Processing and plasticity within the dual olfactory pathway in the honeybee brain

Verarbeitung und Plastizität
des dualen olfaktorischen Wegs im Gehirn der Honigbiene

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Zusammenfassung

In ihrem natürlichen Lebensraum sind Lebewesen mit komplexen und hoch dynamischen olfaktorischen Reizen konfrontiert, was eine schnelle und zuverlässige Duft-Verarbeitung sowohl bei Insekten als auch bei Wirbeltieren erfordert. Im visuellen oder auditorischen System wird sensorischer Eingang durch Parallel-Verarbeitung schneller und effektiver an höhere Gehirnzentren übertragen und verarbeitet. Im olfaktorischen System ist generell und im speziellen über Parallel-Verarbeitung noch wenig bekannt. Die Honigbiene stellt jedoch mit ihrer hoch spezialisierten Duftwahrnehmung und ihrem Duft und Pheromon gesteuerten Verhalten aufgrund ihrer Neuroanatomie einen besonderen Modelorganismus für die Erforschung der Duftverarbeitung und insbesondere der olfaktorischen Parallel-Verarbeitung dar.

Honigbienen besitzen „duale olfaktorische Bahnen“, die ausschließlich in Hymenopteren (Bienen, Ameisen, Wespen) als Merkmal ausgeprägt sind. Gebildet werden sie aus zwei spiegelbildlichen Projektions-Neuronen (PN) Ausgangs-Trakten, die das erste olfaktorische Verarbeitungs-Zentrum, den Antennal-Lobus (vergleichbar mit dem Olfaktorischen Bulbus der Wirbeltiere, OB) mit sekundären Verarbeitungszentren, dem Pilzkörper (MB) und dem lateralen Horn (LH) verbinden. Der mediale Antennal-Lobusprotocerebrale Trakt (m-APT) innerviert erst den MB und dann das LH, der laterale Trakt (l-APT) projiziert in umgekehrter Reihenfolge. Der MB ist bei Orientierung, Lernen und Gedächtnis involviert, über die Funktion des LH ist in der Biene noch wenig bekannt.

Über die Neuroanatomie und Evolution dieser dualen Bahnen wurde viel geforscht, die Funktion und damit ihre Physiologie sind allerdings noch unzureichend aufgeklärt. Die vorliegende Dissertation beschäftigt sich deshalb mit der Duftverarbeitung im Bienengehirn und im Speziellen mit Parallelverarbeitung in der Olfaktorik. Für die Aufklärung wurde eine neu entwickelte und in dieser Dissertation beschriebene Messmethode etabliert (**1. Kapitel**). Mit Hilfe dieser Messapparatur (Multi-Unit Recordings) ist es jetzt das erste Mal möglich, hoch-zeitaufgelöst simultan aus beiden Trakten mehrere PNs auf unterschiedliche Düfte hin zu untersuchen.

Das **2. Kapitel** beschäftigt sich eingehender mit der Analyse von Duftanworten der PN. Die Hauptergebnisse sind, dass beide Trakte auf alle getesteten Düfte regieren, dies aber mit unterschiedlichen Charakteristiken tun. Da gezeigt wurde, dass beide Trakte ähnlichen olfaktorischen Eingang erhalten, die Trakte aber Düfte unterschiedlich verarbeiten, stellen diese Ergebnisse ein erstes Indiz für Parallelverarbeitung im olfaktorischen System der Biene dar. M-APT

PN reagieren mit Zeitverzögerung und duftspezifisch, d.h. selektiver auf Düfte. Dagegen reagieren l-APT PN vergleichsweise schneller und duft-unspezifischer auf die in dieser Arbeit verwendeten Düfte. In einigen PN beider Trakte wurde gefunden, dass die PN Duft-Identitäten über duftspezifische Antwort-Latenzen abgebildet werden können.

Um Aufschluss über die Gesamtdynamik der PN zu gewinnen, wurden l- und m-APT PN Antworten über weite Duftkonzentrationen (10^{-6} bis 10^{-2}) hin untersucht (**3. Kapitel**). Die PN reagierten mit linearen und nicht-linearen Korrelationen. Zudem sind in den meisten Fällen l-APT PN bei schwachen Duftkonzentrationen sensitiver. Die Antwort-Latenz ist zur Duftkonzentration in beiden Trakten negativ-proportional.

Alternative Kodierungsmöglichkeiten und die Ausarbeitung der Hypothese, dass die dualen Bahnen eine Koinzidenzverschaltung auf die nächst höheren Neurone, die Kenyon Zellen (KC), bilden könnten, wird im **4. Kapitel** behandelt. Dazu zeigen Kreuz-Korrelationsanalysen und synchrone Antwortmuster aus beiden Trakten, dass prinzipiell Düfte auch über Zeit-Kodierung verarbeitet werden können. Generell zeigt sich, dass die dualen olfaktorischen Bahnen eine verbesserte Duftkodierung gegenüber einem Trakt gewährleisten.

In einem weiteren Ansatz wurde die alterskorrelierte Plastizität der inhibitorischen GABAergen (gamma-Aminobuttersäure) Innervation im Pilzkörper der Biene während der Adult-Reifung bestimmt (**5. Kapitel**). Inhibition ist für olfaktorische Kodierung sehr wichtig. Eine fast dreifache Reduktion in der Gesamtmenge von GABA wurde während der Adult-Reifung in beiden Zielregionen der dualen olfaktorischen Bahn gleichermaßen gefunden. Dieser Effekt wurde mit einer insgesamt halbierten GABA Innervierung ebenfalls im visuellen Innervationsgebiet des MB gefunden. Die Ergebnisse passen gut in das derzeitige Verständnis von Adultplastizität der Pilzkörper in der Honigbiene, in denen eine Ausdünnung (Pruning) präsynaptischer Endigungen von PN und ein Auswachsen von KC-Dendriten beschrieben wurde.

Aus den neuroanatomischen und physiologischen Eigenschaften der dualen olfaktorischen Bahnen lässt sich schlussfolgern, dass Düfte sowohl über Raten- als auch Zeit-Kodierung bis hin zu Koinzidenz-Verschaltungen verarbeitet werden können. Zudem zeigen derzeitige Arbeiten über analoge Ausgangs-Trakte im OB von Wirbeltieren, dass Parallelverarbeitung im olfaktorischen System ein allgemeines Kodierungsprinzip über weit entfernte Taxa zu sein scheint.

Abstract

In their natural environment animals face complex and highly dynamic olfactory input. This requires fast and reliable processing of olfactory information, in vertebrates as well as invertebrates. Parallel processing has been shown to improve processing speed and power in other sensory systems like auditory or visual. In the olfactory system less is known about olfactory coding in general and parallel processing in particular. With its elaborated olfactory system and due to their specialized neuroanatomy, honeybees are well-suited model organism to study parallel olfactory processing.

The honeybee possesses a unique neuronal architecture - a dual olfactory pathway. Two mirror-imaged output projection neuron (PN) pathways connect the first olfactory processing stage, the antennal lobe (analog to the vertebrates olfactory bulb, OB), with the second, the mushroom body (MB) known to be involved in orientation and learning and memory, and the lateral horn (LH). The medial antennal lobe-protocerebral tract (m-APT) first innervates the MB and thereafter the LH, while the other, the lateral-APT (l-APT) projects in opposite direction.

The neuroanatomy and evolution of these pathways has been analyzed, yet little is known about its physiology. To analyze the function of the dual olfactory pathway a new established recording method was designed and is described in the **first chapter** of this thesis (multi-unit-recordings). This is now the first time where odor response from several PNs of both tracts is recorded simultaneously and with high temporal precision.

In the **second chapter** the PN odor responses are analyzed. The major findings are: both tracts responded to all tested odors but with differing characteristics. Since recent studies describe the input to the two tracts being rather similar, the results now indicate differential odor processing along the tracts, therefore this is a good indicator for parallel processing. PNs of the m-APT process odors in a sparse manner with delayed response latencies, but with high odor-specificity. PNs of the l-APT in contrast respond to several odor stimuli and respond in general faster. In some PN originating from both tracts, characteristics of odor-identity coding via response latencies were found.

Analyzing the over-all dynamic range of the PNs both l- and m-APT PNs were tested over a large odor concentration range (10^{-6} to 10^{-2}) (**3. chapter**). The PNs responded with linear and non-linear correlation of the response strength to the odor concentration. In most cases the l-APT is

comparatively more sensitive to low odor concentrations. Response latency decreases with increasing odor concentration in both tracts.

Alternative coding principles and elaboration on the hypothesis whether the dual olfactory pathway may contribute coincidental innervation to the next higher-order neurons, the Kenyon cells (KC), is subject of the **4. chapter**. Cross-correlations and synchronous responses of both tracts show that in principle odors may be coded via temporal coding. Results suggest that odor processing is enhanced if both tracts contribute to olfactory coding together.

In another project the distribution of the inhibitory neurotransmitter GABA (gamma-aminobutyric acid) was measured in the bee's MB during adult maturation (**5. chapter**). GABAergic inhibition is of high importance in odor coding. An almost threefold decrease in the total amount of GABAergic innervation was found during adult maturation in the l- and m-APT target region, in particular at the change in division of labor during the transition from a young nurse bee to an older forager bee. The results fit well into the current understanding of brain development in the honeybee and other social insects during adult maturation, which was described as presynaptic pruning and KC dendritic outgrowth.

Combining anatomical and functional properties of the bee's dual olfactory pathway suggests that both rate and temporal coding are implemented along two parallel streams. Comparison with recent work on analog output pathways of the vertebrate's OB indicates that parallel processing of olfactory information may be a common principle across distant taxa.

I General Introduction

Biology of the Honeybee

The European honeybee, *Apis mellifera L.*, is a holometabolous insect within a group of up to 20,000 species of Hymenoptera, derived about 100-120 Mio years ago during the Cretaceous (Grimaldi and Engel, 2005). Besides ants, social wasps and termites, honeybees are fascinating representatives of the group of eusocial insects. Social insects are classified in terms of cooperative brood care, division of labor and overlapping generations within a colony (Wilson, 1971). Although the majority of bee species is solitary, the social system of the honeybees *Apis mellifera* can reach a high degree of organization and complexity with colonies of up to 70,000 individuals.

Honeybees show age dependent polyethism (Rösch, 1925; Robinson, 1992). Freshly hatched bees become the worker caste that fulfills nursing and cleaning duties inside the hive. After 2-3 weeks worker bees mature into foragers that start to fly outside, collect pollen as a protein source for larvae feeding and search for nectar providing energy for workers and foragers (Seeley, 1982). Honeybee societies can respond to environmental and internal changes with adaptive behavior (Robinson, 2002). And honeybees possess a well-established pheromone communication system which ensures intraspecific communication in many behavioral contexts (Sandoz, 2011). For example the queen-mandibular-pheromone has been shown to steer behavior alterations in the worker cast (Le Conte et al., 2001; Vergoz et al., 2007; Le Conte and Hefetz, 2008). Besides pheromone communication, honeybees use their olfactory system for orientation and foraging which necessitates plasticity and cognition (Lindauer, 1949; von Frisch, 1967; Seeley, 1985; Giurfa and Capaldi, 1999; Laska et al., 1999; Galizia and Menzel, 2000a; Menzel, 2001, 2012a; Slessor et al., 2005; Giurfa, 2007; Le Conte and Hefetz, 2008; Galizia and Rössler, 2010).

The olfactory sense -

a comparative survey of insects and mammals

The olfactory world is full of a multitude of odorant molecules emerging from likewise many odorant sources. The understanding of how nervous systems code this complex molecular space into neuronal activity forming a percept in the brain is far from being understood and represents a fascinating research area. Humans have more difficulties in evaluating olfaction compared to other modalities like audition or vision, modalities that have easily accessible measurable units like wavelength of light or the intensity or frequency of sound. Instead, olfaction is a question of proportion and concentration of molecules in the air, it is multidimensional in nature making it far more complicated to analyze. Yet remarkably, animals ranging from insects to mammals are well-equipped to receive and perceive odor information and are able to distinguish thousands of odors and odor mixtures as well as minute odorant concentrations. Noteworthy, vertebrates and insects share similar principles in odor detection and processing, even though their common origin is very distant in phylogeny (Hildebrand and Shepherd, 1997; Laurent, 1999; Strausfeld and Hildebrand, 1999; Ache and Young, 2005; Kay and Stopfer, 2006; Wilson and Mainen, 2006).

The olfactory system of vertebrates and insects can be subdivided by the sequence in which odors are received and processed, resulting in three different levels. The first part is the odor reception at the periphery; the second step is the processing of the converged information in either the antennal lobe (AL) of the insect or the olfactory bulb (OB) of vertebrates. Finally, the third step represents the information processing in higher order brain centers for sensory association, learning and memory (Menzel et al., 2006; Galizia, 2008; Mori and Sakano, 2011).

Olfactory sense of vertebrates

To start with the olfactory periphery, as a common principle odors bind to olfactory receptor proteins (ORs) that are housed in enlarged dendritic parts of olfactory receptor neurons (ORNs) situated in the nasal cavity on olfactory cilia in the olfactory epithelium (Mombaerts et al., 1996; Mombaerts, 2004). Olfactory binding proteins (OBPs), present in the epithelium fluid, likely support increased sensitivity or alternated turnover of odor binding (Tegoni et al., 2000). Thereby an

individual OR is sensitive to more than one odorant (Buck, 1996). The ORs are expressed from about 400 genes in humans and about 1200 genes in mice (Ache and Young, 2005; Benton, 2006). ORs in vertebrates share are G-protein coupled receptors (GPCRs). These GPCRs are also related to veromonasal receptors of the assessor olfactory bulb likely responsible for pheromone detection (Buck and Axel, 1991; Mombaerts, 2004). When odorants bind to the GPCRs, signal transduction cascades activate heterotrimeric G-proteins that activate downstream second messenger pathways. To date there are two second messenger pathways known that lead to signal transduction in the ORNs by opening cation channels and producing action potentials; the cyclic AMP pathway (Adenylatcylase mediated) and the phosphoinositol pathway (Phospholipase C mediated) (Ache and Young, 2005). Odor induced action potentials (APs) are transmitted along the axon to the first processing stage, the olfactory bulb (OB). ORNs terminate into small, discrete spheroidal subcompartments, the so called glomeruli, whereby each ORN expressing the same OR targets the same glomerulus (Buck, 1996; Malnic et al., 1999; Mombaerts, 1999). The olfactory glomeruli are situated in the glomerular layer of the OB and are innervated by several ORNs expressing the same OR leading to a strong convergence in connectivity of about 5000 ORNs that synapse onto 1 glomerulus in rodents (Shepherd, 2004). Interestingly, recent studies show ORNs with the same OR target glomeruli in two separated places of the main olfactory bulb forming a mirror-image symmetric pair (Nagao et al., 2000; Savic et al., 2000; Belluscio and Katz, 2001; Lodovichi et al., 2003; Yan et al., 2008; Bozza et al., 2009). Additionally, it has been shown in mice that different individuals have glomeruli with the identical odor sensitivity and, across species, mouse and rat share many glomeruli with apparently identical odor tuning, arranged in a similar layout (Soucy et al., 2009).

All glomeruli of the OB are interconnected via intrinsic interneurons, the periglomerular neurons (PG). These cells are inhibitory and release GABA (γ-aminobutyric acid), dopamine or both (Shepherd, 2004). Beyond that, PG cells receive direct excitatory input from ORNs or can synapse onto other PG cells leading to a complex odor-dependent activity pattern in the OB (Friedrich and Korsching, 1998; Niessing and Friedrich, 2010). Due to the molecular space of each OR that is distinct from each other, different odors activate different ORNs and thus activate different glomeruli leading to a combinatorial odor processing (Laurent, 1999; Kay and Stopfer, 2006). After the convergence of the odor elicited ORN activity into spatio-temporal combinatorial glomeruli codes, the olfactory information is guided to higher brain centers by mitral and tufted cells (M-/T-cells), that are interconnected with each other or via granule cells in the external plexiform layer of

the OB (Wachowiak and Shipley, 2006). Remarkably, in comparison to other modalities, olfactory information is directed to emotional and cognitive processing stages like the amygdala, enthorinal cortex and to the olfactory cortex directly after the first processing stage (Wilson and Mainen, 2006) supporting the idea that the olfactory sense represents an ancient sensory modality.

Olfactory sense of insects - periphery

In insects as well as in vertebrates odors bind to olfactory receptor proteins (ORs) that are housed in the dendritic part of olfactory receptor neurons (ORNs) situated in cuticular specializations, the olfactory sensillae (Esslen and Kaissling, 1976), on the surface of the insect antenna (Schneider, 1957; Lacher and Schneider, 1963; Vareschi, 1971). A varieties of sensilla types is found in insect antennae that, in addition to olfactory stimuli, also respond to humidity (Riegert, 1960), carbon dioxide (Stange and Stowe, 1999), or temperature (Ruchty et al., 2010). Most olfactory sensillae are hair like sensilla (sensilla trichodea), sensilla basiconica, and poreplate sensillae (sensilla placeodea) (Lacher and Schneider, 1963; Esslen and Kaissling, 1976; Shanbhag et al., 2000; Nishino et al., 2009) containing variable numbers of ORNs (Kelber et al., 2006). Similar to vertebrate ORNs insect ORNs in most cases express only one olfactory receptor (Vosshall et al., 1999; Vosshall, 2000). Recent studies on insect genomes found ~60 OR genes in different fly species (Robertson et al., 2003; Benton, 2006; Nozawa and Nei, 2007), ~80 OR genes in mosquitoes (Hill et al., 2002), ~160 in honeybees (Robertson and Wanner, 2006), and 100-160 genes were found in ants (Bonasio et al., 2010). But recently even ~350 functional OR have been sequenced in the red harvester ant *Pogonomyrmex barbatus* (Smith et al., 2011).

In general individual OR are sensitive to more than one odor (Hallem and Carlson, 2006), but exceptions exist with OR that are tuned to respond to just one odor (Nakagawa et al., 2005), in particular in the case of pheromones (Hildebrand and Shepherd, 1997; Kanzaki et al., 2003). Additionally, as the concentration of the odorant increases, the amount of responding ORs likewise increases (Hallem and Carlson, 2006) and thus indicates a combinatorial odorant code due to recruitment of responding ORs (Kay and Stopfer, 2006). The OR in insects form a large and highly divergent gene family (Vosshall et al., 1999), which little homology to the vertebrates ones. Insect OR and the related gustatory receptors (GRs) (Robertson et al., 2003) form an arthropod specific chemoreceptor superfamily (Hansson and Stensmyr, 2011). Insects OR genes encode for a seven transmembrane-domain protein that is comparable to the vertebrate G-protein coupled receptors (GPCRs) but with inverted configuration. Additionally, insect ORs are always found as heterodimeric

complexes composed of a single ligand-binding OR and an OR co-receptor called ORCO (Vosshall and Hansson, 2011), first described in *Drosophila* and formerly known as OR83b (Vosshall et al., 2000; Larsson et al., 2004; Benton, 2006). Recent work has gained knowledge about the physiology of the OR-ORCO dimers (Sato et al., 2008; Wicher et al., 2008) with ionotropic characteristic most likely serving rapid transduction of high odor concentration while a metabotropic pathway allows highly sensitive odor detection (Hansson et al., 2010). Common principles between insect and mammals can be found in olfactory binding proteins (PBPs) and other supporting proteins that probably provide an increased sensitivity of odor molecule reception in either the sensilla lymph of insects (Fan et al., 2011) or the olfactory mucus in vertebrates (Tegoni et al., 2000).

Olfactory Sense of Insects - Primary processing stage, the AL

From the olfactory periphery ORNs extend axons to the first olfactory processing stage in the insect nervous system, the antennal lobe (AL) analog to the olfactory bulb (OB) of vertebrates (Hildebrand and Shepherd, 1997; Ache and Young, 2005; Wilson and Mainen, 2006). In the AL the ORNs terminate in small spheroidal compartments, the glomeruli (Boeckh and Tolbert, 1993; Hildebrand and Shepherd, 1997; Mori et al., 1999; Laurent, 2002; Mombaerts, 2004; Kay and Stopfer, 2006; Wilson and Mainen, 2006) after they undergo a sorting processes at the entrance of the AL (Rössler et al., 1999). In the fly *Drosophila melanogaster*, it is well established that glomeruli receive input from receptors expressing one OR, and all ORNs expressing a particular OR terminate in one glomerulus (Vosshall et al., 2000; Couto et al., 2005; Fishilevich and Vosshall, 2005). This is confirmed by the correlation of the number of OR genes and the corresponding amount of glomeruli across different insect species, as shown for flies with ~49 AL glomeruli and about 60 OR genes (Robertson et al., 2003; Nozawa and Nei, 2007), and the honeybee with ~160 OR genes and ~160 AL glomeruli (Robertson and Wanner, 2006; Forêt et al., 2007). Although within the insect taxa there are also few species that are known to lack glomeruli (Kristoffersen et al., 2008) or show organizations in the AL different from the typical glomerular structure (Ignell et al., 2001).

It has been shown that each glomerulus is odor specific (Galizia et al., 1999c). Glomeruli thus represent olfactory processing units with unique response profiles (Hallem and Carlson, 2006) that interact via a local network inside the AL. The arrangement and number of glomeruli within the AL are largely species specific (Martin et al., 2011), and the total number of glomeruli vary strongly

from about 49 glomeruli in *Drosophila* (Laissue et al., 1999) to 160 in the honeybee and up to 460 glomeruli in the carpenter ant *Camponotus floridanus* (Arnold et al., 1985; Galizia et al., 1999c; Zube et al., 2008). In *Drosophila* it is also know that different olfactory sensilla types map onto specific groups of glomeruli in the AL (Couto et al., 2005; Fishilevich and Vosshall, 2005). In Hymenoptera ORN axons are grouped into sensory tracts that enter the AL and innervate certain clusters of glomeruli (Arnold et al., 1985; Flanagan and Mercer, 1989a; Galizia et al., 1999c; Kirschner et al., 2006; Zube et al., 2008; Nishino et al., 2012).

In insect ALs also contain glomeruli with specified functions. Especially moth species have a specialized glomerular cluster of enlarged, neighboring glomeruli, the macroglomerular complex (MGC), which is only present in males and processes sex-pheromone information (Christensen and Hildebrand, 2002). In female moth distinct dimorphic regions of specialized glomeruli were found that presumably are involved in processing of volatiles important for oviposition (Rospars and Hildebrand, 2000; Reisenman et al., 2009). In ants an increased volume of special glomeruli, so called macroglomeruli, can be found that are responsible for trail pheromone processing and express a phenotypic plasticity (Kleineidam et al., 2005; Kelber et al., 2009, 2010; Kuebler et al., 2010; Nakanishi et al., 2010; Stieb et al., 2011). Sexual dimorphic differences of the AL can also be found in social Hymenotpera. Male honeybee drones have a lower number of glomeruli compared to female workers and queens (Sandoz, 2006; Groh and Rössler, 2008; Zube and Rössler, 2008). The network of glomeruli is interconnected by local interneurons. These LNs mainly innervate multiple glomeruli inside the AL and can be subdivided in 'homo' LNs that have their neuritis almost equally distributed along the glomeruli, or 'hetero' LNs that have their neuritis only in smaller numbers of specific glomeruli, hence LNs vary in their morphology (Flanagan and Mercer, 1989a; Stocker et al., 1990; Christensen et al., 1993; Sun et al., 1993; Seki and Kanzaki, 2008; Chou et al., 2010; Dacks et al., 2010; Seki et al., 2010; Reisenman et al., 2011; Meyer and Galizia, 2012). LNs also differ in terms of their neurotransmitter and neuropeptide content (Bitterman et al., 1983; Barbara et al., 2005; Nässel and Homberg, 2006; Carlsson et al., 2010; Dacks et al., 2010; Kreissl et al., 2010; Galizia and Kreissl, 2012). In most insects LNs are inhibitory and have GABA or histamine as transmitters (Galizia and Kreissl, 2012) However, in flies excitatory LNs have been found in addition to inhibitory LNs, (Shang et al., 2007; Huang et al., 2010). Most LNs produce odor elicited Na^+ spikes (Christensen et al., 1993; Galizia and Kimmerle, 2004; Wilson and Laurent, 2005; Meyer and Galizia, 2012) except for Orthoptera (locust) LNs (MacLeod and Laurent, 1996) and a subset of Blatteria (cockroach) LNs (Husch et al., 2009) responding to odors with subthreshold membrane oscillations and voltage

activated calcium currents. The AL local networks are thought to also mediate learning-related plasticity (Daly et al., 2004; Thum et al., 2007; Rath et al., 2011). Learning-induced volume increases of individual glomeruli have recently been reported for the honeybee (Hourcade et al., 2009; Arenas et al., 2012).

Olfactory Sense of Insects - Central Processes

From the AL, projection neurons (PNs) guide the pre-processed olfactory information from the AL to higher brain areas, in particular the mushroom bodies (MB). These brain structures are involved in sensory association, learning, memory and orientation (De Belle and Heisenberg, 1994; Mizunami et al., 1998b; Strausfeld et al., 1998; Heisenberg, 1998, 2003; Menzel, 2001; Davis, 2004, 2011; Gerber et al., 2004; Gerber and Stocker, 2007; Giurfa, 2007; Masse et al., 2009; Kahsai and Zars, 2011). The olfactory information is also transferred to the lateral protocerebrum, in particular the lateral horn (LH) (Mobbs, 1982; Hildebrand and Shepherd, 1997; Yasuyama et al., 2003). The LH was suggested to be involved in more 'stereotyped' olfactory information processing (Jefferis et al., 2007) and subsequent modulation of sensori-motor pathways (Mizunami et al., 2004). However, its function is still not fully understood (Hallem and Carlson, 2006; Martin et al., 2011; Sandoz, 2011). Recent recordings in locust described ten different types of LH neurons that are globally responding to odors. There functions are suggested to support extraction of general stimulus features such as odor intensity, mediation of bilateral integration of sensory information, and integration of multimodal sensory stimuli (Gupta and Stopfer, 2012). Whereas the ORNs provide olfactory input into the AL, PNs represent the output of the AL in a way that spatial glomerular activity patterns are translated into a cross-fiber- activity patterns of PN activation (Laurent, 2002).

There are two different types of PNs that can be subdivided depending on their innervation pattern inside the AL. Uniglomerular PNs (uPN) have dendritic branches in exclusively one glomerulus whereas multiglomerular PNs (mPNs) innervate multiple glomeruli (Galizia and Rössler, 2010). The PNs are combined in axonal tracts that share the same routes along the CNS. Recently the nomenclature of these PN tracts was changed from 'antennocerebral tracts' (ACT) into 'antennal lobe protocerebral tract' (APT) (Galizia and Rössler, 2010). With this nomenclature the relative position of the tracts was standardized in medial, medio-lateral and lateral (Galizia and Rössler, 2010). Although APTs of different insects share similar projections they do not necessarily employ homology or similar physiological function (Galizia and Rössler, 2010). The medial APT (m-APT) is the most prominent tract across insects. It can already be found in basal insects like Archeognatha and

Zygentoma (Strausfeld, 2009; Strausfeld et al., 2009) and it is present in flies (where it mainly consists of uPNs) (Diptera), moths (Lepidoptera), ants, bees, wasps (Hymenoptera), and cockroaches (Blatteria) (Figure I.1). In these species the m-APT targets the MB input sites (MB calyx) and the LH. In the locust the m-APT consists of only multi-glomerular PNs (Ignell et al., 2001) which innervate small glomerular shaped structures in the AL that do not necessarily resemble ordinary glomeruli of other insects (Anton and Hansson, 1996). The lateral APT (l-APT) is composed of the multiglomerular PN axons in moths and cockroaches and a mixture of uni- and multiglomerular PNs can be found in flies (for review see Galizia and Rössler, 2010).

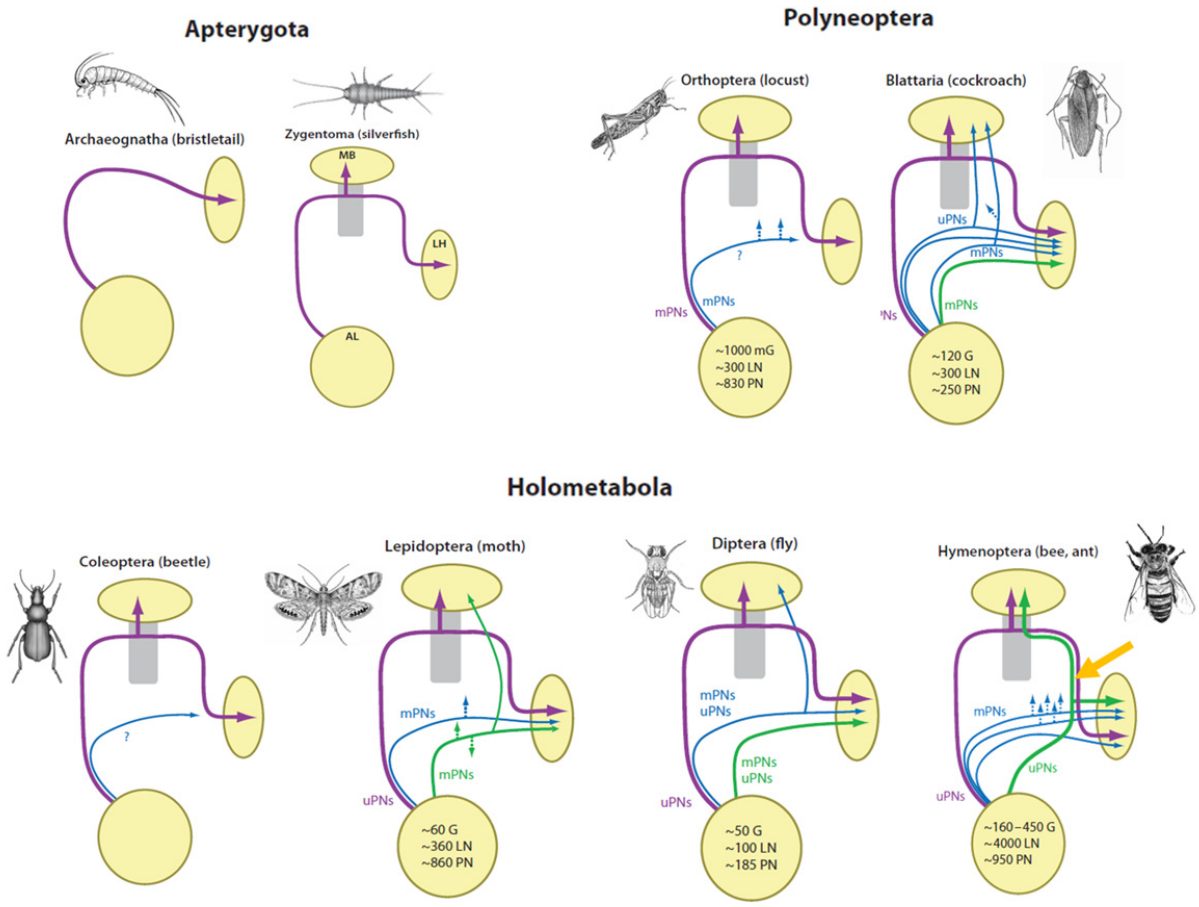


Figure I.1: adapted from (Galizia and Rössler, 2010). Comparison of olfactory tracts in insects. Schematic comparison of multiple parallel projection neuron (PN) pathways connecting the antennal lobe (AL) with secondary, higher olfactory neuropils in the protocerebrum, the mushroom bodies (MBs), and the lateral horn (LH). Whenever known, the approximate range of the numbers of olfactory glomeruli (G, glomeruli; mG, microglomeruli, small glomerulus-like structures in the case of the locust AL), local interneurons (LN), and uni- and multiglomerular projection neurons (uPN, mPN) are indicated in the AL.

In ants and bees uniglomerular PNs from one hemi-lobe of the AL project via the l-APT, constituting a distinct, parallel olfactory system (see next paragraph) thus far described only in the Hymenoptera (Abel et al., 2001; Kirschner et al., 2006; Zube et al., 2008; Nishikawa et al., 2011; Rössler and Zube, 2011). This system is also present in some basal, solitary Hymenoptera and was suggested to be an adaptation to complex olfactory environments and behaviors (Rössler and Zube, 2011).The l-APT in cockroaches and flies terminates in the LH, but in moths, bees, and ants it also projects to the MBCs. Finally, additional, minor tracts compose a medial- lateral APT (ml-APT) that carries axons of uni- or multiglomerular PNs and projects in species-specific patterns to the MBs, LH, or other regions in the protocerebrum (for review see Galizia and Rössler, 2010; Martin et al., 2011).

The olfactory pathway of the Honeybee

Already Kenyon (1896) started to be investigate in detail the neuroanatomy of the honey bee and this interest is preserved until today (Vowles, 1955; Lacher and Schneider, 1963; Mobbs, 1982; Homberg, 1984; Sun et al., 1993; Rybak and Menzel, 1993; Kloppenburg, 1995; Grünewald, 1999a; Strausfeld et al., 2000; Strausfeld, 2002; Fahrbach et al., 2003; Kirschner et al., 2006; Groh and Rössler, 2008; Nishino et al., 2009; Rössler and Zube, 2011). The peripheral odor detection in the honeybee starts at the antenna where the ORNs are situated in the sensilla. Besides prevalent hair like sensilla (sensilla trichoidea) and sensilla basiconica (see paragraphs above) well-known also from other insects, in Hymenoptera especially poreplate sensilla (sensilla placodea) are the main olfactory sensilla that house 5-35 ORNs, each of them innervating a different glomerulus in the AL (Kelber et al., 2006). The ORNs are organized further into two antennal nerves of the flagellum (Nishino et al., 2009) and enter the AL in segregated sensory tracts (T1 - T6). Four tarcts (T1-T4) are olfactory sensory tracts (Galizia et al., 1999a; Kirschner et al., 2006; Figure I.2). Each tract terminates in a distinct set of neighboring glomeruli which are further segmented into the dorsal (T2,3,4) and ventral (T1,3) hemilobes (Kirschner et al., 2006). Honeybees show a sexual dimorphism in the amount of glomeruli in the AL. Workers and queens possess 160-174 glomeruli (Arnold et al., 1985; Flanagan and Mercer, 1989a; Groh and Rössler, 2008), whereas drones have a smaller number of olfactory glomeruli (116), possess four enlarged glomeruli, and lack sensilla basiconica (Arnold et al., 1985; Sandoz, 2006; Groh and Rössler, 2008; Nishino et al., 2009).

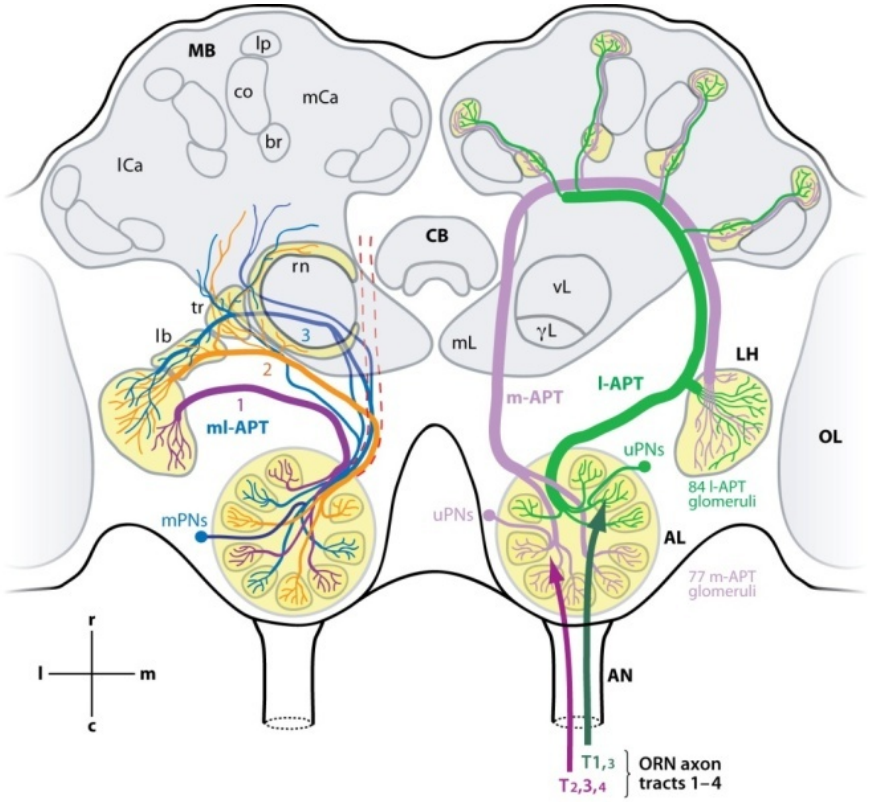


Figure I.2: (from Galizia and Rössler, 2010) Parallel systems in the honeybee olfactory system. Schematic overview of the dual olfactory system in honey bees (right hemisphere). Four antennal nerve (AN) sensory input tracts (T1–4) (note that font size is indicative of the difference in tract size) innervate the olfactory glomeruli in the antennal lobe (AL), as indicated on the lower right side. The left side of the schematic brain shows the projections of multiglomerular projection neurons (mPNs) along three mediolateral antennal lobe - protocerebral tracts (ml-APT 1–3), which innervate many glomeruli across the AL. Their target areas are the lateral horn (LH) and a lateral network in the lateral protocerebral lobe with the ring neuropil (rn), triangle (tr), and lateral bridge (lb). The right side shows medial (m-) and lateral (l-)APTs, which comprise uniglomerular projection neurons (uPNs) receiving input from individual glomeruli in two hemilobes of the AL. The uPN axons from both tracts target specific regions in the basal ring (br) and lip (lp) of the mushroom body (MB)-calyces and separate domains in the LH. Further abbreviations: CB, central body; c, caudal; co, collar; l, lateral; m, medial; mL, medial lobe; ORN, olfactory receptor neuron; r, rostral; vL, vertical lobe; γL, gamma lobe.

In the AL the glomeruli are interconnected by approximately 4000 LNs that can be classified according to their anatomy (Sun et al., 1993; Meyer and Galizia, 2012), physiology (Flanagan and Mercer, 1989b; Fonta et al., 1993; Meyer and Galizia, 2012) or neurotransmitters (Galizia and Kreissl, 2012; Grünewald, 2012). These LNs have also been shown to partly be segregated within the AL hemilobes (Meyer and Galizia, 2012). After preprocessing via LNs (Wilson, 2011), projection neurons (PNs) finally transfer the information from the AL to higher brain centers, in particular the mushroom bodies (MBs) and the lateral horn (LH). The dual olfactory pathway consists of approximately 920 uniglomerular projection (output) neurons (PNs) (Rybak and Eichmüller, 1993;

Rybak, 2012). The PN axons form two separate output tracts, a medial- and lateral antennal-lobe protocerebral tract (m- and l-APT), which connect two sets of glomeruli in the two AL hemilobes with distinct target areas in the MBs and the lateral horn (Abel et al., 2001; Kirschner et al., 2006; Galizia and Rössler, 2010). The m-APT contains ~410 uPNs that innervate the MB calyx and the LH. The l-APT consists of about ~510 PN axons that first innervate the LH and then the MB input (Abel et al., 2001; Kirschner et al., 2006; Rybak, 2012). In the MBs the PN terminals synapse onto MB intrinsic neurons, the Kenyon Cells (KCs), and form so called microglomeruli (Ganeshina and Menzel, 2001). These were further analyzed with antibodies staining pre- and postsynaptic components (Frambach et al., 2004; Groh et al., 2004, 2006; Ganeshina et al., 2006). The MBs contain the KCs and form doubled cup shaped neuropils, the calyces (Vowles, 1955; Strausfeld, 2002). The calyces can further be subdivided into the lip receiving olfactory input from the two APTs (Homberg, 1984; Abel et al., 2001; Müller et al., 2002; Krofczik et al., 2009), the collar receiving visual input (Mobbs, 1982; Gronenberg, 1986; Ehmer and Gronenberg, 2004), and the basal ring receiving input from both modalities in Hymenoptera (Gronenberg and López-Riquelme, 2004). The KCs that form the MBs have their dendritic arborizations within the calyx and their axonal terminals project along the peduncle and finally bifurcate to form the horizontal and vertical MB lobes (Mobbs, 1982, 1984; Strausfeld et al., 2000; Strausfeld, 2002; Farris et al., 2004). The KCs constitute with about 184,000 neurons per hemisphere more than 40% of the total amount of neurons in the honeybee brain (Rössler and Groh, 2012). Within the MB, feedback neurons interconnect the peduncle and the lobes with the input region, the calyx, via GABAergic extrinsic MB neurons (Grünewald, 1999a, 1999b) which have synaptic profiles in the MB microglomeruli (Ganeshina and Menzel, 2001). About 400 extrinsic output neurons (ENs) transmit KC information from the lobes and the peduncle to other regions of the protocerebrum (Mobbs, 1982; Rybak and Menzel, 1993) and have recently been identified to be involved in learning and memory (Okada et al., 2007; Haehnel and Menzel, 2010; Strube-Bloss et al., 2011). One single neuron, PE1, has been found that connects the pedunculus with the LH (Rybak and Menzel, 1993) and was shown to be involved in learning-related plasticity (Mauelshagen, 1993; Menzel and Manz, 2005; Okada et al., 2007). Finally centrifugal neurons have been labeled that project from the MBs to the AL (Kirschner et al., 2006). There is also one neuron identified, called VUMmx1 (ventrally unpaired median cell of maxillary neuromere1), that connects all neuropils of the olfactory pathway in the bee and is known to produce a associative memory trace (Hammer, 1993, 1997; Hammer and Menzel, 1995).

Parallel Processing

The brain has to compute incoming information from the outer world transferred by its sensory organs. To filter relevant information and to direct behavior, this process necessarily needs to work fast and reliably. One example of increasing processing speed in neuronal computation is provided at the periphery. For example the well-known lateral inhibition in the retina which minimizes information flow to the brain – as a tradeoff between necessary loss against spare information - already increases processing speed (Thorpe et al., 2001). Besides the periphery, which has been shown to increase in significance also in olfaction (e.g. Su et al., 2012), in vertebrates parallel up-stream pathways to the cortex increase neuronal processing capacity. Parallel pathways are able to code different tasks (Knudsen et al., 1987) and this is expected to support the speed and accuracy of processing (Nassi and Callaway, 2009).

As parallel streams process different tasks their input demands similarity which can be found in multiple repetitions of neuronal maps in the brain (Young, 1998; Rauschecker and Scott, 2009). Therefore Eric Young (1998) stated: “in most systems, there are multiple repetitions of the map at both subcortical and cortical levels. Because all or most of the receptor sheet is contained in each map repetition, each separate representation is a unit in a system of serial/parallel channels making up the overall system.”

Early investigations of parallel systems were conducted in the somatosensory system. For instance the discovery of different nerve fiber diameters supporting temporally segregated perception of pain and temperature in comparison to touch (Gasser and Erlanger, 1929). The most prominent examples come from the vertebrate visual system with its magno- (M) and parvocellular (P) pathways of the lateral geniculate nucleus that give input to segregated layers of the primary visual cortex V1 (Callaway, 2005; Lennie and Movshon, 2005). Within this pathway image information is subdivided into color (M) and spatial-temporal (P) information that finally can lead to differentiated perception (Livingstone and Hubel, 1988; Merigan and Maunsell, 1993). Segregated pathways of the visual system have also been found in insects ,in particular within the medulla with segregated pathways to higher brain centers (Ribi and Scheel, 1981; Fischbach and Dittrich, 1989; Douglass and Strausfeld, 1996; Strausfeld et al., 2006; Paulk and Gronenberg, 2008; Paulk et al., 2008, 2009). These pathways were demonstrated to divide incoming information into temporal resolution and color vision as well (Paulk and Gronenberg, 2008; Paulk et al., 2008, 2009; for review see Dyer et al.,

2010). Additionally color independent segregated channels for motion detection have been found in the fruitfly (Rister et al., 2007; Yamaguchi et al., 2008).

In the vertebrate auditory system parallel streams have been identified as well: the posterior parietal (the postero-dorsal-) pathway which is suggested to serve spatial processing in audition with the temporal (the antero-ventral-) pathway being hypothesized to contribute to identification of complex pattern or objects (reviewd in Rauschecker and Scott, 2009). In the insect auditory system parallel processing has been suggested in Acrididae (Orthoptera) where auditory information is transmitted from auditory receptor neurons onto projection neurons by information-channels either mediating information on pattern recognition or another mediating directional information. The information is separately transferred to the brain (Helversen and Helversen, 1995). Another example is the sense of electroreception in weak electric fish that show enormous parallel processing capabilities (Young, 1998). Four somatotopic maps of the electroreceptors of the body wall surface have been identified and show a clear separation into distinct behaviors linked to each map (Heiligenberg and Dye, 1982; Metzner and Juranek, 1997). In electroreceptive ictalurid catfish it is also interesting to mention that the electrosensory lateral line together with the mechanosensory lateral line and the auditory/acoustic system project from the periphery to similar brain regions, the torus semicurcularis, in parallel (Finger and Tong, 1984).

