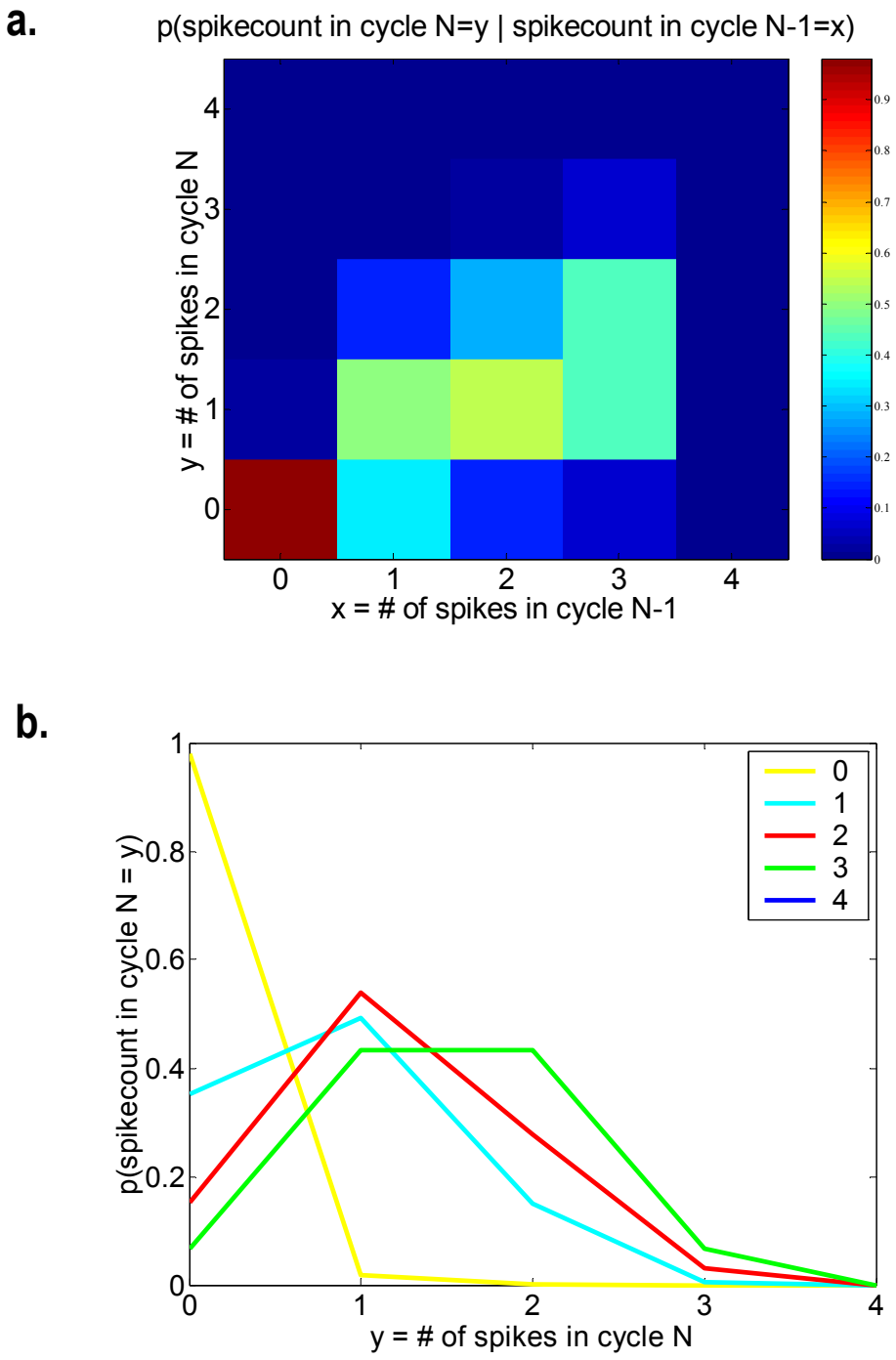

Figure 4.11. PN odor responses are bursty. These plots were computed for a set of 12 PNs different from those in Fig. 4.10 for a single concentration of each odor (data courtesy of Katrina MacLeod). See Fig. 4.10 legend for details.

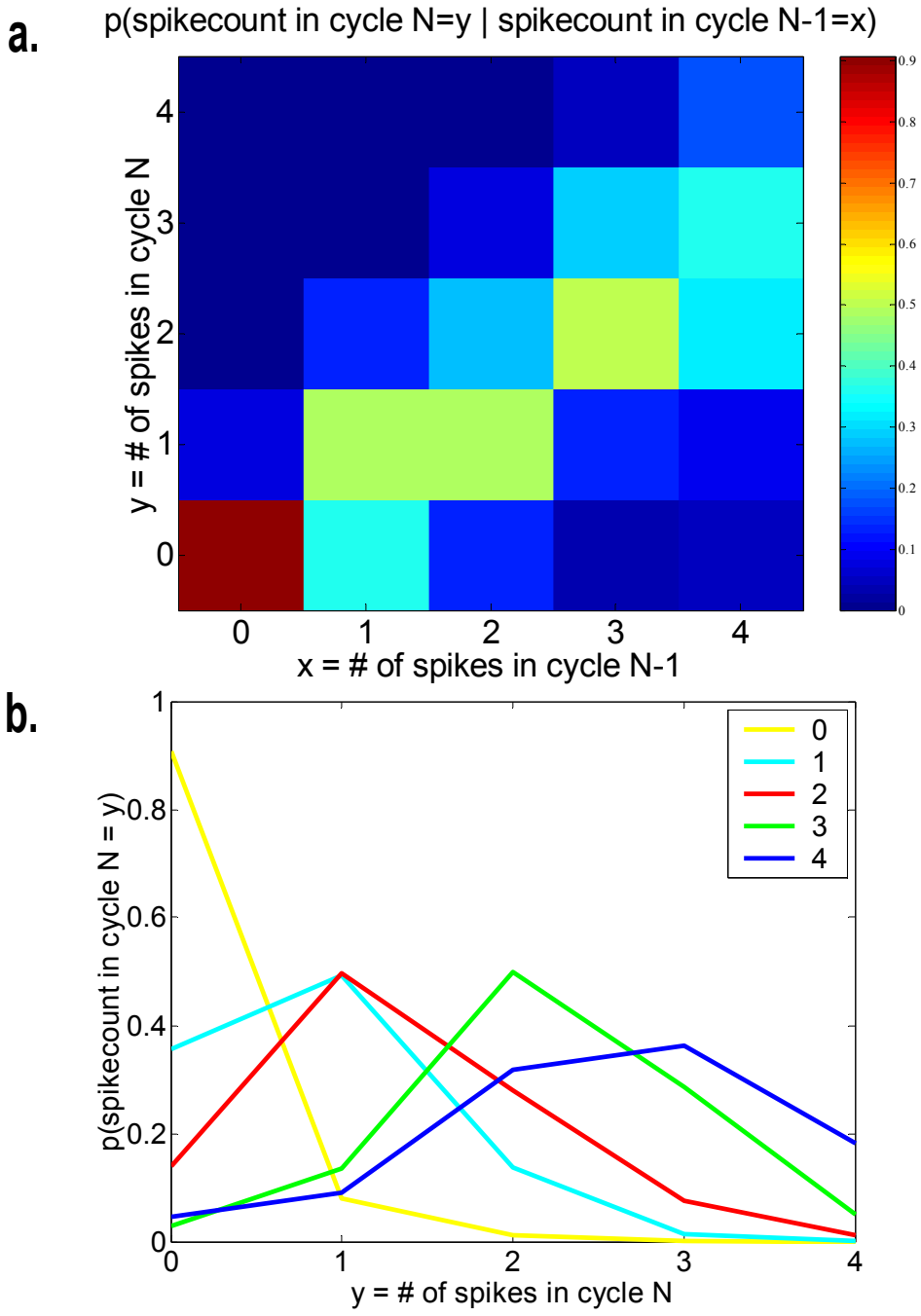

Figure 4.12. PNs' basal activity is bursty: The probability of firing of a PN in any given cycle during basal activity between odor stimulations is significantly enhanced if the PN has fired in previous cycles, and the number of spikes in successive cycles is significantly correlated. a) P(x,y) = p(# of spikes in cycle N=y | # of spikes in cycle N-1=x). **b)** The same data plotted as line plots of the probability distributions of the # of spikes in cycle N-1. Each curve represents a different # of spikes in cycle N (see legend). Note the shift rightward in the curves as the # of spikes in cycle N increases. These plots were calculated for the same cells as Fig. 4.11.

for all odors for the 46 PNs in Fig. 4.10 and during the 1-second period of odor stimulation, the probability of encountering a spike in any 50 msec long time window was 0.04 if there had been no spikes in the preceding 50 msec window, but jumped to 0.61 if there had been one or more spikes in the preceding window (see Table 1).

TABLE 1. Conditional probabilities of PN spike counts in 50 msec windows during 1 sec period of odor stimulation

X	P(0 spikes | X spikes in previous 50-msec)	P(1 spike | X spikes in previous 50-msec)	P(2 spikes | X spikes in previous 50-msec)	P(3 spikes | X spikes in previous 50-msec)	P(4 spikes | X spikes in previous 50-msec)
0	0.9641	0.0313	0.0041	0.0005	0.0000
1	0.4661	0.4056	0.1174	0.0102	0.0008
2	0.1866	0.4359	0.3092	0.0623	0.0060
3	0.0987	0.1947	0.4409	0.2211	0.0445
4	0.0625	0.0625	0.2768	0.3482	0.2500
Row probabilities may not add up to 1 due to windows with 5+ spikes.					Calculated over all trials for all odors for 46 PNs.

To confirm this correlation and measure its timescale, I calculated spike-triggered firing rate averages for 119,351 spikes in 10,730 trials in 377 cell/odor/conc datasets for 46 PNs. Beyond a refractory period, PNs exhibited a large positive autocorrelation with a time constant of several hundred milliseconds both during odor responses (Fig. 4.13a) and baseline firing (Fig. 4.13b).

In the locust, the output of PN assemblies is decoded by Kenyon cells (KCs) in the mushroom bodies[1]. The decoding scheme for individual KCs is quite different from the classification algorithms

1. As well as by Lateral Horn inhibitory neurons.

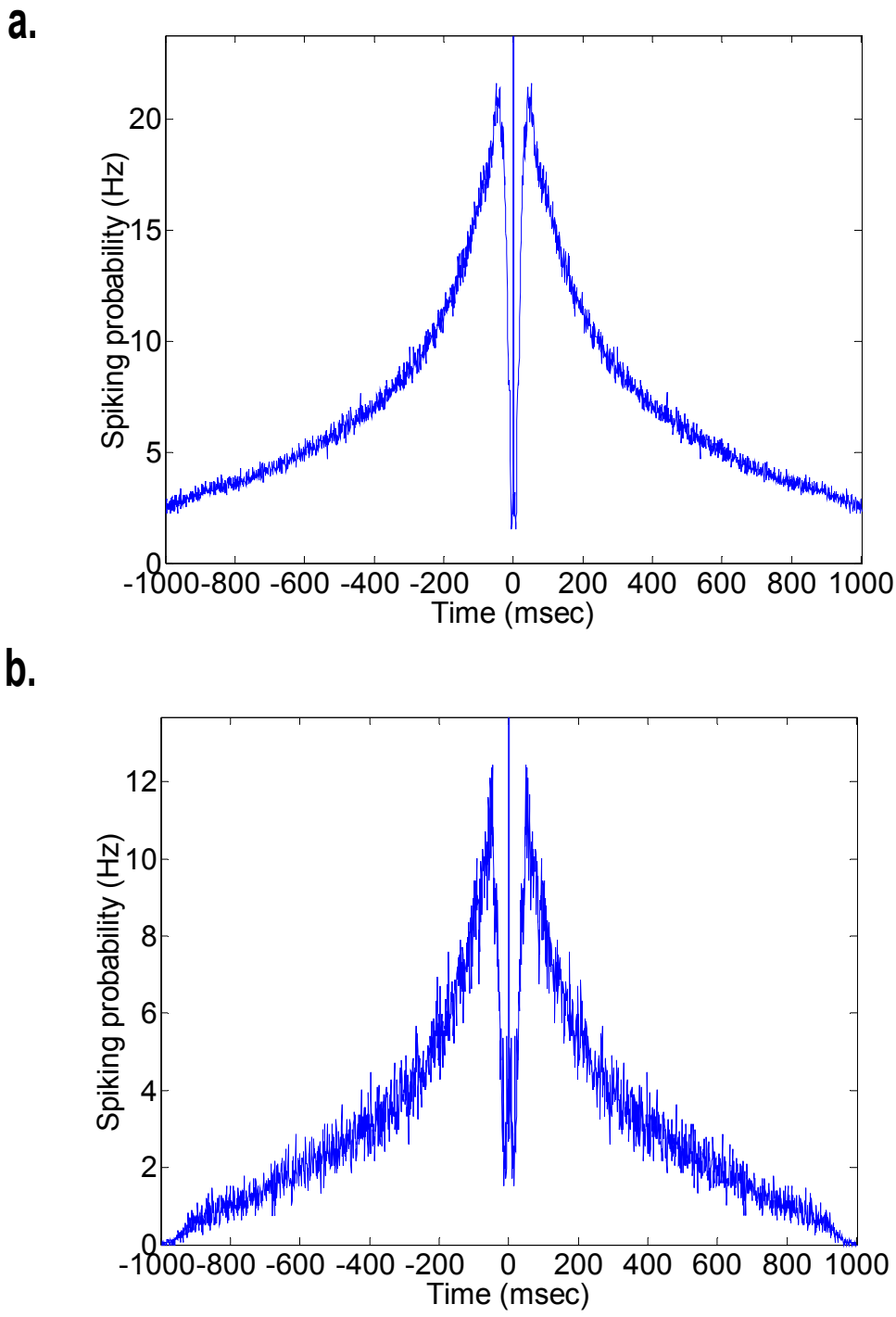

Figure 4.13. PNs exhibit a positive autocorrelation with a time constant of several hundred milliseconds both during odor responses (a) and baseline firing (b).

employed above: KCs exhibit sparse representation of odors, often responding to only one odor. It thus reasonable to expect that the properties of the decoding algorithms are likely to minimize false positives and false negatives in a representation where each KC codes only for the odor that excites it the most, rather than maximizing odor discrimination across the entire spectrum of odors. To study the effect of the timescale of decoding on the reliability of the encoding of single odors by KCs, I created simple model KCs that smoothed each PN's response by convolving it with a Gaussian of standard deviation Tau and then integrated the smoothed inputs of 10 PNs together additively. The number 10 was chosen because combinatorial arguments and existing data on PN-KC connectivity suggest that any one KC integrates inputs from a number of PNs that is less than 20, and that it fires upon activation of a subset of these probably not exceeding 10 (Bäcker and Laurent, unpublished results). On any given trial, the model KC fired if and only if its PN inputs exceeded a threshold value at any point in time. No dynamics were considered for the KC. I then calculated, for each of 16 odors, the threshold value such that all trials of that odor elicited a response from the model KC. The odor which yielded the highest such threshold was selected as the model KC's preferred odor, and the KC's threshold was set to the corresponding threshold value. Then, the proportion of trials for non-preferred odors which elicited a response from the model KC was calculated, and called the proportion of false positives. If the representation is sparse and reliable, this proportion should be low; otherwise, it will be higher. The proportion of false positives was then computed as a function of the timescale Tau at which the PN inputs were smoothed. As was the case with odor discriminability using Victor and Purpura's algorithm above, odor discriminability with this sparse coding scheme also was optimal for timescales on the order of 1-2 sec: the proportion of false positives decreased with increasing Tau (Fig. 4.14). Sensitivity to variations in PN spike trains on a timescale of 1-2 oscillation cycles did not contribute to enhance the reliability and sparseness of the representation.

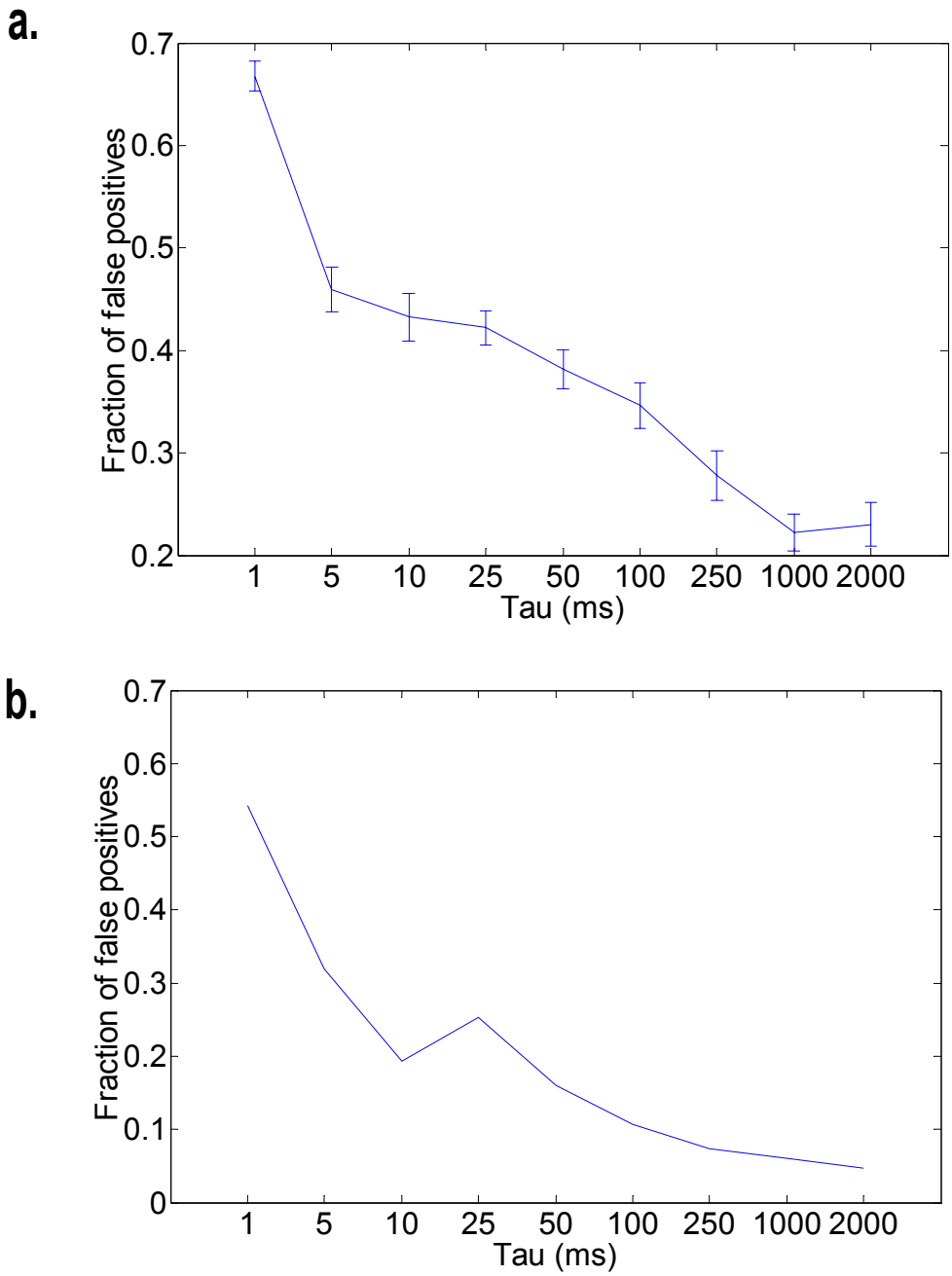

Figure 4.14. The fraction of false positive responses of a model Kenyon cell coding for 1 odor out of 16 and with its threshold set to have no false negatives decreases as the timescale (Tau) of the decoding algorithm is increased (see Text for details). a) Mean fraction of trials which yield false positives over 20 different PN assemblies. **b)** Minimum fraction of trials which yield false positives over 20 different PN assemblies, i.e., fraction of false positive trials for the most discriminating set of PNs.

4.4 Discussion

In summary, I have shown that odor information in single PNs allows classification among several odors significantly above chance levels. This information peaks at odor onset and decays about 500 ms later. The information content of single PN spike trains saturates at 500-1000 ms. Different PNs carry non-redundant information: correct classification rates were significantly higher if I kept track of neuronal identity than if responses were summed over the PN assembly. Increasing the size of the PN assembly increased correct classification rates, saturating at about 10 PNs. No further improvement was seen by increasing the size of the PN assemblies from 10 to 19. The optimal timescale for decoding proved to be of the order of 1-2 seconds, yielding significantly higher classification rates than the timescale of the oscillation cycles of 50 milliseconds. This was true both for single PNs and PN assemblies, and both for discrimination among all odors presented and a sparse coding scheme in which each decoder encoded a single odor. The preferred timescale of decoding can be explained by the observation that PN responses, both during odor presentation and between odor stimuli, are highly correlated on the timescale of several hundred milliseconds.

This correlation over timescales of hundreds of milliseconds is in sharp contrast with the results of Wehr and Laurent (1996), who reported on 4 PNs some of whose firing probabilities changed abruptly from one oscillation cycle to the next for any given odor. Although I have observed such PNs with highly precise and fast-varying firing rates in my data as well (Fig. 4.15), they constitute a minority of all PNs recorded, as evidenced by the analysis across 77 PNs presented here. More representative of the majority is a bursty PN (Fig. 4.16). The differences cannot be explained by a differential recording bias in favor of bursty PNs with high firing rates on my part, because the same degree of correlation in PN responses was observed not only in the recordings of Katrina MacLeod, but also in extracellular tetrode recordings in which the position of the electrodes is not manipulated

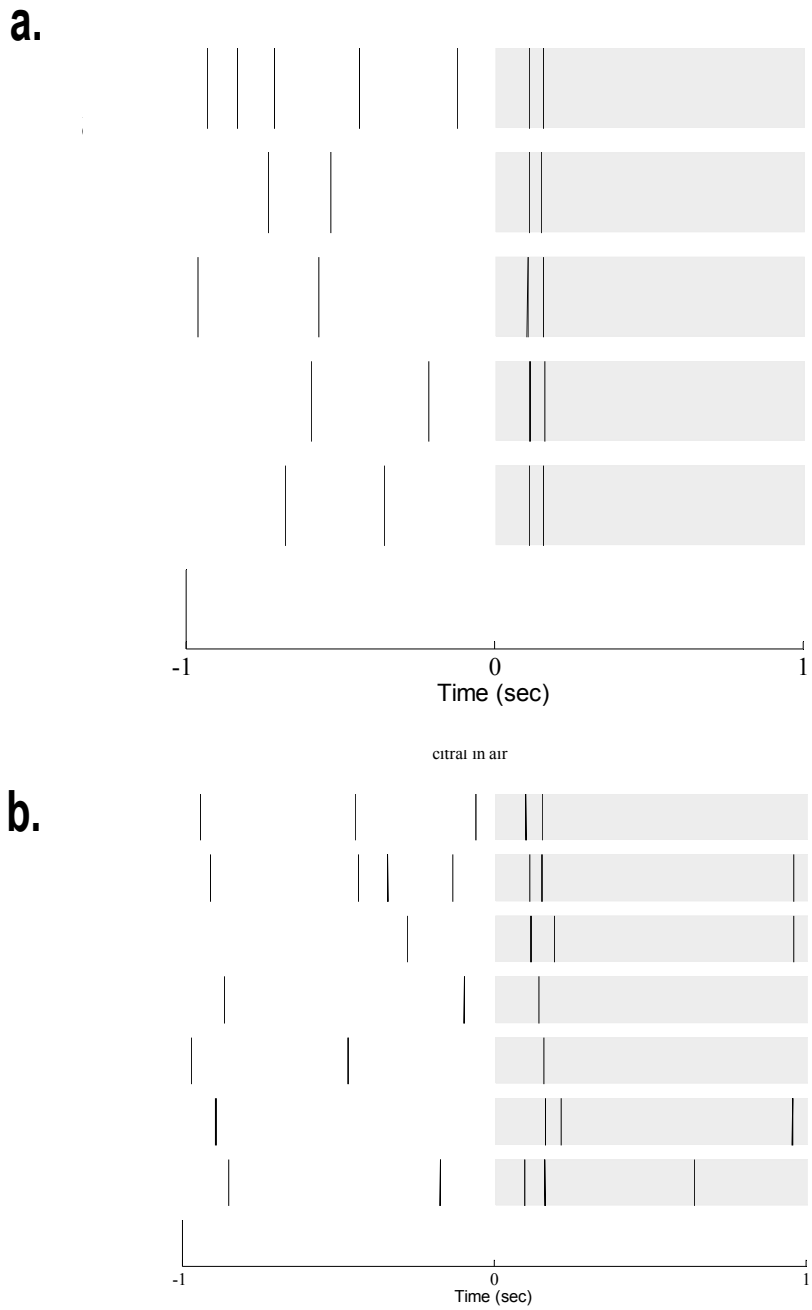

Figure 4.15. The response to cherry (a) and citral (b) of a PN with precise and brief response patterns, as described by Wehr and Laurent (1996). Responses such as these are found in a minority of PNs. They are typically characterized by short latencies, low intertrial variability and short duration. These probably constitute a subtype of PNs, since their responses to all odors all typically fall in the same category of precise, brief, early responses. The odors were presented from t=0 to 1 s.

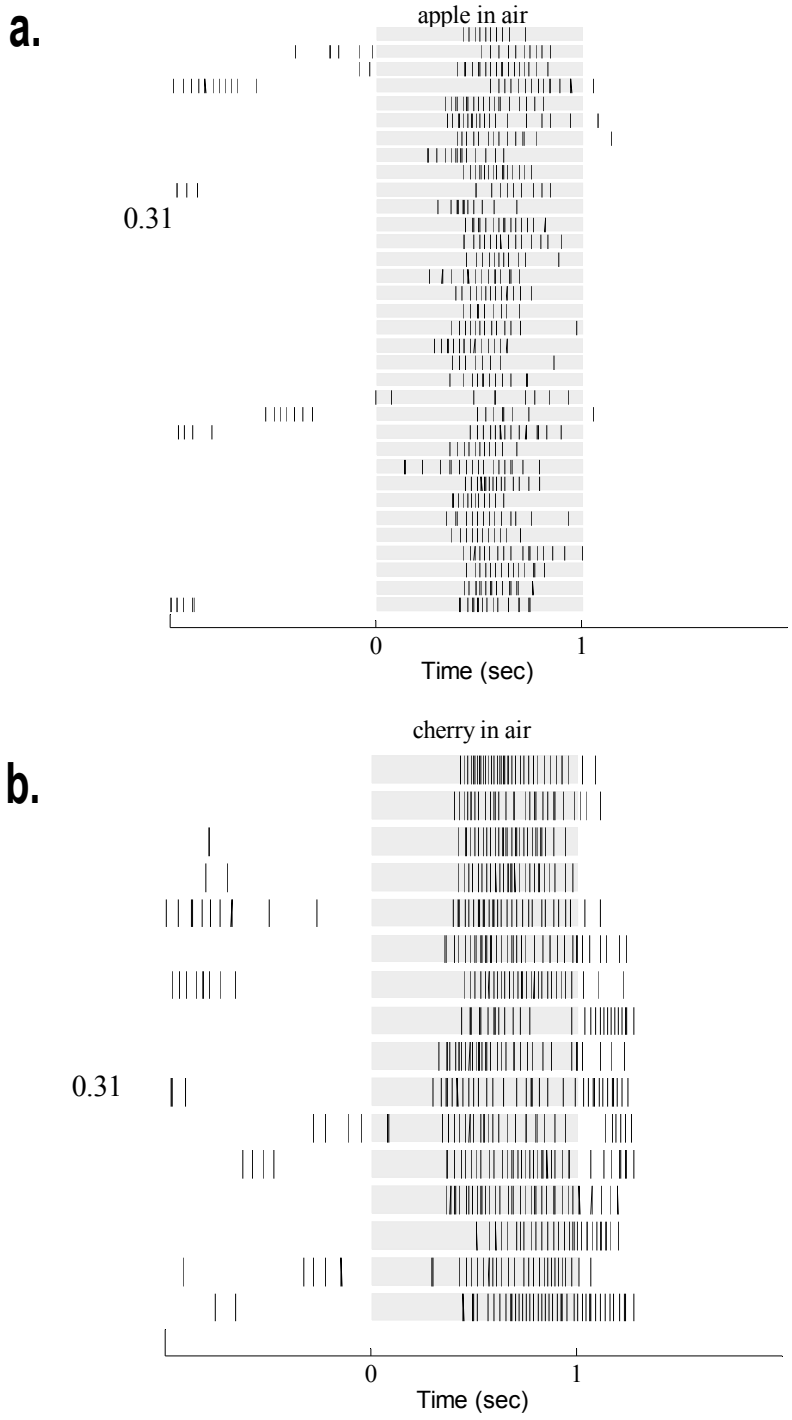

Figure 4.16. The response to apple (a) and cherry (b) of a PN in another locust than that shown in Fig. 4.15. This PN, more representative of the majority of PN recordings than that in Fig. 4.15, responds to odors with bursts of spikes lasting several hundred milliseconds. This type of neuron is responsible for the highly significant slow autocorrelation observed across all 77 PNs analyzed above.

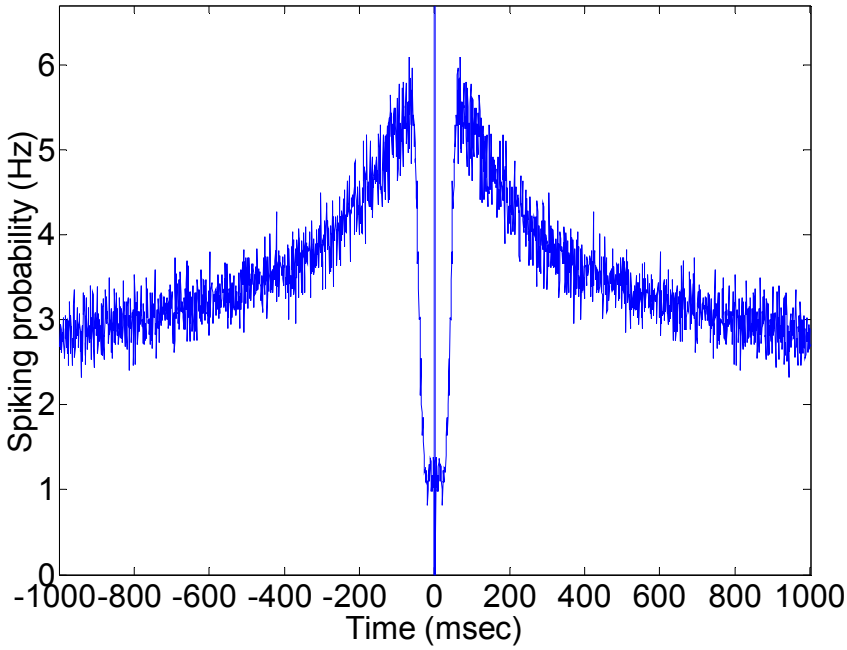

Figure 4.17. Spike-triggered average firing probability for 19 PNs simultaneously recorded with 2 tetrodes (data courtesy of Stijn Cassenaer). As with intracellular recordings (Fig. 4.13), a positive autocorrelation on a timescale of several hundred milliseconds is observed.

for individual cells (Fig. 4.17).

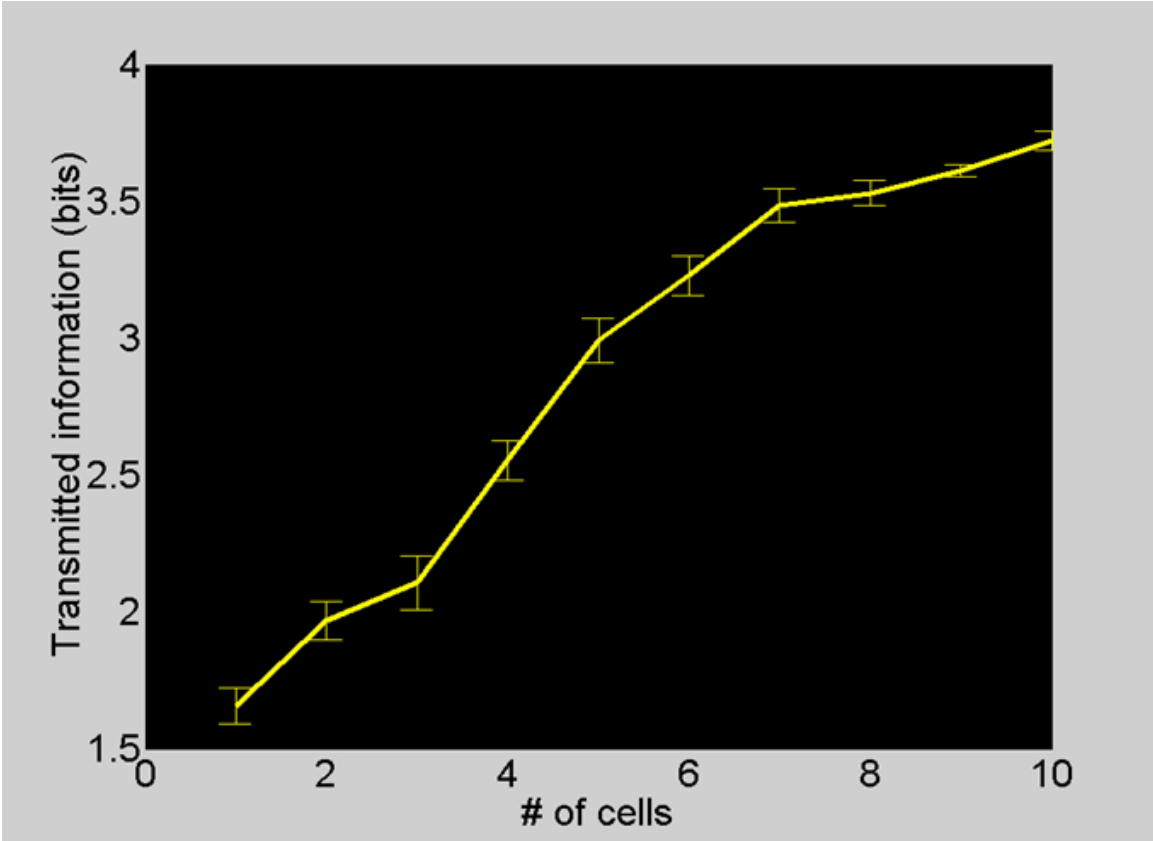

Figure 4.18. Transmitted information as a function of the number of projection neurons used for odor identification.

The work of Stopfer and colleagues (1997) and Chapter 5 of this thesis (part of which has been published as MacLeod et al., 1998) has shown that synchronization on a timescale much smaller than that seen here to be optimal for decoding is required for fine olfactory discrimination and the readout of PN assemblies by downstream neurons. The functional advantage conferred by such selectivity remains unknown, and will be addressed in the first part of the next chapter. It is possible that neuronal biophysics makes it impossible to integrate over timescales of several hundred milliseconds, making the ideal decoding algorithm biologically implausible. Alternatively, it is possible that the analysis of larger numbers of simultaneoulsy recorded PNs, or the analysis of decoding algo-

rithms that approximate Kenyon cells more closely than those used herein, will reveal a role for fast timescales in the decoding of PN assemblies. Finally, it is possible that fast timescales are useful in the encoding of the rapidly varying signals in natural dynamic odor plumes rather than the more uniform odor pulses used in the experiments in this thesis and in the previous work of the laboratory.

4.5 Applications

The method applied here to classify spike trains into the stimulus classes most likely to have given rise to them, and slight variations on it, have been widely applied (MacLeod, 1999; Stopfer and Laurent, 1999; Friedrich and Laurent, 2001; Bhazenov et al., 2001, Ch. 8, this thesis) since the publication of parts of this work (MacLeod et al., 1998).

4.6 Methods

Surgery, Odor delivery and Electrophysiology

See Chapters 7-9, Methods.

Clustering analysis

The clustering analysis is based on the cost-based metric methods (Victor and Purpura, 1997) according to which a ‘distance’ is computed between spike trains. This distance is defined as the cost paid to transform one spike train into the other using three elementary steps: insertion; deletion

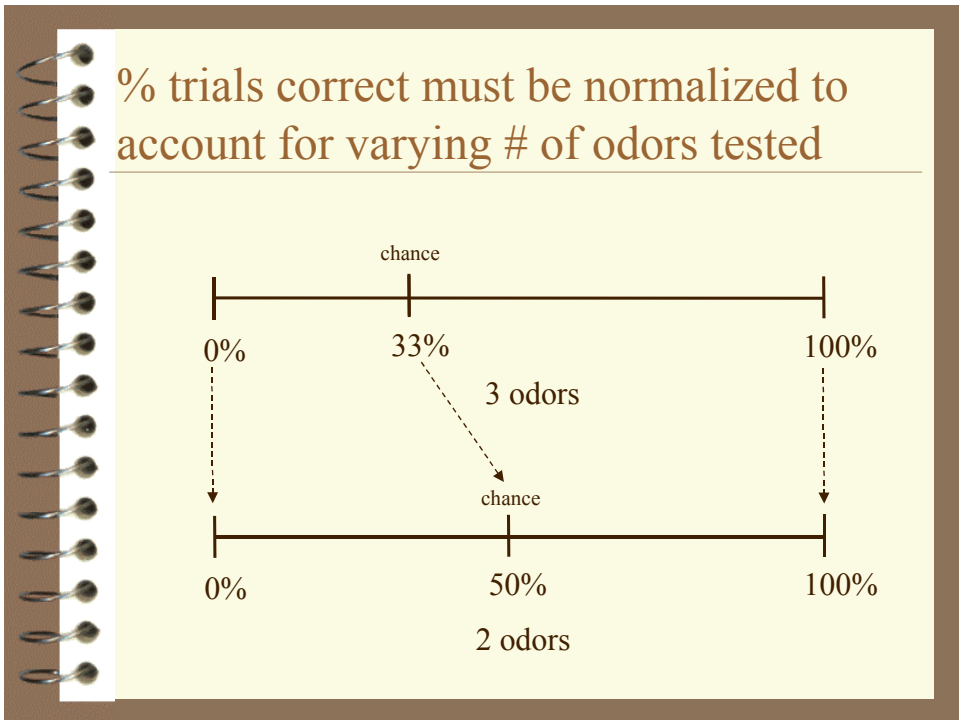

Figure 4.19. Normalization of fraction correct to a two-alternative-forced-choice scenario.

of a spike (each at a cost of 1); and displacement of a spike by 1 ms (cost of 2/*T* for each displacement, where *T* is the maximum separation in ms allowed between the spike time in one train and that in the other). I used a range for *T* between 16 and 4,000, with *T* = 150 providing the best classification overall. Results were not greatly different for 16 <= *T* <= 1000. Classification was carried out using two methods: In one, the mean distance between a spike train and all spike trains of a stimulus class was an arithmetic mean (all points equal); in the second, the mean (M) was geometric, with the exponent set to -15 (less weight to outliers):

$$M = \sum_i d_i^{-15}$$

Percent correct results from choosing among all odors without restriction to pairwise assignments in those neurons that responded to more than two odors. For each neuron *i*, chance level is thus 1/*m*i, where *m*i is the number of odors to which neuron *i* responded. The effective number of odors for the mean percentage correct was calculated as $(<1/mi>)^{-1}$. When mean rates are shown as 50%, mean percentage correct was then normalized to a two-alternative-forced-choice scenario by dividing the difference between the percentage correct observed and the chance level given the effective number of odors, by 0.5, i.e., the maximum improvement above chance possible after normalization. For the lag and duration analyses, in order to obtain a distribution of percentages for all datasets to be able to compare across lag or duration values, percentages of trials correct were normalized for each dataset using a piecewise linear transformation between the space for the actual number of odors for each dataset and a normalized space with 2 odors, such that 0, 100% and chance levels were fixed points and mapped to the corresponding point in the other space, and all other points underwent a linear mapping using the closest fixed points as anchors (Fig. 4.19).

For the estimation of discrimination as a function of latency and spike train duration, this method was then applied to successive sliding windows of the spike trains, varying the duration and lag of the windows with respect to the time of stimulus onset (Fig. 4.20).

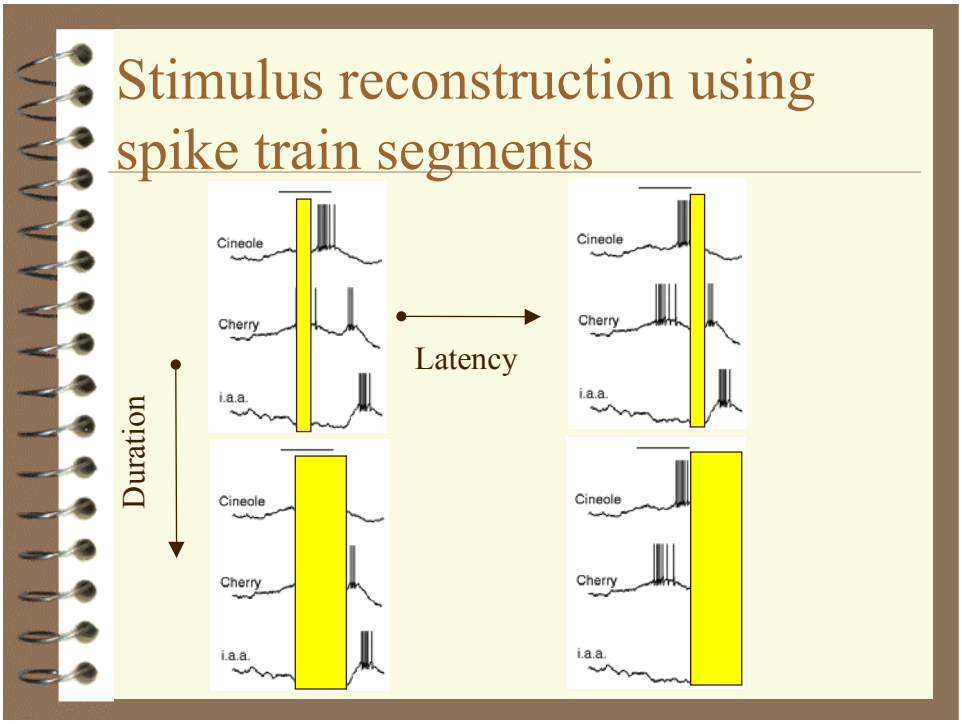

Figure 4.20. Schematic diagram of method used to estimate odor discrimination as a function of latency and spike train duration.

CHAPTER 5

The role of oscillatory synchronization in the decoding of temporal information in PN assemblies

Abstract

Synchronization of neuronal assemblies has been widely found in brain circuits. Its functional significance, however, remains a mystery. In the locust antennal lobe, PNs respond to odors with oscillatory synchronization. Synchronization is mediated by oscillatory inhibition and can be selectively disrupted with picrotoxin, a GABA antagonist (MacLeod and Laurent, 1996). We exploited this manipulation to test four hypotheses for the role of synchronization. We show that synchronization does not reduce PN firing rates and does not decorrelate PN responses in time. Furthermore, the informational value about odor identity of synchronized spikes is not different from that of unsynchronized spikes. Synchronization is nonetheless shown to be essential for the correct decoding of odor information in PN assemblies by downstream neurons. Disruption of synchronization leads to loss of odor-related information in downstream neurons. The informational value in the set of synchronized spikes is shown to be equivalent to that of the set of all spikes, suggesting that downstream neurons sensitive only to synchronized spikes do not miss information on odor identity.

Introduction

This chapter employs a methodology adapted from work in the visual system for use in the olfactory system for the first time, and applies it to study two related problems stemming from previous work of the laboratory.

I. Picrotoxin does not alter stimulus information in PN spike trains

Previous results claiming that picrotoxin selectively disrupts synchronization without affecting slow temporal patterns (MacLeod and Laurent, 1996) have been subject to the criticism that it appeared

arbitrary to say that the slow temporal patterns remained unaffected, given that some degree of change was present whose significance, relative to the variability inherent in neuronal responses, was unknown. Our present results show that an objective criterion, namely the stimulus information present in the spike trains, as measured by the fraction of trials assigned to the correct odor using closest-neighbor classification employing a cost-based metric, renders spike trains of single cells in control and picrotoxin-injected conditions statistically undistinguishable.

II. A role for neuronal synchronization

As Lord Adrian pointed out in 1951, the fact that the differences in the spatial and temporal pattern of excitation produced by different smells can be distinguished by the electrophysiologist does not mean that smells are distinguished in this way by the animal (Adrian, 1951). This chapter provides evidence that, indeed, the olfactory system is unable to read information *present* in neuronal assemblies when those assemblies are desynchronized.

Synchronization is a ubiquitous phenomenon in brain circuits. It has long been known that the mammalian olfactory bulb exhibits oscillatory activity in response to odors (Adrian, 1950). Neurons in the visual system synchronize in response to visual objects (Gray and Singer, 1989). Synchronization has also been found in the somatosensory system (Nicolelis et al., 1995), the motor system (Riehle et al., 1997), the hippocampus (reviewed in Bland and Oddie, 2001) and frontal cortex (Abeles et al., 1993). Recent experiments suggested that synchronization serves a function in the discrimination of similar, but not dissimilar, odors by the bee (Stopfer et al., 1997). But what is this function that has remained elusive for so long?

One hypothesis for the role of synchronization is that the role of the inhibition is to reduce prolonged activity and thus prevent learning. If inhibition is prevented in the accessory olfactory bulb of rats by a bicucculline injection, an olfactory memory for the odor present is created (Brennan et al., 1990). This memory prevents pregnancy blocking by the odor (Brennan et al., 1990). This could potentially explain the lack of synchronizing inhibition in the first trial of exposure to an odor (Stopfer and Laurent, 1999): once an odor is not novel, learning is inhibited.

At the behavioral level, disruption of oscillatory inhibition did not lead to the creation of a more robust memory for the odors presented, but rather, to greater confusion between chemically similar odorants, and thus, presumably weaker memories (Stopfer et al., 1997). On the other hand, "stronger" memories could become more overlapping for similar odorants after disruption of inhibition, and explain the picrotoxin-induced confusion. The results reported in the paper included at the end of this chapter (Macleod et al., 1998), however, show that firing rates do not increase after the disruption of synchronization by picrotoxin (see Fig. 4d in the paper). Furthermore, the mean spike count in 50 msec windows is not affected by picrotoxin ($p>0.36$, Wilcoxon ranksum test).

Whether or not synchronization's role is in learning as opposed to perception, it is conceivable that oscillatory inhibition serves to decorrelate PN responses in time. Since PN responses are bursty (Figs. 4.10-4.13 and 4.17), and if excitation in PNs is enhanced by depolarization, periodic inhibition could serve to hyperpolarize PNs following excitation and prevent the formation of a burst, making the neuronal assembly active in successive cycles more different from one another. This hypothesis can be tested by comparing the correlation in time of PN responses with and without oscillatory

inhibition.

