1 Introduction

1.1 Learning and neuronal plasticity

The ability to store and recall information is one of the most important capacities of higher organisms in a permanently changing world. Learning processes lead to changes in behavior which allow the animals to permanently adjust to the environment. The information obtained by learning is stored as memories over a long period of time and can be used for operating behavior. Learning and memory are based on changes in neurons and their connections. As the ion channels are modulated, diverse enzyme cascades are either activated or inactivated, and/or neuronal structure changed. This neuronal plasticity seems to exhibit functional homologies in vertebrates, as well as in invertebrates (Menzel, 1990; Menzel and Müller, 1996).

The ability to learn and maintain memory is developed in all animals, and the processes underlying learning and memory storage often run in vertebrates and invertebrates in a similar way. So-called learning consists of a learning process itself, which includes acquisition and, following, different phases of memory formation. Despite a big difference in a subject of learning, a general concept was developed, which allows the distinction between different forms of learning. Basically, associative and non-associative forms of learning are considered.

1.2 Non-associative and associative learning

Elementary forms of learning are divided into two general categories: non-associative and associative (Kandel and Schwartz, 1982).

Habituation and sensitization are forms of non-associative learning; they are two reciprocal processes. Habituation is a simple form of non-associative learning, where an animal learns through repeated presentations to ignore a weak stimulus, the consequences of which are neither noxious nor rewarding (Kandel and Schwartz, 1982). On a behavioral level it is expressed as a gradual decline in a behavior response resulting from repeated stimulation of a reflex pathway (Thompson and Spenser, 1966; Groves and Thompson, 1970).

Sensitization is an elementary form of non-associative learning in which an animal learns to strengthen its defensive reflexes and to respond vigorously to a variety of previously neutral or indifferent stimuli after it has been exposed to a potentially threatening or noxious stimulus. On the behavioral level, sensitization reflects an increase in reflex responsiveness that follows the presentation of a novel stimulus.

Associative learning includes classical and operant conditioning. For associative learning to take place, two stimuli or a stimulus and a response must be temporally associated. For example, in classical conditioning an initially indifferent weak or ineffective conditioned stimulus (CS) acquires novel behavioral significance only after it has been paired with a strong unconditioned stimulus (US), reward or shock. After an animal has been conditioned it behaves as if the CS predicts the US. Pavlov in 1927 performed experiments on dogs, in which he conditioned the salivating reflex. After exposure to a ringing bell (CS) the dogs were given a piece of meat (US). After several repetitions of this CS-US pairing, the animals started to salivate in response to the bell (Pavlov, 1927). Thus, in the classical conditioning animals learn about causal relationships in the environment. In contrast, non-associative learning does not require temporal pairing of stimuli and does not teach animal to expect any relationship between stimuli (Kandel and Schwartz, 1982).

Operant conditioning is another form of associative learning. It implies an active participation of an experimental animal, whereby animals learn to associate between their actions and meaningful stimulus. As well as in the classical conditioning, the stimulus can be either appetitive or aversive. By the execution of an action, the animal learns to precipitate or avoid the occurrence of the stimulus. There are some other, higher, forms of associative learning, as imprinting learning and insight. In both of these forms, the impulse comes not from the outside but is the internal actual state of organism, such as curiosity, sense of harmony or expectance. This is one of the reasons why such learning processes are hard accessible to the neuronal and cellular analysis.

Behavior obtained in the associative learning is saved in the memory. It should be concerned as a dynamic process in which the information is saved in diverse ways and under involvement of different neuronal structures. Dynamic of the memory can be split into at least two temporal phases: short-term memory (STM) and long-term memory (LTM).

The STM is built shortly after learning process and is characterized by its limited memory capacity and susceptance to failure against new events and amnestic influence. At the same time existing proteins and signal cascades were modified. The STM conveys into a late period, LTM, through the process of consolidation. The LTM is protein-dependent because consolidation of the LTM is based on reconstruction of existing and setting up the new synapses (Bailey and Kandel, 1993).