Compared to our knowledge on parallel processing in the above mentioned sensory systems, parallel processing in olfactory systems is far from being understood (Galizia and Rössler, 2010). Functional specializations of sensory processing within the olfactory system have only recently been appreciated. In mammals there are at least four olfactory processing areas: the main olfactory system, the vomeronasal system, the septal organ and the Grueneberg organ (Breer et al., 2006). Different subsystems are also present in amphibians (Manzini et al., 2002; Manzini et al., 2007), fish (Kotrschal, 2000) and insects (Galizia and Rössler, 2010; Martin et al., 2011), suggesting their adaptive value in olfaction. With the exception of fish, that do not possess a veromonasal organ but express veromonasal-type odorant receptor genes co-expressed in the same OE (Hashiguchi and Nishida, 2006).

Besides segregation of olfactory information into different neuronal maps, parallel streams have been found within different taxa (mammalians, fish, insects). Parallel olfactory processing means the extraction of different feature from a similar input. An example of specialized pathways was found in the fish olfactory system where it has been suggested that three parallel pathways project from

the glomeruli in the OE to higher olfactory centers. A medial olfactory tract mediates alarm reactions, the lateral part of the medial olfactory tract mediates reproductive behavior, and the lateral olfactory tract mediates feeding behavior (for review see Hamdani and Døving, 2007). In mammals, receptors sharing the same cell adhesion molecules project onto two mirror-image sensory maps with to date unclear functional relevance (Nagao et al., 2000). Besides this mirror-image projection, output streams from the OB to the olfactory cortex have been the matter of recent research on parallel processing. Recent studies analyzed the M/T cells (mitral and tufted cells) sequentially either by whole cell patch-clamp recordings (Fukunaga et al., 2012) or by juxtacellular recordings with sharp electrodes and consecutive neuronal staining and tracing (Igarashi et al., 2012), or with tungsten electrodes from the respective target areas (Payton et al., 2012). Due to these differing experimental approaches results are partly contradicting regarding olfactory processing and need to be verified by further studies. Additionally even in humans a parallel segregation of olfactory percepts along the ventral and dorsal pathway in the cortex has now been suggested (Frasnelli et al., 2012).

Insects are outstanding models to study parallel processing in olfaction. Especially honeybees with their dual olfactory pathway and a segregation of glomeruli into an almost fifty-fifty distribution (84 l-APT glomeruli, 77 m-APT glomeruli, Kirschner et al., 2006) within the AL are most suitable (Galizia and Rössler, 2010). The honeybee not only possesses a dual olfactory pathway with segregated hemilobes in the AL and mirror-imaged uniglomerular PN tracts, but also doubled MBs (reviewed in Galizia and Rössler, 2010; Martin et al., 2011). The evolution of the dual olfactory pathway has recently been linked to an early emergence in the hymenopteran evolution (Rössler and Zube, 2011), whereas the evolution of the doubled MB calyx was suggested to be associated with the emergence of a parasitoid lifestyle (Farris and Schulmeister, 2011). To investigate functional properties of the dual olfactory pathway in the honeybee, recent calcium imaging studies analyzed the input to the segregated AL (Carcaud et al., 2012; Galizia et al., 2012). In other studies sequential intracellular recordings were performed from the l- and m-APT PNs (Sun et al., 1993; Abel et al., 2001; Müller et al., 2002; Krofczik et al., 2009). An imaging study in ants linked processing of social odors to parallel processing in the AL (Brandstaetter and Kleineidam, 2011). Especially the analyses of the bee's l- and m-APT PN pathways was extended with calcium imaging studies at the level of presynapses of PNs in the MB calyx (Yamagata et al., 2009). These studies revealed partly contradicting odor extraction features along both pathways and partly suffer from low numbers of

recorded animals, PNs and/or odor stimulus repetitions. Most importantly, none of these studies was able to simultaneously record from both olfactory information streams.

Olfactory coding

Neuronal coding of a sensory input is characterized by the conversion of the stimulus input of the external world into neuronal activity. The CNS consecutively computes and filters the incoming information to finally translate the information in behaviorally relevant or irrelevant aspects, stores the information, and/or triggers behavior. Neurons contributing to the CNS are coarsely described as integrate and fire modules, incorporating an analog input - the post synapse that integrates neuronal excitation over several incoming presynapses - and the digital output - the axon which fires in an all-or-none phenomenal fashion (Gerstner and Kistler, 2002). The AP rate can directly be correlated to the neurons intrinsic excitation state. This is, of course, just a coarse simplification. There are both new and old discussions about implications of applied experiments in neuroscience and information theories to understand biological neural networks. Additionally, several feedback mechanisms by, for instance, neuromodulators or intrinsic molecular conditions can change the synaptic transmission. This includes, for example, Hebbian synaptic plasticity, which changes the rate of synaptic vesicles exocytosis at the presynapse, resulting in long-term potentiation (LTP) or long-term depression (LTD) (Caporale and Dan, 2008; Glanzman, 2010).

Regarding the olfactory system, different kinds of coding mechanisms and concepts for analyses were adapted from other modalities. To introduce olfactory coding, the following topics are considered: combinatorial code, non-linear processing within the AL, rate- latency- and temporal code, synchrony and sparsening.

Rate coding

The mechanism of rate coding assumes that neurons change their activity levels in response to a presented stimulus (Hubel and Wiesel, 1968). For determination of the neuron's activity level the firing frequency of APs is a valuable characteristic. Especially using electrophysiological methods, the rate of APs that change during stimulus presentation is a robust method to evaluate the read

out of neuronal activity. Rate code allows for analyses in populations and improves precision. In the olfactory system this is a well-known value and additionally facilitates comparison between studies; for example within the honeybee olfactory system (Abel et al., 2001; Müller et al., 2002; Krofczik et al., 2009). Additionally, rate coding also enables the implementation of tuning measures like signal-to-noise analyses (e.g. Strube-Bloss et al., 2011), lifetime and population sparseness (Willmore and Tolhurst, 2001), directional tuning (Hubel and Wiesel, 1968), and Euclidean distance as population measure (e.g. Krofczik et al., 2008).

Combinatorial code:

Focusing on the insect olfactory system, ORNs innervate glomeruli in the AL. As odorants activate different ORNs, these neurons will activate different glomeruli according to their OR specificity or molecular receptive range. Odors are mainly bouquets of several odorants thus an odor will lead to the activation of several glomeruli in an odor-specific pattern. Additionally, since different chemical characteristics - as for example vapor pressure and odorant concentrations - contribute to an odor bouquet, this will likely lead to a temporally diverse response onset in different ORNs. The elicited odor-specific glomerular pattern thus differs not only in its spatial distribution but also in temporal aspects as can be seen in the insect AL (Joerges et al., 1997; Galizia et al., 1999b, 2000; Sachse et al., 1999; Vosshall et al., 2000; Carlsson, 2002; Hallem and Carlson, 2006; Zube et al., 2008). This was termed a “combinatorial code” and was found in insects as well vertebrates (Sullivan et al., 1995; Friedrich and Korsching, 1998; Malnic et al., 1999; Rubin and Katz, 1999; Wachowiak and Cohen, 2001; Manzini et al., 2007; Bozza et al., 2009). Based on combinatorial coding mostly imaging data were discussed and linked to behavior (for example Zube et al., 2008; Strauch et al., 2012).

Non-linear processing within the AL

There are several different principles of odor processing in the AL. Gain control mechanisms mediate the relationship between ORN input to a glomerulus and PN output which is facilitated by non-linear transformation of odor evoked neuronal excitation (Bhandawat et al., 2007; Jarriault et al., 2010; Olsen et al., 2010; Kuebler et al., 2012; Meyer and Galizia, 2012). For instance, gain control prevents the olfactory system from saturation at high odor concentrations to maintain sensitivity. Gain

control is determined as the slope of the function of the relationship between odor concentration and PN output, which can be altered by neuromodulators (for review see Martin et al., 2011). Other components of non-linear odor coding are sharpening and broadening effects of the population output from the AL. Lateral inhibition can produce activity-dependent inhibition of glomeruli (Sachse and Galizia, 2002; Wilson and Laurent, 2005; Girardin et al., 2012). The inhibition leads to sharpening of the representation of the stimulus input to the output, which prevents weak but behavioral relevant stimuli from being underrepresented in the neural network. In analogy sharpening effects are well-known from the contrast enhancement of visual stimuli in the vertebrate retina (e.g. Cook and McReynolds, 1998).

On the other hand lateral excitation spreads activity throughout the AL, increasing the response of some PNs, and in some cases producing output in PNs that receive no input from their cognate ORNs (Shang et al., 2007; Chou et al., 2010). Consequently the population response in PN output layers is ‘‘broadened’’ with respect to the ORN input (Wilson et al., 2004; Schlief and Wilson, 2007). This excitation can be additionally mediated by electrical synapses (Huang et al., 2010; Yaksi and Wilson, 2010). Nevertheless it has to be mentioned that there are also labeled line systems known, which do not undergo any non-linear transformation, for example CO_2 processing in the fruitfly (e.g. Suh et al., 2004).

Latency coding

Response latency was suggested as an elaborated coding principle in other sensory systems and was suggested to increase processing speed (Thorpe et al., 1996). The concept of a latency code is simple in a way that a population of neurons is activated in a specific temporal order for one stimulus and in a different order for a different stimulus (Thorpe et al., 2001; Chase and Young, 2007; Nawrot, 2012). For example, the visual world of vertebrates has to be compressed to be sent from the retina to the cortex. Since the optic nerve has limited capacity for axon guidance, alternative coding principles from rate coding like latency coding and rank order coding were suggested to facilitate image processing in retinal ganglion cells (Thorpe et al., 2001; Gollisch and Meister, 2010).

In olfactory research temporal coding is discussed controversially for the vertebrate olfactory system (Bathellier et al., 2008; Junek et al., 2010; Smear et al., 2011). Spors et al. (2006) found odor-specific response latencies already at the level of OB input. In insects, temporal odor

representations, e.g. oscillations or synchrony in PN activity, was demonstrated in locusts and moths (Laurent, 1996; Stopfer et al., 1997; Christensen et al., 1998; Ito et al., 2009; Kazama and Wilson, 2009; Lei et al., 2009; Riffell et al., 2009b; Raman et al., 2010; Assisi et al., 2011). Quantitative and qualitative analysis of single PN activity suggest that response latencies may be odor specific (Müller et al., 2002, Krofczik et al., 2008; this can also be seen in figure 1 of Perez-Orive et al., 2002 and in figure 1,2 of Wilson et al., 2004). The same odor stimulus can evoke neuron-specific response latencies in a PN population suggesting that latencies are potentially used for coding odor identity (see Figure 2 in Wilson et al., 2004 and Figure 2 in Namiki and Kanzaki, 2008). This was proposed for olfactory processing in bees (Müller et al., 2002; Krofczik et al., 2009) and moths (Belmabrouk et al., 2011; Kuebler et al., 2011). It remains to be shown, whether the brain actually uses this parameter for odor recognition. In rodents, initial evidence was found to link odor response latency differences to behavior (Smear et al., 2011).

Temporal coding

While latency coding can be explained with the timing relationship between different odor responses, temporal coding focuses on individual neuronal events and their synchronous activity (Lei et al., 2002; Riffell et al., 2009a). Temporal coding is defined by Theunissen and Miller (1995) as an implementation of a rate or temporal encoding scheme to represent a continuously varying sensory signal, characterized by a one-to-one correspondence between the time of occurrence of a sensory event and the time of occurrence of a sensory corresponding neuronal response. For instance, cross-correlation analyses evaluate the relationship of spike trains of two neurons and has for example successfully been used in analyses of the cochleus nucleus (Aertsen et al., 1989) or analyses of thalamo cortical projections (Alonso et al., 1996).

Temporal coding is also existent in the olfactory system. Especially in the locust a temporal code was linked to synchronous activity of neuron populations inducing synchrony or oscillations (Laurent, 1999). Mainly AL PNs orchestrate their activity and induce LFP in the locust MB (Laurent et al., 1996). The behavioral relevance of oscillatory activity in olfaction, however, has remained unclear. A transition from rate code to temporal code at the output has been described in cockroach AL (Lemon and Getz, 2000). Temporal coding is the underlying mechanism for synchrony and oscillation coding and was linked to sparse coding via coincidence detectors.

Synchrony

Synchronized neural activity is believed to be essential for many brain functions, including neuronal development, sensory perception, and memory formation (Galán et al., 2006; Singer, 2009). In vertebrates almost 70 years ago the first odor-evoked OB oscillations have been found (reviewed in Kay et al., 2009). In analyzing the output neurons of the OB (mitral and tufted cells), in several species and experiments odorants evoke precisely sniff-locked activity (Kepecs et al., 2006; Bathellier et al., 2008; Carey and Wachowiak, 2011; Shusterman et al., 2011; Smear et al., 2011) and were shown to be driven by inhibition (Buzsáki and Chrobak, 1995; Friedrich and Laurent, 2004; Friedrich et al., 2004; Lagier et al., 2007; Abraham et al., 2010). In locusts local field potentials that oscillate in the range of 20Hz have been recorded and linked to odor processing (Laurent et al., 1996; MacLeod and Laurent, 1996; Perez-Orive et al., 2002). Studies in moths, bees and flies show that their PN are not fully entrained to their local field potential (LFP), since their PNs are able to fire faster than the respective LFP (Christensen et al., 2000, 2003; Lei et al., 2002; Wilson and Laurent, 2005; Turner et al., 2008; Tanaka et al., 2009). Additionally, their initial response to odors might already be coupled to the onset of odors which, in contrast to oscillation as a coding principle, support coincidence coding. In imaging experiments KCs have been shown to respond to odors only briefly after odor onset (Szyszka et al., 2005). In fact extrinsic MB neurons, only one synapse behind KCs, fire only shortly after odor onset in the ORN (Strube-Bloss et al., 2011). And these extrinsic MB neurons sometimes even elicit responses before the population of AL neurons has concluded the response to an odor stimulus (Strube-Bloss et al., 2012).

Sparsening

Sparse coding is suggested to promote accurate information storage (Olshausen and Field, 2004). Sparse coding is the activity or representation of stimuli of a rather small number of neurons within a population of neurons. For a different stimulus this is a different subset of neurons contributing to the sparse code. Regarding sparse coding in insects, already the neuroanatomical layout of the AL and MB resembles the underlying criteria to support sparse coding. From the glomeruli in the AL, PNs guide odor information to the MB. During the transition from the AL to the MB, PNs diverge and converge onto about ~180,000 KCs in the honeybee (Witthöft, 1967; Mobbs, 1982; Rybak, 2012) and about 4,000 KCs in the fruitfly (Aso et al., 2009). Different odors activate certain subsets of KCs. This sparse coding in the MB was found in several insect species - in the honeybee (Szyszka et al., 2005),

the locust (Perez-Orive et al., 2002, 2004; Brown et al., 2005; Broome et al., 2006; Jortner et al., 2007), moth (Ito et al., 2008), and in the fruitfly (Turner et al., 2008; Honegger et al., 2011).

In contrast to the divergent and convergent innervation of PNs onto KCs in the MB calyx, the convergence found in ORN innervating the glomeruli at the first processing stage is in the range of 1:5,000 in the mouse OB (Shepherd, 2004) and about 1:375 in the honeybee (Mobbs, 1982). This described convergence is more likely supporting signal-to-noise criteria for odor detection of low concentration (Rabinovich et al., 2006). In analogy, convergence in fruitflies from the lobula to glomerular-like structures in the lateral protocerebrum have been found (Otsuna and Ito, 2006) that were recently described to code different visual stimuli (Mu et al., 2012). These authors suggest that the optic glomeruli and the AL glomeruli resemble the same ancestral anatomical and functional ground pattern (Mu et al., 2012).

In the locust sparse coding is mediated by feedback inhibition from a unique, giant GABAergic neuron back-projecting from the output to the input of the MB (Papadopoulou et al., 2011). Additional GABAergic LH neurons were believed to mediate feed-forward inhibition to the MB calyx (Perez-Orive et al., 2002; Assisi et al., 2007; Jortner et al., 2007) until Gupta and Stopfer (2012) classified 10 different LH neurons but did not find any neuron type being responsible for the supposed feed-forward inhibition to the MB. In the honeybee sparseness is believed to emerge from GABAergic pre- and postsynaptic influences within the MB calyx (Szyszka et al., 2005) based on the finding that reciprocal microcircuits exist between the MB microglomeruli (Ganeshina and Menzel, 2001). On the other hand, due to KC recordings in the cockroach, spike frequency adaptation processes were found and believed to be sufficient for the phasic and sparse KC responses (Demmer and Kloppenburg, 2009; Nawrot, 2012).

Thesis Outline

Major Objectives

Having described the different olfactory systems in vertebrates and different insects comparatively and having described all the different coding principles that have been found in olfactory systems in the general introduction, it is now of high importance to observe how the dual olfactory pathway is in fact coding different odors. Consecutively many questions arise:

Will we record odor-elicited oscillations in the bee's brains, as has been mentioned earlier (Stopfer et al., 1997)? Or do we experience synchronous firing PNs as has been found in moth and locusts (Lei et al., 2002; Riffell et al., 2009a; Raman et al., 2010)? Will a new method be able to confirm former electrophysiological recordings of l- and m-APT PNs (Abel et al., 2001; Müller et al., 2002; Krofczik et al., 2009)? Is it possible to answer the question about the significance of a derived doubled PN-tract layout unique to Hymenopterans? Which are the advantages and disadvantages related to this layout? Does the dual olfactory pathway represent a delay-line model to possess coincidental innervation to KCs? What may be possible indications of this?

Aims and Questions:

Correspondingly, the thesis focuses in detail on the following questions and aspects:

Chapter 1:

- Which method is best-suited to gain knowledge of the function of the dual olfactory pathway?
- What are the setup objectives and the related requirements?

Chapter 2:

- Do the l- and m-APT code odors in a "dual segregated" fashion (different odors in different tracts) like pheromonal and general-odor subsystems in moths (Martin et al., 2011) or flies (Schlief and Wilson, 2007) - or in a "dual parallel" fashion (similar input, differential feature extraction) (Galizia and Rössler, 2010)?
- Are the l- and m-APT PNs capable to code odor-identity only via AP-rate or are there other coding principles involved?

Chapter 3:

- What is the over-all dynamic range of the l- and m-APT? Is one tract coding odors at different odor-concentrations differently?
- If the tracts code odors in parallel, is this also valid at different concentrations?

Chapter 4:

- Is there temporal processing within a single or across both APTs?
- Does the dual olfactory pathway provide olfactory processing via a coincidental delay-line model onto KCs?
- What are possible benefits of possessing a doubled PN tract layout?

Chapter 5:

- Is the olfactory system plastic? And does the significant GABAergic innervation change during adult maturation?
- Does the GABAergic innervation follow similar pruning and growing principles as has been shown for the MB microglomeruli in bees and desert ants (Groh et al., 2004, 2006; Stieb et al., 2010, 2012; Rössler and Groh, 2012)?

Thesis Outline

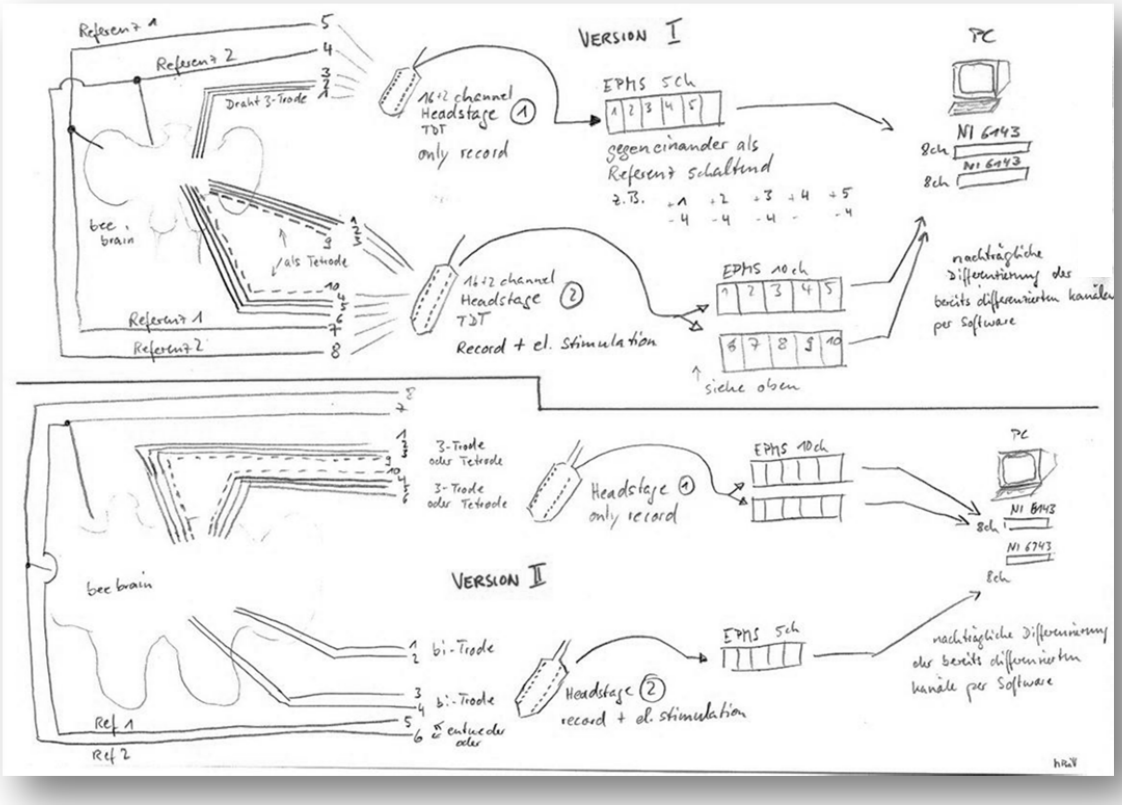
This dissertation is divided into 5 chapters, framed by a general introduction to the research topics at the beginning and a general discussion, conclusions together with an outlook at the end. Note that Figure numbers do not resemble chapter numbers!

The outline is as follows:

- A general introduction to honeybee biology with a comparison to vertebrates and other insects in terms of neuroanatomy and function of the olfactory system. Additionally, an introduction into parallel processing and olfactory processing in particularly is given.
- **1. Chapter**: A methodological chapter highlighting my work on the development of a new extracellular multi-channel, multi-unit recording setup with the description of the applied spike-sorting procedures and an outline of the construction and design of a 16-channel multi-unit recording setup with additional self-written data acquisition software.
- **2. Chapter**: Parallel processing in the dual olfactory system is analyzed in detail based on simultaneous multi-unit recordings of the l- and m-APT in 14 bees using a panel of olfactory stimuli. This chapter is mainly based on the publication: Brill et al. 2013 J Neurosci (in press) and describes the feature extraction and odor coding by PNs of the l- and m-APT. The results are highly supportive for parallel processing in the olfactory system of honeybees.
- **3. Chapter**: Concentration coding is the object of the third chapter based on results from simultaneous recordings in seven honeybees stimulated with three different odors over a wide range of odor concentrations.
- **4. Chapter**: This chapter investigates temporal coding, which is discussed based on recordings in individual bees. Hypothetical temporal coding strategies are discussed based on simultaneous recordings of PNs in both information streams of the dual olfactory pathway.
- **5. Chapter**: Finally, in the fifth chapter adult maturation of the GABAergic system is described and discussed with special regard to synaptic and behavioral plasticity in Hymenoptera.
- General discussion: This chapter represents a general discussion with final conclusions and suggests future perspectives and experiments based on these studies.

II - Chapter 1:

Multi-Unit Recording Setup



Introduction

Trying to understand the mechanisms and functions of the honeybee olfactory neuronal circuitry is challenging, especially regarding intracellular electrophysiology. The number of publications using sharp electrode recordings is rather sparse. Small sample sizes are mostly due to the small size of neurons in miniaturized nervous system (Chittka and Niven, 2009; Chittka and Skorupski, 2011). Certainly, sharp electrode recordings, if successfully applied, are of high quality and deliver highly important information about single neuron physiology together with high temporal precision (Hammer and Menzel, 1995, 1998; Grünewald, 1999b; Abel et al., 2001; Müller et al., 2002; Galizia and Kimmerle, 2004; Okada et al., 2007; Meyer and Galizia, 2012) and patch-clamp studies are able to gather knowledge about the intrinsic properties of olfactory neurons (Laurent et al., 2002; Grünewald et al., 2004; Wüstenberg et al., 2004; Dupuis et al., 2010). On the other hand, since optical recordings became feasible (Galizia et al., 1997; Joerges et al., 1997), analyses of spatial aspect in olfactory coding in general (Galizia et al., 1999c; Sachse, 2002; Sachse and Galizia, 2002), its concentration dependency (Sachse and Galizia, 2003; Yamagata et al., 2009), coding of odorant and mixtures (Deisig et al., 2006, 2010; Yamagata et al., 2009), pheromones (Sandoz, 2006; Sandoz et al., 2007), circuit interactions (Guerrieri et al., 2005b; Szyszka et al., 2005; Galizia and Szyszka, 2008), and learning dependent changes in coding (Faber et al., 1999; Galizia and Menzel, 2000a; Sandoz et al., 2002; Szyszka et al., 2005, 2008; Peele et al., 2006; Fernandez et al., 2009; Haehnel and Menzel, 2010; Rath et al., 2011) became possible. However, analyses of temporal properties need to combined with knowledge about spatial aspects to fully understand the power of olfactory coding (Scanziani and Häusser, 2009). Extracellular multi-unit recording became a favorable tool to achieve this, especially since activity of multiple neurons can be recorded in parallel at high temporal resolution. These methods were first applied in mammals (Rasmussen and Malven, 1981; Gray et al., 1995) and later also in insects (Christensen et al., 2000) (Christensen et al., 2000; Daly et al., 2004; Dacks et al., 2008; Ritzmann et al., 2008; Riffell et al., 2009b; Strube-Bloss et al., 2011). Substantial progress was achieved in the development and improvement of extracellular multi-channel recording techniques (Stevenson and Kording, 2011). This, for example, includes the development of new electrodes (Blanche et al., 2005; Du et al., 2009; Piironen et al., 2011; Viventi et al., 2011) or novel spike sorting and clustering algorithms (Lewicki, 1998; Pouzat et al., 2002; Buzsáki, 2004; Quiroga et al., 2004; Schmitzer-Torbert et al., 2005; Delescluse and Pouzat, 2006; Borghi et al., 2007; Joshua et al., 2007).

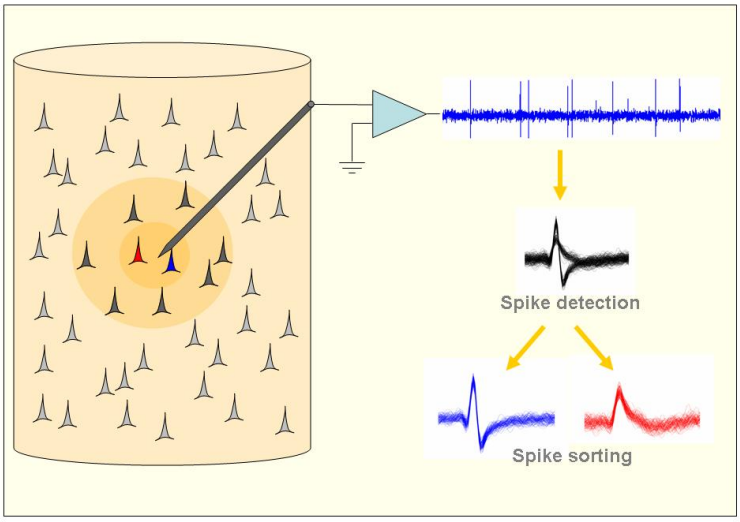


Figure I.1: Schematic drawing of an extracellular wire-electrode placed in vertebrate nervous tissue that gets amplified and bandpass filtered. The closer the recorded neurons are positioned to the electrodes, the stronger and better the signal gets (colored neurons). If neurons are farther away from the electrode tip, their spike shape diminishes hence their signal becomes less sortable constituting to a population responses or multi-unit activity (MUA) (dark grey neurons) until neurons are so far away that they become background noise (grey neurons); adapted from (Quiroga et al., 2004; Quiroga, 2007)

General methods of extracellular multi-unit recording techniques are well described elsewhere (Lewicki, 1998; Buzsáki, 2004; Harris, 2005; Quian Quiroga and Panzeri, 2009) and will be only briefly summarized (Figure II.1) (Quiroga, 2007). Each neuron has spikes of a characteristic shape, which is mainly determined by the amount and types of ion channels, the morphology of their dendritic trees and the distance and orientation relative to the recording electrode (Gold et al., 2006). Since wire electrodes have lower impedance due to their relatively large diameter compared to the recorded neurons, they record information from several neurons simultaneously (Gray et al., 1995). If more than one wire-electrode is used, the gained spike shapes from different neurons surrounding the electrodes will again elicit different spike shapes (Gold et al., 2006). Neurons that are closer to the electrode recording site give better signal-to-noise ratios. Spikes from neurons farther away cannot be detected and contribute only to background noise (Quiroga et al., 2004). Proper amplification of the neuronal activities the range of several micro Volt is a crucial prerequisite.

The purpose of my project was to analyze olfactory processing along two segregated neuronal pathways, the honeybee dual olfactory system. The system had previously been analyzed

anatomically in great detail (Abel et al., 2001; Kirschner et al., 2006; Zube and Rössler, 2008; Zube et al., 2008; Rössler and Zube, 2011). The physiology had been studied in previous work using either sequential single cell recording techniques (Abel et al., 2001; Müller et al., 2002; Krofczik et al., 2009), sequential calcium-imaging (Yamagata et al., 2009), or modeling approaches (Schmuker et al., 2011). To overcome the shortcomings of the previously used physiological methods, one main goal of my work was to develop a novel technique for simultaneous multi-unit recordings from both tracts. In the following I present the construction of a low noise 16-channel µV extracellular multi-unit recording setup suited for these simultaneous recordings from multiple projection neurons (PNs) in a small brain. Additional I will introduce self written data acquisition which was mainly accomplished under my guidance by Tobias Rosenbaum.

Objectives

Analyzing the function of the dual olfactory pathway in honeybees, it was necessary to record from both uniglomerular PN tracts as mentioned earlier. Additionally, the postsynaptic cells of the PN-tracts, the KCs, are of high importance since there is little knowledge about their physiology in honeybees besides their proposed relevance in learning and memory. Therefore the setup was primarily designed to record from the output of the AL inside the PN tracts, and future extracellular recordings of the KCs inside the MB calyx at the same time either using wire electrodes or glass electrodes (Figure II.2). The honeybee is stimulated with odors to verify the proper recording position of the electrodes inside the tracts. The design of the recording setup also included that at at these recording positions electrical stimuli can be applied for artificial time-controlled stimulation.

Setup requirements

The amplifier was designed to amplify 16 channels differentially up to 10k times together with high- and low-pass filtering combined with a short circuit layout and just view component parts to reduce noise amplification. For electrical stimulation, the amplifier needs to have inputs to guide stimulation patterns, and it also needs to integrate isolated stimulators and enable the steering of switchable headstages. For data-acquisition, requirements are computer cards that sample up to 16 channels simultaneously with high temporal precision with more than a 20kHz sampling rate, high

resolution of more than 12bit and data output to control the stimulation apparatus. The acquisition system needs to be PC-software independent including on board timing to reassure accurate recording.

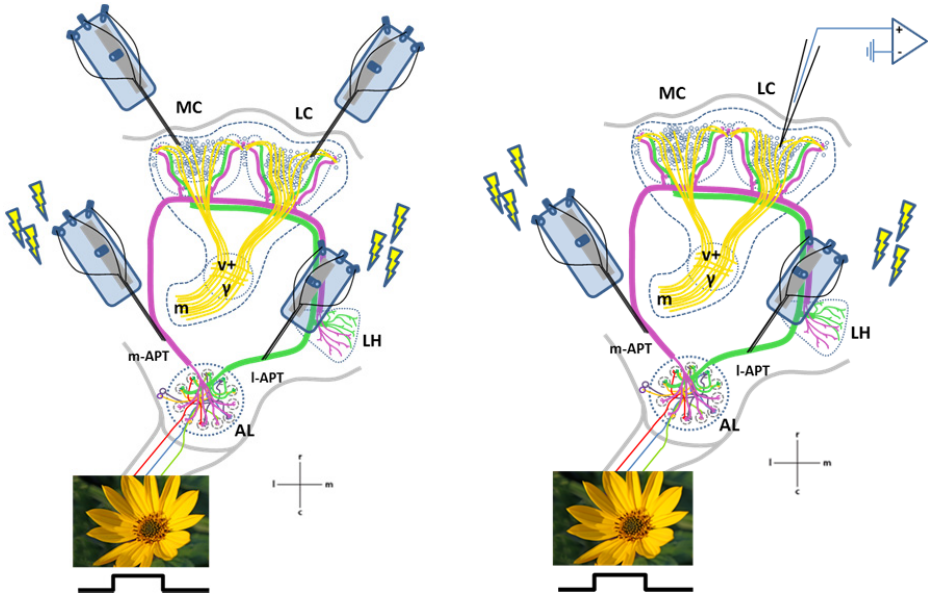


Figure II.2: Schematic drawing of the recording situation with wire-electrodes in the uniglomerular PN-tracts (l- and m-APTs) and wire-electrodes placed in the medial (MC) and lateral Calyx (LC) of the MB (left side) or extracellular recording of KC activity with a glass-electrode (right side). The setup was designed to provide either olfactory stimuli to the antennae or electrical pulses via the wire-electrodes directly into the l-and m-APT (indicated with yellow flashes).

Electrode Design

Several types of electrodes were tested for extracellular PN recordings. First I chose commercially available electrodes. Besides tungsten electrodes from Thomas Recordings (Germany i.e. used in (Plachta, 2003) or Science Products (Germany, bipolar metal electrodes), which are mainly used in vertebrates, I also tested silicon probes (Drake et al., 1988) provided formerly by the Centre of Neural Communication Technology (CNCT, Michigan, USA) and presently are offered by NeuroNexus (Michigan, USA). These electrodes are often used in vertebrates (i.e. Csicsvari, 2003) but were also used in insect such as the moth (Christensen et al., 2000; Lei et al., 2004; Dacks et al., 2008; Riffell et al., 2009a), locust (Stopfer et al., 2003; Brown et al., 2005; Broome et al., 2006) or cricket (Ritzmann

et al., 2008). Silicon probes are obviously well suited for rather large insects as the hawk moth or the locust. Honeybees are comparably small, hence recordings with these electrodes yielded in poor signal-to-noise ratios. Thin wire electrodes were used instead in several studies (i.e. in vertebrates (Reichinnek et al., 2010) or insects (Mizunami et al., 1998b; Perez-Orive et al., 2002, 2004; Hustert and Baldus, 2010)) and earlier extracellular recordings in honeybees were successfully applied with wire electrodes (Okada et al., 2007; Strube-Bloss et al., 2011). I tested tiny copper wires with a diameter of 15µm (polyurethane-coated copper wire with 15µm diameter, Elektrisola, Germany). Preparation of the electrodes was adopted from Ryuichi Okada (Mizunami et al., 1998a; Okada et al., 1999, 2007) and the methods was adapted with the help of Martin Strube-Bloss (Strube-Bloss et al., 2008). To obtain neuronal activity from more than one electrode, each electrode shank consisted of three copper wires glued together with melted dental wax and fixed on a glass capillary. The copper wires all together had a diameter of under 50µm (EM picture Figure II.3B). The glass capillaries with the fixed wires were attached to a small Plexiglas plate providing contact to the preamplifier.

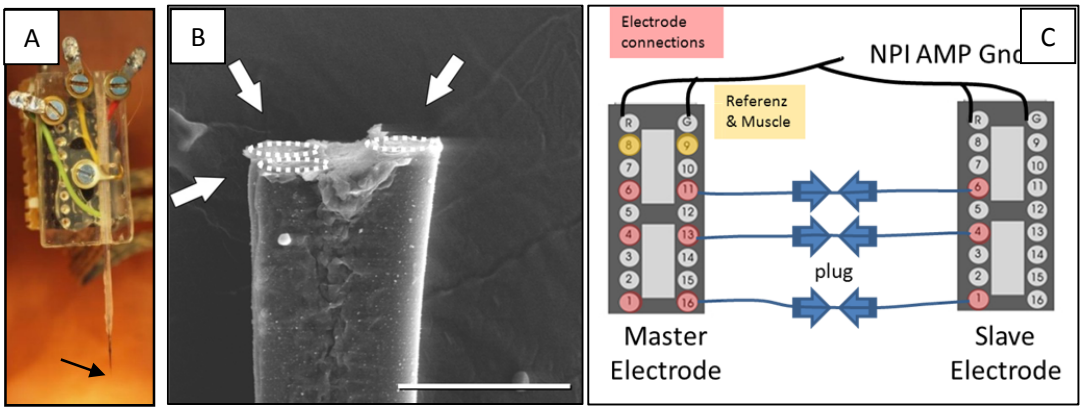


Figure II.3: Images of the wire electrodes used for recordings. A) Photograph of the assembled electrode holder with electrode shaft incorporating three copper wires (arrow). B) Raster electron microscope image of the wire electrode shaft consisting of 3 copper wires of 15µm diameter and additional 1µm polyurethan insulation (each electrode wire is indicated by white arrows and dotted circles) all glued together with dental wax; scale bar: 50µm. C) wiring diagram of the headstage connections to the electrodes. (electrode soldering in red, reference- and muscle-electrode connections in yellow).

Headstage

Since the dual olfactory pathway had to be recorded simultaneously, at least two electrode recordings from different places inside the brain at the same time were needed. To fulfill these requirements one electrode holder was constructed as “master” that was directly connected to the headstage, the other was designed as “slave” electrode holder. Wires from the “slave” electrodes were bypassed to the “master” electrode-holder, where also wires from the muscle and reference Ag-wire-electrodes were connected to. As described above it was planned to electrically stimulate the PNs. Therefore, a switchable headstage was purchased that either could record or electrically stimulate via the recording electrodes (SH16, Tucker-Davis-Technologies, USA). To toggle the headstage from recording to stimulation status we (Tobias Rosenbaum under my guidance) designed a program in LabView (v8.6, National Instruments) that has an easy to use interface and is called “headstager”. The headstage requires three connectors to the amplifier system. One connector provides the transmission of the recorded channels to the amplifier; another connector provides the headstage with the electrical stimulation patterns from a stimulus isolator and a third connector transmits the steering pattern for toggling. The steering pattern is divided in three (DIO) channels, one channel gives the underlying clock-pattern in the range of up to 25kHz, the second channel gives a digital latch signal and the third one is responsible for transmission of the digital toggle status with either 1 or 0 setting. Digital pulses are delivered as 5V TTL-pulse (Figure II.4).

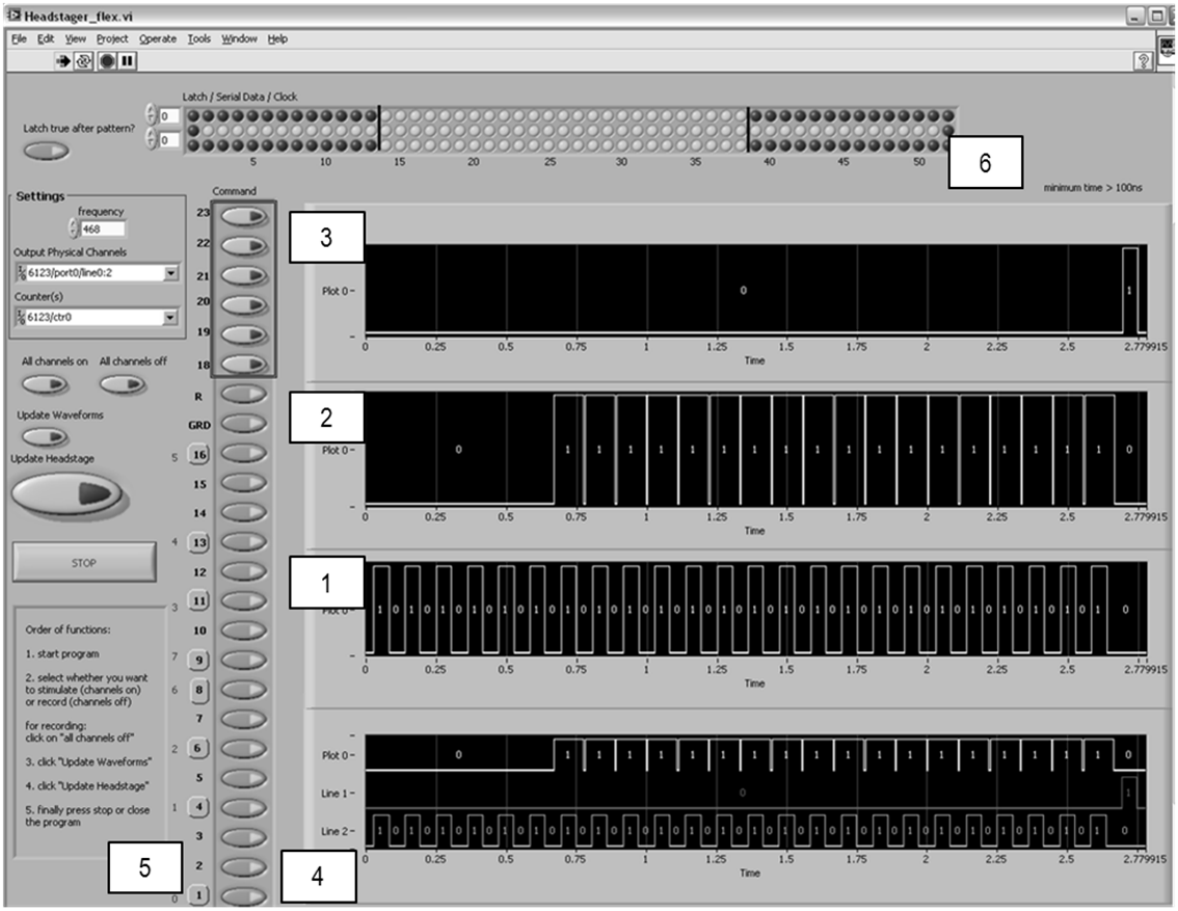


Figure II.4: Screenshot of the LabView program "Headstager" that toggles the headstage between recording and stimulation status on each single electrode channel. The headstage is steered by three channels providing different digital patterns, 1) provides a clock rhythm, 2) is the actual status information that can be either 1 or 0 for stimulation or recording status, respectively or finally 3) the latch signal that concludes the transmission to the headstage. With the toggles (5) each electrode channels can be chosen and the view-box (4) gives feedback about the transmitted pattern. (6) If fine adjustment is needed, all 52 bit of the TTL pulse of channel number 2 can be set.