In fact, the opposite was true: the absolute differences in spike count between successive 50 msec windows were slightly but significantly lower in control trials than after picrotoxin injection (mean in controls = 0.067 +- 0.001 spikes vs. 0.072 +- 0.001 spikes after PCT injection, $p<0.008$, Wilcoxon ranksum test). Together with the fact that mean spike counts are not affected (see above), this indicates that synchronization actually causes a slight increase in the correlation of successive 50 msec windows in PN spike trains. Fig. 5.1 shows the spike-triggered firing rate average in control and after PCT injection. Fig. 5.2 shows the correlation between the spike counts in successive 50 msec time windows in control and after PCT injection.

A third hypothesis sustains that synchronization serves as a filter to differentiate spikes with high informational content from spikes with low informational content ('noise'). To test this, I compared the informational content of synchronized spikes (defined by a phase variance of less than **X** radians; this synchronization threshold was selected so that an equal number of synchronized and unsynchronized spikes were present) versus that of unsynchronized spikes, by classifying spike trains consisting only of the corresponding spike type into the odor corresponding to the closest cluster (see methods, below, and Fig. 5.3). The fraction of trials correctly classified for each PN using synchronized spikes was not significantly different from that using an equal number of unsynchronized spikes (Fig. 5.4, $p>0.1$ for every T-value (10, 25, 50, 100, 250, 500, 1000, 2000 and Inf) and z-value (-15 and 1) combination tested, n=46 PNs, Wilcoxon ranksum test). This result is consistent with our previous result showing that odor information in PNs is not disrupted by desynchronization with PCT (see Nature paper below). A caveat must be noted in that the classification was

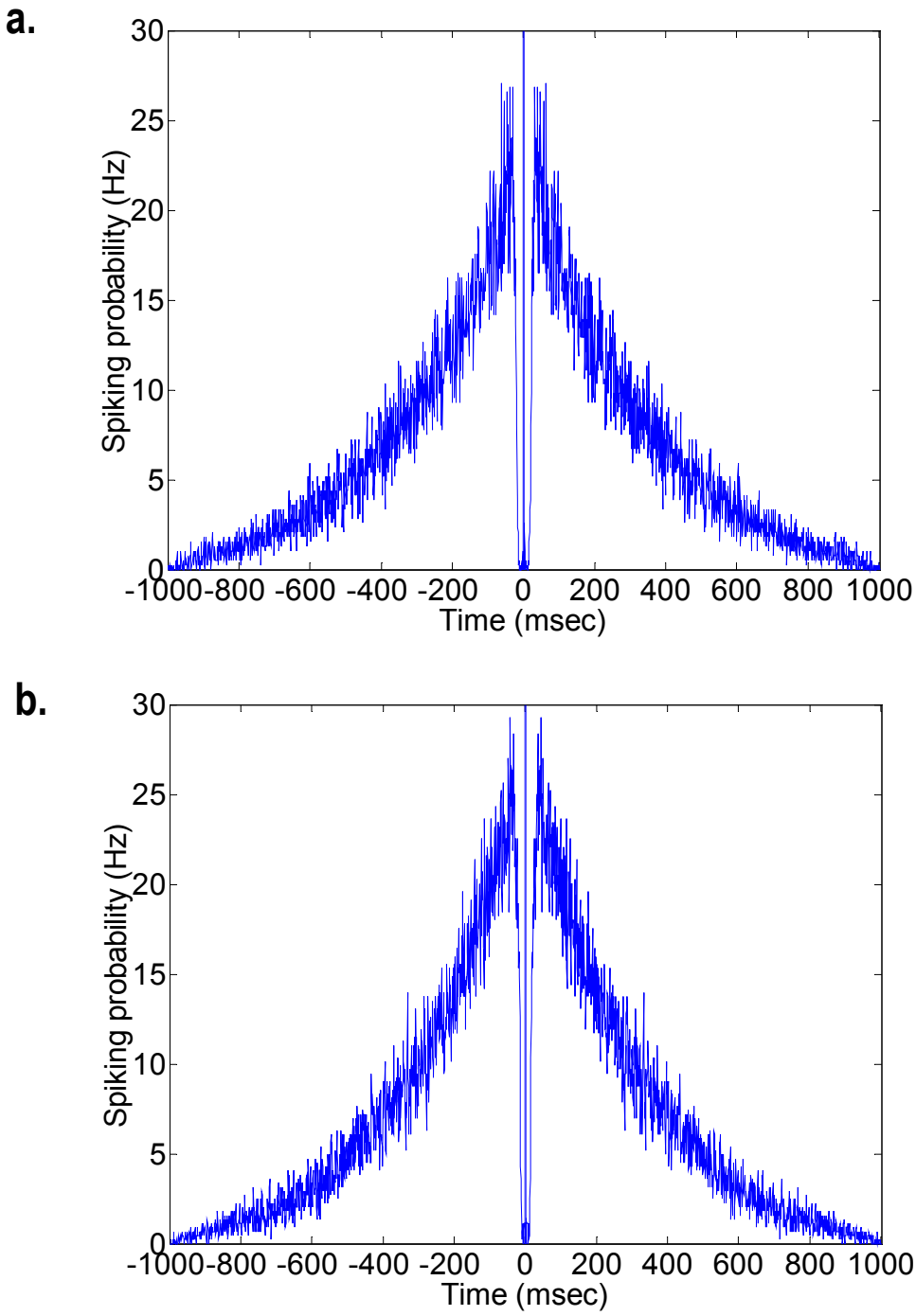

Figure 5.1. Spike-triggered average firing rate before (a) and after (b) PCT injection. Computed for the set of 12 PNs in Fig 4.11 (data courtesy of Katrina MacLeod).

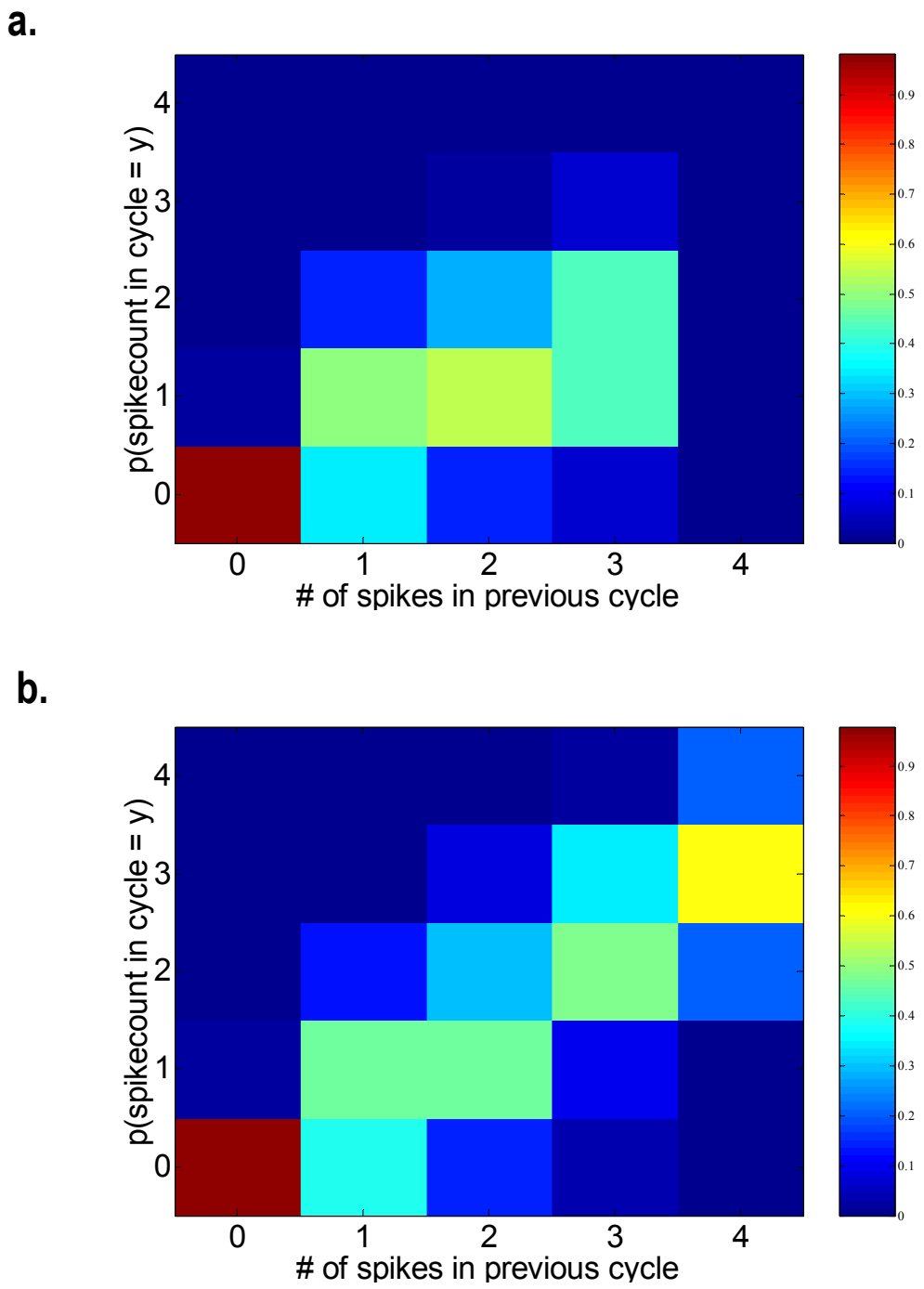

Figure 5.2. PN odor responses are bursty with and without synchronization: The probability of firing of a PN in any given cycle during the 3 sec following the onset of odor presentation before (a) and after (b) PCT injection is significantly enhanced if the PN has fired in previous cycles, and the number of spikes in successive cycles are significantly correlated. P(x,y) = p(# of spikes in cycle N=y | # of spikes in cycle N-1=x). Computed for the set of 12 PNs in Fig. 5.1 (data courtesy of Katrina MacLeod).

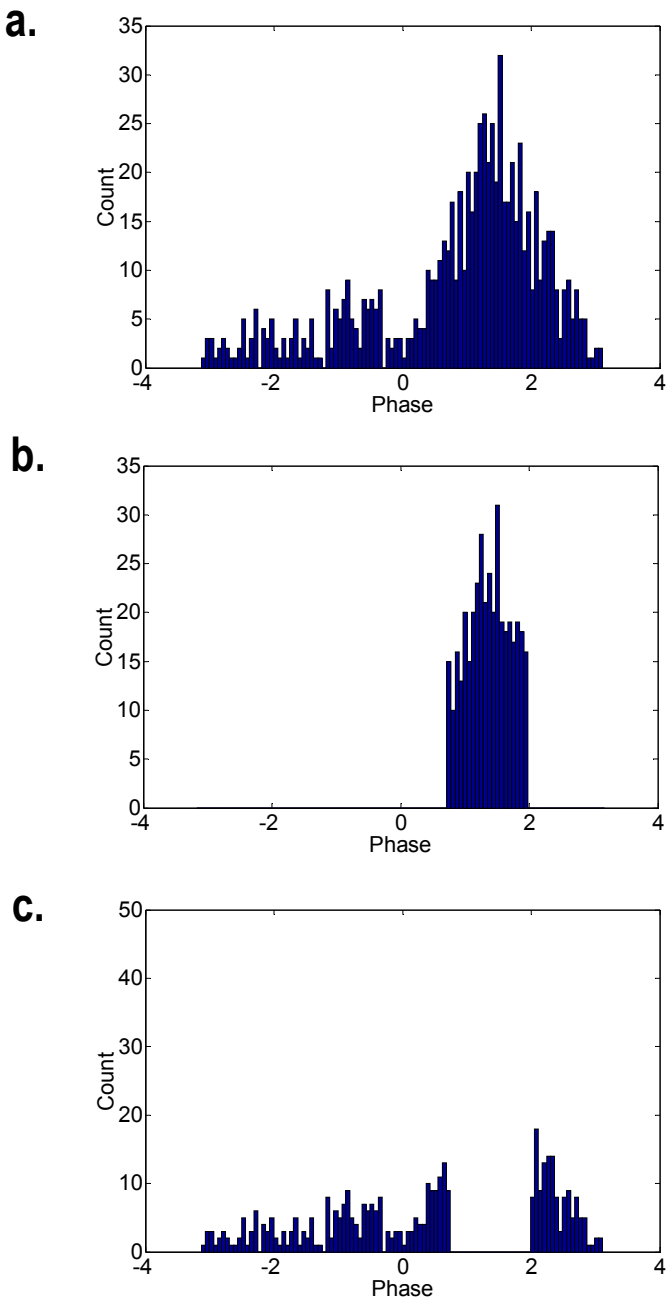

Figure 5.3. Method used to separate spikes into synchronized and unsynchronized spikes. The entire phase distribution for the responses of a given PN-odor pair at a given concentration (a) is fed to an algorithm that calculates the mean (vector) phase and the phase range centered on the mean phase that divides all spikes in two sets with equal number of spikes. That closest to the mean phase is termed the synchronized half (b) and the other one is termed the unsynchronized one (c).

not performed simultaneously using spike trains from multiple PNs —a multi-cell analysis of a set of simultaneously recorded PNs is ongoing. The finding that, for single PNs, synchronized spikes are as informative as those which are not, however, suggests that synchronized spikes do not carry an intrinsic informational value higher than that of unsynchronized spikes.

Classification using synchronized spikes was itself not significantly different from classification using all spikes (Fig. 5.4, $p>0.04$ for every T-value (10, 25, 50, 100, 250, 500, 1000, 2000 and Inf) and z-value (-15 and 1) combination tested, n=46 PNs, Wilcoxon ranksum test not corrected for multiple comparisons). This suggests that Kenyon cells do not miss out on odor information by reading out only synchronized spikes.

A final hypothesis holds that synchronization is important for downstream neurons to decode signals from neuronal assemblies, which may discard spikes unless they are part of a volley of quasi-simultaneous spikes across a cell assembly. In other words, even if synchronized spikes are not intrinsically more informative, they may be read out preferentially by the decoding algorithm employed by cells downstream of PNs. This hypothesis has recently garnered some support from the biophysics of Kenyon cells, which amplify large inputs nonlinearly (Pérez-Orive et al., in press).

The paper that follows, published in the October 15th 1998 issue of *Nature* and reproduced here with the kind consent of the publisher, examines this fourth hypothesis for the role of synchronization, and addresses the question of whether desynchronization impairs the information content of single PNs (see (I) above). The experimental work therein was carried out by Katrina MacLeod. My

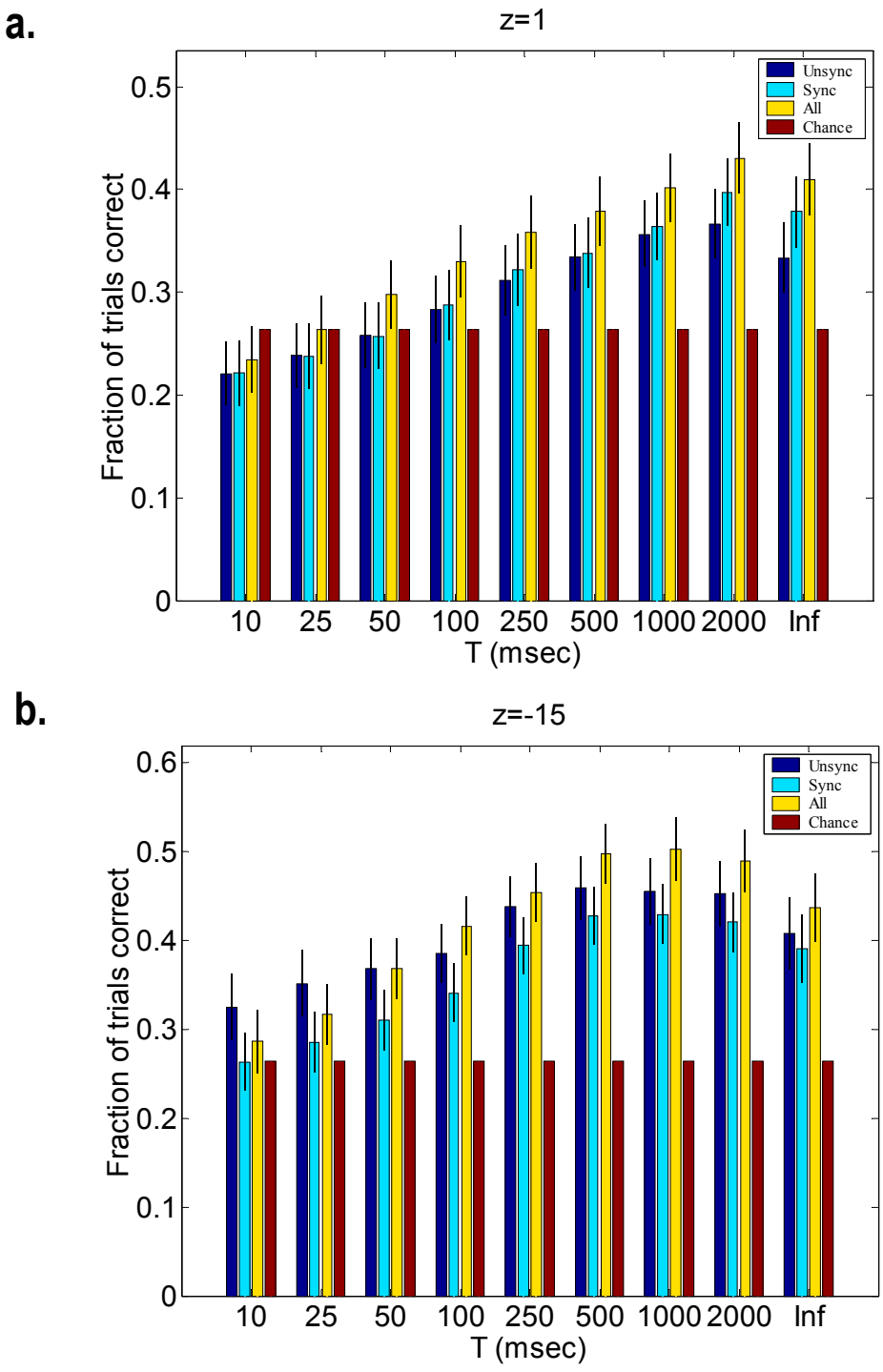

Figure 5.4. Odor discrimination as a function of spike synchronization. Mean fraction of trials correctly classified (and s.e.m.) by 46 PNs as a function of the timescale T of decoding (see Methods) using synchronized (light blue), unsynchronized (dark blue) or all (yellow) spikes, for (a) linear averaging across trials (z=1) and (b) geometric averaging (z=-15). Chance classification levels are shown in red.

contribution was limited to the proposal that a comparative information-theoretical analysis could help elucidate the initially puzzling effect of desynchronization of PNs on downstream neurons, and to the subsequent analysis that classified responses of PNs and downstream neurons into the odor responsible for eliciting the cluster most similar to each.

A note on methods

The paper employs two methods to classify spike trains into clusters corresponding to odors. Below is a brief comparison between the two. This may be of particular interest given that later papers (Stopfer and Laurent, 1999; Friedrich and Laurent, 2001; Bhazenov et al., 2001) have all employed the method that is less favored by the considerations below.

Differences between vector clustering method used by Katrina MacLeod (called #1 in the paper using a reverse chronological convention) and the clustering method using Victor and Purpura's cost-based metric I employed (#2) (MacLeod, Bäcker and Laurent, 1998):

A. Theory

1. #1 uses binning, which results in general in a loss of resolution. This can be solved by applying convolution of the spike train with a gaussian prior to binning.

2. #1 used an average spike train template, which may not be typical at all (as a crude example, the average of the points on a circumference does not lie in the circumference at all). #2 avoids having

to do that and looks at the distances between all individual spike trains. This could also be implemented for #1, though.

3. #1 was used in a pairwise comparison, while #2 was applied to a more difficult task that is closer to what the animal needs to do: choosing among all odors experienced.

4. Centroids including the spike train being classified, as done with method #1, eliminate independence of the classification because the distance is biased toward the right answer through the self-inclusion. This can be remedied by recalculating the centroid for each classification, excluding the corresponding spike train for each. Even better, centroids can be done without altogether (see #2 above).

5. The main and irreconcilable difference is that #2 takes the continuous nature of spike trains into account, while #1 deforms the one-dimensional nature of spike trains by mapping time bins onto Euclidean space. In doing so, #1 treats each bin as orthogonal to each other. Consequently, two spike trains with one spike each, in which one spike falls in one bin and the other in an adjacent bin, are just as different for method #1 as two spike trains with single spikes many seconds apart. In #2, in contrast, the distance between spike trains is directly related to the time difference between corresponding spikes.

B. Empirical comparison

A comparison of the results yielded by the vector clustering algorithm and the Victor and Purpura method (V&P) for the data in the paper below yields a simple yardstick by which to measure both methods (see Fig. 4b and c in the paper). For both PNs and ß-lobe neurons, the answer of the clas-

sification method using V&P's distance seems to agree more with our visual inspection of the data and the results we reported in the paper than the vector-based one: For ß-LNs, V&P is more sensitive in picking up the changes induced by picrotoxin (PCT), yielding a p-value twenty times smaller (i.e. more significant) than the vector method, even though V&P significance was assayed with a two-tailed test which is more conservative than the one-tailed test used for the vector method in that it does not assume that classification can only be impaired by PCT. This advantage of the V&P method in sensitivity was present despite the fact that V&P was applied to all datasets, while the vector method was applied only to datasets which showed good odor classification in the controls, a selection bias that, if anything, would tend to *increase* the impairment in correct classification due to PCT.

For PNs, V&P is able to classify PN responses just as well for PCT as for the controls (p=0.93!), but the vector method yields a clearly visible, yet not significant, decrease in correct classification. Without any extra information, it is impossible to determine which of these represents the data more closely. This is not made any easier by the fact that the cluster method was applied to pairwise classification, while the V&P method was used to classify among all odors to which the neuron was exposed, so the fraction of trials correctly classified cannot be compared between the two. The visual inspection we later made of the data suggested to us, however, that the information in PNs to discriminate odors was unchanged by PCT application. Once again, the datasets used with the vector method were a selected subset, advancing a potential explanation for the nonsignificant decrease in information seen with that method but absent in the analysis with the V&P method that included all datasets.

The most biologically relevant alternative, of course, were our knowledge complete, would be to use the actual decoding algorithm of the neurons that constitute the real decoders of PNs and ß-LNs.

stereo plaids appeared in separate 180-ms intervals separated by a 400-ms blank period. Observers selected the interval containing the more distant of the two test plaids. The plaids consisted of a 15° positive-disparity grating and a 45° zero-disparity grating, each with 5% contrast. They appeared at slightly different depths on the near side of the plane of fixation, as shown in Fig. 4. The two 15° gratings had disparity phase angles of 31.0° and 18.6°, yielding a horizontal disparity difference of 8′. The disparity of the adapting pattern was either midway between the disparities of the 15° component gratings appearing in the two test intervals or midway between the disparities of the 2D features appearing in the two test intervals. There were four adapting stimuli: a 15° grating, a 105° grating, a plaid with components at 15° and 45°, and a plaid with components at 105° and 135°. The horizontal disparities of the adaptors were set to correspond to the depths of adaptors A and B in Fig. 4. For plaid adaptors, both components (and the 2D features) had the same horizontal spatial disparity. Adapting gratings had spatial frequencies of 1.0 cycle per deg, the same as the test gratings; they appeared in a circular window with a diameter of 9°, to ensure complete retinal overlay of the 7.85° test window. Each run of 20 trials began with an adapting period of 40 s, and each trial began with a 4 s 'topping-off' adaptation period, with 0.4 s separating adaptor offset and the first test interval. Adaptors underwent smooth harmonic motion at 1.0 cycle per s to minimize retinal adaptation. Adaptation and no-adaptation conditions were identical except for the adaptor grating contrast (10 and 0%, respectively). Adaptor contrast and duration were identified by systematic sampling to favour disparity adaptation at the expense of contrast adaptation; relatively brief and low-contrast adaptors tended to yield this result. Contrast adaptation and disparity adaptation have opposite expected consequences, the former inhibiting and the latter facilitating depth discrimination. Observers were instructed to maintain fixation on the central square throughout each trial and to respond by clicking one of two on-screen buttons to indicate the interval containing the test plaid more distant from the observer. Of three observers, one was naive; all had corrected-to-normal monocular and stereo acuities. Each data point was based on 40 trials.

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Who reads temporal information contained across synchronized and oscillatory spike trains?

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Our inferences about brain mechanisms underlying perception rely on whether it is possible for the brain to 'reconstruct' a stimulus from the information contained in the spike trains from many neurons[1–5]. How the brain actually accomplishes this reconstruction remains largely unknown. Oscillatory and synchronized activities in the brain of mammals have been correlated with distinct behavioural states or the execution of complex cognitive tasks[6–11] and are proposed to participate in the 'binding' of individual features into more complex percepts[12–14]. But if synchronization is indeed relevant, what senses it? In insects, oscillatory synchronized activity in the early olfactory system seems to be necessary for fine odour discrimination[15] and enables the encoding of information about a stimulus in spike times relative to the oscillatory 'clock'[16]. Here we study the decoding of these coherent oscillatory signals. We identify a population of neurons downstream from the odour-activated, synchronized neuronal assemblies. These downstream neurons show odour responses whose specificity is degraded when their inputs are desynchronized. This degradation of selectivity consists of the appearance of responses to new odours and a loss of discrimination of spike trains evoked by different odours. Such loss of information is never observed in the upstream neurons whose activity is desynchronized. These results indicate that information encoded in time across ensembles of neurons converges onto single neurons downstream in the pathway.

The function of oscillations and synchronization in information processing, perception and action is difficult to establish directly. Studies in mammals have correlated the degree of neural synchronization with specific behavioural or cognitive tasks, such as segmentation[8], rivalry[9], and sensorimotor tasks[10,11], suggesting a functional link. In locusts, stimulation by odours evokes synchronized firing in dynamic and odour-specific ensembles of projection neurons in the antennal lobe, a region analogous to the vertebrate olfactory bulb[16–18]. This synchronization relies critically on fast GABA (γ-aminobutyric acid)-mediated inhibition, and can be selectively blocked by local injection of the GABA receptor antagonist picrotoxin[19]. Picrotoxin spares the slow modulation of individual projection neuron responses[19] but desynchronizes the firing of the odour-activated assemblies[19] and impairs fine odour discrimination[15]. These results establish a causal link between synchronization and perception. They do not, however, determine where information is lost when projection neurons—the information channels—are desynchronized. One possibility is that no single neuron between sensory and motor/cognitive areas is, on its own, sensitive to input synchronization. The behavioural deficit caused

by desynchronization of projection neurons induced by picrotoxin would thus be a result of collective neural activity only. Another possibility is that injection of picrotoxin leads to a loss of information in the responses of individual projection neurons due, for example, to jittering in their spike times. Desynchronization of projection neurons would thus be a by-product of picrotoxin treatment, but not the actual cause of the behavioural deficit. Finally, perhaps individual projection neuron responses undergo no loss of information after picrotoxin treatment, but responses of neurons downstream from them do. This result would indicate that information contained across projection neurons[16] is indeed crucial, and it would identify the downstream neurons as decoders of this relational/temporal information.

We searched for such putative neural elements downstream from the antennal lobe projection neurons, which project to the mushroom body, where they make divergent connections onto ~50,000 mushroom body intrinsic neurons (Fig. 1a). Odours cause oscillatory activity in these neurons, but they evoke spiking responses only in very sparsely distributed ones[20], making the sampling of odour-responsive neurons very difficult. We thus focused on a smaller population of neurons directly postsynaptic to the intrinsic neurons of the mushroom body[21,22], two synapses downstream from the projection neurons (Fig. 1b). These neurons, the β-lobe neurons, receive convergent input from thousands of mushroom body intrinsic neurons and have clear odour-specific responses (Fig. 1c–e). This makes them a suitable 'read-out' of signals processed in the early olfactory system. We recorded intracellularly from the dendrites of these β-lobe neurons. Dye injection showed discrete and dense (presumed dendritic) arbors in the β-lobe and sometimes also within the pedunculus (Fig. 1b, left). Sparser, varicose (presumed axonal) fibres projected to the α-lobe and sometimes also within the pendunculus (Fig. 1b, left). Many morphological types were found, indicating a heterogeneous population. Morphologically identical examples of a type (for example, Fig. 1b, left) could have different physiological profiles in different animals, indicating either many exemplars of a type in each animal, or an animal-specific tuning history. Many, though not all, β-lobe neurons responded to at least some of the ten odours that we presented and about half of them showed significant phase-locking of their spikes to the odour-evoked local field potential (LFP) oscillations. Responses were usually stimulus-specific but less complex than those of the projection neurons of the antennal lobe[18]. They consisted of phasic or phasotonic increases in firing rate and, in a few cases, of a suppression of firing (Fig. 1c–f). Whereas spiking responses could be brief, a subthreshold synaptic drive consisting of both excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) was usually maintained throughout the duration of the stimulus and mushroom body LFP oscillation (Fig. 1e).

To determine whether responses of the β-lobe neurons depend on synchronization of the projection neurons, a β-lobe neuron was

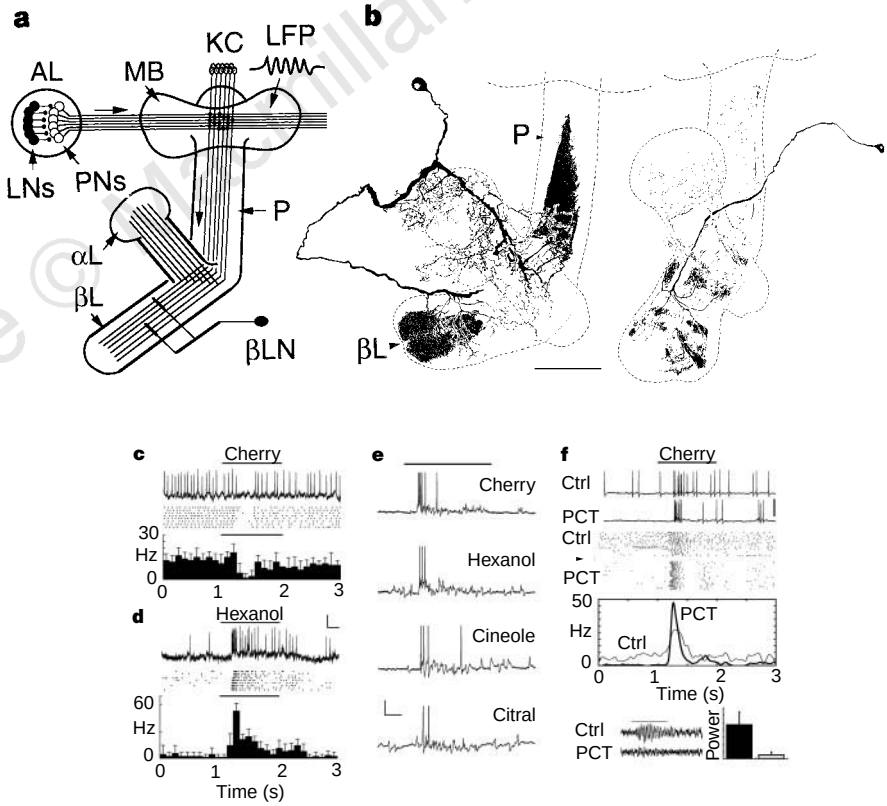


Figure 1 β-lobe neurons (βLNs). **a**, The insect olfactory pathways. Antennal lobe (AL) projection neurons (PNs) receive excitatory input from peripheral olfactory receptor neurons and GABA-mediated inhibitory input from local neurons (LNs)[19,25]. PNs project to the mushroom body (MB) calyx, where oscillatory local field potentials (LFPs) can be recorded in response to odours. Intrinsic neurons of the MB (the Kenyon cells, KCs) receive direct excitatory input from PNs[20,25], and bifurcate to the α- and β-lobes (αL, βL)[20]. βLNs receive input from thousands of KCs. **b**, Left and right, cobalt hexamine fills of two βLNs, each from a different animal. P, pedunculus of the MB. Calibration, 100 μm. **c**, Suppression of activity of the left βLN in **b** in response to a cherry odour. Top, intracellular; centre, rasters; bottom, PSTH; mean firing ± s.d. All odour pulses lasted for 1 s; 7–10 s between trials. Calibrations, 5 mV, 0.2 s. **d**, Prolonged excitatory response of the right βLN in **b** in response to the odour hexanol. Calibrations, 2 mV, 0.2 s. **e**, Transient excitatory responses of a third βLN to four odours. Note subthreshold activity during stimulus. Calibrations, 10 mV, 0.2 s. **f**, Effect of picrotoxin injection on a fourth βLN. Top to bottom: intracellular, rasters, smoothed PSTHs, LFP (bottom left), and normalized integrated power of LFP (bottom right) over 15–30 Hz, mean ± s.d., before (black) and after (open) picrotoxin injection. Calibration, 50 mV.

CHAPTER 6

A computerized odor delivery system for arbitrary time-varying concentrations and mixtures

6.1 Abstract

The behavior of a sensory system is only as rich as the set of stimuli it is faced with. Sensory physiologists are thus faced with the challenge of generating a set of stimuli as rich as possible in a controllable manner. As compared to vision and hearing, where computer screens and synthesizers provide great control and flexibility, the study of olfaction has suffered from a relative lack of flexible odor delivery systems. Here, I present a computerized odor delivery system capable of delivering arbitrary concentrations controlled in real time, binary mixtures in arbitrary ratios, and the potential to deliver arbitrary discrete or continuous stimulus waveforms.

6.2 The need for a novel odor delivery system: Features

There have been many successive improvements in the design of odor delivery systems (Tucker, 1963; Kauer, 1974; Dravnieks, 1975 and references therein; Kauer and Shepherd, 1975; Meredith, 1986; Vigouroux et al., 1988; Perritt et al., 1993) –often called olfactometers rather than olfactogens, a nomenclature that appears no more appropriate than calling a computer screen a photometer. For the purpose of our experiments, though, none of them possessed all of the features we required. In addition, our system is relatively inexpensive, particularly for users who already have a computer.

Concentration in liquid does not equate concentration in vapor

Some, though not all (see references above), of the odor delivery systems in use control the concentration delivered only indirectly, by selecting among flasks each of which has a liquid solution of the odorant in a solvent, such as mineral oil, at a different concentration. While varying the concentration in the solution certainly changes the concentration of the vapor in equilibrium above it, the dependence is not quite straightforward, and depends both on the volatility of the odorant and the

nature of solute-solvent interactions. Indeed, varying the concentration in a solution can change the concentration in the vapor with a log linear relationship or a more complex one, depending on the solvent used (Brockerhoff and Grant, 1999). Changing the concentration in the solution by tenfold can change the concentration in the vapor by 1500-fold. The situation is even less desirable when the odor solution is placed on a filter paper, since the filter paper acts as a chromatographic column to some degree, separating solute from solvent, and thus making the concentration of the odor in the vapor phase more independent of the quantity of odorant introduced, to the extreme that, if separation is complete, the concentration of the odor in the vapor phase will be the odorant's vapor pressure, independently of the quantity of odorant introduced[1]. For direct control of the concentration in the vapor phase, and especially for its quantification, gaseous dilution is preferable over liquid dilution.

Short-term plasticity mandates repeatability across trials

The concentrations delivered by systems which vary the concentration of a solution in filter paper are notoriously time dependent (Brockerhoff and Grant, 1999). The recent discovery that odor responses in the antennal lobe of the locust undergo plasticity in the timescale of a few trials (Stopfer and Laurent, 1999) requires, for the study of such a system, a delivery system known to present repeatable stimuli across trials. In order to achieve that, the system must achieve a steady state before delivery begins, a property shared only by continuous flow systems.

The capability to deliver arbitrary concentrations

Given that we were interested in the coding of odors at varying concentrations, control of the con-

1. As long as the quantity is enough to ensure the headspace of the container can be saturated in odor.

centration was a critical requirement of our system. A majority of previous studies had focused on the *differences* in neuronal responses for different concentrations. Given that one of our interests lies in understanding how the olfactory system achieves *invariance* to concentration, we were especially interested in the ability to vary concentration continuously until we encountered a change in a neuron's response. Many of the odor delivery systems previously described, however, allow only a few discrete concentration steps. The system described here allows dilution to virtually any concentration value in between the minimum and maximum allowed, its resolution limited by the computer's ability to control voltage: a 12-bit card then allows control to better than 1/1000th of the dynamic range, and a 16-bit card provides for better than 1/16,000th. As described below, this ability to deliver similar yet distinct concentrations turned out to be critical for the discovery of abrupt transitions in neuronal responses to concentration.

Real-time online stimulus choice

Related to the ability to delivery arbitrary concentrations was our need to select the concentrations online during an experiment. Thus, while a simple system consisting of delivering the air above one of several odorant flasks pre-diluted in the liquid phase allows the selection of concentrations as close or as different as desired, the choice of concentrations must usually be done *before* the experiment begins. In finding an olfactory threshold or an abrupt transition in response, though, it is paramount to be able to adjust the concentration steps dynamically during the experiment. Controlling the stimulus using a computer allowed for real-time stimulus control.

Stationary vs. non-stationary flow

One of the problems with the simple odor delivery systems used in many previous studies of olfaction is that the pulsed (discontinuous) nature of the flow through the odorant flasks, combined with

the small volume of the flask, cause the odor concentration during even short, 1-second-long pulses non-stationary. This occurs because in the period between odor pulses, the air in the flask reaches equilibrium (in the case of a pure liquid odorant, it becomes saturated with odor), but because the volume of odor delivered during a pulse is greater than the capacity of the flask, the initial phase of high concentration odor is followed by a subsequent phase of a lower concentration, whose concentration is determined not by thermodynamics but rather by the dynamics of a process out of equilibrium. This introduces an uncontrolled and unmeasured temporal dimension to the stimulus which can confound the origin of temporal patterns in neuronal responses.

Our system solves this problem by reaching a steady-state flow before the beginning of the first pulse and by bubbling incoming air through tall enough columns of liquid odorant so that the air emerges saturated in odor regardless of the amount of time since the previous pulse.

The composition of the vapor of solutions of mixtures is not stable over time or concentration

Because more volatile components evaporate more rapidly than less volatile ones, solutions of mixtures produce varying headspace compositions depending on the concentration of the solution and the length of time during which the components of the mixture are allowed to evaporate (Brockerhoff and Grant, 1999). At high concentrations, the composition of the headspace above the solution was almost identical to that of the solution, but as the concentration of the solution decreased, the proportions of the more volatile compounds decreased, until only the least volatile component was left, the more volatile compounds having evaporated soon after the solution was made.

The system presented here avoids these problems; because each odorant is kept undiluted and in large volumes, and the headspace of each odorant is allowed to reach saturation to ensure its composition is identical to that of the source.

Long-term stability in concentration delivered

Finally, previous systems that utilize a liquid dilution on a filter paper have the additional disadvantage that they lack long-term stability in the concentration delivered, given that the odorant quantities are relatively small compared to the amount delivered in a single series of pulses and that the fact that they operate out of equilibrium makes odorant quantity a factor in the concentration reached in the finite amount of time of a puff. This probably causes a slow decline in the concentration which accumulates over trials, an effect that is particularly harmful when studying plasticity with a relatively slow timecourse (see Chapter 9, for example).

Ensuring that the height of the column of liquid odorant is kept well above that required for saturation, combined with the large quantities of odorant used in each of our bubblers, achieves a constant concentration independent of the exact amount of odorant in the bubbler at any point in time. In other words, the concentration of the odorant coming out of each of our bubblers (see below) depends on a thermodynamic equilibrium rather than being dependent on the length of time for which the air stream has been allowed to be in contact with the odorant.

6.3 Design

Mechanical artifact prevention

A diagram of our odor delivery system is shown in Fig. 6.1. Air at a pressure of approximately 30 PSI is filtered with a charcoal filter and, if desired, dried by passage through anhydrous calcium sulphate (Drierite, Xenia, OH). This air is then separated into three streams. One of the streams provides pure air, at a flow rate equal to that of the odor-carrying stream (see below), to the animal in between odor pulses, ensuring that the animal is exposed to constant air flow. An electromagnetic

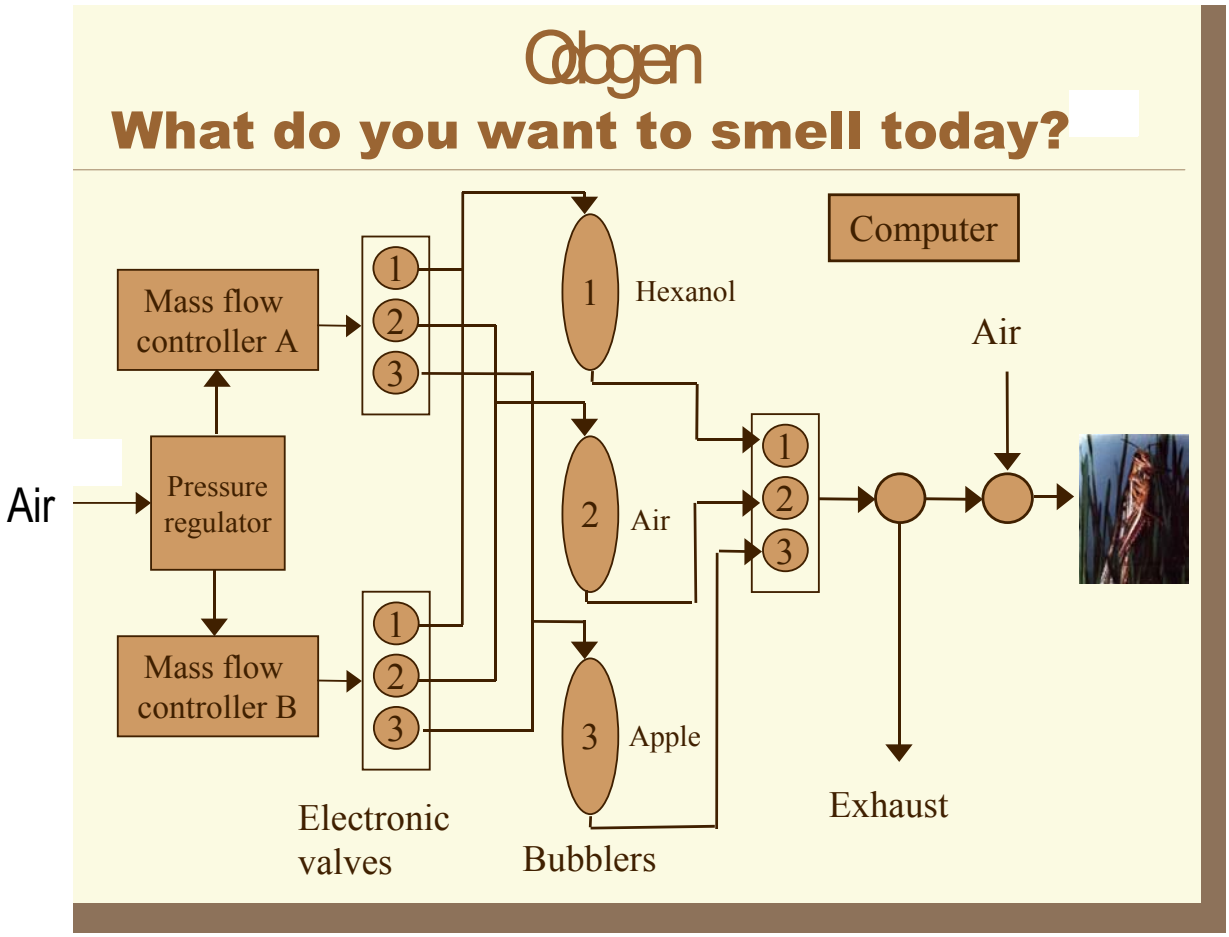


Figure 6.1. Diagram of the odor delivery system. For simplicity, only 2 of 7 odors are shown.

valve (Pneutronics or Clippard) switches between this stream and the odor-carrying stream. This eliminates the mechanosensory component of odor puffs[1].

Gaseous dilution to generate arbitrary concentrations of one of several pure odors or a combinatorial diversity of binary mixtures

The other two air streams each go through a separate mass flow controller (MFC) (Pneutronics divi-

1. A brief and smaller mechanical stimulus may persist due to the switching time of the valve switching between the stream carrying odor and that carrying air. This was reduced by placing a widening nozzle downstream of the valve to low-pass filter the stream reaching the animal.

sion of Parker Hannifin, Hollis, New Hampshire; or Unit Instruments, Kinetics division of USFilter, Dublin, Ireland), calibrated for flows between 0 and 1 liter per minute (lpm). We have verified their accuracy to at least 1 part in 100 of full scale (Figs. 6.6-6.7). Flows of less than approximately 0.02 lpm, though, are limited by inability of the air flow to form bubbles at the bottom of the bubbler under the weight of the liquid odorant column (see below). Each MFC works by setting the desired flow rate using an input voltage, supplied by the computer, and then using a feedback loop to adjust the size of an orifice within until the flow, defined as the mass flowing per unit time, measured within the MFC, equals the desired flow rate.

After exiting the MFCs, each of the air streams enters a separate diverting manifold which consists of eight electronic valves (Pneutronics or Clippard Minimatic, Cincinnati, Ohio), each of which leads to a bubbler with a different odor –one of the bubblers is empty to allow the use of pure air as one of the components of the binary mixture to ensue. The system can readily be expanded to accommodate more than seven odor components by adding bubblers and replacing the manifolds with larger ones consisting of more valves. Of the eight valves in each manifold, one is open at any given time to determine the odor that the corresponding air stream will carry.

Each of the eight bubblers was made out of glass (Rick Gerhart, Caltech Glassblowing Shop) and consists of a cylinder of ~1.5 cm diameter and ~40 cm height, with a sphere of ~5 cm diameter at the top. The bubblers are filled with undiluted liquid odorant up to a level that ensures that the odorant fills the bubbler during flow without overflowing. The air stream enters the bubbler at the bottom of the liquid column of odorant and exits it at the top, saturated in the odor. The height of the bubblers was designed so that it exceeds the height of odorant required for saturation of the air (Christine Chee-Ruiter, personal communication; Brett Doleman and Eric Severin, personal communication). This height can be measured by mass loss experiments, in which the bubbler is weighed at periodic intervals of time, increasing the height of the odorant column at the end of each interval until the mass loss during the constant intervals ceases to increase with the height of the column, demonstrating that additional fluid height no longer contributes to increased concentration

of the odor in the outgoing air stream due to saturation.