1.3 Models to study the processes of learning

Marine mollusk *Aplysia californica* has always been one of the favorite models for investigating synaptic plasticity, learning and memory. It offers three important advantages: its nervous system is made up of a quite small number of neurons (about 20 000, whereas the mammalian brain has a trillion central nerve cells); many of these are gigantic; and many are uniquely identifiable (Frazier et al., 1967; Kandel, 2001). The simplest behaviors of *Aplysia californica* that can be

modified by learning may directly involve less than 100 neurons. (Kandel, 2001). Synaptic facilitation of the sensory-motor (sensory neuron-motor neuron; SN-MN) synapses, a cellular model for sensitization in *Aplysia californica*, exists in at least three temporally and mechanically distinct phases (Sharma et al., 2003a). Sensitization of *Aplysia* gill and siphon withdrawal reflex is a simple form of learning (Bernier et al., 1982).

Drosophila melanogaster is another model object to investigate learning and memory processes. It allows easy breeding in the lab and because of its relatively small genome and short reproductive cycles, *Drosophila* became one of the favorite model animals for genetic experiments. Not only non-associative but also associative forms of learning can be observed in this species. In the experiments with courtship conditioning, the males learn to reduce their courtship behavior in relation to a recently mated female (McBride et al., 1999). Another well established example is that of olfactory avoidance learning. (Quinn et al., 1974). In this task, two consecutive odors are presented to the flies. The presentation of the first odor (CS+) is paired with multiple electric shocks (US). Flies learn to associate between the odor and the shock, which is tested in a T-labyrinth, where both the odors are presented and fly chooses, in which direction to move. Animals which learn the association significantly avoid the CS+ differently from the control group.

The developing of such learning paradigms made possible the identification of those gene products which are crucially involved in learning and memory processes. It was possible to show four different phases of the memory formation in *Drosophila*: the acquisition, or learning, short-term memory (STM), mid-term memory (MTM) and long-term memory (LTM) (Dubnau and Tully, 1998).

In rats and mice, the long-term, NMDA receptor-dependent potentiation at Schaffer collateral/comissural synapses in area CA1 of the hippocampus has been widely studied as a model for associative memory. The long-term potentiation (LTP), originally defined by Bliss and Lomo (1973), is a use-dependent increase in synaptic efficiency (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). The LTP can be viewed as a manifestation of two physiological processes: an increase

in synaptic strength and increased excitability in the postsynaptic neuron (Sweatt, 1999).

The expression of LTP can be temporally divided into two stages. Early LTP (eLTP), which lasts 60-90 min, is subserved by persistently activated protein kinases and does not require ongoing protein synthesis. A later stage, late LTP (lLTP), which lasts >3 h, is dependent on changes in gene expression and is sensitive to blockers of transcription and translation (Huang et al., 1996; Giovannini et al., 2001).

There is also a third phase of LTP, which is actually an initial stage of LTP, generally referred to as short-term potentiation (STP), which lasts 30-45 min and is independent of protein kinase activity for its induction and expression. It is a persistent form of NMDA receptor-dependent synaptic plasticity, induced by LTP-inducing tetanic stimulation and is a prelude to eLTP and lLTP (Roberson et al., 1996). The biochemical mechanisms for initial-LTP induction, maintaince, and expression are not well understood at this time (Sweatt, 1999). As core mechanisms of LTP the following protein kinase pathways are considered: PKA, PKC, CaMKII and MAPK cascades.

1.4 Molecular mechanisms of learning and memory

In both vertebrates and invertebrates, long-term memory differs from short-term memory in requiring protein synthesis during training (Dash at al, 1990).