Low noise µV amplifier system

The amplifier system consists of custom made amplifiers and connector modules, which the company of Hans Reiner Polder, NPI (Tamm, Germany) designed in close interaction with me. Since the switchable headstage had to be steered, the headstage is wired to a connection module INT-03M (Figure II.5(2), II.6A) that has toggles to freely switch the reference channels as being one of the electrodes or the input of the headstage, respectively. The connection module also provides the power supply for the headstage, transmits the digital steering pattern, and conveys electrical stimuli

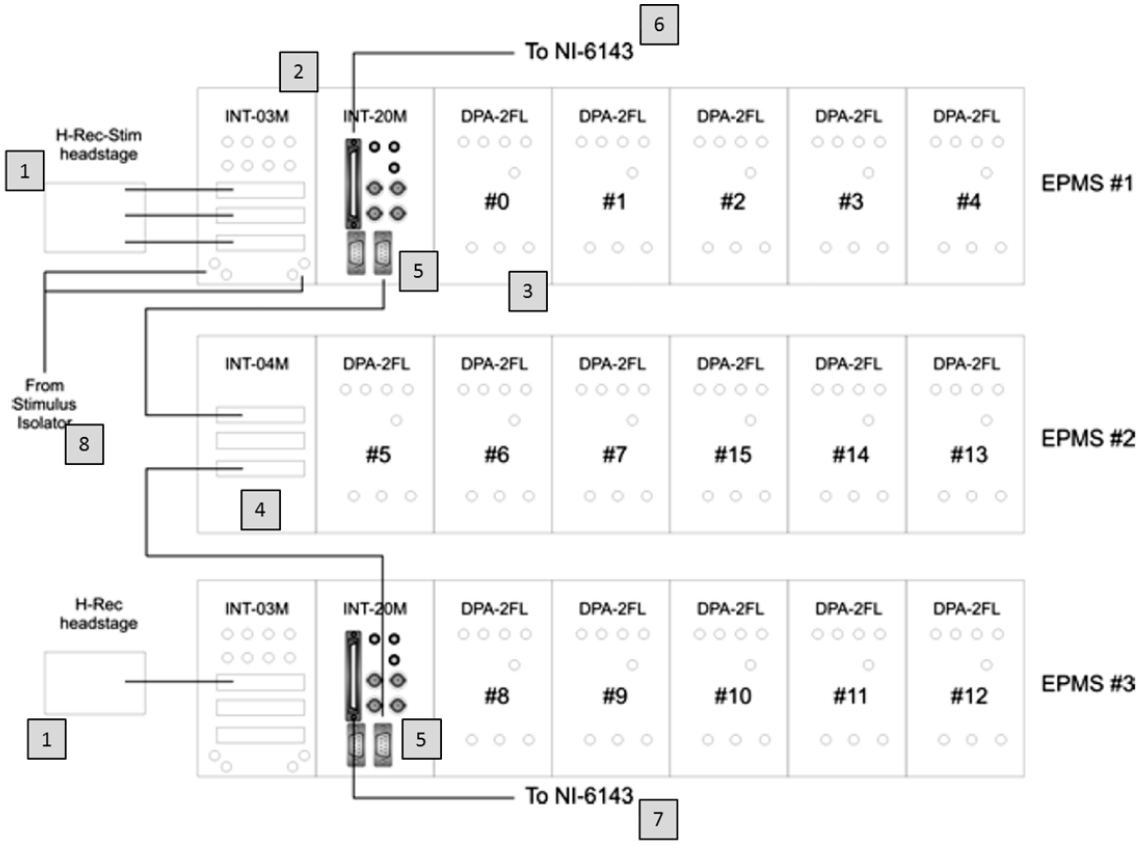


Figure II.5: Overview and wiring schematics of the recording amplifier setup. Signals coming from the electrodes are guided from the headstage (1) to the connection module INT-03M (2) that enables free selection of the reference channel. The module provides the headstage with power and if the switchable headstage SH16 is connected, it provides the headstage with a digital steering pattern or trans passes the electrical stimuli coming from stimulus isolators (8) to the headstage. Each electrode is amplified in a separate differential low noise amplification modules DPA-2FL which are interconnected (3). Each second differential input of the amplification module is wired to the free selectable toggle switch in (2) and can be enabled as the common ground or reference. To handle the 16 channels, the modules housed in three 19" racks are interconnected via the connection module (4). Since the data acquisition cards NI-6143 and NI-6123 (7) only handle 8 channels simultaneously each, two brake-out modules (5) were designed.

coming from isolated stimulators to the headstage. Each recorded electrode channel is amplified differentially by the DPA-2FL module (Figure II.5(3), II.6B). Each of the modules is interconnected with their second inputs forming the reference channels that can be chosen from the headstage-connection module. The amplifier modules comprise high- and low-pass filtering of the signal, offset settings and gain control of up to 10k times (Figure II.6B). The setup was designed to record 16 channels from two to four different places inside the bee's brain. Up to two headstages are needed that require power supply and digital steering patterns for switching (see before). As the rack mount cabinets EPMS (npi) has limited capacity to handle all amplification modules, three EMPS cabinets

for two headstages and the additional supply were required. The data acquisition cards (NI-6123, NI-6143, National Instruments, Germany) can only handle 8 channels synchronously, hence the 16 required amplifier modules need to be converged to two output streams via the break-out boxes INT-20M (Figure II.5). These requirements were fulfilled by connecting the EPMS cabinets via connection modules (INT-04M) as it is depicted in Figure II.11 in the appendix.

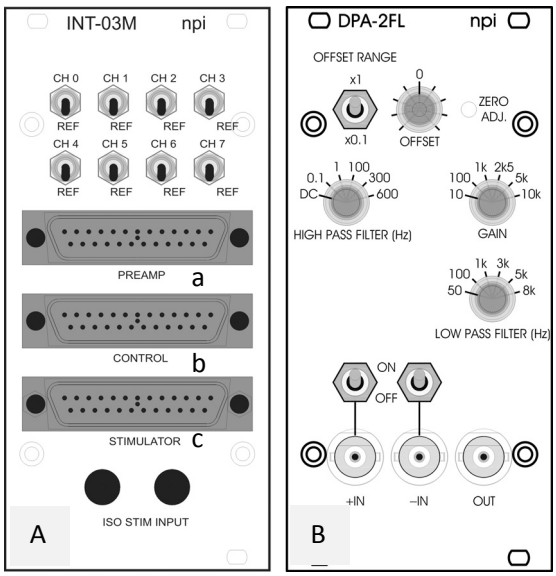


Figure II.6: Schematics of the connection module INT-03M (A) and the low-noise differential amplification module DPA-2FL (B). The switches of the connection module INT-03M (A) enable the toggling of each electrode to be the reference channel of all others. Three connectors provide the headstage with power via the "preamp" connector (connector a). The digital steering pattern is provided via the "control" connector (connector b) and the conveyance of the electrical stimuli from the stimulus isolators inputs is done via the "stimulator" connector (connector c). B) The amplifier modules DPA-2FL are differential amplifiers with 2 inputs. One input gets the signal from the electrode channels, the other is toggled by the connection module being the reference. Potentiometers adjust the signal with high- and low-pass filtering, offset setting and gain control.

Connectivity to peripheral instruments (valves, stimulus isolators)

Since the amplifier setup was designed to record multiple channels simultaneously, combined with interactively producing output signals for steering peripheral instruments as the solenoid valve, the digital input/output (DIO) channels of the acquisition boards (NI-6123, NI-6143, National Instruments, Germany) were bypassed representing the headstage digital steering pattern (see above, DIO 0 – 2). The other DIO-channels were accessible via connectors on the break-out boxes INT-20M (Figure II.5(5), II.11) that were fed in the peripheral instruments. The DIO 3–4 are either a toggle channel or power supply for a solid state switch that provides the steering of the solenoid valve which provides air delivering odor stimulation. DIO 5-6 are meant to steer the stimulus isolators.

Data acquisition

Regarding data acquisition I decided to choose low-cost high-efficiency data acquisition boards from National Instruments (Germany) that record each channel via separated multiplexers with high temporal accuracy, high resolution and with an own timing device (clock) independent from the computers own software based pulse algorithms. Therefore, the acquisition cards NI-6123 and NI-6143 were purchased. Both cards enable recordings of each channel with 31,250Hz sampling rate (feature factory-based conditions of up to 250kHz per channel) and a resolution of 16bit. Both cards incorporate their own timing device (clock) additionally the former one also facilitates on-board memory storage (64MB) that is necessary to produce complex digital output pattern. The PCI-cards are mainly accessed by the factories own software package named LabVIEW (v8.6, National Instruments). It is visual-based programming software that is predominantly used in technological science or for industrial applications. LabView is a graphical interface system design suited to handle huge amounts of data in parallel. Mainly Tobias Rosenbaum constructed the data-acquisition software under my guidance, instructions and recommendations (Figure II.7).

With this program up to 16 channels can be acquired and digitized as well as stored automatically on hard-drive devices (HDD) in “ascii” file format. Settings are subdivided into channel, timing, stimulation, file and arbitrary parameters that can easily be set. Digital pulses of custom designed patterns can be generated. Data can be viewed online either combined with all recorded channels or in higher magnification of a single channel. Each channel can be amplified and its offset can be set software-based. In addition to the already differentially recorded signals provided by the amplifier system (see above) and to assure focal recordings directly from the tip of the electrodes, cross-differentiation of a recorded channel in respect to an arbitrary one can be applied according to the programs own toggle interface (Figure II.8). Since extracellular recordings are prone to hum and noisy interferences from the surroundings, online power spectrum analyses of each channel are given (Figure II.7(7)).

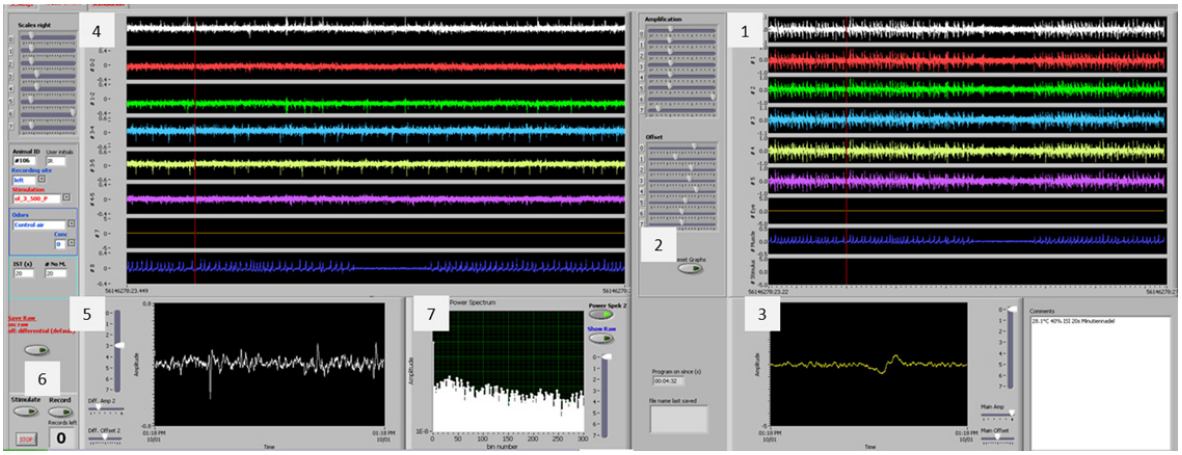


Figure II.7: Screenshot of an acute multi-channel recording from the bee's dual olfactory pathway acquired with the custom designed program "Neuromaster" v28 (neuromaster is not a registered trademark and is not intended to compete with purchasable products of other companies) written in LabView (National Instruments). (1) Each recorded channel is acquired and plotted in different colors, (2) and can be adjusted in setting the software based gain and offset. To analyze the recordings in more detail each channel can be magnified and adjusted (3). Focal recordings from the direct electrode tip are assured by online cross-differentiation of each electrode channel with an arbitrary one (4) and viewed again in color code that can be adjusted software-based. Also the focal recordings can be magnified in time and resolution with additional analyses of the power-spectrum (7) to confirm proper recordings without interference from hum and noise. The program is steered by pull-down windows and insertion remarks (6).

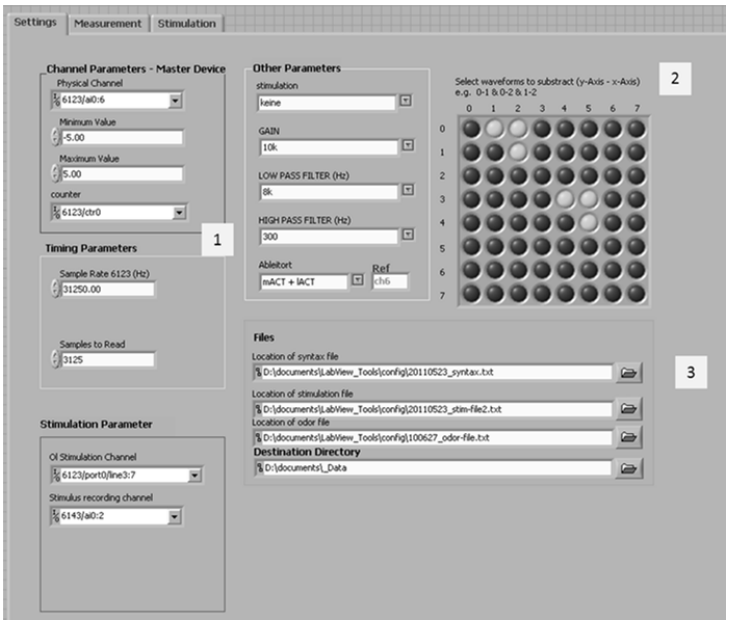


Figure II.8: Screenshot of the Neuromaster user-interface. (1) Any important setting can be done with pull-down queries or by typing the information i.e. the sampling rate. Settings are subdivided into channel, timing, stimulation, file and arbitrary parameters. To assure focal recordings online cross differentiation of the channels is provided by a toggle array (2). File parameters link the program to ascii files that compose easy to use stimulation protocols and syntax files (3).

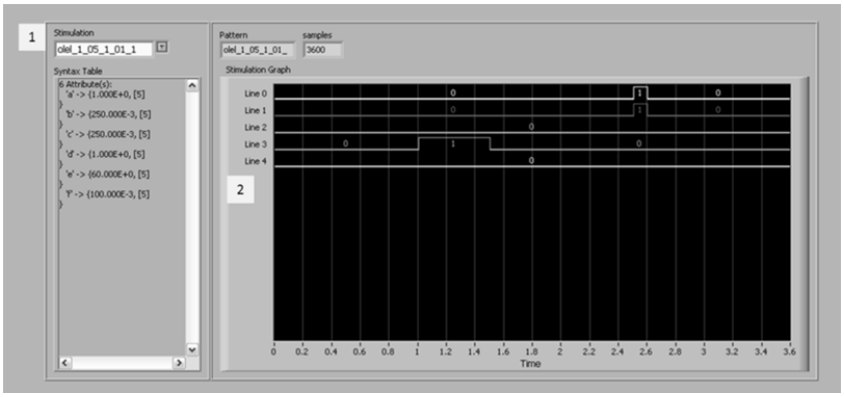


Figure II.9: Screenshot: monitoring of the custom designed stimulation patterns that are generated with easy to use syntax files. (1) Each generated stimulation pattern can be evaluated. Parallel stimulation on several DIOs are possible (2) whereas in this view every DIO channel gets its own color.

The program uses an easy to use syntax handling, implemented by Tobias Rosenbaum; for instance storing of odorant names that are listed and numbered in an ascii files as database. For stimulations, which are designed out of digital TTL pulses (0 or 5V rectangular pulses respectively), a syntax file can be composed that resembles time periods of a certain length and decodes it with certain letters. These letters can further on be catenated and stored as a specific stimulation paradigm. Since digital stimulation patterns are used for more than one channel the catenated letters are labeled according to the specific DIO. The program allows monitoring the custom designed stimulation pattern (Figure II.9).

As the recordings of up to 16 channels with a resolution of 31,250Hz require hardware storage of about 3.2MB per second, it was also necessary to establish a RAID-system based hardware storage with two parallel working hard drives. Finally the setup was running, as can be seen in a successful bee recording the Figure II.10.

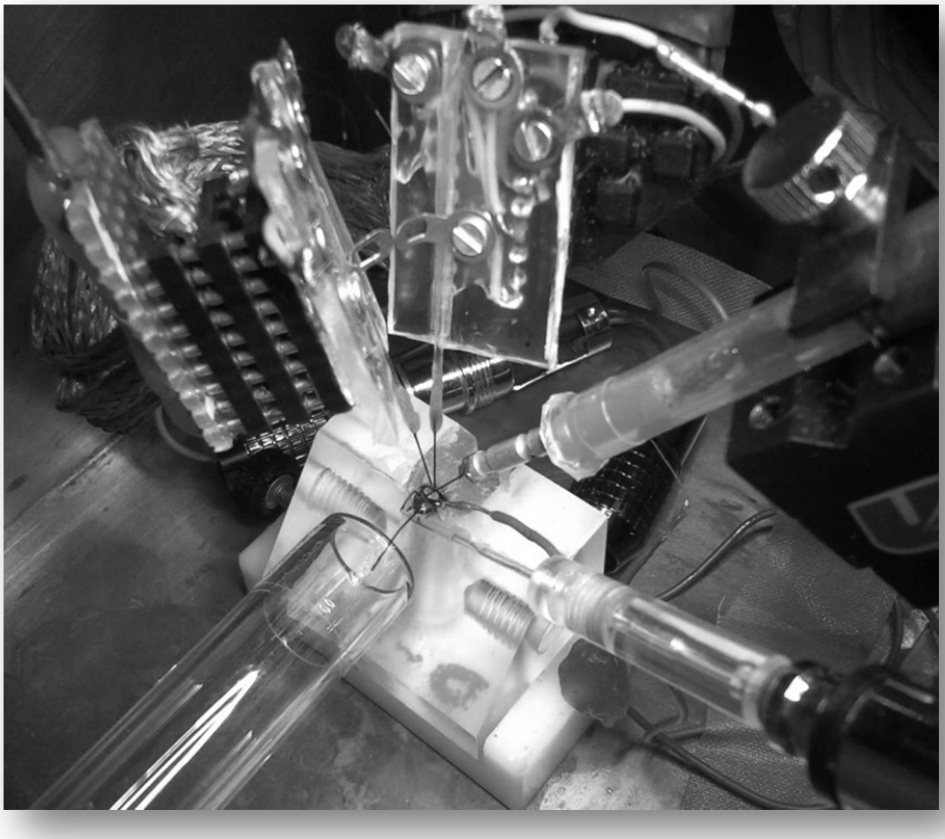


Figure II.10: Image showing a simultaneous recording of the olfactory induced activity (EAG, glas electrode & tungsten electrode as reference) in PNs (l- and m-APT PN with multi-channel wire electrodes attached to the headstage and holder) and the brains output (proboscis extension, silver wire in the head capsule) in response to floral odor stimulation.

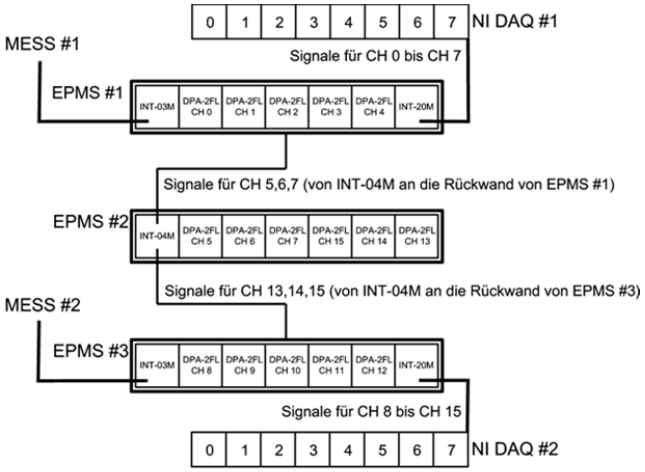
Appendix

Soldering protocols specified for each number of EPMS rack mount cabinet of the amplifier and connection modules (written in German, adapted from NPI, Tamm, Germany) (Figure II.11-II.13)

DPA-2FL im EPMS #1 und im EPMS #2

interne Verbindungen im Modul

DPA-Verstärker Nr.	I/O Bezeichnung	nach Bus-Stecker Pin	Position EPMS #
0	+IN	3	1
0	-IN	7	1
0	OUT	1	1
1	+IN	11	1
1	-IN	12	1
1	OUT	5	1
2	+IN	14	1
2	-IN	15	1
2	OUT	9	1
3	+IN	16	1
3	-IN	18	1
3	OUT	13	1
4	+IN	19	1
4	-IN	20	1
4	OUT	17	1
5	+IN	22	2
5	-IN	23	2
5	OUT	21	2
6	+IN	24	2
6	-IN	26	2
6	OUT	25	2
7	+IN	27	2
7	-IN	31	2
7	OUT	29	2



DPA-2FL im EPMS #2

interne Verbindungen im Modul

DPA-Verstärker Nr.	I/O Bezeichnung	nach Bus-Stecker Pin	Position EPMS #
5	+IN	3	2
5	-IN	7	2
5	OUT	1	2
6	+IN	11	2
6	-IN	12	2
6	OUT	5	2
7	+IN	14	2
7	-IN	15	2
7	OUT	9	2
13	+IN	22	2
13	-IN	23	2
13	OUT	21	2
14	+IN	24	2
14	-IN	26	2
14	OUT	25	2
15	+IN	27	2
15	-IN	31	2
15	OUT	29	2

DPA-2FL im EPMS #3 und im EPMS #2

interne Verbindungen im Modul

DPA-Verstärker Nr.	I/O Bezeichnung	nach Bus-Stecker Pin	Position EPMS #
13	+IN	22	2
13	-IN	23	2
13	OUT	21	2
14	+IN	24	2
14	-IN	26	2
14	OUT	25	2
15	+IN	27	2
15	-IN	31	2
15	OUT	29	2
8	+IN	3	3
8	-IN	7	3
8	OUT	1	3
9	+IN	11	3
9	-IN	12	3
9	OUT	5	3
10	+IN	14	3
10	-IN	15	3
10	OUT	9	3
11	+IN	16	3
11	-IN	18	3
11	OUT	13	3
12	+IN	19	3
12	-IN	20	3
12	OUT	17	3

Figure II.11: Overview of the soldering pattern and channel connectivity schematics of the 16channel amplification system. Each EPMS rack mount cabinet consists of the amplification modules DPA-2FL. To ensure differential amplification with a switchable reference channel, the second inputs of each DPA-2FL modules are interconnected. Each rack mount cabinet EPMS containing a limited capacity of modules needs to comprise steering and powering of two headstages as well as 16 channel amplification; hence 16 modules that again need to converged again onto two-times 8-channel data-acquisition cards (NI-6123, NI-6143), therefore the second EPMS cabinet is subdivided according to the schematics.

INT-03 im EPMS #1

Connector A

Kanal Nr.	Pin Nr. SUB-D-Stecker B	Arbeitsgang	nach Bauteil
GND	15	Leitung löten	Netzteil GND
GND	16	Leitung löten	Netzteil GND
+1,5V	14	Leitung löten	Netzteil +1,5V
-1,5V	17	Leitung löten	Netzteil -1,5V
1	1	Leitung löten	Schalter CH0 Pin 2
4	4	Leitung löten	Schalter CH1 Pin 2
6	20	Leitung löten	Schalter CH2 Pin 2
11	10	Leitung löten	Schalter CH3 Pin 2
13	11	Leitung löten	Schalter CH4 Pin 2
16	25	Leitung löten	Schalter CH5 Pin 2
8	21	Leitung löten	Schalter CH6 Pin 2
9	9	Leitung löten	Schalter CH7 Pin 2

Connector B

Kanal Nr.	Pin Nr. SUB-D-Stecker B	Arbeitsgang	nach Bauteil
GND (0V)	5	Leitung löten	Netzteil GND
VCC (+3V)	7	Leitung löten	Netzteil +3V
DATA	6	Teiler 2k/3k (5V nach 3V)	Bus-Stecker Pin 28
LOAD	18	Teiler 2k/3k (5V nach 3V)	Bus-Stecker Pin 30
CLOCK	19	Teiler 2k/3k (5V nach 3V)	Bus-Stecker Pin 32

Kanal Nr.	Pin Nr. SUB-D-Stecker POWER OUTPUT	NI Bezeichnung	von Bauteil
DATA	1	DIO 0	J23 Pin 1
LOAD	2	DIO 1	J23 Pin 2
CLOCK	3	DIO 2	J23 Pin 3
verfügbar	4	DIO 3	J23 Pin 4
verfügbar	5	DIO 4	J23 Pin 5
verfügbar	6	DIO 5	J23 Pin 6
verfügbar	7	DIO 6	J23 Pin 7
verfügbar	8	DIO 7	J23 Pin 8
	9	GND	

Connector C

Kanal Nr.	Pin Nr. SUB-D-Stecker C	Arbeitsgang	nach Bauteil
1	1	zu-sammen-löten	ISO STIM INPUT #1 positiv
2	2		
3	3		
4	4		
5	7	zu-sammen-löten	ISO STIM INPUT #1 negativ
6	20		
7	8		
8	21		
9	9	zu-sammen-löten	ISO STIM INPUT #2 negativ
10	22		
11	10		
12	23		
13	11	zu-sammen-löten	ISO STIM INPUT #2 positiv
14	24		
15	12		
16	25		

Figure II.12: Overview of the wiring schematics of the connection module INT-03 in the rack mount cabinet EPMS #1 that contains the steering connectors for the switchable headstage SH16 as well as transmission of electrical stimuli coming from stimulus isolators. Connector A powers the headstage and conveys the electrode channels to the amplifier system, connector B provides the switchable headstage with the required digital steering pattern for toggling between stimulation and recording status. Connector C enables bypassing of electrical currents from stimulus isolators to the headstage.

INT-04 im EPMS #2

BUS SIGNAL Connector #1

Kanal Nr.	Pin Nr. SUB-D Stecker #1	Arbeitsgang	nach Bauteil
5	1	Leitung löten	Bus-Stecker Pin 3
5	2	Leitung löten	Bus-Stecker Pin 7
5	6	Leitung löten	Bus-Stecker Pin 1
6	3	Leitung löten	Bus-Stecker Pin 11
6	4	Leitung löten	Bus-Stecker Pin 12
6	7	Leitung löten	Bus-Stecker Pin 5
7	5	Leitung löten	Bus-Stecker Pin 14
7	9	Leitung löten	Bus-Stecker Pin 15
7	8	Leitung löten	Bus-Stecker Pin 9

BUS SIGNAL Connector #2

Kanal Nr.	Pin Nr. SUB-D-Stecker #2	Arbeitsgang	nach Bauteil
13	1	Leitung löten	Bus-Stecker Pin 22
13	2	Leitung löten	Bus-Stecker Pin 23
13	6	Leitung löten	Bus-Stecker Pin 21
14	3	Leitung löten	Bus-Stecker Pin 24
14	4	Leitung löten	Bus-Stecker Pin 26
14	7	Leitung löten	Bus-Stecker Pin 25
15	5	Leitung löten	Bus-Stecker Pin 27
15	9	Leitung löten	Bus-Stecker Pin 31
15	8	Leitung löten	Bus-Stecker Pin 29

Verbindungskabel INT-20 nach INT-03 (2x)

9-pol. SUB-D-Buchse INT-20 Pin Nr.		25-pol. SUB-D-Stecker INT-04 Pin Nr.
1		1
2		2
6		6
3		3
4		4
7		7
5		5
9		9
8		8
Schirm		Schirm

INT-03 im EPMS #3

Connector A

Kanal Nr.	Pin Nr. SUB-D-Stecker B	Arbeitsgang	nach Bauteil
GND	15	Leitung löten	Netzteil GND
GND	16	Leitung löten	Netzteil GND
+1,5V	14	Leitung löten	Netzteil +1,5V
-1,5V	17	Leitung löten	Netzteil -1,5V
1	1	Leitung löten	Schalter CH0 Pin 2
4	4	Leitung löten	Schalter CH1 Pin 2
6	20	Leitung löten	Schalter CH2 Pin 2
11	10	Leitung löten	Schalter CH3 Pin 2
13	11	Leitung löten	Schalter CH4 Pin 2
16	25	Leitung löten	Schalter CH5 Pin 2
8	21	Leitung löten	Schalter CH6 Pin 2
9	9	Leitung löten	Schalter CH7 Pin 2

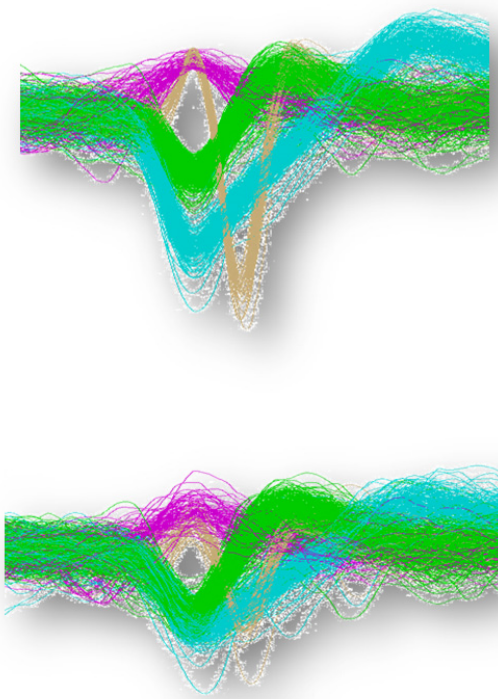
INT-20 im EPMS #3

Kanal Nr.	Pin Nr. SUB-D-Stecker POWER OUTPUT	NI Bezeichnung	von Bauteil
DATA	1	DIO 0	J23 Pin 1
LOAD	2	DIO 1	J23 Pin 2
CLOCK	3	DIO 2	J23 Pin 3
verfügbar	4	DIO 3	J23 Pin 4
verfügbar	5	DIO 4	J23 Pin 5
verfügbar	6	DIO 5	J23 Pin 6
verfügbar	7	DIO 6	J23 Pin 7
verfügbar	8	DIO 7	J23 Pin 8
	9	GND	

Figure II.13: Overview of the wiring schematics of the connection modules INT-04 (left) and INT-03 (right) in the rack mount cabinets EPMS #2 (left) and EPMS #3 (right). The connection module INT-04 enables the connectivity of the EPMS#1 and EPMS#3 with EPMS#2 and guides the amplified signals and reference channels to the break-out boxes. Connection module INT-03 provides powering and connection of a non-switchable headstage.

III - Chapter 2:

Olfactory processing: Parallel processing



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Author contributions:

M.F.B., W.R., C.J.K. designed research, M.F.B., T.R., I.R., performed research, M.F.B., T.R., I.R., M.P.N. analyzed the data, M.F.B., , W.R., M.P.N., C.J.K. wrote the manuscript

Abstract

In their natural environment animals face complex and highly dynamic olfactory input. This requires fast and reliable processing of olfactory information, in vertebrates as well as invertebrates. Parallel processing has been shown to improve processing speed and power in other sensory systems and is characterized by extraction of different stimulus parameters along parallel sensory information streams. Honeybees possess an elaborate olfactory system with unique neuronal architecture - a dual olfactory pathway comprising a medial and lateral projection-neuron (PN) output tract (m-, l-APT) connecting the olfactory lobes with higher order brain centers. We asked whether this neuronal architecture serves parallel processing and employed a novel technique for simultaneous multi-unit recordings from both tracts. The results revealed response profiles from a high number of PNs of both tracts to floral, pheromonal and biologically relevant odor mixtures that were tested over multiple trials. PNs from both tracts responded to all tested odors, but with different characteristics indicating parallel processing of similar odors. Both PN tracts were activated by widely overlapping response profiles, which is a requirement for parallel processing. The l-APT PNs had broad response profiles suggesting generalized coding properties, whereas m-APT PNs responded comparatively weaker and less frequent, indicating higher odor-specificity. Comparison of response latencies within and across tracts revealed odor-dependent latencies. We suggest that parallel processing via the honeybee dual olfactory pathway provides enhanced odor processing capabilities serving sophisticated odor perception and olfactory demands associated with a complex olfactory world of this social insect.

Introduction

Olfaction is of paramount importance for most animal species. Insects have evolved sophisticated olfactory systems for communication, orientation, nest-mate recognition, and predator avoidance (e.g. Martin et al., 2011; Hansson and Stensmyr, 2011). For olfactory processing, nervous systems are confronted with an enormous chemical coding space (Guerrieri et al., 2005b; Schmuker and Schneider, 2007), but also face the need to process dynamic sensory input fast and efficiently (Geffen et al., 2009; Nawrot, 2012). Sensory processing along parallel pathways supports this task (Nassi and Callaway, 2009). Compared to knowledge on parallel processing in other sensory

modalities (auditory, visual, somatosensory), parallel processing in olfactory systems is far from being understood (Galizia and Rössler, 2010).

In insects, olfactory receptor neuron (ORN) axons transfer input from antennal sensilla to glomeruli, spheroidal neuropil units in the antennal lobe (AL), the analogue to the vertebrate olfactory bulb (OB) (e.g. Hildebrand and Shepherd, 1997; Kay and Stopfer, 2006). After AL processing (Martin et al., 2011; Wilson, 2011), odor information is transferred via projection (output) neurons (PNs) as spatio-temporal response patterns (Sachse and Galizia, 2002; Wilson and Laurent, 2005; Arenas et al., 2009; Krofczik et al., 2009) to higher brain centers in the mushroom bodies (MBs) and lateral horn (LH) (Mobbs, 1982). Hymenoptera (ants, bees, wasps) possess a unique dual olfactory pathway (Abel et al., 2001; Kirschner et al., 2006; Zube et al., 2008; Galizia and Rössler, 2010; Nishikawa et al., 2011; Rössler and Zube, 2011) with a medial- and lateral AL-protocerebral-tract (m- and l-APT). Both tracts comprise axons from ~920 uniglomerular PNs (~410 m- and ~510 l-APT PNs) (Rybak, 2012). M-APT PNs connect glomeruli in the upper AL-hemilobe with the MB and the LH, whereas l-APT PNs connect glomeruli in the upper AL-hemilobe in reverse order with the LH and MB.

This dual pathway represents a favorable system to address fundamental questions in parallel olfactory processing. It is still unclear whether the tracts code odors in a “dual segregated” fashion (different odors in different tracts) like pheromonal and general-odor subsystems in moths (Martin et al., 2011) or flies (Schlief and Wilson, 2007) - or “dual parallel” (similar input, differential feature extraction) (Galizia and Rössler, 2010). Recent calcium imaging studies support that the two AL subsystems receive similar sensory input (Carcaud et al., 2012; Galizia et al., 2012). Independent (sequential) recordings in different individuals (electrophysiology, calcium imaging) indicate that m- and l-APT PNs may differ in physiological properties (Abel et al., 2001; Müller et al., 2002; Krofczik et al., 2009; Yamagata et al., 2009). However, simultaneous PN recordings from both tracts with high temporal precision are still lacking.

We used thin-wire multi-electrodes for simultaneous double-tract recordings to obtain response profiles from many uniglomerular PNs over multiple trials to floral, pheromonal and social odors. The results show highly overlapping odor specificity, but distinct differences in response properties between m- and l-APT PNs, which strongly supports parallel odor processing. Consistent odor-dependent response latencies of PNs within and across tracts support temporal coding of odor identity via PNs of both tracts.

Material & Methods

Experimental animals and preparation

We caught foragers of the European bee *Apis mellifera L.* in the morning from a feeder filled with saccharose solution (Apiinvert, 50%) and anesthetized them on ice. The legs were cut above the coxae. We fixed the bees in custom-made plexiglas holders using dental wax (surgident periphery wax, Heraeus Kulzer) and fed with saccharose solution ad libitum.

Antennae were gently fixed at the pedicellus with dental wax. We dissected the brain by cutting open the head capsule between the compound eyes, the ocelli and the antennal sockets. To diminish movements, mandible-muscle apodemes were cut. We gently coated the ventro-dorsal abdomen and wings with liquid dental wax. Glands and trachea were moved aside, and the neurolemma was removed at the insertion site before electrode placement. Two Ag-wires (150 µm diameter, AGT05100 WPI, Germany) served as reference electrode and for M17 proboscis muscle recordings, which is involved in proboscis extension (Rehder, 1987). We inserted the M17 recording electrode between the central ocellus and the compound eye. The reference electrode was introduced in the ipsilateral complex eye. For the AL output tract recordings, we inserted electrodes from anterior, dorsally to the AL, using prominent landmarks. The m-APT electrode was placed between the AL and the medial lobe of the MB using the vertical MB lobe, pharynx and the AL as landmarks (Figure 1A).We inserted the l-APT recording electrode into the lateral-caudal protocerebrum between the AL and LH - at the position where the l-APT exits the AL (cp. Figure 1, location no.3,4 in Kirschner et al., 2006; and schematic Figure 1A). Following electrode placement, the brain was either covered with 2 component low viscosity silicone (Kwik-Sil, WPI, USA) or left untreated.

Odor stimulation

Odors were delivered by an olfactometer providing a laminar airstream using a 1 cm outer diameter glass tube placed 1cm in front of the bee's antennae. The olfactometer was supplied by two independent analog flow controllers (2-65-B P-067, Brooks Tube) that delivered moist air through a charcoal filter (AK 02/05, UltraFilter, Germany; 0.5 l/min air flow each). We applied a constant air stream to adapt tactile sensillae. Odor delivery into this constant air stream was controlled by a solenoid valve (LFAA1201618H, Lee Co., CT) and bypassed through 10ml glass vials (CZT, Germany) that contained the test odors, dissolved either in 100µl paraffin oil (Sigma Aldrich, Germany), in pure water or presented as solid objects.

For the first group of bees, we used twelve different odors, including five pheromones plus control (pure solvent) (Table 1). As general odors we used limonene, hexanal, 1-pentanol, 1-octanol, 2-octanone and two floral bouquets, clove oil and orange oil, as well as citral and geranylic acid as pheromones from the Nassanoff gland (Shearer and Boch, 1966; Pickett et al., 1980), isoamylacetat and 1-hexanol, as alarm pheromone components of the Koschevnikov gland (Boch et al., 1962; Collins and Blum, 1983), and 2-heptanone, as alarm pheromone from the mandibular glands (Shearer and Boch, 1965) (all purchased from Sigma Alldrich, Germany). For a recent review on pheromones see Sandoz et al. (2007). For the second group of bees, we used 5 odors from the bees' natural environment for stimulation: 200mg cleaned bees wax, 200mg used and mixed bees wax from several hives, 200mg abandoned brood combs, 200mg honey diluted in 2ml water, and 3 freshly killed bees. In order to provide temperature conditions that match conditions in the hive, stimulations took place at 32-33°C (Seeley, 1985; Tautz et al., 2003). Glass vials were put in a copper mesh and heated by a peltier-element (Conrad electronics, Germany). We delivered all odors at a concentration of 1:100 and delivered for 500ms, with either a single pulse or 10 trials with 20s inter-stimulus interval (IST), or as 3 pulses (500ms with 500ms ISI) for 20 trials with 20s IST.

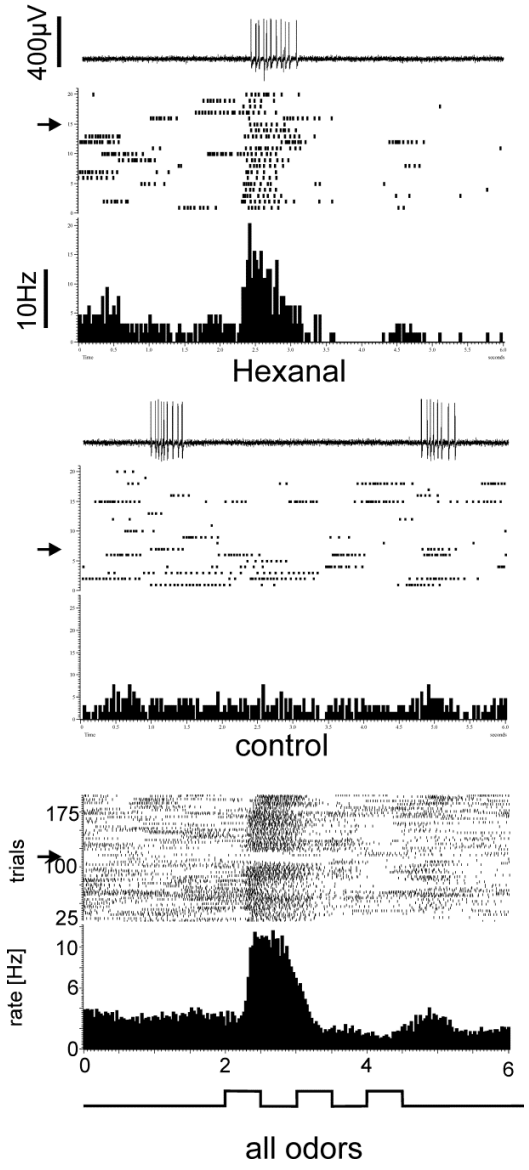


Figure III.1: Recordings of the proboscis extension muscle (M17) and its response to the tested odors (Table 1). The recordings show a naïve bee that responded to all odors except to the solvent which gives a behavioral evidence for appropriate odor stimulation. Given are PSTHs of 20 odor trial stimulations to a three-pulse stimulus (500ms pulse with 500ms ISI, 20s inter pulse pattern interval). On top of the upper and middle PSTH the raw recording of the M17 can be seen which is in turn indicated by the arrow left to the raster. The lower PSTH depicts the M17 recordings in response to all tested odors. The arrow to the left indicates the 20 trials of the solvent.

Table III.1 Tested odors with their specifications group in respect to the tested bee groups

bee group	odor	abbr.	conc	floral	phero-monal	natural mix	CAS	CID
1	control air	ctr-Air	1					
1	control oil	ctr-Oil	1				8012-95-1	
1	citral		1:100	x	x		5392-40-5	638011
1	hexanal		1:100	x			66-25-1	6184
1	geranylic-acid	ger-acid	1:100	x	x		459-80-3	9989
1	isoamyl-acetate	IAA	1:100	x	x		123-92-2	31276
1	(+) limonene	lim	1:100	x			5989-27-5	440917
1	clove oil		1:100	x		x	8000-34-8	12658395
1	orange oil		1:100	x		x	8008-57-9	
1	1-pentanol	1-pent-ol	1:100	x			71-41-0	6276
1	1-hexanol	1-hex-ol	1:100		x		111-27-3	8103
1	1-octanol	1-oct-ol	1:100		x		111-87-5	957
1	2-heptanone	2-hept-ne	1:100		x		110-43-0	8051
1	2-octanone	2-oct-ne	1:100	x			111-13-7	8093
2	control H2O		1					
2	control air	ctr-Air	1					
2	clean bee wax	wax clean	200mg			x	8012-89-3	
2	bee’s wax mix	wax mix	200mg			x		
2	brood combs		200mg			x		
2	honey in water		1:100			x		
2	freshly killed bees		3 bees			x		

Electrophysiology and recording setup

Multi-unit recordings

We placed the plexiglas holder with a mounted bee inside a small copper cube (12 x 12 x 12 cm copper-plate cube) situated within a custom made faraday cage on an air-cushioned laboratory table (Spindler & Hoyer, Germany) (Figure III.2). Air exhaust was provided by the building's ventilation system. We adapted electrode fabrication from Okada et al., (1999, 2007) and Strube-Bloss et al. (2011). Each electrode shank consisted of three copper wires (polyurethane-coated copper wire with 15µm diameter, Elektrisola, Germany) glued together with melted dental wax (diameter < 50µm; Figure 2B inset). For double recordings, both electrodes were connected to a switchable headstage (SH16, Tucker-Davis-Technologies, USA) and fixed on micromanipulators (Leitz, Germany). The Ag-wires (150µm diameter, AGT05100 WPI, Germany) were connected to the headstage and served as reference and M17 recording electrode.

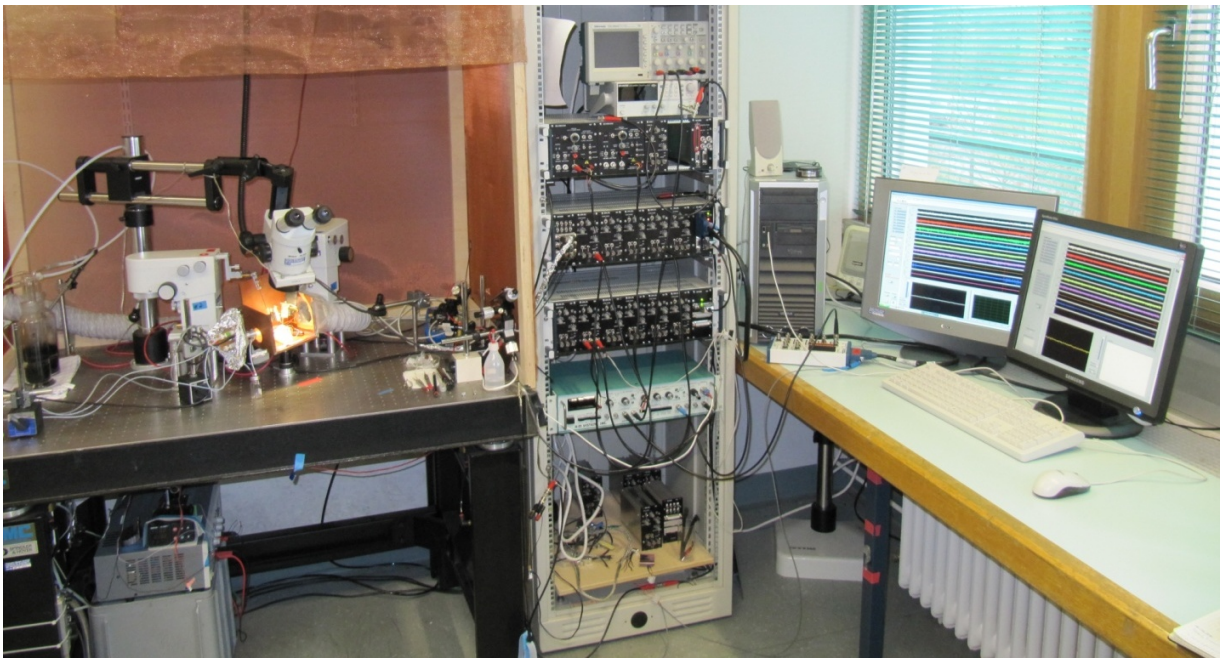


Figure III.2: Photograph of the recording setup. In the Faraday cage situated on an air-cushioned table the coppery recording chamber is situated in between a binocular and two manual micromanipulators carrying the headstage. On the left odor-stimulation and wash bottles are visible. Right to the Faraday cage, the amplifier rack supports a control digital oscilloscope, and stimulus generator (top), three levels of the 16-channel extracellular amplifier system (middle), and an intracellular amplifier (bottom). Next to the amplifier rack is the acquisition computer.