Upon exiting the bubbler[1], the stream of odorized air travels through Teflon tubing to prevent absorption of the odor by the tubing walls. As an additional measure to reduce purging time, the distance between the odor selector and the target was minimized. The two streams, each with a different odor or with air and each flowing at a potentially different rate, enter a selector manifold of eight valves with eight inputs –one from each bubbler— and one output. This output leads to a glass mixer (Caltech Glassblowing Shop), where both streams are fully mixed.

In our experiments, the two flow rates are regulated so that their sum remains constant at all times, ensuring the flow of air to the animal remains at a constant rate despite variations in its odor content. The proportion of the total flow going through each of the two streams regulates the concentration of the odor in air, or in the case of binary mixtures, the relative concentrations of the two components.

Controlling concentrations as fractions of an odorant's vapor pressure has the advantage that different odorants have approximately equal thermodynamic activities at the same fraction of the corresponding vapor pressure (Ferguson, 1939; see Chapter 2). Substances at the same activity have approximately equal effectiveness as odorants, as measured by olfactory detection thresholds (Mullins, 1955; see Chapter 2).

Constant flow to eliminate non-stationarities

The output of the mixer flows through an electromagnetic valve that switches the odor stream from flowing to the animal during odor pulses to flowing to an exhaust tube in between them (Fig. 6.2). This design serves the purpose of making the flow through the bubblers continuous over the time

1. It is important to seal each of the connections in the air path (e.g., with Teflon tape), particularly downstream of the MFCs, since minute leaks can alter the flow rate and concentration of the odor delivered.

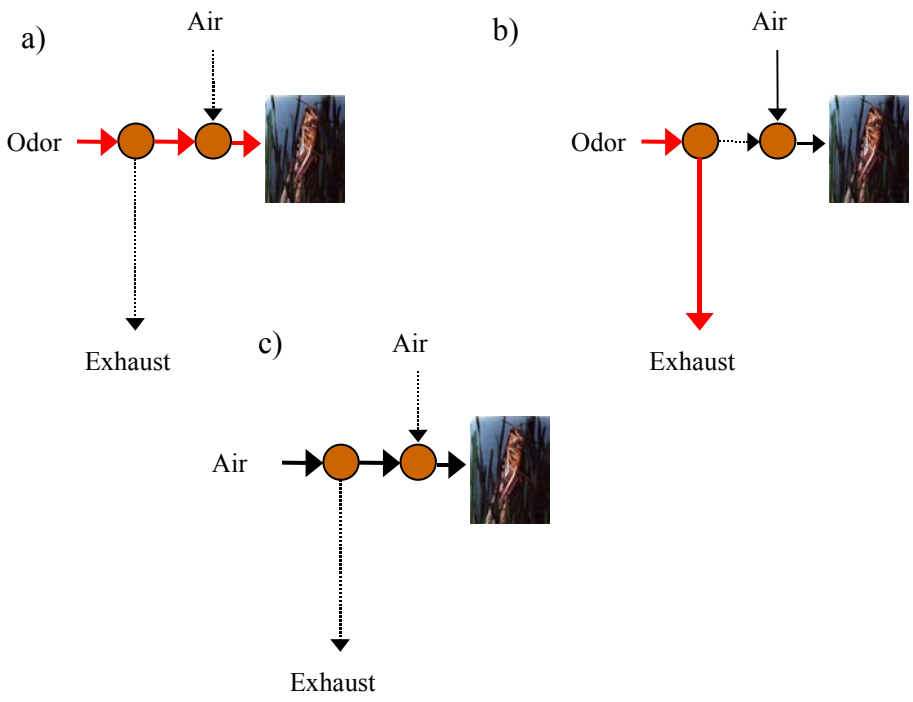


Figure 6.2. Design for the prevention of mechanical artifacts and to obtain continuous flow.
a) Circulation during odor pulses. **b)** Circulation during inter-pulse interval and during pre-circulation at the beginning of an odor series. **c)** Circulation during odor purging at the end of an odor series.
Red arrows indicate the flow of odorized air. Continuous black arrows indicate the flow of clean air. Broken arrows indicate airways not in use.

during which a given odor concentration is delivered, rather than pulsed with the delivery to the animal. This in turn serves to make the odor pulses homogeneous (Fig. 6.3).

Purging to prevent hysteresis

In an earlier design, I employed separate nozzles for each odor. This had the advantage of avoiding any contamination of tubing with a previously used odor, but had the disadvantages that i) the different angle of approach of each nozzle contributed to a difference between responses to different odors besides the one due to odor identity (M. S. Wehr and A. Bäcker, unpublished observations),

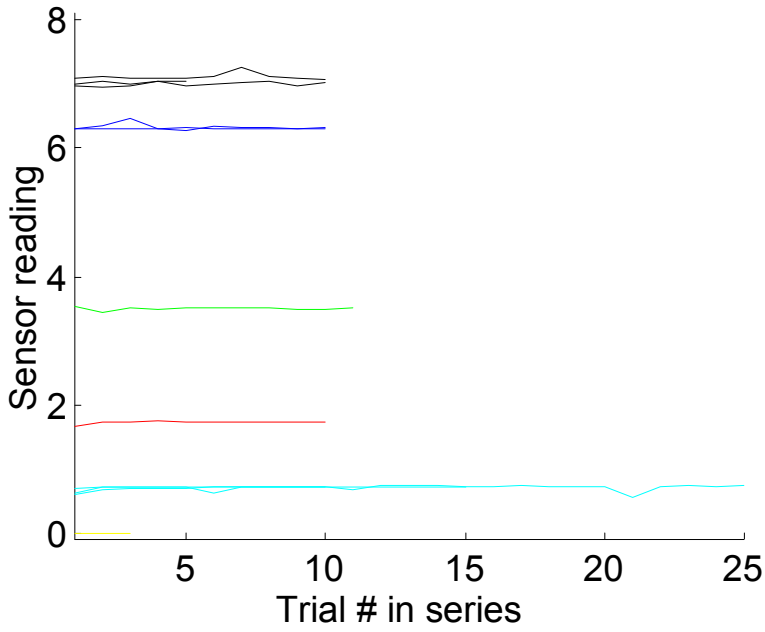


Figure 6.3. There is no hysteresis across trials: the concentration delivered is independent of the trial number in the series for each concentration (each represented by a different color). Each color represents a different concentration. Superimposed lines of the same color represent different series at the same concentration. Each point represents the mode of the sensor readings during the odor response for one trial, which correspond to the plateau level reached by the sensor for the trial.

and ii) that odors could not be mixed to deliver a blend. The first problem was solved by including a step motor to rotate the nozzles so that the active nozzle was always in the same position. This had the disadvantage of introducing a noisy electromagnetic device in the proximity of the target, though –inconvenient if the target is an animal from which electrophysiological recordings are being made. Furthermore, the need for purging was not avoided if we were to vary the concentration, and it would have been impossible to have a separate line for each concentration if we wanted the ability to delivery an arbitrary number of concentrations.

I thus opted for the present design, and implemented a purging system that proved to prevent any

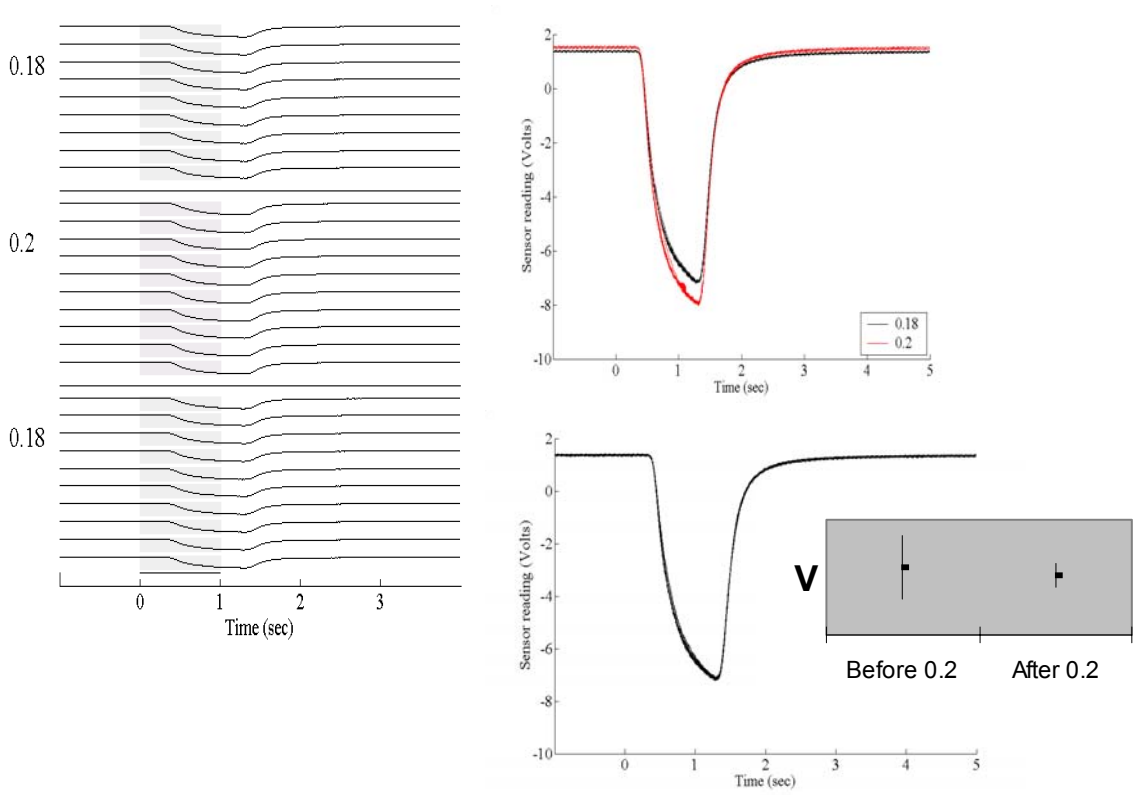


Figure 6.4. Timecourse of 1 sec long odor pulses shows no hysteresis across consecutive trials or nonconsecutive series. The sensor exhibits a negative signal in response to CO_2 . **Left:** The gray boxes show the period during which the pulsing valve was on, illustrating the delay between the switch and the sensor downstream. **Right, top:** Overlaid traces for ten trials of each of concentrations of 0.18 and 0.2 are perfectly discriminable. **Right, bottom:** CO_2 tracer delivers undistinguishable traces before and after the delivery of a higher concentration ($p>0.8$, Wilcoxon rank-sum test). All 20 traces are overlaid. Inset: Mean and standard deviation of mean CO_2 reading before and after delivery of a different concentration overlap.

hysteresis or contamination across odor series (Fig. 6.4 and Fig. 6.6). In between odor series, when the odor or the concentration is changed, the part of the system downstream of the odor selector, which is common to all odors, is purged by flowing clean air through the nozzle to the animal for approximately 30 seconds. Then, before the beginning of a new stimulus series to the animal, the new odor or concentration is pre-circulated to the exhaust for another 30 seconds to reach a steady state after purging the air in the system. This pre-circulation does not reach the nozzle. Instead,

clean air flows through the nozzle before and in between the odor pulses, purging its small volume completely in between pulses so that all pulses are identical to each other. This design dictated that the valves that switch air flow between the animal and exhaust, and between clean and odorized air, be located close to the target in order to minimize the volume downstream of them.

Computer control and the capability to deliver arbitrary stimulus waveforms

All of the parameters of the system are controlled by a Macintosh personal computer using an analog and digital input/output card (National Instruments, Austin, Texas), custom-made multichannel current amplifier and indicator cards[1], Labview (National Instruments) and a software program, Odomix, written by the author for this purpose and available upon request[2]. These parameters include the flow rate for each of the mass flow controllers[3], which dictate the concentration and composition of the odor delivered as well as the flow rate of the overall stream, the selection of odor(s) in the blend delivered[4], the duration and frequency of the odor pulses[5], the number of pulses in each series, and the purging times. A separate program can be used to deliver continuously varying stimulus waveforms, a capability that could do much for our understanding of the processing of more natural odor plumes while retaining the control and understanding of the stimulus which is harder to

1. The current drawn by the valves exceeds the current sourcing capability of the computer card's digital input/output lines. In order to solve this, I constructed a Darlington circuit for each valve. This circuit used the current drawn from the computer card as a switch to turn on a circuit that drew on an autonomous current source to drive the valve. Eight of these current amplifiers were placed in each of several printed circuit boards, which also included LEDs to signal which valve was active at any point in time. Similar cards may be commercially available (SBX/TTL module, Pneutronics).
2. Email: abacker@alum.mit.edu.
3. Each MFC is controlled by one analog output line.
4. Each electromagnetic valve is controlled by a digital TTL output line (two valves per bubbler).
5. The two switching valves near the nozzle are controlled by two TTL timer lines for accurate timing control.

achieve with natural uncontrolled odor sources. This can be achieved by delivering the desired waveform as a control voltage to one of the MFCs and a complementary waveform to the other MFC to keep total flow constant, keeping the pulsing valves continuously in the odor delivery configuration (Fig. 6.2a).

The use of the computer to control the stimulus sequence also serves to keep an electronic record of the entire stimulation history[1], and could be used to program entire automatic experimental sequences. Of course, it also allows for real-time stipulation of the concentration or blends desired given the responses observed.

6.4 Testing

The performance of the system was tested using a portable gas chromatography mass spectroscopy (GCMS) system, a tracing system with a capnograph or CO_2 detector (Godart, Holland), polycaprolactone/carbon black (80:20 wt/wt) composite polymers (Lonergan et al., 1996) that change their resistance in direct response to odorants, and an insect brain.

Timecourse of the signal

To evaluate the timecourse of the odor pulses, one of the mass flow controllers was fitted with a cylinder delivering a mix of carbon dioxide in air (Fig. 6.5). The mixture of that line was mixed with the stream of the other mass flow controller, carrying pure air. The nozzle was connected to the input of a carbon dioxide sensor that works by shining light through the gas in the sensor and measuring the

1. In addition to keeping the stimulation history in a file in the computer, the stimulus specification for each series of odor pulses is encoded by the computer with a series of fast TTL pulses and output concurrently with the stimulus itself, for storage with electrophysiological data in a digital tape recorder (Biologic, France).

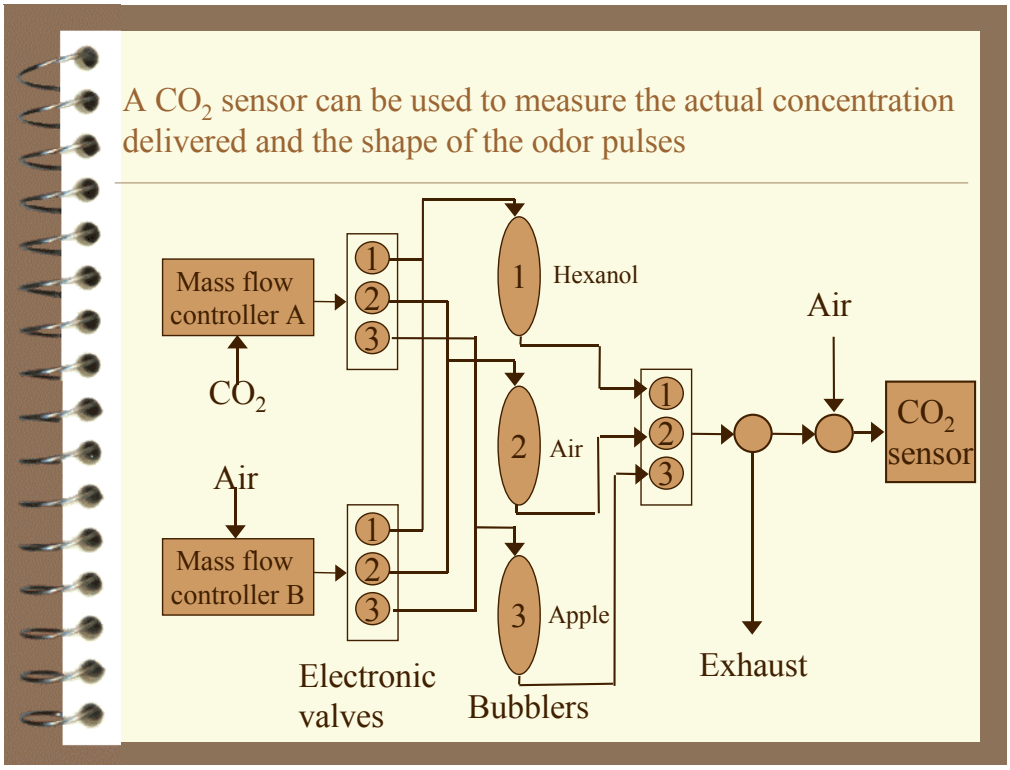


Figure 6.5. Using carbon dioxide as an odor tracer.

amount of absorption at a frequency characteristic of CO_2. The shape of the signal, filtered through the properties of the CO_2 detector, thus observed is shown in Fig. 6.4a. This timecourse was independently verified with carbon black composite polymers (Lonergan et al., 1996) that change their resistance in direct response to odorants.

Repeatability across trials

The repeatability of the odor pulses delivered can be seen in Fig. 6.4 and Fig. 6.7. The standard deviation of the mode concentration measured during pulses varied from 4.7% of the signal at a concentration of 2% of saturation to 0.6% at a concentration of 20% of saturation[1].

Repeatability across series

In order to verify the effectiveness of the purging procedure, the repeatability comparing identical stimuli delivered before and after the delivery of a different concentration was measured using the CO2 sensor. To ensure that the readings obtained for any concentration were repeatable and that the differences observed between different concentrations were not caused by hysteresis, I presented a series of 10 trials at a concentration of 18% of saturation, followed by 10 trials at 20%, and then another 10 trials at 18%. The variability across series of the same concentration (variance/mean for all 20 trials from both series at 18% = 2.7×10^{-4}) was well below the mean difference between readings for different concentrations: when decreasing the specified concentration from 20% to 18%, the mean readings decreased by exactly 10.0% (Fig. 6.4).

As an independent test to verify the return of the odor signal to baseline after the delivery of an odor pulse, GCMS was used to measure the odor concentration directly before, during and after the delivery of several odorants. The GCMS device acted as a low pass filter, but within approximately one minute the concentration reported decreased back to the baseline registered before the odor pulse (Fig. 6.6).

Linearity

Besides being repeatable, it is important that the concentrations delivered be both discriminable from each other and predictable. Figure 6.7 shows that the system's response is linear and that the

1. Concentrations above 20% saturated the CO_2 detector at the gain used and were thus excluded from the analysis. The trend observed, though, was that the fractional error consistently decreased with increasing concentrations.

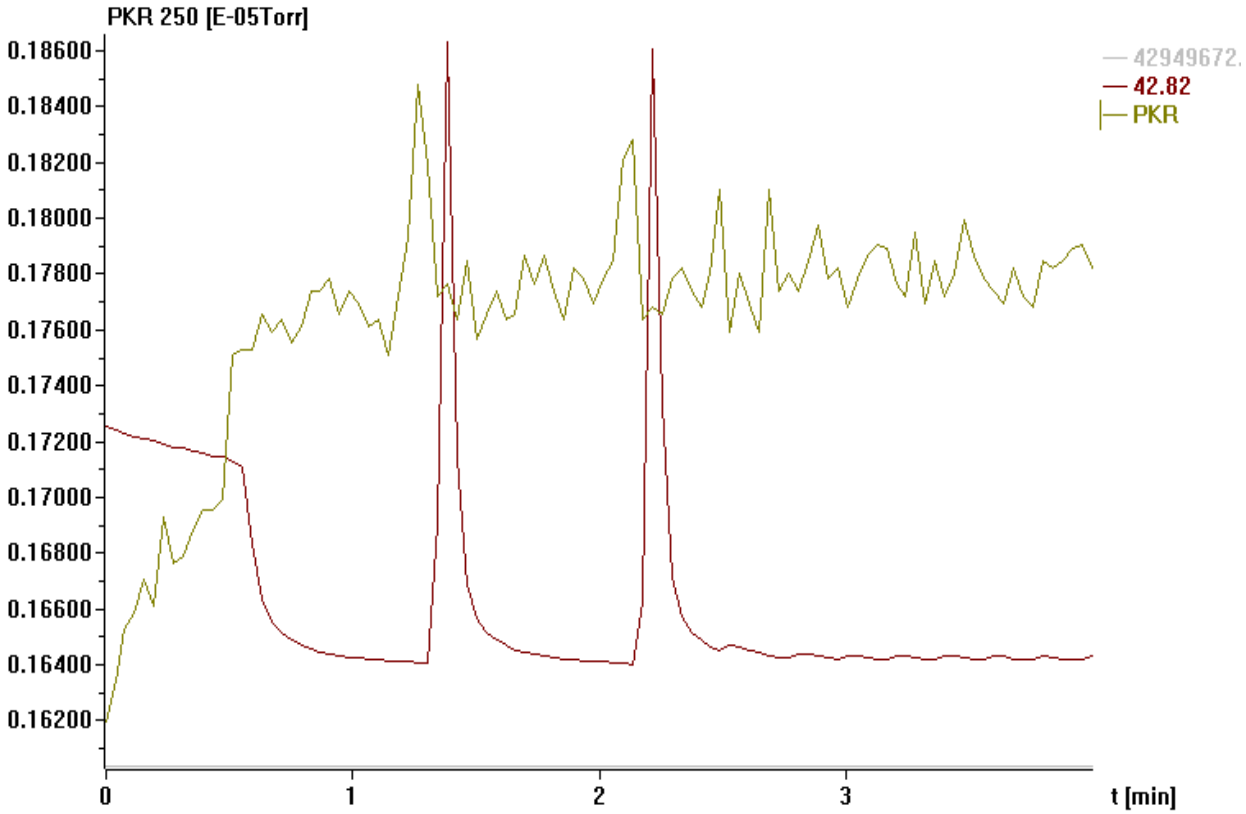


Figure 6.6. GCMS tests directly for hysteresis: Concentration returns to baseline after purging following an odor pulse (red trace).

concentration is highly repeatable and highly discriminable from others, complying with both of these requirements, even when the concentrations compared are close together (see also Fig. 6.4b).

Physiological relevance of small concentration changes

Finally, I show an application of the system that serves both to demonstrate once again the reliability of the system, and equally importantly, to illustrate that the small concentration changes that this system is capable of generating are relevant to physiology and important to understand the olfactory

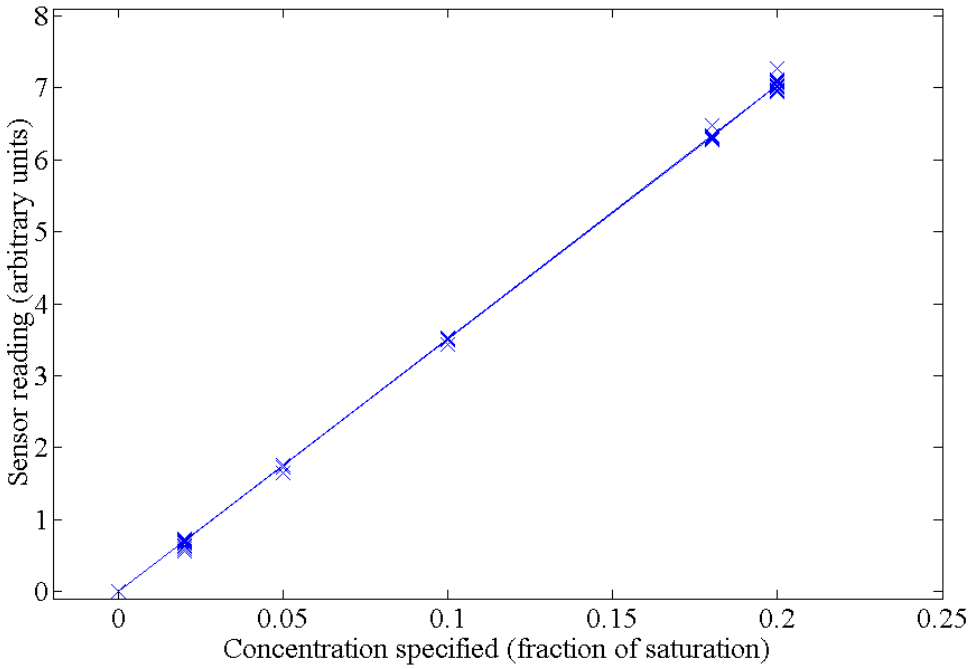


Figure 6.7: Linearity of the system. Each point represents the mode of the sensor readings during the odor response for one trial.

system. Intracellular recordings were performed of single projection neurons (PNs) in the antennal lobe of adult live awake locusts as described previously (Laurent and Davidowitz, 1994; Laurent et al., 1996). The PN shown in Fig. 6.8 responded vigorously and repeatedly to citral at a concentration of 28% of saturation, but did not respond to the same odor at 27% of saturation[1]. Such an abrupt threshold can only be found if the concentration can be specified to arbitrary values online, adjusting it dynamically toward the threshold as a neuron's responses are monitored in response to varying concentrations.

1. Figures 6.5a and 6.6 independently confirm that the odor delivery system is well able to deliver well-discriminable concentration differences in that magnitude range.

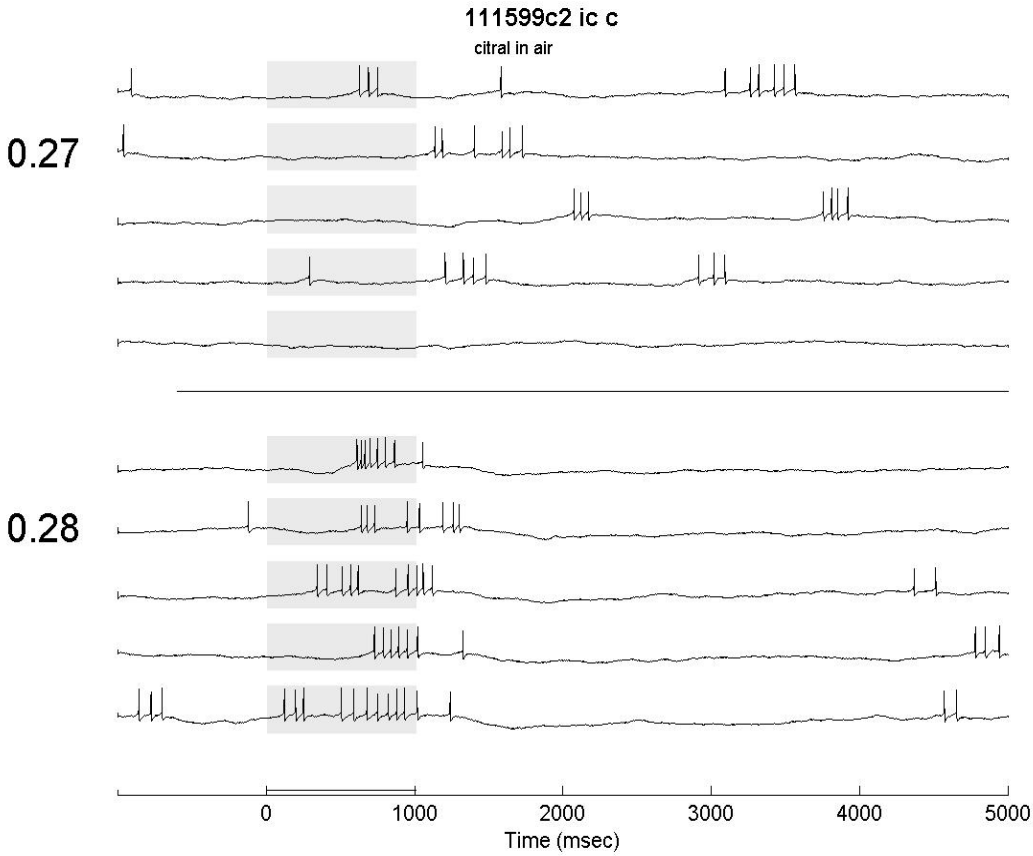


Figure 6.8: Relevance of small concentration changes for the insect olfactory system: The response of a projection neuron in the antennal (olfactory) lobe of the locust to citral at 27% of saturation and 28% of saturation.

6.5 Closing remarks

It is my hope that the use of systems such as the one described here will lead to the study of responses to continuous plume-like odor waveforms and blends of varying compositions to understand the processes that mediate the striking and at present unpredictable differences between the responses of the olfactory system to a blend versus those to their individual components.

6.6 Acknowledgements

Special thanks go to Ari Hershowitz, who contributed to an early version of the system, Yingzhong Tian, who helped in its construction, Tim Heitzmann of Caltech's Bioelectronics shop for help with the Darlington circuits, Christine Chee-Ruiter and Tom Meade for providing her expertise, Greg Sotzing for providing carbon black composite polymers, and Peter Green and Hui-Ming Hung for invaluable help with GCMS testing.

CHAPTER 7

Multiplexing odor identity and concentration information with a population temporal code

Abstract

The nervous system faces a dual task in the representation of sensory stimuli. On the one hand, exquisite sensitivity to differences in the stimulus requires different representations for each different stimulus. On the other hand, robust recognition of a stimulus under varied conditions requires invariance to changes in intensity, position, … In olfaction, psychophysical studies show that individual odors can be perceived as identical over significant ranges of concentrations (Gross-Isseroff and Lancet, 1988; Bhagavan and Smith, 1997). And yet some compounds are edible at low concentrations and toxic at high concentrations (McKechnie and Morgan, 1982; Van Delden 1982; Chakir et al., 1993), so the ability to discriminate concentrations has a selective advantage. How the brain deals with this trade-off is currently unknown. While odor identity information has long been thought to be encoded by the identity of the neurons responding and by slow temporal response patterns (Laurent and Davidowitz, 1994; Wehr and Laurent, 1996), how intensity information is multiplexed with the identity signal has remained controversial. One hypothesis proposes that odor identity and odor concentration information is multiplexed in the same neurons by having the phase of action potentials with respect to the local field potential code for concentration (Hopfield, 1995). Here, I record simultaneously from individual projection neurons in the antennal lobe of the locust and the local field potential from their target area, the mushroom body calyx, and find that the phase is constant across concentrations, contrary to the prediction of the model. A second hypothesis proposes that concentration information is carried in the response to initial exposure, but that the changes induced by short-term plasticity (Stopfer and Laurent, 1999) might make subsequent trials are less sensitive to concentration (Stopfer and Laurent, 2000). My data shows this not to be the case either: concentration information is contained in all trials (exposure to high concentrations, though, makes responses more similar across concentrations; see Chapter 9). A third hypothesis proposes that increasing concentrations introduce additional spikes that are not locked to the local

field potential (Stopfer and Laurent, 2000). This hypothesis has the attractive feature that the extra spikes could be filtered out by Kenyon cells selective for synchronized activity to achieve concentration invariance. My data, however, argues against this hypothesis too: PNs do not on average respond to higher concentrations with extra spikes, and even those that do show tighter synchronization with increasing concentrations, contrary to what one would predict. Based on my data, I present a fourth hypothesis, namely, that concentration is coded by the tightness of synchronization across PN assemblies.

Introduction

Behaviorally relevant concentrations range from detection threshold —for a dog following a faint track— to saturated vapor pressure very close to the source —inside a flower for a bee or against a potential mate's behind for a dog. Fruit flies feed and deposit eggs on fermenting plant materials in which ethanol is the most abundant short-chain alcohol (McKechnie and Morgan, 1982; Van Delden 1982). The ability to detect ethanol is thus important for chemotaxis toward food sources. However, adult flies are also susceptible to intoxication and death in high ethanol environments (Chakir et al., 1993). Therefore, there is a selective advantage for the ability to avoid environments with dangerously high alcohol concentrations. In Drosophila, for example, the LUSH gene has been determined to be required specifically for the response to high concentrations (Kim et al., 1998). This illustrates the selective advantage conferred by the ability to discriminate concentrations of an odor which have different behavioral relevance.

While large changes in odor concentration are known to change the identity of the neurons activated (Kauer, 1974), how the system encodes smaller changes in concentration, over which the

identity code must remain invariant to allow for perceptual recognition, remains unknown.

Results

A recent hypothesis proposes that odor concentration is encoded by the phase of action potentials with respect to an oscillatory drive (Hopfield, 1995). To test this hypothesis, I carried out intracellular recordings from PNs simultaneously with the local field potential (LFP) in the calyx of the ipsilateral mushroom body while delivering various concentrations of 7 odors. I computed the phase of each spike with respect to the LFP (see Methods). Contrary to the prediction of the Hopfield model, the phase remained constant not only for different odors (Fig. 7.1; see also Laurent and Davidowitz, 1994; Wehr and Laurent, 1996), but also for different concentrations of any one odor (phase for maximum concentration not significantly different from that at minimum concentration tested, $p>0.2$ both in naive animals and after exposure to higher concentrations, Wilcoxon rank-sum test, n=165 PN-odor pairs, see Fig. 7.2).

Contrary to a previous report (Laurent and Davidowitz, 1994), the peak phase at which PNs synchronized to the LFP varied significantly across recordings (compare Figs. 7.1, 7.2 and 7.5), and did not always occur during the rising phase of the LFP (see, for example, Fig. 7.5). Whether these differences were due to PN identity or to the position of the LFP electrode remains to be determined.

A second hypothesis put forth based on preliminary evidence (Stopfer and Laurent, 2000) is that information about concentration could be contained in the intensity of bursts upon initial exposure to an odor, but reduced by the effect of short-term plasticity on PN responses (Stopfer and Laurent, 1999). To test this hypothesis, I quantified the difference between responses to different concentrations for initial as well as later exposures to each of seven odors. Responses were signifi-

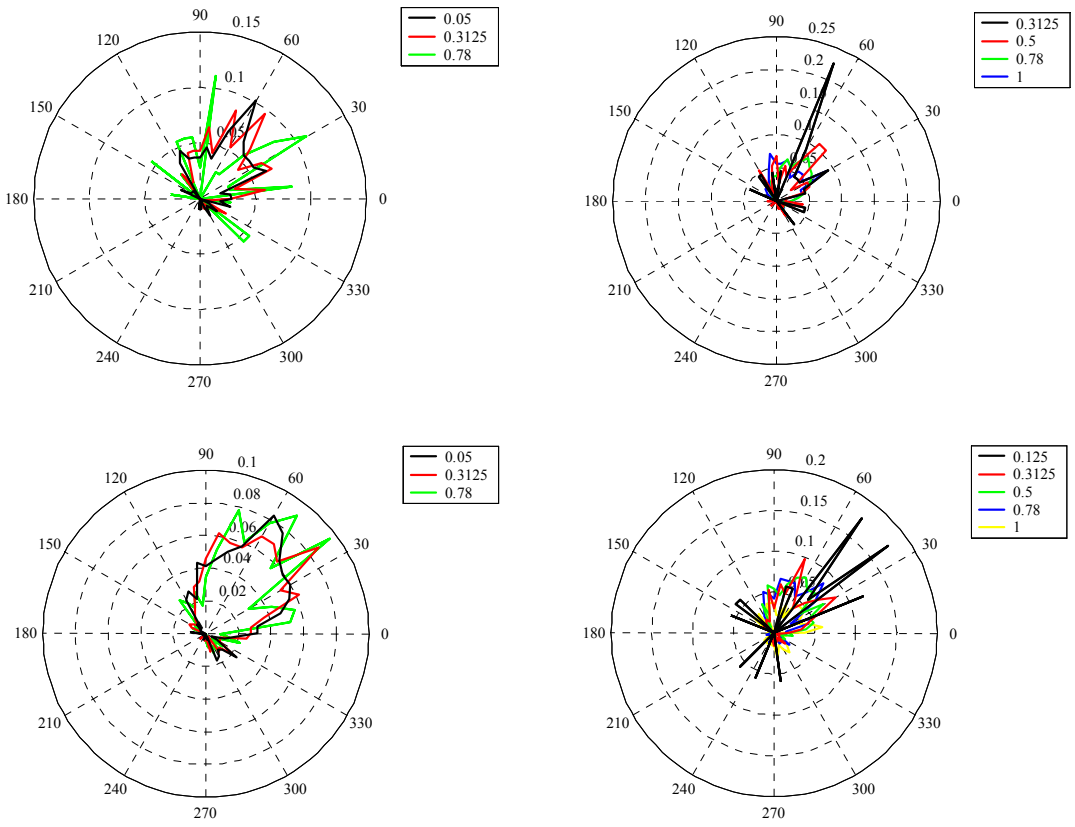


Figure 7.1. A PN synchronizes to the LFP at the same phase for different odors. From top left and clockwise, the same PN's response to cineole, citral, geraniol and octanol.

cantly different for different concentrations both for initial and later responses. In fact, trial number had no significant effect on the difference between responses to different concentrations, both for lower concentrations ($p>0.9999$, ANOVA, n=51 PN-odor pairs, see Fig. 7.3) and higher concentrations ($p>0.8935$, ANOVA, n=51 PN-odor pairs). Note that a different kind of plasticity induced by exposure to higher concentrations of the same odor, though, can indeed reduce the dependence of PN responses on concentration (see Chapter 9).

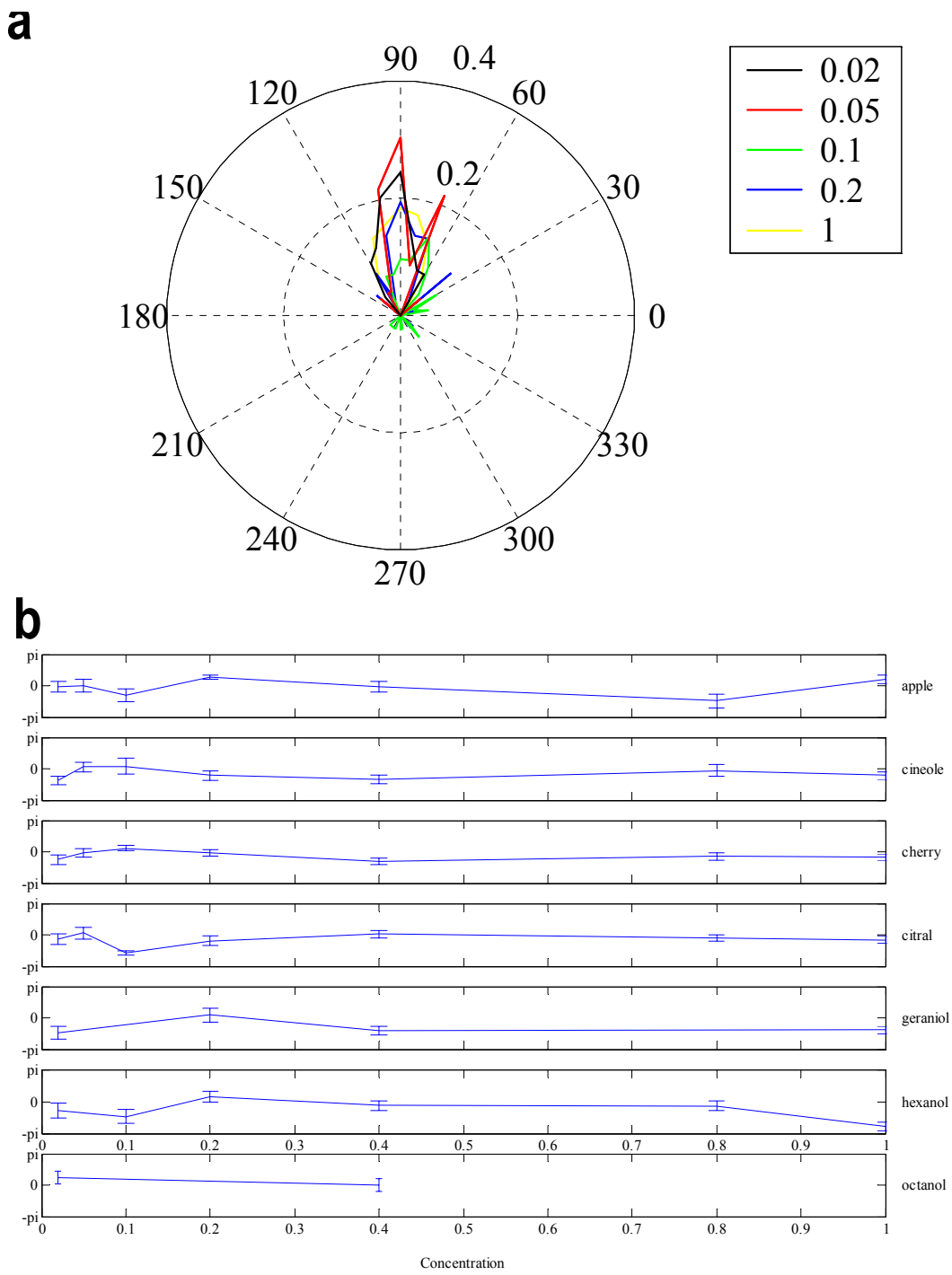


Figure 7.2. The phase of PN spikes with respect to the LFP remains constant across concentrations. a) Polar plot of a PN's spikes' phases in response to apple at various concentrations. **b)** Mean phase (and s.e.m.) for different concentrations of each of seven odors, averaged across 170 PN-odor pairs.

A third hypothesis suggested by preliminary evidence is that information about concentration is encoded in extra spikes that appear at higher concentrations in between the spikes that are locked to the FP (Stopfer and Laurent, 2000). This hypothesis has the attractive feature that a downstream neuron that was sensitive only to synchronized spikes would be invariant to concentration changes, while one which was sensitive to all spikes would be sensitive to concentration. This would enable the animal to retain information about concentration while achieving the invariance necessary for robust recognition. This hypothesis predicts that the degree of synchronization between PNs and the LFP will decrease with increasing concentration, as extra spikes ar**v**e elicited. My data, however, indicate that the opposite is true: the proportion of spikes that are synchronized to the LFP increases with increasing concentration (Figs. 7.4 and 7.6). Some PNs' (33% of the 71% that showed synchronization at any time) spikes were synchronized to the LFP at all times, during, before and after odor responses (Figs. 7.5 and 7.7). No such synchronization was present for randomly generated spike trains or uniform spike trains with a spike every millisecond. Synchronization could be measured even though the LFP's amplitude was signficantly smaller outside of the periods of odor responses. For these PNs, synchronization did not change with concentration. Other PNs (67% of those that showed synchronization at any time) synchronized only in response to odors. For these, synchronization increased with increasing concentration (Fig. 7.4 and 7.6). I never observed the synchronization of the spikes in an odor response decrease with increasing concentration (n=170 PN-odor pairs in 46 PNs). Over all PNs, the proportion of spikes between -pi/2 and pi/4, where 0 is defined as the peak of the LFP, was signficantly larger for the maximum concentration than for the minimum concentration tested ($p<3x10-4$, Wilcoxon ranksum test, n=170 PN-odor pairs in 46 PNs, Fig. 7.6).

If synchronization indeed codes for concentration, an important question arises: is the concentration sensitivity of synchronization eliminated by priming (see Chapter 9), or is it robust to

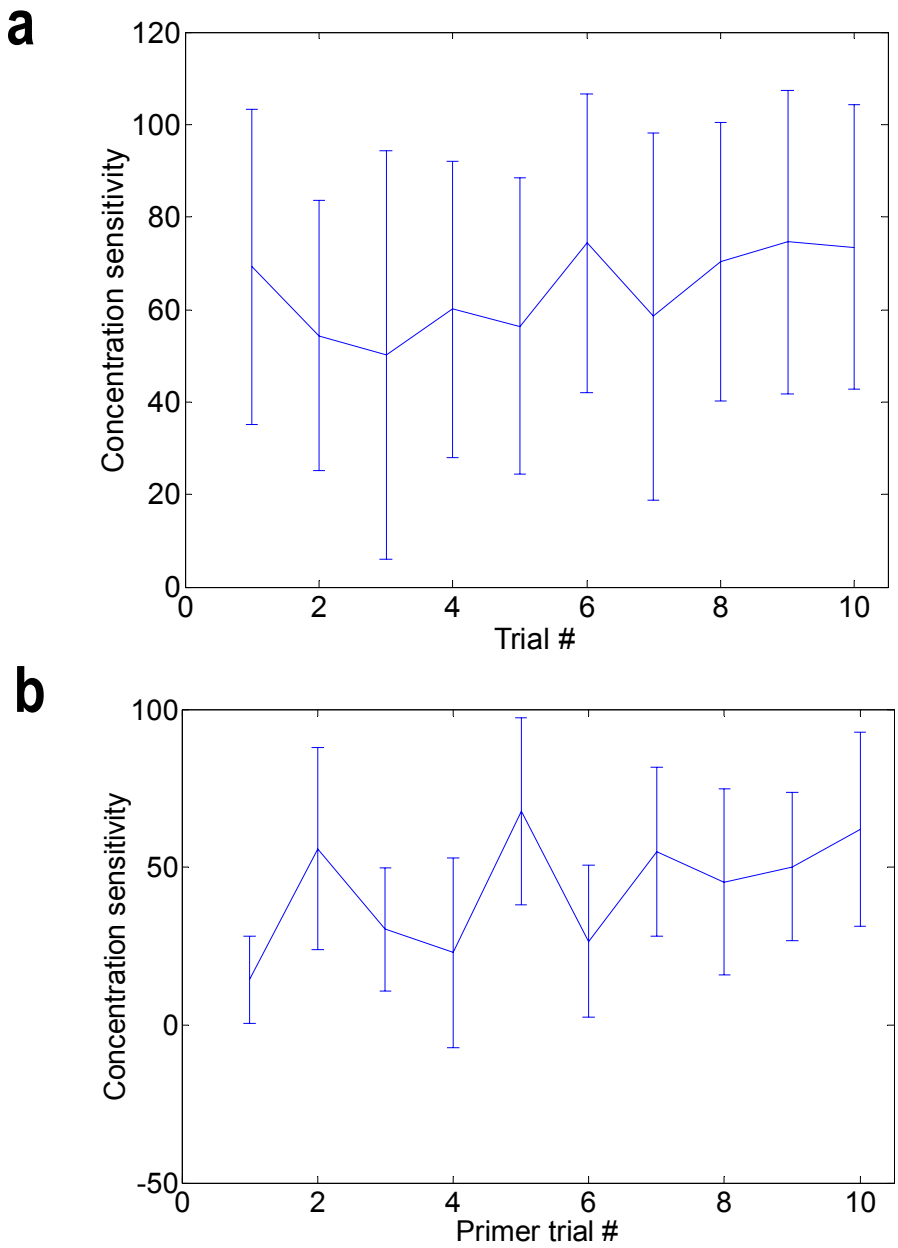


Figure 7.3. Concentration sensitivity is not affected by succesive exposures to the same odor stimulus. Concentration sensitivity (CS) for trial #N was defined as CS=Cross - Self, where Cross is the mean distance between trial #N at the **(a)** lower or **(b)** higher of two concentrations and all 10 trials in a series at the other of the two concentrations, and Self is the mean distance between trial #N at the **(a)** lower and **(b)** higher concentration and all the other trials for the same concentration. Plots show means over 55 PN-odor pairs and s.e.m. The series at low concentrations were presented before those at high concentrations since responses were affected by exposure to higher but not to lower concentrations (see Chapter 9).