One of the possible mechanisms of short-term memory is the regulation of transmitter release (Kandel, 2001). In short-term facilitation (LTF) in *Aplysia*, lasting from minutes to hours, a single tail shock causes a transient release of serotonin that leads to covalent modification of pre-existing proteins. Serotonin acts on a transmembrane serotonin receptor to activate the enzyme adenylyl cyclase (AC), which converts ATP to the second messenger cyclic AMP (cAMP). That leads to increased excitability and a broadening of the action potential by reducing specific K^+ currents, allowing greater Ca^{2+} influx into the presynaptic terminal with each action potential (Klein and Kandel, 1980). The greater Ca^{2+} influx in turn

could contribute to the enhanced transmitter release. cAMP produces its action in the brain through the cAMP-dependent protein kinase (PKA), by activating it (Castellucci et al., 1980) and thus, causing phosphorylation of the K^+ channel by activating PKA (Klein and Kandel, 1980). Castellucci with colleagues demonstrated that the active catalitic subunit of PKA by itself produced broadening of the action potential and enhancement of glutamate release (Castellucci et al., 1980). Thus, serotonin leads to an increase in presynaptic cAMP, which activates PKA and leads to synaptic strengthening through enhanced transmitter release produced by a combination of mechanisms (Kandel, 2001).

Long-term facilitation in *Aplysia* leads to the modification of gene expression. In the *Aplysia* LTF, repeated stimulations (5x 5-HT or 5x tail shocks) causes the level of cAMP to rise and persist for several minutes. The repeated stimulations activate PKA, which in turn recruits MAPK p44/42. Both translocate to the nucleus where they activate a transcriptional cascade beginning with the transcriptional factor CREB-1 (cAMP response element binding protein-1) so called because it binds to a cAMP response element (CRE) in the promoters of target genes (Dash et al., 1990). Bartsch and colleagues cloned *Aplysia* CREB-1a and showed that injection of the phosphorylated form of this transcription factor by itself could initiate the long-term memory process (Bartsch et al., 1998). Alberini et al. (1994) and Bartsch et al. (2000) found in *Aplysia* two additional positive transcription regulators, the CAAT box enhancer binding protein (ApC/EBP) and activation factor (Ap/AF). CREB-1 activates this set of immediate response genes, which in turn act on downstream genes (including elongation factor 1α, EF1α) to give rise to the growth of new synaptic connections (Dash et al., 1990; Schacher et al., 1988; Kaang et al., 1993; Bailey and Kandel, 1993; Martin et al., 1997), which converts the short-term to long-term processes. Bailey and Chen (1988) showed that the growth of new synaptic connections may represent the mechanism underlying the LTM.

It has also been shown that the LTF requires not only activation of memory-enhancer genes, but also inactivation of memory-suppressor genes (Bartsch et al., 1995; Michael et al., 1998). One of these, the transcription factor ApCREB-2,

can repress ApCREB-1a mediated transcription. Relieving this repression lowers the threshold for the long-term process.

In contrast to the activators ApC/EBP and CREB-1, the transcription factor CREB-2 appears to act as a repressor of LTF. While CREB-2 lacks PKA phosphorylation sites, it can be phosphorylated *in vitro* by apMAPK p38 (Michael et al., 1998). Thus, MAPK p38 may also contribute to LTF by phosphorylating CREB-2 and thereby relieve its ability to inhibit the long-term processes. Guan and team (2003) demonstrated that the apMAPK p38 facilitates long-term depression in *Aplysia*. ApMAPK p38 mediates also the short-term depression, via activating of phospholipase A_2. They also found that inhibitory neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFa) activated the MAPK p38 and 5-HT inhibited the MAPK p38, in contrast to the MAPK p44/42, which is activated by both 5-HT and FMRFa (Michael et al., 1998; Guan at al., 2003).

In *Drosophila melanogaster*, the early evidence of the role of the second messenger cascades in learning and memory came from two mutants: *dunce* and *rutabaga*. In these two mutations, the cAMP-metabolism is defected, so that the STM formation is affected (Tempel et al., 1983; Levin et al., 1992). Another mutant *amnesiac*, with a defect in a neuropeptide gene, has a selective decrease of the MTM several hours after training (Quinn et al., 1979; Feany and Quinn, 1995). The transgenic flies with the reduction of PKC activity show no learning during courtship conditioning but they can develop normal memory (Kane et al., 1997).