The headstage output was fed into a custom designed connection module (INT-03M, NPI, Germany) that provides freely selectable reference channels and supplies the switchable headstage with power and digital steering patterns. The headstage input-module was connected to a custom-made amplifier system consisting of 16 custom designed low noise differential amplifier modules (DPA-2FL, NPI, Germany). We differentially amplified 5k recordings from all channels to the reference electrode and band-pass filtered (300Hz - 8,000 Hz). The recorded and amplified signals were conveyed to synchronous data acquisition cards (NI6123, Ni 6143, National Instruments, Germany) with a sampling rate of 31,250Hz and 16bit resolution each channel. We stored data via self-written data-acquisition software (mainly Tobias Rosenbaum; LabVIEW v6.8, National Instruments, Germany). The software enabled monitoring of each channel with amplification, offset settings, single channel magnification view and online power spectrum analyses. Furthermore, it allowed an electrode-wise, user-defined online cross differentiation of each recording channel. Software-based online cross differentiation allowed focal recording of the extracellular area between the electrode tips and thus elimination of noise and interference from muscle potentials or unrelated neuronal activity (comparable to the analog differentiation of the recorded channels from Strube-Bloss et al., 2011, Figure 1B). Since recording with two electrode shafts could produce shaft-specific bias, we recorded both tracts alternating with one or the other shaft, and observed no bias of any of the electrode shafts in pre-tests.

Electroantennogram (EAG) recordings and analyses

For EAG measurements, we recorded the ipsilateral antenna differentially at the tip with low-resistant (<0.5MOhm) borosilicate electrodes (1B100F-3, WPI, USA) pulled with a horizontal filament puller (DMZ Universal Puller, Germany) and filled with 0.5M KCl solution. A tungsten electrode was inserted as reference below the scapus of the ipsilateral antenna. We 10x amplified signals using an intracellular amplifier (Model 1600, A-M-Systems, USA), and fed the signal in the amplifier setup to be further amplified (100x) and bandpass filtered (0.1 – 100Hz). We acquired EAG-signals with the same recording setup as mentioned above. In addition to the tract recordings, activity of the proboscis extension muscle M17 was monitored simultaneously to monitor the reliability of odor controls and possible influences of muscle potentials to the tract recordings (100x amplification, 1-100Hz band-pass filtered). Recording duration of each stimulation trial was 7s, with 2s before and 4.5s post stimulus onset. Data were imported in Spike2 software (v7.04, Cambridge Electronic Design, UK) for off-line spike sorting and cluster cutting. We measured the onset of odor response in

the ORNs by EAG recordings in 5 bees, similar to a recently published study (Meyer and Galizia, 2012). EAGs were captured with the complete odor panel (Table 1), filtered offline with the smooth algorithm in Spike2 (time constant 32µs) and averaged over 6 trials. We defined onset of odor response as the time point of the steepest negative slope of the potential drop.

Post recording visualization of electrode and tract positions

For visualization of electrode tracks from penetrating brain tissue, electrodes were dipped in fluorescent dyes (Alexa 568 hydrazide; A-10437, Molecular Probes) prior to the recordings. After successful experiments, brains were rinsed with fresh bee-ringer solution (37mM NaCl, 2.7mM KCl, 8mM Na2HPO4, 1.4mM KH2PO4, pH 7.2), glands and trachea were removed, and crystals of MicroRuby (Tetramethylrhodamin Dextran, Invitrogen, Germany) were inserted in the recorded AL to trace the m- and l-APT (Zube et al., 2008; Rössler and Zube, 2011). After 30-45min, brains were dissected and immediately rinsed in 4% formaldehyde in 0.1M PBS overnight at 4°C. Following two rinses in 0.1M PBS for 10min, the brains were washed (3x, 20min each step) in 0.1M PBS with 0.2% Triton-X 100 (1:250), and incubated thereafter in 0.5% Lucifer Yellow in 0.1M PBS overnight at 4°C. After washing three times (10min each) in 0.1M PBS, brains were dehydrated in an ascending alcohol series (30%, 50%, 70%, 90%, 95% and 3x 100% Ethanol) and finally transferred in methylsalicylate (Sigma-Aldrich Chemie, Steinheim, Germany). We viewed brains as whole-mount preparations with a confocal laser scanning microscope (Leica TCS SP2 AOBS, Leica Microsystems, Germany). For image acquisition, a HC PL APO (10x0.4 NA imm) objective was used, and optical sections were taken every 2 to 5µm. Finally, we 3D-reconstructed brain neuropils and electrodes (Amira 5.2.0, Mercury Computer Systems, Berlin, Germany; Figure 1C).

Off-line spike-sorting

Multi-electrode recordings allow simultaneous acquisition of multiple neurons at high temporal resolution (Gray et al., 1995). The method benefits from recording neuronal activity with more than one electrode to improve spike sorting (Lewicki, 1998; Gold et al., 2006). Spike sorting is often subject to potential misclassification (Harris et al., 2000; Joshua et al., 2007; Quiroga, 2012), therefore we took specific care in this task. We used a well-established commercial software (Spike

2, v7.4, Cambridge Electronic Design, England) for spike sorting that has successfully being used in several other studies (Brierley, 2003; Hoare et al., 2008; Strube-Bloss et al., 2011; Payton et al., 2012). We preprocessed each channel using the implemented "smooth" algorithm, which FIR filters the input with a time constant of 80µs (comparable to a low-pass-filter of 6,250Hz) and "DC-remove" with a time constant of 3.2ms leading to offset adjustment (comparable to a high pass Filter of 312.5 Hz).

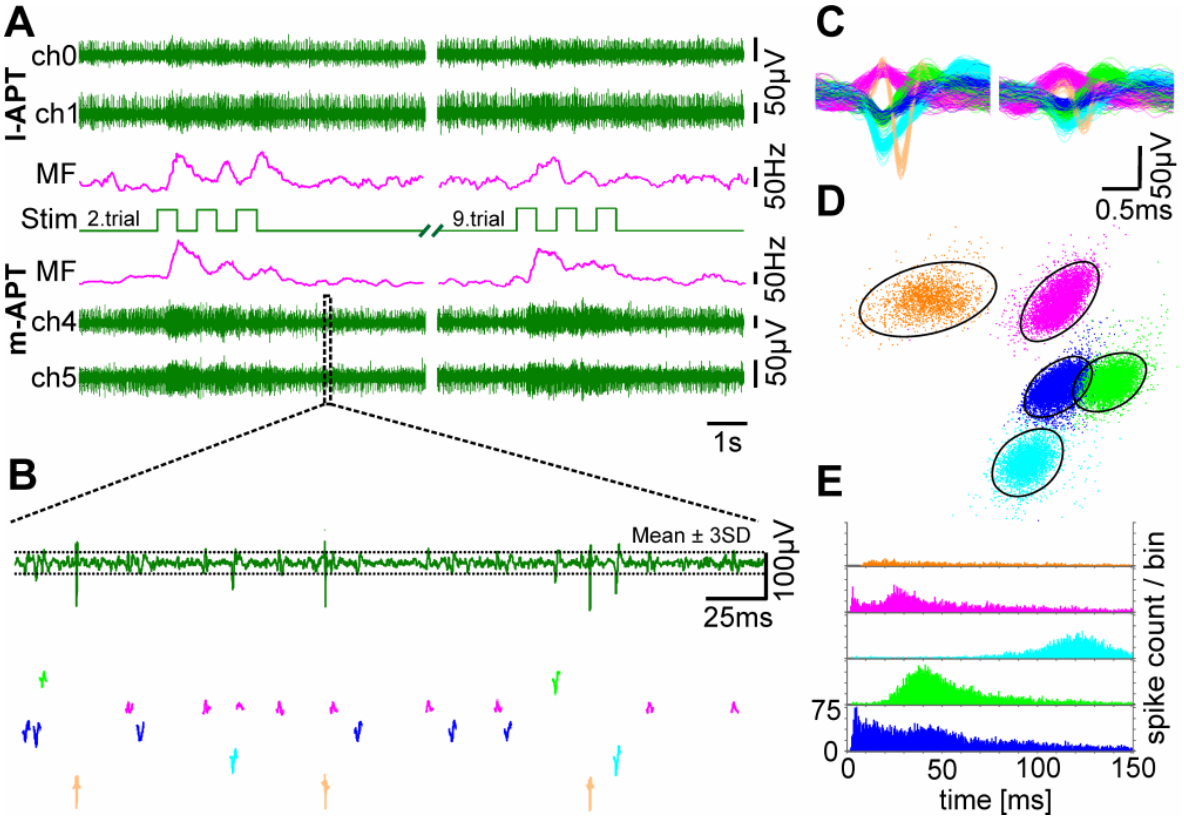


Figure III.3: Spike-sorting of extracellular multi-electrode recordings. A) Three-pulse odor stimulation (illustrated as 3 squares, Stim.) with honey in water (1:100) at 32-33°C elicit neuronal responses recorded from the l-APT and m-APT with three wire electrodes in each case, two of them shown as differential (pairwise subtracted) channels as the upper- or lowermost two traces, respectively. Odor elicited responses are shown for the second (left) and 9th trial (right) of a 20 trial recording as mean spike frequency for each tract (MF respectively for each tract, time constant: 301ms). B) High magnification of spike shapes of four sorted units color coded below one of the raw data channels. Thresholds for template-matched spike sorting are illustrated as dotted lines: mean ± 3 fold SD of recordings during spontaneous activity, (template-matched spike-sorting, window width 1 - 1.4ms). C) The sorted units depicted as overlay show differing spike shapes that cluster distinctly after Principal-Component-Analysis (PCA) shown in D). The clustered units are surrounded by 3.5 times the Mahalanobis distance (a multidimensional version of standard deviation) and demonstrate almost no overlay. D) Inter-spike interval histogram for each sorted unit reveals different spike time regimes.

For spike sorting, we either used two or three of the recorded channels. If neuronal signals on all three recorded channels demonstrated adequate signals with good signal to noise ratios (SNR), we copied one channel and all conjoined channels were analyzed as a tetrode. If only two channels with good SNRs were available, we analyzed these as stereotrodes. For semi-automated template-matched spike sorting, we set the amplitude threshold to + and - 3 standard deviations of the mean signal amplitude of the recording over at least 1min spontaneous neuronal activity recorded previous to the test trials (Figure III.3B).

For semi-automated template formation, we set the time window to -0.4ms before and 0.8ms after either positive or negative peak amplitude. After template formation, we clustered and sorted units by applying the Spike2 built-in clustering dialogues. First principal component analysis (PCAs) with components based on waveforms were separating the units (Figure III.3C) and additional feature extraction like slope of spikes, amplitude, peak area helped to further classify the data. After cluster analysis, we analyzed and compared interval histograms of each single unit (see Figure III.3C,D), in order to prevent hum-interferences or false positive sorting. Successful dual-tract recordings are characterized by good SNRs and acceptable spike sorting (Figure III.3).

Additionally to the implemented offline sorting algorithm in Spike2 (CED, United Kingdom) we tested two other open source sorting programs (Rosenbaum, 2011):

Wave Clus Unsupervised detection and sorting

Wave Clus is a toolbox for Matlab (Quiroga et al., 2004) that can be freely downloaded from the University of Leicerster's homepage (http://www2.le.ac.uk/departments/engineering/research/bioengineering/neuroengineering-lab/spike-sorting, April 2010).

SpikeOMatic

SpikeOMatic is a toolbox for the free R project for statistical computing and is developed amongst others mainly by Christoph Pouzatt (Pouzat et al., 2002; Delescluse and Pouzat, 2006). The program can be freely downloaded from the website of the University of Paris (http://www.biomedicale.univ-paris5.fr/physcerv/C_Pouzat/SOM.html#download, April 2010).

With both automated spike-sorting procedures we had difficulties to align our data. Either the data volume was overfloating the program (SpikeOMatik) or our Data had SNR values not

suitable to work with the program (Wave Clus). Based on these experiences we decided to analyze all our data with the well-known spike-sorting program Spike2 (CED, United Kingdom).
We excluded units that did not respond to any of the tested odors or that showed technically induced (e.g., influence from power-supply, artifacts coming from solenoid valves) response characteristics: 45 l-APT units and 29 m-APT units. In the following we use the term PN for classified unit activity, and we are well aware that these units do not necessarily equate to a single neurons in all cases.

Data analyses

As a first quality control, we performed analyses of single PNs with Spike2 (v7.4, Cambridge Electronic Design). We automatized final analyses of the complete population in Matlab (v2009b, The MathWorks, Inc.) including different functions from the FIND toolbox Version 1.1 (Meier et al., 2008). We performed statistical analyses additionally in Statistica (v10, StatSoft, Inc.).

Identification of odor response profiles in PNs

Each PN was classified in a fully automated fashion as being responsive or non-responsive to each test odor. Briefly, we tested over repeated odor stimulations whether the mean firing rate was different between pre- and peri-stimulus intervals. In a pre-test, we identified responses from mean trials to a given odor. If a response was indicated here, the procedure was repeated on single trial level to verify the odor-response. If at least half of all single trials showed stimulus correlated modulation, the PN was classified as responsive to the given stimulus. Trials without any response were excluded from further analysis.

In detail, we proceeded in the following way: 1) to detect responses from mean trials, we re-sampled to bins of 1ms, averaged and generated a representative, natural spike train of a single PN in response to one odor. 2) To estimate the rate function of this representative trial, we convoluted with a symmetric smoothing filter (Savitzky and Golay, 1964, polynomal odor 0, 300ms width) 3) Baseline firing rate was estimated over an interval of 600ms before stimulus onset. 4) Responses were screened within a time window between 0 and 600ms post stimulus onset. Neuronal activity was defined as response if it was different ± two standard deviations from baseline over a duration of at least 50ms. Positive deviations above threshold correspond to excitations, and negative deviations correspond to inhibitions. If a response was indicated in the averaged trial, we repeated

the same procedure, but this time on the basis of genuine single trial spike trains. For all odors tested on average responses to 14.1 ± 2.5 trials of the l- and 13.5 ± 3.1 of the m-APT out of 20 stimulus repetitions were beyond a 50% threshold. All data was additionally calculated with 25% and 75% threshold criteria, which did not qualitatively change the results regarding latency, lifetime sparseness and recruitment rates. For analyses on PN population response latency differences see Figure III.13.

Estimation of latencies and rate functions

Analyses of latencies and rates were restricted to PNs that were excited by odor stimulation. Latency and rate changes evoked by different stimuli may be described on different levels:

A) On the level of a single PN, which responds to a given stimulus in repeated trials (PN latency/rate).

B) On the level of an odor to which a group of neurons respond each with their individual latency (Odor latency/rate).

C) On the level of each tract where different odors elicited activity in a population of PNs (Tract latency/rate).

We estimated rates and latencies throughout each level with the same method based on the derivative of the trial-aligned firing rate (Meier et al 2008). This method has been repeatedly used in related studies (Krofczik et al., 2008; Meyer and Galizia, 2011). It processes data in three successive steps: 1) Estimation of the derivative of input spike trains by convolution with an asymmetric Savitzky-Golay filter (polynomal order 1, 301ms width, Welch windowed). 2) Alignment of spike trains by maximizing the average pair-wise cross correlation (Nawrot et al. 2003). The estimated time-shifts correspond to each trial's *relative latency*. Their standard deviation σ gives a measure for the across-trial latency variability. 3a) To estimate the *absolute latency* of the input data, aligned spike trains are merged and again convolved with the same asymmetric Savitzky-Golay Filter. We defined latency as that point in time where the slope of the firing rate was steepest, that is the first derivative's maximum. 3b) To estimate the average rate function of the input data, the merged spike train is normalized by the number of contributing trials and convolved by a symmetric smoothing filter (as described in section: response detection). For a simplified description of rate

changes, we calculated three descriptive values: baseline firing rate, maximum rate increase, and average evoked rate, being the averaged spike rate during the stimulation period. Baseline firing rate was defined as the mean rate within 500ms before stimulus onset. Maximum rate is the peak rate from which baseline is subtracted. Finally, evoked rate was defined as the mean rate within a response window from 0 to 600ms post stimulus onset from which the baseline was subtracted.

We applied this method sequentially to each of the levels defined above:

A) On the PN level, single-trial spike trains of a given PN responding to a single odor were aligned and merged. Based on the merged spike train we estimated the PNs rate function and its (absolute) latency. These measures describe a PN's response to one single type of stimulus. The standard deviation across time shifts as produced by the alignment procedure describes the variation between single trials, which is the relative PN latency.

B) On the odor level, we aligned averaged spike trains from different PNs assessed at the PN level analysis. By aligning and merging these average trials from PNs, which responded to a given odor, we could estimate an odor specific rate function and absolute latency. Here, the standard deviation across time shifts, measured in the alignment procedure, describes the variation between single PNs responsive to the same odor, which is relative odor latency.

C) On the tract level, latency and rate illustrate the representative activity across different odors elicited in a population of PNs gathering in the same tract. To estimate tract rate and absolute latency, we aligned and merged the averaged odor spike trains assessed in the odor level analysis. Thus, time shifts derived from the alignment procedure correspond to differences between odor stimulations within a PN population in one tract. Their standard deviation is a measure for the relative tract latency.

Tuning measures

Tuning measurements were performed according to recent publications (Krofczik et al., 2009; Strube-Bloss et al., 2011; Nawrot, 2012).

For each single unit, we computed the lifetime sparseness (LS).

$LS = 1 - \left(\frac{\sum|r_j|}{N}\right)^2 / \sum(\frac{r_j^2}{N})$, where N = 12 denotes the number of tested odors. r_j is the PN's response rate to stimulus *j* computed as the trial-averaged mean firing rate within the response interval (100 ms, 600 ms) after stimulus onset, minus the trial-averaged mean baseline rate within the 500ms interval before stimulus onset. Sparsely responding PNs show clear responses only for a small subset of all stimuli, while the majority of the stimuli evoke no or only very weak responses (Vinje and Gallant, 2000; Willmore and Tolhurst, 2001). LS has been repeatedly used to quantify the response sparseness of neurons in the insect olfactory pathway (Perez-Orive et al., 2002; Wilson et al., 2004; Strube-Bloss et al., 2011).

In order to evaluate tuning strength of single PNs according to their latency, we computed the signal-to-noise ratio (Mehring et al., 2003): SNR = $\sigma^2_{signal}/\sigma^2_{noise}$ on the level of evaluated latencies (see above). σ^2_{signal} denotes the variance of the trial-averaged latency deviation, calculated across each of the individual stimuli. σ^2_{noise} denotes the variance across all single trial latencies, after subtraction of the stimulus-specific latency and thus quantifies the trial-by-trial variability of the PN's response latency. This constitutes the noise. We then compute the $SNR_{empiric}$ by dividing $\sigma^2_{signal}\sigma^2_{noise}$. For further details see Strube-Bloss et al. (2011).

Statistical Analyses

Data underwent statistical analyses (Statistica v10, StatSoft, Inc.), first by testing for normal distribution. In case of normal distribution we used parametric t-test to compare two independent groups (l- and m-APT PN responses). In cases where data was not normally distributed, non-parametric tests were used (Wilcoxon rank-sum tests). For comparison of PNs within the same tract, response changes to different odors were again tested for normal distribution. In case of normal distribution, single factor ANOVA was used and non-parametric Wilcoxon ANOVA for not-normally distributed data. For comparison of binary values across tracts, x^2 test was used. In cases of comparison of several independent binary values (recruitment rates to different odors within tracts) a non-parametric ANOVA based on binary data was used (Cochran's Q-test). For correlations we used the Kendall rank correlation coefficient.

Results

Using the simultaneous dual-tract recording technique, we were able to record from a total of 14 bees in which both tracts were hit and acquired a large set of PNs (63 m-APT PNs, 59 l-APT PNs, 260 odor trials per PN) for floral and pheromonal odor stimulations with 20 trials per stimulus and subsequent quantitative data analysis. Recordings from 4 bees (9 m-APT PNs, 9 l-APT PNs, 70 odor trials per PN) were analyzed for their responses to stimulations with social odors over ten trials. The first group of odors (Table III.1) and the associated responses were selected to facilitate comparison to results from earlier intracellular recordings (Sun et al., 1993; Abel et al., 2001; Müller et al., 2002; Krofczik et al., 2009) and calcium-imaging studies (Joerges et al., 1997; Sachse et al., 1999; Sandoz et al., 2003; Guerrieri et al., 2005a; Szyszka et al., 2008; Deisig et al., 2010; Galizia et al., 2012). Since one recorded bee responded to all tested odors but to the controls with a PER, we were ascertained about adequate odor-stimulation (Figure III.1) The second group of odors (Table III.1) are odors from the bee's direct environment and were applied in the hive's temperature range the bees are confronted with (Seeley, 1985; Tautz et al., 2003). These types of context dependent odors were chosen to analyze whether all odors and odor types are responded along both tracts, and whether APTs are odor-specific.

Post recording verification of electrode and tract position

We tracked the electrode depth using the micromanipulator positions (m-APT: 177 ± 25µm, l-APT: 306 ± 19µm). Recorded depths were in accordance with the relative location of the APTs caudal to the AL (Brandt et al., 2005; Kirschner et al., 2006). Additionally, we established a novel double labeling technique to verify the electrode recording positions in relation to the m- and l-APT after successful dual tract recordings (Figure III.5). The electrodes were bathed in adhesive fluorescent dye which sticks to the dental wax surrounding the electrode wires. The fluorescent dye penetrated the tissue surrounding the electrodes, and the APTs were anterogradely labeled after successful recordings. Double staining was confirmed in whole-mount preparations using confocal image stacks and 3D AMIRA reconstructions (n=9; Figure III.5C).

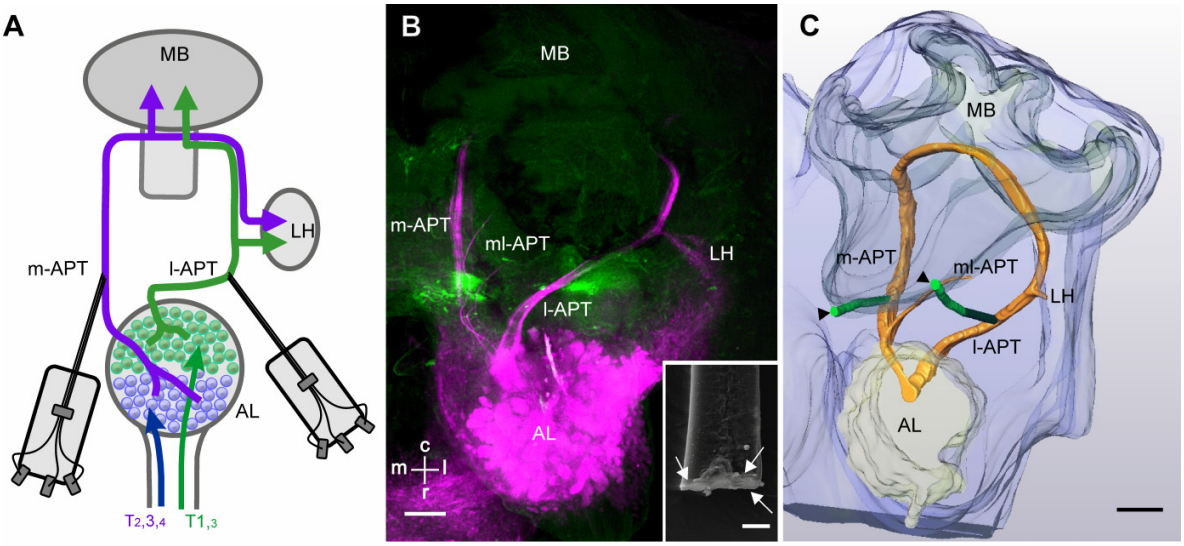


Figure III.5: Simultaneous dual-tract olfactory PN recordings using thin-wire electrodes. A) Schematic overview of the recording position in uniglomerular PN pathways of the honeybee. The AL is innervated by axonal olfactory receptor neuron tracts (T1-T4) that segregate in glomeruli of the dorsal (magenta) and ventral (green) AL hemilobes. After preprocessing in the AL two antennal-lobe protocerebral tracts, the medial (m-APT) and lateral (l-APT) connect glomeruli in the two hemilobes of the AL with the mushroom bodies (MB) and lateral horn (LH) in opposite order. Wire electrodes (each shaft comprising 3 copper wires, each 15 µm diameter, see inset in 1B, indicated by arrows) were used to record PNs from the m-APT and l-APT. B) Staining of electrode insertion sites (green) and anterograde staining of the l- and m-APT (magenta).C) Reconstructions of electrode positions (either by fluorescent staining or identification of electrode tracks in the tissue) and APTs proved that on the m-APT side, the electrode was in close vicinity to the m-APT, above the branching point of the ml-APT. On the l-APT side the electrode was positioned in the lateral-caudal protocerebrum between vertical lobe and the AL (see Figure 1 in Kirschner et al., 2006, positions 3 and 4). m: medial, l: lateral, c: caudal, r: rostral, ml-APT: medio-lateral APT, scale bar in B,C: 100µm, inset in B: 25 µm

Odor response profiles of individual PNs

First we analyzed odor response profiles (tuning curves) of individual APT PNs. Visual inspection of the PN's activity indicated that in a bee we found a single l-APT PN (raster plot, Figure III.6A) being responsive to most of the tested odors whereas a simultaneously recorded single m-APT PNs from the same bee was activated by comparably few odors (Figure III.6A). We quantified PNs response profiles by calculating the odor evoked spike rate, which is the average PNs spike rate during odor stimulation above background, in response to 12 different floral and pheromonal odors (Table III.1, Figure III.6B). Four representative examples of single PNs from each tract confirm the finding of the raster plot that l-APT PNs respond broadly to many odors (Figure III.6B). In contrast m-APT PNs were activated by comparably fewer odors (Figure III.6B). We computed the lifetime sparseness (LS) for

each single PN based on its response profile. This measure is designed to quantify stimulus specificity of individual neurons (Willmore and Tolhurst, 2001; see Materials and Methods) and allows comparison across different neuronal populations. This analysis, again, supports that m-APT PNs have a higher odor-specificity compared to l-APT PNs (Figure III.6C) (l-APT: 0.42 ± 0.21, m-APT: 0.60 ± 0.24; t-test, $p<0.001$). Although individual m-APT PNs have comparably high odor specificity, on the population level m-APT PNs cover the whole odor spectrum, similar as the population of l-APT PNs (Figure III.6B).

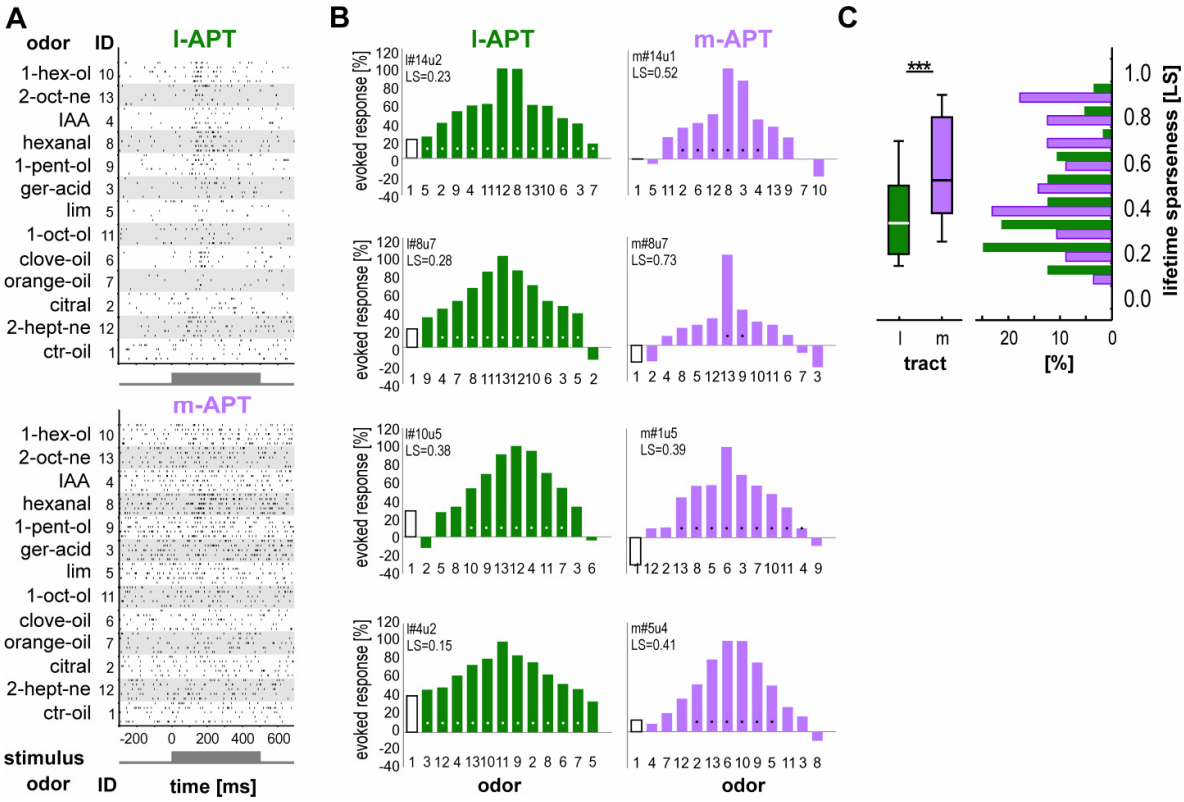


Figure III.6: Odor response profiles indicate higher odor-specificity for m-APT PNs. A) Raster-plots of two simultaneously recorded PNs from the l- and m-APT of one bee stimulated with 12 different odors indicating higher odor-specificity in the m-APT PN compared to the l-APT PN which responds to more odors. The first 5 out of twenty trials are depicted together with odor name and its respective odor ID number. Stimulus duration of 500ms is indicated as grey box. B) Odor-response profiles of four representative PNs from the l- and m-APT (green: l-APT; purple: m-APT) are shown. Normalized evoked response strength is given as normalized spike rates during stimulus duration (500ms) above background in response to 12 different odors (filled bars) and one control (empty bar). Single PN life-time sparseness is shown in insets (LS) together with PN identity. Above-threshold firing response levels are indicated by white or black dots, respectively. Odor ID numbers are indicated and correspond to the odors shown in A. C) Lifetime sparseness (LS) collected from all recorded PNs from the l- and m-APT (green and purple, respectively) illustrated as box-plot (left) and histogram (right). The medians of all PNs LS indicate the m-APT PNs to be significantly higher odor specific compared to l-APT PNs (non-parametric Kolmogorov-Smirnov, $p<0.001$); box plot settings: line=median, box: 1st and 3rd quartiles, whisker: 10th and 90th quartiles)

Odor-specific PN recruitment

Odor-specificity in m-APT PNs was significantly higher than in l-APT PNs. In turn this suggests that a given odor stimulus may activate more l-APT PNs than m-APT PNs. These expectations were confirmed qualitatively by viewing raster plots from all recorded APT PNs of all tested bees stimulated with one odor (Figure III.7A) illustrating higher numbers of activated l-APT PNs compared to less activated m-APT PNs. This is confirmed in the 2-D plot (Figure III.7D). We counted all PNs that were excited by the tested odors and in total found significantly higher proportion of activated l-APT PNs than m-APT PNs (l-APT: 51.0 ± 7.9%, m-APT: 34.0 ± 8.4%; Chi^2, p=0.0011) (Figure III.7B, dashed lines; Figure III.7D). These proportions of activated vs. non activated PNs in the population of all PNs are well suited to maximize the local computation of odors in the AL as it was discussed elsewhere (Nawrot, 2012). Odors that elicited responses in increasing numbers of l-APT PNs also activated increasingly higher numbers of m-APT PNs (Kendalls Tau =0.59, p=0.007). In both tracts, 1-octanole and 2-octanone activated the highest number of PNs followed in decreasing order either by 1-hexanol and 1-pentanol in the l-APT or hexanal and 1-hexanol in the m-APT. Interestingly, within both tracts, the odor dependent recruitment rates significantly differ between odors (Cochran's Q-Test; l-APT p<0.040; m-APT p<0.007). These analyses match the finding of odor-specific activation of glomerular activation patterns as observed in calcium imaging studies (e.g. Joerges et al., 1997; Sachse et al., 1999; Galizia and Menzel, 2001; Szyszka et al., 2005; Fernandez et al., 2009; Carcaud et al., 2012). Our results, counting the number of effective odors per PN (Figure III.7C), conclusively confirm the higher odor-specificity of m-APT PNs compared to the odor-generalization properties in l-APT PNs shown on a low number of neurons in previous studies.. On average, a single m-APT PN was predominantly activated by about $1/3^{rd}$ of the tested odors. In contrast, a single l-APT PN on average responded to about half of the 12 odors tested (l-APT: 6.4 ± 3.3, m-APT: 4.1 ± 2.8, t-test, p>0.001) (Figure III.7C, Table III.2).

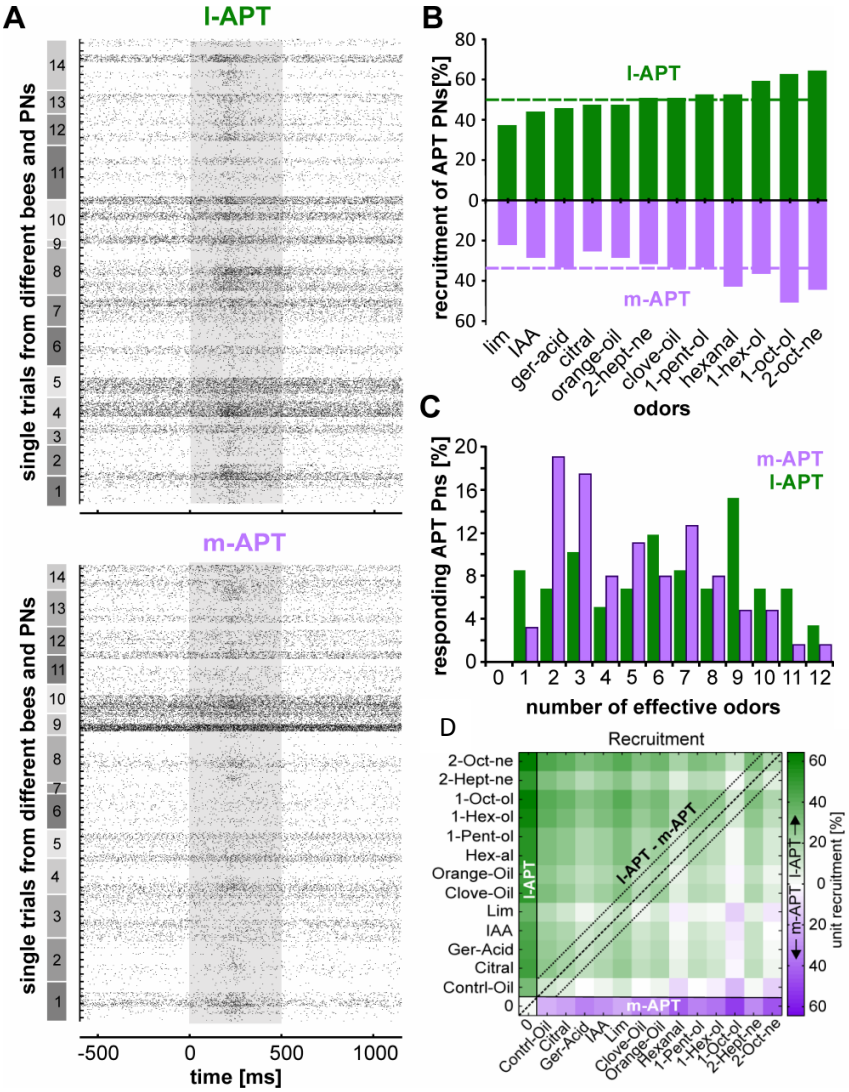


Figure III.7: Odor response spectra of l- and m-APT populations. A) Global firing pattern of all simultaneously recorded m- and l-APT PNs from in total 14 bees. All PN responses recorded from the l- and m-APT (A: 59 PNs in l-APT, B: 63 PNs in m-APT) which were stimulated with a 500 ms pulse of 1-octanol (1:100 in paraffin oil; stimulus is indicated as grey box). The responses are sorted along both tracts by the same sequence of bees (numbers on grey boxes to the left) with the first 5 out of 20 tested trials illustrated as raster-plots. B) Recruitment rates (percentage of odor-activated PNs per odor) are depicted tract-wise (green: l-APT, purple: m-APT) in response to 12 odors. Profiles correlate significantly (Kendalls Tau =0.59, p=0.007). Mean recruitments rates are indicated as dashed line. C) Higher odor-specificity in the m-APT is indicated by the proportion of effective odors per APT PN (12 odors tested, l-APT, green bars: on average 6.4 ± 3.3 odors per PN, m-APT, purple bars: 4.1 ± 2.8 odors on average), which is significantly different in both tracts (M-W U-test, p<0.001). D) 2 dimensional diagram illustrating the recruitment differences along both tracts by subtracting the odor-wise recruitment rate of the l-APT (left, vertical line, grenish) from the recruitment rate of the m-APT (bottom, horizontal line, pruple) which results in the framed diagonal.

Comparison to previous studies

In order to provide comparison, previous studies relied on low numbers of sequentially intra-cellular recorded neurons. They found in 37 m-APT PNs and 15 l-APT PNs a rate of 73% activated l-APT PNs and 35% m-APT PNs with about half of all tested odors (n=5-8) eliciting responses in the l-APT, 1/4th in m-APT PNs (Müller et al., 2002). More recent recordings of 23 m-APT PNs and 7 l-APT PNs stimulated with 3 odors found a rate of 56% activated l-APT PNs and 65% m-APT PNs (Krofczik et al., 2008).

Response latency on individual PNs

Recently, it has been suggested that odors may be encoded by response latencies (in vertebrates: Junek et al., 2010; Smear et al., 2011; in insects: Müller et al., 2002; Krofczik et al., 2008; Kuebler et al., 2011). In our study, we address the question whether latency may indicate odor identity. And whether our recordings confirm previous findings for the bee, that is single APT PNs vary in their response latencies (Müller et al., 2002; Krofczik et al., 2009). We determined odor response onsets in antennal ORNs by EAG recordings (Material and Methods). We found the EAG response latency, on average, to be 99ms from stimulus onset in the olfactometer (valve-switching) to the onset of EAG responses. This setup specific odor transmission delay was subtracted from further latency measurements in PN recordings. To exclude that vapor pressure or water solubility affect odor response latencies, we correlated these parameters with the response onset of EAGs. No correlation was found for both vapor pressure ($r^2 = 0.095$, $p = 0.308$) and water solubility ($r^2 = 0.085$, $p=0.358$) for the odors used.

We found response latencies differing between different odor stimulations within single APT PNs and between different APT PNs within one tract (Figure III.8A). To be a reliable source of information, response latencies to different odors would need to exceed inter-trial latency variations to the same odors. To quantify the reliability of a latency code we calculated the signal to noise ratio (SNR) based on latencies (Figure III.8B). But with the addition, that SN ratios need to pass unity, ensuring odor coding by response latencies (see materials and methods). We found SN ratios exceeding unity in about 1/4th of the l-APT PN (25.0%, 12/48 units) and more than 1/3rd of all the m-APT PNs (36.8%, 14/38 units; Figure III.8B). Although the SN ratios across the entire l- and m-APT PN

populations on average did not exceed one and additionally do not differ between tracts (Wilcoxon rank-sum test,, p=0.28).

We asked whether the response latencies are odor dependent and found 8 out of 12 tested odors eliciting faster responses in l-APT PNs whereas two pheromonal odors IAA and 2-heptanone and two natural odor bouquets elicited faster responses in the m-APT than in the l-APT (Table III.2). Across all individual APT PN response latencies (Figure III.8C) we found general latency differences between tracts, that is the l-APT PNs, on average, respond by about 14ms earlier compared to m-APT PNs (l-APT: 169.7ms ± 76.0ms; m-APT: 183.8ms ± 78.6ms; t-test, p<0.01; Table III.2).

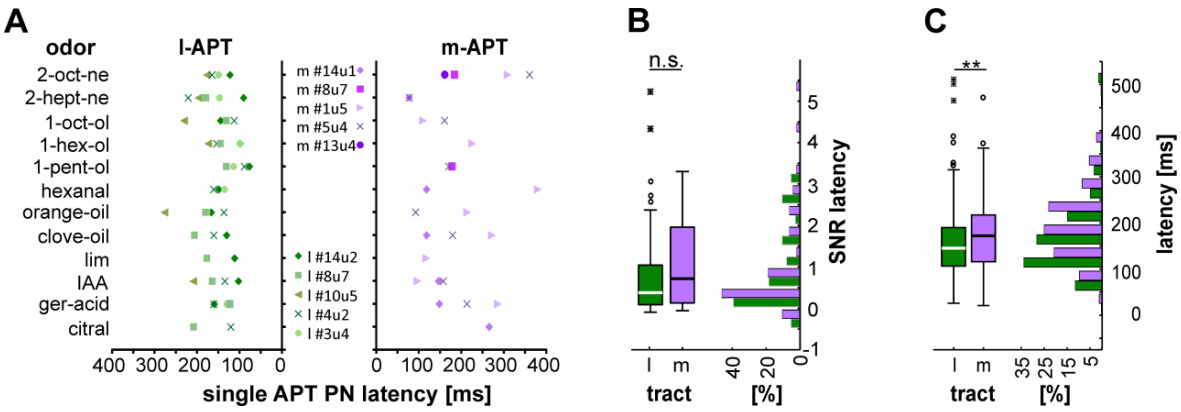


Figure III.8: Response latencies of single PNs differ for different odors, PNs and tracts and support a latency code along the l- and m-APT. A) Response latency of three representative APT PNs from either the l- or m-APT are shown for different odors (PNs correspond partly to those shown in Figure 3B). Note that individual PNs did not respond to all odors. B) Distribution of the signal to noise ratio (SNR) indicating the variation of latencies between odors relative to the variation across trials of identical stimuli. C) Box-plots and histogram of tract-wise odor-dependent latencies show that l-APT PNs respond faster compared to m-APT PNs (M-W U-test, ** p=0.003). Box line: median, box: 1st to 3rd quartiles, whisker: ± 1.5 times the box ± the box, outliers above whisker).

Population response of l- and m-APT PNs

To address coding at the PN population level as has been done in calcium-imaging studies which revealed combinatorial odor-coding (Sachse and Galizia, 2002), we calculated rate functions - averaged firing rate profiles over time. Instead of looking at individual rate functions based on odor-evoked trial-averaged and aligned single PN responses as done above, we averaged and aligned all

odor evoked PN responses and condensed them in odor-dependent rate functions (Figure III.10-III.13)(method after Krofczik et al., 2008; Strube-Bloss et al., 2011; Meyer and Galizia, 2011; Nawrot, 2012). In addition, the alignment process reveals precisely time-matched rate functions suitable for population latency measurements.

Population rate codes

We averaged all odor responses tract-wise and for each tested odor (Figure III.9,III.10). This analysis revealed phasic-tonic response characteristics in both, the l- and m-APT with mostly strong phasic responses to the odor onset and subsequent weak tonic firing patterns over the time of odor stimulation. Our results show that the l-APT has in tendency slightly higher background activity (l-APT: 15.2 ± 17.8Hz, m-APT: 12.4 ±17.9Hz; Wilcoxon rank-sum test, $p>0.1$, see Figure III.7A), a result comparable to previous intracellular recordings of PNs (12Hz, Sun et al., 1993; 11Hz in m-APT and 3,5Hz in l-APT, Müller et al., 2002; 7-12Hz in both tracts Krofczik et al., 2008, with slightly higher rates in l-APT PNs, personal communication M. Nawrot). Odor-evoked peak rate across all responsive l-APT PNs is on average 24.6Hz and about one third higher compared to the population of m-APT PNs with 16.2Hz (median: l-APT= 16.4Hz (3.5Hz-107.9Hz); m-APT: 13.7Hz (2.2Hz-62.4Hz); Wilcoxon signed rank test, $p<0.001$).

We plotted the rate functions of l- and m-APT PNs in response to 12 different odors (Figure III.10A,B). The rate functions in response to different odors show surprisingly high similarities across both tracts with some odors resulting in longer transient from phasic to tonic than others (for example IAA, Figure III.10B). In some cases, we found off responses (citral). An observation which matches data from previous intracellular recordings remarkably well (compare Figure 3 in Abel et al., 2001; or Fig. 5 in Müller et al., 2002). For the vast majority of odor responses, l-APT PNs are activated stronger compared to m-APT PNs. However, one third of all tested odors elicited similar AP rates in both tracts, indicating that the found differences are not due to a generally lower responsiveness of m-APT PNs. We additionally asked whether odor evoked AP rates are different between different odors tested. We normalized the responses which revealed that l- as well as m-APT PNs are able to encode odors by AP rates (non-parametric Kruskal-Wallis ANOVA, l-APT: $p<0.001$, m-APT: $p<0.001$). This fact is also visible in the normalized depiction of the relative response strength in a 2D plot (Figure III.9A,B).