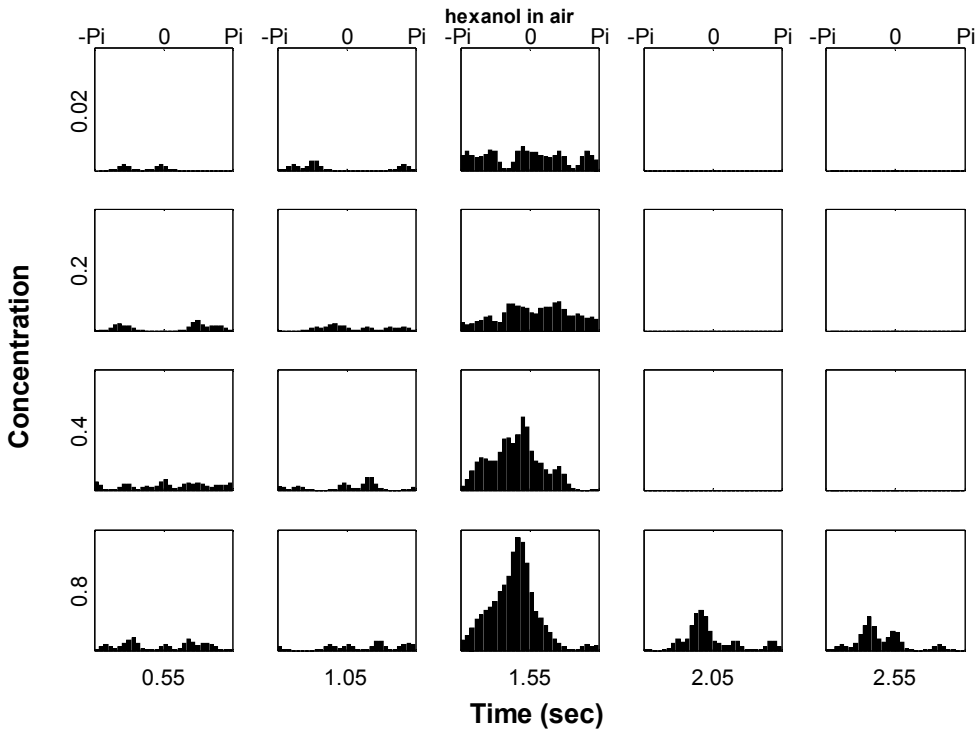


Figure 7.4. Synchronization of a PN's spikes to the LFP gets tighter with increasing concentration. Each plot shows a histogram of phases for a 500 ms window centered at the indicated time. The odor was presented between t=1s and t=2s. Notice only spikes during the odor response are synchronized, and then only at high concentrations.

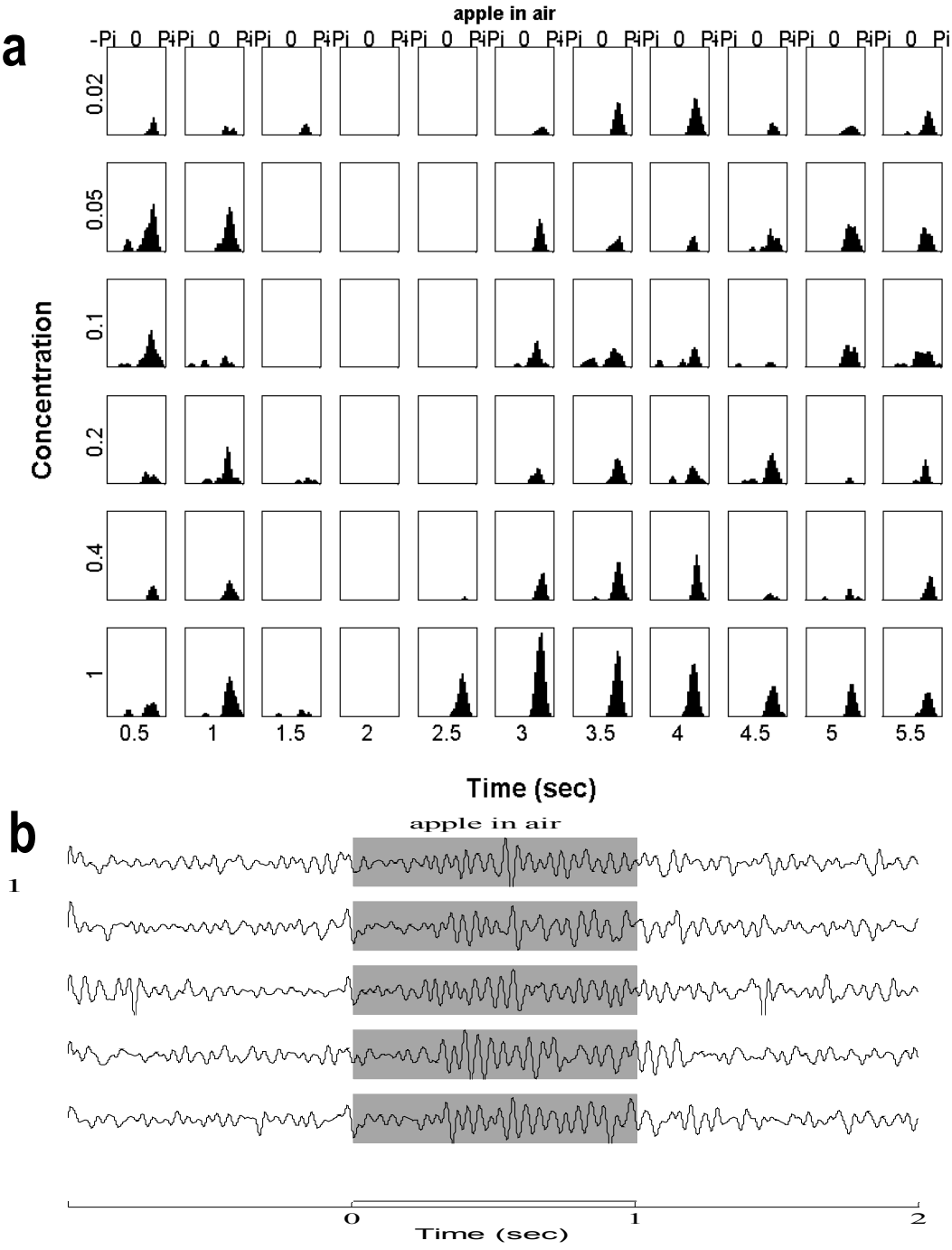


Figure 7.5. Some PNs are continually synchronized to the LFP. a) Synchronization does not vary with concentration. The phase is also constant throughout the recording and for all concentrations tested. Each plot shows a histogram of phases for a 500 ms window centered at the indicated time. The odor was presented between t=1s and t=2s. **b)** Filtered LFP traces for 5 of the trials of 100% concentration. Note that cycles are sometimes visible even in the absence of odor presentation.

exposure to high concentrations? Interestingly, I found that the effect of concentration on synchronization is robust to exposure to higher concentrations: contrary to the effect of priming on the concentration sensitivity of single neuron responses, the concentration sensitivity of synchronization is, if anything, enhanced by exposure to higher concentrations (Fig. 7.6b).

Conclusions

By multiplexing odor quality information in the slow temporal patterns with concentration information in the synchronization across neurons, the olfactory system might effectively solve the problem of achieving invariance to concentration while keeping concentration information as well. Furthermore, this dissociation allows the system to manipulate odor identity and concentration information separately, as priming does (see Chapter 9).

How would such an encoding scheme be decoded? The answer depends on whether the decoder's goal is to recognize odor identity, independent of concentration, or odor concentration. Animals are presumably interested in both (McKechnie and Morgan, 1982; Van Delden 1982; Chakir et al., 1993; Kim et al., 1998). Interestingly, PNs have two known distinct targets: Kenyon cells in the mushroom body, and lateral horn interneurons (LHIs). Kenyon cells (KCs) are odor selective and fire only during part of each LFP cycle (Laurent and Naraghi, 1994; Perez-Orive et al., submitted). Furthermore, KCs appear to exhibit a prolonged hyperpolarization after every spike, and their odor responses are very sparse, often responding with a single spike in any one oscillation cycle, or even throughout an entire odor response (Laurent and Naraghi, 1994; Perez-Orive et al, submitted). LHIs, on the other hand, respond vigorously to odor stimulation and show very limited odor-selectivity. The LHI population fires spikes during over half of each cycle, and sends inhibitory projections to

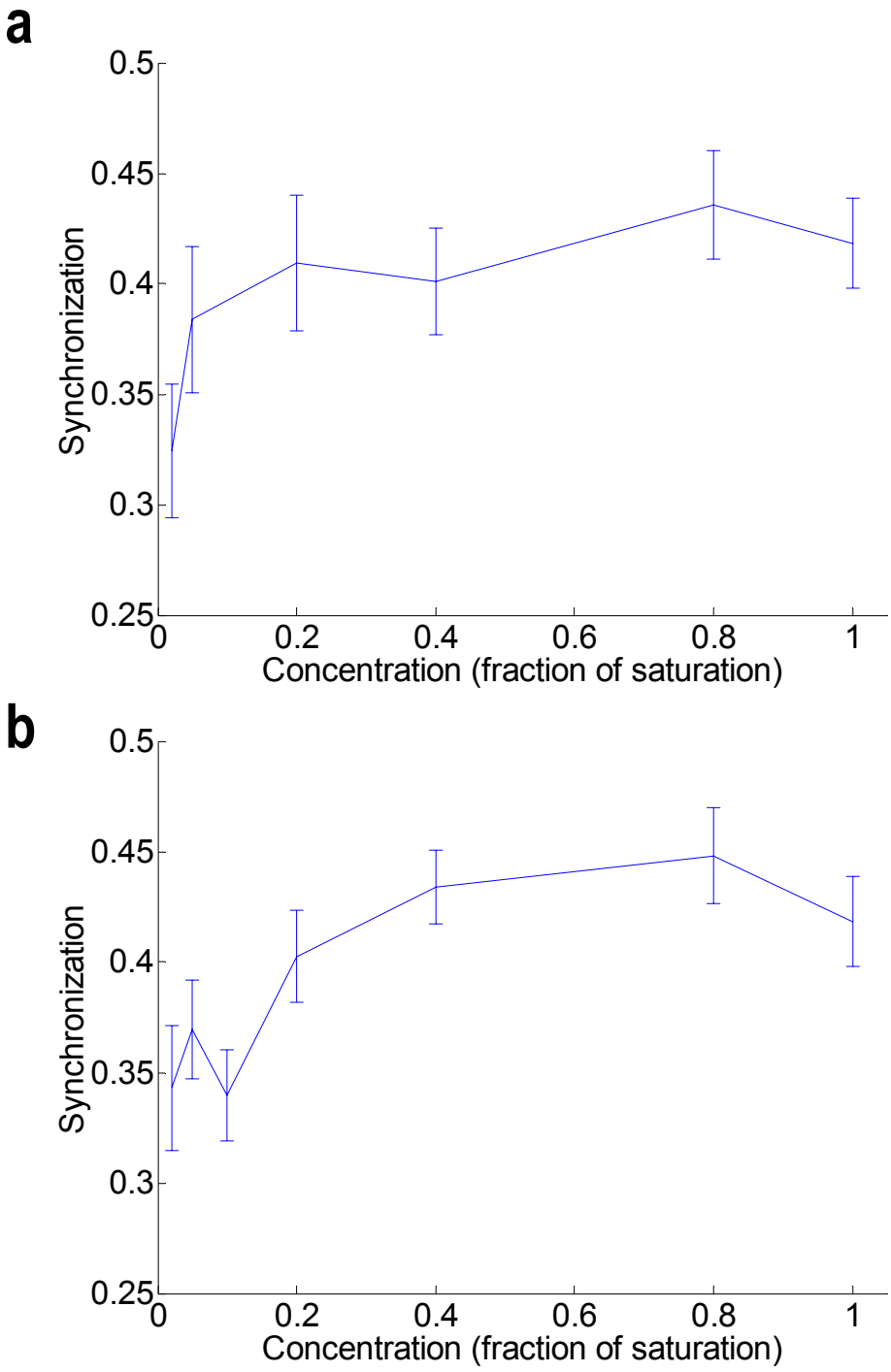


Figure 7.6. Synchronization between PNs and the LFP increases as a function of concentration, both in naive animals (a) and after exposure to higher concentrations (b). Mean fraction of spikes that fall between pi/2 before LFP peaks and pi/4 after them, averaged over 170 (a) and 165 (b) PN-odor pairs, and s.e.m.

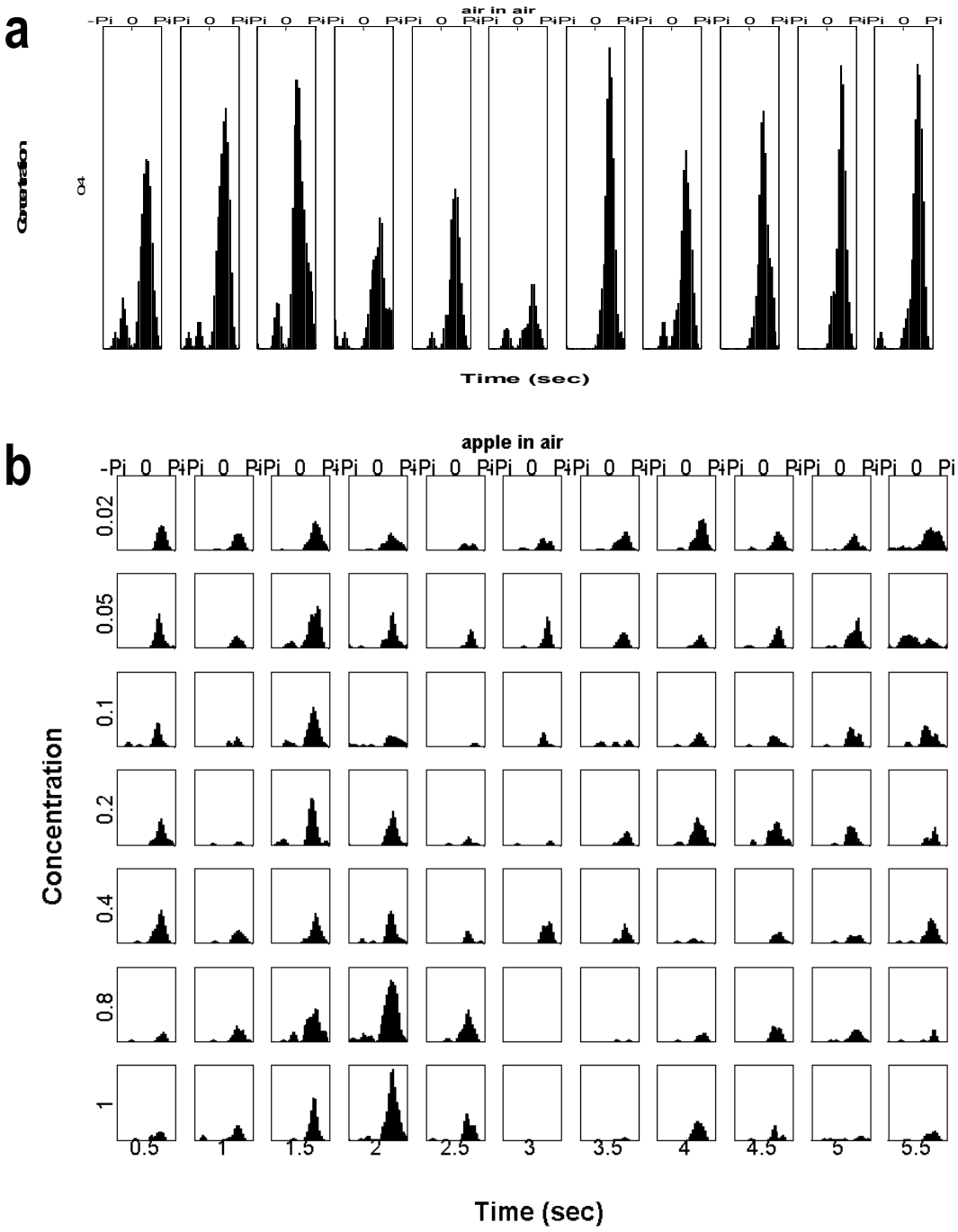


Figure 7.7. A different PN from that in Fig. 7.5 is continually synchronized to the LFP during baseline firing and responses to air (a) and apple (b). Synchronization does not vary with concentration. The phase is also constant throughout the recording and for all concentrations tested. Each plot shows a histogram of phases for a 500 ms window centered at the indicated time. The odor was presented between t=1s and t=2s.

the mushroom body. It is tempting (though premature) to speculate that Kenyon cells might respond with a single spike whenever the corresponding set of PNs fires synchronously enough to exceed threshold, and thus be relatively invariant to suprathreshold concentrations, since one a KC's threshold for firing is reached, further synchronization would not make them fire more than one spike. LHI's, on the other hand, might respond more vigorously to a volley of PN input if it is more synchronized, as it would be for higher concentrations. The ensuing stronger inhibition feeding back on KCs might itself act as a mechanism of gain control, keeping the output of KCs invariant to concentration. Preliminary recordings from LHIs suggests that LHIs indeed respond more robustly, and in a more periodically synchronized way, to higher concentrations (Glenn Turner, personal communication).

A caveat to note is that it is conceivable that the higher degree of synchronization observed is due to the higher amplitude of the local field potential oscillations at higher concentrations, which elevates the signal-to-noise ratio and might make synchronization measurements less noisy. To test this possibility, I computed the spike-triggered average of the spike trains for each concentration across all odors and the 46 cells, to test whether any periodicity became more pronounced and/or more tightly locked as concentration was increased. No significant periodicity was observed at any concentration. I then computed the autocorrelation of the intracellular membrane voltage for all odor responses as a function of concentration. Once again, although periodicity was observed in some individual datasets, no periodicity was observed in the averages across PNs, possibly because the frequency of the oscillatory drive to PNs might change slightly across different cells or possibly even from trial to trial. A potential test to control for the LFP amplitude being responsible for the observed increase of synchronization with concentration would be to record from multiple PNs simultaneously and measure the number of spike coincidences as a function of concentration. It is unlikely that the FP amplitude can account for the differences observed in synchronization, however, since tight synchronization was clearly observable even at the very low LFP amplitudes present in the absence of

odor stimulation for PNs which exhibited permanent synchronization (see, for example, Fig. 7.5).

The demonstration that PNs are synchronized to the LFP over a range of concentrations spanning two orders of magnitude suggests that this coding scheme is not restricted to high concentrations (see also Chapter 8).

Methods

Specimens, odor stimulation and electrophysiology

Intracellular recordings were obtained from 180 cell-odor pairs in 46 PNs of 21 locusts, *Schistocerca americana*. Surgery and recordings were performed as previously described (Laurent and Davidowitz, 1994; Laurent et al., 1996). Delivery of seven odors, including pure compounds as well as ethologically relevant blends, was performed using a gaseous dilution computerized odor-delivery system capable of delivering arbitrary concentrations by mixing a stream carrying saturated odor vapor with a second stream carrying pure air. The concentration of the odor delivered was regulated by controlling the relative flow rates of both streams (see Chapter 6). The system was purged between presentation of different stimuli. The stimulus sequence was delivered to an initially naïve animal, that is, one that had no prior experience with the odor tested. Successive stimuli presentations were spaced 10 seconds apart. No adaptation was observed between successive presentations.

The odor timecourse and magnitude was measured by using CO_2 as a tracer in the air line carrying the odor while the diluting stream carried ambient air, and measuring the CO_2 concentration at the nozzle (see Chapter 6). Direct measurement of the odorant concentration with GCMS also showed that concentration returned to baseline within 1 minute of purging.

Data were digitized at 20 KHz using a digital tape player (DAT, Biologic) and then acquired into a personal computer at 4 kHz using an analog/digital input/output card (National Instruments). Analysis was conducted on raw intracellular voltage traces as well as spike rasters. Spike isolation was conducted by voltage thresholding and visual inspection.

Phase analysis

LFP traces were resampled to 1 kHz with MATLAB's resample function and filtered with a 13-30 Hz 5th order Butterworth non-causal bandpass filter. Filtered traces were processed to find local maxima and minima. Maxima (minima) were identified by locating zero-crossings that coincided with a positive (negative) second derivative. The phase of spikes was defined as

$$\text{phase} = \text{pi} * M + \text{pi} \,.\, f,$$

where f is the fraction of time elapsed between the previous FP extreme (maximum or minimum) and and the time of occurrence of the spike:

$$f = (t_{spike} - t_{previousextreme}) / (t_{nextextreme} - t_{previousextreme})$$

and M was 1 if the previous FP extreme was a minimum and 0 if it was a maximum. I also experimented defining phases with respect to every quarter cycle and every full cycle, but half cycles proved the most consistent across trials.

Naive vs. experienced animals

For every dataset, a trial was defined as naive if the animal had not been exposed to the same odor at a higher concentration before, and post-high-concentration if the animal had been

exposed to the same odor at a higher concentration before (see Chapter 9).

CHAPTER 8

Gain control in early olfactory circuits

Abstract

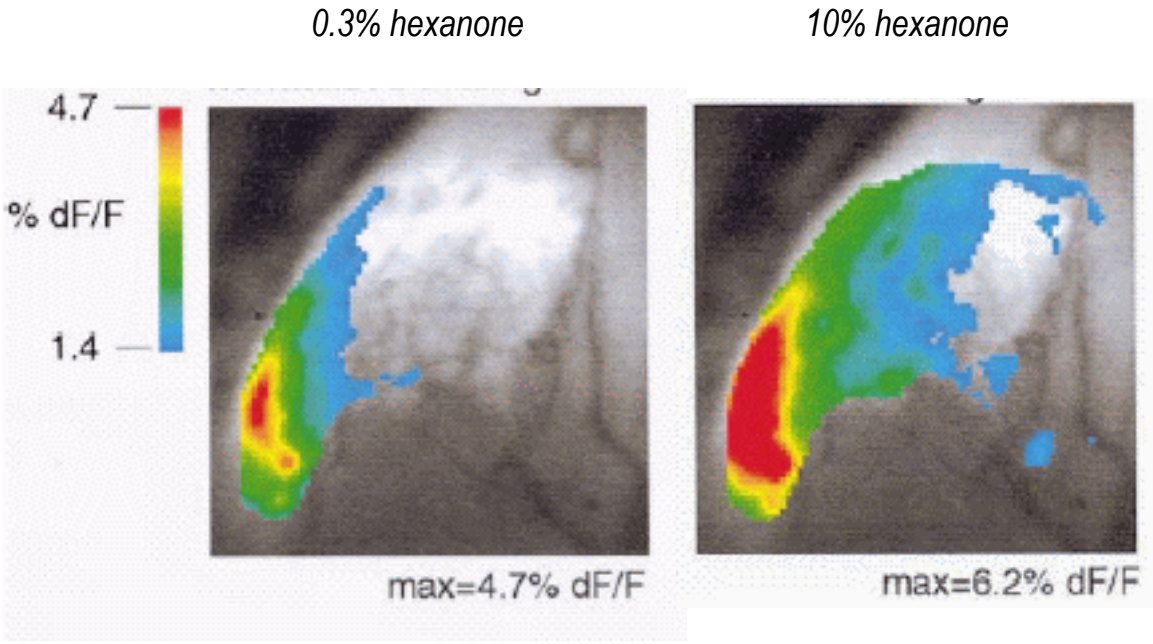
It has been known for some time that an increase in odor concentration causes a monotonic increase in the active area of the vertebrate olfactory bulb and insect antennal lobe as measured with calcium imaging, suggesting that downstream targets may have to deal with a large dynamic range of activity levels. On the other hand, response of mitral cells to increasing odor concentration has traditionally presented a less clear picture, with non-monotonic changes in firing rate. How are these pictures of input and output reconciled? Here, we record intracellularly from locust PNs and show that the mean firing rate across PNs is relatively invariant to odor concentration, due to a dynamic balance of excitation and inhibition acting as a gain control: as odor concentration increases, inhibition is strengthened in parallel to excitation. This effect, which is missed by calcium imaging techniques, may provide a solution to the conundrum of how complex odor blends do not evoke the percepts corresponding to all of their components, but rather a different percept altogether (Laing and Francis, 1989) despite the fact that receptors' responses are monotonic in concentration: the increase of inhibition with increasing concentration may allow detectors to be inhibited with the addition of additional blend components, allowing for cells to be tuned to particular odors. These may constitute dual roles of slow inhibition, whose role is controversial (Laurent, 2000), in the antennal lobe: tuning PN responses to respond specifically to particular odors, exhibiting lower responses to supersets of their preferred stimuli, and gain modulation, to keep PN firing rates within PNs' dynamic range and that of the decoders.

Introduction

The fundamental role of the olfactory system is, of course, to detect and recognize odors. Because information for rapid action is ultimately encoded in the nervous system by action potentials, there must be sets of neurons in the olfactory system which fire selectively in response to particular odors. Responding selectively to an odor, say apple, requires, in turn, responding to the presentation of apple while, at the same time, *not* responding to blends of apple with something else. There is behavioral evidence that animals indeed fail to recognize the components of complex odor mixtures (Laing and Francis, 1989).

Yet odorant receptor proteins have monotonic response functions of concentration: the higher the odorant concentration, the higher the proportion of receptors bound, and the larger the signal (Malnic et al., 1999). The number of olfactory receptor neurons (ORNs) responding to a given odorant increases with increasing concentrations of ligand (see Fig. 8.1, and Cinelli et al., 1995; Friedrich and Korsching, 1997; Joerges et al., 1997; Rubin and Katz, 1999; Wachowiak et al., 2000; Wachowiak and Cohen, 2001; Ziesmann et al., 2001). Some ORNs display a steep dependence of the mean instantaneous spike frequency (MISF) on stimulus strength saturating within a 10-fold increase in odor concentration (Ziesmann et al., 2001). Other ORNs display dose-response curves with a dynamic range covering concentrations of several orders of magnitude (Ziesmann et al., 2001).

Furthermore, optical imaging studies have shown that activity in the vertebrate olfactory bulb and its homologue in insects, the antennal lobe, increases monotonically with increasing concentration (Galizia and Menzel, 2001; Meister and Bonhoeffer, 2001). The dependency of the input to the olfactory bulb on odor concentration has been characterized with a simple formalism for ligand bind-



The response of olfactory receptor neurons in the three-toed box turtle, measured with Calcium Green-1 dextran 10 kD, is not concentration invariant, but rather increases with increasing concentrations (from Wachowiak et al., 2000).

ing (Holy et al., 2000; Meister and Bonhoeffer, 2001). If the olfactory system responds with increasing activity every time an odor is added, however, how does the system achieve odor-selectivity, which requires *not* responding to blends that include a response-eliciting component?

The antennal lobe, the insect analog of the vertebrate olfactory bulb, is the site of projection of olfactory receptor neurons. In locusts, it forms a compact (830 output neurons, or PNs, the insect analog of mitral cells), complete and dynamic representation of odors (Laurent et al., 2001). In order to characterize both excitatory and inhibitory inputs to PNs as well as PN output, I carried out intracellular recordings from PNs in the antennal lobe of awake locusts, while presenting series of 1-second-long odor puffs of varying concentrations using a computerized delivery system (see Chapter 6). Simultaneous recordings were carried out of the local field potential (LFP) in the mushroom body, the target of PNs.

Results

The field potential represents a measure of the population activity of synchronized PN assemblies (refs Laurent 94). The LFP showed increased power in the 13-30 Hz band during odor responses for all concentrations tested (Fig. 8.2a-b). The LFP's peak frequency remained constant across concentrations at 16-18 Hz (median peak frequency across trials for t=0-1 s was not significantly different for 2% concentration than for 100% concentration, p>0.4, Wilcoxon ranksum test, Fig. 8.2e).

Increasing odor concentration caused the peak-to-peak amplitude of the field potential during odor responses to increase, except for the highest concentration step, at which saturation, or even a slight decrease in LFP amplitude, occurred ($p<<10^{-6}$, n=133 PN-odor pairs, see Methods, Fig. 8.2a,c,d). This dependency was present both for naive animals (coefficient of correlation = 0.52, $p<<10^{-6}$, Pearson test and univariate Anova, n=133 PN-odor pairs) and for those that had been previously exposed to higher concentrations of the odor tested (coefficient of correlation = 0.61, $p<<10^{-6}$, Pearson test and univariate Anova, n=104 PN-odor pairs) (see Chapter 9). This was also reflected in an increase in the LFP's power in the 13-30 Hz window (Fig. 8.2a). This prompted the question of whether the increase in the LFP power was caused by an increase in PN firing rates, by a tightening in PN synchronization, or both.

Surprisingly, although mean PN firing rates increased in naive animals as a function of concentration (rate of minimum concentration tested, 2% of saturation, was not significantly different than that for maximum concentration, 100% of saturation; p<0.008, Wilcoxon ranksum test, n=152 PN-odor pairs), PN firing rates were not significantly affected by odor concentration after exposure to a higher concentration (rate of minimum concentration tested, 2% of saturation, was not significantly different than that for maximum concentration, 100% of saturation; p>0.8, Wilcoxon ranksum test, n=154 PN-odor pairs, Fig. 8.3) (see Chapter 9).

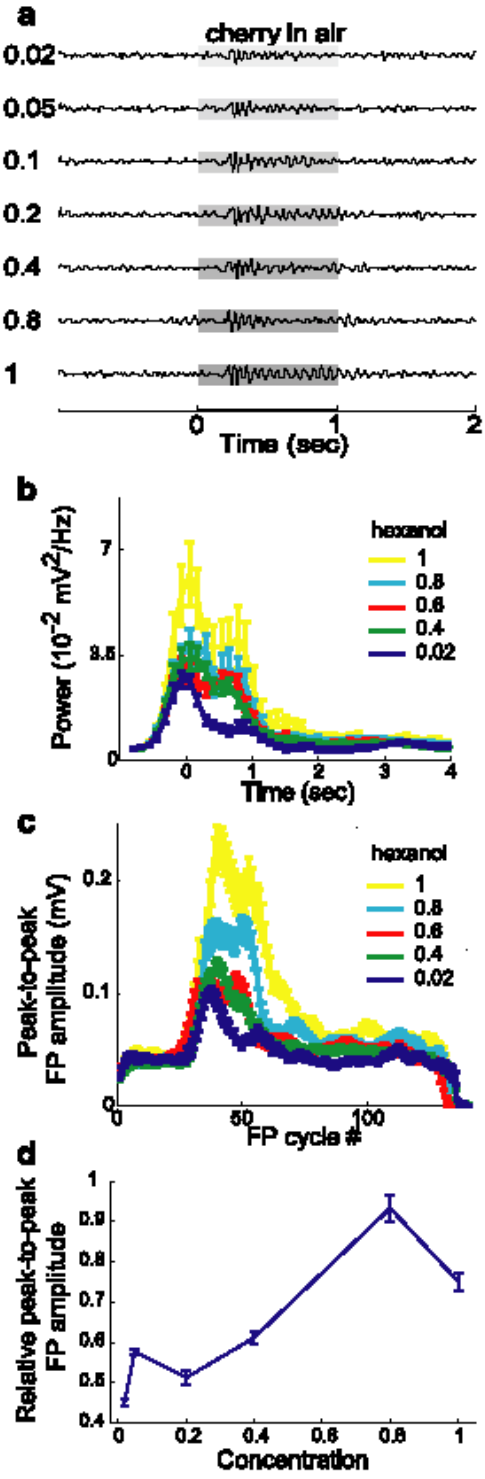
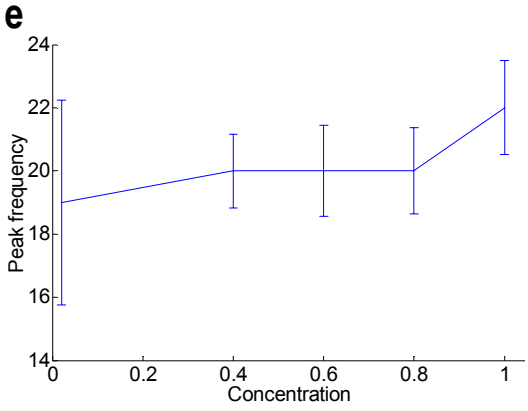


Figure 8.2 The local field potential's (LFP) peak-to-peak amplitude and power in the 13-30 Hz band increase as a function of concentration. a, LFP traces during exposure to a 1 sec pulse of varying concentrations, presented at t=0-1 s. **b,** Median power and s.e.m. in the 13-30 Hz band as a function of time and concentration for a typical PN-odor pair. Power was evaluated for 500-ms sliding windows. odor was presented at t=0-1 s. **c,** Median peak-to-peak amplitude (and s.e.m.) for every LFP cycle of the PN-odor pair in (b). **d,** Median peak-to-peak amplitude and s.e.m. averaged over cycles #30-80 and 133 PN-odor pairs.Cycle #1 was 1s before odor onset. e, Median peak frequency in the 0-100 Hz band for t=0-1 s for the PN-odor pair in (b) and (c).



A more detailed analysis of the intracellular recordings revealed that the lack of an effect of concentration on mean firing rate hid conflicting effects of concentration on excitation and inhibition. odor responses increased in contrast relative to baseline with increasing concentration. Increasing concentrations strengthened excitatory responses ($p<10^{-4}$, n=62 PN-odor pairs, see Methods), lengthened inhibitory responses (14 of 19 inhibitory responses were longer for higher concentrations, and mean duration was higher for higher concentrations, $p<0.003$, Wilcoxon ranksum test) and deepened inhibitory responses (18 of 19 inhibitory responses were more hyperpolarizing for higher concentrations, and mean hyperpolarized potential was more negative for higher concentrations, $p<<10^{-6}$, Wilcoxon ranksum test) of subthreshold inhibitory periods in naive animals (see Methods) (Fig. 8.4). After priming, the length of inhibitory responses was not significantly different for different concentrations ($p>0.05$), but inhibition remained significantly more hyperpolarizing for higher concentrations ($p<10^{-3}$). After priming with high concentrations, then, the strengthening of excitatory and inhibitory responses balanced each other out, leaving no net change in mean firing rates across PN assemblies as a function of concentration (Fig. 8.3).

The effect of concentration on the temporal response patterns of PNs was complex and varied for different PNs. All changes observed with increases in concentration could be explained by a strengthening of excitation, a strengthening of inhibition, or both. In no case did I observe a response present at any given concentration disappear at higher concentrations with no concomitant increase in excitation or inhibition. Some of the most typical responses are shown below. In some PNs, excitation and inhibition were strengthened approximately equally, and thus showed the same approximate temporal response patterns across the range of concentrations to which they responded (Fig. 8.5). In others, the duration of inhibitory responses increased with increasing concentration (Fig. 8.6). This systematically increased the latency of excitatory responses following the inhibition period. In PNs which exhibited early excitatory responses and no inhibition, the excitation

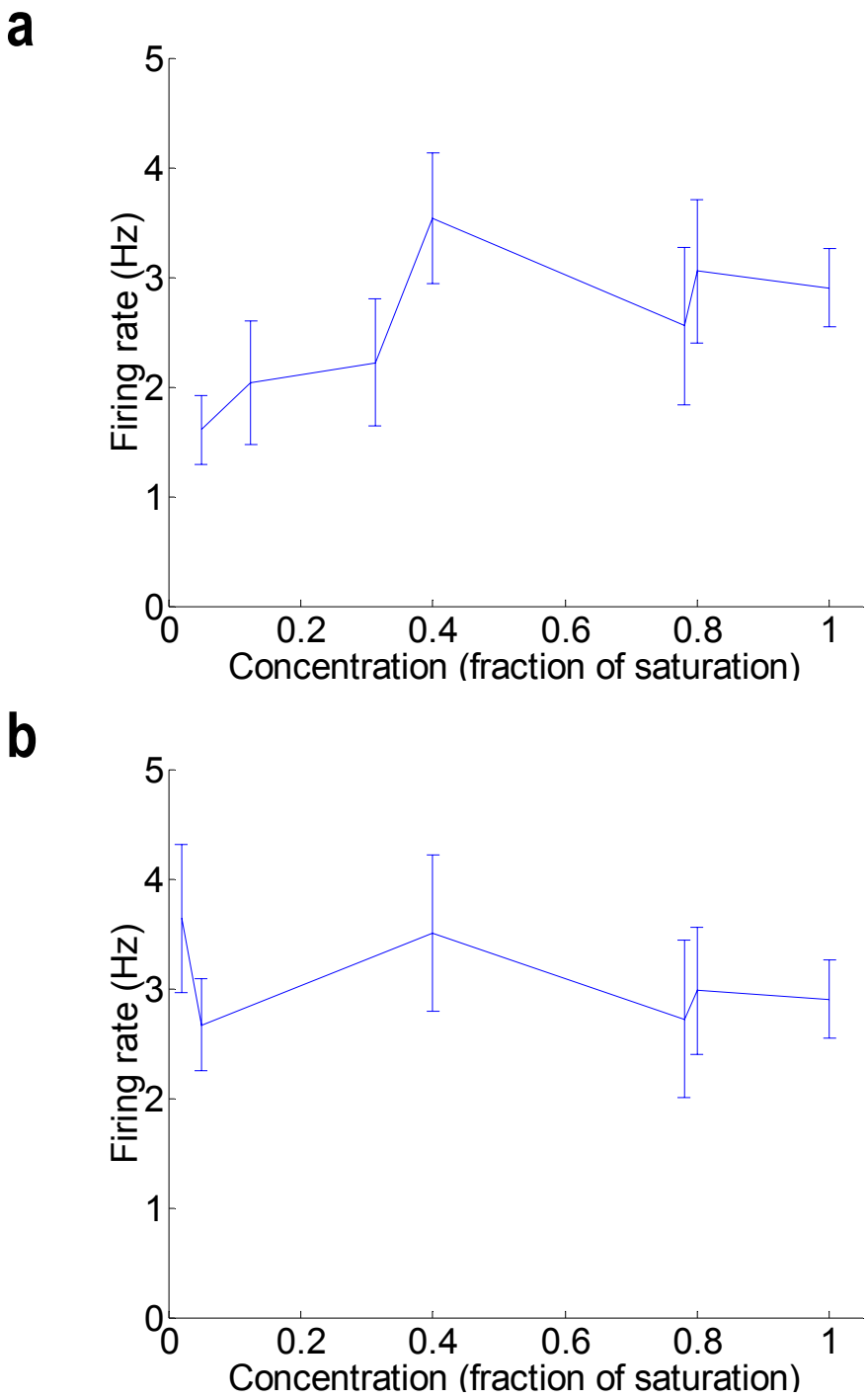


Figure 8.3 PN mean firing rates in naive animals increase slightly with concentration (a) but become independent of concentration after exposure to higher concentrations (b). Mean firing rates and s.e.m. for t=0-3 sec after odor presentation, averaged over 152 (a) and 154 (b) PN-odor pairs. S.E.M.s are large due to the heterogeneity of PN response patterns; the plot illustrates the overall average tendency of PNs to keep firing rates relatively constant over 2 orders of magnitude of concentration.

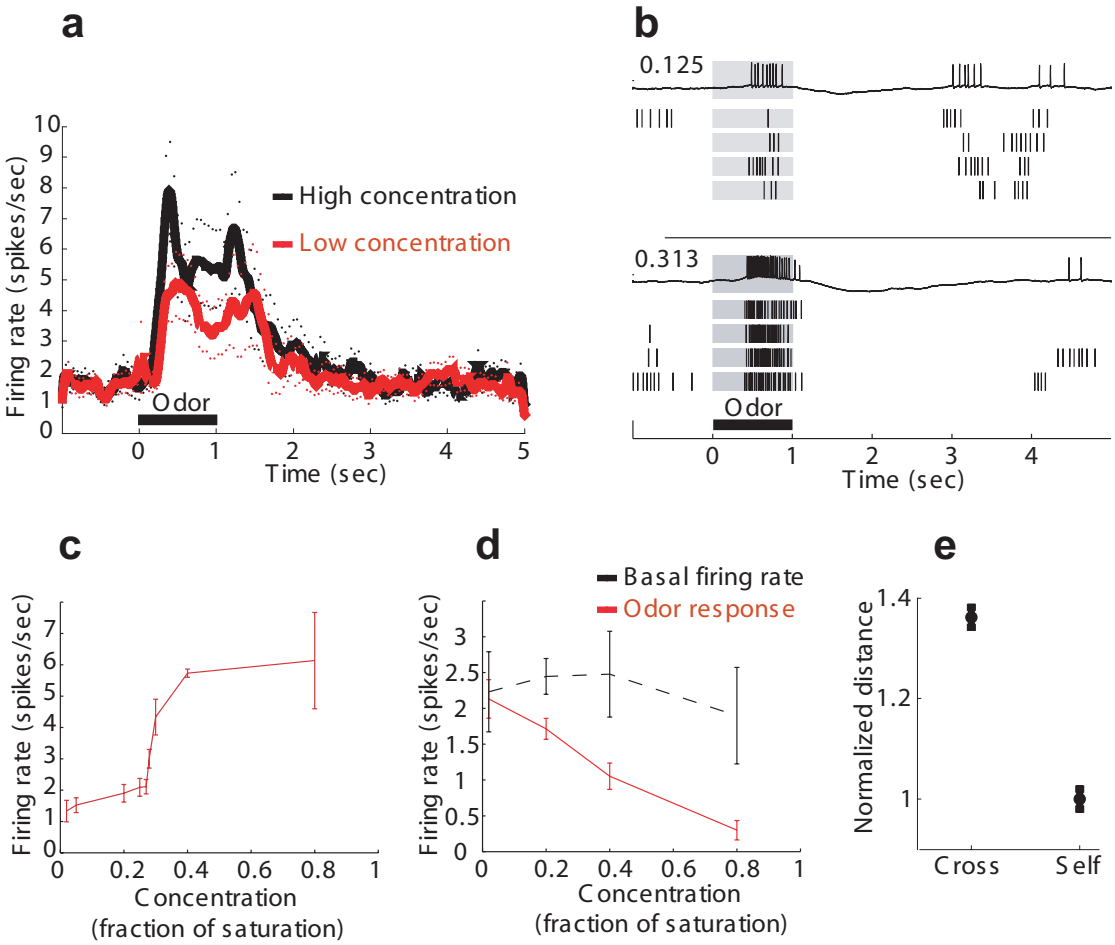


Figure 8.4 Response strength increases with concentration. a) The firing rate of PNs' excitatory responses increases with increasing odor concentration (maximum rates for higher concentration significantly higher than maximum rates for lower concentration, $p<10^{-4}$, n=62). Peri-stimulus time histogram (PSTH) of the mean and s.e.m. over all trials for 62 cell-odor pairs containing only excitatory responses, smoothed with a Gaussian (25 ms SD), for the highest and lowest nonzero concentration tested for each cell/odor pair. S.E.M.s are large due to the heterogeneity of PN response patterns. **b)** PN excitatory and inhibitory responses become more intense with increasing odor concentration. One PN's response to cherry over multiple trials at concentrations of 12.5% (top) and 31.3% (bottom). **c)** Another PN's response to citral as a function of concentration (mean firing rate and standard error of the mean, calculated from t=0.1-3.1 sec). **d)** Mean firing rate (and s.e.m.) of the PN in C as a function of concentration during the inhibitory response to apple (t=0.1-3.1 sec) and background activity during the 1-second period preceding odor presentation. **e)** Responses to different concentrations of the same odor are significantly different from each other ($p<<10^{-6}$, Distance test –see Methods—, n=62 cell-odor pairs). Cross: mean *difference between* low and high concentration series (and s.e.m.). Self: *variability within* series.

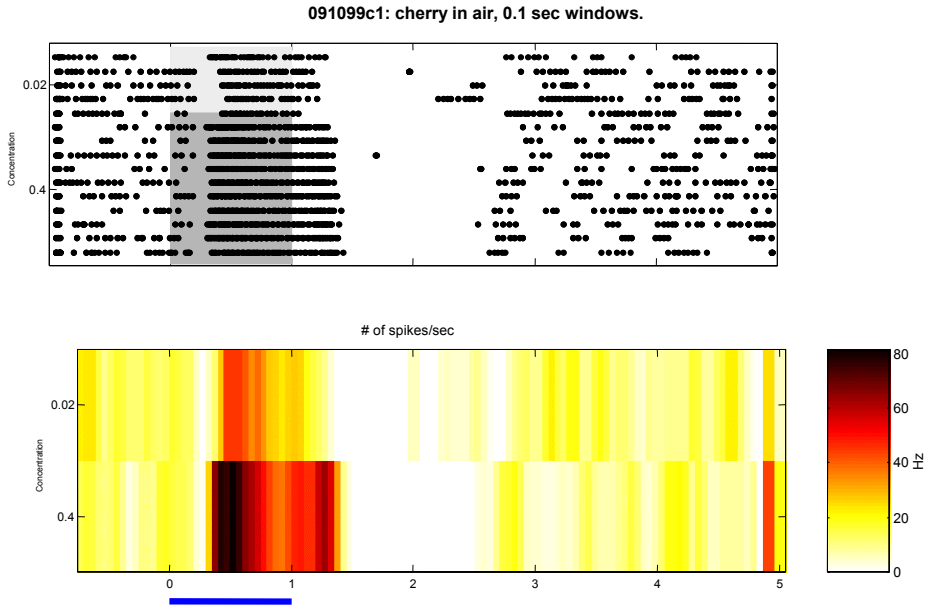


Figure 8.5 A PN's response to 2% and 40% of saturated cherry vapor. a) Spike rasters. **b)** PSTHs. Both excitatory and inhibitory responses are more pronounced at the higher concentration.

was usually strengthened, decreasing its latency and increasing firing rates (Fig. 8.7). Other PNs were recruited to an odor's representation for higher concentrations only, exhibiting excitatory and/or inhibitory responses absent at lower concentrations (Fig. 8.8). Concentration tuning (Kauer, 1974) was not found. It is conceivable that over larger concentration ranges, the strengthening of excitation could predominate at lower concentrations, followed by a predominant strengthening of inhibition at higher concentrations; this combination would produce an excitatory response only at intermediate concentrations, as described by Kauer for concentration tuned neurons (Kauer, 1974).