The induction phase of LTP in the rat hippocampus is N-Methyl-D-Aspartate (NMDA) receptors-dependent. Only when the glutamate binds this receptor and the voltage-dependent magnesium blockade is simultaneously cancelled through the depolarization of postsynaptic membrane, the NMDA-channel is permeable for Ca^{2+} ions (Bliss and Collingridge, 1993). Ca^{2+} influx into postsynaptic cells through activated NMDA-channels leads to transient activation of various second-messenger cascades, in particular PKC and CaMK (Silva et al., 1992). The late phase of LTP is characterized by sustained activation of the PKM (Powell et al., 1994). As the LTF in *Aplysia*, lLTP depends on gene expression (Frey et

al., 1988). Enhanced Ca^{2+} influx activates not only CaMK but also PKA. As described above, PKA is activated by cAMP.

Thus, activity of different protein kinases contributes to processes of learning and memory formation. Since these kinases have different temporal dynamics, they can be implicated in formation of different memory phases.

1.5 The honeybee as a model to study the processes of learning

Insects have always been favorite subjects for neurobiological studies. Their nervous system is relatively small and contains many identifiable cells. It is well compartmentalized, with clear separations between multisensory higher-order neuropiles in the brain and neuropiles serving sensory-motor routines in the ventral cord. The rich behavior of insects includes orientation in space and time, visual, chemical, and mechanical communication, and complex motor routines for flying, walking, nest building, defence, and attack (Hammer and Menzel, 1995). Bees perform all these tasks with a rather small brain of approximately 1 mm^3 and fewer than 960 000 neurons (Witthöf, 1967). Many neurons of the cental nervous system are individually recognizable and large enough to be recorded intracellularly during ongoing behavior, including learning. (Menzel and Müller, 1996).

Foraging behavior of bees includes learning of local cues (such as color, odor, shape, location, handling), and responding quickly and effectively according to the reward (nectar, pollen) they provide (Menzel, 1999). Olfactory learning of the honeybee can be initiated in a laboratory context.

1.6 Classical olfactory conditioning of the proboscis extension reflex (PER) in the honeybee (The Olfactory Conditioning Paradigm)

Many insects extend their tongues (proboscis) reflexively when the sucrose receptors at the antennae, mouth parts, or tarsae are stimulated. Kuwabara (1957) and Takeda (1961) found that the proboscis extension reflex (PER) of bees can be conditioned to visual and olfactory stimuli if the bees are allowed to suck sucrose solution following the presentation the conditioned stimuli (Menzel and Müller, 1996). It has been shown that odors are associated with sucrose much faster (Menzel et al., 1974; Vareschi 1971) than visual stimuli (Masuhr and Menzel, 1972).

In the laboratory, restrained bees can be conditioned to olfactory stimuli. Each bee is restrained in a tube such that it can freely move only its antennae and mouthparts (mandibles and proboscis). The antennae are the main chemosensory organs of the bee. When the antennae of a hungry animal are touched with sucrose solution, the bee will reflexively extend its proboscis to reach out towards the sucrose and suck it. Odors and their stimuli to the antennae do not release such a reflex in naive animals. If, however, an odor is presented immediately before sucrose solution (so called forward pairing), an association is formed which enables the odor to release the PER in a successive test. This affect is clearly associative and involves classical, and not operant, conditioning. Thus, the odor can be viewed as a conditioned stimulus (CS) and sucrose solution as an unconditioned stimulus (US). (Bitterman et al., 1983; Menzel and Müller, 1996; Hammer and Menzel, 1995; Hammer, 1997).

1.7 Memory dynamics and memory contents in the honeybee

Memory dynamics in the honeybee after single- or multiple-trial appetitive learning both in color learning by free-flying bees and in olfactory conditioning of PER has been well described (Menzel and Müller, 1996; Menzel 1999). For both kinds of learning, it was discovered that a single conditioning trial (CS-US pairing) leads to a dual-phase retention function with high retention at very short (<1 min) and at longer (>5 min) intervals, with a dip around 3 min, indicating a consolidation process in the time range of several minutes after learning. The initial high response level is dominated by a non-associative, sensitization component, because a single US alone also arouses the animal for a short period of time, leading to a transient increase of response, including proboscis extension response to many stimuli, including the CS. Consolidation of the associative component requires minutes to develop, and during this process memory becomes more specific (Menzel, 1990; Hammer and Menzel, 1995). Multiple learning trials facilitate consolidation into long-lasting memory (Menzel et al., 2001). LTM in bees can last for several months, surviving the winter rest of the colony (Lindauer 1963) and in summer lasting the life span of a forager (about two weeks), even after only three learning trials (Menzel, 1968).

