1 General Introduction

Learning can be defined as changing the potential of a behavior to occur in response to individual experience (Thompson, 1986). Animals need to learn which environmental stimuli predict biological meaningful aversive or appetitive, reinforcing stimuli. The nervous system must store representations of the value of stimuli and must be able to retrieve these representations to regulate adaptive behavior (e.g. approach, retreat) (Hammer, 1997). Thus, memories are internal representations, which are neuronally encoded models of the world that could guide behavior (Dudai, 2002).

A memory is retrieved to make predictions of the immediate future. A mismatch between what is expected and what is experienced mostly leads to new learning. Therefore, memories need to be stable to be maintained but at the same time they need to be plastic to update information of a changing environment. How this is achieved is not well understood.

An objective of this thesis was therefore to investigate the role of retrieval in memory formation. For this approach an olfactory learning paradigm in honeybees was used. First I will introduce the general concepts of learning, retrieval and the processes which are thought to be induced by retrieving a memory. Subsequently the honeybee as a model system will be briefly presented and finally the main questions are introduced.

Classical Conditioning

To investigate learning and memory processes and their neuronal substrate several paradigms in several model systems from invertebrates to vertebrates were developed. One of the simplest forms of learning is classical conditioning.

In the late 19th century the Russian physiologist Pavlov discovered that environmental events that previous had no relation to a given reflex could come through experience to trigger the reflex. Animals possess a number of reflexes which are innate and do not dependent upon experience; Pavlov called them the unconditioned reflexes. A biological important stimulus triggers a reflex, for example food in the mouth triggers salvation. The triggering stimulus is referred to as unconditioned stimulus (US). Pavlov termed the reflex response itself, in this case salvation, the unconditioned response (UR). If an a

priori meaningless or neutral stimulus is contingently paired with the appearance of the US the animal becomes conditioned to it. Hence a neutral stimulus turns into a conditioned stimulus (CS). In anticipation of the US the animal displays a response which mostly, but not always, resembles the UR. This response is then termed the conditioned response (CR). It is thought that an association of the CS-US is formed. Pavlov termed the formation of a CS-US association as excitatory conditioning, independent of the nature of the US (e.g. appetitive or aversive) the CS comes to excite a CR. (Pavlov, 1927). One temporal pairing of CS and US is often referred to as one trial.

The strength or intensity of a CR increases steadily from trial to trial until an asymptotic level is reached. The asymptotic level is influenced among others factors by the interval between the CS and US (inter-stimulus interval), CS intensity, US intensity and salience of the stimuli (Rescorla & Wagner, 1972). It is shown that a so called delayed conditioning is most effective, here CS onset precedes US onset. The termination of the CS occurs with or during US presentation. It is supposed that animals have a predisposition to associate certain stimuli to a specific US (Seligmann, 1970). The likelihood that a particular neutral stimulus will elicit the CR after conditioning reflects the salience of a neutral stimulus. The salience of a stimulus is species-dependent.

Consolidation

Consolidation refers to a post-acquisition stabilization process of a memory (Dudai, 2002). Freshly acquired associations are sensitive to interference shortly after learning and become resistant to it over time (Ebbinghaus, 1885). Pilzecker & Müller suggested that this sensitive time interval reflects the process during which associations are stabilized or 'consolidated' into memory (Müller & Pilzecker, 1900). In support of this theory many studies in neuroscience research reported that amnesic treatment shortly after acquisition leads to amnesia, but do not disturb memory formation if applied later (McGaugh, 1966). The time window of susceptibility depends on the paradigm and the type of interference (Dudai, 2004).

Reports that synthesis of RNA and proteins in the brain are necessary for the formation of a long-term memory (LTM) (Agranoff & KLINGER, 1964) influenced many researchers to investigate the effect of protein-synthesis inhibitors on LTM in different behavioral paradigms. The general conclusion was that protein-synthesis during or

shortly after training is necessary for consolidation of LTM, but not for short-term memory (STM) (Davis & Squire, 1984). Since then STM and LTM were dissected by their susceptibility to consolidation blockers. Furthermore, the term consolidation is now predominantly used to refer to the time interval during which formation of LTM can be blocked by inhibitors of protein or RNA synthesis.

