1 Introduction

1.1 Neuronal representations

A fundamental function of nervous systems is processing of information of the environment of an animal. Environment is regarded here as the external world and internal milieu of an organism. Information about the environment is neuronally encoded in internal representations. Encoding is accomplished in such a way, that information is represented distinct, while at the same time representing the structural relationship between the information. A neural code is a system of rules and mechanisms by which a signal carries information. However, the mere fact that a signal carries information does not turn the signal into a neural representation. The signal has to be read out by a target, e.g. neurons, muscles, or other effector organs. Therefore, a neural representation is not just a mirror-image of the environment. Neural representations have always a content and a function (deCharms and Zador, 2000). With other words internal representations were described as structured versions of the world, which can potentially guide behaviour (Dudai, 1989). The latter argument is important, as it separates epiphenomenal neural signals occurring in correlation with sensory stimulation, from those signals which are read by the system and are therefore relevant.

Coding, on the one hand, can involve many different brain structures, on the other hand, the code can be different at different levels of the brain. In part, this always depends on the level of the perspective taken, single cells, micro-circuits, brain areas, or the whole nervous system. At any level, the representation is encoded following level specific rules (syntax), which produces a meaning (semantic). The semantics at one level might then be only syntactic at another level. This is important to note because examining nervous systems, often requires dissection of complex phenomena into their elementary components. When pursuing such a reductionistic approach, the interpretation of the results should be clearly discussed in respect to the analyzed level (which will be a critical argument in the discussion section below). In the end,

however, it is always the whole organism which is represented in the whole nervous system.

1.2 Simple and central neural sensory representations

As representation is an elemental function of all nervous systems, internal representations vary in their complexity. Generally, internal representations are encoded in spatial and temporal parameters of neuronal activity. Neuronal representations are often distinguished into simple and complex representations. Important characteristics of complex central sensory representations are parallel processing of different stimulus qualities like form, color, motion in vision (Vanessen and Gallant, 1994); serial hierarchical processing which synthesizes the information, for example the orientation and direction of motion of visual stimuli (Hubel and Wiesel, 1962); reciprocal multistage integration to produce coherent information; topographically or computationally mapped sensory space (Merzenich et al., 1987).
Complex central representations have been well described for somatosensory (Pons et al., 1987; Welker and Van der, 1986) electro receptive (Heiligenberg and Bastian, 1984; DeYoe and Van Essen, 1988) visual (Hubel and Livingstone, 1987; Zeki and Shipp, 1988) and auditory systems (Konishi, 2003).
In contrast to the complex central representation, simplicity in representations is defined by identified neurons to which the representation of, for example a sensory-to-motor neuron path, can be traced to. Those neurons are dedicated and committed to deal with a particular type of internal representation as is the case for example in the gill withdrawal reflex in Aplysia. All significant neurons of the circuit have been located in the abdominal ganglion; 24 central mechanosensory receptor cells innervate the skin and synapse onto 6 gill motor cells; and a group of excitatory and inhibitory interneurons (Byrne et al., 1974; Byrne et al., 1978; Castellucci et al., 1970; Hawkins et al., 1981).

1.3 Plasticity in internal representations

Modifications of neural representations have been described in great detail in different animals and at different levels. Many studies demonstrate plasticity during rigid developmental programs, like activity dependent establishment of ocular dominance columns in the visual cortex (Hubel et al., 1977). Plasticity due to lesions or injury are also well documented, like for example neocortical reorganizations following stroke (Jenkins and Merzenich, 1987).

The environment of an organism is dynamic and survival depends on adaptive behavior. Neural plasticity has been identified to underlie behavioural adaptation and learning can be considered as experience dependent generation or modification of enduring internal representations (Dudai, 1992). There are a variety of examples of experience dependent plasticity of neuronal representations at the cortical level of vertebrates. Topographic representations of the hands in the cortex of owl monkeys have been shown to alter following a discrimination task (Recanzone et al., 1992). Modulation of cortical motor output maps during development of declarative knowledge of a motor task was reported in humans (Pascual-Leone et al., 1994). The neural code for stimulus magnitude in primary auditory cortex is shaped by associative learning (Polley et al., 2004).

1.4 An enigmatic example of changes in neural representation: olfactory learning?

Changes of central presentations in distance from the primary percept as well as “isolated” reflex circuits devoted to one function are conceptually unproblematic. However, in complex sensory systems, for example, learning should not alter the encoding of neural representations in such a way, that the reliable distinctiveness and the structural relationship between representations are disrupted. If one brain area conveys a particular type of information to another downstream target area, then the information of the second area is represented in some fashion in the first area as well.