STM

Within the time interval of 2-3 minutes after a single conditioning trial (CS-US) unspecific memory is strongly reduced but specific associative memory is not yet finally established. Memory at this period is also still highly sensitive to amnestic treatment, such as brain shaking, drugs, or electric shock (Menzel at al., 1974; Erber et al., 1980), and the effectiveness of trial repetition to induce LTM is low for intermediate intertrial interval (ITI) of 3 min but is high for shorter and longer ones (Gerber et al., 1998). The tests performed by Menzel and the others (Menzel and Sugawa, 1986; Smith, 1991; Menzel et al., 1993) indicate that animals not only behave differently during the early phase of memory but that the

consolidation status of memories changes over time. The capacity of additional information, either conflicting or affirmative, to induce LTM is then dependent on the consolidation status of these earlier memories (Menzel, 1999).

The early phase of memory (STM) neither depends on transcription and translation (Grünbaum and Müller, 1998) nor can be affected by inhibition of nitrogen oxide (NO) (Müller, 1996). Physiologically STM is characterized by a fast transient wave of PKA activation, lasting 1-2 min (Müller, 2000).

MTM

At the beginning of MTM, bees behavior is controlled by consolidated, highly specific memory. At the MTM stage memory is more resistant to extinction, conflicting information and elapsing time. Some information about the context may have already been stored (Menzel, 1999).

Physiologically mid-term memory is characterized by the first wave of PKC activity. A learning-triggered protease-dependent process leads to cleavage of PKC resulting in constitutive active PKM in the AL (Grünbaum and Müller, 1998). The primary neuropile may be therefore a substrate for MTM. Since the MB appears to be the critical substrate for consolidation during STM, the MTM trace in the AL can be established under the control of MB. The MB provides the information which relates the memory traces in the primary sensory neuropiles to context stimuli across modalities (Menzel et al., 1994). Output neurons of the MB feed back to the AL and other sensory neuropiles (Rybak and Menzel, 1993) and may carry this information. For example, a particular neuron of the MB output ,the Pe1, demonstrates associative plasticity during lSTM only (Mauelshagen, 1993).

LTM

In contrast with STM, LTM after multiple conditioning trials is protein-synthesis- and NO-dependent (Müller, 1996; Wüstenberg et al., 1998; Grünbaum and Müller, 1998).

It is usually distinguished between two forms of LTM: early LTM (eLTM), which lasts 1-2 days and depends on translation, and late LTM (>2 days), which depends both on translation and transcription (Müller, 2002). The translation from STM to both eLTM and lLTM is independent of MTM, because inhibition of the characteristic substrate of MTM (protease-dependent enhancement of PKC activity) does not prevent formation of eLTM and lLTM (Grünbaum and Müller, 1998). It has been shown that induction of LTM requires prolonged activity of PKA. Using injections of inhibitors and antisense-oligonucleotides, it was shown that the activity of the PKA during learning is important for the formation of the LTM 24 hours later (Fiala et al., 1999; Müller 2000).

1.8 The Mitogen-activated protein kinase (MAPK) family and its implication in learning and neuronal plasticity

1.8.1 Study on the contribution of the MAPK activation to learning and memory processes

To understand the role of MAPK signaling in the protein-dependent long-lasting forms of synaptic plasticity and memory consolidation, the contribution of numerous potential downstream effectors regulated by MAPK must be investigated.