How is consolidation thought to be implemented on the neuronal level? Already in 1894 Cajal proposed that memories might be formed by strengthening the connections between existing neurons in order to communicate with each other more effectively (Squire & Kandel, 2000).The famous Hebb synapse is a widely used model for learning-related but also developmental plasticity. According to Hebb, the efficacy of a synapse will change due to the coincident activity of two neurons (Hebb, 1949).The synaptic changes are probably due to transient covalent changes on proteins in the short term range (Goelet et al., 1986). For a LTM the integration of new proteins into synapses in order to modify their properties is supposed. It is presumed that extracellular signals initiate intracellular signal cascades which subsequently can activate gene expression (Goelet et al., 1986). In cell cultures of *Aplysia* neurons it could be shown that long-term facilitation, which is thought to reflect LTM, depends on protein synthesis and is associated with the growth of new synaptic connections (Bailey & Kandel, 1993).

Retrieving a Memory

Memory can only be measured indirectly through behavior. To measure memory retention the CS is presented without the US (CS-only trial) and the latency, probability or strength of the CR are recorded. The presentation of the CS is also termed retrieval trial. Retrieval is the use of memory in neural and behavioral operations ((John, 1967) in (Dudai, 1992)). It should be emphasized that the absence of the CR does not necessarily mean the absence of a memory. Critical is also the motivation of the animal to react to the CS, which depends on the internal state of the animal. However, retrieval is a dynamic process and can in turn render the memory and/or initiate new learning.

Extinction

If, after an association has been formed, a CS is repeatedly presented without US, the probability or intensity of occurrence of the CR decreases (Pavlov, 1927). It is widely accepted that extinction represents a new learning process about the CS-noUS

association. That extinction does not destroy the excitatory CS-US association, but is new learning instead, is suggested by several behavioral phenomena like renewal, reinstatement, rapid reacquisition and spontaneous recovery (Myers & Davis, 2002; Bouton, 2004). Renewal is the reappearance of an extinguished CR in a new context. This is probably the strongest argument that the CS-US association can not be destroyed by extinction. The presentation of the US alone results in a partially recovery of the extinguished CR, this phenomenon is termed reinstatement (Rescorla & Heth, 1975). Furthermore, in rapid reacquisition the animal is subjected to a further CS-US pairing following extinction. Often the development of a CR is more rapid than in the original acquisition. The last phenomenon, spontaneous recovery of extinction refers to the return of the CR in time without additional training or presentations. Again it was Pavlov (1927) who first observed this phenomenon. He proposed that the CS-US association is temporarily suppressed by an 'internal inhibition' (Pavlov, 1927).

Recently it has been shown that extinction is a protein synthesis-dependent process. This supports the hypothesis that CS-only trials induce new learning about the CS-noUS association (Pedreira & Maldonado, 2003; Berman & Dudai, 2001; Sangha et al., 2003b).

Reconsolidation

The term reconsolidation was first used by Spear (1973) and describes processes that are induced after retrieval of a memory (Spear, 1973). According to the reconsolidation hypothesis retrieval returns the memory to a labile, sensitive state similar to the one after acquisition. The memory can then be modified, changed or even erased (Nader, 2003). This is in contrast to the consolidation theory, where a once consolidated memory is thought to remain in a stable state that is insensitive to amnestic treatments.