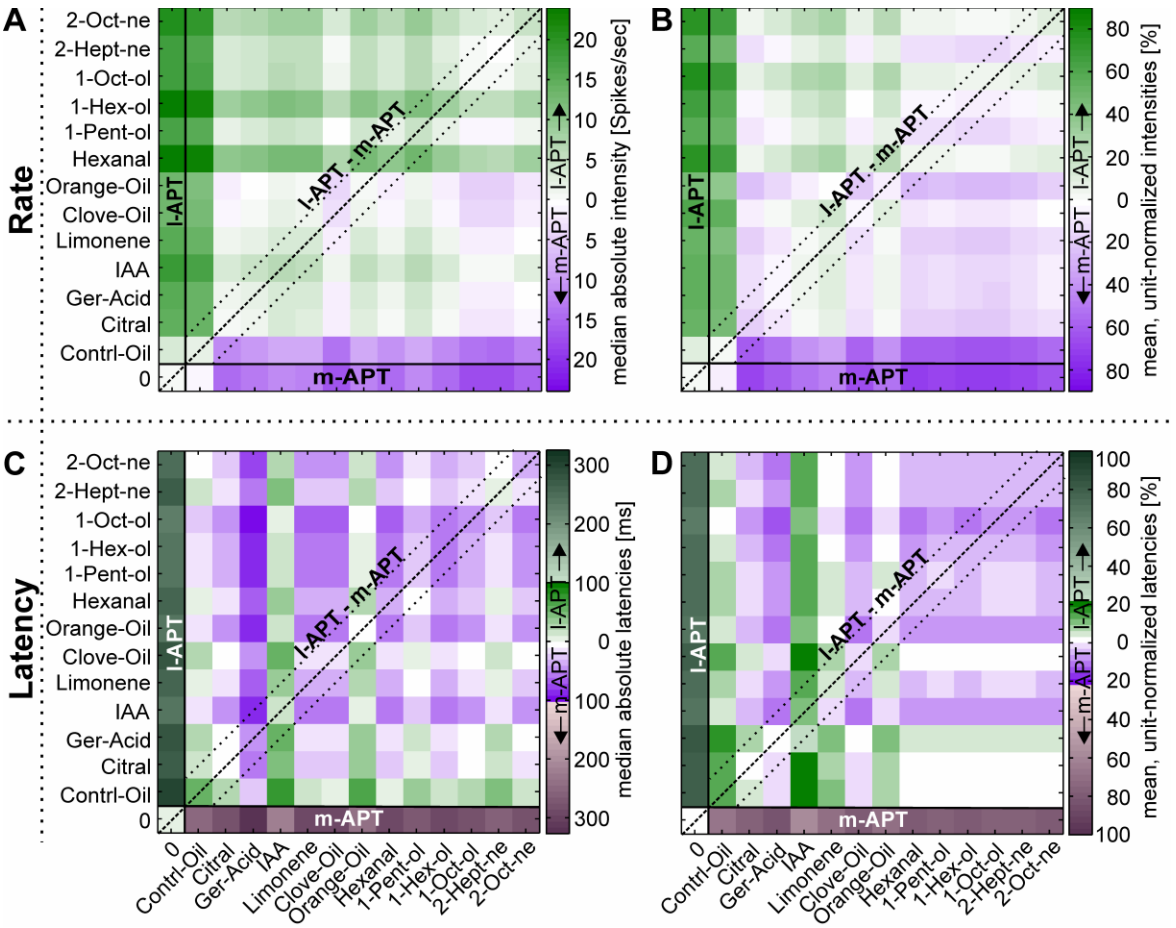


Figure III.9: Quantitative differences of odor-elicited responses in both, the l- and m-APT, regarding rate code differences (A,B9 and response latency differences (C,D). A) Median absolute intensity and B) relative response intensity of 65units from the m-APT (bottom line) and 65 units from the l-APT (left row) normalized to each bee at a time (15 in total) with simultaneous dual tract recordings in respect to 12 odors and 2 controls. The difference of each odor response is illustrated as false color coded as 2D-plot. The recruitment or median of the relative intensity change of a single odor is depicted in color code in the diagonal framed by two grey lines. The absolute response strength is enhanced within the l-APT whereas the median of relative intensity change of both tracts shows almost the same intensities for both tracts. C,D) Evaluation of the response latency of single APT PN from both tracts illustrates that in absolute as well as normalized data, the l-APT has, in most cases, shorter response latency.

Population response latencies

Since some APT PNs showed odor-dependent differences in response latencies (Figure III.8A,B), we analyzed response latencies at the population level (Figure III.10). To reduce inter-individual latency variations, we used an alignment algorithm of odor-response in time (Nawrot et al., 2003) (see material and methods). Comparison of the population response latencies with those of individual

APT PNs (previous section) confirmed that l-APT PNs responded on average ~13ms faster than m-APT PNs, although for each tested odor we found overlapping latency deviations. The alcohols 1-pentanol and 1-octanol elicited the fastest population responses in the l-APT, whereas the pheromonal odors IAA and citral elicited the fastest population responses in the m-APT (Table III.2). At the level of population responses, some odors generate consistent latency differences if PNs of both tracts are considered (ANOVA, $p<0.05$).

Since honeybees encounter odor stimuli in the environment that naturally underlie turbulences and thus change their odorant concentration immediately (Vetter et al., 2006), besides the stimulation of a single 500ms pulse we additionally stimulated the olfactory system with triple odor stimuli pulses of 500ms with additional 500ms ISI and analyzed the population responses (Figure III.11). Already in the raster, the responses to the triple pulses of the l-and m-APT PNs follow the responses by phasic responses, but with decreasing intensity to the second and third pulse with in total comparably weaker responses in the m-APT PNs. This is likely caused by habituation, although the ORNs are precisely able to follow the stimulus presentation (see EAG in Figure III.11). The l-APT PNs respond more strongly to the given stimulus (2-octanone in that example, Figure III.11) compared to the m-APT PNs, which is additionally reflected in the proposed habituation.

To evaluate whether the calculated PN population response latency is biased by our threshold criterion, which is that 10 of 20 trials need to respond to odors over threshold, we additionally analyzed the PN population response latency for thresholds of 5 and 15 trials (25%, 50%, 75% respectively; Figure III.12) which resulted in similar qualitative response latency differences. Still the l-APT elicits faster responses.

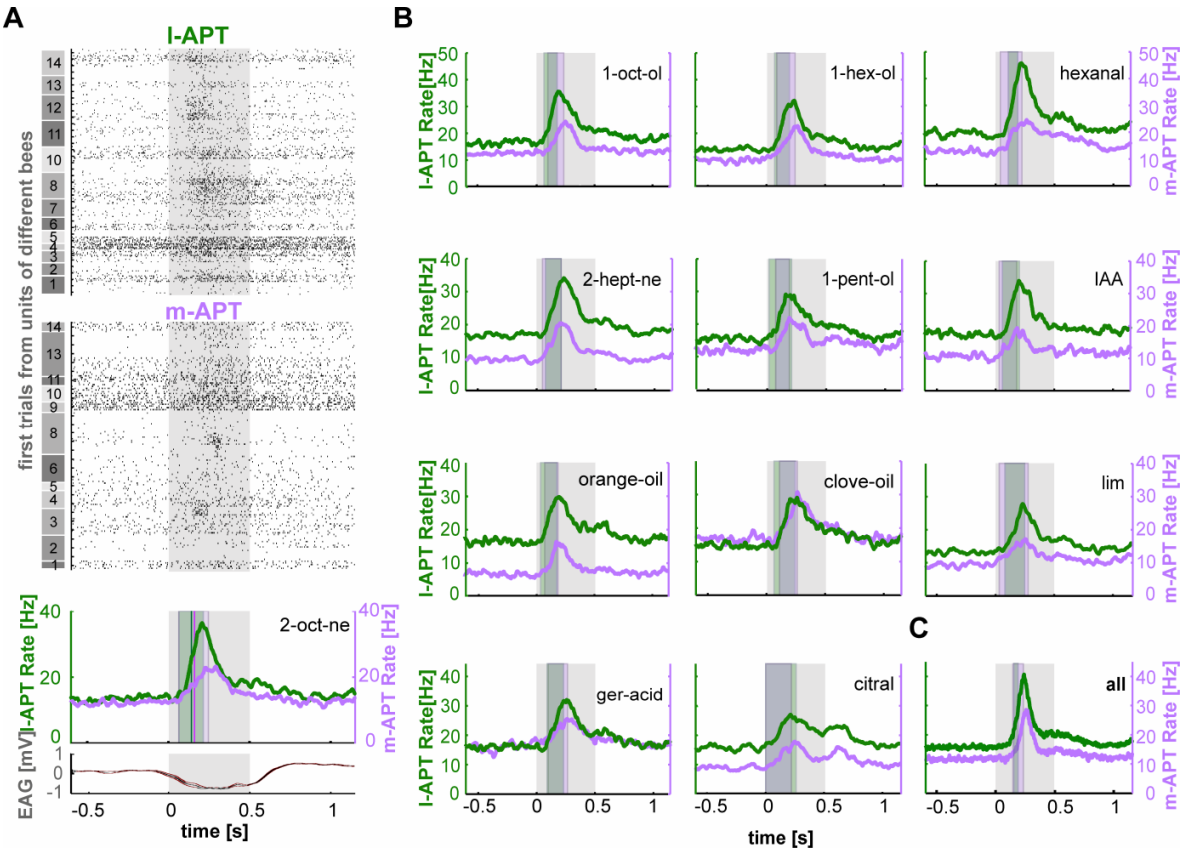


Figure III.10: PN population response characteristic of the m- and l-APT. A) Raster plots illustrate spike patterns of l- and m-APT PNs stimulated with a 500ms odor pulse (grey box; 2-octanone; 1:100). The responses are aligned to their average peak-response for latency measurements and sorted in the same sequence for the different bees (numbers on the left side). The responses are averaged and shown tract-wise as spike response profiles (green: l-APT, magenta: m-APT). Response latency was adjusted to the olfactory receptor neuron (ORN) response determined by the odor onset in EAG measurements (lowest trace; EAG with SD is shown) averaged over 6 trials. The population response latency is indicated by vertical lines, and the respective latency deviation as light colored boxes (green: l-APT, magenta: m-APT). Raster shows only the first 5 out of 20 odor-stimulation trials. B) Population response profiles illustrated as rate functions over time calculated from the PNs (responses to 2-octanone shown in A) in response to 11 different odorants (Table 1). The respective latency deviations are shown as light colored boxes. Response latency deviations in population responses, in most cases, are largely overlapping supporting potential coincidence activation of postsynaptic neurons. C) Population response profiles of all l- and m-APT PNs averaged over all tested odors (Table 1) illustrated as rate functions over time. Over all tested odor the l-APT responded faster and comparably stronger to the tested odors.

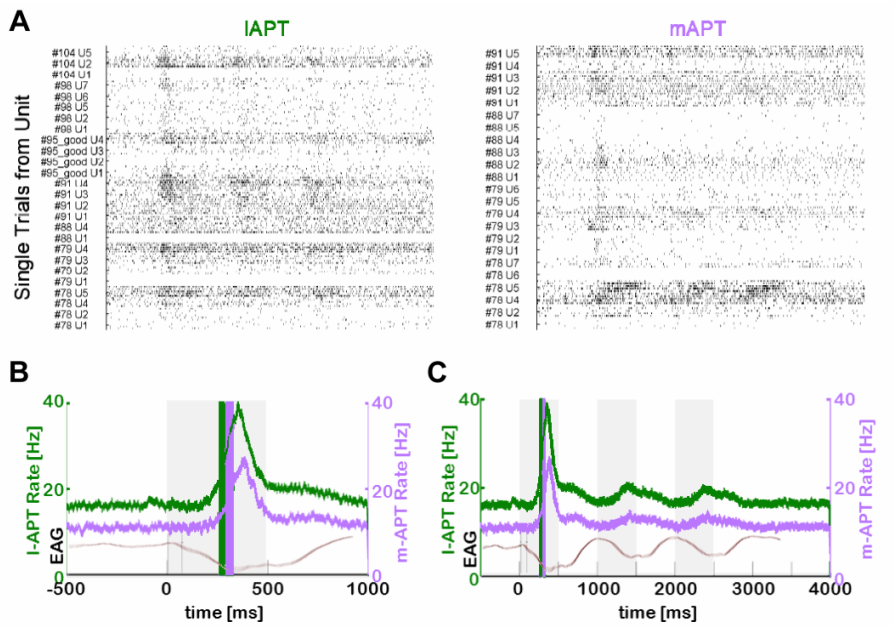


Figure III.11: Comparison of single odor-pulse stimulations versus three successive odor-pulse stimulations. A) Raster plots illustrate spike patterns of l- and m-APT PNs stimulated with a 500ms odor pulse (grey box; 2-octanone; 1:100). The responses are aligned to their average peak-response for latency measurements and sorted in the same sequence for different bees (bee ID together with PN number is indicated next to the raster). Raster shows only the first 5 out of 20 odor-stimulation trials. The responses are averaged and shown tract-wise in B) as spike response profiles (light gray: l-APT, dark gray: m-APT). Response latency was adjusted to the olfactory receptor neuron (ORN) response determined by the odor onset in EAG measurements (lowest trace; EAG with SD is shown) averaged over 6 trials. The population response latency is indicated by vertical lines and the respective latency deviation as light colored boxes (green: l-APT, magenta: m-APT). C) Indicates the same odor response profile of both tracts but for three successive odor pulses of 500ms, 500ms ISI (indicated as grey boxes).

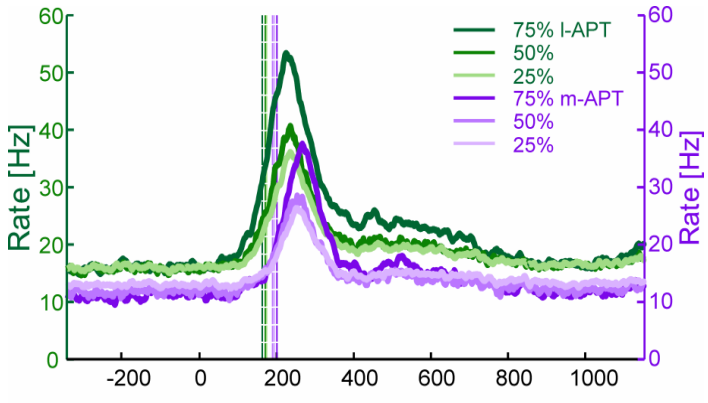


Figure III.12: PN population response profiles of all l- and m-APT PN pooled in response to all tested odors (Table1). Different criteria of used trials in the data analyses (25%, 50%, 75%, of the acquired 20trials) do not qualitatively change the properties. At all cutoff criteria the l-APT PN population responded earlier to the odor stimuli in contrast to the m-APT PN population.

Responses to biologically relevant odors

Odor specificity of m-APT PNs is not well described and it is still under debate whether this tract, presumably the m-APT, may be adapted to code for socially relevant odors and/or pheromones. To explore this possibility, we used natural odor mixtures with high biological relevance: a mixture of old bees wax, cleaned bees wax, abandoned brood combs, honey solved in water, and freshly killed bees (Table III.1). All mixtures were presented at a temperature range close to natural conditions in the hive (Seeley, 1985; Tautz et al., 2003). The results clearly show that information about all natural odor mixtures is transferred in parallel along both PN tracts (Figure III.13). Population responses of l- and m-APT PNs (n=4 bees: l-APT 9 PNs, m-APT 9 PNs, except for freshly killed bees n = 3 bees, l-APT 6 PNs, m-APT 7 PNs) reached comparable AP rates in both tracts (Figure III.13A,B).The odor mixtures evoked similar rate functions in PNs of both tracts (Figure III.13B). The high AP rate in response to the natural odor mixtures probably is due to a higher vapor pressure of the tested odors (Carcaud et al., 2012). The insignificant difference in AP rate between tracts might be due to the small sample size (median: l-APT= 23.8Hz (4.8Hz-76.2Hz), m-APT= 24.4Hz (13.9Hz-42.7Hz); Wilcoxon Rank-sum test, p=0.86).

The highest AP rates in both tracts were elicited in response to freshly killed bees (median: l-APT= 22.2Hz (7.9Hz-65.4Hz), m-APT= 24.4Hz (14.0Hz-24.4Hz)) suggesting a high sensitivity or high concentration of the related odors (Seeley, 1982; Robinson et al., 1999; Spivak et al., 2003). Compared to latency differences in single APT PNs (Figure III.8), in response to the hive odors we measured latency differences of l-APT PNs being activated 20.6ms earlier compared to the m-APT PNs in this group of bees, but probably due to low numbers not being significantly different (Table III.2, Wilcoxon rank-sum test, p>0.1).

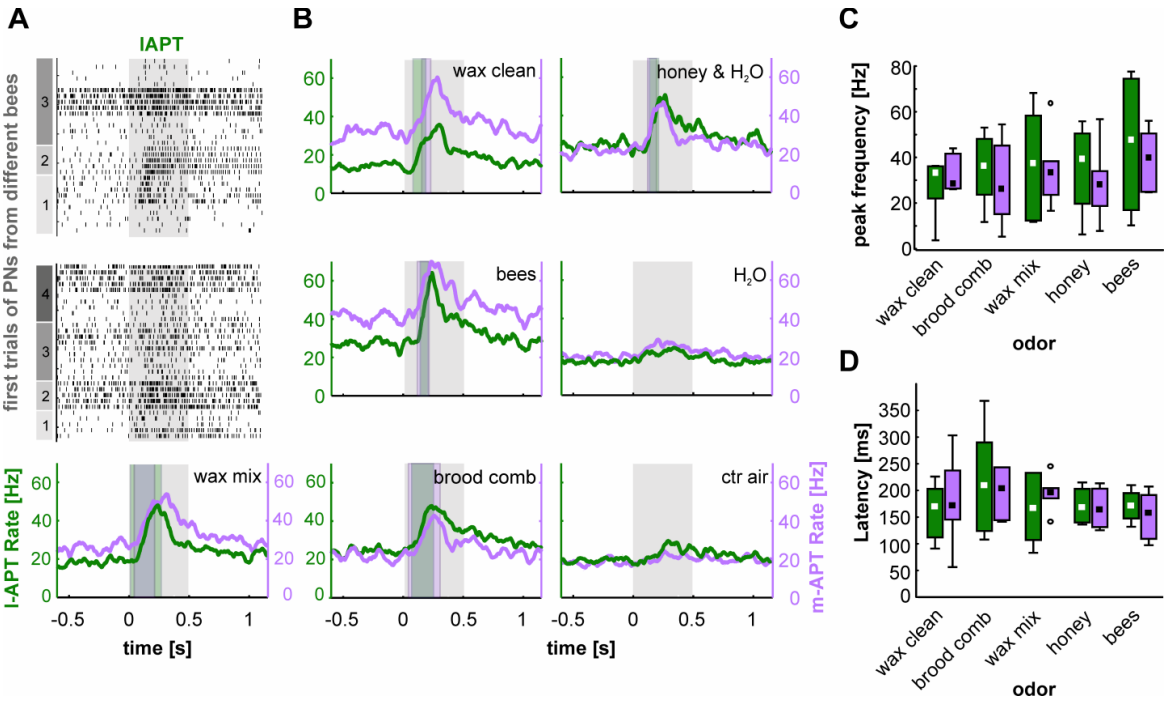


Figure III.13: APT PN responses to biologically relevant odor mixtures. A) As an example, raster plots illustrate spike patterns of beyond threshold responses in APT PNs (l-APT upper and m-APT lower plot) from 4 bees (500ms stimulus duration, grey bars; wax-mix at 32-33°C, see methods section for details). Responses are aligned to the average peak-response and sorted for the different bee (grey boxes to the left; only the first 5 out of 10 trials are shown). Bottom plot: averaged spike frequency profiles of l- and m-APT PNs (green: l-APT, purple: m-APT) illustrate population response to the bee's wax mixture. Population response latencies are calculated via the n-shift algorithm and indicated as latency deviation in light colored boxes (green: l-APT, purple: m-APT). B) Population response profiles illustrated as rate functions over time (calculated as in A) to 4 natural odor mixtures (clean bee's wax, freshly killed bees, brood combs, honey solved in water, and two controls: air and water) heated to 33°C to meet natural hive conditions. Strong phasic population responses in both APTs with almost similar odor onsets are visible. Notice only three bees were recorded in response to freshly killed bees.

Table III.2: APT PN population response latencies. The response latencies of APT PNs are shown partitioned into tested bee groups. Response latencies with standard deviation (SD) are plotted odor-wise for the number of responding PNs (n) and the latency differences of m- versus l-APT (m-l) .

bee group	odor	l-APT			m-APT			
		n	latency [ms]	SD [ms]	n	latency [ms]	SD [ms]	m - l
1	all	357	169.7	76.0	254	183.8	78.6	14.1
1	IAA	25	157.1	59.4	17	133.2	66.8	-23.9
1	citral	27	212.6	124.5	16	191.9	109.3	-20.7
1	orange-oil	28	153.0	59.6	18	141.4	56.5	-11.6
1	clove-oil	30	201.2	89.5	21	194.3	75.0	-6.9
1	1-pent-ol	31	167.3	95.0	21	174.4	58.3	7.1
1	2-hept-ne	30	164.9	66.2	20	173.6	79.1	8.7
1	lim	20	192.9	60.4	13	205.2	107.2	12.3
1	ger-acid	26	191.7	66.7	21	211.4	82.2	19.7
1	1-hex-ol	35	152.2	62.5	23	183.9	78.0	31.7
1	hexanal	31	163.7	39.6	26	195.5	84.7	31.8
1	1-oct-ol	37	143.4	55.7	31	184.4	53.6	41.0
1	2-oct-ne	37	160.1	70.0	27	201.5	80.9	41.4
2	all	19	164.5	49.4	27	186.3	63.8	21.8
2	bees	4	164.8	31.3	5	152.2	48.6	-19.1
2	honey	5	175.6	63.0	7	188.1	53.3	12.5
2	wax mix	4	162.0	49.2	6	192.0	89.3	30.0
2	wax clean	2	149.0	4.2	6	189.2	56.4	40.2
2	brood comb	4	164.5	60.0	3	210.0	71.5	45.5

Discussion

We recorded, for the first time, simultaneously odor induced activity from projection neurons (PNs) of two separate output pathways (lateral and medial APT) of the primary olfactory centers in the honeybee, taking advantage of the distinct anatomy in the honeybee's olfactory pathway (Kirschner et al., 2006). The most important findings are that information about all tested odors (including natural mixtures) is transferred along both tracts, and that comparison of single PN response profiles and population responses revealed systematic differences in representations of individual odors and odor mixtures across both tracts. We conclude that this differential processing of the same odors along two olfactory pathways matches the criteria of parallel processing, comparable to other sensory systems (auditory: e.g. Yu and Young, 2000; Rauschecker and Scott, 2009; visual: Livingstone and Hubel, 1988; Strausfeld et al., 2006; Paulk et al., 2008; Nassi and Callaway, 2009; somatosensory: Ahissar et al., 2000; electrosensory: Metzner and Juranek, 1997). In the visual system, for example, the magno- and parvocellular pathways from the lateral geniculate nucleus mediate different elemental properties of the same visual scene such as color and spatio-temporal patterns (Livingstone and Hubel, 1988). In rodents parallel odor processing was recently found in mitral and tufted cells targeting the olfactory tubercle and piriform cortex and showing similar odor response magnitudes and profiles but different signal-to-noise ratios (Payton et al., 2012).

Segregated vs. parallel coding of olfactory information

The honeybee dual olfactory pathway is anatomically separated in PNs that innervate the MBs and LH in reverse order (Mobbs, 1982; Abel et al., 2001; Kirschner et al., 2006; Zube et al., 2008; Rössler and Zube, 2011). This pattern provoked different hypotheses about its functional significance -"dual segregated" (different odors in different tracts) or "dual parallel" (similar input, differential feature extraction) (Galizia and Rössler, 2010). Two recent calcium imaging studies indicate that sensory input to l- and m-APT glomeruli is remarkably redundant (Carcaud et al., 2012; Galizia et al., 2012) with only slight coding preferences for chain length and functional group or variations in response strengths between the two subsystems. Representation of colony odors in the ant AL (Brandstaetter and Kleineidam, 2011) suggests that the two subsystems either receive similar sensory input or sensory input is locally distributed across both hemilobes. Our study strongly supports that PNs of

both APTs receive largely similar input, although we cannot exclude that certain odors may be transferred only via one APT, especially considering the large odor space bees are confronted with (Guerrieri et al., 2005b; Haddad et al., 2008, 2010; Chen et al., 2011). Future experiments will have to expand analyses within the behaviorally relevant odor space.

Odor specificity range

The lifetime sparseness (LS), the recruitment rate of PNs per odor, and the average number of odors activating PNs indicate higher odor-specificity in m-APT PNs than in l-APT PNs. This large data set resolves differing results from previous studies (Müller et al., 2002; Krofczik et al., 2009) with intracellular recordings of low numbers of neurons and odors tested. In contrast to our finding, a sequential imaging study of PN boutons in the MB calyx (Yamagata et al., 2009) found higher odor-specificity in l- compared to m-APT boutons. This may indicate that calcium activity in PN boutons may be influenced by MB microcircuits, in particular inhibitory feedback and neuromodulation (Grünewald, 1999a, 1999b; Ganeshina and Menzel, 2001; Yasuyama et al., 2002; Okada et al., 2007; Rybak and Menzel, 2010; Sandoz, 2011), possibly related to learning and memory (Stopfer et al., 1997; Heisenberg, 2003; Gerber and Stocker, 2007; Okada et al., 2007; Haehnel and Menzel, 2010, 2012; Gauthier and Grünewald, 2012).

Both physiological and modeling studies suggest that lateral inhibition and gain control mechanisms in the AL are mediated by different types of local interneurons (Martin et al., 2011; Wilson, 2011; Assisi et al., 2012). A recent modeling study indicates that this allows variable tuning of odor specificity and concentration dependency in honeybee PNs (Schmuker et al., 2011). Transferred to our results the m-APT would undergo stronger lateral inhibition and gain control mechanisms compared to the more broadly tuned l-APT.

Response latency differences across tracts

In rodents latency differences exist between parallel OB output streams (mitral (M) and tufted (T) cells) (Igarashi et al., 2012). Higher order olfactory parallel processing was also suggested to remain

separated in humans (Frasnelli et al., 2012). Anatomically, in rodents M/T cells target different areas in the olfactory cortex, whereas both l- and m-APT PNs in honeybees target the MB calyces and LH. However, innervation within the MB and LH shows segregated and overlapping zones (Kirschner et al., 2006; Zube et al., 2008). Previous intracellular recordings revealed latency differences between PNs of both tracts (Müller et al., 2002). Our dataset shows a significant overall latency difference between both APTs. Is this latency difference relevant for odor coding? We hypothesize that the broadly tuned l-APT delivers fast and global information about the timing of an odor, whereas the m-APT provides more specific information about odor identity. In analogy to "what-" (object vision) and "where-"channels (spatial vision) in the inferior temporal and the posterior parietal cortex (Mishkin et al., 1983; Merigan and Maunsell, 1993; Milner and Goodale, 2008), the m- and l-APT system may provide "what-" and "when" olfactory information.

Odor-specific response latencies

Temporal coding is discussed controversially for the vertebrate olfactory system (Bathellier et al., 2008; Junek et al., 2010; Smear et al., 2011). Spors et al. (2006) found odor-specific response latencies already at the level of OB input. In insects, temporal odor representations, e.g. oscillations or synchrony in PN activity, was shown in locusts and moths (Laurent, 1996; Stopfer et al., 1997; Christensen et al., 1998; Ito et al., 2009; Kazama and Wilson, 2009; Lei et al., 2009; Riffell et al., 2009b; Raman et al., 2010; Assisi et al., 2011). Quantitative and qualitative analysis of single PN activity suggest that response latencies may be odor specific (Müller et al., 2002, Krofczik et al., 2008; this can also be seen in figure 1 of Perez-Orive et al., 2002 and in figure 1,2 of Wilson et al., 2004). The same odor stimulus can evoke neuron-specific response latencies in a PN population suggesting that latencies are potentially used for coding odor identity (see Figure 2 in Wilson et al., 2004 and Figure 2 in Namiki and Kanzaki, 2008). This was proposed for olfactory processing in bees (Müller et al., 2002; Krofczik et al., 2009) and moths (Belmabrouk et al., 2011; Kuebler et al., 2011) and is supported by our study. It remains to be shown whether the brain actually uses this parameter for odor recognition. In rodents, initial evidence was found to link odor response latency differences to behavior (Smear et al., 2011).

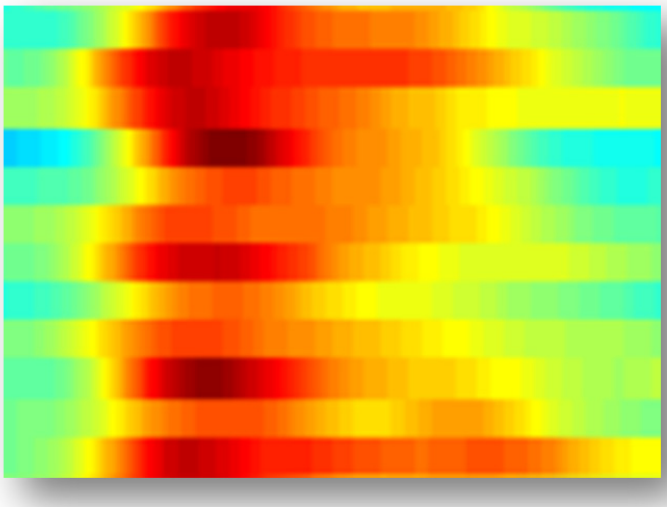
Are odor-specific temporal differences in both PN streams in the honeybee used to extract odor identity in higher centers? KCs require convergent and synchronous (coincident) synaptic input to be excited (Perez-Orive et al., 2002, 2004; Demmer and Kloppenburg, 2009). Sparse coding was shown in the honeybee (Szyszka et al., 2005), locust (Perez-Orive et al., 2002; Brown et al., 2005; Broome et al., 2006; Jortner et al., 2007), moth (Ito et al., 2008) and fruitfly (Turner et al., 2008; Honegger et al., 2011) and was suggested to promote accurate information storage (Olshausen and Field, 2004). Physiological recordings and modeling studies in the locust suggest that temporal divergence in PNs is mainly based on local AL processing (Stopfer et al., 2003; Assisi et al., 2011; Assisi and Bazhenov, 2012). In the honeybee KCs may receive input from multiple PNs. A Golgi study (Strausfeld, 2002) shows that spiny class I KCs and class II KCs have dendritic arbors that span across both m- and l-APT PN target regions (Kirschner et al., 2006). We found latency variances within and across tracts with sufficient temporal overlap for coincidence (Figure 6, III.14). Consequently, odor specific combinations of temporal PN input patterns from both tracts may be transferred in a coincidence pattern of KC excitation. The enormous divergence from PN boutons to KCs provides large computational capacity (Witthöft, 1967; Schürmann, 1974; Mobbs, 1982; Strausfeld, 2002; Groh et al., 2012).

The role of the LH is much less understood in the honeybee. In the locust Gupta and Stopfer (2012) recently categorized ten types of LH-neurons. In contrast to sparse coding and learning-dependent plasticity in KCs (Perez-Orive et al., 2002; Cassenaer and Laurent, 2007, 2012), the majority of LH-neurons was multimodal with broad odor response profiles. In honeybees broad responding l-APT PNs contact the LH first, which fits into this picture. Future analyses of PN spike synchrony within and across tracts together with simultaneous recordings in MB and LH target-neurons are needed to further understand the nature and significance of temporal olfactory coding.

Combining anatomical and functional properties of the honeybee dual olfactory pathway suggests that both rate and temporal coding are implemented along two parallel streams. This may represent an adaptation to serve the multitude of olfactory demands associated with the importance of a complex olfactory world for a social insect.

IV - Chapter 3:

Olfactory Coding: Concentration Coding



Introduction

Odor detection is of significant behavioral importance for insects (Anton et al., 2007; Hansson and Stensmyr, 2011). The olfactory system therefore needs to ensure the reception and perception of an odor under natural conditions. Odors are transported by the air which is of constant movement and flux. Thus, odors are dispersed, mixed and diluted by the surrounding air and additionally intermixed with background odors (Vetter et al., 2006), which finally leads to filamentous plumes of the source odor in differing concentrations (reviewed in Martin et al., 2011). Animals therefore rely on olfactory systems that are suitable to track, receive and perceive the odors over a wide range of concentrations. In pheromonal communication, one most intriguing example is the silk moth *Bombyx mori*, in which it was demonstrated that a single molecule of the female pheromone bombycol potentially elicits behavior in the perceiving male (Kaissling and Priesner, 1970). This astonishing ability of insects to receive tiny amounts of odor molecules engaged researchers to work on the olfactory system over several decades. Recent studies, for example, analyzed the periphery in the fruitfly and suggested that insect OR themselves are able to code for different odor-concentrations mediated by the newly discovered ionotropic and metabotropic characteristics of the insect OR dimers (see general introduction, Hansson et al., 2010). Concerning concentration coding it has been shown that increasing odor concentration broaden the receptive range of ORN (Vareschi, 1971; Akers and Getz, 1993; de Bruyne et al., 2001). It is well-known that glomeruli in the AL elicit odor-specific pattern in a spatio-temporal manner (Galizia and Menzel, 2000b) which changes with increasing odor concentrations by activation of additional glomeruli (Sachse and Galizia, 2003; Wang et al., 2003; Carcaud et al., 2012; Strauch et al., 2012) in an odor dependent manner (Zube et al. 2008). Additionally, different odor concentrations have been shown to affect the local network of the first processing stage, the AL in insects (Sachse and Galizia, 2003; Stopfer et al., 2003; Carcaud et al., 2012; Meyer and Galizia, 2012). Both physiological and modeling studies support that lateral inhibition and gain control mechanism mediate odor detection by increasing sensitivity via excitatory LN or by suppressing non relevant background odors via inhibitory LN in the AL (Martin et al., 2011; Schmuker et al., 2011; Wilson, 2011; Assisi et al., 2012; Girardin et al., 2012). Since in vertebrates it has been shown that mitral cell firing frequency does not scale monotonically with those of the OSN input (reviewed in Cleland and Linster, 2005), we were interested to analyze whether the output neurons of the AL, the l- and m-APT PNs, code different odor concentrations differently. Additionally we wanted to analyze whether there are differences along the dual

uniglomerular PN tracts. Especially since imaging studies in the PN boutons at the synaptic input site of the MBs found rising concentration dependencies in m-APT PNs and less odor concentration dependency in l-APT PNs (Yamagata et al., 2009). These findings were introduced into a dual pathway model inspired by the honeybee's olfactory system, suggests that the l-APT undergoes strong lateral inhibition and strong gain control mechanism whereas the m-APT is influenced by weak lateral inhibition and weak gain control mechanism (Schmuker et al., 2011).

Material & Methods

All experiments were done as described in the former sections (chapter 1, 2). Changes to the methods are mentioned in this section as well as regarding odor stimulation and/or data analyses.

In a pilot study we analyzed coding properties of l- and m-APT PNs in response to different odor concentrations. We tested one bee (#46) with three different odors (citral, 1-hexanol, 2-heptanone) in four different log odor concentrations (10^{-5} to 10^{-2}) in ten trials each condition and analyzed the responses of two m-APT PNs and one l-APT PN (Fig. IV.1).

Furthermore we expanded the stimulation paradigm with one order of magnitude and additionally exchanged one odor (hexanal instead of 1-hexanol) to enable the representation of three different odorant groups (floral-, pheromonal-odor or a mixture out of both, Table III.1). Accordingly we tested three odors (citral, hexanal, 2-heptanone) in 5 different concentrations (from 10^{-6} to 10^{-2}). We recorded simultaneously from both tracts and acquired data from seven bees resulting in 43 units (l-APT: 22, m-APT: 21). Same recordings and stimulus apparatus were used as in the former section.

For response latency measurements with respect to different concentrations, we analyzed one bee (#60) as a pilot experiment. To determine the latency we either used the n-shift criterion and all consecutive analyses (see above) or determined the response as AP increase above 1.5 SD above background. The APs were counted in 5ms bins in a PSTH (Fig. IV.4).

Data Analysis

Since we tested odor responses of APT PNs with odor concentrations that likely will lead to subthreshold responses, we decided not to use any threshold to detect odor responses. Instead, we

counted the APs during stimulus time above background (see evoked responses) or plotted PSTHs (Fig. IV.1). To evaluate differences along the odor concentration range, we performed linear regression analyses and performed ANCOVA statistical tests in Statistica (StatSoft).

Linear regression was analyzed with Excel (Microsoft) in response to latency measurements. Heatplot creation and MATLAB analyzing is done by Tobias Rosenbaum under my guidance.

Results

To analyze coding properties of l- and m-APT in response to different odor concentrations, we tested bees with three different odors (citral, 1-hexanol, 2-heptanone) in four different log odor concentrations (10^{-5} to 10^{-2}) in ten trials with each condition (Figure IV.1, IV.2)

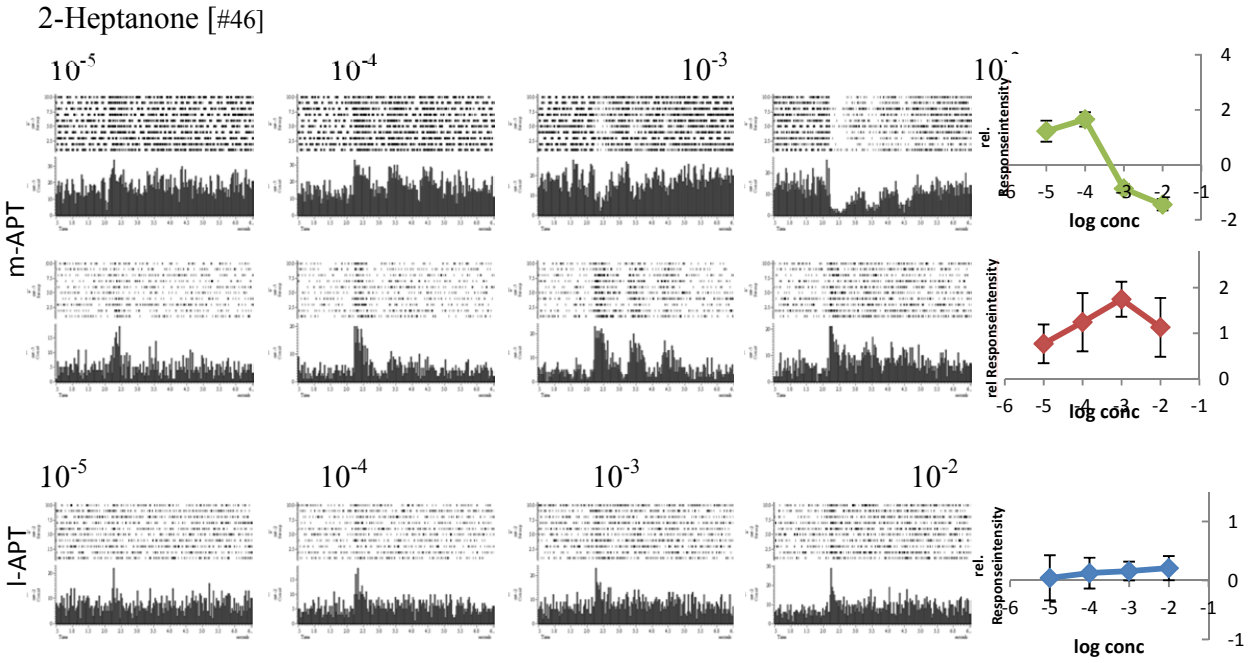


Figure IV.1: PSTHs of three simultaneously recorded PNs from the l- and m-APT (upper and lower box, respectively) in response to four successive 2-hepatnone concentrations ranging from 1:100,000 to 1:100 solved in paraffin oil. Relative response strength, analyzed as AP during stimulation versus background activity, is given on the right along each row. The bee was stimulated ten times with each odor concentration. One m-APT PN indicates clear concentration dependency with strong responses at a concentration of 1:10,000 that switches into clear inhibitoric responses at the highest presented odor concentration. In contrast, the recorded l-APT PN (bottom) fired phasically and elicited similar response strengths to each given odor concentration. Odor pulses are delivered in three pulses of 500ms duration with 500ms ISI and 30s interval between each odor stimulation pattern.

In one bee (#48), mainly in the m-APT, we found two odor responsive PNs that were either excited at lower odor concentrations or switched their response behavior to inhibition with respect to increased odor concentrations. In another PN we found the PN being excited by the lower odor concentrations and increased response strength according to the increasing odor concentration (Figure IV.1). In that bee the l-APT PN fired without weak increasing AP rate to the increasing odor concentration (in detail illustrated for stimulation with citral Figure IV.1). To compare between the three different odors we analyzed the relative response strength, which is the AP rate during the odor-evoked stimulus period divided by the background activity (Figure IV.2).

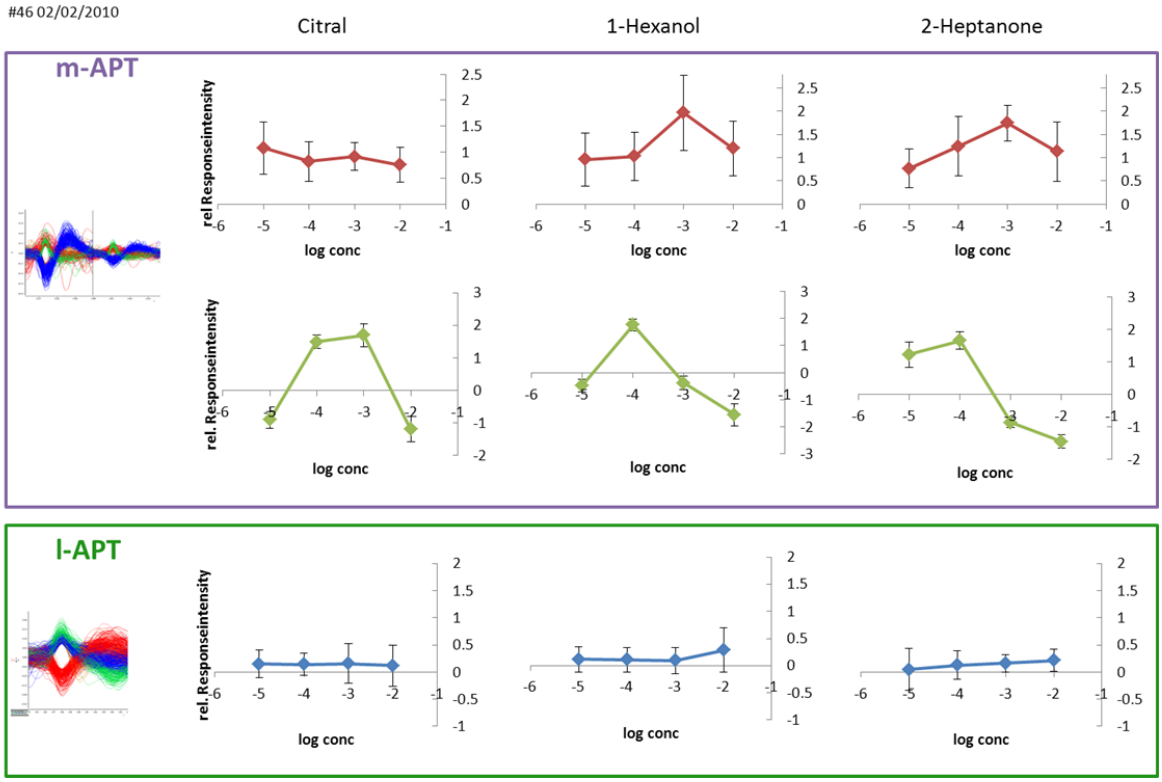


Figure IV.2: Relative response strength of three APT PNs from the m- and l-APT (upper and lower box, respectively) is plotted in response to three different odors (citral, 1-hexanol and 2-heptanone) in four increasing log dilutions ranging from 10-5 to 10-2. Relative response strength is given on the right along each row. The wavelets of the recorded PNs are given to the left in each box representing the l- or m-APT. Odor pulses are delivered in three pulses of 500ms duration with 500ms ISI and 30s interval between each odor stimulation pattern.

Similar as for 2-heptanone-stimulation the bivalent m-APT PN (green, Figure IV.1, IV.2) elicited similar responses for 1-hexanol and a bell-shaped response profile for citral. The other m-APT PN did

not change response strength (citral) or was most active at the concentration of 10^{-3} (1-hexanol). The l-APT PN only showed weak response increase to the other odors.