It is well known that storage of LTM requires new mRNA and protein synthesis (Davis and Squire, 1984; McGaugh, 2000). In contrast, STM is insensitive to inhibitors of transcription and translation. Long-lasting forms of synaptic plasticity, such as lLTP, exhibit a similar dependence on macromolecular synthesis, whereas more transient modifications of synaptic strength, such as eLTP, can be established in the absence of new mRNA and protein synthesis (Kandel, 2001).

Molecular mechanisms by which the MAPK pathway may regulate the temporal phases of LTP and memory may involve MAPK-dependent regulation not only at the transcriptional, but also at the translational level (Kelleher et al., 2004).

In *Aplysia* the effect of long-term facilitation is due in part to MAPK phosphorylation of a specific isoform of a cell-adhesion molecule (apCAM). Phosphorylation at MAPK sites leads to internalization and degradation of apCAM, lifting inhibitory constraints and allowing for synaptic reorganization (Bailey et al., 1997).

Studies in *Aplysia californica* show that activation of the *Aplysia* MAPK (apMAPK) by applying five repeated pulses of serotonin (5-HT) or by the cAMP pathway is important for intermediate-term facilitation (ITF) (Sharma et al., 2003) and long-term facilitation (LTF) of the connections between the sensory and motor neurons of the gill-withdrawal reflex (Kornhauser and Greenberg, 1997; Michael et al., 1998) but not necessary for short-term facilitation (Michael et al., 1998; Orban et al., 1999; Sharma et al., 2003a).

Long-term, but not short-term facilitation, requires new protein synthesis and is reflected in an altered level of expression of specific proteins regulated through the cAMP second-messenger pathway (Kaang at al., 1993). During this 5-HT induced facilitation MAPK p44/42 translocates into the nucleus of the presynaptic but not the postsynaptic cell (Martin et al., 1997). Sharma and colleagues investigated the role of MAPK p44/42 in three temporally and mechanically distinct phases of memory for sensitization in *Aplysia californica*, using tail shock: short-term memory (STM), intermediate-term memory (ITM) and LTM (Sharma et al., 2003a). They showed that five tail shocks, as well as five pulses of 5-HT, produce a sustained activation of MAPK p44/42 in ventrocaudal cluster of sensory neurons (SNs). Repeated tail shocks induce ITM and LTM to sensitization, that also leads to sustained MAPK p44/42 activation in the SNs. Activation of endogenous tyrosine kinases enhances the induction of long-term memory for sensitization, and this enhancement also requires MAPK p44/42 activation. Thus, tyrosine kinases, acting through activation of MAPK p44/42, play a pivotal role in LTF and LTM formation (Purcell et al., 2003). This MAPK p44/42 activation, or phosphorylation, at the membrane is important for the internalization of apCAMs and, thus, may represent an early regulatory step in the growth of new synaptic connections that accompanies long-term facilitation (Bailey et al.,

1997).

In rodents, MAPK p44/42 has been localized both pre- and post-synaptically in the hippocampus, providing an abundant source of potential targets. This targets include synaptic vehicle proteins (Jovanovic et al., 1996), second messenger signaling systems (Lin et al., 1993), cytoskeletal proteins (Brugg and Matus, 1991) and some voltage-gated ion channels, such as Kv4.2 (Adams et al., 2000).

In rats and mice many studies demonstrate that the MAPK p44/42 cascade plays a crucial role in differentiation, growth, apoptosis, development and stress. However, only a few investigations point to a function of MAPK 44/42 in neuronal plasticity and learning. Thus, it has been demonstrated that activation of the MAPK p44/42 kinase, or MEK, is required for induction of long-term potentiation (LTP) in the rodent hippocampus (Orban et al., 1999). Long-term potentiation is a robust and long-lasting form of synaptic plasticity that is the leading candidate for a cellular mechanism contributing to mammalian learning and memory (Sweatt, 2001). Initial studies in the area of investigating the role of MEK in LPT focused on NMDA receptor-dependent LTP in hippocampal area CA1, using slices *in vitro* (English and Sweatt, 1996 and 1997; Wu et al., 1999). It has also been shown that MAPK p44/42 activation is necessary for the induction of NMDA receptor-independent LTP and also for LTP in the dentate gyrus *in vitro* (Coogan et al., 1999), LTP *in vivo* (Davis et al., 2000; Rosenblum et al., 2000) and LTP at the amigdalar inputs into the insular cortex (Jones et al., 1999).