Misanin et al. (1968) reported that retrieving a consolidated memory in conjunction with electroconvulsive shock (ECS) results in amnesia for the original learning (Misanin et al., 1968). If the retrieval trail was omitted the ECS did not affect the memory and no amnesia was induced. The authors speculated that the memory must be 'evoked' to be blockable by ECS and presumably also other amnesic agents. Therefore, they distinguished between active and inactive memories. Learning always occurs in the presence of specific stimuli and in a contextual environment. If these stimuli or the context occurs again the memory will be reactivated. Thus, during original learning and retrieval memories are active or reactivated and therefore susceptible to disruption.

Subsequently, the results were confirmed by many studies (Sara, 2000), but contrary, negative findings have also been obtained (Dawson & McGaugh, 1969; Squire et al., 1976) in (Cahill et al., 2001). The topic got into the focus again by a publication of Nader et al. (Nader et al., 2000),who reported that injection of anisomycin, a translation inhibitor, into the lateral and basal nuclei of the amygdala of a rat results in loss of the acquisition memory in cued fear conditioning.

Why should a memory become instable again with the risk of being lost? It is thought that reconsolidation is necessary to maintain and/or up-date the memory (Nader, 2003). Interestingly, one explanation for the decay of memory is that the non-use of memory leads to forgetting. (McGeoch, 1932)

Classical Conditioning in Honeybees

Honeybees are well-known for the impressive repertoire of behavior they can perform. During foraging bees easily learn to associate odor, color and shape of the visited flowers with the reward they receive (Menzel & Müller, 1996; Menzel, 1990). Even more abstract features like symmetry of patterns can be learned (Giurfa et al., 1996). To analyze the neural mechanisms of these behavior patterns requires an experimental approach, which reduces the number of variables, but preserve characteristics of the behavior. With restrained bees features of reward learning can be studied in laboratories. Hungry honeybees extend their proboscis reflexively if the antennae or the proboscis are stimulated with sucrose. This reflex-like behavior is used in the proboscis-extension-response (PER) paradigm. During conditioning honeybees learn to associate an odor (CS) with a sucrose reward (US) (Kuwabara, 1957b; Menzel et al., 1974), Bitterman et al., 1983).

This paradigm is extensively used to investigate the neuronal and molecular basis of learning and memory formation. Delayed conditioning, where the CS precedes the US and both stimuli overlap, leads after few trials to an asymptotic increase in response probability. If CS and US are not paired no conditioning of the CR is observed, instead it results in inhibitory learning (Hellstern et al., 1998). In this case the CS is associated with the absence of the US. PER conditioning shows most of the basic features of classical conditioning in vertebrates, e.g. extinction, differential conditioning, second-order conditioning (Bitterman et al., 1983).

At the cellular level, stimulus association is reflected in the convergence of excitation of the CS and US pathways (Hammer, 1995). Three convergence sites of these pathways are known for olfactory learning: first, at the primary olfactory neuropil, the antennal lobes; second, the secondary olfactory integration areas, the lip region and the basal ring of the mushroom bodies; and third, on the output region of the brain, the lateral procerebrum lobes. These sites are innervated by a neuron called VUMmx1 (ventral unpaired median neuron) that plays a central role in US processing (Hammer, 1993).

Memory phases in honeybees

Reward learning in the bee initiates multiple memories, which are sequential and parallel processed (Menzel, 2001). One CS-US pairing results in an early form of short-term memory (eSTM) in the seconds range. This memory is highly dominated by appetitive arousal and sensitization induced by the US and therefore quite unspecific (Menzel, 1990). The formed association needs minutes to consolidate and to become specific. Retention of this memory reaches its maximum one hour after acquisition and decreases over time to a level of ~20% (Menzel, 1999). This memory is independent of translation and transcription (Grünbaum & Müller, 1998; Friedrich et al., 2004).

Three CS-US pairings lead to a robust memory. According to the susceptibility to inhibitors three forms of memory are differentiated: a mid-term memory (MTM), an early long-term memory (eLTM) and a late long-term memory (lLTM) (Menzel, 1999).