Furthermore, we expanded the stimulation paradigm with one order of magnitude and additionally exchanged one odor (hexanal instead of 1-hexanol) to enable the representation of three different odorant groups (floral-, pheromonal-odor or a mixture out of both, Table 1). Accordingly we tested three odors (citral, hexanal, 2-heptanone) in 5 different concentrations (from 10^{-6} to 10^{-2}) (Figure IV.3A,B).

Again, in an example bee (Figure IV.3A,B), no inhibition in response to higher odor concentrations as compared to the former pilot experiment (Figure IV.2) was detectable. Instead the relative intensity change correlated with the odor intensity in a way that the higher the intensity increased, the stronger the PNs fired. Similar as seen before, at a concentration of 10^{-3} highest responses were elicited in the recorded l-APT PNs to 2-heptanone and hexanal. Regarding citral, the response strength did not change between 10^{-3} and 10^{-2}. The m-APT PN did not show inhibitory responses to higher or lower odor concentrations and fired with an similar firing behavior, although with in total fewer APs (Figure IV.3B).

A more global view is provided by the population response of 43 units (l-APT: 22, m-APT: 21) from 7 bees (Figure IV.3C). The relative intensity change of the normalized response strength revealed similar response profiles with either positive correlations or no correlation of odor concentration and response strength. Similar to the single bee shown above (Figure IV.3A,B) predominantly the l-APT elicited stronger responses compared to the m-APT. For statistic analyses the responses underwent linear regressions. Except the responses of the m-APT to citral, all other tract-odor combinations showed significant correlations with slopes different than 0 (citral l-APT: r^2= 0.22, ANCOVA, F=11.97, p=0.0013; hexanal: l-APT: r^2= 0.08 , ANCOVA, F=4.655, p=0.0355; m-APT: r^2= 0.187, ANCOVA, F=9.427, p=0.0038; 2-heptanone: l-APT: r^2= 0.08 , ANCOVA, F=4.239, p=0.0447; m-APT: r^2= 0.22, ANCOVA, F=13.23, p=0.0007). Although the different odors and the different applied concentrations elicited stronger responses in the l-APT, no significant difference between the tracts could be evaluated (citral; ANCOVA, F=1.7174, p= 0.194; hexanal; ANCOVA, F=0.3912, p=0.5332; 2-heptanone: ANCOVA, F=0.2944, p=0.5887). Since the slopes were not significantly different, tests on different elevations or intercepts also revealed no significant difference between the tracts (citral F=2.338, n=78 p=0.1303; hexanal: F=2.397, n=95, p=0.1249, 2-heptanone: F=3.004, n=97, p=0.0862).

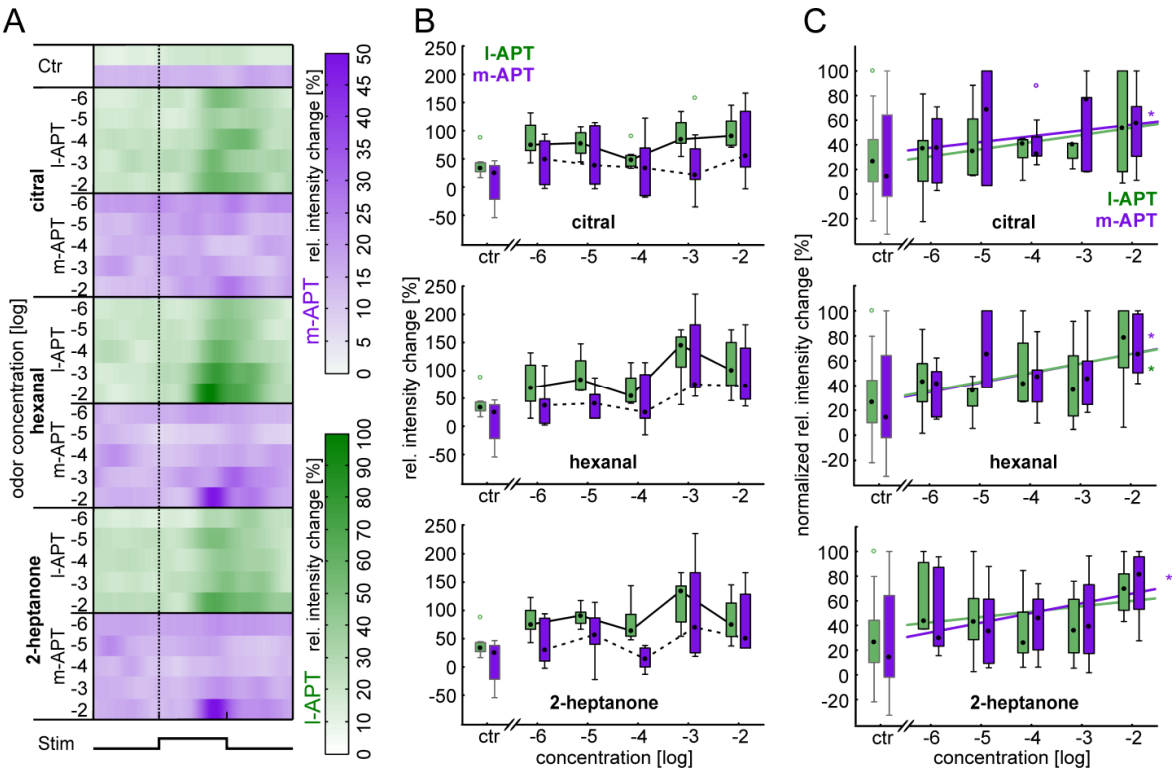


Figure IV.3: Concentration dependent firing pattern of simultaneously recorded units from the l- and m-APT in response to three different odors (citral, hexanal and 2-hepatnone) in 5 different concentrations (in logarithmic steps from 10-6 to 10-2). A) tract-specific color-coded heatplots of two single units coming either from the l- or the m-APT (green or purple, respectively). Analyses of the responses in A) are shown in B). For each tested odor the l-APT unit responds more strongly compared to the m-APT units. C) Responses of 22 l_APT units and 21 m-APT units recorded from 7 bees in total with each unit normalized to its strongest response. Stimulated with hexanal l- and m-APT units show significant positive concentration dependency (l-APT: r=0.39, p= 0,003; m-APT: r=0.39, p=0.0097), citral elicited positive odor-concentration dependency only in l-APT units (r=0.31, p=0.036) whereas 2-heptanone lead to positive odor-concentration relationship only in m-APT units (r=0.36, p=0.0128).

Besides the analyses of the response strength, we were also interested in response latency analyses. As visualized in Figure IV.3A, IV4 we found that in both the l- and m-APT PNs response latencies decreased with increasing odor concentration: the higher the odor concentration the shorter the response onset. In one representative example bee (Figure IV.4) the latency difference is about 81.5ms from the lowest to the highest concentration tested for citral (194.5ms ± 49ms at a concentration of 10^{-2} to 276ms ± 34.8ms at 10^{-5}), which elicited highly significant concentration dependent changes in the l-APT. In the m-APT 2-heptanone elicited strongest differences of about 60ms (227ms ± 41.1ms at a concentration of 10^{-4} to 160ms ± 47.1ms at the next concentration range

of 10^{-3}) and only about 19.5ms difference between the maximal concentration distance still leading to significant changes (Kruskal Wallis ANOVA l-APT: citral p<0.001, 1-hexanol: p=0.297, 2-heptanone p=0.897; m-APT: citral p=0.144, 1-hexanol p=0.177, 2-heptanone p<0.05).

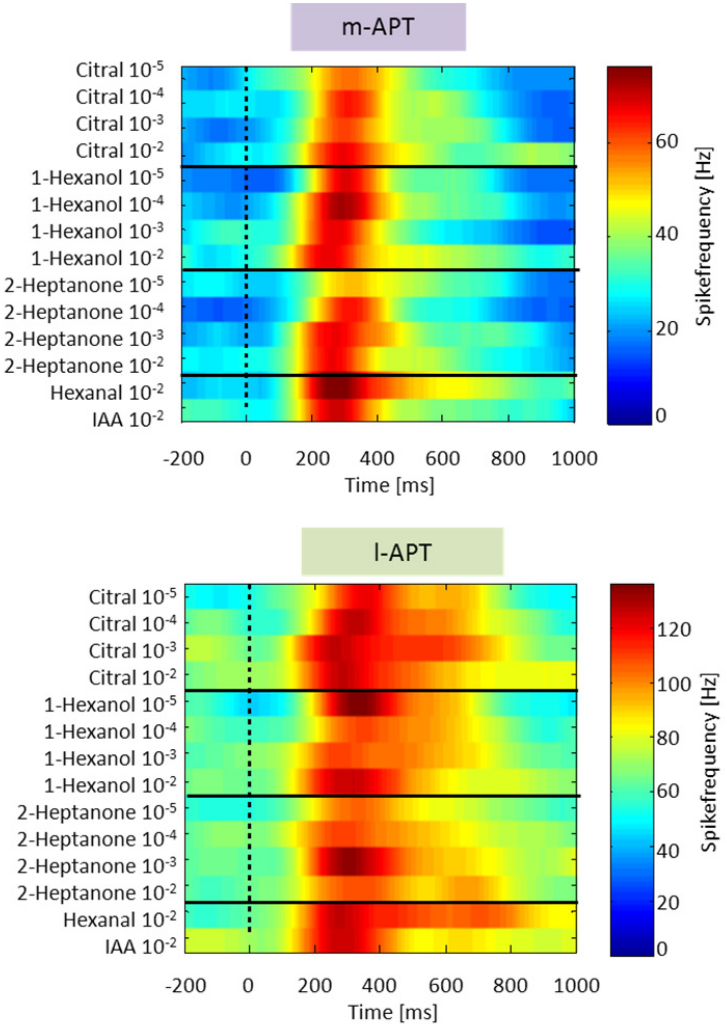


Figure IV.4: Response latency differences of one l- and one m-APT PNs (bee #60) in response to three different odors (citral, 1-hexanol and 2-heptanone) delivered in 4 successive concentrations (1:10^{-5} to 1:10^{-2}) and stimulated ten times with three pulses of 500ms duration, 500ms ISI and 30s interval between each odor stimulation pattern. Response strength of one l- and one m-APT PNs (bee #60) is given as heatplot illustrating increasing response latencies in response to increasing odorant concentrations.

In the m-APT highly significant linear regression was found for stimulation with 2-heptanone (R^2 = 0.195, p<0.01) significantly different to citral (R^2 = 0.15, p<0.05) and to a statistic trend in 1-hexanol

(R^2 = 0.097, p=0.05). The l-APT responded only to citral (R^2 = 0.37, p<0.001), with highly significant linear regression, whereas 1-hexanol and 2-heptanone did not follow linear correlation. Of course these data need to be handled with care, since it is the response of a single bee, although a similar trend is also visible in the other example bee (Figure IV.3A).

Discussion

To analyze the capacity of the bee's olfactory system to cope with different odor concentrations, we stimulated bees (7 bees and an additional 2 bees for pilot experiments) with different pheromonal and plant odors in the range of 5 (pilot experiments 4) orders of magnitude in concentration.

In the majority of the recorded PNs we found that PNs in both tracts show firing characteristics with clearly concentration dependent positive correlations: the higher the odor concentration the higher the AP rate with slightly stronger responding l-APT PNs. Recent imaging studies, which recorded from the ventral as well as from the dorsal AL, indicate a similar odor concentration behavior regarding the input of both APTs with a slightly stronger activated ventral hemilobe, i.e. l-APT (Carcaud et al., 2012; Strauch et al., 2012). This positive correlation was also observed in other species like the moth magcroglomerular complex (MGC) (Carlsson, 2003; Jarriault et al., 2009; Deisig et al., 2012)and the fruitfly (Wilson et al., 2004; Bhandawat et al., 2007; Schlief and Wilson, 2007; Silbering et al., 2008; Asahina et al., 2009; Olsen et al., 2010).

On the other hand we find steady state firing behavior over up to four orders of magnitude on both tracts, the l- and on the m-APT, which previously has been shown solely for T1 glomeruli innervating the l-APT (in bees: Sachse and Galizia, 2003; in ants: Zube et al., 2008). This non-linear rate code steady state firing behavior likely results from inhibitory and excitatory networks inside the AL which is indicated by the discovery that ORN and PN are not correlated in a linear manner in their response strength (Bhandawat et al., 2007; Jarriault et al., 2010; Olsen et al., 2010; Kuebler et al., 2012; Meyer and Galizia, 2012). AL preprocessing of PN responses provides the olfactory system with olfactory information over a long range of concentrations by using gain control mechanism, sharpening (lateral inhibition) (Sachse and Galizia, 2003) or broadening (lateral excitation) of olfactory information (for review see Martin et al., 2011; Wilson, 2011). This steady state coding

mechanism might also be responsible for concentration invariant odor identity-coding (Sachse and Galizia, 2003; Stopfer et al., 2003). It was recently hypothezised that odor is kept as generalized odor identity over several concentrations due to pattern continuity and a smooth transitions between patterns for concentration level of the same odor (Strauch et al., 2012). Nevertheless it has been mentioned that honeybees behaviorally perform better in odor discrimination tasks if the applied odor concentration is increased (Wright et al., 2009), which is also shown in physiology (Strauch et al., 2012). This astonishing AL modulation and processing system has been implemented in computer models (Schmuker et al., 2011; Assisi and Bazhenov, 2012; Assisi et al., 2012).

An earlier imaging study from the PN boutons of both, the l- and m-APT at the MB input site, the MB calyces, found positive as well as negative correlations especially at high concentrations to the increasing odor concentrations (Yamagata et al., 2009). As discussed already in earlier chapters, this study is likely influenced by GABAergic feedback neurons (like the PE1) within the MB that mediate feed forward inhibition to the microglomeruli in the MB calyx (Grünewald, 1999a, 1999b; Haehnel and Menzel, 2010). Additionally the observed difference probably accounts for the fact that we stimulated with rather low odor concentrations (up to 1:100 solution) whereas the imaging study tested with up to pure odorants which might have strongly influences lateral inhibition already at the AL level (Stopfer et al., 1997, 2003; Sachse and Galizia, 2002; Girardin et al., 2012). However, we cannot exclude non-linear relationships in concentration coding, since our recordings indicate partly sigmoidal distribution of response strength. Additionally, at least in one bee (#46) we found inhibition to highest odor concentrations as well. Probably this transition from strong excitation to strong inhibition (Figure IV.1) resembles the analyzed behavioral transition found in PER studies that found odor concentrations being able to change odor identity perception in bees (Wright et al., 2005). However, we did not find inhibitory responses within the remaining APT PN recordings. Whether this finding is based on the used recordings methods (extracellular recordings, pattern recognition during searching mainly biased towards excitatory PN responses) requires additional future experiments on this topic with expanded odor concentration ranges to pure odors to facilitate comparisons to earlier studies.

Response latency differs with different odor concentrations

We find rising concentration dependency in response strength (Figure IV.3) and a negative correlation of the response latency (Figure IV.4) of l- as well as m-APT PNs in at least one studied bee: the higher the odor concentration the shorter the response latency. This fact can be explained by the ORN's own molecular receptive range. At low concentrations only odor specific ORNs mediate signal transduction. As the odor concentration increases more and more odor molecules will activate secondary non-odor-specific receptors (Vareschi, 1971; Akers and Getz, 1993; de Bruyne et al., 2001).

Latency differences in relation to differing odor concentrations can be seen by visual inspection of recorded ORNs and PNs of *Drosophila* in response to differing odor over 7 log concentrations (Asahina et al., 2009) or has been analyzed in moths (Lei et al., 2002; Jarriault et al., 2009). In the locust however, PNs response latencies are switched in terms that increasing odor concentrations prolong the response latency to the first PN spike, which is likely due to increasing inhibitory interplay in the AL (Stopfer et al., 2003).

Behavioral significance

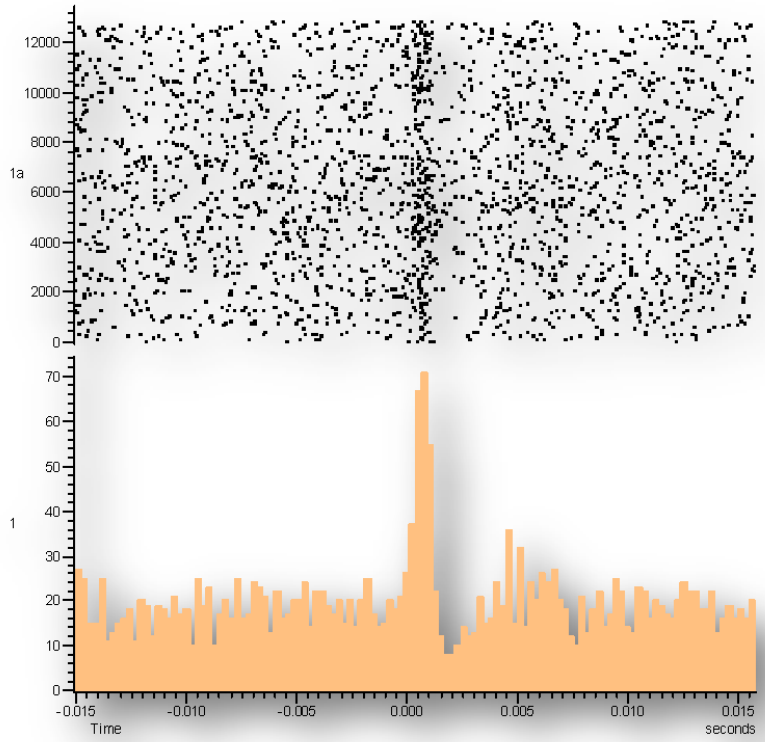
Honeybees encounter different odorant concentrations for instance during nestmate recognition which is especially important for a social insect (Brandstaetter et al., 2008, 2011). Nestmate recognition is primarily dependent on the relative amount (concentration) of odorants or chemical compounds gathered in a species-specific bouquet (Page et al., 1991; Smith et al., 2009). Besides this behavior, the recognition of flowers to feed (foraging) or familiar scents of the nest bouquet (homing) are of high interest for the insect. In the environment these odors are encountered by the bee in various different concentrations.

The result of earlier behavioral studies using classical conditioning paradigms like the proboscis extension response (PER) show that low odor concentrations do not lead to odor discrimination disabilities, but instead lead to prolonged learning performances (Wright et al., 2005, 2009). Since in our tests the lowest odor concentrations still elicited detectable responses in the l-APT, odor-identity might already be able to be coded with solely one APT. But interestingly the lack of odor-identity information of the other tract obviously decreases processing speed and learning

acquisition which supports the significance of both APT to provide parallel processing (see former chapter).

V - Chapter 4:

Olfactory Coding: Temporal Coding



Introduction

In the insect nervous system olfactory information may be processed by several types of coding principles. In the past, due to newly established recording methods, studies have tried to verify different coding principles emerging in the olfactory system (see e.g. Bargmann, 2006; Galizia and Szyszka, 2008; Hansson and Stensmyr, 2011; Martin et al., 2011; Nawrot, 2012). For instance, gain control mechanism, transition from the input to output of the AL in terms of broadening and sharpening of olfactory information, synchrony of responding neurons and the decoding of temporal stimuli have been analyzed and linked to their behavioral significance (Wilson, 2010; Martin et al., 2011).

After rate codes, response latency, population response rate and latency analyses have been evaluated and linked to the dual olfactory pathway of the honeybee (see former chapters 2, 3), it is highly interesting to test whether this system might incorporate additional coding mechanisms that may increase the trade-off between odor-detectability and odor-discriminability. Mainly in the moth, fruitfly, and locust olfactory system, electrophysiological recordings gave insight into temporal coding which was mainly observed in synchronously firing PNs or odor-induced oscillations. For example in the sphinx moth Lei et al. (2002) found pheromone-sensitive PNs leaving a single glomerulus of the MGC that are strengthened in synchrony compared to pheromone-sensitive PNs originating from two different glomeruli indicating a behavioral meaning of this synchrony. The authors suggest this synchrony might reflect an increased signal-to-noise ratio, comparable to the high convergence rate of ORN innervating glomeruli, which strengthens odor-detectability with increased sensitivity. More recently similar results of increased synchrony in PNs have also been observed with mixtures in general-odor responsive PNs (Riffell et al., 2009a). In analogy, this might also happen in the bee where about 5-7 PNs likely originate from a single glomerulus, as female honeybee workers posses about 160 glomeruli and about 900 uniglomerular PNs in the AL (Kirschner et al., 2006; Rybak, 2012). Synchrony of PNs has been extensively studied in the moth (Christensen et al., 2000, 2003; Lei et al., 2002; Ito et al., 2009; Riffell et al., 2009a) and in the fruitfly *Drosophila melanogaster* (Wilson and Laurent, 2005; Turner et al., 2008; Tanaka et al., 2009). Whether synchrony necessarily also leads to oscillations is still under debate. To date oscillations have been found in the moth *Manduca* (Ito et al., 2009), the fruitfly *Drosophila* (Tanaka

et al., 2009), the locust (Laurent and Naraghi, 1994; MacLeod and Laurent, 1996; Perez-Orive et al., 2002), and in the honeybee (Stopfer et al., 1997).

Fortunately, due to the simultaneous recording technique introduced in the first chapter, we were able to acquire a multitude of PN responses from PNs of two tracts in the honeybee brain, the l- and m-APT. Since we recorded the neurons simultaneously, we now have the possibility to test their combined responses in a temporally precise manner. We specifically were interested to see, (1) whether we find synchronous activity within one uniglomerular PN tract and (2) whether we find synchrony across PNs of both the l- and m-APT.

In case we find synchronously firing PNs, we wanted to test the hypothesis whether the mirror-imaged l- and m- APTs may mediate coincident activation of KCs within the MB calyx. Since we found odor-dependent response latency differences of different APT PNs, these differences may consecutively lead to stimulus dependent coincidental activation of KC within the MB. This is likely possible since whole-cell patch-clamp recordings from cockroaches revealed that KC require strong synaptic input (Demmer and Kloppenburg, 2009). This strong synaptic input could easily be mediated by coincidental activation from PNs of both streams to the MB. Considerations based on neuroanatomical features are supportive for potential coincidental activation of KCs. Data from Golgi stainings by Strausfeld (2002) and comparison with the PN target areas (Krischner et al. 2006) indicate that both segregated and converging inputs in different KC populations is possible. KCs, hence, could get input from either the l- or m-APT, or from both. Physiologically, this hypothesis might be strengthened by the fact that Szyszka et al. (2005, 2008) found sparsely activated KCs at the input site of the MB calyx. Spatially sparse activation of KCs in the MB calyx is what would be expected since only a certain amount of PNs from the l- and m-APT will propagate coincidental excitation. With our synchronously recorded APT PNs we now can ask whether the two uniglomerular PN pathways may mediate coincidental activation of postsynaptic KCs and whether synchronously firing PNs are correlated to an odor stimulus.

Material & Methods

We analyzed synchronously firing PNs of either the same PN tract (Figure V.1, V.2B,C) or across both APTs (Figure V.2D,E). First we combined the APs of single PNs in a 5ms bin width and plotted the

estimated synchronous firing frequency in time (Figure V.1A). Since the background activity influences the stochastic coincidental activity of the synchronous firing PNs (Ts'o et al., 1986; Melssen and Epping, 1987), in a second approach the rate function (see previous sections, (Rosenbaum, 2011)) was evaluated (Figure V.1B). The net synchronous activity was archived by subtracting the synchronous activity from the rate function which results in pure synchronous activity without any bias by the spike frequency itself (Figure V.1C).

We applied this approach to the entire series of the synchronously recorded l- and m-APT PN pairs of two bees, #104 and #107 (Figure V.2). Since we correlated every acquired PN within one tract or across both tracts, we acquired about 190 correlations in bee #104 and 136 analyses for bee #107. The analyses were done in MatLab (v2009b, The MathWorks, Inc). For all three analyses: synchronous firing within a bin width of 5ms, the mean firing rate of PN pairs, and the pure synchronous activity above background activity underwent a filtering process with a triangular filter to create a smooth estimated firing rate function with time constant of 301ms (Nawrot et al., 1999). This filtering leads to a prolonged activity profile with zero activity at the borders and highest synchronous activity in the middle.

Synchronously l- and m-APT PN recorded bees were stimulated with a triple pulse (500ms stimulus, 500ms ISI, 30s ITI) of odors which facilitate comparison with former experiments (see chapter 2) or with increasing odor concentrations ranging from 10^{-6} to 10^{-2} (see chapter 3).

Cross-correlation (CC) analyses were performed in Spike2 (CED, United Kingdom) by accumulating a histogram of time differences between the occurrence of spikes in the two recorded cells. Therefore the spikes of a reference channel of one PN were correlated to the spikes of another channel from the opposite APT (Figure V.3,4). The left group of bins in the histogram represents the negative times, when the second cell (l-APT) fired prior to the first (m-APT). The center, or zero, bin of the histogram accumulates the number of instances when the two cells fired precisely together. The right group of bins, the positive times, account for the various time intervals that the first cell fired prior to the second cell. The temporal resolution of the CC was 30ms with a bin width of 250µs. The CED script "CrossChanCorrelation" (CED 17/09/2007) was adjusted to observe CCs within the recordings of a bee where each PN of one tract (APT) was correlated with a PN of the other tract (Figure V.3). To analyze stimulus triggered correlation changes, a certain stimulus period was chosen for evaluation, e.g. pre stimulus 0.05 - 2 s or peri stimulus: 2.1 - 2.6s (Figure V.4A). To observe the

correlation changes in response to an odor, we analyzed the CC within the period of control stimulation (solvent) or within odor stimulation (Figure V.4B).

Results

We wanted to analyze synchronous firing activity of APT PNs. Figure V.1 shows the analyses of responses from two PNs of the m-APT. We evaluated the amount of APs during 5ms bins of two m-APT PN pairs which fired during the same time window and traced the synchronous spike rate in time (Figure V.1A). We found synchronous activity during odor stimulation. The odor-elicited excitatory response is depicted by the averaged mean spike rate of the PN pair in time (Figure V.1B). It has to be mentioned that the excitatory odor-response to the tested odor is delayed in comparison to other odors or individuals (as shown in former chapter), and is about 400ms after odor onset or about 300ms after EAG adjustment (Figure V.1B). As expected the synchrony of the responding PN pair increased during the evoked odor stimulation period (Figure V.1A). However, analyzing synchrony may lead to false positive detection since spontaneous firing rate and the neuron's stimulus response may increase the stochastic process of synchronous events. To circumvent this problem, we calculated the quotient of the estimated rate-function of synchronous events (Figure V.1A) divided by the mean estimated firing rate of both PNs (Figure V.1B). The gained pure synchronous activity results in a qualitatively weaker increase in synchronous activity during the odor response (Figure V.1C). Interestingly the estimated synchronous activity rises during odor stimulation and decreases after odor stimulation - similar as the rate function or the unfiltered synchronous rate function in Figure V.1A,B.

To evaluate whether synchrony is odor-dependent, we did the same analyses as described before but with the entire odor stimulus protocol for one bee (#107, 6 l-APT PNs and 7 m-APT PNs) (Figure V.2). We stimulated the bee with 12 different odors (see chapter 2, Table III.1) and three odors in five successive odor concentrations ranging from 10^{-6} to 10^{-2} in solvent. Additionally, we plotted the mean background activity for 6 l-APT PNs and 7 m-APT PNs (Figure V.2A). In the m-APT we did not find any synchronous activity in pairs of PNs neither in the given example PN pair

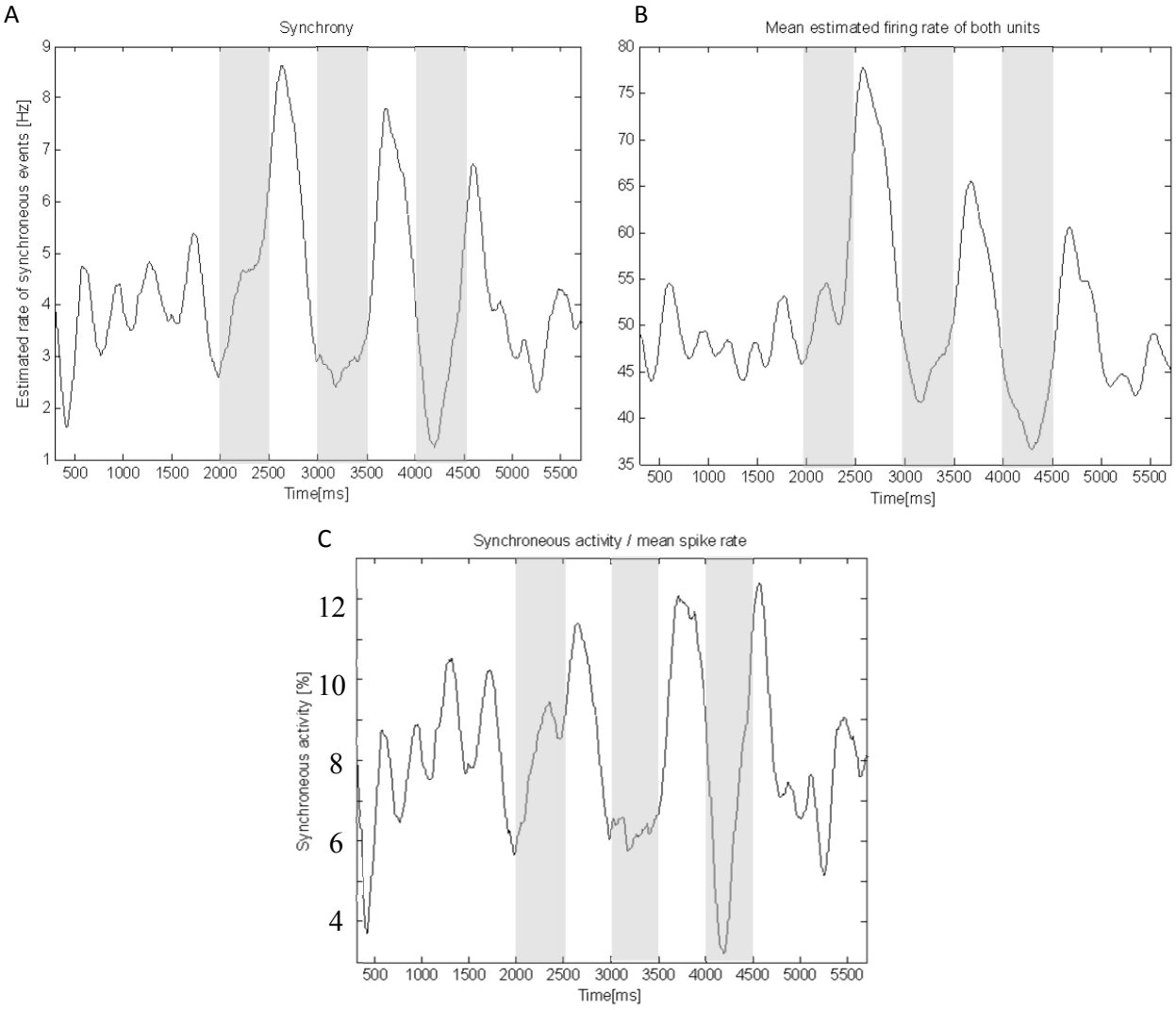
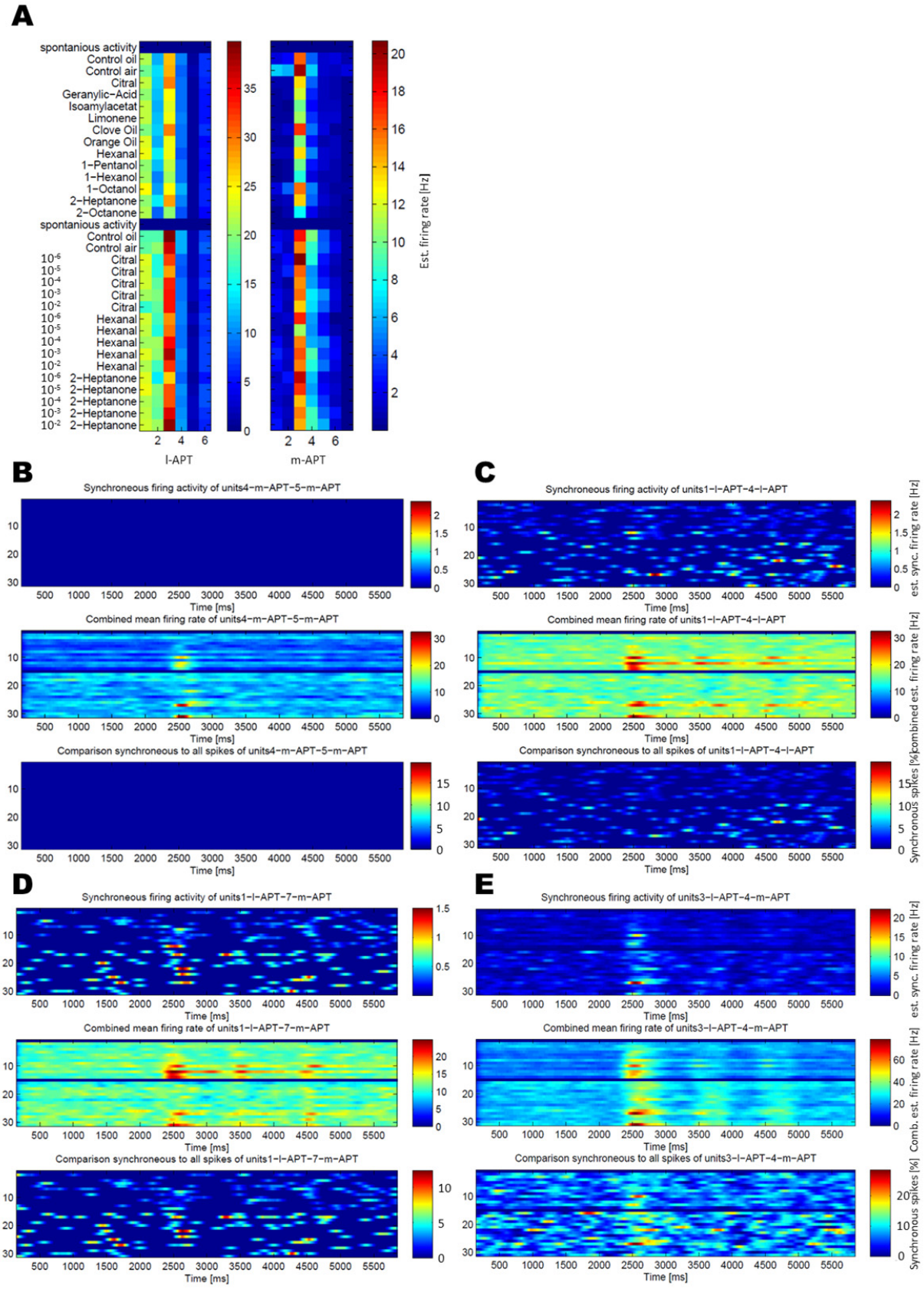


Figure V.1: Estimation of synchronously firing APT PNs simultaneously recorded from within one tract (m-APT). A) All spikes of two odor-sensitive PNs elicited in both PNs within a time window of 5ms were aligned and plotted as estimated synchronous rate function. The odor stimulus was a triple 500ms pulse of 2-heptanone B) Rate function of the same odor-responsive PNs as in A) but now plotted as the averaged rate functions . C) As a reliable comparison of synchronous activity we aligned the synchronous activity in respect to the firing rate of the PNs, given in percentage of synchronously fired APs. (grey boxes indicate odor stimulation: tripple pulse of 500ms, 500ms ISI, 20 trials, 20s inter stimulus pattern interval).

(Figure V.2.B) nor in all possible m-APT PNs pairs. Although, during odor stimulation combined mean firing rate in time (rate function) indicates a clear response to the given odor stimuli (middle trace, Figure V.2B). In contrast, in the l-APT PN pair weak synchronous activity is visible in the synchronous rate function as well as in the unbiased synchronous firing activity plot (upper and lowest plot Figure V.2C), although it has to be mentioned that not all l-APT PN pairs elicited synchronous activity.

Astonishingly, if we now analyze these data with APT PNs from the l- and m-APT combined, synchronous activity is immediately visible and partly stimulus correlated (two examples are depicted, Figure V.2D,E). Synchrony is not only locked to the odor panel with the 12 highly volatile odors and pheromones but also visible during stimulation with increasing odor concentrations. Mainly the first odor-pulse of the triple odor-pulse stimulation induces a response of the APTs, which are similar conditions to those we can observe in the synchronous activity plots.

Figure V.2 (right side): Heatplots of several synchronously recorded APT PNs from either the l- or m-APT with estimated synchronous firing activity. To abolish stochastically biased synchronous activity via background activity from the observed PNs, heatplots are drawn to indicate background activity during odor stimulation to several high volatile plant and pheromonal odors with additional stimulation of three odors (citral, hexanal, 2-heptanone) in different concentration (from 10^{-6} to 10^{-2}). A) Heatplots indicate the background activity of the recorded PN outside odor stimulation as indication for general background activity. B-E) Whether synchronal excitation is limited to the l-, the m-APT or both is graphically analyzed in the following figures. Comparable to Figure V.1A-C first the rate of APs of two PN pairs summed and binned in 5ms intervals. Their rate is drawn in time and color coded (upper graph, each sub-figure). The combined mean firing rate illustrates the summed spike rate of the respective PN pair (middle trace/graph, each sub-figure) and finally the stochastically unbiased synchronal firing activity of the respective PN pair is drawn. B) As in this example no synchronous activity is acquired in the m-APT. C) Weak synchronous activity of two PNs can be found in the l-APT but combining PNs of the l- and the m-APT increases the synchronous activity that is mainly strengthened during odor stimulation (D-E).


A
spontanious activity
Control oil
Control air
Citral
Geranylic−Acid
Isoamylacetat
Limonene
Clove Oil
Orange Oil
Hexanal
1−Pentanol
1−Hexanol
1−Octanol
2−Heptanone
2−Octanone
spontanious activity
Control oil
Control air
10-6 Citral
10-5 Citral
10-4 Citral
10-3 Citral
10-2 Citral
10-6 Hexanal
10-5 Hexanal
10-4 Hexanal
10-3 Hexanal
10-2 Hexanal
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10-4 2−Heptanone
10-3 2−Heptanone
10-2 2−Heptanone
2 4 6
l-APT
2 4 6
m-APT
Est. firing rate [Hz]
B
Synchroneous firing activity of units4−m−APT−5−m−APT
Combined mean firing rate of units4−m−APT−5−m−APT
Comparison synchroneous to all spikes of units4−m−APT−5−m−APT
C
Synchroneous firing activity of units1−l−APT−4−l−APT
Combined mean firing rate of units1−l−APT−4−l−APT
Comparison synchroneous to all spikes of units1−l−APT−4−l−APT
est. sync. firing rate [Hz]
combined est. firing rate [Hz]
Synchronous spikes [%]
Time [ms]
D
Synchroneous firing activity of units1−l−APT−7−m−APT
Combined mean firing rate of units1−l−APT−7−m−APT
Comparison synchroneous to all spikes of units1−l−APT−7−m−APT
E
Synchroneous firing activity of units3−l−APT−4−m−APT
Combined mean firing rate of units3−l−APT−4−m−APT
Comparison synchroneous to all spikes of units3−l−APT−4−m−APT
est. sync. firing rate [Hz]
Comb. est. firing rate [Hz]
Synchronous spikes [%]
Time [ms]

Another way to analyze the alternative coding principles in the bee's dual olfactory pathway is possible via calculation of cross-correlations (CC) of the spike trains of one l- against one m-APT PN. With this method we were able to see maximal increase in CC and calculate the delay between one spike of the m-APT PN and the correlated l-APT PN (Figure V.3). In about 80% (8 of 10 bees) of the tested bees this kind of CC maxima can be found along several PN pairs coming from the l- and m-APT. Since these CCs are still stochastically biased and not filtered these have to be regarded as preliminary qualitative results.

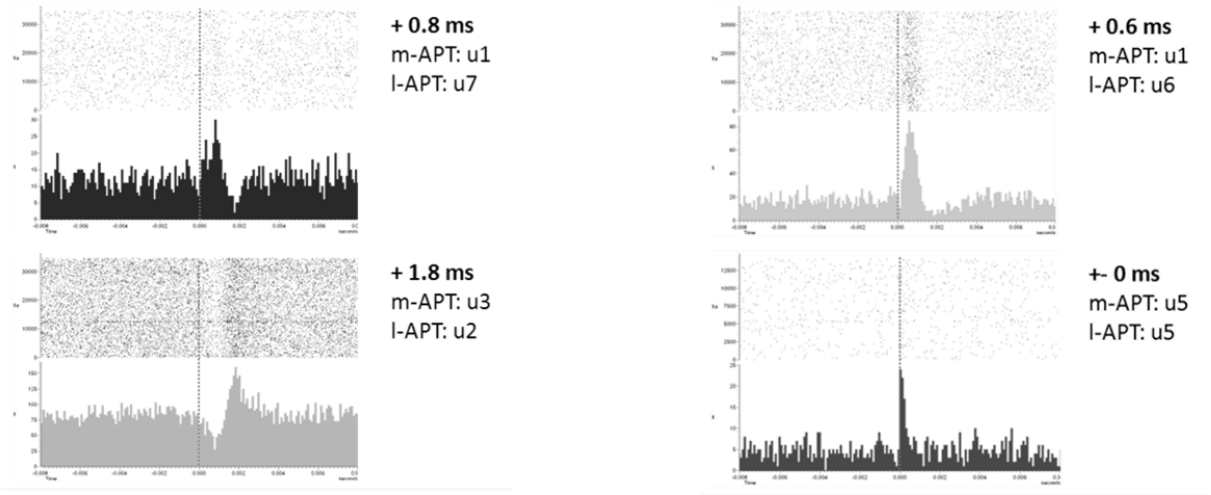


Figure V.3: Cross Correlation (CC) of simultaneously recorded odor-responses from the l- and m-APT of one bee (#98) during odor stimulation. For CC analyses one m-APT PN (time 0ms, indicated as dashed line) is the reference channel to one l-APT PN. The maximal CC indicates the temporal delay between the m- and the l-APT. In the four given PN pairs the m-APT PN is leading in time in a range of 0 to 1.8ms. PN identity and tract identity is indicated, bin size: 0.25ms.

Since we found maximal CC significantly rising above background activity in the CC (Figure V.4A), we were interested to see, whether we find odor induced CC. Therefore, we measured the delay of a reference m-APT PN to the respective l-APT PN and found a slight increase in the delay during odor stimulation (triple pulse (2-5s) compared to the time interval before stimulation (0-2s) (Figure V.4A). To evaluate whether already a single odor may change the CC of l- and m-APT PN pairs, we took an APT PN pair from both tracts and tested the correlation during control stimulation (solvent) and during odor stimulation (IAA, 10^{-2}) (Figure V.4B).

We found a weak increase of correlated spiking activity around 0-1ms. Unfortunately this odor-induced CC-change was so far only detected in a few analyses and hence need to be treated as preliminary results, but certainly gives rise to future directions.

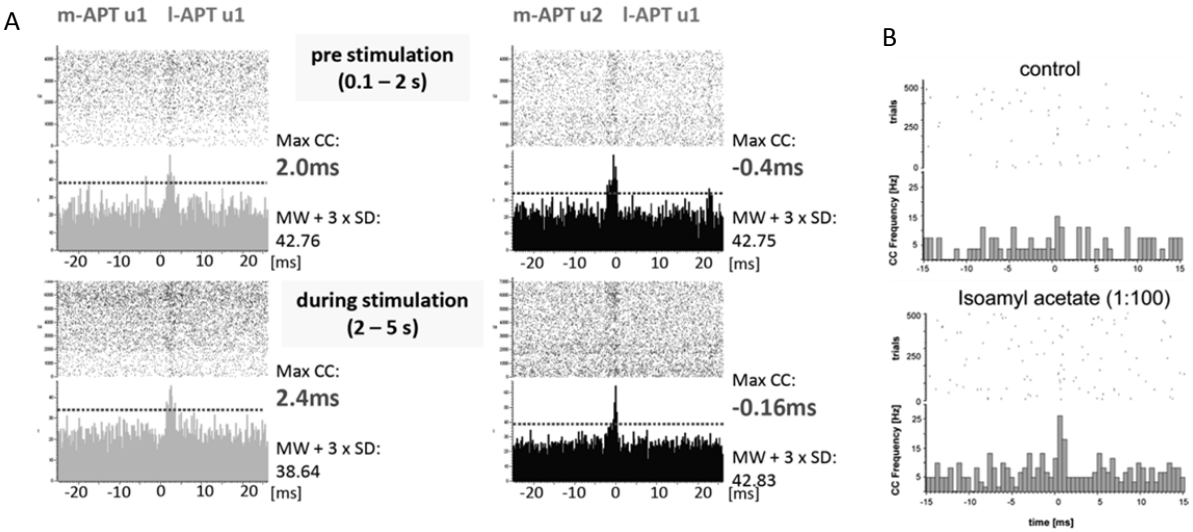


Figure V.4: Qualitative cross correlation analyses in the dual olfactory pathway of the honeybee. A) Estimation of delay in the cross-correlation of two pairs of l- and m-APT PNs (left or right panel). The distance between the spike events of a single m-APT PN to one l-APT PN increases during odor stimulation (0.1-2s) compared to the time period before odor stimulation (2-5s). Maximal cross-correlation is indicated and calculated by taking the mean of the CC which rises above the background CC (mean + 3SD). B) Qualitative observation of cross correlations of one l-APT PN and one m-APT PN in response to the control (paraffin oil, upper CC) and IAA (1:100, lower CC). During odor stimulation a weak increase in the CC around 0 - 1 ms is visible.