In rats, the MAPK p44/42 activity is required for formation of long-term memory (LTM) in fear conditioning (Lu et al., 2001). Furthermore, it has been found that the MAPK cascade is involved in spatial memory, another type of hippocampus-dependent LTM (Atkins et al., 1998; Adams and Sweatt, 2002).

The role of MAPK activation in gene expression may also be linked to the MAPK involvement into learning and memory processes. It is known that MAPK p44/42 regulates a number of transcription factors including Elk-1 (Treisman, 1996; Berman et al., 1998) and CREB (Frank and Greenberg, 1994; Impey et al., 1996, 1998, Roberson et al., 1999). Moreover, MAPK p44/42 appears to

mediate the phosphorylation of CREB by other kinase systems such as PKA and PKC (Impey et al., 1998; Roberson et al., 1999). Thus, activation of PKA by application of forskolin to hippocampal slices results in MAPK activation in area CA1 (Selcher et al., 2003). This also elicits increased CREB phosphorylation. The CREB phosphorylation stimulated by forskolin can be blocked by inhibition of MEK1/2 (Roberson et al., 1999). Thus, these data indicate that the activation of MAPK results in increased CREB phosphorylation in area CA1 and that cAMP pathway utilizes the MAPK p44/42 cascade as an obligatory intermediate in regulating CREB phosphorylation in hippocampal area CA1.

Not only PKA but also PKC can regulate MAPK in area CA1. Roberson and colleagues demonstrated that application of phorbol diacetate to hippocampal slices resulted in a robust increase in CREB phosphorylation, indicating that activation of PKC in area CA1 results in increased CREB phosphorylation. The inhibition of MEK1/2 significantly attenuated phorbol ester-elicited increase in CREB phosphorylation, demonstrating that MAPK p44/42 is a necessary component for maximal coupling of PKC activation to CREB phosphorylation in area CA1.

In other words, CREB activation produced by both forskolin and phorbol esters requires MAPK, as inhibition of MAPK p44/42 activation blocks the activation of CREB in area CA1 of hippocampal slices (Selcher et al., 1999).

To study the question of possible involvement of MAPK signaling in the protein synthesis-dependent phases of memory and LTP, different mouse models have been used. Thus, in 1996 Impey and team constructed a transgenic mouse that allows the monitoring of CREB activation through the insertion of a CRE-driven β-galactosidase gene into the animal's genome. Using this model, they demonstrated CREB activation in area CA1 of the hippocampus after LTP-inducing stimulation. The tetanus-induced LTP in these transgenic animals is NMDA receptor-independent. Thus, these results provide a strong implication that CREB activation is also involved in NMDA receptor-independent LTP.

Kelleher and team (2004) generated mutant mice in which a dominant-negative form of the MEK1 (dnMEK1) is expressed selectively in the postnatal forebrain.

This dominant-negative form of MEK1 bears a K⟶M substitution in the ATP binding site, abolishing its kinase activity but preserving its ability to interact with MAPK p44 and MAPK p42, thereby inhibiting their MEK-dependent activation (Mansour et al., 1994, Kelleher et al., 2004). They found a specific spatial memory (tested in the Morris water maze) deficit, a specific impairment in the protein-synthesis dependent phase of hippocampus-dependent contextual memory (fear conditioning) in dnMEK1 mice.

1.8.2 General organization of the MAPK cascades

The mitogen-activated protein kinase cascades are membrane-to-nucleus signaling modules highly conserved from yeast to vertebrates and involved in multiple physiological processes. Originally the MAPK cascades were discovered in 1987 by Ray and Sturgill as a critical regulator of cell division, differentiation and proliferation. They characterized a MAP2 (microtubule-associated protein 2) kinase from 3T3-L1 adipocytes stimulated by insulin that was distinct from any other insulin or growth factor-sensitive protein kinases that had been described in detail (Ray and Sturgill, 1987).