However, if the representation upstream is changed, how can the reliable representation be maintained downstream? This is particular of importance if complex structural relationships between different representations are necessary. On the one hand, this is required for accurate and reliable perception of complex stimuli, which requires the integration of a variety of sensory cues. On the other hand, contextual and configural learning, for example, depend on the selective combinations of different stimulus features, which therefore need to be reliably represented in different circuits (Menzel, 2003).

In this respect olfactory learning provides us with a conundrum. Behavioural studies have convincingly demonstrated that vertebrates as well as many invertebrates can be conditioned to olfactory stimuli (Davis, 2004; Wilson and Stevenson, 2003). The structural and functional neural plasticity underlying the behavioural adaptations due to learning appears to be dispersed between different areas of the brain (Hammer and Menzel, 1998a; Gluck and Granger, 1993; Tully et al., 1994). Amongst others, neural plasticity due to olfactory learning has been found already at the level of the first processing station in the olfactory pathway, in the vertebrate olfactory bulb (OB) (Brennan et al., 1998; Sullivan and Wilson, 1995; Woo et al., 1987) and the insect antennal lobe (AL) (Daly et al., 2004; Faber et al., 1999b; Yu et al., 2004).

Changes at the first odor processing level are obviously affecting the neural representation of an odor. However, if the neural representation of an odor is changing, how can the animal learn to respond to a particular odor? Moreover, as stated above, behavioural phenomena like contextual, structural and configural learning require a stable representation of stimulus features (for example that of an odor) in different circuits that are selectively and specifically combined (Deisig et al., 2001; Gerber and Menzel, 2000; Giurfa and Menzel, 2003; Sandoz and Menzel, 2001).

In the following thesis I will investigate the issue of stable odor coding in the honeybee AL. The comprehension of the possible neurobiological changes which occur in learning requires understanding the codes of neuronal representations, the subserving neuronal circuit and the resulting behaviour. Therefore, before I introduce

the experimental methods, describe and discuss the results, I will provide a brief summary of olfactory coding in the AL and the OB in general, followed by a review of the olfactory system, and olfactory learning in the experimental animal, the honeybee *Apis mellifera*.

1.5 Olfactory coding

Olfactory information is processed in the vertebrate OB and its insect analogue, the AL (Strausfeld and Hildebrand, 1999). Both are subdivided into functional processing units, the olfactory glomeruli. Odors are believed to be represented in specific spatio-temporal combinatorial patterns of activated glomeruli in vertebrate OBs (Astic and Cattarelli, 1982; Cinelli and Kauer, 1992; Friedrich and Korsching, 1997; Hildebrand and Shepherd, 1997; Johnson et al., 1998; Meister and Bonhoeffer, 2001; Rubin and Katz, 2001; Sharp et al., 1975; Spors and Grinvald, 2002; Wachowiak et al., 2004) and invertebrate ALs (Fiala et al., 2002; Galizia et al., 1999b; Joerges et al., 1997; Ng et al., 2002; Sachse et al., 1999) A particular glomerulus receives afferent input by a specific olfactory sensory neuron type (OSN). All or the great majority of OSNs that express the same receptor also converge onto the same glomerulus (Gao et al., 2000; Mombaerts et al., 1996; Ressler et al., 1994; Vosshall et al., 1999). Local neurons within the bulb or the AL modify the incoming activity (Malun, 1991; Mori et al., 1999; Sachse and Galizia, 2002b; Yokoi et al., 1995), and the processed information is relayed to higher order brain centers by vertebrate mitral/tufted (M/T) cells or the insect projection neurons (PNs). M/T cells project to the olfactory cortex including the piriform cortex, the olfactory tubercle, the anterior olfactory nucleus, and parts of the amygdala and entorhinal cortex (Shipley and Ennis, 1996; Zou et al., 2001). Insect PNs transmit the processed information to higher order olfactory neuropiles, the mushroom bodies (MB) and the lateral protocerebrum (LP) (Abel et al., 2001; Marin et al., 2002; Mobbs, 1982; Müller et al., 2002; Tanaka et al., 2004; Wong et al., 2002).