Discussion

Synchrony

Synchrony is a result from internal coordination of spike timing of a population of neurons and is believed to be essential for many brain functions, including neuronal development, sensory perception, and memory formation. Coherent neuronal excitations in response to odor stimulations induce oscillations (Laurent and Naraghi, 1994; Perez-Orive et al., 2002; Friedrich and Laurent, 2004; Friedrich et al., 2004). Oscillations are suggested to play an important role in information processing and learning in several parts of the brain (Singer, 1999, 2009; Galán et al., 2006). For example, analyzing the output neurons of the OB of vertebrates, the mitral and tufted cells, in several species and experiments, odorants evoke precise sniff-locked activity (Bathellier et al., 2008; Carey and Wachowiak, 2011; Shusterman et al., 2011; Smear et al., 2011) which were shown to be driven by inhibition (Buzsáki and Chrobak, 1995; Lagier et al., 2007; Abraham et al., 2010). Oscillation in the olfactory system of insects have been reported in the locust, moth, fruitfly and honeybee and their origin was related to inhibition similar to those observed in the vertebrate system (MacLeod and Laurent, 1996; Stopfer et al., 1997; Lei et al., 2002; Ito et al., 2009; Tanaka et al., 2009; Assisi and Bazhenov, 2012). Although Stopfer et al. (1997) found oscillations in honeybees using local field potentiaql recordings in our recordings we could not observe global oscillations. Nevertheless it has to be mentioned that we found neurons not responding to any odor stimulus but that fired with stereotyped frequency which could potentially contribute to global oscillations (data not shown, see Reus, 2011).

We found synchronously activated PNs within a single uniglomerular PN tract as well as across both l- and m-APTs PNs. We found synchrony mainly increasing during stimulus presentations and almost complete vanishing of synchrony after stimulus onset (Figure V.1C). In the moth *Manduca sexta* synchrony rises during stimulus presentation and vanishes after odor stimulation (Lei et al., 2002; Riffell et al., 2009a). In analogy to the insect olfactory system stimulus triggered correlations and decorrelations have been found in the fish (Friedrich et al., 2010; Wiechert et al., 2010) or mammalian olfactory system (Lledo et al., 2005; Miura et al., 2012; Spors et al., 2012). Decorrelation is suggested to support the differentiation of chemically similar odors in vertebrates or in general to

reduce the overlap of similar sensory representation which improves the perceptual differentiation of physically similar stimuli (Cleland, 2010; Cleland and Linster, 2012).

In our analyses synchronous activity was calculated by subtracting background and stimulus-correlated spiking activity, since these activities lead to a stochastic increase in coincidental events (Ts'o et al., 1986; Melssen and Epping, 1987). Future analyses need to circumvent this problem by introducing elaborated analyses as for example have been done in cross-correlation analyses with shift-predictor methods in the moth (Riffell et al., 2009b). Whether the detected synchrony of APT PNs within the bee's olfactory pathway mediates odor-identity coding and whether the synchrony will engage the differentiation of odor identity over a wide concentration range will be the matter of future quantitative analyses. Nevertheless, we found synchrony emerging from PNs of both tracts which finally was improved compared to synchrony originating solely from within one APT. This indicates the significance of the dual olfactory pathway in odor processing. Additionally the results indicate that the l- and m-APT contribute to parallel odor processing (as discussed in chapter 2).

Coincidence coding

The anatomical layout of the hymenopteran dual olfactory pathway is unique (Kirschner et al., 2006; Zube et al., 2008; Rössler and Zube, 2011). The trajectories of the uniglomerular PN pathways, the medio- and lateral antennal-lobe protocerebral tracts, are running in a different sequence. The m-APT originating from the dorsal AL hemilobe, reaches the MB first and later the LH - in contrast the l-APT starting from the ventral AL hemilobe innervates the LH first and then the MB (Kirschner et al., 2006; Galizia and Rössler, 2010). This peculiar PN layout, to a certain extent, reflects the layout of delay-lines in other sensory modalities like known from the auditory system.

The best known example for this is the Jeffress delay-line model which is implemented to understand directional hearing in the auditory brainstem of vertebrates (Jeffress, 1948; for review see Joris et al., 1998). It has its experimental origin from barn owl recordings and echo-locating bats (Knudsen et al., 1987; Knudsen, 1999). Auditory-nerve input is relayed from both ears to cells which act as coincidence detectors that fire maximally when action potentials from both ears arrive simultaneously. The inputs arrive along nerve-cell axons which vary systematically in their length on their way to the different target cells. Because longer axons take more time to transmit their signals,

they act like delay lines and introduce cellular time delays. These delays lead to maximal firing at that particular interaural time difference which is represented in the owl and forms a topographic representation of the auditory azimuth (Köppl, 2009). Hence, temporal intervals can be converted to spatial representations utilizing neuronal delays.

Another neuronal layout hypothetically comparable to the dual olfactory pathway is the Hassenstein and Reichardt movement detector model in the visual system, which has first been suggested and analyzed in the beetle (Hassenstein and Reichardt, 1951). This detector consists of two mirror-symmetrical subunits, since a movement detector needs at least two inputs. If one channel gets a stimulus input that is moving across to the second input, the excitation is transmitted via the first channel (axon) to a higher order neuron but delayed on the way. Meanwhile the stimulus arrives at the second input which transmits the stimulus information to the next higher order neuron but without delay. If both inputs mediate their stimulus information with the same arrival time at the next order neuron, the coincidence detector, the excitation is summed up and results in excitation of the target neuron. This set would function only for a certain direction of stimulus flux along the inputs. For example, if the stimulus would have the opposite direction, the stimulus input would reach the second input first, the neuronal transmission along the un-delayed axon would reach the target cell solely before any information is coming from the first, delayed input. Input from only one cell will lead to only subthreshold excitation in the target cell (Borst and Egelhaaf, 1989). Since Hassenstein and Reichert (1951) have discussed the movement detector, different derived models were under discussion together with observations of movements detectors in living organism (Borst and Euler, 2011). Despite the astonishingly simple architecture of the Hassenstein and Reichardt movement detector, neurogenetic research in fruitflies indicates a much more complex neuronal circuitry in motion detection (Rister et al., 2007).

Both examples demonstrate that circuits suited to detect minute time differences depend on some form of delay lines and coincidence detectors that respond maximally when they receive simultaneous inputs (Carr, 1993). Transferred to the dual olfactory pathway of the honeybee this may suggest that the mirror-imaged layout of the APTs supports detection of minute time differences in olfactory coding. In an attempt to to shed light on this hypothesis, we correlated our simultaneous recorded spike trains of the l- and m-APT to evaluate whether we will find some kind of temporal correlation. We found maximal cross correlations which were in the range of 0-3ms (Figure V.3). This correlation is slightly shifting during odor stimulation or is almost absent during

control stimulation (Figure V.3A,B). These results lead to the suggestion that KCs in fact receive coincidental input by the APT PNs of both tracts, which is also indicated by our analyses of synchrony that was intensified as l- and m-APT PNs are considered together (Figure V.2C,D).

As illustrated in the model (Figure V.5), if a PN's AP will run along its axons to the KCs with an hypothesized same neuronal conduction velocity of 20 to 25cm/s (Oleskevich, 1997), by tracking the length of the l- and m-APT PNs within an optical slight, PNs of both tracks will target KC in the medial calyx simultaneously. Regarding the LH, an m-APT PN would first innervate the MB and later on reaches the LH with delay in comparison to an l-APT PN that targets the LH first. The resulting delay would be in the range of 3 to 4ms. Even within the calyx slight delay differences of incoming synchronous PN excitations will lead to excitation of different sets of KC.

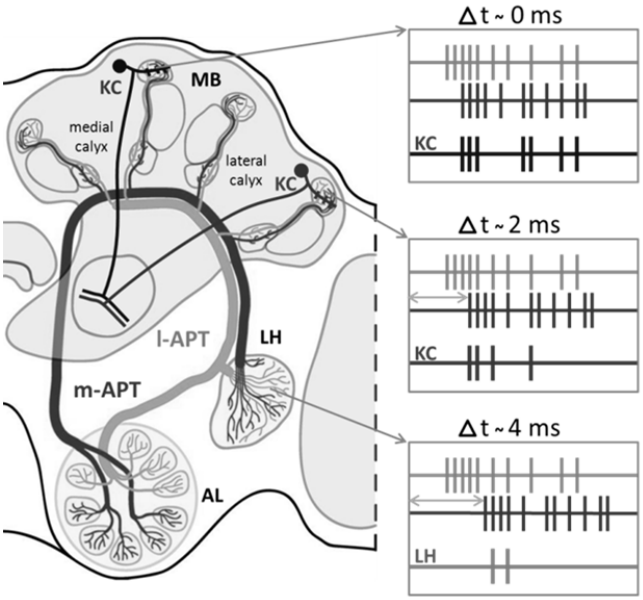


Figure V.5: (schematic drawing with courtesy from Wolfgang Rössler): Model of coincidental activation of target cells in the MB (KC) and LH innervated by PNs from the l- and m-APT. Presumed neuronal conduction velocity of about 20cm/s combined with a calculated distance PNs need to travel, their AP leads to a 1:1 arrival time of AP from a single l- as well as from a single m-APT PN at the medial MB calyx. The arrival time difference between the two tracts increased according to the target region since m-APT PN run in clock-wise direction whereas the l-APT PN innervate the LH first and later on the MB in counter-clock-wise direction.

Additionally, as introduced in the 2. chapter, we find the dual olfactory pathways contributing to odor processing with a significantly overall faster responding l-APT PN population. On the single PN level we found PNs of both tracts being able to code odors by their response latency. Taken these two observations into account, individual odors elicit PN responses with different response latencies. This will consecutively results in another set of coincidentally activated KCs leading to the well described sparse activity pattern of KC, which would change in respect to different odor stimulations. Hence coincidental activation of KC is very likely to be be stimulus dependent and will

promote access for neuromodulator influence and increases odor processing capacity and capacity of multi stimulus associations (Smith et al., 2008).

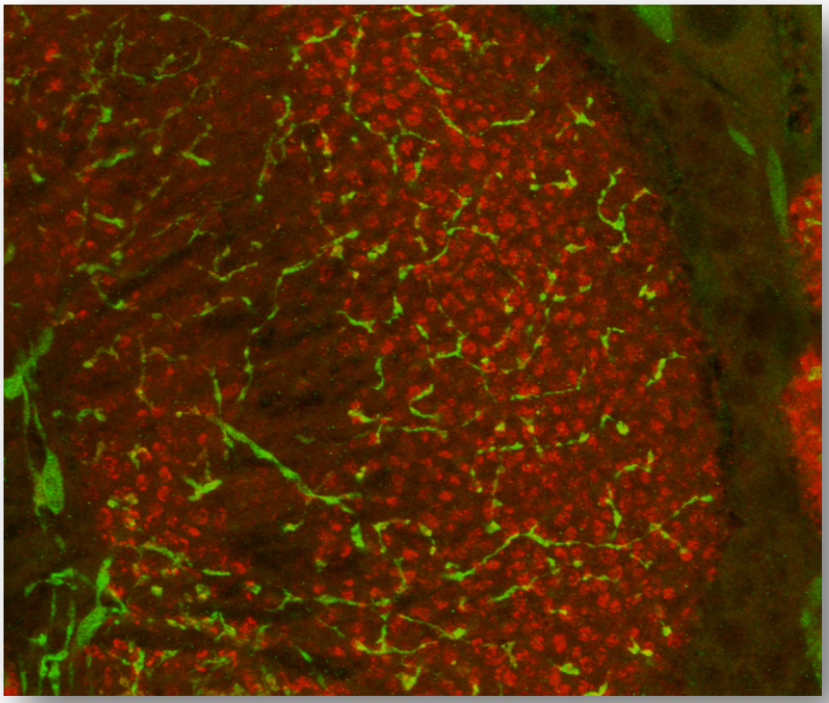
Experimental proof for the existence of a sparse odor code within the MB calyx has been given by studies in the bee (Szyszka et al., 2005, 2008), moth (Ito et al., 2008), locust (Perez-Orive et al., 2002, 2004), or fruitfly (Wang et al., 2004). In the locust Perez-Orive et al. (2002, 2004) described KCs being coincidence detectors, which are sensitive to the synchronized input coming from the AL. The authors suggest that feed-forward inhibition mechanism mediated by LH neurons is responsible to enhanced sparsening in the KC code (discussed in Theunissen, 2003). However a recent study, performing intracellular recordings characterized about 10 different types of LH neurons in the locust. The study described the LH neuron odor range and innervation pattern, but did not find any LH neuron type that could account for the suggested inhibition (Gupta and Stopfer, 2012). Since *in-situ* patch-clamp analyses in the cockroach KCs found strong Ca^{2+} dependent inward and outward currents that require strong synaptic input (Demmer and Kloppenburg, 2009), the KCs are perfect coincidence detectors solely based on their intrtinsic electrical properties. The well described intrinsic properties of the KCs suggest spike-frequency adaptations, which rejects the function of inhibitory LH neurons but promotes sparse coding (Gupta and Stopfer, 2012; Nawrot, 2012).

In summary, the dual olfactory pathway represents a likely neuronal substrate to perform sophisticated odor processing leading to enhanced odor perception properties.

VI - Chapter 5:

Plasticity:

Maturation of the GABAergic system



Brill MF, Wegener S, Rössler W. *in prep*

Author contributions:

MFB, WR designed research; MFB, WS performed research; MFB, WS analyzed the data, MFB, WR, SW wrote the manuscript

Introduction

Nervous systems have the astonishing capacity of being able to adapt to external or internal factors; they are plastic. Social insects perform various tasks in changing environments throughout their lifetime. This temporal polyethism represents one of the most intriguing examples of ontogenetic neuroplasticity (Withers et al., 1993). High genetic relatedness and the rich behavioral repertoire of honeybees have evoked correlations between environment, age and status of individuals and their neuroanatomy, reflecting these various influences on the level of synaptic connectivity (Rössler and Groh, 2012). Environmental factors like rearing temperature or other external sensory stimuli have been shown to induce measurable synaptic changes in Hymenoptera (Masson and Arnold, 1984; Groh et al., 2004, 2006; Krofczik et al., 2008; Seid and Wehner, 2009; Stieb et al., 2010, 2012; Rössler and Groh, 2012). In the course of their lifetime, nervous systems undergo adult maturation asspcoiated with changes in synaptic connectivity (Fahrbach et al., 1995; Seid et al., 2005; Groh et al., 2006, 2012; Seid and Wehner, 2008; Stieb et al., 2010). In contrast to pure volume measurements of neuropils, more recent studies have started to quantify adult neuronal maturation by analyzing synaptic plasticity on the level of pre- and postsynaptic changes in neurons using specific labeling of synaptic proteins (Groh et al., 2004, 2006, 2012; Seid and Wehner, 2008, 2009; Stieb et al., 2010, 2012). The connectivity between neuropils in the olfactory as well as in the visual system of, for example bees, is well-known (Gronenberg, 2001; Kirschner et al., 2006; Paulk and Gronenberg, 2008).

The mushroom body is the most likely candidate to study synaptic plasticity as it represents a second order center for sensory integration and association. In fact the MB comprise ~40% of the total amount of neurons in the honeybee brain (Rössler and Groh, 2012). The MB is considered to be involved in associative learning and memory (Hammer and Menzel, 1995, 1998; Davis, 2004, 2011; Menzel, 2012a). The MB intrinsic neurons, the Kenyon cells (KCs) (Figure VI.1)(KCs), divide the MB in both anatomically and functionally distinct units (Kenyon, 1896). Within the MB calyx PN boutons and KC spines form microglomerular (MG) complexes. Each MG contains a presynaptic PN bouton as its central core, surrounded by a shell of KC dendritic spines (Gronenberg, 2001; Rössler et al., 2002; Yasuyama et al., 2002; Frambach et al., 2004; Groh et al., 2004, 2006, 2012; Krofczik et al., 2008; Stieb et al., 2010). KC axons guide excitation to the vertical- or gamma-, and the medial lobes (Strausfeld, 2002). The calyces receive their input from the primary sensory neuropils and

show a functional segmentation according to the processed modalities (Gronenberg, 2001). The lip region gets olfactory information from the antennal lobes, the collar is supplied with visual information and gets segregated input from visual PNs of the lobula and medulla that mediate color or spatio temporal information (Paulk and Gronenberg, 2008; Paulk et al., 2008). The basal ring receives mixed unilateral chemosensory and bilateral visual information (Gronenberg, 2001; Schröter and Menzel, 2003; Kirschner et al., 2006). In the honeybee there are also known gustatory and mechanosensory inputs to the MB calyx (Schröter and Menzel, 2003).

In nervous systems, besides excitatory connections, inhibition in general is highly important. Inhibition is mainly mediated by GABAergic neurons. GABA is known to open chloride channels that consecutively lead to an inhibition of synapses. Nevertheless, it has to be mentioned that in the developing vertebrate brain GABA was demonstrated to elicit excitatory responses (Gaiarsa and Ben-ari, 2002; Ben-Ari and Gaiarsa, 2007). GABA is of high importance in olfactory coding and behavior which can be observed in various insects. For example, in locusts GABAergic mediated inhibitions in the MB are supposed to influence odor elicited oscillations (Perez-Orive et al., 2004) and were suggested to be important for odor discrimination (Stopfer et al., 1997). The GABAergic inhibition of olfactory elicited KC activity is mediated by a giant feedback neuron from the MB output back propagating to its input (Papadopoulou et al., 2011). In the locust sparsening of olfactory coding at the level of KCs was believed to be a product of feed forward inhibition from GABAergic LH neurons (Perez-Orive et al., 2002) until Gupta and Stopfer (2012) characterized ten types of LH neurons and did not find any neuron resembling the required feed-forward inhibition.

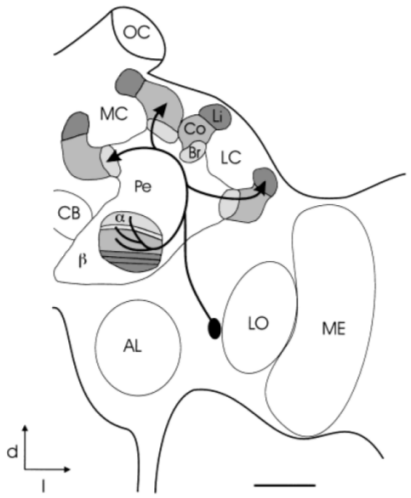


Figure VI.1: adapted from (Grünewald, 1999a): schematic overview of one hemisphere of the honeybee brain with the prominent MB. In black is shown an extrinsic MB feedback neuron that gets its input from the vertical lobe (α lobe) and back-projects to the MBs input site, the calyces. The corresponding input and output regions of the MB are shaded in the same grey value. The extrinsic feedback is GABAergic, has its somata located besides the LH in the latereal protocerebrum. The MB consists of the lateral and medial calyx (LC, MC). Each calyx is composed of the lip, collar and basal ring (Li, Co,Br) at its input and α and β lobes at its output below the peduncle (Pe); dorsal (d) and lateral (l) directions are indicated. Oc: ocellus, CB: central cody, AL: antennal lobe, LO: lobula, ME: medulla. Scale bar: 100µm

In the moth GABA is distributed in many areas of the brain (Berg et al., 2009). It was shown to mediate inhibition in olfactory PNs (Waldrop et al., 1987; Christensen et al., 1998). Moths exhibit thriphasic response profiles with alternating excitatory and inhibitory responses that are species-specific (Jarriault et al., 2009; Lei et al., 2009; Chaffiol et al., 2012). Due to behavioral experiments, GABA was found to be responsible for odor pulse tracking capability (Christensen et al., 1998; Mwilaria et al., 2008; Lei et al., 2009). In the fruitfly *Drosophila* fast synaptic currents in KCs were demonstrated to be driven by alpha-Bungarotoxin sensitive picrotoxin-sensitive GABA receptors (Su and O'Dowd, 2003). In honeybees GABA application elicits Cl^- currents through ionotropic picrotoxin sensitive GABA receptors in cultured KCs (Déglise et al., 2002; Grünewald et al., 2004) and AL neurons (Barbara et al., 2005). GABA immuno-reactivity was found within the bee brain (Bicker et al., 1985; Schäfer and Bicker, 1986; Bicker, 1999). Prominent GABAergic neurons have their somata near the lateral protocerebrum and extend their neurites to the mushroom body lobes and calyces (Figure VI.1), probably also forming synapses in the peduncles. Part of a fast inhibitory feedback loop constituted of feedback neurons from the MB output project back to the input at the calyx (Bicker et al., 1985; Gronenberg, 1986; Grünewald, 1999a; Okada et al., 2007). Besides these feedback-inhibitoric loops recurrent inhibitoric connectivity is visible in ultra structural analyses of microglomeruli in the MB calyx (Ganeshina and Menzel, 2001).

As GABAergic neurons play important roles inolfactory processing, we were interested in age-related effects on the synaptic organization of the honeybee mushroom bodies with respect to its GABAergic innervation. Particularly, we wanted to know whether the GABAergic system underlies adult maturation that correspond to changes in the organization of microglomeruli as shown for the MBs in ants or bees (Groh et al., 2006, 2012; Stieb et al., 2010). To answer this question, we analyzed GABAergic innervations within the MB calyx of individual bees that were part of a cohort study and thus of a defined age (one day after adult eclosion to up to four weeks). We performed qualitative as well as quantitative analyses to determine the degree of colocalization of GABA-immunoreactive (IR) branches (visualized with GABA-immunostaining) and microglomeruli (distinct synaptic complexes between projection neurons and Kenyon cells stained with an antibody to synapsin); and whether the abundance of GABAergic neurites within the mushroom body calyx underlies changes during adult maturation.

Material & Methods

Cohort study

Frames containing pupae of the European honeybee *Apis mellifera carnica* were removed from a source colony and kept in an incubator (33°C, 80% RH). Within 24 hours after adult eclosion, about 250 bees were marked and all but six individuals (day 1 samples) were re-introduced to a colony. The one and two week old bees were taken out of the hive, while the subsequent samples (bees aged three weeks) were collected at the hive entrance. To ensure the forager status and thus full maturation of the bees, only those with either loads of pollen on their hind legs or a distended abdomen were captured. Unfortunately it was not possible to find more than one marked forager after four weeks. Thus, the remaining marked bees at that time point were collected from the hive and dissected although they did not show foraging activity.

Immunohistochemistry

Brains were dissected in ice-cold Ringer and fixed in cold 2% glutaraldehyde in 0.1M PBS at 4°C overnight or up to 44 hours. After washing in 0.1M PBS, brains were embedded in 5% LMP-Agarose and sectioned at 80µm in PBS (except for the first group: one day old bee brains were sectioned at 60µm.) Slices were shortly pre-incubated in PBS with 1% sodiumborohydride $NaBH_4$, washed in 0.1M PBS and blocked in PBS with 0.2%Triton-X 100 and 5% NGS for 1 hour. Incubation in primary antibodies mouse anti-synapsin (1:20; SYNORF monoclonal; with courtesy from Erich Buchner University of Würzburg) and rabbit anti-GABA (1:4000; polyclonal antiserum, # 1013GE, MoBiTec; Göttingen) was done in PBS with 0.2% Triton-X100 and 5% NGS at 4°C for four days. Subsequently, slices were washed in 0.1M PBS and incubated in secondary antibodies Alexa 488 goat anti-rabbit (1:250) and Alexa 568 goat anti-mouse (1:250) in PBS with 1% NGS for 2-4 hours at room temperature. Slices were rinsed in PBS and incubated in 60% Glycerol in 0.1M PBS at 4°C for at least 30 minutes. Slices were mounted in 80% Glycerol in 0.1M PBS on slides and sealed with nail polish.

Confocal microscopy

Preparations (Figure VI.2) were viewed with a confocal laser-scanning microscope (Leica TCS SP2; Leica Microsystems AG, Wetzlar, Germany) equipped with an argon/krypton and an orange laser. Scans were made with an oil HCX PL APO objective lense: UV 63.0x1.40 (working distance 0.1 mm with oil immersion, Leica). The excitation wavelength for Alexa Fluor 568 and 488 were 568 nm and

488 nm, respectively. For quantitative analyses, series of optical sections comprising a total z-depth of 3 µm were captured in 0.5 µm steps. For both quantitative and qualitative analyses the two channels were scanned sequentially for each optical section in combination with a 2.5x digital zoom. For each channel a different false colour was assigned (red for Alexa 568 and green for Alexa 488). The data were viewed either as single orthoslices for qualitative and as projection views of several consecutive orthoslices for quantitative analyses.

Quantification

Quantitative analyses were performed in ImageJ (National Institue of Health). From each set of orthoslices channel wise z-projections were made (maximum intensity of each slice, Figure VI.2). The regions of interest (ROIs) were set in the Synapsin-channel (see Groh et al., 2006; Hourcade et al., 2010; Stieb et al., 2010). The GABA-channel underwent manual and automatized binary threshold setting (see Figure VI.3) to "analyze particles" in the GABA-channel. Evaluation between manual and automatized threshold setting revealed no obvious differences. The area under investigation comprised 800 µm^2 in the lip (two circles 400 µm^2 each) and 1200 µm^2 in the collar (three circles 400 µm^2 each) (Figure VI.3). The amount of above-threshold Alexa 488-immunoflourescent pixels in the ROIs was determined and used as a measure for the density of GABAergic innervation. Statistic evaluation was performed with Wilcoxon rank sum test and Mann-Whitney U-test. Bonferroni correction was applied where demanded.

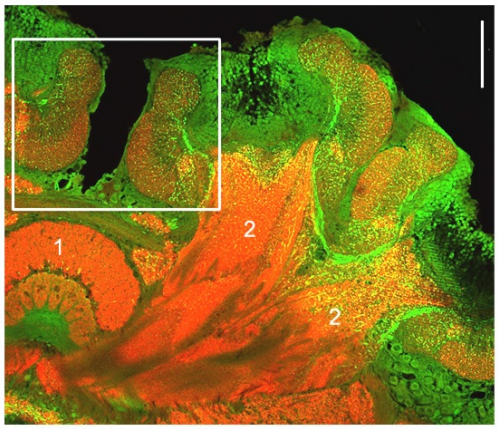


Figure VI.2: Confocal microscope immunofluorescence image of parts of the honeybee brain. The medial and lateral Calyx of the right hemisphere and the medial Calyx of the left hemisphere together with the central body (CB) are visible. Presynaptic profiles are labelled with an antibody staining against Synapsin (SYNORF I, anti synapsin, red) and anti-GABA (green). For analyses only medial calyces were scanned. Scale bar: 100µm

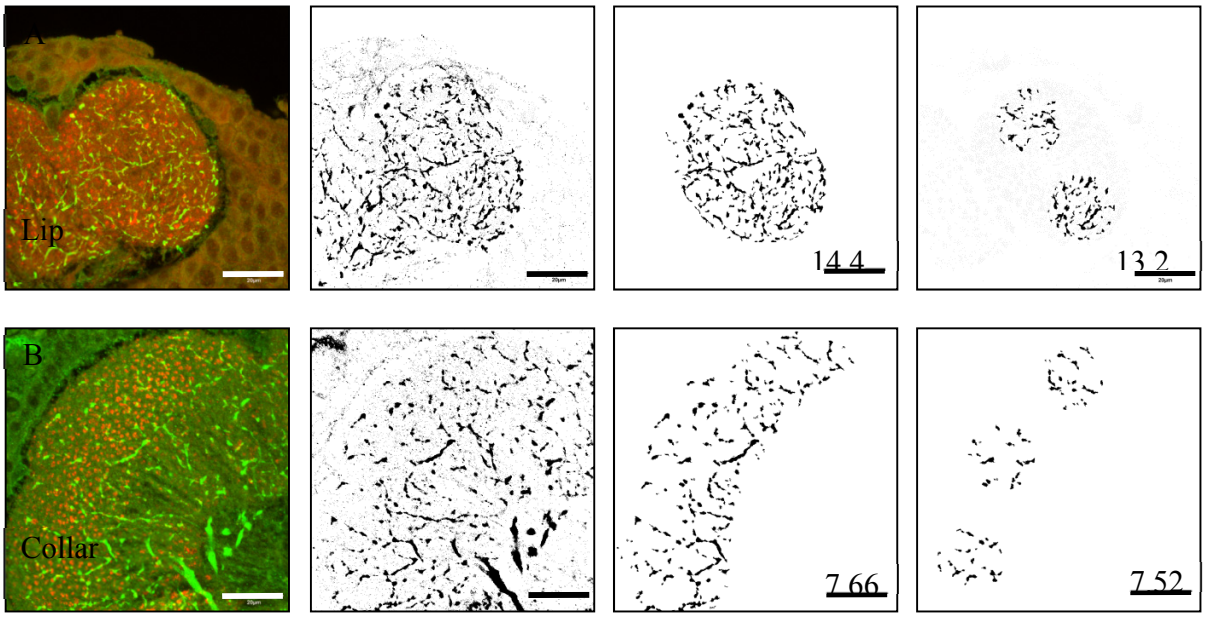


Figure VI.3: Quantification of GABAergic innervation in the mainly olfactory input (lip) and prevalently visual input side (collar) of the bee's MB. For quantification the images underwent image processing in Image J. Explicitly the lip (A) or collar (B) regions were chosen by verifying their origin in the synapsin labeled color channel. The amount of GABAergic processes were evaluated by counting the pixels in either two (A) or three (B) circles with a diameter of 400µm. Thereafter, the percentage of pixels within the circle was calculated and gives a fast overview of GABAergic innervation. Scale bar: 20µm

Results

Qualitative analyses

The co-localization study of GABAergic boutons and anti-Synapsin labelled microglomeruli was performed in lip and collar regions of the honeybees MG (Figure VI.2,3). The analyses of these synaptic microstructures revealed the following characteristics:

- a single microglomerulus may be found adjacent to several GABAergic boutons (Figure VI.3B).
- a single GABAergic bouton may contact several microglomeruli (Figure VI.3A, VI.4B) or just one microglomerulus (Figure VI.4A).
- GABAergic varicosities emerge frequently along PN axons, resembling a beads-on-string motif (Figure VI.3A,B; VI.4A,B).
- finally, rather atypical structures are large boutons that are almost equally stained with anti-synapsin and anti-GABA most likely resembling GABAergic presynapses (Figure VI.3B) but could also be due to coarse optical resolution.

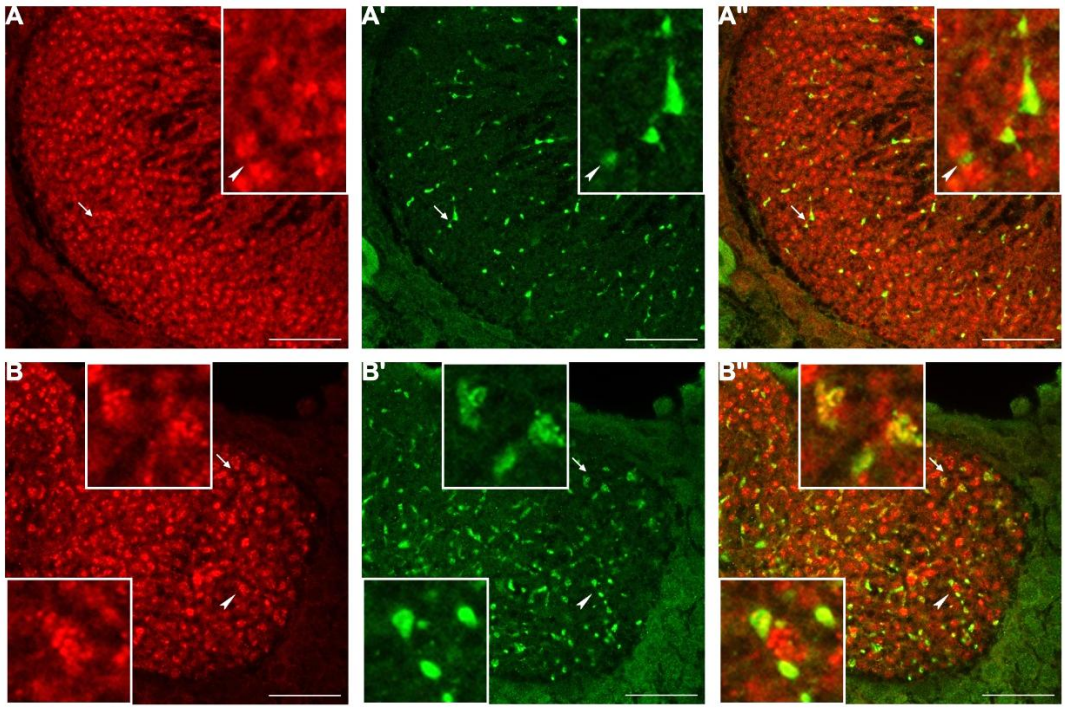


Figure VI.4: Detailed analyses of anti-synapsin (red) and anti-GABA (green) staining reveal colocalization around bouton like structures within microglomeruli. GABAergic profiles surround the presynaptic profile indicating that GABAergic profiles come from MB feedback neurons as described by (Grünewald, 1999a). Stainings in the collar of a one week old bee (A) or from the lip of a bee at the age of four weeks (B). Scale bar: 1.25µm

Quantitative analyses

We examined the density of GABAergic innervation in mushroom body lip and collar region in terms of "area fraction", a simplified measure for the extent to which a given volume contains GABAergic processes (Figure VI.2, VI.3). In the examples of lip and collar regions of fife specimen, a qualitative decrease of the over-all amount of GABAergic innervation is clearly visible (Figure VI.5A;B). For further analyses, we pooled the data from the right and left hemisphere of every bee to increase sample size (Figure VI.2; Table VI.1) as both hemispheres were not significantly different, except for one bee (lip, 1d). In all other groups, the Wilcoxon rank sum test was not significant (Table VI.1). The data indicate that the areas of GABAergic innervation in the lip and collar region of the mushroom body calyx decline during maturation of the honeybee (Figure VI.6,VI.7).

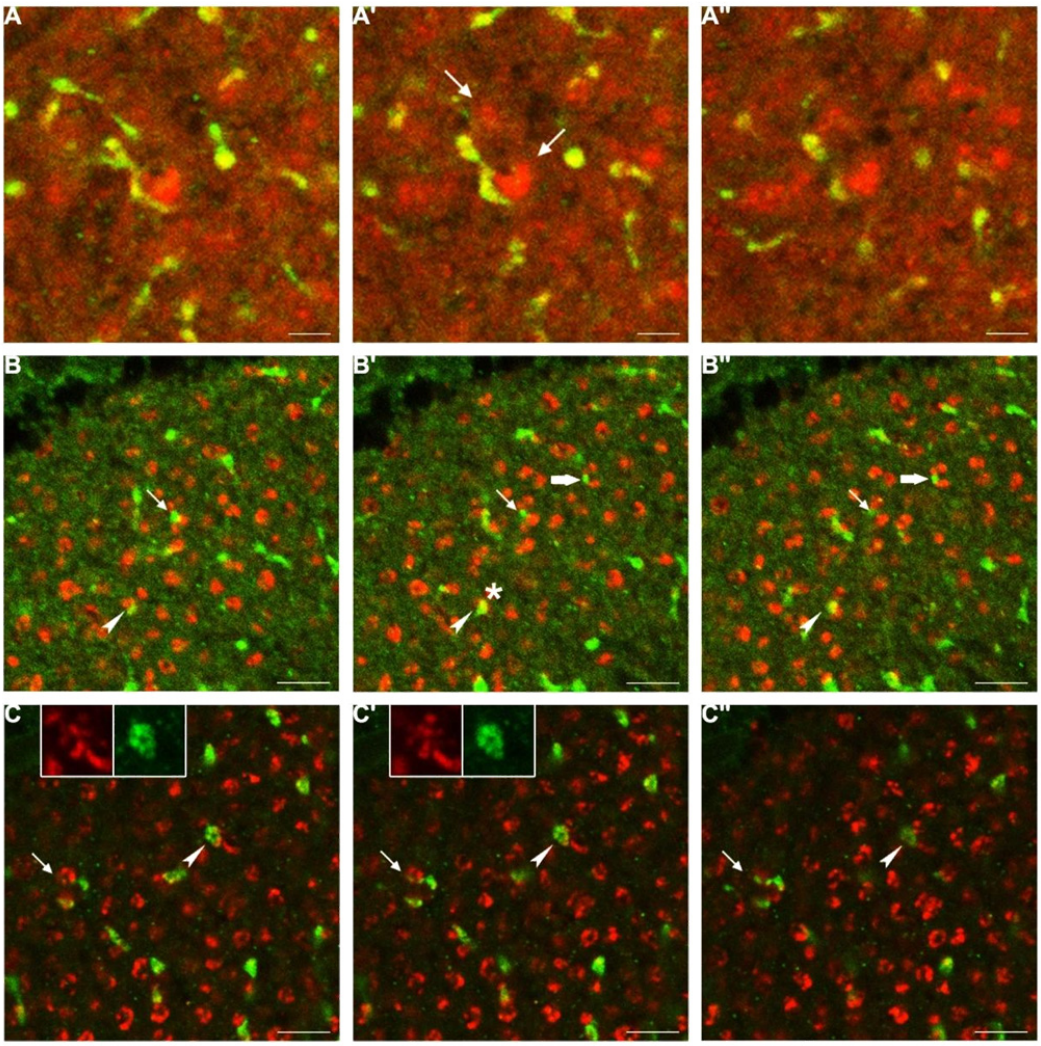


Figure VI.5: images of consecutive orthoslices (d=0.43µm) (A) Lip region of a one day old bee. Scale bar: 2µm. Arrows indicate beads on string profiles as well as distinct MGs. (B) Collar of a two week old bee. Arrows indicate possible MG innervation sites. Scale bar in B,C: 5µm. (C) Collar from a four week old bee. Arrows indicate double labeling of boutons. Inset show single channel 2.5fold magnification.

This effect is similarly established in the collar (1d: 13.7% ± 2.4%; 28d: 5.6% ± 2.9%) and lip region (1d: 8.7% ± 1.6%; 28d: 3.0% ± 0.9%), but with an almost doubled enrichment of GABAergic distribution in the lip compared to the collar. For both regions, we found significant differences between nurses (1d, 7d) and foragers (21d,28d), while bees at the age of two weeks (= 14d) represent an intermediate state (see Table VI.1; Figure VI.7).

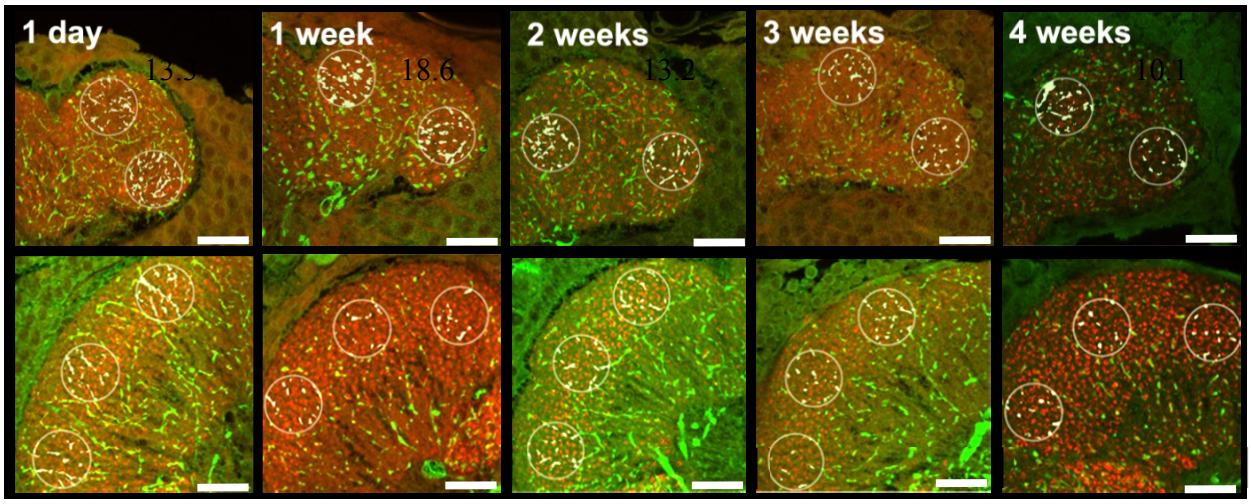


Figure VI.6: Quantification of GABAergic innervation in the lip (upper row) and collar (lower row) region of bees of different ages indicates a decrease of GABAergic innervation within the lip and more prominently in the collar region. Presynaptic profiles are labeled against SYNORF I (anti synapsin, red) and GABAergic profiles labeled with an antibody against GABA (green). The circles (400µm², two for the lip, three for the collar) are indicated and highlight the pixel-based analyses of the GABAergic innervation. The percental amount as well as the age is indicated.

Table VI.1: Comparison of GABAergic innervation between the medial calyces of the left and right bee's hemisphere with additional non-parametric statistical analyses.

Lip	**one day (1d)**		**one week (7d)**		**two weeks (14d)**		**three weeks (21d)**		**four weeks (28d)**	
hemisph.	left	right	left	right	left	right	left	right	left	right
#1	15,8	16,6	16,3	13,8	12,2	9,7	7	11,3	n.a.	n.e.
#2	10,6	15,9	15,8	12,2	10,6	7,6	7,5	9,4	10,1	9,4
#3	10,1	12,3	12,0	18,5	13,2	11,2	6,4	n.e.	4,1	n.e.
#4	13,3	15,4	n.e.	9,9	16,6	11,4	8	8,4	4,1	2,7
#5	11,7	15,1	16,6	n.e.					4,4	4,6
#6			12,8	17,2						
Median	**11,7**	**15,4**	**15,8**	**13,8**	**12,7**	**10,5**	**7,3**	**9,4**	**4,4**	**4,8**
W rank-sum, p=	*0.043		0.465		0.068		0.109		0.285	
Collar	left	right	left	right	Left	right	left	right	left	right
#1	8,6	6,8	5,6	4,9	n.e.	3,9	4,9	2,6	n.e.	n.e.
#2	n.e.	9,9	5,9	6,7	6	7,2	2,5	3,3	3,9	3,9
#3	8,2	11	3,2	4,6	7,5	4,9	2,5	n.e.	1,7	n.e.
#4	9	8,1	4,3	5,6	7,8	5,5	4,0	4,5	3,9	2,6
#5	6,2	10,1	7,1	n.e.					2,6	2,2
#6			7,2	5,2						
Median	**8,4**	**9,9**	**5,8**	**5,2**	**7,5**	**5,2**	**3,3**	**3,3**	**3,3**	**2,6**
W ranksum, p=	0.465		0.686		0.285		1		0.285	0.465

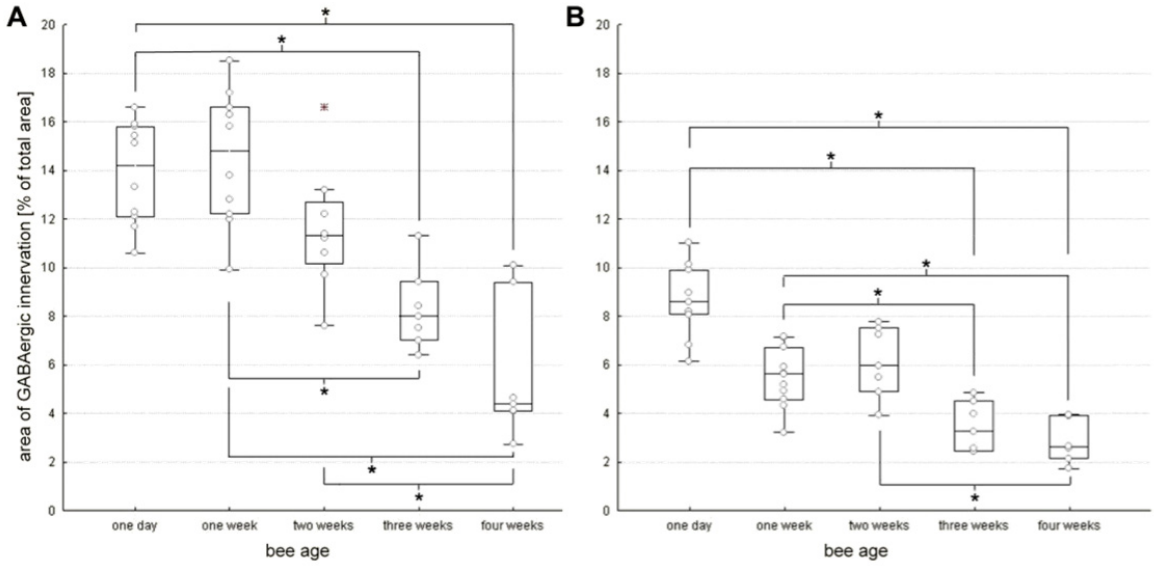


Figure VI.7: Maturation of GABAergic innervation separately plotted for the lip (A) and collar region (B). The comparisons are based on pixel-based analyses of anti GABA stainings in honeybee cohorts. Non-parametric Wilcoxon sum-rank test for comparisons are applied to the data (* = p<0.05).