In contrast to the complex effects of concentration on PN firing rates, PN synchronization to the LFP, measured as the fraction of spikes during the 3-second period following presentation of odor whose phase with respect to the LFP fell within 3/8ths of a cycle of an LFP peak (from pi/2

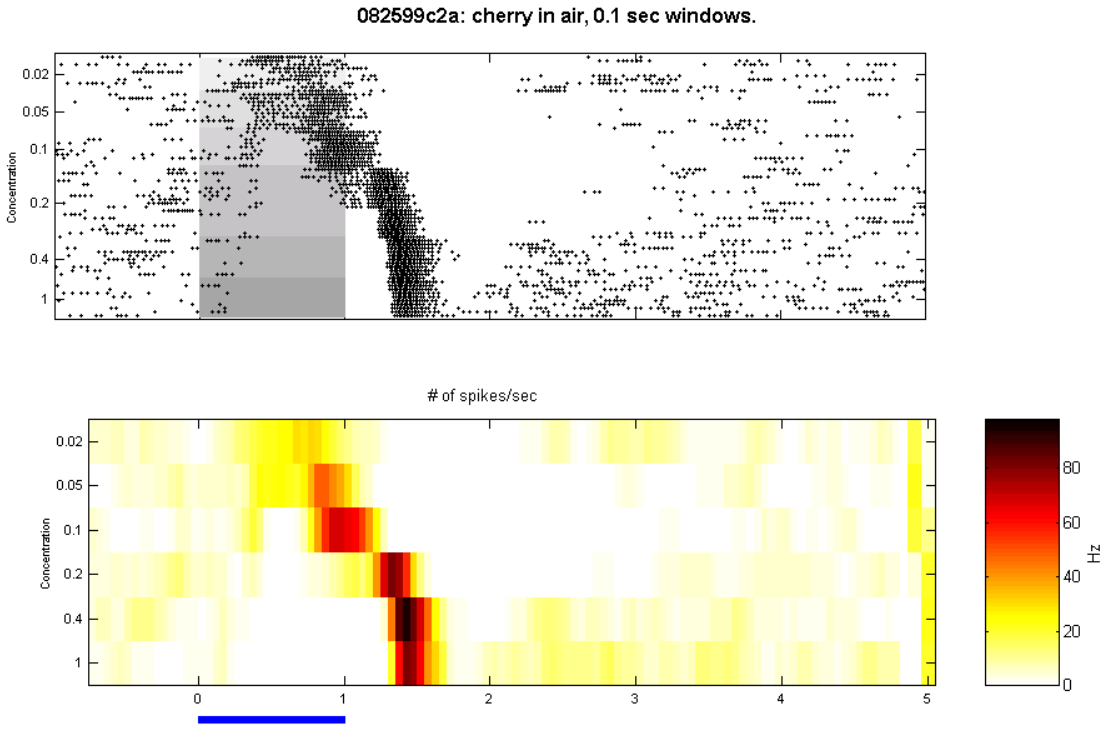


Figure 8.6 Increasing concentration often lengthens periods of hyperpolarization, increasing the latency of post-inhibition excitatory responses. a) Spike rasters**. b)** PSTHs.

before a peak to pi/4 after), increased significantly with increasing odor concentration (synchronization was significantly higher for maximum concentration (100% saturation) than for minimum concentration used (2% of saturation), $p<3x10^{-4}$, Wilcoxon ranksum test, n=144 PN-odor pairs, see Figs. 7.3 and 7.5). This effect was present in naive animals and was robust to exposure to higher concentrations (compare Fig. 7.5a to 7.5b).

In summary, we have demonstrated that odor-induced synchronization of PNs, measured as an odor-induced increase in the 20 Hz band of the field potential (Laurent and Naraghi, 1994; MacLeod and Laurent, 1996; Stopfer et al., 1997), is robust across a range of concentrations spanning two orders of magnitude. Second, we showed that increased odor concentrations strengthen the response of synchronized PN assemblies, as seen in an increase in the power and amplitude of

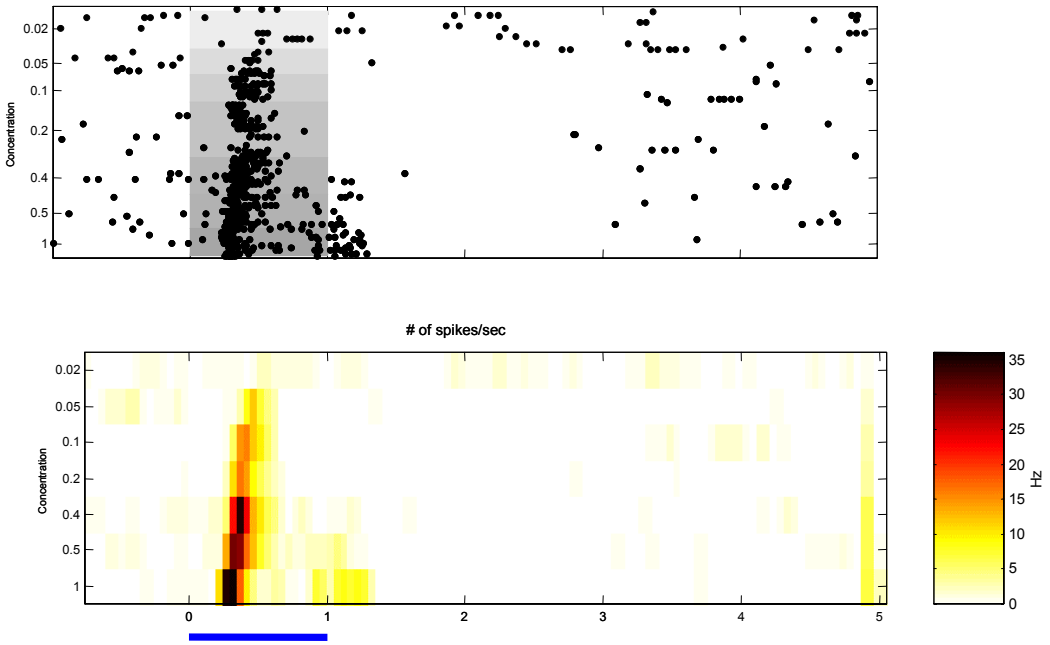


Figure 8.7 Increasing concentration often reduces the latency and increases the firing rate of early excitatory responses. a) Spike rasters. **b)** PSTHs.

the LFP. Third, we showed that these changes are not underlain by corresponding changes in mean firing rates, for both excitatory and inhibitory responses are strengthened and these effects balance each other out on average after exposure to high concentrations. Instead, the changes in the LFP as a function of concentration were primarily caused by a change in the degree of synchronization between PNs and the LFP. Finally, we explained the seemingly complex changes seen in PN temporal response patterns as combinations of two underlying tendencies, that of the strengthening of excitatory and inhibitory responses alike with increasing concentrations. This simple trend also provides an explanation for the concentration tuning observed by other workers in the olfactory bulb.

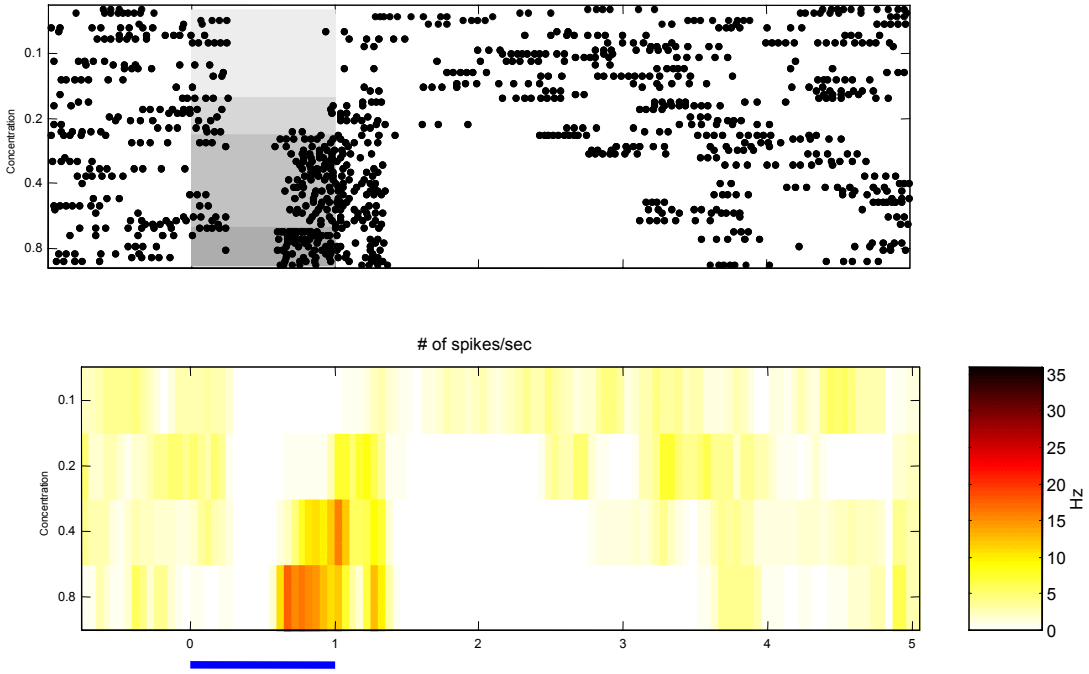


Figure 8.8 Some PNs get recruited to the odor representation at higher concentrations only. Note that both excitatory and inhibitory responses appear as concentration is increased. a) Spike rasters. **b)** PSTHs.

Discussion

Our results have several functional implications. First, they suggest that the odor-induced synchronization of assemblies of projection neurons in the antennal lobe of the locust (Laurent and Davidowitz, 1994; Laurent and Naraghi, 1994) is not restricted to high concentrations but rather extend over at least two orders of magnitude of concentration.

Second, the demonstration that the LFP was reliably strengthened with increasing odor concentrations suggests that odor concentration is encoded in the degree of synchronization of PN assemblies (see Chapter 7).

Third, the demonstration that both excitation and inhibition are strengthened by increasing

concentrations suggests that odor concentration is likely not encoded by a rate code, but rather by one involving identity and timing of spikes across neuronal assemblies. The finding that synchronization between PNs and the LFP increases with increasing odor concentration is consistent with this hypothesis. Of particular interest is the fact that this synchornization code for concentration endures after priming (see Chapter 9). This suggests that although priming enhances concentration invariance, this does not come at the expense of concentration information, which is maintained in the degree of synchronization across PN assemblies.

Fourth, the stark contrast between our results using intracellular recordings and those of optical imaging of the insect antennal lobe and vertebrate olfactory bulb brings into question the validity of optical imaging as a tool to study the output of neuronal assemblies. Recently, the source of optical imaging signals has been brought into question (Meister and Bonhoeffer, 2001; Galizia and Menzel, 2001). It has recently been suggested that calcium imaging signals may represent an overestimation of the output of the olfactory bulb (Galizia and Menzel, 2001). In the mushroom bodies too, the input to Kenyon cells, measured with optical imaging (Wang et al., 2001), is significantly more widespread than the output of these (Laurent and Naraghi, 1994). Our results suggest that indeed, the dependence of olfactory bulb and antennal lobe response on concentration seen with optical imaging is not paralleled in the output of single neurons. A potential explanation of the source of the discrepancy between calcium imaging and electrophysiological studies is that because calcium is released by presynaptic terminals regardless of whether their effect is excitatory or inhibitory, calcium imaging studies are likely to present a rectified version of the input to the area under study. This would explain the fact that, whereas intracellular recordings show the concentration-dependent strengthening of both excitation and inhibition balancing each other out, optical imaging studies see simply a strengthening of the input.

Fifth, the balancing of excitation and inhibition we observe may well serve the purpose of

gain control, allowing the antennal lobe to reduce the dynamic range of its output in the face of large changes in its input.

Finally, the functional role of slow inhibition in the antennal lobe and olfactory bulb has been the subject of controversy (Laurent, 1997, 1999). It has been hypothesized that this inhibition is the result of lateral inhibition serving the purpose of increasing the difference of activity between the least and most active glomeruli for each odor to yield efficient coding and increase odor discriminability (Rospars and Fort, 1994; Yokoi et al., 1995). The role of lateral inhibition has been questioned recently, though (Laurent, 2000). The results presented here, showing the monotonous increase in both excitatory and inhibitory responses to increasing concentration, suggest a different and perhaps more fundamental role for inhibition in PN responses. If the presence of odors is decoded by KCs detecting a particular combination of active PNs, and if a blend of odors is *not* detected as the sum total of all subsets of odors in the mixture but rather as a new odor in and of itself (Laing and Francis, 1989) –the chemical signature of an event—, then an inhibitory mechanism is needed to prevent particular KCs from being activated in response to a subset of components occurring in a blend, ensuring that only the KCs that signal for the whole blend are activated (see Appendix II). When the output is which PN combinations are active simultaneously and which combinations are *not*, the role of inhibition in signaling is just as important as that of excitation.

Our results lead to the prediction that selectively blocking slow inhibition will elicit novel PN responses to blends containing a response-evoking odor, and lead to an increase of false positive behavioral responses to high concentrations and odor blends containing a component to which an animal has been trained to respond selectively.

Methods

Specimens, odor stimulation and electrophysiology

Intracellular recordings were obtained from 180 cell-odor pairs in 46 PNs of 21 locusts, *Schistocerca americana*. Surgery and recordings were performed as previously described (Laurent and Davidowitz, 1994; Laurent et al., 1996). Delivery of seven odors, including pure compounds as well as ethologically relevant blends, was performed using a gaseous dilution computerized odor-delivery system capable of delivering arbitrary concentrations by mixing a stream carrying saturated odor vapor with a second stream carrying pure air. The concentration of the odor delivered was regulated by controlling the relative flow rates of both streams (see Chapter 6). The system was purged between presentation of different stimuli. The stimulus sequence was delivered to an initially naïve animal, that is, one that had no prior experience with the odor tested. Successive stimuli presentations were spaced 10 seconds apart. No adaptation was observed between successive presentations.

The odor timecourse and magnitude was measured by using CO_2 as a tracer in the air line carrying the odor while the diluting stream carried ambient air, and measuring the CO_2 concentration at the nozzle (see Chapter 6). Direct measurement of the odorant concentration with Gas Chromatography Mass Spectroscopy also showed that concentration returned to baseline within 1 minute of purging.

Data were digitized at 20 kHz using a digital tape player (DAT, Biologic) and then acquired into a personal computer at 4 kHz using an analog/digital input/output card (National Instruments). Analysis was conducted on raw intracellular voltage traces as well as spike rasters. Spike isolation was conducted by voltage thresholding and visual inspection.

PN sensitivity to concentration

Each PN-odor pair was challenged with multiple trials (n>=5; n>=10 for most datasets) at each of at least two concentrations between 2 and 100% of saturated vapor pressure. The firing rates for different concentrations were compared using a paired T-test comparing maximum (over time) mean (over trials) firing rates for the highest and lowest concentration tested for each cell-odor pair during 4 sec following odor onset, for the 65 cell-odor pairs with only excitatory responses in the spike trains. A response was defined as excitatory if one or more epochs (300 or 500 msec, shifted in 100 msec steps, to account for short responses with high temporal precision as well as longer responses with more variance in spike times) exhibited a firing rate increase to at least 3 standard deviations above the mean baseline rate.

The low baseline firing rates of PNs (2-6 Hz) prevented an accurate evaluation of inhibition using firing frequency alone. Instead, the intracellular voltage traces for successive trials of the same concentrations were aligned on the mean voltage during the second preceding odor delivery for each trial. An odor was said to elicit an inhibitory response at a given concentration if there was any period (evaluated with sliding windows of 500 msec shifted in 100 msec steps) during which firing was suppressed and the mean voltage was at least 3 standard deviations below the mean voltage during the period preceding odor delivery. 35% of datasets showed some period of inhibition and individual responses often contained a period of excitation and a period of inhibition. The depth and duration of inhibition were quantified for each concentration. Depth was defined as the mean voltage during all inhibited epochs. Duration was defined as the total duration of all epochs during which inhibition was detected.

Peri-stimulus time histograms (PSTHs) were computed by calculating the mean firing rate for successive 100 ms bins.

LFP sensitivity to concentration

LFP traces were resampled to 1 kHz with MATLAB's resample function and filtered with a 13-30 Hz 5th order Butterworth non-causal bandpass filter. Filtered traces were processed to find local maxima and minima. Maxima (minima) were identified by locating zero-crossings that coincided with a positive (negative) second derivative. A cycle's peak-to-peak amplitude was defined as the absolute difference between the corresponding maximum and minimum. To ensure robustness of the effects observed, measurements were done both with all trials and with trials with the single greatest amplitude discarded as outliers for every concentration, with similar results.

Phase analysis

The phase of spikes was defined as

$$\text{phase} = \text{pi} * M + \text{pi} \,.\, f,$$

where f is the fraction of time elapsed between the previous FP extreme (maximum or minimum) and and the time of occurrence of the spike:

$$f = (t_{spike} - t_{previousextreme}) / (t_{nextextreme} - t_{previousextreme})$$

and M was 1 if the previous FP extreme was a minimum and 0 if it was a maximum. We also experimented defining phases with respect to every quarter cycle and every full cycle, but half cycles proved the most consistent across trials.

Naive vs. experienced animals

For every dataset, a trial was defined as naïve if the animal had not been exposed to the same odor at a higher concentration before, and post-high-concentration if the animal had been exposed to the same odor at a higher concentration before (see Chapter 9).

CHAPTER 9

Priming contributes to concentration invariance in early olfactory circuits

Object recognition requires both specificity, to ensure that stimuli with distinct behavioral relevance are distinguished, and invariance, to ensure that different instances of the same stimulus are recognized as the same under varied conditions (intensity, pitch, position, etc.). In olfaction, the question of stimulus specificity has received considerable attention[1-13]. Psychophysical studies also show that individual odors can be perceived as identical over significant ranges of concentrations[14,15]. Whether concentration invariance results, at least in part, from low-level neural phenomena rather than cognitive grouping is so far unknown. Using locusts, we found that projection neurons in the antennal lobe (the first olfactory relay) are sensitive to odor concentration differences, but often in a history-dependent manner. In ~50% of recordings, exposure to a high concentration modified subsequent responses to lower concentrations such that responses to lower concentrations became more similar to responses to the higher concentration recently experienced. This hysteresis, which we called priming, was odor-specific, lasted 15 to 30 minutes, and was not disrupted by exposure to different odors. Priming might provide a mechanism to 'tune in' or bias responses towards an odor likely to be sampled in the near future and contribute to the invariance of neuronal responses across concentrations.

Olfactory systems operate effectively over a very wide dynamic range of concentrations. Insects, for example, can sometimes detect single molecules[16], but also discriminate odors at saturated vapor concentration, such as inside a flower[17]. Odors at different concentrations are sometimes perceived as different[14] and can even acquire a different hedonic valence[18]. Electrophysiological studies in the olfactory bulb (OB) of vertebrates, indeed, indicate that mitral cells (MC) responses generally change with stimulus concentration[13,19-21]. Nevertheless, behavioral studies also show that animals can normally recognize an odor across a range of concentrations[14,15]. Concentration invariance has so far not been described at the level of single neurons.

The antennal lobe, the insect analog of the vertebrate olfactory bulb, is the site of projection of olfactory receptor neurons. In locusts, it forms a compact (830 output neurons) and dynamic representation of odors[22]. We carried out intracellular recordings from projection neurons (PNs), the insect analog of mitral cells, in the antennal lobe of awake locusts, while presenting series of 1-sec-long odor puffs of varying concentrations using a computerized delivery system. The stimulus sequence was delivered to an initially naïve animal, that is, one that had no prior experience with the odor tested.

PNs in naïve locusts generally proved to be concentration sensitive (responses to two concentrations were significantly different in 58 out of 62 responsive odor/cell pairs tested, Distance test, see Methods). Responses increased in contrast relative to baseline with increasing concentration. Increasing concentrations strengthened excitatory responses ($p<10^{-4}$, n=62, see Methods) and inhibitory responses as seen in a lengthening and deepening of subthreshold inhibitory periods ($p<10^{-3}$ for both, Wilcoxon test, n=49; see Methods) (Fig. 8.3; see also Figs. 9.1-9.4).

We observed, however, that PN response differences across concentrations depended on the animal's past history of stimulation. Exposure to between five and ten 1-sec-long pulses of high concentration induced significant changes in odor responses to lower concentrations in 28 out of the 49 concentration-sensitive PN-odor pairs tested ($p<<10^{-6}$, Fig. 9.1a). This proportion is a lower bound, for only a small set of concentrations was tested with each PN-odor pair. These changes included the induction of responses to previously ineffective concentrations (Fig. 9.1a) as well as changes in preexisting responses (Fig. 9.1b). Priming affected both inhibitory and excitatory responses (Fig. 9.1b). Each PN's response was affected in ways more complex than a simple increase or decrease in firing rate; instead, priming made the response to lower concentrations seemingly more similar to the response to a higher concentration (Fig. 9.1b and see below). We call these high concentration-induced changes priming. Priming was not due to hysteresis in the odor delivery system, because responses of a detector to the same low concentration presented before or after high concentration

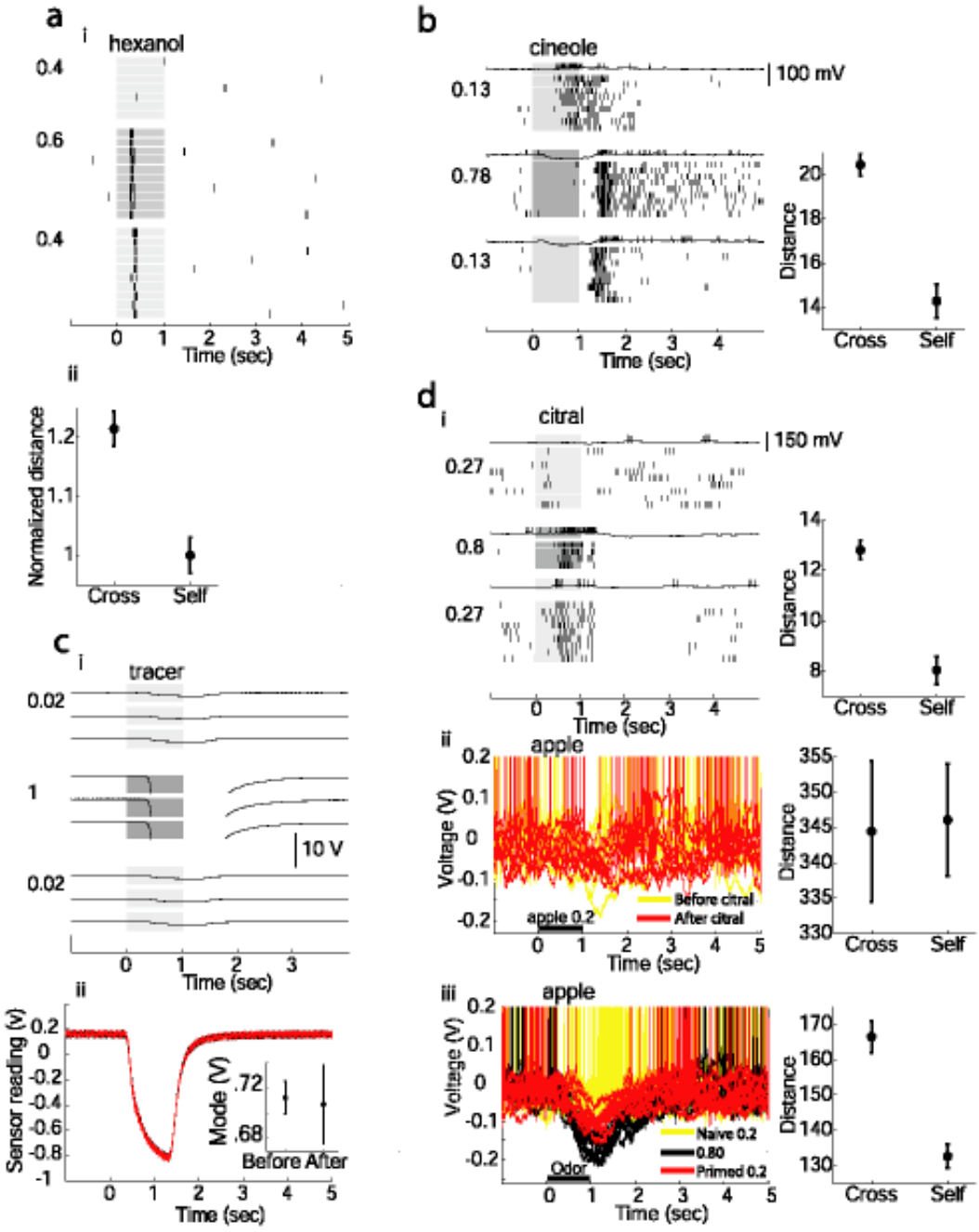

a
i
hexanol
0.4
0.6
0.4
Time (sec)
ii
Normalized distance
Cross
Self
b
cineole
100 mV
0.13
0.78
0.13
Distance
Cross
Self
Time (sec)
c
i
tracer
0.02
1
0.02
10 V
Time (sec)
ii
Sensor reading (v)
Mode (V)
Before After
Time (sec)
d
i
citral
150 mV
0.27
0.8
0.27
Distance
Cross
Self
Time (sec)
ii
apple
Voltage (V)
apple 0.2
Before citral
After citral
Distance
Cross
Self
Time (sec)
iii
apple
Voltage (V)
Odor
Naive 0.2
0.80
Primed 0.2
Distance
Cross
Self
Time (sec)

Figure 9.1. **Exposure to a higher concentration primes PNs' excitatory and inhibitory response to lower concentrations in an odor specific manner. a,** Exposure to a suprathreshold concentration sensitizes PNs to previously subthreshold concentrations. **i,** Trials were presented (top to bottom) with 10 seconds between the onset of each trial and 1 minute in between concentrations (0.4 and 0.6, fration of saturated vapour) for purging the odor delivery system. **ii,** Responses after priming with a suprathreshold concentration were significantly different from responses to the same stimulus before priming ($p«10^{-6}$, Distance test, n=49 cell-odor pairs with significant concentration sensitivity in the naïve state tested for priming). **b,** Priming affects inhibitory components of responses as well as excitatory ones. A different PN's response to cineole before and after priming with a stimulus at a higher concentration. Responses to 0.13 before and after priming (computed for t=0-1 s and t=1-2 s periods) were significantly different ($p<10^{-4}$, Distance test). **c,** Priming is not due to hysteresis in the odor delivery system. **i,** Concentrations delivered in response to identical pulses of 2% of saturation (0.02) before and after a series of saturated vapour (1) pulses are statistically indistinguishable ($p>0.6$, Welch test and T-test). Signal caused by high concentration stimulus off scale. **ii,** Overlaid detected sensor responses from 16 trials at a concentration of 0.02 before (black) and after (red) delivery of high concentration. **Inset,** Mean and standard deviation of mode concentration readings for pulses at 0.02 concentration, before and after delivery of high concentration are overlapping. **d,** Priming is odor-specific and excitatory and inhibitory responses to different odors can be independently primed in the same cell. Exposure to citral at a concentration of 0.8 primes excitatory responses of another PN to citral at a concentration of 0.27 (i) but does not prime responses to apple (ii). The same PN can be primed to respond with inhibition to apple by exposure to apple at a concentration of 0.8 (iii). **Left**: **i,** Intracellular traces (top) and rasters obtained in response to 0.27 citral in a naïve animal, followed by 0.8 and then 0.27 again. **ii,** Superimposed intracellular voltage traces for ten trials of 0.2 apple before (yellow) and after (red) exposure to 0.8 citral. **iii,** Superimposed intracellular voltage traces for ten trials each of 0.2 apple before (yellow) and after (red) exposure to ten trials of 0.8 apple (black). Inhibition of priming affects both hyperpolarization and firing rates: notice spikes present during the odor response in the naïve animal (yellow) disappear after priming (red). **Right**: Distance test (see Methods): (i) $p<10^{-4}$; (ii) $p=0.9$; (iii) $p<10^{-5}$.

pulses were statistically indistinguishable (Fig. 9.1c). Both gas chromatography-mass spectroscopy (GCMS) and polycaprolactone/carbon black (80:20 wt/wt) composite polymers[23] confirmed this result. Priming was specific to odor-responses and could not be attributed to changes in basal firing rate (the changes observed over odor responses were significantly greater than any trends in basal period, $p<<10^{-6}$, Distance test, n=49 cell-odor pairs).

To test whether priming is odor-specific, we presented the following stimulus sequence: A low → B low → B high → B low → A low → A high → A low, where X low stands for odor X at the lower concentration and X high stands for odor X at the higher concentration. Priming was odor-specific: presentation of one odor at the higher concentration caused PNs to respond to previously ineffective concentrations of that odor ($p<0.0005$, n=12 cell-odor pairs, Distance test, Fig. 9.1di), while leaving responses to similar (or even greater) concentrations of a second odor unchanged ($p>0.1$, n=12 cell-odor pairs, Distance test, Fig. 9.1dii). This was true even when responses to the second odor could be primed by subsequent high concentration exposure with that odor ($p<10^{-5}$, Distance test between lower concentration of second odor before and after exposure to higher concentration, Fig. 9.1diii). Priming did not extend across chemically similar (citral (an aldehyde) and its corresponding alcohol, geraniol; hexanol and octanol) or structurally different odorants. Thus, different responses to multiple odors could be enhanced independently in the same neuron. Indeed, a given neuron could exhibit priming of excitation for one odor and priming of inhibition for nother (n=9 odor pairs in 5 cells; see Fig. 9.1d), suggesting that priming is not caused by an intrinsic change in PN excitability.

To quantify whether priming contributed to creating a representation of odor identity that is less dependent on concentration, we asked whether primed responses to low concentrations were more similar to the responses to the (higher) priming concentration trials than the initial responses to low concentrations were. We assayed similarity between responses by calculating the mean distance, using a cost-based metric[24], between spike trains of individual trials for each concentration, in the primed and naïve conditions. After priming, PNs' responses were more similar across different concentrations ($p<0.005$, Distance test, n=49 cell-odor pairs; see Fig. 9.2c), both in firing rates (Fig. 9.2a) and interestingly, in response patterning (Fig. 9.2b-c and see below). Exposure to a high concentration decreased PNs' sensitivity to concentration and thus contributed to concentration invariance. The extent of priming, measured as the across/within-groups distance ratio for odor responses divided by that for the baseline period preceding odor present, was significantly and positively correlated with the concentration sensitivity in the naïve neuron ($r=0.87$ for all 51 cell-odor pairs, $r=0.93$ for concentration-sensitive datasets): the more different responses were to different concentrations in the naïve animal, the more the response to lower concentrations changed in response to priming (see also Fig. 9.2d). None of the PN-odor pairs that were not sensitive to con-

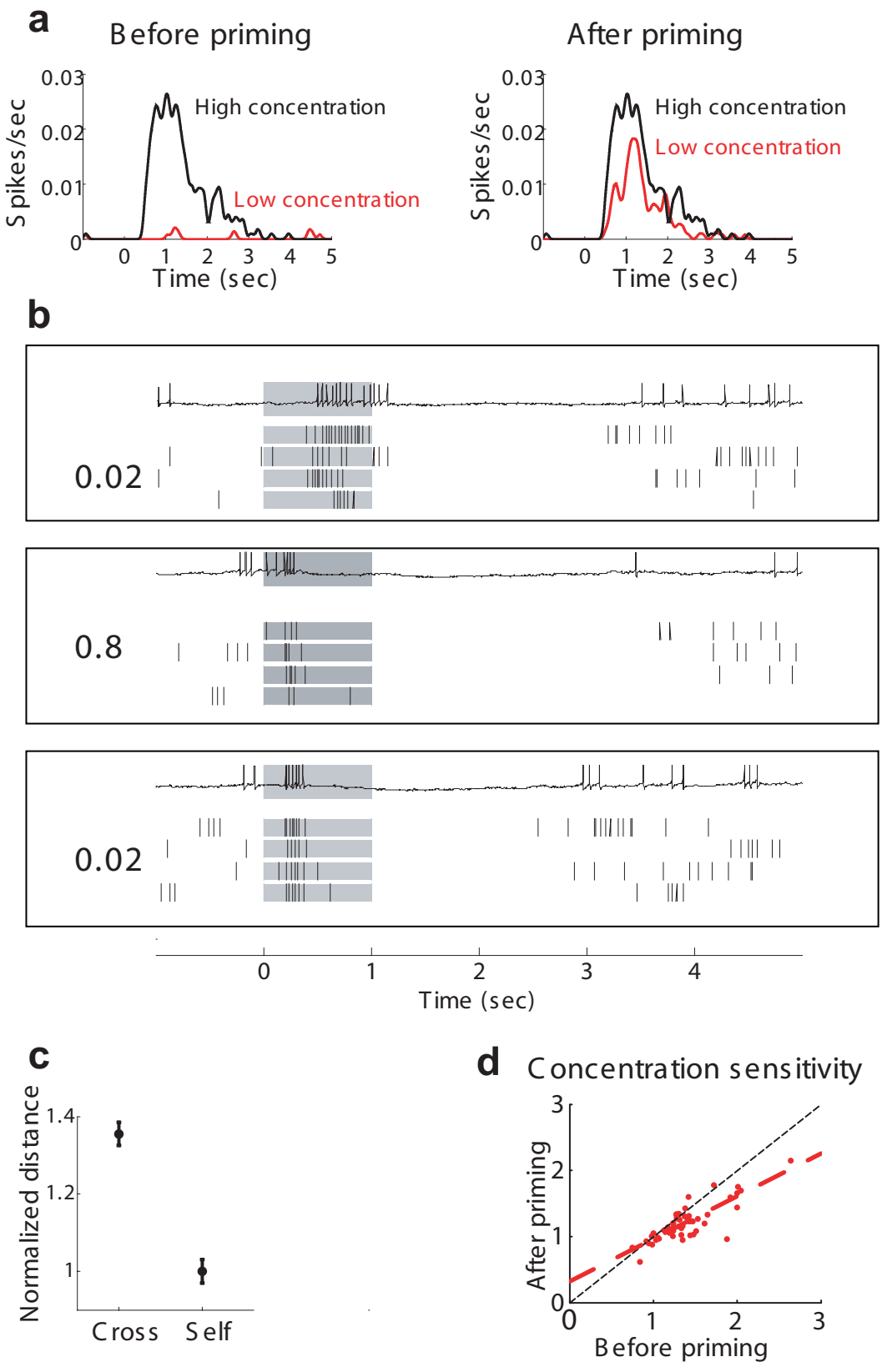


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Figure 9.2. **Priming makes a PN's response more invariant to concentration**. **a,** A PN's firing rate at low and high concentration before and after priming. **b,** Priming makes the response pattern of a PN to lower concentrations more similar to that at higher concentrations ($p<10^{-4}$, Distance test). **c,** Response patterning is affected by priming even when firing rate information is eliminated by normalization ($p<<10^{-6}$, Distance test, n=51 PN-odor pairs). **d,** Scatter plot of the concentration sensitivity of each PN-odor pair before (x-axis) and after (y-axis) exposure to high concentration. All 51 datasets are shown, regardless of whether they were affected by priming or not. Concentration sensitivity is defined by the mean ratio of across-group/within-group distances over all trials (see Methods). The black diagonal line denotes identity (y=x). Note that the population of PN-odor pairs is shifted toward the right of the diagonal, indicating a shift toward greater concentration invariance (concentration sensitivity was significantly reduced by exposure to high concentration, $p<0.005$, Wilcoxon ranksum test, n=51). The red dashed line represents the best linear fit to the data. Its intercept with y=x at x=0.97 indicates that the concentration sensitivity of PN-odor pairs that are initially not concentration-sensitive is not affected by priming. Its slope (0.32) indicates that the most concentration-sensitive PN-odor pairs are affected the most by priming. See also Figs. 9.1 and 9.3.

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centration in the naïve state (i.e., which did not respond to the odor at any concentration when first exposed or whose responses to the two concentrations were not significantly different) were affected by priming (see also Fig. 9.2d). We then tested explicitly whether priming could contribute to identification of odor identity robustly across concentrations. We classified the response of each trial as indicating the odor whose responses across all concentrations tested were on average most similar to those of the trial being classified[25], using the cost-based metric[24] to assay similarity. We found that priming significantly improves classification among odors presented at multiple concentrations ($p<0.05$, n=28 cell-odor pairs that underwent significant priming, as defined by exhibiting primed responses significantly different from naïve responses).

To quantify whether the temporal patterns in PN responses were affected above and beyond the effect on mean firing rates, we calculated a peri-stimulus time histogram (PSTH) for every trial (using a range of s.d. values from 25 to 250 msec), normalized so that the mean firing rates of all trials were identical, and then assayed priming as described above, using the sum squared difference between PSTHs as the distance metric. Exposure to high concentration significantly changed the temporal response patterns evoked by low concentrations ($p<<10^{-6}$, Distance test, n=51 PN-odor pairs, Fig. 9.2c).

To assay whether priming was due to the intervening block of high concentration trials or to the passage of time alone, we carried out experiments presenting three successive blocks of trials of the same odor at the same low concentration (low→low→low) and compared the responses obtained in the third block of trials to those obtained, with the same neuron, after exposure to a high concen-

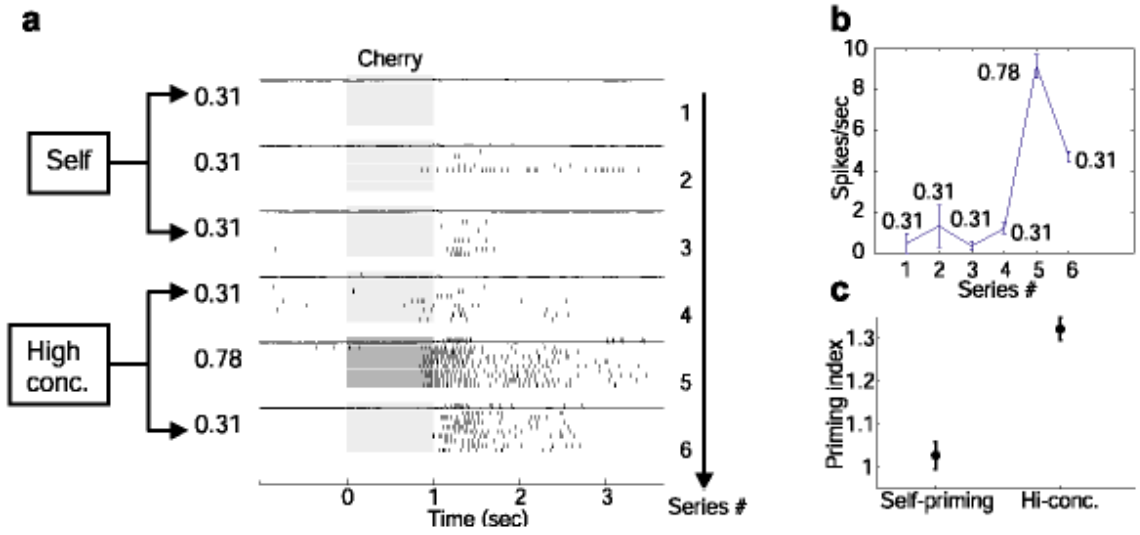

Figure 9.3. **Repeated presentation of an odor at the lower concentration is not sufficient to elicit the priming caused by exposure to a higher-concentration primer. a,** 40 trials of cherry at a concentration of 31% of saturation were presented (left panel), followed by 10 trials of a concentration of 78% of saturation, followed by a final series at 31%. **b,** Mean firing rate during the response period (computed over 0.5-3.5 s interval) for each series on the left panel. **c,** The change in response to the lower concentration, measured by a priming index equal to the ratio of mean across-series distance over mean within-series distance, is significantly larger after exposure to a higher, suprathreshold concentration (right bar) than it is after an equivalent time period with exposure to the lower concentration (left bar) ($p<<10^{-6}$, Distance test, n=6 cell-odor pairs).

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tration (low→high→low) (Fig. 9.3). Neither time nor repeated presentation of the odor at low concentration was sufficient to elicit the changes induced by exposure to high concentration ($p<10^{-5}$, binomial test, n=6, see Methods). Even after equating the total quantity of odorant to which the animal was exposed in each condition by prolonging the number of exposures to low concentration (low-low-low-low-high-low), exposure to high concentration was more effective in inducing priming than a more prolonged exposure to a lower concentration ($p<10^{-4}$, Distance test). Exposure to a higher concentration thus appears to be needed for rapid priming. Conversely, experiments were carried out where the sequence of concentrations presented in the three successive trial blocks was reversed (high-low-high). Priming by the low concentration was never observed under these circum-

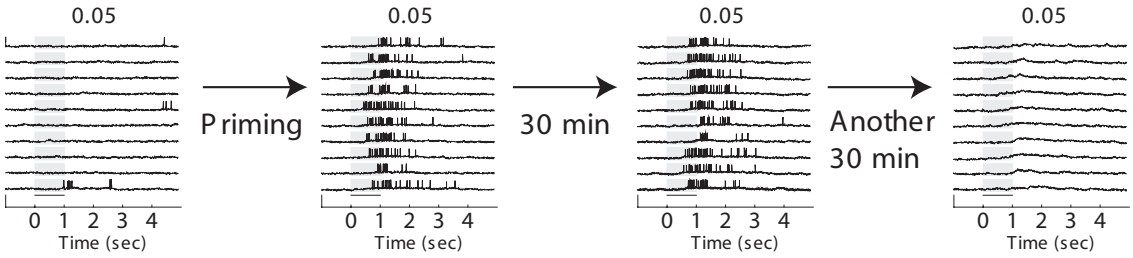

Figure 9.4. **Priming persists for half an hour, but is for the most part reversed 1 h after exposure to high concentration.** A PN's response to the odor geraniol at 0.05 concentration before, immediately after, 30 and 60 minutes after exposure to a higher concentration (0.31). After the neuron stopped firing in response to geraniol at 0.05, the higher concentration continued to evoke suprathreshold responses (data not shown).

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stances ($p>0.38$, Distance test, n=20 concentration-sensitive cell-odor pairs). Priming is thus induced on low concentration responses by exposure to higher concentrations and not the reverse.

We next investigated the duration of this memory. An animal was challenged with a set of low concentration trials, primed with higher concentrations and then stimulated with another set of low concentration trials after variable delays. The effect of priming on PNs was present 15 to 30 minutes after exposure to the primer ($p<<10^{-6}$, Distance test, n=5 cell-odor pairs) but was generally gone an hour later (priming absent 52±32 min after exposure to high concentration, $p>0.3$, Distance test, n=6 cell-odor pairs, all originally significantly primed) (Fig. 9.4). Responses to the higher concentration were present throughout the recording session, ensuring that the loss of the priming effect was not due to deteriorating recording quality. Moreover, priming was not disrupted by intervening exposure to a different odor ($p<<10^{-6}$, Distance test, n=7 cell-odor pairs).

In summary, exposure to a high concentration was found to prime responses to lower concentrations, including previously ineffective ones, in an odor-specific manner. Priming could enhance both excitatory and inhibitory responses or response phases and affected the timing and patterning of responses, making odor responses more invariant to concentration. The effects lasted over 15 minutes but disappeared within approximately 1 hour.

Our results have several implications. Olfactory sampling is serial and intermittent, due both to the turbulent nature of odor plumes[33] and to periodic sampling given by sniffing[28,29] in vertebrates or by

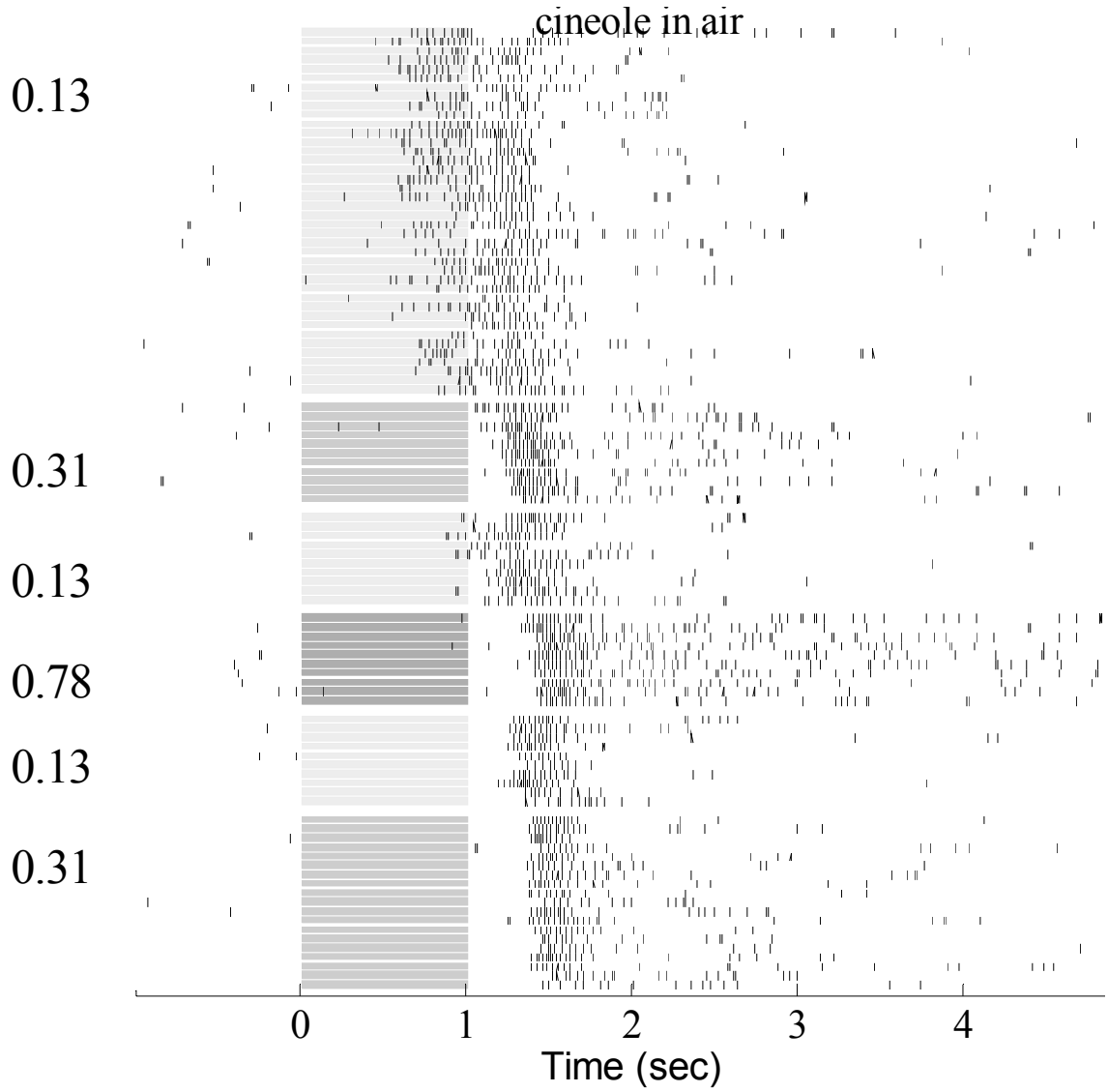

Figure 9.5. **Odor pulses of cineole at varying concentrations, shown in order of presentation from top to bottom.** Note the relative similarity of responses to different concentrations after priming compared to naive responses.

antennal movements in arthropods[30]. Clearly, taking the results of previous samples into account can be advantageous. In natural odor plumes, filaments of higher odor concentration alternate with ones of lower concentration[26,27]. By lowering response thresholds to an odor detected with certainty in the recent past, priming might provide a way to 'tune in' or bias responses towards correct identification of an odor likely to be sampled in the near future.