1.6 The olfactory system of the honeybee

1.6.1 Overview

The ALs are the primary olfactory neuropile in insects (Mobbs, 1985). The AL consists of spherical structures of neurites and synapses, ensheathed in glial cells, the olfactory glomeruli. In the honeybee, there are between 156-166 glomeruli (Arnold et al., 1985; Flanagan and Mercer, 1989). By means of their characteristic shape, size and positions, individual glomeruli can be compared among animals with the help of a morphological atlas (Flanagan and Mercer, 1989; Galizia et al., 1999a). Olfactory input is detected by the ORNs, transmitted to the AL, locally processed by LNs, and the output is relayed by the PNs.

1.6.2 Olfactory sensory neurons (OSN)

On each antenna of the honeybee there are 60.000 OSNs, which project to the ipsilateral AL (Esslen and Kaissling, 1976). 96% of all antennal sensory neurons are olfactory and the remaining 4% are thermo-, hygro- and mechanosensory receptors (Lacher V, 1994). All antennal sensory neurons project into the head capsule, where the antennal nerve splits into six branches. Four tracts (T1 to T4) innervate the AL, while two (T5 and T6) project to the dorsal lobe, which most probably does not receive olfactory input (Suzuki, 1975). T1 and T3 each innervate between 70 and 80 glomeruli, and T2 and T4 each innervate 7 glomeruli (Arnold et al., 1985; Flanagan and Mercer, 1989; Galizia et al., 1999a). Glomeruli can be divided into the outer cap (also referred to as their cortex) and the inner core region. Only the cap region of each glomerulus is innervated by the OSNs, with the exception of T4 OSNs (Mobbs, 1982). Acetylcholine is the putative transmitter of the OSNs (Kreissl and Bicker, 1989; Scheidler et al., 1990). Each ORN only branches in one glomerulus (Brockmann and Brückner, 1995), which gives rise to convergent input of about 400 ORNs.

1.6.3 Local interneurons (LN)

There are about 3750 LNs, which branch only within the AL (Witthöft, 1967). GABA immunoreactivity has been reported in about 750 LNs, which suggests them to be inhibitory (Schäfer and Bicker, 1986). The transmitter used by the remaining non-GABAergic LNs remains unknown. LNs can be devided into heterogeneous, homogenous and a third subpopulation of LNs (Abel et al., 2001a; Fonta et al., 1993; Sun et al., 1993). The majority of LNs are heterogeneous and branch densely in only one glomerulus. Homogeneous LNs branch diffusely in large areas of the AL (60-100 glomeruli). The third subpopulation of LNs branch diffusely in the AL and the dorsal lobe.
Furthermore, multiglomerular interneurons connecting the two ALs have also been reported (Arnold et al., 1985; Fonta et al., 1993; Mobbs, 1985).

1.6.4 Projection neurons (PNs)

PNs are the output neurons of the AL. They transmit the information from the AL to the lateral protocerebrum (LP) and to the mushroom bodies (MB). 500 – 1000 PNs (Bicker, 1993; Hammer, 1997; Rybak, 1994). PNs project by means of five tracts, the lateral, the median, and three mediolateral antennocerebral tracts (l-, m-, and ml-ACT, respectively) to the MBs and the lateral protocerebrum (Abel et al., 2001a).
The PNs running in the lACT are generally uniglomerular (Abel et al., 2001a; Müller and Berg, 2001). Somata of the lACT PNs are found in the anteroventral, dorsoventral and lateral part of the AL (Abel et al., 2001b; Schäfer et al., 1988). LACT PNs receive input from the T1 glomeruli (Bicker et al., 1993). PNs of the mACT are also generally uniglomerular, but innervate the glomeruli of the T2, T3 or T4 group (Bicker et al., 1993). Their somata are reported in the ventro-lateral and dorsal soma clusters of the AL (Bicker et al., 1993). PNs of mlACT are multiglomerular, with branching in many glomeruli (Abel et al., 2001a; Fonta et al., 1993). The m-ACT connects the MB and the lateral protocerebrum (LP) with the AL. Similarly, axons of the lACT innervate the LP and MB but in a reverse sequence: first

the LP and then the lip region of the MB calyces. The mlACT does not project to the MB, but innervates the LP and the neuropil around the α-lobe. In conclusion, the MB receives input only from uniglomerular PNs, but the LP from both uniglomerular and multiglomerular PNs.

Besides the anatomical differences between l- and mACT PNs, their distinct response characteristics indicate important functional differences between the PNs of the two tracts. Whereas lACT neurons respond to many odors with excitation, mACT neuron responses are complex with phases of excitation and inhibition (Müller et al., 2002b).