The behavior from 1 to 6 hours after acquisition is controlled by a highly specific MTM. It is physiologically characterized by a first wave of protein kinase C (PKC activity. This protease-dependent PKC activity was measured in the antennal lobe after olfactory PER conditioning. LTM does not depend on MTM, since the inhibition of PKC activity does not inhibit the formation of LTM (Grünbaum & Müller, 1998).

The eLTM which lasts 1-2 days depends on protein kinase A (PKA) and the inhibition of PKA by antisense oligonucleotids leads to an impaired memory retention one day after acquisition (Fiala et al., 1999). In addition, three conditioning trials lead to a prolonged activation of PKA shortly after acquisition. If the PKA activity is artificially increased a single pairing of the CS-US results in LTM. Normally a single trial does not induce LTM (Müller, 2000). Potential targets of the PKA are the mitogen-activated protein kinases (MAPK). Recently, is has been shown that a subfamily of the MAPK, the extra-cellular regulated kinases (Erk 1/2), are involved in the formation of LTM.

Systemic inhibition of the phosphorylation of ERK 1/2 during acquisition results in an impaired retention 2 days after acquisition (Plekhanova, 2005).

The lLTM (> 2 days) is dependent on translation and transcription; this was demonstrated by the systemic application of anisomycin, a translation inhibitor, and actinomycin D, a transcription inhibitor (Wüstenberg et al., 1998; Menzel et al., 2001). If the consolidation process is disturbed by these inhibitors an impaired retention memory is observable 3 and 4 days after acquisition. Important to notice is that 24 h after acquisition the memory is no longer susceptible to protein-synthesis inhibitors (Wüstenberg et al., 1998). Recently, it has been reported that the application of another translation inhibitor emetine leads to an impaired memory already one day after acquisition, which was prolonged over the next days (Friedrich et al., 2004).

Objectives

In this thesis I performed behavioral experiments with restrained bees to analyze what kind of processes are induced by retrieving a LTM. A simple form of learning, classical conditioning, was used in order to reduce the number of parameters. The above described phenomena of extinction and reconsolidation were known to depend upon protein-synthesis. On account of this I used two translation inhibitors, anisomycin and emetine, to interfere with post-retrieval induced consolidation processes, since inhibitors have been successfully used in the bee to disturb the consolidation of acquisition memory. Furthermore, anisomycin injected systemically inhibits the translation of proteins in the brain to 80% for 2 h (Wittstock, 1986).

As mentioned above the retrieval of a consolidated memory by the CS alone can induce two different processes: reconsolidation or extinction. The behavioral outcomes of these processes are contrary: no reduction in the CR after reconsolidation or a reduction of CR after extinction.

Reconsolidation is predominantly investigated in aversive paradigms (Sara, 2000a; Dudai & Eisenberg, 2004) and in addition, there are also reports of failure to demonstrate reconsolidation (Hernandez & Kelley, 2004; Cammarota et al., 2004) In Chapter I, I analyzed if the phenomenon of reconsolidation could also be observed in an appetitive paradigm in the bee. I studied different time windows to interfere with the reconsolidation process and started to look into the different action of the used inhibitors

In Chapter II the focus is on extinction. Until now extinction in honeybees is only investigated in STM (Bitterman et al., 1983; Grünewald, 1995; Sandoz & Pham-Delègue, 2004). Here I studied the induction of extinction processes in LTM and the dependency on the number of presentations of the unrewarded CS and consolidation of extinction memory.

Studies in medaka fish and rats (Eisenberg et al., 2003) have demonstrated that the strength of the acquisition memory is an important parameter for the induction of either reconsolidation or consolidation of extinction memory. In their studies the numbers of acquisition trials were changed in order to vary the strength of the memory. In bees one CS-US presentation is independent on protein-synthesis and three trials already induced a strong memory, which already reaches the asymptotic level in retention tests. Therefore, the length was varied to alter the strength of the acquisition memory. In Chapter III the influence of the US length during acquisition on the retrieval induced processes of extinction and reconsolidation was analyzed.