Prolonged exposure to an odor has been previously shown to enhance sensitivity and discrimination both at the behavioral[31-37] and peripheral neural[38-40] levels. Such a sensitization, however, occurred over days to weeks rather than seconds to minutes as in our experiments. Exposure for 20 continuous min/day for a week, on the contrary, has been seen to decrease responsiveness of mitral/tufted cells in the rat olfactory bulb non-specifically[41,42]. The difference between these results and ours, using brief pulsed stimuli, suggests that intermittent odor stimuli, like those encountered in natural odor plumes[26,27], may be differentially recognized by early olfactory circuits. It also suggests that olfactory circuits likely change over many different time scales, each adapted to input statistics.

Our results suggest that the response of early sensory circuits to a stimulus can be influenced for relatively long periods by the recent history of stimulation, and that exposure to a stimulus can change subsequent neuronal responses not only to the same stimulus[43], but also to weaker stimuli with the same odor identity. Note that the short-term plasticity previously described by Stopfer and Laurent[43] in the same neurons is quite different from that described here: the former affects the synchronization of PNs over repeated trials at one concentration, while priming reduces the variance of response patterns across concentrations. The timescales of the two phenomena are also different: the effects on synchronization last less than 12 minutes, while priming persists for at least 15-30 min. Whether the changes we describe here are particular to the olfactory system or are more general remains to be seen (but see ref. [44]); because priming has been observed behaviorally both with vision and hearing[45,46], similar cellular effects might be found in those systems as well.

Our results also suggest a solution to the tradeoff between representing odor concentrations and identity. Over all PNs, sensitivity to odor concentration is greater in naïve PNs than after repeated exposure. After exposure to high concentrations, the system may switch to a mode at once more sensitive (response threshold is lowered) and more invariant to concentration. Because priming was seen to affect only 57% of tested PN-odor pairs, it is also possible that different subcircuits within the antennal lobe subserve different roles.

The mechanisms behind priming are, as of now, unknown. Our results appear to contradict what one would predict from knowledge of the adaptation of olfactory receptor neurons[47-50], mitral cells[51]

and olfactory perception[52]. An explanation is that adaptation is a more rapid phenomenon (both on and off)[50]. Adaptation and priming may coexist. Indeed, 3 PN-odor pairs exhibited adaptation in our experiments. These PNs were the same that exhibited priming, both for other odors and for the odor that caused adaptation. Adaptation lasted only seconds to a few minutes, and affected concentrations lower than those that were affected by priming.

Our results lead to testable behavioral predictions. If the behavior of projection neurons is echoed by downstream neurons involved in recognition by the animal, one would expect that exposure to an odor at high concentration would rapidly, transiently and specifically lower the animal's detection threshold for that odor, enhance recognition of that odor in a noisy environment, and do so in a transient manner. Such behavioral improvement of detection in the face of noise after exposure to a stronger version of the stimulus is well known in visual psychophysics[53], but to our knowledge remains to be tested in olfaction.

Methods

Specimens, odor stimulation and electrophysiology

Intracellular recordings were obtained from 46 PNs (150 PN-odor pairs) of 21 locusts, *Schistocerca americana*. Surgery and recordings were performed as previously described[54,55]. Delivery of eight odors, including pure compounds as well as ethologically relevant blends, was performed using a gaseous dilution computerized odor-delivery system. We could deliver arbitrary concentrations by mixing a stream carrying saturated odor vapor with a second stream carrying pure air. The concentration of the odor delivered was regulated by controlling the relative flow rates of both streams[56]. The system was purged between presentation of different stimuli.

The odor timecourse and magnitude was measured by using CO_2 as a tracer in the air line carrying the odor while the diluting stream carried ambient air, and measuring the CO_2 concentration at the nozzle[56]. Direct measurement of the odorant concentration with GCMS also showed that concentration returned to baseline within 1 minute of purging.

Analysis of sensitivity to concentration

Each cell-odor pair was challenged with multiple trials (n_5; n_10 for most datasets) at each of at least two concentrations between 2 and 100% of saturated vapor pressure. The firing rates for different concentrations (Fig. 1a) were compared using a paired T-test comparing maximum (over

time) mean (over trials) firing rates for the highest and lowest concentration tested for each cell-odor pair during 4 sec following odor onset, for the 62 cell-odor pairs with purely excitatory responses. A response was defined as excitatory if one or more epochs (300 or 500 msec, shifted in 100 msec steps, to account for short responses with high temporal precision as well as longer responses with more variance in spike times) exhibited a firing rate increase to at least 3 standard deviations above the mean baseline rate.

The low baseline firing rates of PNs (2-6 Hz) prevented an accurate evaluation of inhibition using firing frequency alone. Instead, the intracellular voltage traces for successive trials of the same concentrations were aligned on the mean voltage during the second preceding odor delivery for each trial. An odor was said to elicit an inhibitory response at a given concentration if there was any period (evaluated with sliding windows of 500 msec shifted in 100 msec steps) during which firing was suppressed and the mean voltage was at least 3 standard deviations below the mean voltage during the period preceding odor delivery. 35% of datasets showed some period of inhibition and individual responses often contained a period of excitation and a period of inhibition. The depth and duration of inhibition were quantified for each concentration. Depth was the mean voltage during all inhibited epochs. Duration was defined as the total duration of all epochs during which inhibition was detected.

Distance test

To further quantify the degree to which each PN was sensitive to the concentration of a novel odor (Fig. 1e), we calculated, for each cell-odor pair, the mean distance between each trial and all other trials for the same concentration (*within-group or self-distance)* and that between each trial and all trials of a different concentration (*across-groups or cross-distance*). The responses to two concentrations were said to be significantly different if the mean across-groups distances, measuring the effect of concentration, were significantly greater than the mean within-group distances, measuring the variability of responses within trials with one concentration. Significance was evaluated with a Wilcoxon ranksum nonparametric test. Distances were calculated using a cost-based metric[24], which measures the difference between two spike trains taking both the number and timing of spikes into account. This distance is defined as the cost paid to transform one spike train into the other using three elementary steps: insertion; deletion of a spike (each at a cost of 1); and displacement of a spike by 1 ms (cost of 2/*T* for each displacement). T thus dictates how far two spikes can be for them to be considered 'similar' in timing. We used a range for *T* between 10 and Infinity, with qualitatively similar results for T>=25 ms (data shown for T=250 throughout the text)[31]. For inhibi-

tory responses, traces were low-pass filtered with a cutoff of 20 Hz, aligned on their mean voltage during the period preceding stimulation, and the distance between two traces taken to be the mean square difference between the traces. To discount any nonspecific change in a neuron's firing rate, two conditions were considered significantly different only if their cross/self distance ratios were significantly greater for the odor response than for the baseline period preceding odor stimulation. For brevity, we call these tests the Distance test throughout the text. Charts show the mean self- and cross-distances for the groups compared and the standard error of the means.

We applied the same test to quantify the extent to which priming had modified responses to lower concentrations (Fig. 2a,c,d and 4c), and the degree to which priming diminished the concentration sensitivity of PNs (Fig. 3c). In this case, the two conditions compared were responses before priming vs. after priming, rather than responses to different concentrations.

Effect of time vs. concentration

All 6 cell-odor pairs showed more change after a high concentration primer than after an equal period with repeated exposures to the same odor at low concentration. For 5 of the 6, the mean across/within distance ratio was significantly larger for the experimental condition with an intervening high concentration than for the control condition without one ($p<10^{-5}$, binomial test). Additionally, a paired T-test on the mean cross/self distance ratios for each dataset showed those ratios to be significantly higher for the experimental condition than for the control one ($p<0.005$, paired T-test).

Decrease in concentration sensitivity

Concentration sensitivity is defined as

$$S = \frac{< \text{across - series distance} >}{< \text{within - series distance} >}$$

where the across-series distance is computed between naïve low and primer high before priming, and between primed low and primer high after priming, and the within-series distances are averaged across the two corresponding series.

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CHAPTER 10

Conclusions, Discussion and Directions

Introduction

I have included the conclusions and discussion relevant to each chapter in the chapters themselves. The functional context for the work described in this thesis has been described in Chapter 1. I will not reiterate either of those here. I will use this space simply to link together some of the findings of the thesis, and to discuss important questions that remain to be answered in the future.

Gain control and invariance in the olfactory system

One thread running throughout this thesis is the olfactory system's tendency to offset changes in the environment irrelevant to its behavioral output. This homeostasis, which parallels Le Chatelier's principle in chemistry, is implemented by gain control mechanisms throughout the system. In Chapter 1, I suggested, based on Zhao and Reed's recent results (2001), that developmental mechanisms may function normally even in the face of severely reduced odorant-induced activity, as long as the competitive balance between neurons is not offset. Thus, development of the ORN-glomerular projection pattern is invariant to absolute levels of odorant-mediated activity. In a beautiful demonstration of the importance of competition, and thus relative levels as opposed to absolute ones, all the way from behavior to neurophysiology, Zhao and Reed's OCNC1-negative mutant mice, whose mutant neurons die out in a competitive environment but thrive in a noncompetitive one, do not survive themselves in a competitive litter environment but go on to reach adulthood normally if their littermates are removed, thereby removing competition for the mother's milk (Zheng et al., 2000). In Chapter 2, I showed that chemoreception is invariant to odorants' volatility, a phenomenon which greatly reduces the dynamic range of concentrations faced by the system. In Chapter 8, I showed that excitation and inhibition in the antennal lobe increase in parallel in response to increasing odorant concentrations, offsetting the effects of concentration on receptor binding. In Chapter 9, I showed that exposure to high concentrations of an odorant trigger mechanisms of sensitization that,

once again, offset the effects of concentration on the response of naive projection neurons, making their responses more invariant to concentration differences. Finally, in Chapter 7, I suggest that LHI's inhibition of Kenyon cells may act as a further mechanism of gain control, showing increased and more synchronized inhibition at higher concentrations.

Functional analysis of coding in the locust antennal lobe

I have addressed the functional roles of three salient features of the representation of odors in the antennal lobe: its dynamic nature, its oscillatory synchronization, and its plasticity. The dynamic nature of the representation was shown in Chapter 4 to improve odor discrimination compared to a mean firing rate neural code. Oscillatory synchronization of PNs was shown in Chapter 5 to be important for the decoding of odor identity information by downstream neurons. Short-term plasticity was shown in Chapter 9 to contribute to the invariance of PN responses to concentration.

Kenyon cells' obliviousness to unsynchronized spikes does not diminish information on odor identity or concentration

The combined findings that PN phase with respect to the local field potential does not code for concentration and that synchronized PN spikes convey as much information about odor identity as all spikes combined suggest that Kenyon cells sensitive only to synchronized spikes do not miss concentration or odor identity information.

Biological decoding shows constraints not related to optimal stimulus reconstruction

The demonstration in Chapter 5 that synchronization is required for the correct decoding of odor information in PN assemblies despite the fact that the disruption of synchronization does not appear to disrupt odor information in the PN assemblies themselves, and despite the fact that synchronous spikes are not more informative than unsynchronized spikes even in synchronizing assemblies of PNs, suggests that neural decoding is subject to constraints unrelated to achieving optimal stimulus reconstruction.

Short-term plasticity truly short-term?

It is worthwhile noting that while short-term plasticity in locust antennal lobe PNs wears off after ten minutes (Stopfer and Laurent, 1999), it may well be that its effects last longer downstream in the olfactory pathway. Indeed, Vanderwolf and Zibrowski (2001) recently reported a similar increase in the power in the 20 Hz range over the first 5-10 trials of exposure to a novel odor in the rat pyriform cortex, whose duration extended for at least five days.

Alternatively, given that Stopfer and Laurent measured the effect ten minutes after initial presentation on the same day and did not record from the same animal on the following day, and Vanderwolf and Zibrowski, in contrast, did not record on the same day after the initial 15 trials but instead recorded on following days, it is possible that the effects wear off initially to reappear later in a more robust, long-lasting state. This would not be without precedent for the acquisition of a memory, and has been demonstrated both in Aplysia (Brandes et al., 1988; Wright et al., 1996; Marcus and Carew, 1998; Muller and Carew, 1998; Sutton and Carew, 2000; Carew, 2000; Carew and Sutton, 2001) and in the honeybee (Menzel, 1987; Menzel, 1999; Menzel et al., 2000).

Finally, it is possible, of course, that the durations of the effect are completely different in rodents than in insects.

A similar note can be made for the duration of priming. Only truly long-term testing can establish whether the effects of priming do not reappear after wearing off initially.

Three notes of caution: Methodological relevance to electrophysiology

A final common theme that emerges throughout this thesis is a note of caution for electrophysiologists, reminding us that simplistic approaches will not work for understanding neural coding. Firstly, the demonstration that temporal coding in assemblies of neurons carries information not detected with a firing rate code adds to previous evidence that stimulus information is encoded in the response dynamics of single neurons (see, for example, Bialek et al., 1991; Wehr and Laurent, 1996). Yet much of the neurophysiological literature continues to be analyzed and published in terms of mean firing rates over relatively long periods. Many a paper shows no glimpse of raw spike trains at all, condensing the richness of neural dynamics into a single number for the mean firing rate. The present findings should serve to encourage authors, reviewers and editors alike to insist that readers be presented with some of the raw data before assumptions are made on what elements of them are important and which are not.

Second, the demonstration that synchronization across neuronal assemblies is essential for the decoding of information present in neurons calls for simultaneous recordings of multiple neurons and/or the local field potential: single cell recordings miss out on important information available to the animal.

Lastly, the discovery of priming in neuronal responses affecting different concentrations of the odor

presented suggests that there is hysteresis in neuronal responses even in the absence of conditioning. This dependence on the history of sensory stimulation opens the field for a whole new set of experimental investigations, and should encourage neurophysiologists to be attentive to the sequences in which stimuli are presented and reviewers to ensure that such information is presented in publications. In particular, it suggests it is not reasonable to assume stationarity in neuronal responses, and calls for a reexamination of receptive fields after an animal has been exposed to a barrage of suprathreshold stimuli.

Synopsis

Finally, this thesis suggests a revision of some views on the olfactory system. First, Chapter 2 throws into question so-called corrections for volatility for the concentrations of odorant solutions, suggesting the olfactory system may be invariant to volatility. Second, the abrupt cycle-to-cycle changes in PN odor responses shown by Wehr and Laurent (1996) have been shown to be the exception rather than the rule, with PN responses highly correlated from cycle to cycle for the majority of PNs (Chapter 4). Third, Chapter 7 provides evidence against three previous hypotheses for the encoding of concentration in the antennal lobe. Fourth, Chapter 7 shows that the phase relationship between PNs and the local field potential (LFP) may not be as constant across PNs and/or LFP positions as previously believed. Fifth, Chapter 7 shows that some PNs are synchronized to the LFP even in the absence of odor stimulation. Sixth, Chapter 8 suggests that the large increase in antennal lobe activation in response to increasing odorant concentrations seen by other workers with optical imaging techniques may be the result of an artifact of half-wave rectification of inhibitory activity, since excitation and inhibition both increase in parallel in response to concentration and tend to roughly cancel each other out, acting as a gain control mechanism which keeps the output level of the antennal lobe significantly more constant across concentrations than one would be led to

believe if the role of inhibition is ignored. Finally, the reinterpretation of the literature in Chapter 1 suggests that activity dependent competition may play a larger role in shaping the map between olfactory receptor neurons and the olfactory bulb than has previously been believed, and that Zhang and Sejnowski's (1999) conclusions regarding stimulus dimensionality and tuning curve widths may need to be reevaluated for finite assemblies of neurons.

On a less discordant note, Chapter 3 puts on a behavioral footing the physiological investigations into the locust's ability to identify odors. Chapter 4 provides a first estimate of the odor identity information provided by each additional projection neuron in the antennal lobe, and of the total bandwidth of the antennal lobe, and shows that there is significant information encoded in the neuronal identity of each spike. Chapter 5 provides, in work done jointly with Kate MacLeod, a physiological basis for the behavioral impairment in fine odor discrimination seen in insects after the disruption of synchronization in the antennal lobe. Chapter 6 describes a new odor delivery system whose applications this thesis has only begun to exploit. Finally, Chapter 9 describes the effect of a novel type of plasticity discovered in PN responses and discusses its contribution to invariance to concentration in PN responses.

Open Questions

Further work is clearly needed in realistic decoding schemes for neuronal assemblies —a simulation of virtual Kenyon cells receiving input from real PN spike trains acquired experimentally is on the works but outside the scope of this thesis.

Regarding priming, questions that remain for the future include the dependence of priming on the number of primer exposures, its dependence on primer concentration, the full scope of the concentrations affected, whether priming by pure odors affects mixtures containing the primer and vice

versa, as well as the mechanisms responsible for it and their site of action. Furthermore, behavioral work lies ahead to test the predictions made in Chapter 9.

One of the most promising lines of future research is the combination of molecular biology tools, which allow the identification of neurons with particular genetic profiles, with physiological investigations. I cannot help but thinking that current electrophysiology, in its exploration of neurons whose identity the researcher ignores, pooling together results from neurons that no doubt have different genetic, anatomic and functional profiles (see Reich et al., 2001, and Chapters 4 and 7 of this thesis), makes the current age still the prehistory of neurobiology. Even when results are not pooled across neurons, the inability to repeatedly record from the same neuron and the lack of information about its genetic, and sometimes its anatomical, profiles, severely hampers advance. An important exception in this regard is the investigation of identifiable neurons, of which several have been found in insect brains —yet another encouraging reason for the prospect of understanding insect brains before we achieve an understanding of the much larger mammalian brain.

An important line of research that remains unexplored in olfaction concerns noisy natural environments. Olfactory researchers customarily deliver single isolated stimuli in a clean environment, but animals must routinely recognize odors in the presence of multiple contaminants. An elegant model by Hopfield (Hopfield, 1991; Hendin et al., 1994) for decomposition of a mixture of signals using temporal variations in their relative intensities remains untested.

Finally, I would like to point out that none of the *hard* problems in olfaction —natural behaviors in natural environments— have yet been successfully addressed. Not only have we not figured out yet how the brain recognizes odors or *why* synchronization is used for decoding by brain circuits — problems toward which we are at least on our way. We scarcely have any idea at all regarding what determines the valence of odors: why some things smell good and others stink. And common olfactory behaviors, such as how a dog is able to, with just a few sniffs (Steen and Wilsson, 1990; Thesen et al., 1993), identify the direction of a track laid many minutes earlier, have barely begun to be

addressed. The task has only just begun.

Most likely, nobody every will, but if by some chance of destiny you were to read through here, your efforts should not go unrewarded: In a tribute to my advisor's techniques, the first person to write to me requesting the prize will get twenty dollars, or enough to buy a Big Mac --enduring symbol of our times--, whichever is bigger at the time.

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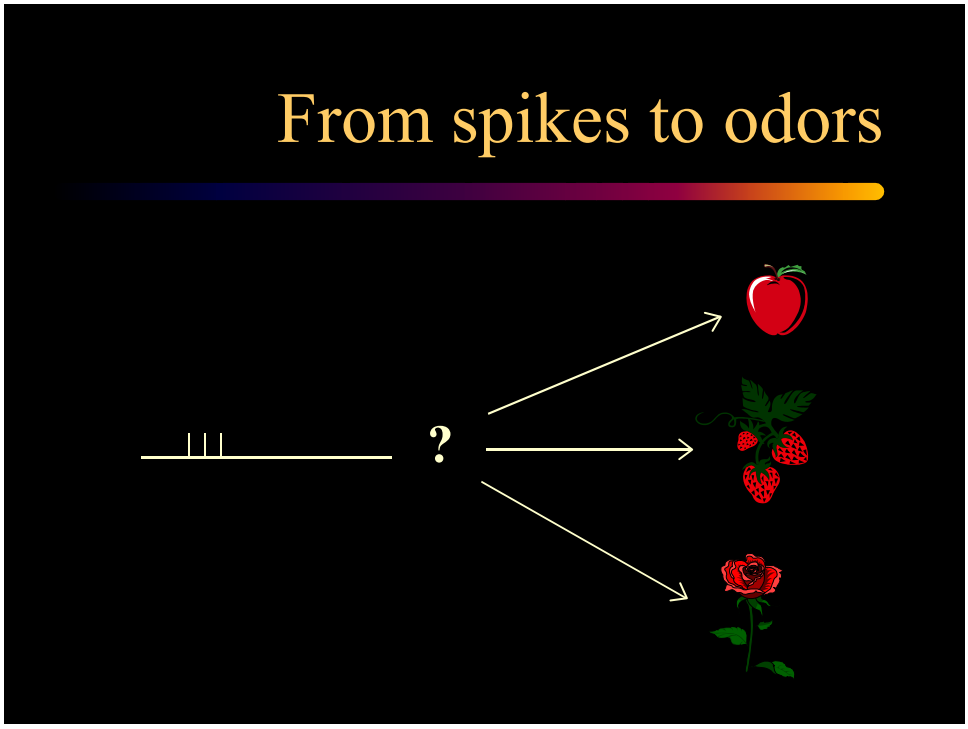
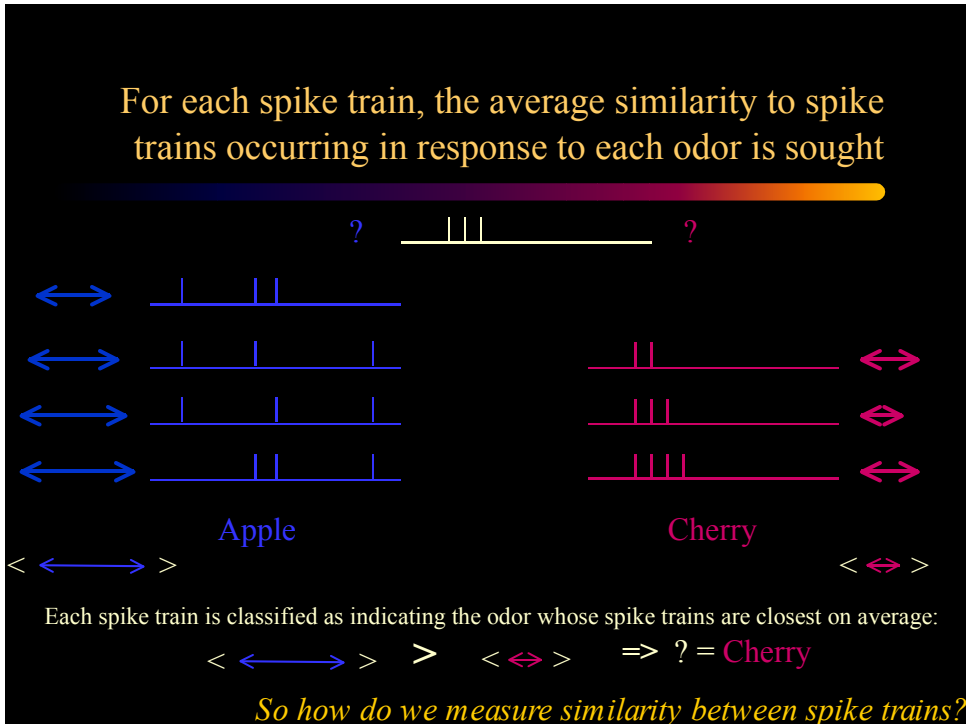
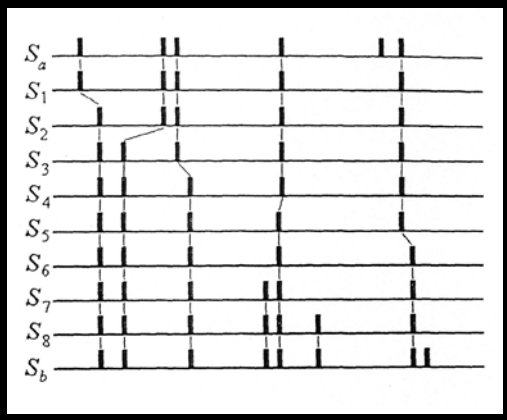
APPENDIX I

A presentation on a cost-based metric to compare spike trains (Victor and Purpura, 1997)


From spikes to odors
?


For each spike train, the average similarity to spike trains occurring in response to each odor is sought
? ?
Apple
Cherry
< >
< >
Each spike train is classified as indicating the odor whose spike trains are closest on average:
< > > < > => ? = Cherry
So how do we measure similarity between spike trains?

A conversion-based metric to measure similarity between spike trains



Elementary step:

} Spike deletion

} Spike shift

} Spike insertion

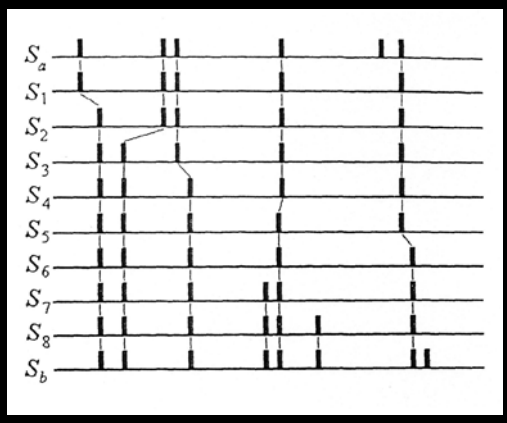
Victor & Purpura (1997) based on Sellers (1974)

What is the relative cost of each elementary step in the cost-based metric?

Due to symmetry property of metrics:
Cost of insertion = cost of deletion $\equiv 1$

q $\equiv$ cost of shifting a spike 1 bin

T $\equiv$ 2/q
= time difference in bins between two spikes such that it is more cost-effective to delete one and add the other than shifting one to the other
= time range at which two spikes share no timing information, i.e. when there is no more information about the two than the number of spikes



So how do you calculate the minimum-cost distance?

The minimality requirement imposes constraints

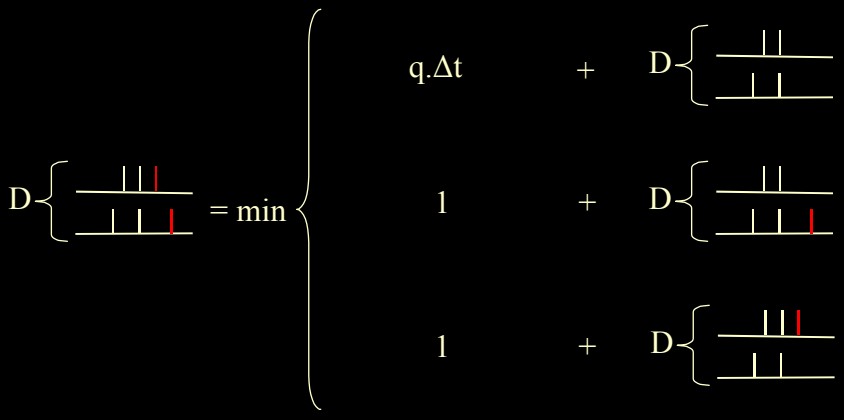
- Each spike will be subject to ***only one*** of three operations: insertion, deletion, or a single unidirectional shift.
- Lines representing spike shifts will not cross each other (or you'd rather switch which spike ends up where --all spikes are born equal).

A matching problem

- The problem is analogous to that of finding which pairs of spikes should be connected by a shift in order to achieve a minimal cost path.

How do we determine whether a spike is to be shifted or deleted?

Induction allows a recursive solution

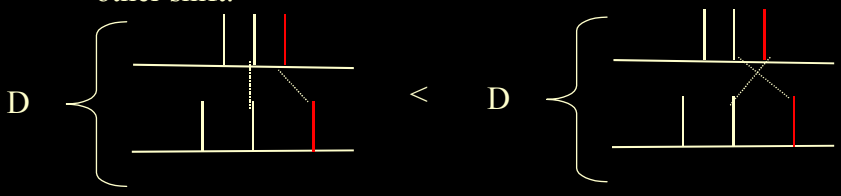


Recursiveness breaks down global optimality problem into local decisions.

How do we know those 3 alternatives are the only possibilities?

i.e. What ensures that a shift cannot connect the last spike of a spike train with a spike before the last in the other spike train?

If the last spike of A were connected by a shift to a spike in B before the last one, the last spike in A must suffer a deletion, or else its shift path would cross the path of the other shift.



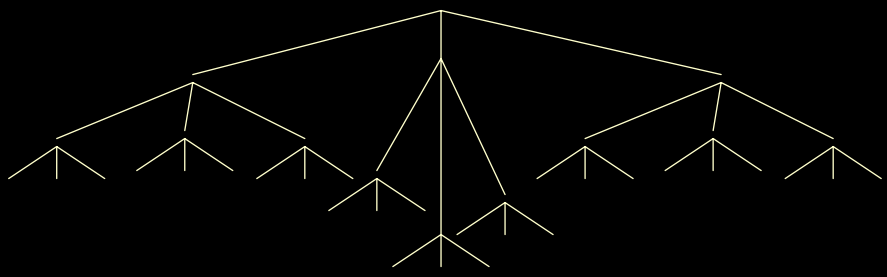
Writing the induction with equations

The cost of converting the spike train consisting of the first i spikes in A into that consisting of the first j spikes in B can be calculated recursively as:

$$C(i,j) = \min\{C(i-1,j)+1,\ C(i,j-1)+1, C(i-1,j-1)+M(e_i,f_j)\}$$

where $M(e_i,f_j)$ is the cost of moving spike e_i to f_j , namely $q.|\ e_i-f_j\ |$,
$C(0,0)= 0$,
$C(0,i)=i$ and
$C(i,0)=i$.

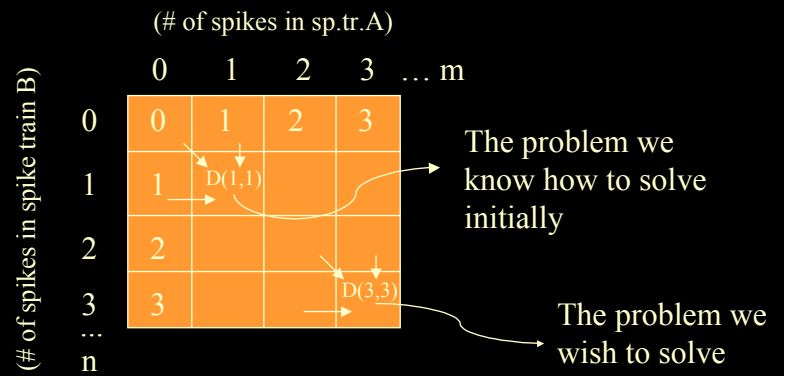
Recursive solution would be order 3^{n+m}



But many of the nodes found would actually be identical to each other.
How can we take advantage of that to make the computation faster?

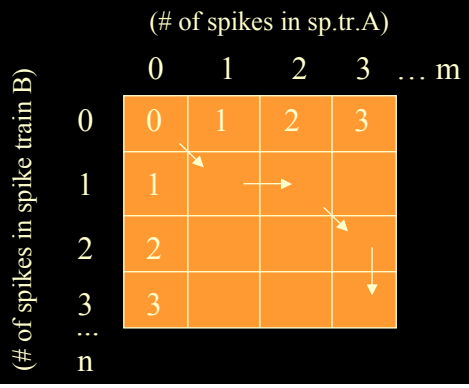
Dynamic programming

How many different nodes are there, and what are they?
They are all the pairs of spike trains consisting of the first i spikes in A and the first j spikes in B with i=1…m and j=1…n. Exactly m x n nodes.



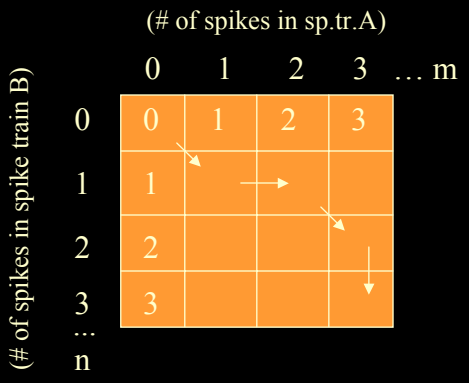
Obtaining the fate of each spike

It is of course possible to ascertain whether each spike suffered a shift, an insertion or a deletion in the minimum-cost transformation connecting both spike trains.



From the distance to the transformation

1. The/A minimal cost path traversing the matrix must be found by finding which of the 3 squares above and to the left of each square, added to the cost of the corresponding transition (insertion/deletion/shift), led to the value of the square being considered.



2. A diagonal arrow in the matrix path pointing at square (i,j) indicates that the i^{th} spike of spike train A and the j^{th} spike of spike train B are connected by a shift.

3. Every spike in A (B) that is not connected with another by a shift must be deleted in order to transform A (B) into B (A).

From individual transformations to an evolutionary path?

- With the fates of each individual spike known in the transformation, it is possible to come up with a series of possible evolutionary paths linking spike train A to spike train B, each differing from the rest in the order of the individual steps, which cannot be recovered from A and B alone.

NB: For a *branching* evolutionary process such as molecular evolution, the most parsimonious evolutionary tree *can* actually be recovered given *multiple* end sequences derived from a common ancestor.

APPENDIX II

Attractors in the representations of odors in the antennal lobe have sharp boundaries

Sufficiently similar inputs to the olfactory system lead to equal behavioral responses, and thus presumably to equal neural responses at some level. This means that the time-varying responses to odors must act as dynamic attractors in neural dynamics space. An open question in olfactory research is what the shape of the basins of attraction that lead to the various dynamic attractors coding for odors in the antennal lobe is. A preliminary investigation of intracellular recordings of PNs during presentation of odors at varying concentrations as well as binary mixtures in various proportions revealed sharp transitions in odor responses with minute changes in odor composition and concentration. These changes were equally abrupt in spike rasters and intracellular voltage traces, suggesting the non-linearities were not the result of PN firing thresholds. These results suggest that the basin of attraction for the representation of an odor in the antennal lobe has sharp boundaries in composition and concentration space.

To test the extent to which projection neurons respond to mixtures of components to which they are responsive, and more importantly, the shape of the transition in response patterns as a mixture is morphed from one odor to another, I recorded intracellularly from individual projection neurons in the antennal lobe while delivering various ratios of binary mixtures. What I found was quite surprising. Rather than exhibiting gradual transformations between one odor's representation and another's as their relative concentrations was varied systematically, PN response patterns often displayed abrupt transitions in concentration space (Fig. II.1-II.2) and blend composition space (Fig. II.3-II.4). The abruptness of the changes in response patterns as concentration was changed was present both in extracellular (Fig. II.1) and intracellular traces (Fig. II.2), indicating that the nonlinearity of response patterns is not due to the firing threshold.

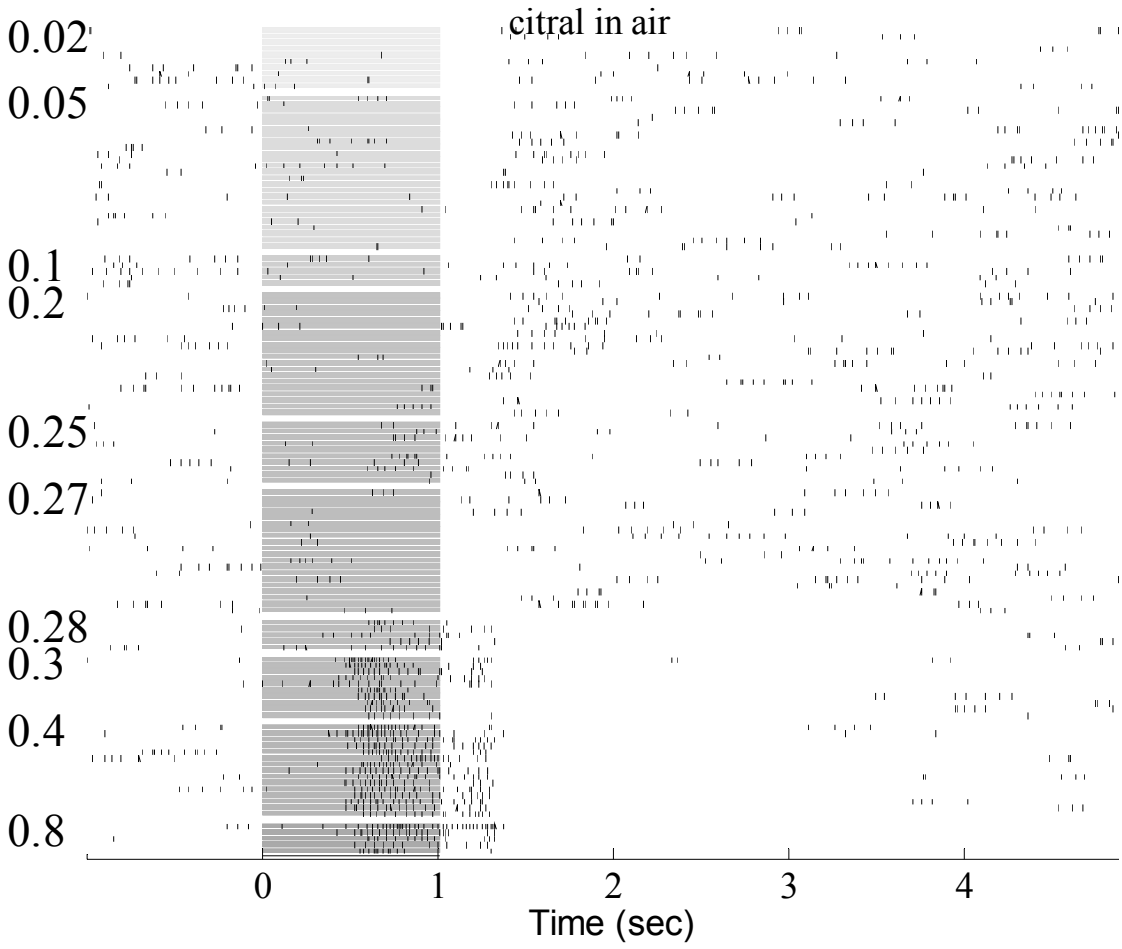


Figure II.1. A PN's response to citral has an abrupt response threshold between 27 and 28% of saturated vapor pressure.

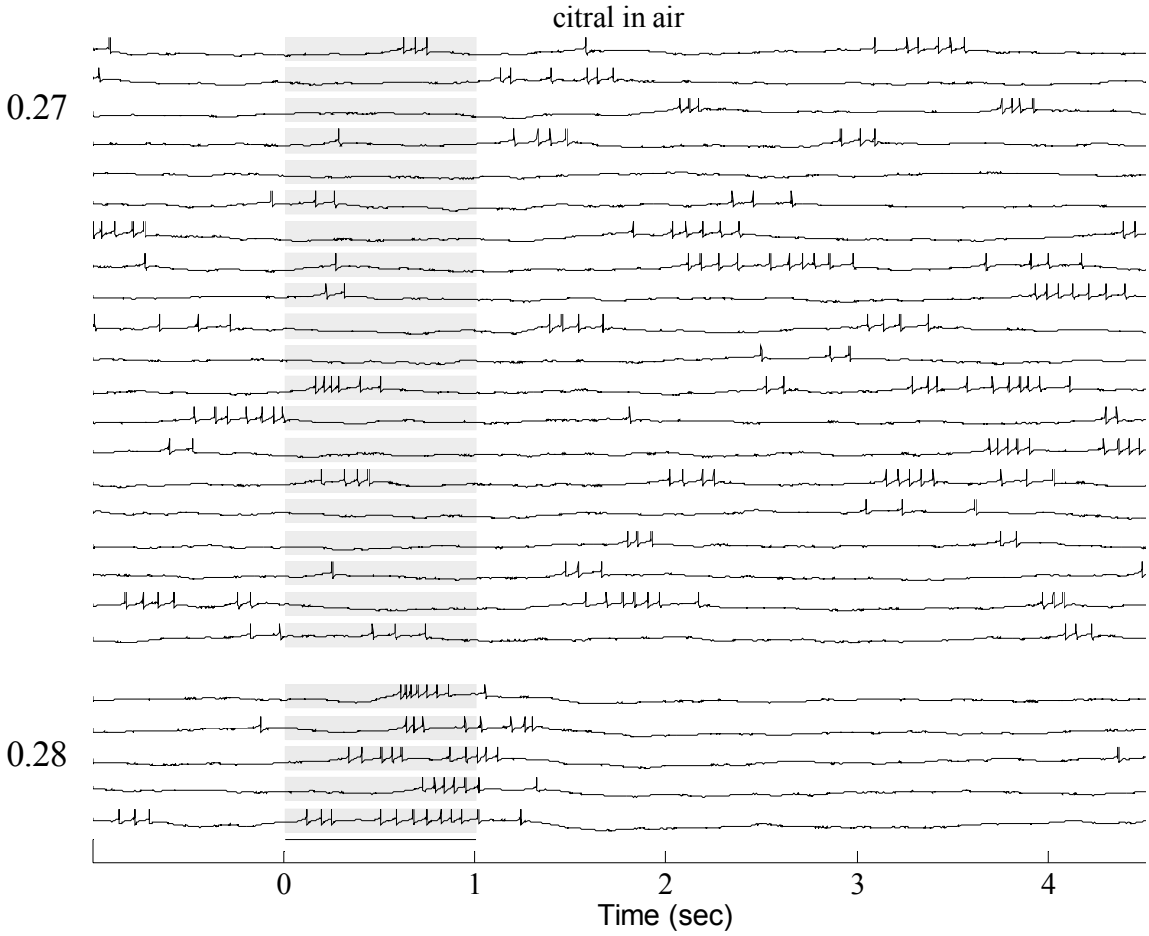


Figure II.2. Intracellular recordings of the same PN as in Fig. I.1 reveals no subthreshols response to the threshold deemed subthreshold with spike rasters.

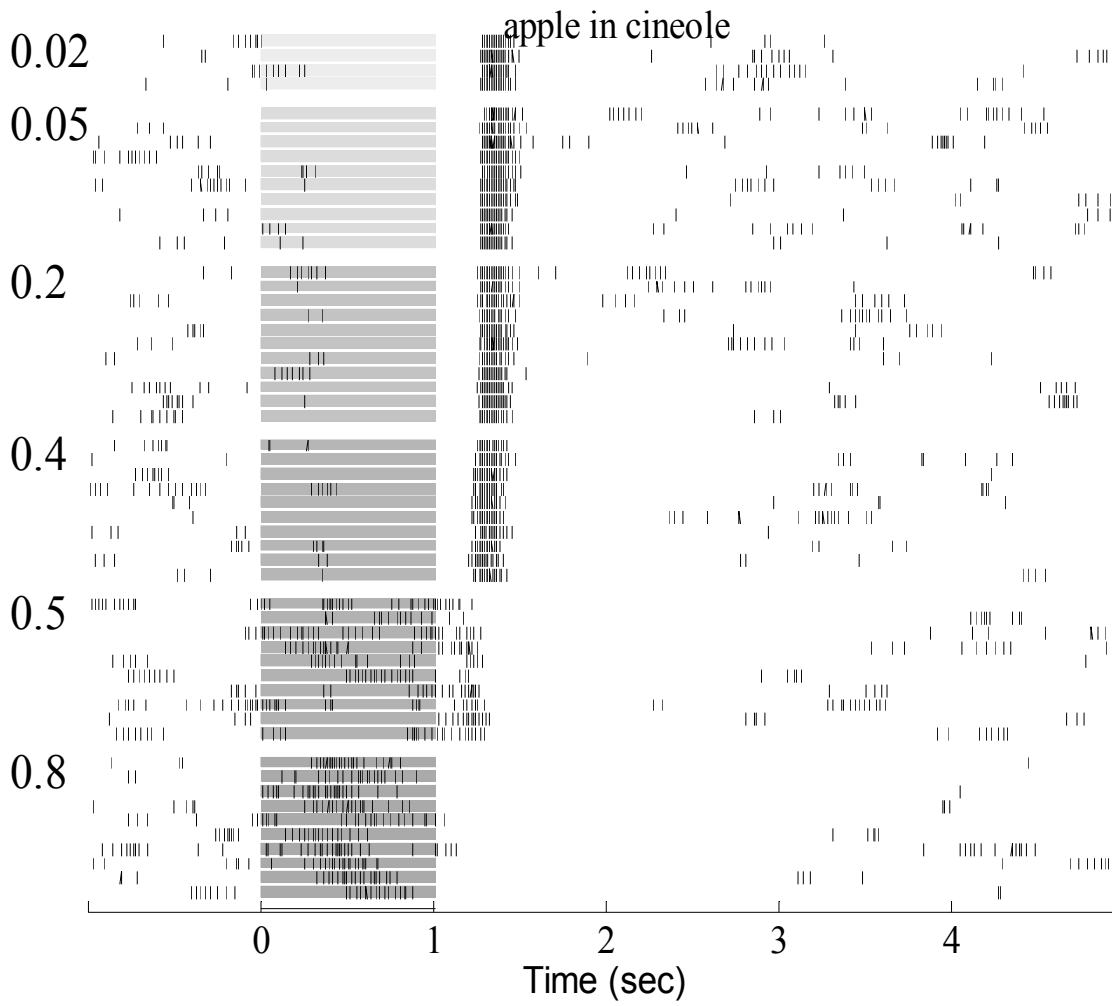


Figure II.3. PN responses exhibit abrupt transitions as a function of mixture composition. Odor mixtures were presented from t=0 to 1 sec.