1.7 Olfactory learning and memory in the honeybee

Sucrose stimulation of antennal or proboscis chemoreceptors of a hungry bee leads to the proboscis extension reflex (PER)(Bitterman et al., 1983; Kuwabara, 1957). In the olfactory learning paradigm (PER conditioning), an odor, the conditioned stimulus (CS), is paired with a subsequent sucrose reward as the unconditioned stimulus (US). The animals form an association between the two, so that an odor stimulation alone elicits PER (conditioned response) previously elicited only by the US. Differential conditioning is also possible, thus bees can learn in addition that a different odor is no predictor for reward. This effect is clearly associative and involves classical, but not operant, conditioning (Bitterman et al., 1983). PER conditioning shows most of the basic characteristics of classical conditioning: among others, acquisition and extinction, differential conditioning and reversal learning, stimulus specificity and generalisation, dependence on odor as well as on reinforcement intensity, dependence on the temporal interval between stimulus and reinforcement, and dependence on the temporal interval between learning trials (Menzel, 1999). The number of conditioning trials applied to the honeybee induces different memories, which exhibit different properties. The memory induced by a single conditioning trial decays over several days and is sensitive to amnestic treatments. This memory is independent of translation and transcription. In contrast, multiple conditioning trials induce a stable, long-lasting memory depending on translation and transcription (Menzel, 1999).

1.8 Representation of the unconditioned stimulus

An identified neuron, VUMmx1 has been shown to mediate the unconditioned stimulus, the sucrose reward (Hammer, 1993). Depolarisation of the VUMmx1 neuron shortly after the CS presentation can substitute the sucrose US. The VUMmx1 neuron arborizes in the ALs, MBs and the LPs, neuropiles involved in processing of olfactory information. Thus, the reward pathway converges with the olfactory pathway in those neuropiles. Octopamine is the putative transmitter of VUMmx1 (Kreissl et al., 1994), suggesting an important role of octopamine in processing of the US. This function has been demonstrated, since pairing of an odor with subsequent local octopamine injections into either the ALs or the MBs induce an olfactory memory (Hammer and Menzel, 1998b).

1.9 Rationale of this study

The intent of this study was to test the hypothesis of learning induced modified neuronal odor representation at the level of the AL. To this end, I combined a variety of appetitive olfactory conditioning paradigms with *in vivo* calcium imaging of the output of the AL (Sachse and Galizia, 2002). I focussed on one subpopulation of the olfactory PNs, the lACT PNs. I measured calcium signals in the uniglomerular lACT in the AL before, during and following associative olfactory learning. I attempted to integrate the knowledge about the different effects of the various conditioning protocols in the experimental design.

Recent data of a calcium imaging study has been interpreted as modification of the odor representation in the PNs due to differential conditioning (Weidert, 2003). However, the design of this experiment did not allow for an odor and glomerulus specific analysis, both of which is necessary to reveal the logic behind the modification. In accordance with this study, I compared odor responses during a pre-test phase and a test-phase beginning 5 minutes following conditioning. However, throughout the whole study I always reinforced the same odor. Moreover, I did not

randomize odor combinations to allow the interpretation of possible learning effects in the background of a controlled odor experience. I was particularly concerned to control for effects of successive odor stimulation and compared the conditioning paradigms with unrewarded control groups.

In a parallel approach I followed the argument that if the odor responses were indeed changing, this should be somehow related to intracellular signalling cascades involved in learning and memory. Three trial forward pairing has been shown to induce long term memory whereas a single forward and three trial backward pairing does not (Müller, 2000). A single odor/sucrose forward pairing leads to a transient increase in PKA activity in the antennal lobes that returns to basal levels 60 seconds after the conditioning trial. This transient elevation of PKA activity is drastically prolonged after the third odor/sucrose forward pairing, which induces long term memory (Menzel, 1990; Muller, 2000). In contrast, three backward sucrose odor pairings induce the same temporal PKA activation as a single US stimulation. In behavioral experiments multiple-trial backward conditioning resulted in conditioned inhibition, which is maximal for a 15-sec interval between US and CS (Hellstern et al., 1998). However, my main concern was to relate possible effects to the second messenger cascades. Thus, in accordance with these studies I investigated one and multiple forward, and respectively backward pairings, of an odor with sucrose. Following the temporal dynamics of the intracellular signalling cascades, I tested the odor responses during the period of prolonged PKA activity and at later points in time after conditioning.