Table VI.2: Statistical evaluation of the decrease of GABAergic innervation in the lip or collar region during adult maturation (1d, 7d, 14d, 21d, 28d). P-values of a Man-Whitney-U-test are given

.

lip Mann-Whitney U-test	7d	14d	21d	28d
1d	(9,11) 0.473	(9,7) 0.062	(19,7) **0.002	(9,7)**0.001
7d		(11,7)* 0.045	(11,7) **0.002	(11,7) **0.001
14d			(7,7) *0.020	(7,7) **0.004
21d				(7,7) 0.086
collar Mann-Whitney U-test	7d	14d	21d	28d
1d	(10,11) **0.001	(10,8) **0.007	(10,7)**0.001	(10,7)**0.001
7d		(11,8) 0.298	(11,7) **0.004	(11,7) **0.001
14d			(8,7) 0.298	(8,7) **0.003
21d				(7,7) 0.371

Discussion

The present study is a first immunohistochemical account to investigate the maturation-dependent plasticity of GABAergic innervation in the mushroom body calyx of honeybee workers. Previous studies have demonstrated that MB calyx neuropils increase in volume during adult maturation (Withers et al., 1993, 2008; Durst et al., 1994; Fahrbach et al., 1998, 2003; Farris et al., 2001). Immunohistochemical studies reported that the density and total number of microglomeruli in the MB lip and collar decrease and, at the same time, dendritic processes of KCs largely increase and grow between individual MG in foragers compared to nurses (Groh et al., 2012) which has also been observed in the desert-ant *Cataglyphis fortis* (Stieb et al., 2010, 2012).

We found an over-all decrease in GABAergic innervation in the lip as well as in the collar region during behavioral transition from nursing to foraging. Additionally, we found the lip region contained about doubled amounts of GABAergic profiles compared to the collar region. Similar results have been gained from cohort studies using antibody staining against the vesicular GABA transporter (vGAT), although in individual specimen the opposite may be possible (personal communication Claudia Groh, Nancy Butcher). Combining both data-sets will increase the reliability of the analyses of GABAergic innervation. Additional computer aided 3D-reconstructions may further improve quantitative analyses of GABAergic innervation.

How does the decrease of GABAergic innervation on the one hand and an increase in MB calyx volume fit together with a decrease in the desnity of microglomeruli during adult maturation? The decrease in GABA-IR processes is likely to represent maturation of GABAergic input into microglomeruli of the mushroom body calyx: while nurse bees feature abundant synapses all along GABAergic axons, foragers have fewer but more prominent GABAergic processes. Recent EM studies indicate that the amount of PN boutons decrease while at the same time the number postsynaptic partners in individual boutons increased (Groh et al., 2012). According to to this finding our current hypothesis is that during adult maturation mainly dendritic neurites grow in between the MGs which leads to a neuropil expansion (Rössler and Groh, 2012). The outgrowth of KC dendrites and the associated calycal synaptic changes were shown to be triggered by changes in sensory exposure, in particular using light stimulation, suggesting that sensory experience plays a significant role (Farris et al., 2001; Krofczik et al., 2008; Withers et al., 2008; Stieb et al., 2010, 2012).

An electron microscopic (EM) study in the mushroom body lip proposed that calycal microcircuits employ negative feed-forward as well as feedback loops which are realized by GABAergic synapses that occurred pre- as well as postsynaptically in microgomlerular circuits (Ganeshina and Menzel, 2001). Similar results have been reported for the fruitfly (Yasuyama et al., 2002). Consistent with our light microscopic analyses, Ganeshina and Menzel (2001) found single GABAergic synapses in contact to several microglomeruli, as well as the reverse: single microglomeruli contacting several GABAergic synapses. These resulting recurrent inhibitory microcircuits may facilitate sparse coding in the MB calyx (Szyszka et al., 2005).

Bernd Grünewald (Grünewald, 1999b) showed well elaborated recordings of GABAergic feedback neurons to the MB calyx and described their potential function in odor learning. He recorded the inhibitory feedback neurons that decreased their firing frequency already after a single conditional trial which indicates their influence in neuronal plasticity. Together with these and the former described findings our study indicates that GABAergic circuits in the MBs undergo functional changes during synaptic maturation or learning-mediated plasticity at behavioral transition from nurse bees to foragers. This will be an interesting aspect for future electrophysiological and imaging studies using nurse bees and foragers. It will be particularly interesting to find out whether and how these changes affect olfactory processing, perception and learning and how this relates to the social structure in honeybee colonies.

VII - General Discussion

General discussion

The present dissertation mainly focuses on processing of odor stimuli within the honeybee dual olfactory pathway connecting the first olfactory processing stage, the AL, with the second stages, the MB and LH, via mirror-image PN pathways. Due to the newly established recording method (simultaneous extracellular multi-unit-recordings) it was possible to gain important knowledge about system properties within the olfactory system of the honeybee. The advantage of this study was the fact that incoming odor information can be tracked as well as circuit properties recorded simultaneously, and even the behavioral output of the percept is measurable via the proboscis extension (muscle recordings). Timing sensitive activity at different node points within this olfactory circuit can be recorded within the same animal.

This general discussion summarizes the major outcomes of this dissertation. Specific parts were discussed within the discussion of single chapters. This chapter will conclude by combining the results from different chapters.

Parallel Processing

Parallel processing supports fast and reliable sensory processing (Nassi and Callaway, 2009). A most intriguing example of parallel processing can be found in the vertebrate visual system: the magno- and parvocellular pathways from the lateral geniculate nucleus mediate different elemental properties of the same visual scene such as color and spatio-temporal patterns (Livingstone and Hubel, 1988). In the olfactory system knowledge about parallel processing is weak or lacking (Galizia and Rössler, 2010). In the vertebrate olfactory system parallel streams, the OB output pathways (mitral/tufted cells), have only recently been appreciated (Fukunaga et al., 2012; Igarashi et al., 2012; Payton et al., 2012). In insects, especially the honeybee with its unique neuroanatomical layout of the mirror-imaged propagation of the AL output PN tract - the medial APT (antennal-lobe protocerebral tract) first innervating the MB and later the LH, and the lateral APT first innervating the LH and consecutively targeting the MB - is a fascinating model system to analyze parallel processing. Early studies, already about two decades ago (Sun et al., 1993), and more recent studies recorded sequentially from both uniglomerular PN tracts, but unfortunately due to the

methodological limitations, only small numbers of recorded neurons and stimulus repetitions produced controversial results (Abel et al., 2001; Müller et al., 2002; Krofczik et al., 2009). Recently sequential imaging studies of glomerular activation in the l- and m-APT hemilobes gave insight into the input to the two subsystems, the ventral and dorsal AL hemilobes. These studies reported that the subsystems process similar olfactory input but slightly differ, for example, in processing of odorant carbon chain-length or active molecular groups (Carcaud et al., 2012; Galizia et al., 2012). Until now, studies could not really answer the question whether the honeybee dual olfactory pathway codes odors in a "dual segregated" (different odors in different tracts) or in "dual parallel" fashion (odors responded in both tracts but with differing feature extraction).

Based on the newly established recording setup and the adjusted methods we were the first who managed to record from the two uniglomerular PN tracts simultaneously. This method gained high numbers of odor stimulations and, more importantly, facilitated multiple odor stimulations with high repetition rates (10 - 20). We analyzed the recorded data systematically and automatized the analyses to minimize anthropogenic affected results. We tested not only floral and pheromonal odors that have been used in the former studies, but additionally stimulated with behavioral relevant odors. Since we took this demanding approach, we now gained confident knowledge that the dual olfactory pathway of the honeybee indeed processes many odors in parallel. To filter out specific differences in parameter extraction along both information streams, we used new methods to analyze the recordings for tuning measures like neuron stimulus specificity (lifetime sparseness), the ratio of odor-responding neurons (recruitment rate), response latency differences (single and population response latencies) and response profile of l- and m-APT PN populations (population rate functions) and found:

These analyses indicate that m-APT PNs process odors in a sparse manner and with a delayed response, but with high odor-specificity. The l-APT PNs, in contrast, are more broadly tuned (less odor specific) and respond with shorter latency. In some PNs from both tracts we found evidence for odor-identity coding via odor-specific response latencies (SNR measures).

Taken together the results indicate that odor processing along the two APTs share coding principles to increase processing capacity (rate and response latency code). This indicates that the olfactory pathway in the honeybee is adapted to serve the multitude of olfactory demands associated with the importance of a complex olfactory world for a social insect. According to the vertebrate visual system with "what-" (object vision) and "where-"channels (spatial vision) in the inferior temporal

and the posterior parietal cortex (Mishkin et al., 1983; Merigan and Maunsell, 1993; Milner and Goodale, 2008) we now hypothesize that the l- and m-APT system provide a “what-” (the sparse and odor-specific coding m-APT) and a “when-“ channel (fast and global responding l-APT).

Concentration Coding

Odor detection is of high behavioral importance for insects in general (Anton et al., 2007; Hansson and Stensmyr, 2011). The olfactory system, therefore, needs to ensure reception and perception of various concentrations of an odor under natural conditions. Odors are transported via the air with a constant movement and flux. Thus odors are dispersed, mixed and diluted in air and additionally intermixed with background odors (Vetter et al., 2006). This finally leads to filamentous plumes of the source odor in differing concentrations (reviewed in Martin et al., 2011). Animals therefore rely on olfactory systems that are suitable to track, receive and perceive the odors over a wide range of concentrations and temporal structures. The sensitivity of some systems is intriguing like the silk moth *Bombyx mori* which was shown that only few molecules of the female pheromone bombycol are sufficient to elicit behavior in the perceiving male (Kaissling and Priesner, 1970).

We analyzed the concentration coding capacity of the dual olfactory pathway to investigate, whether the APT PNs are capable to code odor-identity over a wide range of odor concentrations. Bees were stimulated with different volatile plant- and pheromonal- odors in the range of 5 orders of magnitude in concentration.

In the majority of the recorded PNs we found concentration-dependent positive correlations: the higher the odor concentration, the higher the AP rate with slightly stronger responding l-APT PNs. Recent imaging studies of glomeruli in the ventral as well as the dorsal AL indicate similar odor concentration characteristics regarding the input of both APTs with a slightly stronger activated ventral hemilobe (l-APT) (Carcaud et al., 2012; Strauch et al., 2012). A recent imaging study from Yamagata et al. (2009) found positive as well as negative correlations especially at high odor concentrations via calcium imaging from PN boutons of both, the l- and m-APT at the MB input site, the MB calyces. Unfortunately these results are difficult to compare with our recordings as changes in intracellular calcium were measured at the synaptic bouton within the MB calyx, which could be

strongly influenced by GABAergic feed-back neurons and recurrent GABAergic connectivity within the MB calyx (Grünewald, 1999a, 1999b; Ganeshina and Menzel, 2001; Haehnel and Menzel, 2010).

We found steady state and non-linear coding during increasing odor concentrations. Steady-state firing has previously been shown solely for T1 glomeruli innervating the l-APT (in bees: Sachse and Galizia, 2003; in ants: Zube et al., 2008) and likely results from interaction with inhibitory and excitatory influences inside the AL. AL preprocessing of PN responses provides the olfactory system with olfactory information over a long range of concentrations by using gain control mechanism, sharpening (lateral inhibition) (Sachse and Galizia, 2003) or broadening (lateral excitation) of olfactory information, (for review see Martin et al., 2011; Wilson, 2011). This steady state coding mechanism might also be responsible for concentration invariant odor identity-coding (Sachse and Galizia, 2003; Stopfer et al., 2003; Zube et al., 2008). It was recently supposed that odor is kept as a generalized odor identity over several concentrations due to pattern continuity and smooth transitions between pattern for concentration levels of the same odor (Strauch et al., 2012).

We find rising concentration dependency in response strength and a negative correlation of the response latency of l- as well as m-APT PNs in at least one studied bee: the higher the odor concentration, the shorter the response latency. This fact can be explained by the ORN's own molecular receptive range. At low concentrations only odor specific ORNs mediate signal transduction. As the odor concentration increases more and more odor molecules will activate secondary non-odor-specific receptors (Vareschi, 1971; Akers and Getz, 1993; de Bruyne et al., 2001) that consequently activates additional glomeruli in the AL which leads to an increase in excitation of PNs that elicit faster response onsets.

The result of earlier behavioral studies with classical conditioning tests (PER) show that low odor concentrations do not lead to odor discrimination disabilities, but instead lead to prolonged learning performances (Wright et al., 2005, 2009). Since in our tests the lowest odor concentrations still elicited detectable responses in the l-APT, odor-identity might already be coded with one APT. But interestingly the lack of odor-identity information of the other tract obviously decreases processing speed and learning acquisition, which supports the significance of both APTs to provide parallel processing.

Temporal Coding

Besides rate- and response latency coding principles found in the honeybee olfactory system it is of high interest to evaluate whether coding principles on the level of spike events of single PNs might enhance odor identity coding.

First we tested whether in APT PNs synchronous activity can be found and synchronously activated PNs within a single uniglomerular PN tract or across both the l- and m-APT. We found synchrony mainly increasing during stimulus presentations and almost completely vanishing after stimulus termination (Figure V.1C). Similarly observations were made in the moth *Manduca sexta* (Lei et al., 2002; Riffell et al., 2009a). Stimulus triggered correlations and decorrelations were also found in the fish (Friedrich et al., 2010; Wiechert et al., 2010) and mammalian olfactory systems (Lledo et al., 2005; Miura et al., 2012; Spors et al., 2012). Decorrelation is suggested to support the differentiation of chemically similar odors in vertebrates or to the overlap of similar sensory representation reduce in general, which improves the perceptual differentiation of physically similar stimuli (Cleland, 2010; Cleland and Linster, 2012).

Whether the detected synchrony of APT PNs within the honeybee olfactory pathway codes for odor-identity and whether the synchrony improves differentiation of odor identity over a wider concentration range and within odor mixtures will be the matter of future quantitative analyses. The fact that we found synchrony across PNs recorded from both tracts that even improved compared to synchrony across PNs within one APT indicates the potential significance of the dual olfactory pathway in temporal coding of odors. This gives further support to parallel processing properties of the l- and m-APT.

Coincidence coding

The anatomical layout of the hymenopteran dual olfactory pathway is unique (Kirschner et al., 2006; Zube et al., 2008; Rössler and Zube, 2011). The trajectories of the uniglomerular PN pathways, the medio- and lateral antennal-lobe protocerebral tracts, are running in a mirror-image direction. The

m-APT, originating from the dorsal AL hemilobe, reaches the MB first and later the LH. In contrast the l-APT, starting from the ventral AL hemilobe, innervates the LH first and then the MB (Kirschner et al., 2006; Galizia and Rössler, 2010). This PN layout, to a certain extent, reflects the layout of delay-lines in other sensory modalities.

Best known example is the Jeffress delay-line model which was implemented to understand directional hearing in the auditory brainstem of vertebrates (Jeffress, 1948; for review see Joris et al., 1998). Auditory-nerve input is relayed from both ears to cells which act as coincidence detectors, themselves firing maximally when action potentials from both ears arrive simultaneously. The inputs arrive along nerve-cell axons which vary systematically in their length on their way to the different target cells. Because longer axons take more time to transmit their signals, they act like delay lines and introduce cellular time delays. These delays lead to maximal firing at that particular inter-aural time difference which is represented in the owl and forms a topographic representation of the auditory azimuth (Köppl, 2009). Hence, temporal intervals can be converted to spatial representations utilizing neuronal delays.

This examples demonstrate that circuits suited to detect minute time differences depend on some form of delay lines and coincidence detectors that respond maximally when they receive simultaneous inputs (Carr, 1993). Transferred to the dual olfactory pathway of the honeybee may suggest that the mirror-imaged layout supports detection of minute time differences in olfactory coding. Recently it has been shown with calcium imaging that bees can behaviorally and physiologically differentiate incoherent odor mixtures with a time delay of just 6ms (Szyszka et al., 2012). To start to test this hypothesis we correlated simultaneously recorded spike trains of the l- and m-APT to analyze temporal correlations. We found maximal cross correlations within the time window of 0-3ms (Figure V.3). The correlations slightly shifted during odor stimulation and became almost absent during control stimulation (Figure V.3A,B). These results support the idea that KCs can receive coincidental input by APT PNs from both tracts. This is also indicated by our analyses of synchrony in l- and m-APT PNs (Figure V.2C,D).

If we assume that a PN AP is running along its axons to the KCs with the same neuronal conduction velocity of 20 to 25cm/s, as it has been determined in the honeybee (Oleskevich, 1997), by tracking the lengths of the l- and m-APT PNs within a optical sections, AP forms PNs of both tracts should reach in the medial calyx simultaneously. APs from m-APT PNs would first arrive in the MB and later the LH with considerable delay in comparison to l-APT PNs that target the LH first. The resulting

delay would be in the range of 3 to 4ms. Between the medial and lateral calyx slight delay differences of incoming synchronous PN excitations are likely to result in the excitation of different sets of KC. With our knowledge about spike synchrony in PNs within and across tracts this system appears to be well suited to improve analysis of temporal properties of odor stimuli.

We found that the l-APT PN population responds faster compared to the m-APT population. On the single PN level we found that PNs of both tracts code odors by differences in response latency. Taken these observations into account, odors would elicit PN responses with different response latencies. This will consecutively lead to another set of coincidentally activated KCs leading to the well described sparse activity pattern of KC, which would change in correlation to different odor stimulations. Hence the coincidental activation of KC might be stimulus dependent and will likely promote access for neuromodulator influence and increases odor processing capacity and capacity of multi sensory association (Smith et al., 2008).

Experimental proof for the existence of a sparse odor code within the MB calyx has been given by studies in the honeybee (Szyszka et al., 2005, 2008), moth (Ito et al., 2008), locust (Perez-Orive et al., 2002, 2004), or fruitfly (Wang et al., 2004). Since *in-situ* patch-clamp analyses in the cockroach KCs found strong Ca^{2+} dependent inward and outward currents that require strong synaptic input (Demmer and Kloppenburg, 2009), the KCs are prominent coincidence detectors but also enable intrinsically mediated sparse activation. The well described intrinsic properties of the KCs suggests spike-frequency adaptations which refuse the idea of inhibitory LH neurons suggested by Perez-Orive et al. (2004) for the locust, instead rather due to their intrinsic properties KC contribute to sparse coding (Gupta and Stopfer, 2012; Nawrot, 2012).

In summary the dual olfactory pathway may be a well suited neuronal layout to perform sophisticated odor processing both regarding odor specificity and temporal structure which is likely to result in enhanced odor perception capabilities.

Adult plasticity of GABAergic innervation

Social insects experience various tasks in changing environments throughout their lifetime, resembling one of the most intriguing examples of ontogenetic neuroplasticity (Withers et al., 1993). During lifetime, nervous systems undergo adult maturation that changes synaptic connectivity (Fahrbach et al., 1995; Seid et al., 2005; Groh et al., 2006, 2012; Seid and Wehner, 2008; Stieb et al., 2010). Especially in the MB calyx PN to KC synaptic complexes, microglomeruli, were demonstrated to undergo pruning, which leads to decreased MG concentration in the MB and, at the same time, massive outgrowth of KC dendrites (Stieb et al., 2010; Groh et al., 2012).

We were interested to evaluate how the inhibitory system of the MB changes during this adult synaptic maturation as GABAergic feedback neurons were demonstrated to be highly important in olfactory coding and behavior.

GABA-IR was found at various sites in the honeybee brain (Bicker et al., 1985; Schäfer and Bicker, 1986; Bicker, 1999). GABA application elicits Cl^- currents through ionotropic picrotoxin sensitive GABA receptors in cultured KCs (Déglise et al., 2002; Grünewald et al., 2004) and AL neurons (Barbara et al., 2005). Prominent GABAergic neurons have their somata near the lateral protocerebrum and extend their neurites to the mushroom body lobes and calyces, probably also forming synapses in the peduncles and part of a fast inhibitory feedback loop constituted of feedback neurons from the MB output back to the input at the calyx (Bicker et al., 1985; Gronenberg, 1986; Grünewald, 1999a; Okada et al., 2007). Besides these feedback-inhibitoric loops recurrent inhibitoric connectivity is visible in ultrastructural analyses of MB calyx microglomeruli (Ganeshina and Menzel, 2001).

We found an over-all decrease in GABAergic innervation in the lip as well as in the collar region during behavioral transition from nursing to foraging. Additionally, we found the lip region containing about double the amount of GABA compared to the collar region.

The decline of anti-GABA stained areas reflects maturation of GABAergic input into the MB calyx: while nurse bees feature abundant synapses all along GABAergic axons, most of those are pruned as maturation proceeds (which may account for the general sparsening of GABAergic processes) and is most likely due to sensory experience in foragers. This hypothesis would be consistent with a recent EM study (Groh et al., 2012). Due to this finding the current hypothesis is that during adult

maturation mainly dendritic neuritis grow in between the MGs (Rössler and Groh, 2012). KC dendrite outgrowth was shown to be triggered by sensory experience (Farris et al., 2001; Krofczik et al., 2008; Withers et al., 2008; Stieb et al., 2010, 2012).

Based on the recent studies and our findings, synaptic plasticity during the behavioral transition of nurse bees to foragers is an interesting aspect for analyzing olfactory coding in the honeybee brain and for future physiological studies on olfactory processing and learning.

Conclusion

Social insects like the honeybee are fascinating and have been examined by many researchers. Already a century ago, in 1914, Karl von Frisch, Nobel-Prize winner of 1973, published his discovery about color vision in the honeybee (von Frisch, 1914). Until today researchers are fascinated by the surprising sensory and cognitive skills studied in this tiny animal (Menzel, 2012a). Besides the discovery of the complex associational skills in the bee (Chittka and Niven, 2009; Chittka and Jensen, 2011; Chittka and Skorupski, 2011), already in the 1850s Dujardin studied the brain of the honeybee and was intrigued by its miniaturization and its beauty (reviewed in Strausfeld, 2012). In 1897 Ramon y Cajal depicted the first neuronal wiring of honeybee brains (reviewed in Menzel, 2012). Behavioral and neuroanatomical knowledge about the honeybee has increased the interest to collect information about the underlying physiological properties that use the neuroanatomical layout and finally control behavior.

In my PhD thesis I have tried to add knowledge about olfactory coding in the honeybee to the research community. Especially the important new approach of simultaneous recordings (first chapter) has opened a new field in analyzing circuit properties and system characteristics (Nawrot, 2012). One of the major open questions, whether the dual olfactory pathway in the honeybee processes odor information in a “dual segregated” or in a “dual parallel” fashion, has now been answered: I describe clearly differing coding properties in this thesis, although the input to the two subsystems is remarkably similar, (Carcaud et al., 2012; Galizia et al., 2012). This is a very powerful indicator for parallel processing (Nassi and Callaway, 2009). The main findings are that the m-APT responds sparsely with high odor-specificity (proven via lifetime sparseness and recruitment rate)

but evokes responses that are delayed in direct contrast to the l-APT (with fast response latency, fires more frequently, has a global response profile) (chapter 2)). I could demonstrate that both tracts respond to increasing odor concentrations either with correlated response strength or with a non-linear response characteristic (chapter 3). These results give insight into the astonishing coding property of the AL that most likely mediates these non-linear processing transitions (Meyer and Galizia, 2012). Hypotheses about additional temporal coding principles besides rate- and response latency codes can now be addressed experimentally (chapter 4). I could give indications that the dual olfactory pathway mediates synchronous activity within the uniglomerular PN tracts that is enhanced, if PNs in both fire synchronously. This again strongly supports a special role of the dual olfactory pathway in elaborated olfactory coding in this social insect. Most interesting is the idea that KCs are considered to resemble coincidence detectors. The mirror-imaged lateral and medial APTs provide a well-suited neuronal layout for this. With this model, delay lines contributed by the different axonal trajectories and innervation patterns of the opposing tracts, may lead to spatial and temporal sparsening of coincidentally activated KCs. This hypothesis is strengthened by the fact that I could provide information about differences in response latencies across l- and m-APT PN populations and within single APT PNs (SNR). Additionally, cross-correlations revealed that individual l- and m-APT PNs fire in synchrony, most likely supporting coincidental input to the KCs. This hypothesis still needs experimental support with sophisticated analyses and procedures including simultaneous KC recordings, but the results already give insight in an exciting new field of alternative coding principles that could be responsible for elaborated concentration invariant odor identity coding, in particular for complex odor mixtures.

Finally, organisms are plastic. During adult maturation brains continue to develop, for example by axonal pruning and/or dendritic growth. I could demonstrate that the GABAergic inhibitory system, significant for olfactory coding, underlies adult maturation (5. chapter). The distribution of GABAergic processes in the MB calyx decreases during the behavioral transition from a young nurse bee to a forage about threefold in the lip (olfactory input) and collar (visual input) region of the MB calyx, with a doubled amount of GABAergic processes in the lip compared to the collar. This first step is important as it provides an interesting background for novel physiological experiments on maturation of olfactory coding that take these changes into account.

Outlook

Opening a new field is a long-lasting approach. Especially if one starts from scratch with the construction of experimentally suited electrodes, amplifier systems, adjustments of combination of different amplifier systems, establishment of self-written software aided data-acquisition, self-written headstage controller software, development of basal and advanced data analyzing software, and the establishment of a new preparation for dual tract recordings in a life insect with a mini brain. However, after this accomplishment, the entire system is transparent to the user without any black-box feeling. Of course these approaches are not manageable in all respects solely by a single person, therefore especially software-guided elaborated analyzing algorithm demand help from specialists, for example from neuroinformatics.

Extending existing experiments.

As already mentioned within individual chapters, some experiments necessitate increased numbers of sample sizes (concentration coding, temporal coding).

Regarding experiments on parallel processing (2. chapter):

- Although the experiments provide good evidence for parallel processing, more odors need to be considered, in particular behaviorally relevant classes of odors. It is especially interesting to chose odors systematically that are distant in the odor space (Haddad et al., 2008) or that elicit similarity in the odor percept (e.g. Chen et al., 2011).
- Since honeybees are social insects, the experiments should include stimulation with additional pheromones like brood-pheromones (Sandoz et al., 2007).
- In the present studies odor stimulations were mainly static, although still much shorter than in comparison to earlier studies which facilitates a more naturalistic stimulation paradigm. However, future stimulations should consider flexible odor stimulations (e.g. Geffen et al., 2009).
- Finally, odor mixtures are in most cases most relevant and should be investigated systematically in the future using this approach (e.g. Szyszka et al., 2012).

Regarding concentration coding (3. chapter):

- The experiments used stimulation over a wide odor-concentration range but lack especially high concentrations (1:10, and pure odors). Future experiments necessarily need to include this odor concentration range.
- Additionally in the data shown, odors in differing odor-concentrations were delivered with ten trial repetitions. Solid analyses are required which need recordings that include even higher repetition rates.

Regarding analyses on temporal coding (4. chapter):

- Higher numbers of PN recordings allow a more detailed analysis in particular on temporal coding. Cross-correlations often lack suitable amounts of recordings, especially since m-APT PNs fire more sparsely. More recordings need to care of this concern.

Regarding adult maturation in the GABAergic system (5. chapter):

- The experiment on GABAergic adult maturation in honeybee MBs cohorts needs to be combined with ultrastructural data and studies including other antibody stainings.
- Anatomical quantification of immunostainings is difficult and needs further improvement using 3D-reconsturctions software.

Future projects:

Due to the large amount of data from dual tract recordings, clustering of APT PN populations were performed to give detailed insight in differences between the two populations. This kind of analysis was first introduced to segregate non-identifiable neuronal response profiles into AL neuron classes (LN vs. PN) (personal communication, Anneke Meyer).

We will be able to find deeper insight in the differing features contributing to olfactory coding within both PN tracts if we search for feature extraction based on computer-aided sorting algorithms like the hierarchical clustering (Figure VII.1). For hierarchical clustering separation was based on spiking features like CV2 (measure of spiking irregularity), FanoFactor (a measure of across trial spike-count variability), latency, lifetime sparseness (measure of stimulus-specificity), background rate, maximal

firing rate, evoked rate, surprise criterion (change in firing properties), burst duration, and number of bursts. Future analyses will need to include "support vector machine" classification which might increase separation quality.

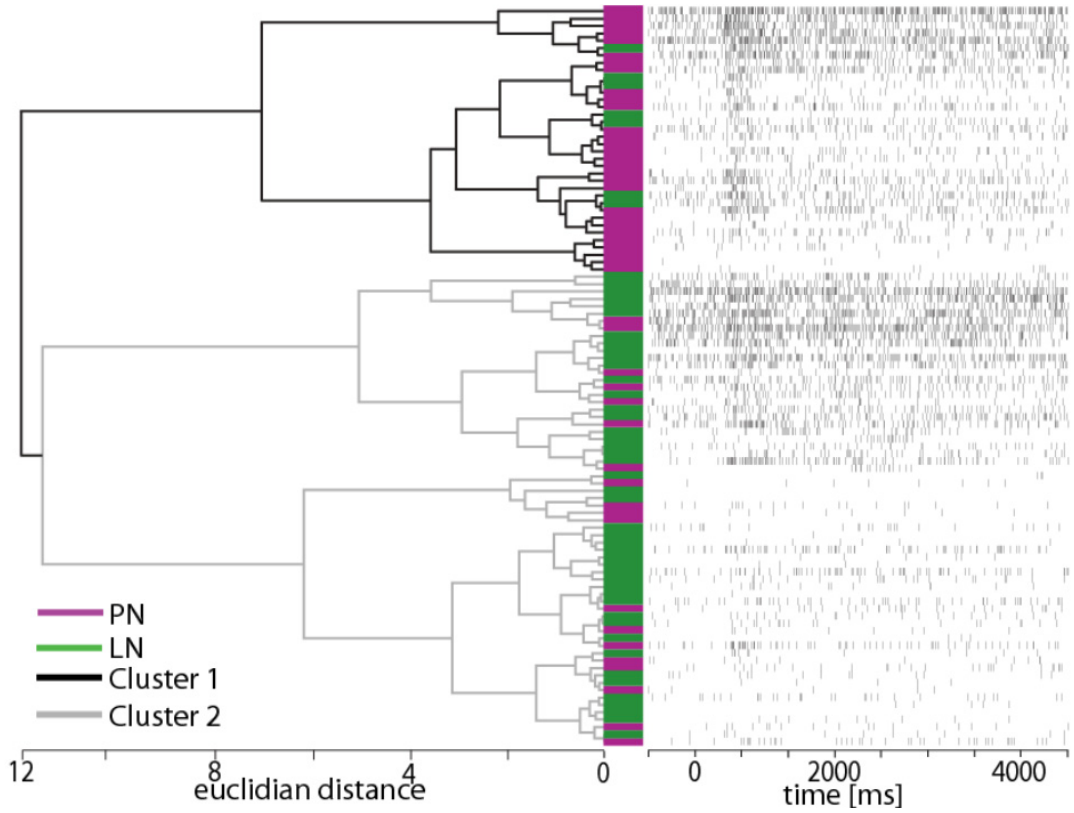


Figure VII.1: Hierachical clustering of simulatenously recorded PNs (n=122) of the l- and m-APT (green and purple, respectively), acquired from 14 bees. Ten different features (CV2, FanoFactor, latency, lifetime sparseness, background rate, maximal firing rate, evoked rate, surprise criterion, burst duration, and number of bursts) were choosen. The APT PN cluster mainly in two populations; Matthew correlation 0.47 (confidence level of 95%: 0.19).

Analyzing temporal coding properties is demanding. To evaluate synchronous events within several PN recordings over long time periods requires sophisticated automized analyses, but also new experiments are neseccary. The setup was designed to also allow for electrical stimulation. Best evidence on the potential function of the dual olfactory pathway as a delay-line system that promotes coincidence coding in KC via the l- and m-APT will be enabled by (1) recording extracellularly from the tracts and additionally from KCs, for example using glas-electrodes. If this is sucessful, eletrical stimulation can be used to elicit excitation to the PN tracts in precise temproal

patterns. With systematic stimulation paradigms, KCs might respond only to particular temporal stimulus constellations. (2) Dual recordings of a single APT at different sites will give more insight into the neuronal conduction velocity. The results could afterwards be implemented into model analyses. (3) Modeling studies should evaluate whether the supposed delay-line and coincidence coding machinery is feasible and could make predictions for futuree experimnetal approaches.

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IX - Abbreviations

OB	Olfactory Bulb
OE	Olfactory Epithelium
AL	Antennal Lobe
m	medial
l	lateral
ml	medio-lateral
r	rostral
c	caudal
APT	antennal lobe protocerebral tract
ACT	antenna cerebralis tract
MB	mushroom body
MC	medial calyx
LC	lateral calyx
CC	Cross Correlation
LS	lifetime sparseness
KC	Kenyon Cell
M/T	mitral & tufted cells
OR	Olfactory Receptor
ORN	Olfactory receptor Neuron
ORC	Olfactory Receptor Cell
OBP	Olfactory Binding Protein
GPCR	G-protein coupled receptor
AP	action potential
MGC	Macroglomerular complex
LN	Local Neurons
PN	Projection Neuron
uPN	uniglomerular PN
mPN	multiglomerular PN
CNS	Central Nervous System
G	Glomeruli
LH	Lateral Horn
T1-T6	sensory tracts
EN	extrinsic MB Neurons
ORCO	Olfactory Receptor Co-Receptor
DIO	Digital Input/Output
VUMmx1	ventrally unpaired medial cell of maxillary neuromere 1
ISI	Inter Stimulus Interval
PSTH	Peri-Stimulus Time Histogram
SD	Standard Deviation

X - Curriculum Vitae

Martin Fritz Brill *13.04.1980 in Bad Karlshafen

Dipl. Biol.

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Scientific Education

since 04/2007	PhD candidate (Dr. rer. nat. cand.) at the University of Würzburg and graduate student in the Graduate School of Life Sciences, University of Würzburg, section: Integrative Biology (2008-2012) Graduate Studies supported by the DFG: SFB 554 (A8) & SPP 1392 **PhD thesis:** "Processing and plasticity within the dual olfactory pathway in the honeybee brain" in the department of Behavioral Physiology and Sociobiology, Julius-Maximilians University of Würzburg *Supervisor: Wolfgang Rössler, Christoph Kleineidam (Uni Konstanz), Peter Kloppenburg (Uni Köln)*
11/2006	Diploma in Biology, Georg-August University of Göttingen
11/2005-10/2006	**Diploma thesis**: "Intrazelluläre Untersuchungen der Netzwerkeigenschaften im thorakalen auditorischen System der Grille" in the department of Neurobiology, Johann-Friedrich-Blumenbach Institute for Zoology and Anthropology, Georg-August University of Göttingen *Supervisor: Andreas Stumpner, Norbert Elsner*
09/2002-08/2003	ERASMUS (European Union exchange program) at Utrecht University, the Netherlands, main studies in the department of functional Neurobiology
10/2000-11/2006	Biology Studies (Diploma) at the Georg-August University Göttingen main subject: Zoology minor subjects: Biochemistry, Human Genetics

Research Internship

05/2009	Dept. of Neuroscience, Prof. John Hildebrand, University of Arizona, Tucson, Arizona, USA

Awards & Grants:

01/2012	Travel grant (800 €) provided by the DFG priority program on "Integrative Analysis of Olfaction", SPP 1392 to participate in the XVI. International Symposium on Olfaction and Taste in Stockholm, Sweden
09/2008	1st prize (5000 €) at the occasion of the poster presentation contest at the Scientific Summer Party of the Graduate School of Life Sciences, University of Würzburg

Scientific Initiatives:

05/2008-07/2009	Organizational committee of the 20th Neuro-DoWo 2009 (neurobiology PhD student workshop) in Würzburg
06/2012-07/2012	Organizational committee of the international symposium: "olfaction in insects under debate: from receptors to behavior" Würzburg

Teaching Experience

since 2007	Supervision of bachelor/master/diploma courses in Neurobiology and Animal Physiology; Co-supervision of student internships, and Bachelor, Master and Diploma theses, University of Würzburg
2005-2006	Teaching assistant in practical courses for bachelor students in Biology and teaching assistant in practical courses for master students in electrophysiological methods, University of Göttingen

Memberships

since 2010	Deutsche Zoologische Gesellschaft e.V. (DZG) - *German Zoological Society*
since 2010	Neurowissenschaftliche Gesellschaft (NWG) - *German Neuroscience Society*
since 2012	Society for Neuroscience (SfN)

Würzburg, 3.01.2013 Martin Brill

Date Signature

XI - Publications

Peer-reviewed Journal Publications

Brill M.F., Rosenbaum T., Reus I., Kleineidam C.J., Nawrot P.M., Rössler W. (2013) Parallel processing via a dual olfactory pathway in the honeybee . Journal of Neuroscience (in press)

Oral Presentations

Brill M.F., Reus I., Rosenbaum T., Kleineidam C.J., Nawrot P.M., Rössler W. (2012) Parallel processing in the olfactory system of honeybees. Department Seminar talk in the Dept. of Cellular Neurobiology, University of Göttingen Prof. M. Göpfert, Prof. A. Stumpner, Prof. R. Heinrich

Brill M.F., Rosenbaum T., Reus I., Kleineidam C.J., Rössler W. (2011) Integrative analysis of multiple pathways in the honeybee olfactory pathway - Parallel processing in the honeybee olfactory pathway. Berlin, Symposium des Schwerpunktprogramms SPP 1392 “Integrative Analysis of Olfaction” June 6.-8.

Brill M.F., Rosenbaum T., Reus I., Kleineidam C.J., Rössler W. (2011) Parallel processing in the honeybee olfactory pathway. Rauischholzhausen Seminar: Development and Plasticity of the Insect Nervous System, Rauischholzhausen, Germany

Brill M.F., Rosenbaum T., Reus I., Rössler W. (2011) Simultaneous recordings from the dual olfactory pathway of the honeybee. Seminar talk, Department of Neurobiology Prof. G. Galizia, habil. Kleineidam University of Konstanz, Germany

Brill M.F. (2010) Odor processing in the honeybee. 5th internation Symposium organized by the students of the Graduate School of Life Sciences, Würzburg, Virchow-Center

Brill M.F., Rosenbaum T. and Rössler W. (2010) Multi-unit recordings in the dual olfactory pathway of the honey bee. SFB 554 Final Meeting Bronnbach, Germany

Brill M.F., Rosenbaum T. and Rössler W. (2010) Multi-unit recordings in the dual olfactory pathway of the honey bee. Rauischholzhausen Seminar: Development and Plasticity of the Insect Nervous System, Rauischholzhausen, Germany

Brill M.F., Rosenbaum T. and Rössler W. (2010) Multi-unit recordings in the dual olfactory pathway of the honey bee. Dept. Seminar Talk, Prof. R. Menzel FU Berlin, Germany

**Brill M.F. (2010) Biologie als Beruf. Berufsinformationstag Albert-Schweizer Schule Hofgeismar, Germany

**Brill M.F. (2009) Biologie als Beruf. Berufsinformationstag Albert-Schweizer Schule Hofgeismar, Germany

Brill M.F., Kleineidam C.J. and Rössler W. (2008) Plasticity in the dual olfactory pathway of the honeybee. SFB 554 Meeting Field Station Fabrikschleichach, Germany

Brill M.F., Kleineidam C.J. and Rössler W. (2008) Processing and Plasticity within the dual olfactory pathway in the honeybee brain. Rauischholzhausen Seminar: Development and Plasiticity of the Insect Nervous System, Rauischholzhausen, Germany

Brill M.F. and Rössler W. (2008) Processing within the dual olfactory pathway of the honeybee brain. 19th Neuro-DoWo (neurobiology PhD student workshop), Saarbrücken, Germany, p46

*Brill M.F. and Stumpner A. (2007) Photoablation von auditorischen Interneuronen. Lab Seminar Talk, Prof. P. Kloppenburg Uni Kolone, Germany

*Brill M.F. and Stumpner A. (2007) Photoablation von auditorischen Interneuronen. Lab Seminar Talk, Prof. W. Rössler Uni Würzburg, Germany

Published and unpublished Poster Abstracts

Brill M.F., Meyer A., Nawrot M.P., Rössler W. (2013) Odor identity coding reveals parallel processing within the bee's dual olfactory pathway. 10th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany,

Meyer A., Brill M.F., Rössler W., Nawrot M.P. (2013) Linking physiology and morphology in two types of honeybee projection neurons. 10th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany,

Brill M.F., Meyer A., Nawrot M.P., Rössler W. (2012) Parallel processing within a dual olfactory pathway in the honeybee. Neuroscience Meeting, New Orleans, USA, 259.30

Brill M.F., Meyer A., Nawrot M.P., Rössler W. (2012) Parallel processing within the bee's dual olfactory pathway. 105th Annual Meeting of the German Zoological Society (DZG), Konstanz, Germany. 137

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Brill M.F., Reus I., Rosenbaum T., Nawrot M.P., Rössler W. (2012) Parallel odor processing in the honeybee favors synaptic coincidence coding. XVI International Symposium on Olfaction and Taste (ISOT), Stockholm, Sweden, 7

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Rosenbaum T., Brill M.F., Rössler W., Nawrot M.P. (2011) Do antennal lobe output neurons employ a latency code? 9th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany, T19-30A

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Goldberg E., Bürklein M., Brill M. and Mlynski R. (2008) Laser-Doppler-Vibrometrische Untersuchungen am BAHA-Knochenanker im Felsenbeinmodell. 79th Annual Meeting of the German Society of Oto-Rhino-Laryngology, Head and Neck Surgery, Bonn, Germany, 08hno49

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*Brill M.F. and Stumpner A. (2007) Directional Processing of crickets differs from that in bushcrickets. 7th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany, T18

* Presentations during my Diploma thesis

** non-scientific presentations

XII - Affidavit

I hereby confirm that my thesis entitled "Processing and plasticity within the dual olfactory pathway in the honeybee brain" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor similar form.

Würzburg, 3.01.2013
Place, Date

Martin Brill
Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Verarbeitung und Plastizität des dualen olfaktorischen Wegs im Gehirn der Honigbiene" eigenständig, d.h. insbesondere selbstatändig ohne Hilfe eines kommerziellen Promotionsberaters angefertigt und keine anderen als die vo mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 3.01.2013
Ort, Datum

Martin Brill
Unterschrift

XIII - Acknowledgment

Für die letzten 6 Jahre meines Lebensabschnittes in Würzburg würde ich mich gerne bei den Menschen bedanken, die mich auf den Weg meiner Dissertation unterstützt und begleitet haben:

Meinem **Supervisorcommittee** bestehend aus **Prof. Dr. Wolfgang Rössler, Dr. habil. Christoph Kleineidam** und **Prof. Dr. Peter Kloppenburg** möchte ich für die langjährige Betreuung und den damit verbundenen regen wissenschaftlichen Austausch bedanken. Sie haben mir auf vielerlei Weise gezeigt, kleine Insekten und ihre famose sensorische und teils soziale Leistungsfähigkeit lieben zu lernen.

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Christoph hat mich mit seiner wissenschaftlichen Arbeitsweise beeinflusst. Ohne Christoph hätte ich wahrscheinlich nicht begonnen, z.B. das Projekt mit LabView gesteuerter Software zu realisieren. Sein Ideenreichtum verschiedene Fragestellungen praktisch anzugehen, sind eine wahre Inspiration. Nebenbei ist Christioph aber auch ein guter Visionär und Gespräche - meist mit Gerstensaft bei Hand - sind immer wieder ein Quell neuer Ideen.

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Exkursionen, Lebensweisheiten und etlichen Besuchen der Würzburger Wein- und Mittelfränkischen Bierkultur sondern auch durch das tolle Arbeitsklima und Geselligkeit den Bayerischen Freistaat kennen und ein bisschen lieben gelernt ;) Es war eine tolle Zeit mit (vorwiegend) Höhen und (ganz wenigen) Tiefen – wie in einer richtigen Familie.

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Eins hab ich leider während meiner Dissertation nur wenig gelernt: *mich kurz zu fassen* … ;)