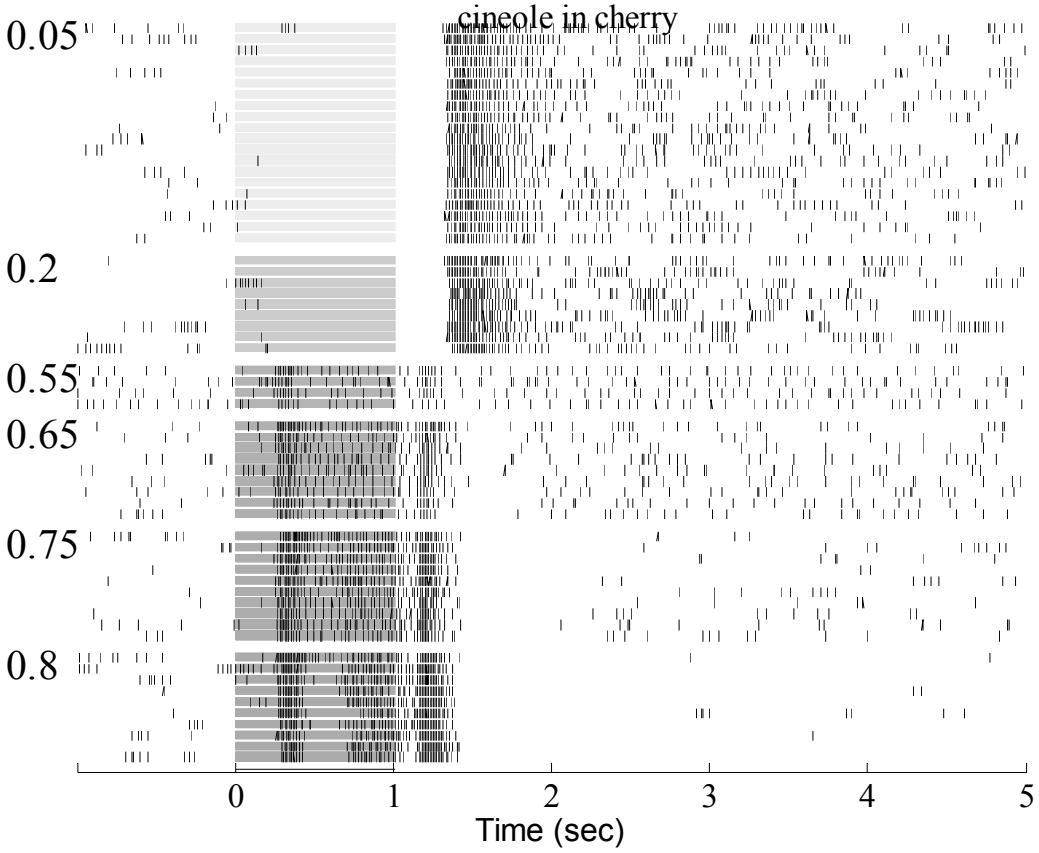


Figure II.4. PN responses exhibit abrupt transitions as a function of mixture composition. A different PN from that in Fig. II.3 is shown. Odor mixtures were presented from t=0 to 1 sec.

APPENDIX III

The response of a PN varies depending on the region of the antenna stimulated

This appendix briefly describes a preliminary experiment performed by Michael S. Wehr and I to test whether a projection neuron (PN) in the antennal lobe of the locust may carry information about the region of the antenna stimulated. To this end, we placed the nozzle of an odor delivery system 2 cm from the antenna and delivered a hexanol pulse of 1 sec in duration, varying the region of the antenna stimulated systematically, from the proximal tip to the distal tip. A PN would respond to an odor regardless of the region of the antenna stimulated, but we found that the PN's temporal response pattern carried information about the region of the antenna stimulated (Fig. III.1). Furthermore, small changes in the area of antenna stimulated could elicit large changes in response patterns (Fig. III.2).

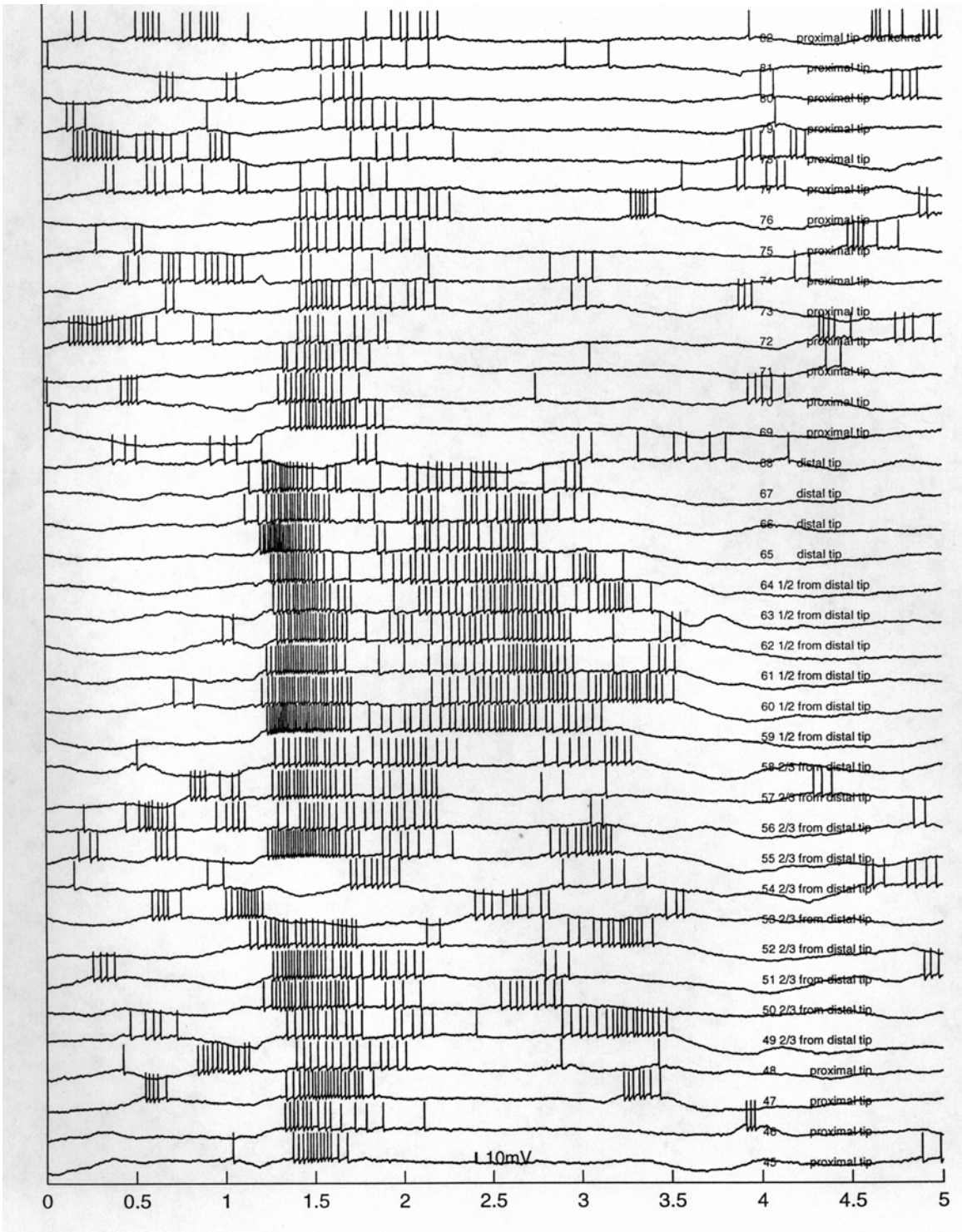


Figure III.1. A PN responds differentially to the same odor applied to different portions of the antenna. The odor was presented between t=1 and 2 sec. Note that responses to stimulation of the proximal tip remain constant throughout the experiment.

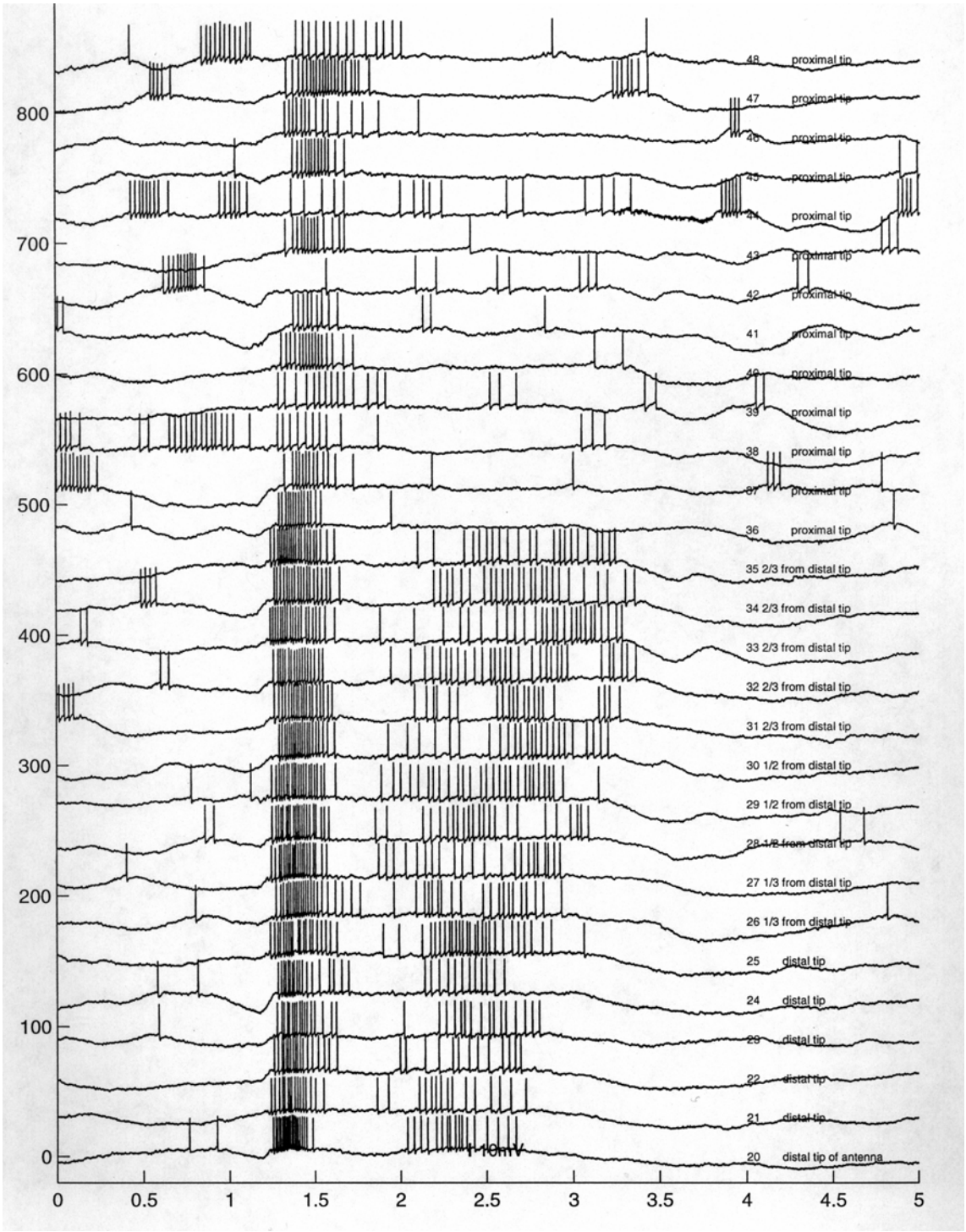


Figure III.2 A PN can exhibit abrupt response differences as a function of the region in the antenna stimulated. Hexanol was presented between t=1 and 2 sec.

APPENDIX IV

The connectivity between the locust antennal lobes and mushroom bodies: Combinatorics of a representation

"If you've made up your mind to test a theory, or you want to explain some idea, you should always decide to publish it whichever way it comes out. If we only publish results of a certain kind, we can make the argument look good. We must publish BOTH kinds of results."

Richard Feynman, Cargo Cult Science, Commencement address, California Insitute of Technology, 1974

Introduction

The connectivity pattern between locust antennal lobe (AL) projection neurons (PNs) and mushroom body (MB) Kenyon cells (KCs), as its vertebrate counterpart from olfactory bulb to piriform cortex, is characterized by massive divergence, from 830 PNs to 50,000 KCs. Furthermore, there is electrophysiological evidence that KCs have relatively high thresholds, requiring the coincidence of several input to fire a spike. Finally, PN firing rates are relatively low (less than 5 Hz across all PNs on average during odor responses). This suggests that interesting mechanisms may be in place to avoid active PNs from being "diluted out" in the sea of KCs, leading to no activity in the MB. The purpose of this exploration is to investigate the kinds of connectivity patterns between PNs and KCs that are consistent with known anatomical and physiological facts.

Notation

Let P equal the number of PNs in one AL, and K equal the number of Kenyon cells in one mushroom body that receive inputs from the AL. Let D equal the divergence ratio, the number of synapses each PN makes on average. Note that this number need not be the number of different postsynaptic neurons per PN, if there is more than one synapse to the same postsynaptic target for any given PN. Let C equal the convergence ratio, or the number of synapses that each KC receives on aver-

age. Let T be the threshold number of EPSP's caused by PNs required on average to produce an action potential in a KC. Let PS be the number of PNs spiking during the uninhibited portion of a given cycle of the local field potential (LFP) oscillations during an odor response. Let N(E) be the average number of KCs that receive E EPSPs during a given cycle of the LFP, and KS be the number of KCs that spike during a given cycle of the LFP.

Data

I will begin by giving, for each parameter, the ranges of values consistent with experimental observations:

P is very close to 830 (Leitch and Laurent, 1996).

K has a maximum of 50,000, since this is the total number of KCs in one MB. If all oscillatory activity in KCs that results from odor stimulation were the result of *direct* PN activation, K would have a minimum value of about 25,000, since this is the estimate for the number of KCs which show activity in intracellular recordings after presentation of a single odor (Laurent and Naraghi, 1994). 42% of KCs also showed extracellular responses to one or more of a panel of an average of 15 odors (Pérez-Orive et al., 2002). Imaging experiments suggest that the majority of the surface of the calyx received olfactory inputs (Wang et al., 2001).

D is about 600: 30 varicosities x 20 synapses per varicosity (Leitch and Laurent, 1996).

C can then be calculated to be:

$$C = (P \times D) / K \qquad > 830 \times 600 / 50{,}000 = 10$$

$$< 830 \times 600 / 25{,}000 = 20$$

So $10 < C < 20$.

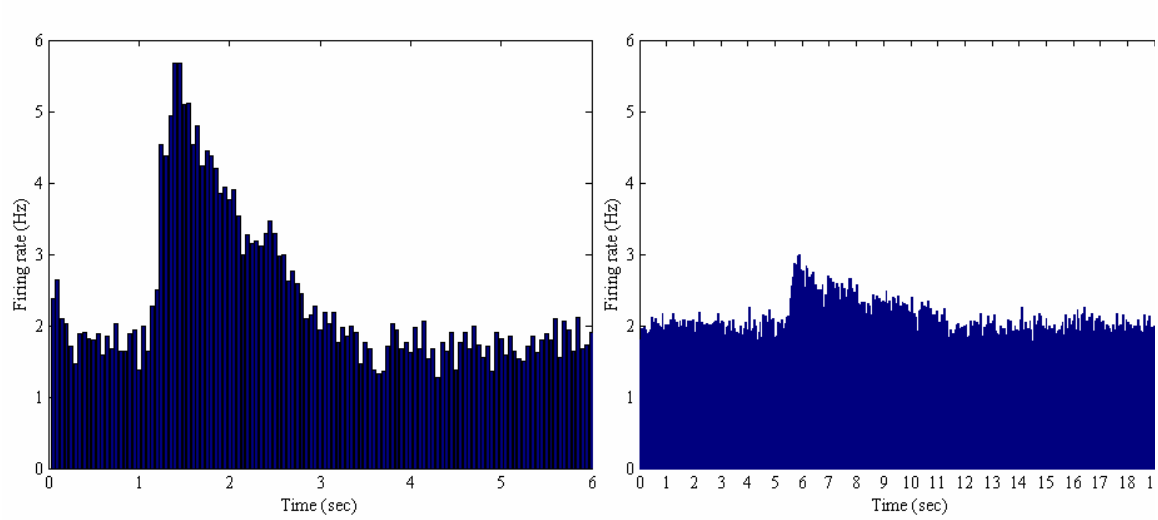


Figure IV.1. Mean PN firing rate during odor responses. Intracellular recordings (54 PNs, left) and tetrode recordings (right, 19 PNs courtesy of Stijn Cassenaer). Odors were presented at maximum concentration from t=1-2 sec at left and t=5-7.4 sec at right.

Experiments with electrical stimulation of the olfactory tract with simultaneous intracellular KC recordings independently suggest that C is at least 10, since different levels of electrical stimulation of the PN axon bundle can cause up to ~10 discrete EPSP levels in a KC (Laurent and Naraghi, 1994).

PS can be calculated as follows: The peak firing rate across all PNs (responsive and unresponsive) is 4-6 Hz (+- 8-11 Hz) (Bäcker, this thesis). That translates into 1 spike/cycle for every 4 PNs. KCs are responsive during approximately one half of the cycle only (Pérez Orive et al., 2001). Taking into account the synchronization of PNs, the fraction of of these spikes occuring during the most active 1/2 of a cycle is less than 2/3. Combining these figures, the fraction of active PNs during a half cycle is no more than 2/3 x 1/4=1/6. One sixth of 830 PNs is 138.

T is above 2-3, because subthreshold activity of KCs is regularly oscillatory, and at least that number of PNs are required to create a summated sinusoidal activity. T can also not be too low, since only 11% of KCs show suprathreshold response to any given odor (summing over the entire response period, Pérez Orive et al., 2002).

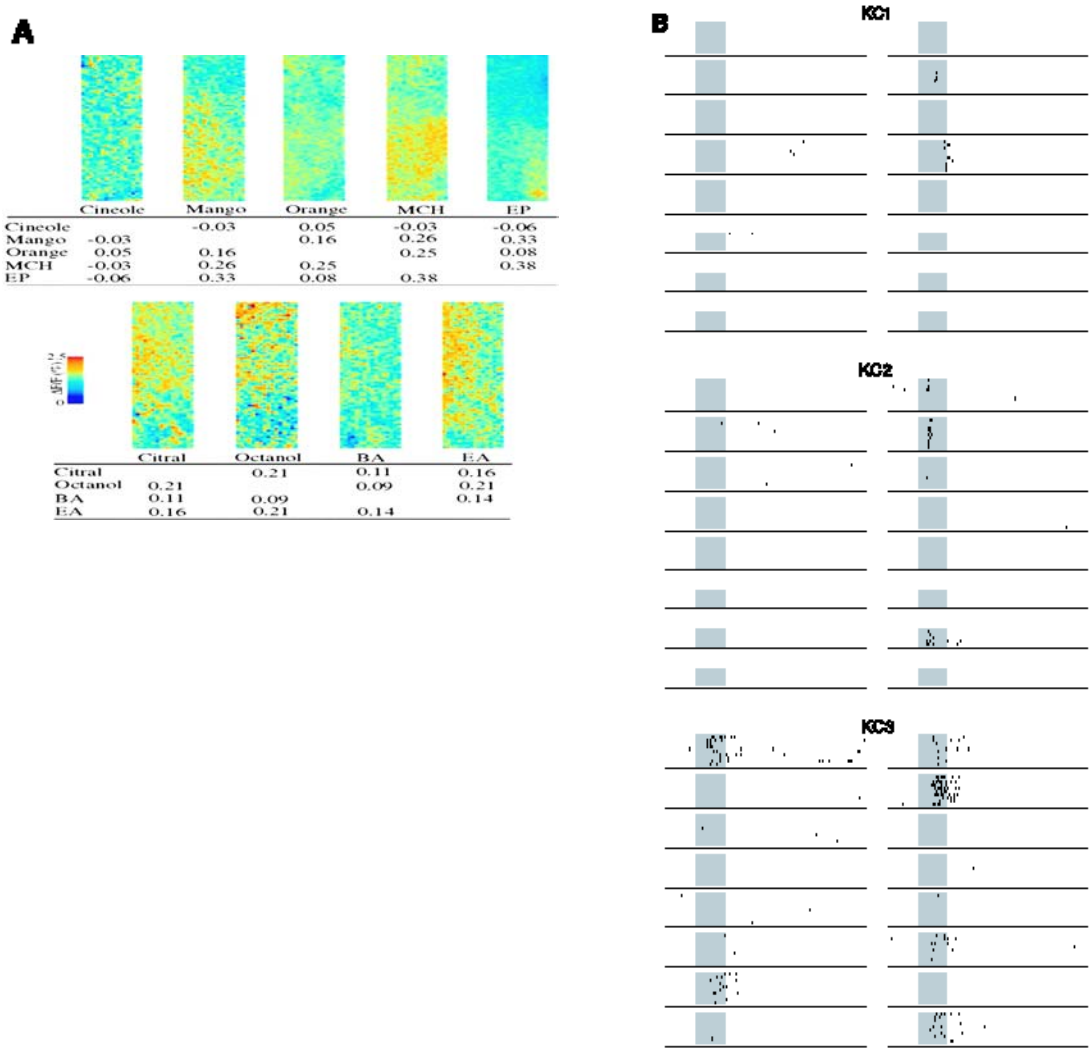


Figure IV.2. There is a striking difference between the broad nature of the inputs to KCs and the sparse nature of KC outputs. A) Spatial distribution of Ca++ signals in the mushroom body, likely to represent inputs to KCs (see Chapter 8) (from Wang et al., 2001). B) Spike trains in response to 1-sec pulses of up to 16 odors for 3 KCs (from Pérez Orive et al., 2002).

KS is on average approximately 250: 50,000 x 0.11 response probability integrated over 1-sec long odor pulses / 20 cycles per second.

Model

I assume random connectivity between PNs and KCs, and calculate the number of KCs receiving n EPSPs during the uninhibited portion of any one cycle as a function of different values for each of the parameters above. I can then compare the values obtained for KS with the experimental one to verify whether the assumption can be discarded or not. The fraction of KCs receiving i EPSPs is given by

```
pr(i) = p^i * q^(C-i) * comb(C,i);
```

where p=PS/P and q=1-p.

The number of KCs firing in response to an odor during a cycle is K times the fraction of KCs receiving less than the threshold number of EPSPs.

Results

The model suggests the threshold may be on the order of 8 synchronous spikes (the requirement for synchronization derives from the assumption of no decay between spikes) assuming that half the Kenyon Cells receive randomly distributed PN inputs and the parameters are as discussed above (Fig. IV.3).

Conclusions

This work suggest a range of values for KC firing thresholds that are consistent with known experimental data, assuming KCs integrate their inputs over approximately half an LFP cycle. Should the threshold be shown experimentally to diverge significantly from this value, one would conclude that a) connectivity is not uniform and/or b) integration mechanisms are very different from a one-com-

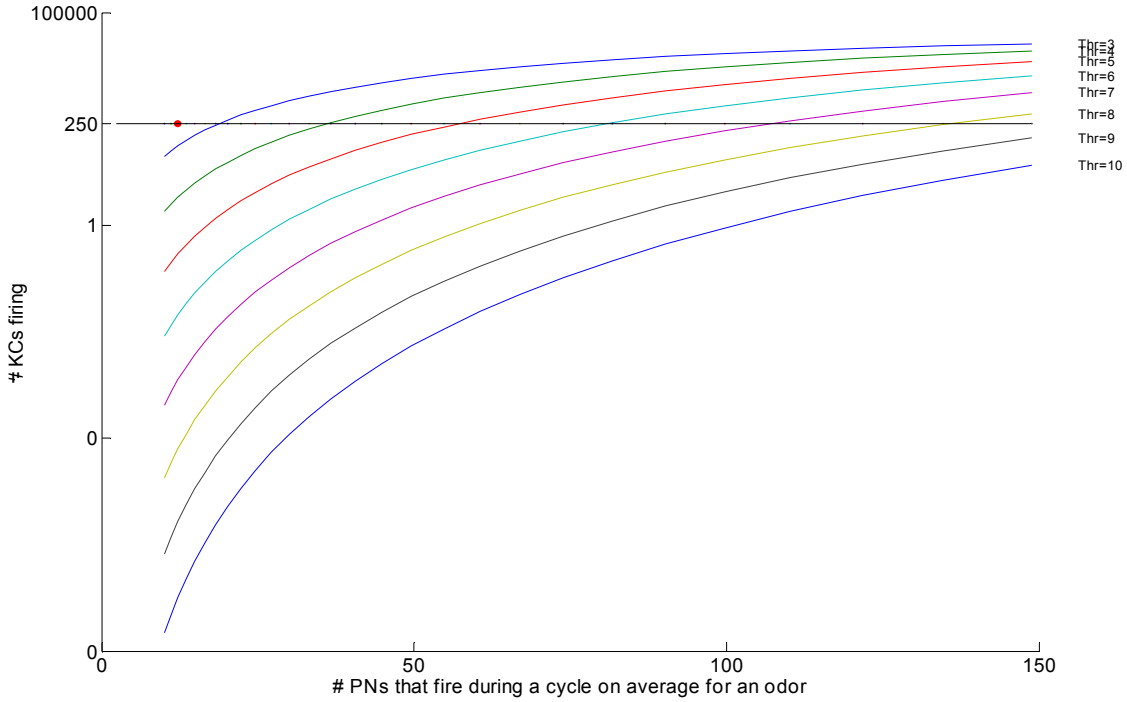


Figure IV.3. Themodel is compatible with the data for KC=25,000 and T=8.

partment coincidence detector.

Acknowledgements

The initial thrust behind this approach and the first calculations for selected parameter values are due to Gilles Laurent.

APPENDIX V

The probability that a single underlying binomial distribution yields two given success proportions: A hypothesis test

The problem

Binomial distributions are ubiquitous. Examples include a coin toss and questions such as, “Does a locust spend more time in the air or in the odor compartment of a Y-maze?” Whenever two such experiments are performed under different conditions, the question arises: Is the difference in the proportion of success observed in two experiments significant? i.e. What is the probability that a difference at least as large as that between the results of two experiments could be produced by chance if the two conditions have the same underlying success probability? For example, does starving the locusts cause a significant change in their chemoattractant behavior?

Notation

Let us call the number of observations in the first experiment *n1* and the number of observations in experiment 2 *n2*. Let us also call the fraction of successes in each experiment *f1* and *f2*. Finally, let us call the success probabilities of the two underlying distributions *p1* and *p2*. What we wish to calculate is the probability that if the null hypothesis was true, i.e. that both experiments have the same underlying success probability, two experiments with n1 & n2 trials, respectively, would yield success proportions as different as those observed or more.

Alternatives

The usual methods to address this question include the normal approximation to the binomial distribution, which is approximately valid only when the number of observations is significantly greater than 30, and assuming that p1=p2=f1, which is approximately valid only when n1>>n2. But today’s computational technology make approximations an unnecessary compromise.

I present a solution to the problem that can be calculated to arbitrary precision. The probability of getting the empirical results or more extreme results given *a* probability for a common underlying distribution is calculated exactly. The only approximation involved in the test below is in the discretization of such success probabilities used to partition the 0-1 interval to integrate over all possible underlying probabilities. This approximation can be carried to arbitrary precision (given by the *precision* parameter below).

The exact hypothesis test

Given an underlying success probability p0 and the success fractions of two experiments, f1 and f2, we want the probability of obtaining a difference between the success fractions of two experiments that is equal or greater than that observed, namely greater than |f1-f2|. This difference, calculated by the MATLAB function BINODIFCDF below, is given by summing the probabilities of obtaining each of the results of the two binomial experiments which yield a difference between success fractions equal or larger than |f1-f2|. The probability of each result can be calculated with the standard binomial formula:

$$p(f1 \mid p=p0) = p0^x \cdot q^y \cdot (n1 \text{ choose } x). \quad (1)$$

where x = # of successes and y = # of failures, given an underlying success probability p0.

The probability of two success fractions f1 and f2 given an underlying success probability p0 is given by

$$p(f1 \,\&\, f2 \mid p=p0) = p(f1 \mid p=p0) \cdot p(f2 \mid p=p0). \quad (2)$$

Since we don't know what the underlying success probability is, we need to calculate the above for every possible p0 value (the 0-1 interval is discretized to a desired accuracy). The process above is then repeated for all p0 values ranging from 0 to 1, and the results integrated. But all values for p0 do not have equal probability given f1 and f2. The weight of each, i.e. the probability that the underlying success probability is p0, given that f1 and f2 are the fraction of successes observed in two experiments with N1 and N2 observations is calculated using Bayes' rule (and taking the prior probabilities to be uniform) as the probability of obtaining f1 and f2 given p0:

$$p(p=p0 \mid f1 \,\&\, f2) = p(f1 \,\&\, f2 \mid p=p0) \quad (3)$$

which can in turn be calculated from (2) above.

MATLAB implementation

An electronic copy of the following code is available at http://www.its.caltech.edu/~alex/code/binomialtest.htm:

```
function [realpval] = binomialanal(p1,p2,N1,N2,precision)
% © Alex Bäcker Aug 01
% NS1 & NS2 (integers) are the two numbers of successes being compared to see if they can come from the same underlying distribution.  They represent the two 'conditions'.
% N1 & N2 are the # of experiments for p1 & p2, respectively. Default N2=N1.
%
% REALPVAL returns the p-value for the null hypothesis that both experiments were generated by the same underlying
```

```
distribution.
%
% Note that REALPVAL is not only a function of abs(p1-p2), N1 and N2, but rather also of p1 & p2, because the relative probabilities of different underlying success probabilities are different for different p1 & p2 pairs even for constant difference: More extreme values of p1 & p2 indicate more consistent processes with smaller variances, and thus the probability of observing the same difference by chance gets smaller as p1 & p2 get away from 0.5
%
% Takes p(underlying prob==X | p1 & p2) = p(p1 & p2 | underlying prob==X)

if nargin<4,
   N2=N1;
end
if nargin<5,
   precision=.1;
end

dif=abs(p2-p1); % prob difference observed

k=0;
for m=0:precision:1, % These are continuous, not discrete, because they are the true underlying distribution, which is independent of N1 & N2
   k=k+1;
   p12=m; % p of getting a head:

   % Prob of getting a larger difference of p's than observed given underlying success prob is p12:
   pvalue(k)=binodifcdf(dif,p12,N1,N2);

   % Prob of underlying success prob being p12 given p1 & p2 observed:
   pp1= binopdfab(p1,N1,p12); % If you use binopdf & binocdf, you get NaN when using p12=0 &/or 1
   pp2= binopdfab(p2,N2,p12);
   pp(k)=pp1*pp2;

end
totp=sum(pp);
realpval=sum(pp.*pvalue)/totp;


function    pvalue=binodifcdf(dif,p,N1,N2)
% BINODIFCDF - Binomial difference cumulative distribution
% pvalue=binodifcdf(dif,p,N1,N2)
% © Alex Bäcker Aug 01
% Yields the cumulative probability distribution that the difference b/w 2 # of heads  is greater than or equal to DIF
% if the underlying probability is P12 and N1 & N2 are the # of observations in each experiment
% Default N2 = N1

if nargin<4,
   N2=N1;
end
if N2<N1, % Ensure N2>=N1
   [N1,N2]=swap(N1,N2);
```

```
end

pvalue=0;
for Nh1=0:min(N1,N2-dif), % Nh1<=Nh2
   for d=dif:N2-Nh1,
      Nh2=Nh1+d;
      pv=binopdfab(Nh1,N1,p)*binopdfab(Nh2,N2,p);
      pvalue=pvalue+pv;
  end
end

if dif==0, % To avoid repeating the situation Nh1=Nh2 above and below, do not count it again. Equiv: dif=max(dif,1);
   dif=dif+1;
end
for Nh2=0:N1-dif, % Nh2<Nh1
   for d=dif:N1-Nh2,
      Nh1=Nh2+d;
      pv=binopdfab(Nh1,N1,p)*binopdfab(Nh2,N2,p);
      pvalue=pvalue+pv;
  end
end
```

function y = binopdfab(x,n,p)

```
% BINOPDFab Binomial probability density function.
% © Alex Bäcker Aug 01
%   Y = binopdfab(X,N,P) returns the binomial probability density
%   function with parameters N and P at the values in X.
%   Note that the density function is zero unless X is an integer.
%
%   The size of Y is the common size of the input arguments. A scalar input
%   functions as a constant matrix of the same size as the other inputs.
%
%   The Mathwork's BINOPDF can give warning Log of zero if X=N, this one does not.

% Initialize Y to zero.
y = zeros(size(x));

% Binomial distribution is defined on positive integers less than N.
q=1-p;
ix=n-x;
k = find(x >= 0  &  x == round(x)  &  x <= n);
if any(k),
   for i=1:length(k),
      y(k(i)) = p(k(i)).^x(k(i)).*q(k(i)).^ix(k(i)).*nchoosek(n(k(i)),x(k(i)));
   end
end

k1 = find(n < 0 | p < 0 | p > 1 | round(n) ~= n);
if any(k1)
  tmp = NaN;
```

```
  y(k1) = tmp(ones(size(k1)));
end
```

Acknowledgments

Thanks to Natalia Caporale for bringing the problem that motivated this appendix to my attention.

APPENDIX VI

Fractional K-nearest neighbors: Dealing with non-uniform sampling

Summary

This appendix briefly describes a novel variation of the well-known k-nearest neighbor (KNN) metric for classification. The new variation is often more useful than its predecessor when the number of exemplars of each class is not the same.

The problem

KNN (Duda, Hart and Storm, 2000) assigns a data point X to the cluster or class with the greatest number of points among the k-nearest neighbors of X. When our sample has a different number of points for each class, and when the number of points we have for each class does not reflect the actual density or probability of each class in the underlying distribution, but rather derives from sampling biases, for example, then the outcome of KNN will be biased toward classes with the largest number of samples, an undesired effect.

Fractional K-Nearest Neighbors

This problem is solved by simply assigning a point X, not to the class with the greatest *number* of points among its k-nearest neighbors, but rather to the class with the greatest *fraction of its members* among X's k-nearest neighbors.

Acknowledgment

Thanks to Pietro Perona for a discussion on this subject.

APPENDIX VII

Asymmetric sliding-window cross-correlation

VII.1 Introduction

Sliding-window cross-correlation is a common method to esimate time-varying correlations between signals (Laurent and Davidowitz, 1994; Laurent et al., 1996; Macleod and Laurent, 1996; Stopfer and Laurent, 1997; Wehr, 1999 (p. 96)). It produces a correlation value betwen two signals (positive or negative) for every (time,lag) pair of values. In principle, the expected value of the correlation for any pair of (time,lag) values must be computed by averaging x(t).y(t+lag) over many realizations of the stochastic process. This is called an ``ensemble average'' across realizations of a stochastic process. Lacking a large enough number of realization over which to average, one must resort to other methods. If the correlations are stationary (i.e. time-invariant), then we may average across time to estimate the expected value. If the correlations are not stationary, we may divide the signals into sliding windows of a size such that the correlations can be considered stationary on the timescale of the window width, and calculate the cross-correlation, as a function of lag, for each window, sliding the window along the signal to obtain correlations for different time values. This is what is called a sliding-window cross-correlogram.

VII.2 The problem

Take, for example, two signals which start off uncorrelated, then show a periodic positive correlation for 1 second, and finally become uncorrelated again. The cross correlogram at the beginning of the 1-second period of correlation should show positive correlation with positive lags, but not with negative lags: the signal is correlated to what will come, but not to what was there before. In the middle of the 1-second period, correlation is positive for negative and positive lags. Toward the end, correlation is positive only for negative lags. And yet, as first noted by my Caltech colleague Stijn Cassenaer (personal communication), none of the sliding-window cross-correlograms in the literature exhibit this asymmetry.

The reason why the asymmetry is lacking is the following: the correlograms were computed by calculating the cross-correlation function for each window separately, using commercial routines such as MATLAB's xcorr function, which slide one signal's vector past the other one for each window. With this method, only the values within the window being used are used for the correlation, and thus whether the window is before, after or in the middle of a period with high correlation makes no difference other than by the correlation present in the window itself. In particular, if two signals, s and s', are perfectly correlated, as in an autocorrelogram, their cross-correlation will be symmetrical for each window (i.e. for each t-value, taken to lie in the middle of each window) by construction:

$$c(t,+lag) = s(t-lag/2).s'(t+lag/2)$$

$$c(t,-lag) = s(t+lag/2).s'(t-lag/2) = s'(t-lag/2).x(t+lag/2)$$

If s is perfectly correlated with s', c(t,+lag)=c(t,-lag), because the asymmetry will show up in one signal at positive lag and in the other signal at negative lag*. In other words, instead of having the correlation at positive lag computed from a comparison with a window shifted in the positive direction and the correlation at negative lag computed from a comparison with a window shifted in the negative direction, the existing method uses the same window for both lags, simply shifting the window in different directions.

That method leads to another, related, problem: the greater the magnitude of the lag, the less data is used. This happens because the edge of the window does not move as the lag is changed, and thus only lag zero allows a comparison between every sample in the window for each signal. For lags of any magnitude, the shift between signals forces the comparison to be done over every decreasing stretches of signal, until at lags of the window length, a single sample from each signal is used. Thus, the traditional method will yield noisy correlation estimates for any lags which are not significantly less than the length of the windows used (see Fig. VII.1), and cannot be used at all for lags greater than the window length. The most powerful cross-correlogram, though, is one

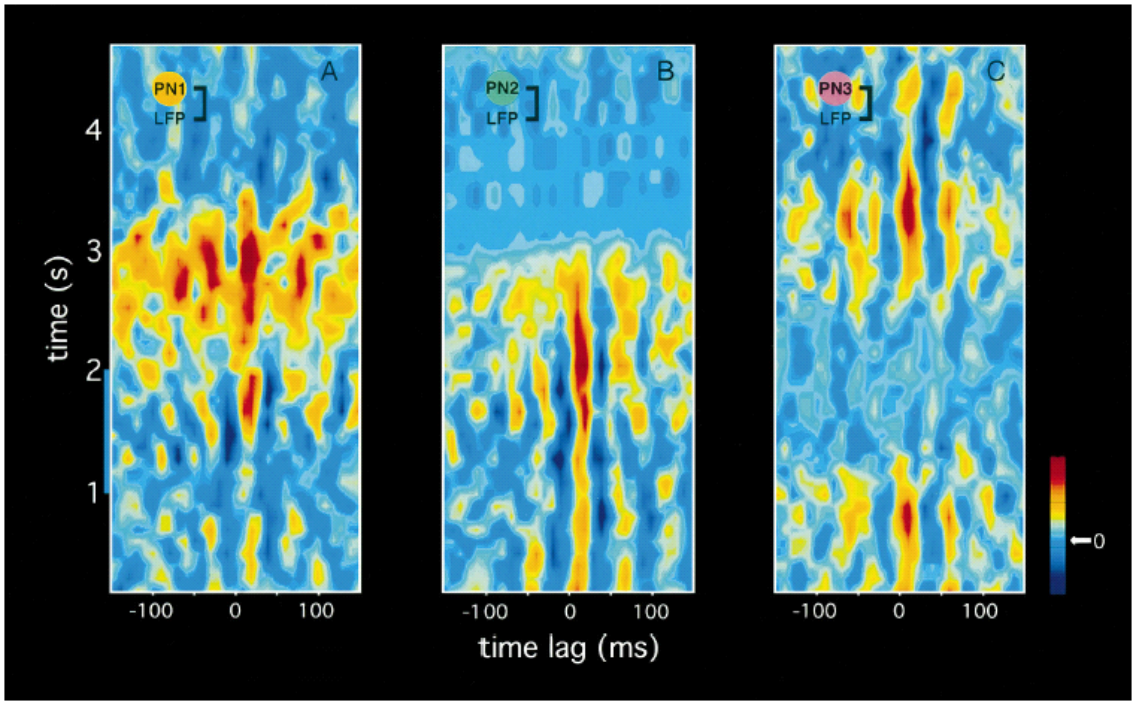


Figure VII.1. Sliding-window cross-correlograms (from Laurent et al., 1996) calculated with previous methods get increasingly noisy with lags of increasing magnitude. Note that even though the correlation lasts on the order of a second or more (see vertical extension of central high correlation bands), estimates of the correlation at lags an order of magnitude smaller than that are quite noisy. This is due to the method of estimation as well as to any aperidocities that can exist in the signal.

which has small window length (so as not to blurr variations in time) and large lag ranges (to observe correlations at any lag). In particular, the windows have to be small compared to the time-course of variations in the correlation. This means that at the onset and offset of the oscillations, the windows should be particularly small. But if the maximum lag is constrained to the length of the window, small windows do not allow seeing the periodic structure of the correlation.

VII.3 A solution

As discussed above, the motivation behind sliding windows is the assumption that signals are relatively stationary on the timescale of the window length. Because there are no hard bound-

aries, the signal is not stationary only within the windows, but rather on the timescale of the window length, and thus samples toward the edge of the window should be correlated with samples of the other signal within a window-length from them. In other words, if one knows the signal surrounding a window, those samples must be taken into account in calculating the mean signal following or preceding samples in the window. Thus, the cross correlation between s and s' at time t is given by:

$$c(t,lag) = < s(t).s'(t+lag) >,$$

where the average is over all t values in the window.

This method, which we term asymmetric cross-correlation, has several advantages over its predecessor, termed symmetric cross-correlation below for comparison: 1) it eliminates the artificial symmetry, 2) it eliminates the reduction of data for increased lag magnitudes, and 3) it allows for small windows concurrently with large lag ranges[1]. This is illustrated in figure VII.2.

1. If enough repetitions are available so as to allow the use of small windows, the length of the sliding window can be reduced even to a single sample with this method, for any lag range desired.

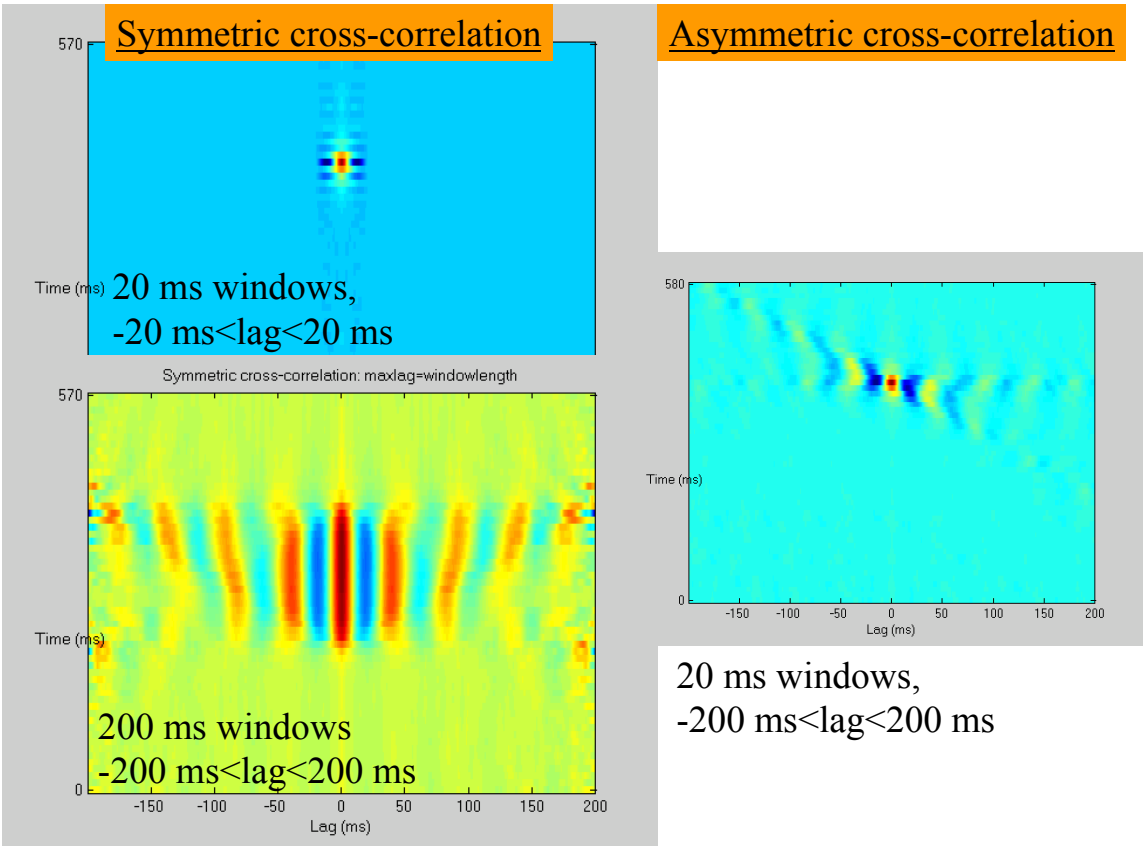


Figure VII.2. Symmetric cross-correlation forces a tradeoff between small windows, allowing for increased sensitivity to nonstationarities, and large lag ranges, allowing a full appreciation of any periodicity or delay in the correlation. Asymmetric cross-correlation allows for large lag ranges concurrent with small windows. The same signal is used for all 3 autocorrelograms above.

APPENDIX VIII

Self-organization of neural networks into winnerless competition

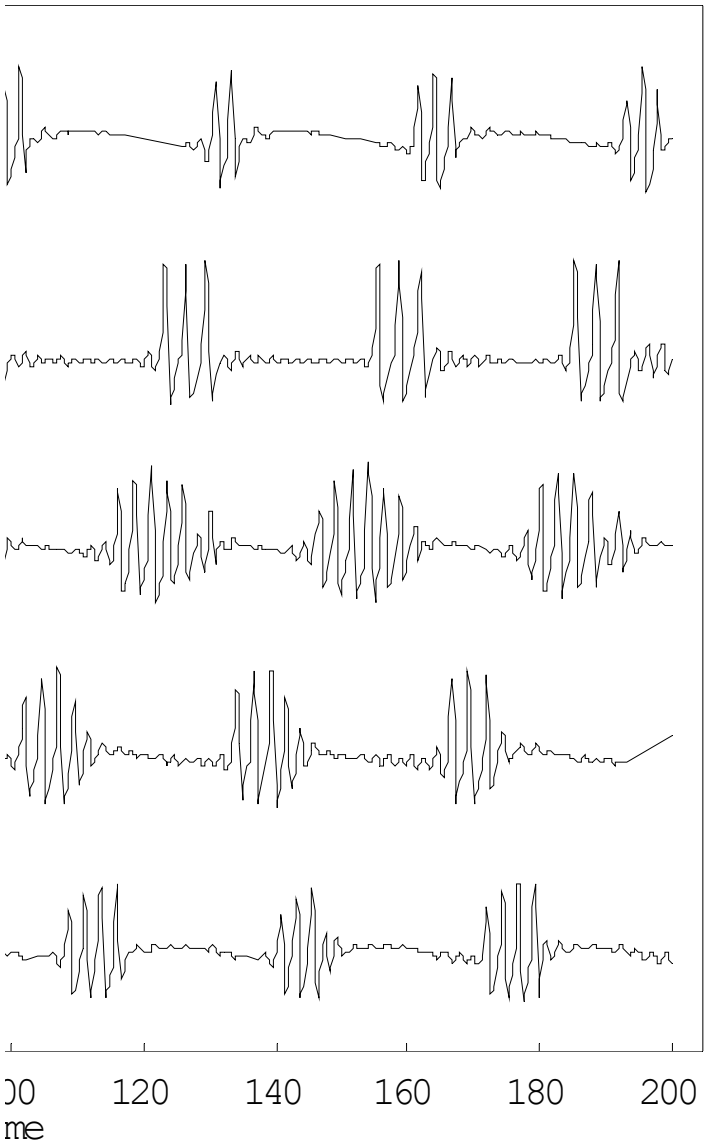
The evolution of neural responses to odors in the antennal lobe of the locust follows temporal dynamics that do not lead to a static attractor or a closed limit cycle. Nevertheless, sufficiently similar inputs to the olfactory system lead to equal behavioral responses, and thus presumably to equal neural responses. This means that the time-varying responses to odors must act as dynamic attractors in neural dynamics space. To model these and other such attractors, Rabinovich et al. (2001) introduced a class of dynamical systems they termed competitive networks, or Winnerless Competition (WLC). These produce deterministic trajectories moving along heteroclinic orbits that connect saddle fixed points or saddle limit cycles in the system's state space. These saddle states correspond to the activity of specific groups of neurons, and the separatrices connecting these states correspond to sequential switching from one state to another.

In its original formulation, WLC was assumed to require closed loops in the network, i.e. closed loops of strong unidirectional connections with weak or no connections in the opposite direction (Laurent et al., 2001). This requirement led to cyclical behavior of the network (Fig. VIII.1), a property that is not shared by the biological networks in the olfactory system. Here, I show that closed loops are not required, and that relaxing that requirement eliminates the cyclical behavior, leading to activity more similar to that observed in biological networks (Fig. VIII.2).

More importantly, how a network can self-organize to produce the connectivity required for WLC remains unknown. In particular, the requirement for asymmetric connectivity suggests an interaction between each synapse and its corresponding synapse with the opposite connectivity, but these two synapses are typically far-removed from each other, making a specific direct interaction between the synapses difficult. I show here that a simple biologically-observed local learning rule suffices to create WLC in initially random networks.

WLC requires that for every pair of neurons (A,B) for which A projects strongly to B, B project weakly (if at all) to A. At a first glance, it seems impossible for a local learning rule to achieve this antisymmetric connectivity pattern, for the strength of a connection A to B depends on that of another synapse, potentially a long distance away. And yet closer examination reveals that a local rule can indeed do the job. Furthermore, the trick is accomplished by a rule that has actually been observed in biology, albeit in slightly different circumstances.

The rule in question was discovered in what have rapidly become classic studies by Markram et al. (1997) and Bi and Poo (1998). It is called spike-time dependent plasticity (STDP), and says that the synapse between neuron A and neuron B is strengthened if a spike in A immediately precedes one in B, but is weakened if a spike in A follows one in B (Fig. VIII.3). I propose that the antisymmetric character of this rule --which induces in the A-B and B-A synapses opposite changes upon a suc-



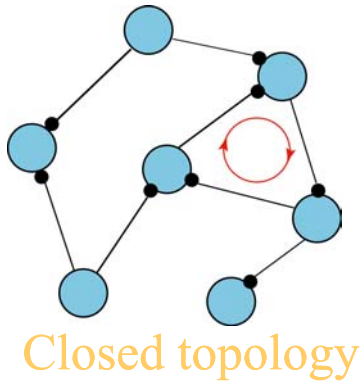


Figure VIII.1. The original formulation of WLC involves a closed topology, i.e. a ring of strong connections, leading to cyclic behavior. But networks in the antennal lobe of the locust to do not exhibit cyclic behavior, even with prolonged stimulus pulses (Wehr, 1999).

cession of quasi-coincident spikes across the neurons-- suffices to create WLC out of an network initially configured with random synaptic weights. Computer experiments by Valentin Zhigulin have confirmed this prediction (Fig. VIII.4).

The stability of the attractors produced by this learning rule remains to be determined. It is likely that

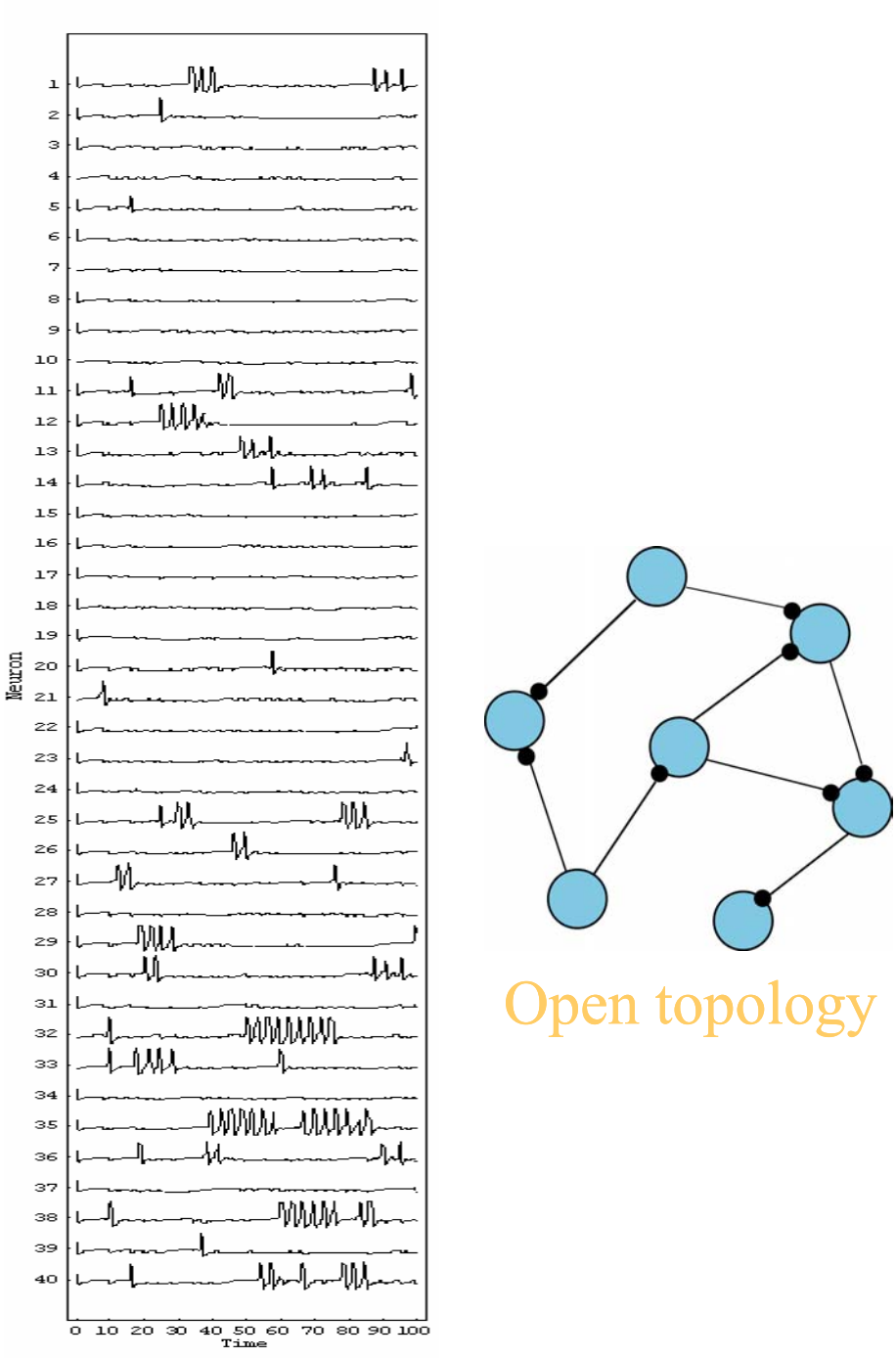


Figure VIII.2. Eliminating closed loops causes network activity to lose cyclic behavior, but preserves the dynamic nature of the network's response, emulating the behavior of biological networks more closely.

10470 J. Neurosci., December 15, 1998, 18(24):10464–10472

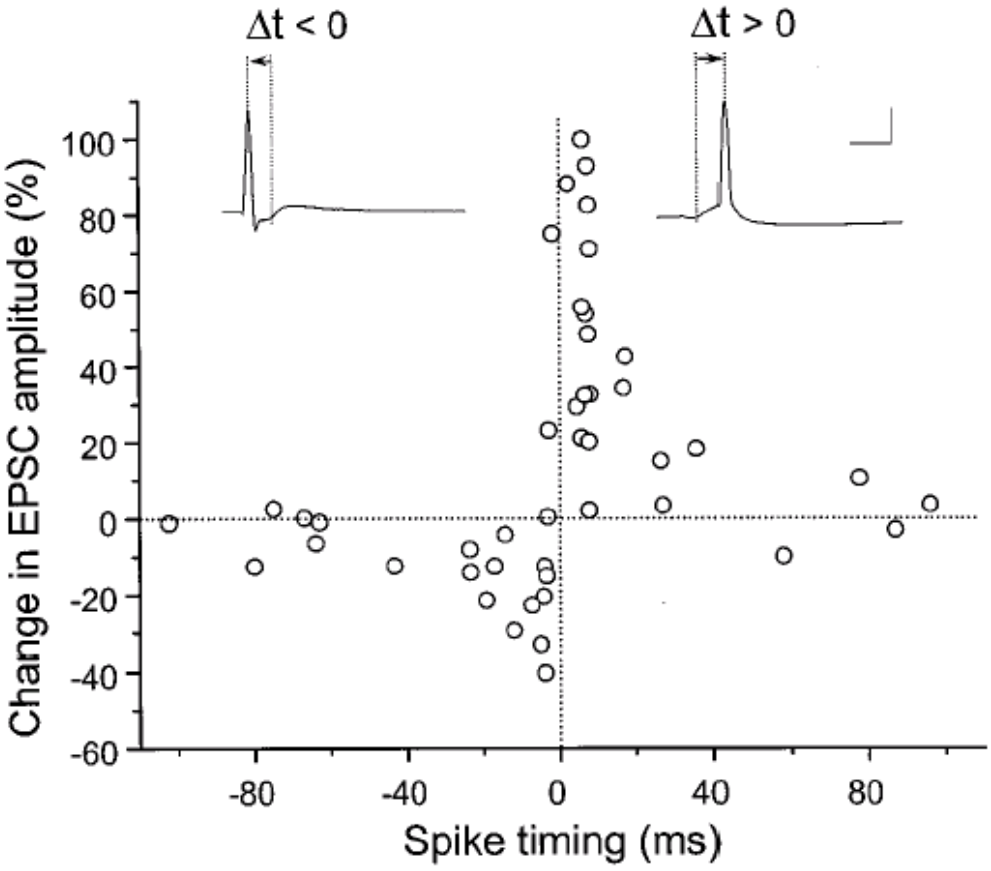


Figure VIII.3. Spike-time dependent plasticity (from Bi and Poo, 1998).

additional contraints, such as a multiplicative factor in the learning rule that makes synapses that are far from their initial values less prone to further modification, need to be introduced for this purpose.

Acknowledgement

The validity of the ideas above was demonstrated experimentally by Valentin Zhigulin.

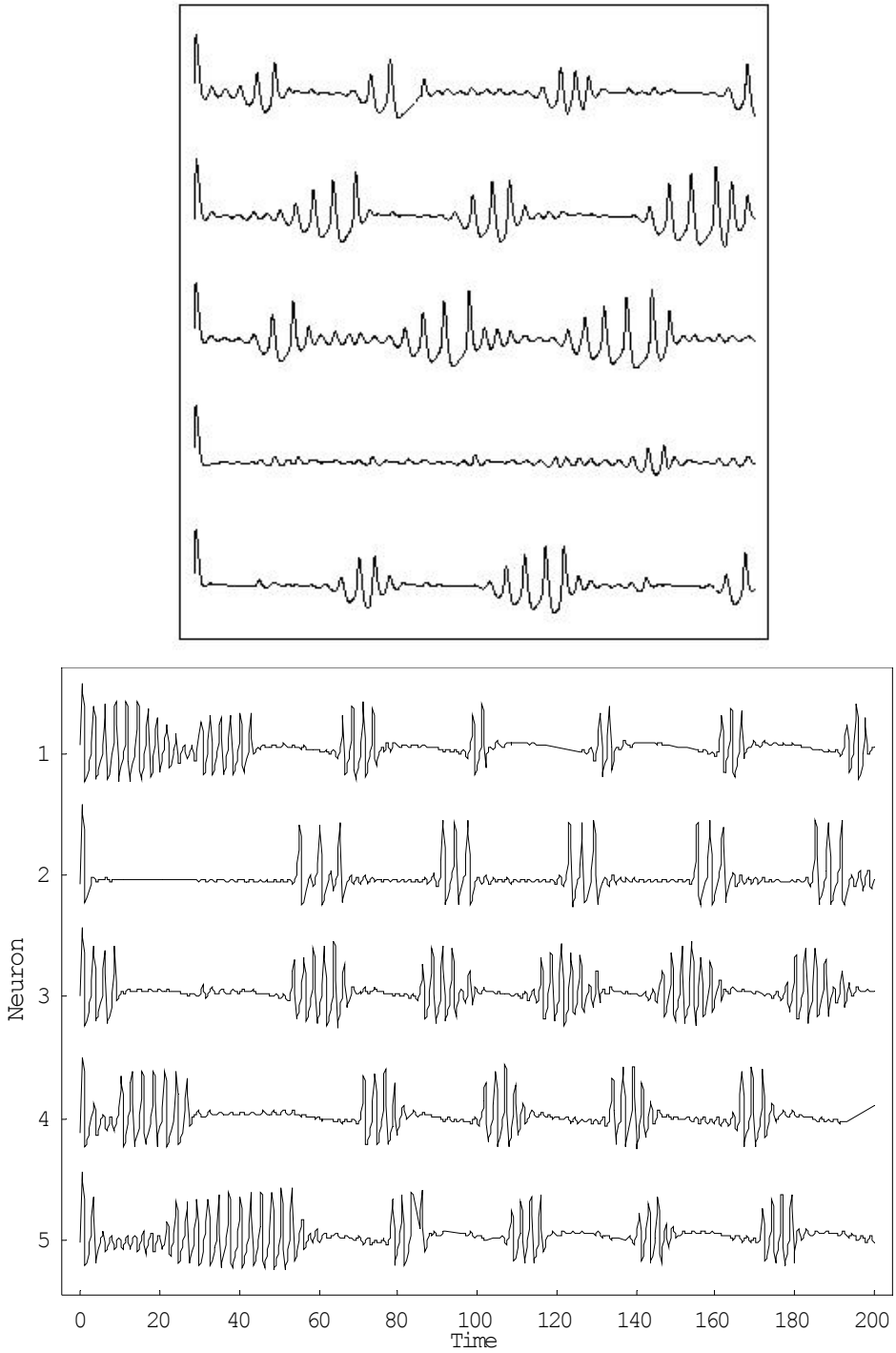


Figure VIII.4. STDP produces WLC in excitatory (top) and inhibitory (bottom) initially randomly connected neural networks.

APPENDIX IX

Response variability is correlated across multiple projection neurons in the antennal lobe of the locust

Odors appear to be represented in the antennal lobe of the locust by odor-specific, but overlapping, evolving assemblies of synchronously firing projection neurons. These projection neurons (PNs) fire in temporal patterns which are odor- and cell-specific. I have examined the variability of the responses of these cells across repeated stimulus presentations of the same odor. I found strong correlations ($p<0.001$) between the variability of spike trains of many, but not all, simultaneously recorded pairs of PNs. In some of these pairs, the firing of the neurons was negatively correlated: i.e. one neuron fired less than on average on trials when the other neuron fired more than on average. In other pairs, the firing was positively correlated. The correlation in some cell pairs was odor-evoked, and, interestingly, happened only at a particular epoche in the dynamically evolving response. I also observed significant correlations during periods of no odor stimulation, which disappeared at the onset of the odor responses. When the variability of the responses was analyzed as a function of time in the trial – assessed as the variance in the firing rate for short time windows – the variability appeared to vary systematically as the response evolved in time, so that a given cell would exhibit periods of high variability and periods of low variability. In summary, we found intertrial variability was not independent neuronal noise and that neurons are coupled at several timescales. These results suggest that the effective connectivity of the antennal lobe varies as the response to odors unfolds in time. From the standpoint of neural analysis, these results suggest that there is much to be gained in analyzing single trials rather than PSTH's

This work, which was presented in Bäcker, Wehr and Laurent, 1997, was carried out on pairs and triplets of PNs recorded simultaneously by Michael S. Wehr.

Methods

Electrophysiological recordings were done *in vivo* in immobilized adult locusts (*Schistocerca americana*).We simultaneously recorded extracellularly from two or three PN's with an equal number of

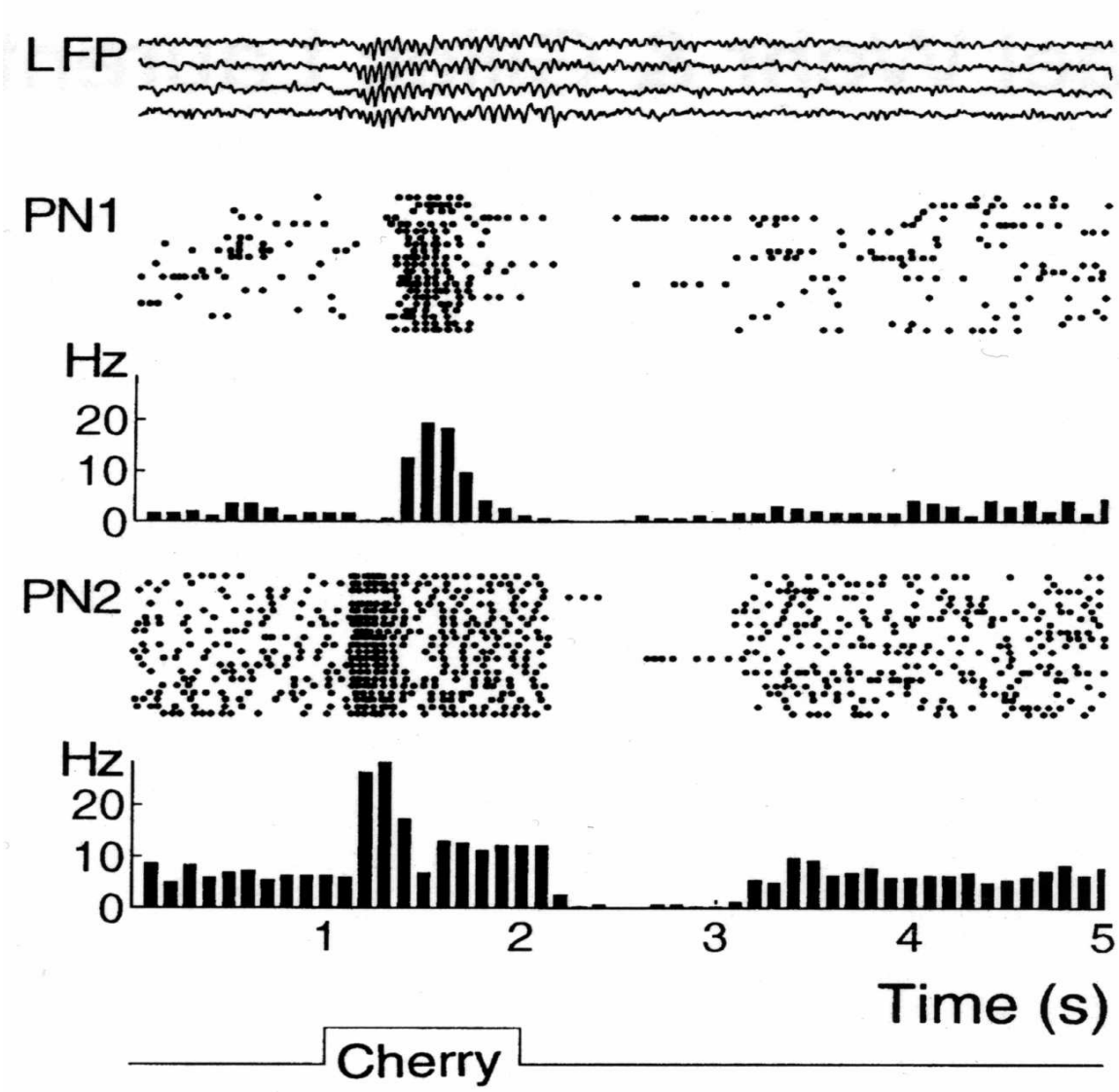


Figure IX.1. Some spikes are reliable; others are not. Odors evoke temporal response patterns of variable reliability in projection neurons (PNs). Notice the extreme degree of repeatability across trials in the first burst in the left diagram, and the much more irregular nature of firing patterns in the second burst or in the diagram on the right. Trials are aligned on the odor delivery pulse onset. Note also the atypical response pattern after odor onset in trial 4 for both PNs. Odor delivery lasts 1 sec, marked 1-2 sec. From Wehr & Laurent (1996), *Nature* 384: 162-166.

glass micropipettes while one-second long puffs of odor were applied to one antenna at regular intervals. We later assessed the independence of the variability of two neurons' responses, i.e. the

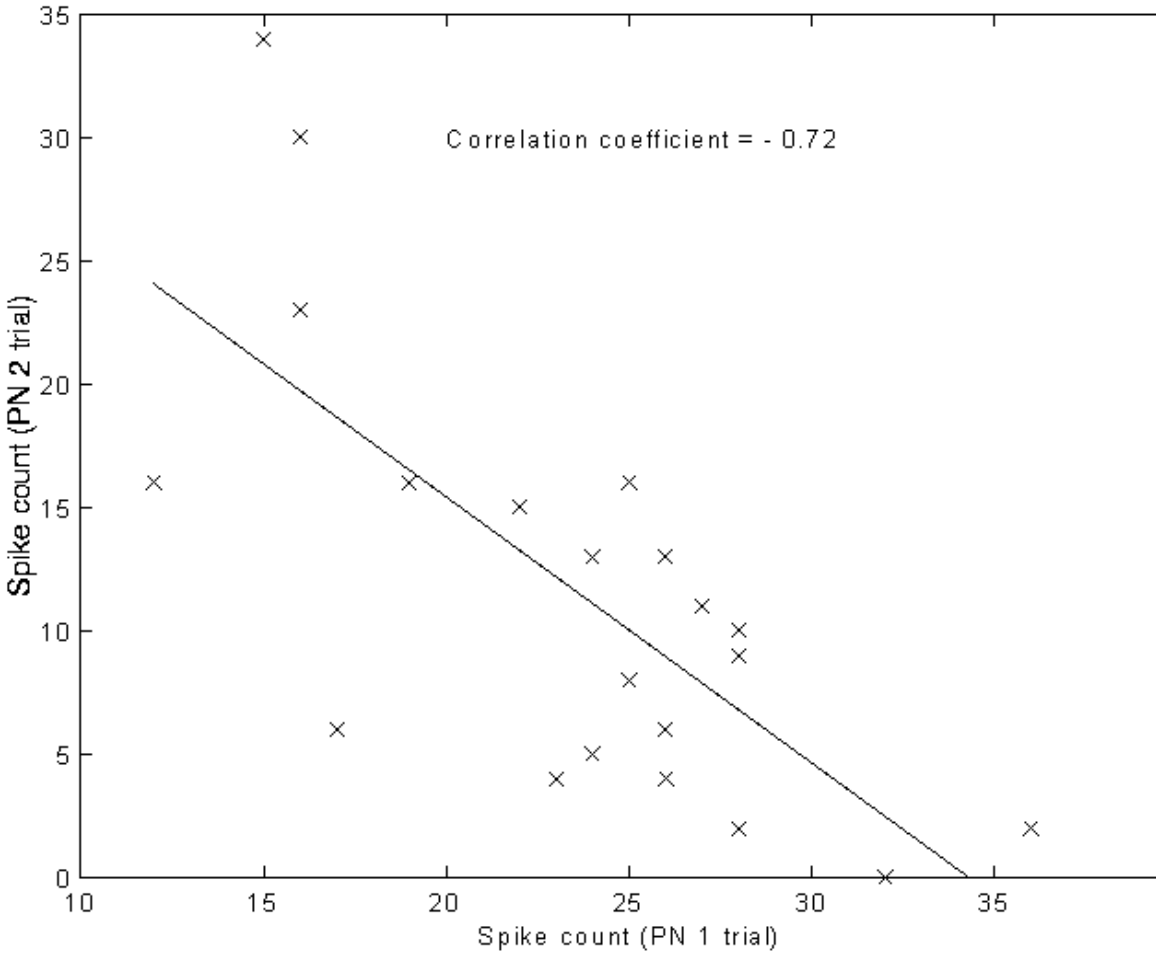


Figure IX.2. There is a tight coupling between two PN's spike count *on a trial by trial basis*. Spike count of each cell during a 3- second time window following odor presentation. Each data point represents the activity of two cells recorded simultaneously during one trial. The line shows the best linear interpolation of the data. The correlation coefficient is –0.72, indicative of a strong negative correlation between the firing of the two cells. Note that it is impossible to derive this information by using only responses averaged over trials.

deviation away from their respective average responses, by computing the correlation coefficient (r) between the number of spikes in each cell for a specified time window in the odor response. This correlation coefficient yields 1 for perfect correlation, 0 for independent variables, and –1 for perfect anticorrelation. We then plotted this correlation coefficient both as a function of time in the trial and of lag between the time windows for both cells. The significance of the numbers thus obtained was

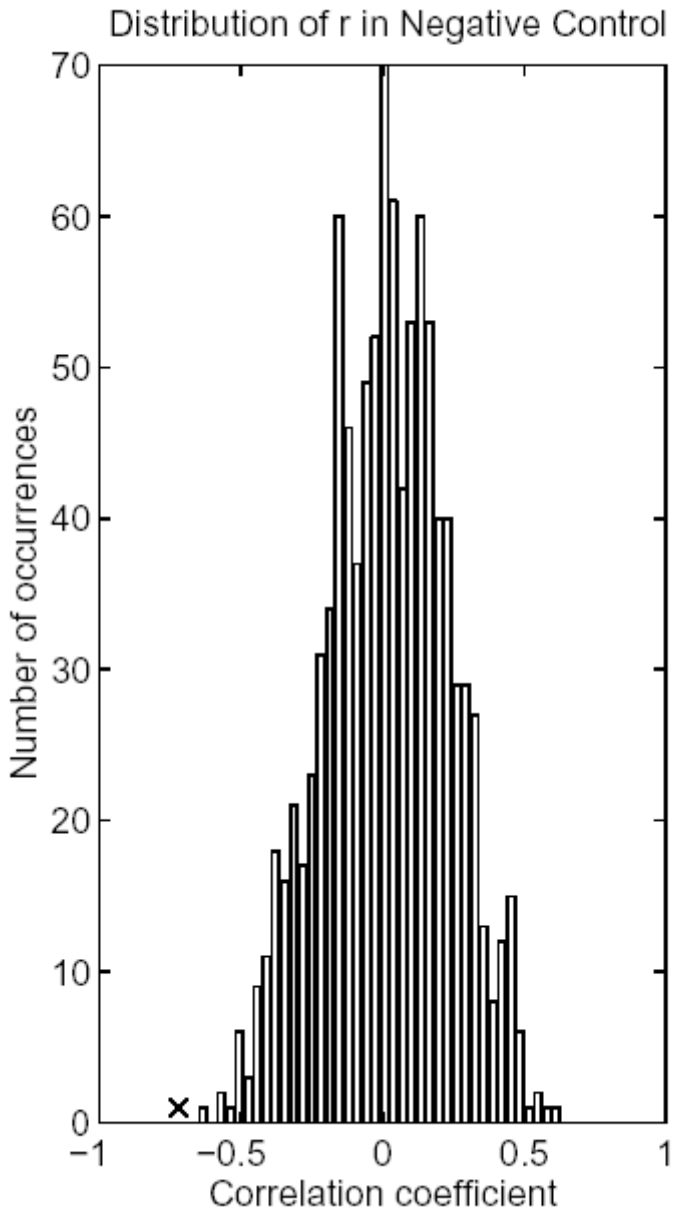


Figure IX.3. The covariation in firing rates across cell pairs is highly significant. Histogram of correlation coefficients between the two cells in Fig. IV.2 obtained with 100,000 random rearrangements of trial order in one of those cells, showing that the probability of obtaining by chance a correlation magnitude as large as or greater than that in Fig. IV.2 is less than 0.0005.

assessed by performing the same computation for a large number of data sets in which the order of the trials had been randomly shuffled. This manipulation preserves the average responses (PSTH)

intact, but eliminates all simultaneity between the records of the two cells.

$$r = \frac{\langle (\#\text{ spikes cell 1}) \cdot (\#\text{ spikes cell 2}) \rangle - \langle \#\text{ spikes cell 1} \rangle \cdot \langle \#\text{ spikes cell 2} \rangle}{\left[\langle (\#\text{ spikes cell 1})^2 \rangle - \langle \#\text{ spikes cell 1} \rangle^2 \cdot \left(\langle (\#\text{ spikes cell 2})^2 \rangle - \langle \#\text{ spikes cell 2} \rangle^2 \right] \right]^{1/2}}$$

Significance of a measurement among multiple comparisons

Determining the statistical signficance of the correlation timecourses shown in the figures in this appendix is not trivial, because each time series presents multiple measurements. This is explained in the general discussion below, together with some proposed solutions for different cases.

Let us assume we are making an experiment in which we are trying to decide if any of a set of measurements under experimental condition A is different from the corresponding set of measurements made under the negative control condition B. For example, we might be trying to tell whether a spike train in response to a stimulus is different from spike trains under a control condition where there is no stimulus; one measurement might be the # of spikes within a time window, the set might be given by a series of successive windows. The null hypothesis is that both sets are indistinguishable: that the response to A is no different from that to B.

If making many measurements and reporting *any* deviation from the value expected given the null hypothesis, the probability of finding *a* value equal or greater than X given the null hypothesis is *not* given by the probability of finding that value if one were performing a single measurement --even if one uses the probability for the measurement that actually gave the deviation.

$$P = p(\text{any of N measurements} >= X) = 1 - p(\text{all N measurements} < X)$$

If all N measurements are independent, we can write

$$P = 1 - p(M_1<X).p(M_2<X)\ldots(p(M_N>=X)$$

Furthermore, if all N measurements are drawn from the same distribution and thus have the same p-values

$$P = 1 - [1-p(\text{one measurement} >= X)]^N$$

Thus if we are making two independent measurements, and we wish to be as strict as if we were doing a single measurement and using a p-value of 0.05, we must make P above equal to 0.05:

$$0.05=1-(1-\text{p value from a single comparison to be reported as significant given multiple comparisons})^N$$

p value from a single comparison to be reported as significant given multiple comparisons = Nth root of 0.05 , and thus report any measurement where p<1-(1-0.05)^2.

But what if we do not know if the measurements are independent, or if we suspect they are not? There are at least two possible empirical solutions:

A. If one has plenty of experiments under the experimental condition (condition A): one can use a subset of the experiments (e.g. half of them) to identify measurements that one believes may be significant, and then formulate a specific hypothesis that those are significant, that one can then test with the rest of the experiments and for which one can obtain a p-value without accounting for multiple comparisons, since there is only one hypothesis being tested.

B. If one has access to plenty of negative controls, but few under the experimental condition (A) so that partitioning the set of condition A experiments is impractical:

1. Calculate the p-value, without accounting for multiple comparisons, for the mea-

surement whose multiple comparisons (MC)-corrected p-value we wish to obtain.

2. For each of the other measurements, calculate, without accounting for multiple comparisons, the level of the measurement that constitutes the same p-value calculated in (1).

3. By analyzing a large number of trials for the negative control, compute the probability that a *set* of measurements (one of each type, e.g. one in each time window) in the negative control yields *any* value more extreme than the corresponding levels calculated in (2). This will be the multiple-comparisons-corrected p-value for the result obtained in the original measurement used in (1).

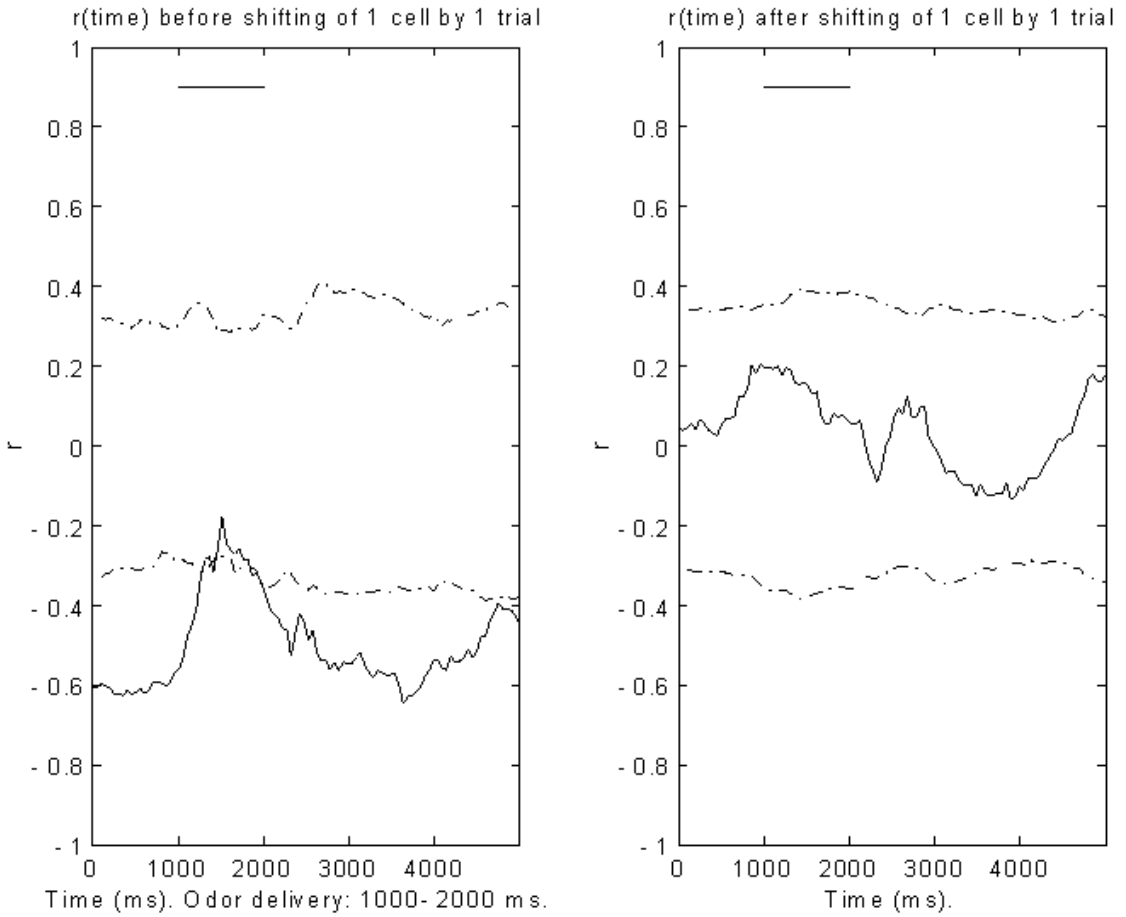


Figure IX.4. There is no correlation between successive trials of a pair of cells. The plot on the right was obtained by shifting all trials of one cell by one trial position. The disappearance of the correlation found on the left-hand plot illustrates the transient nature of thesecorrelations. The dashed lines represent the p=0.05 significance value, measured from the variance of r for sets of randomly shuffled trials (see above).

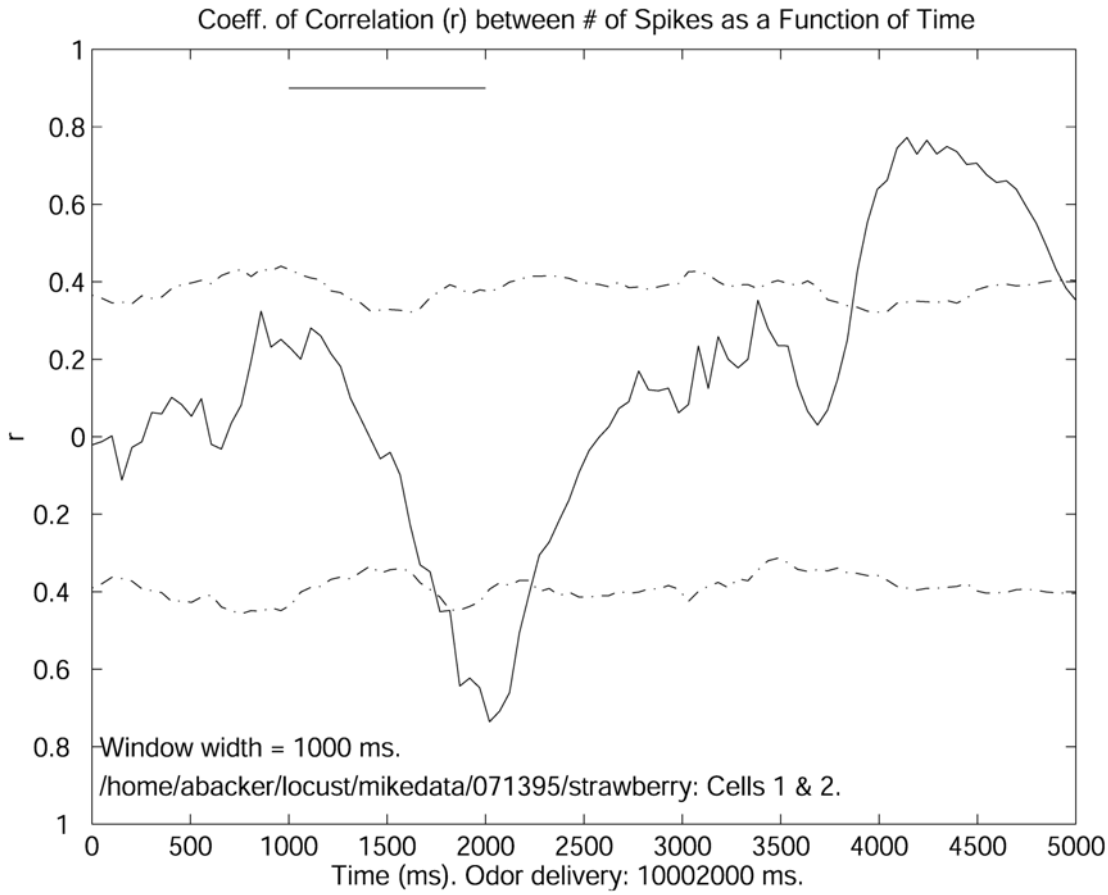


Figure IX.5. Correlations can be positive or negative and occur during specific periods of the odor response. Plot of the correlation coefficient (r) of the spikes in two PN's as a function of time in the trial. Each point represents r for a single time window centered around the point.

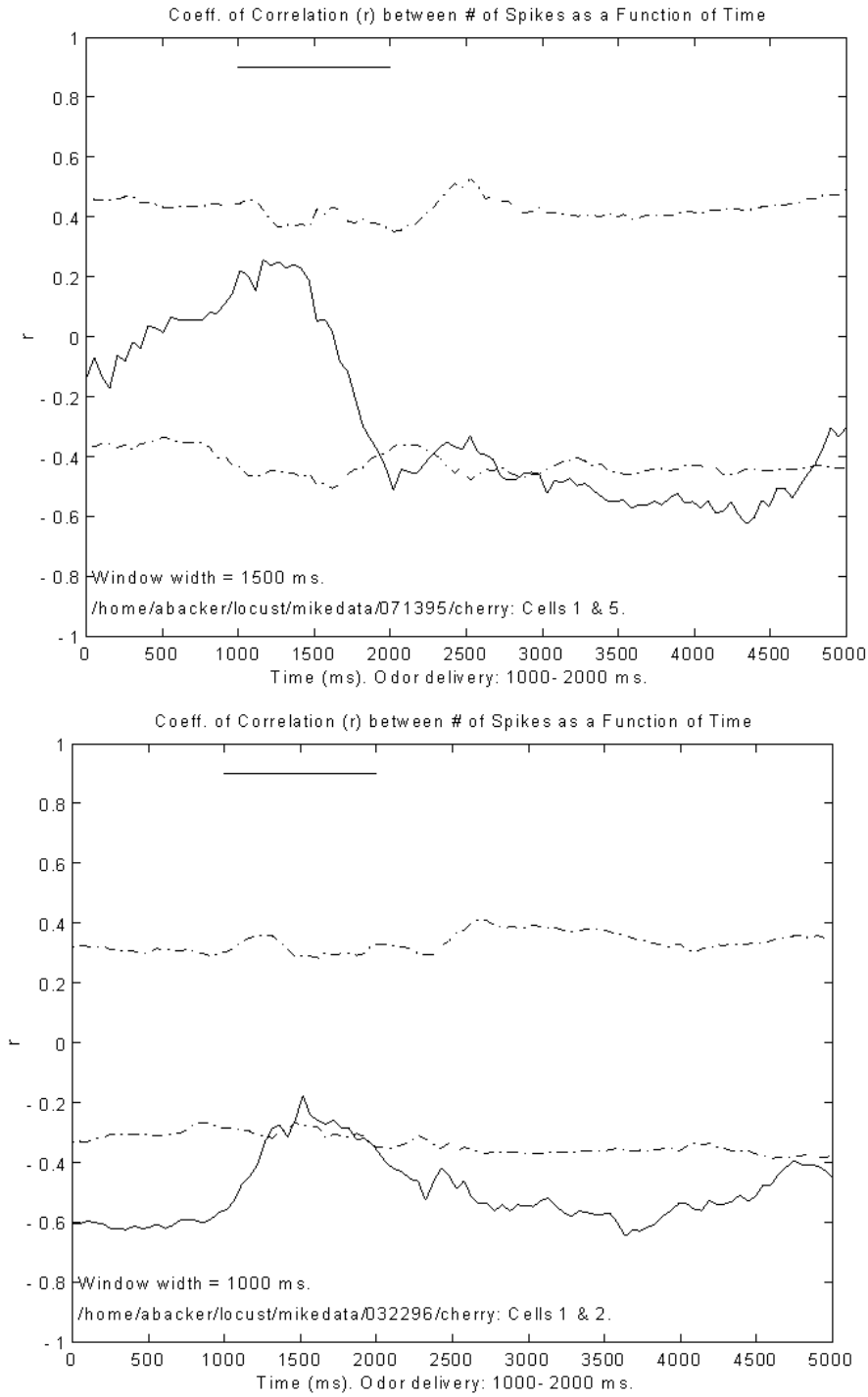


Figure IX.6. Cell pairs can correlate (top) or decorrelate (bottom) in response to an odor. A decorrelation is a change from coordinated variability to independent responses. The two neurons in the bottom plot had highly correlated activity before and after odor presentation, but suffered a marked decrease in their correlation during the duration of the odor pulse.

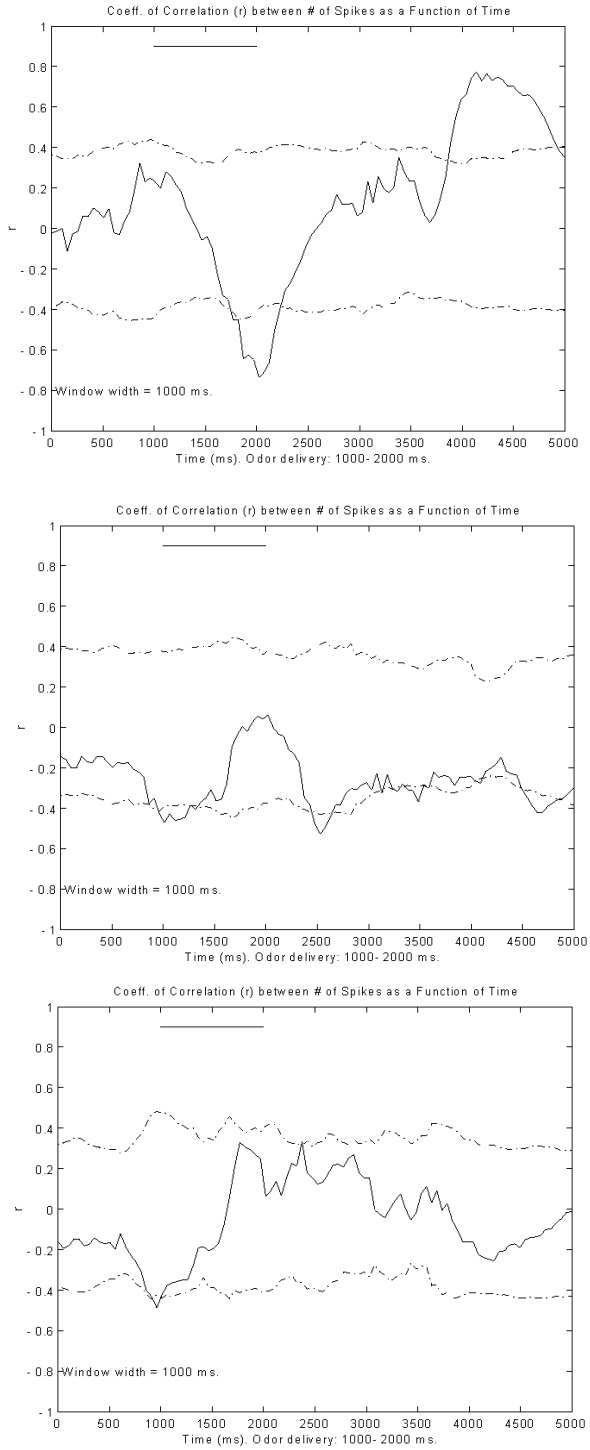


Figure IX.7. Correlations occur within specific neuronal populations. Recordings from triplets of neurons show that correlations and their timecourses are specific to a particular neuronal pair. The triplet above exhibits 3 different correlation patterns in the 3 pairs that compose it (top, center and bottom).

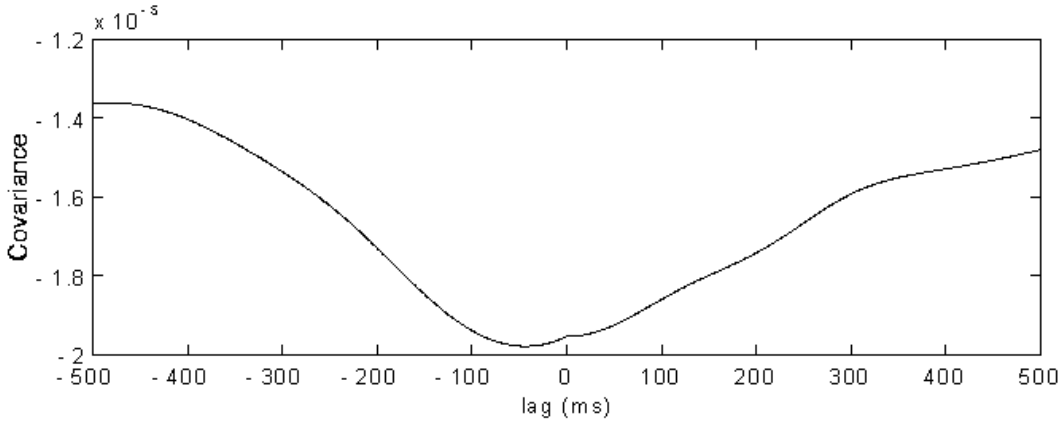


Figure IX.8 The cross-correlation for this pair of PNs exhibits a timeconstant of several hundred milliseconds and a peak at roughly 50 ms lag (i.e. 1 oscillation cycle). The plot above shows the covariance between the number of spikes in one time window for each cell, as a function of the lag of one cell's window with respect to the other cell's.