MAPKs are the superfamily of dual-specific kinases, which are hierarchically organized into three modules (Marshall, 1995; Cobb, 1999), where each module is a particular cascade, consisting of no fewer then three enzymes that activate each other by sequential phosphorylation (Haystead et al., 1992; Robbins et al., 1993; Kornhauser et al., 1997; Sweatt, 2001; Peyssonnaux et al., 2001).

The MAPK superfamily comprises three cascades: MAPK p44/42, or ERK, JNK/SAPK (Jun N-terminal kinase/stress-activated protein kinase) and the MAPK p38 (Fig. 1.1). The latter two can be referred to stress-activated protein kinases. The cascades are activated by distinct stimuli, associated with synaptic activity and plasticity. Most notable of them are membrane depolarization and Ca^{2+} influx (Rosen at al., 1994), neurotrophins (McAllister et al., 1999, Tyler et al., 2002; Wiklund et al., 2002; Thomas and Huganir, 2004), and also growth factor (Hoshi et al., 1988; Ahn et al., 1992), phorbol esters (Hoshi et al., 1988), tumor promot-

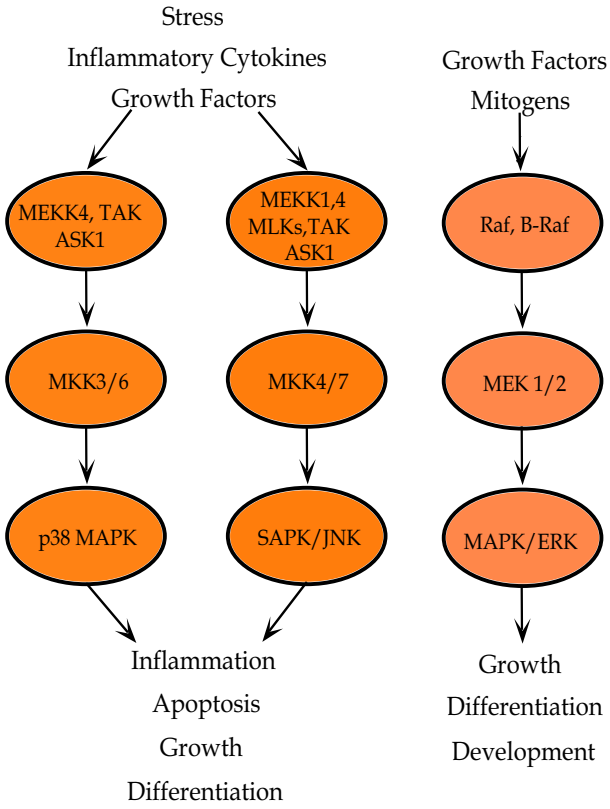

Fig. 1.1: Three modules of MAPK cascades: MAPK p38, JNK/SAPK, and MAPK p44/42, or ERK (extracellular-regulated kinase). Each cascade consists of at least three protein kinases, which consequently activate each other.

ers (Miyasaka et al., 1990; Raingeaud et al., 1995), hormones, such as urocortin, (Schulman at al., 2002), estradiol (Ruifen at al., 2001), aldosterone (Hendron and Stockand; 2002). Forskolin can also activate MAPK cascade (Martin et al., 1997).

Direct activators of MAPKs are so called MAP kinase kinase, also known as MKK. Different MAPK cascades are activated by distinct subsets of MKK. For instance, MEK1 and MEK2 - two members of MKK family - activate the MAPK p44/42, or ERK (extracellular-regulated kinase), pathway (Crews et al., 1992). The MKK4 (or SEK, or JNKK1) and the MKK7 (or JNKK2) are responsible for activating the JNKs (Yan et al., 1994). The MAPK p38 is activated by MKK3 and MKK6 (Raingeaud et al., 1996).

MAPK cascades are known to mediate growth (Hoshi et al., 1988; Ahn and Krebs, 1990;) differentiation (Yamashita et al., 2005), proliferation and development (Seger and Krebs, 1995; Fisher at al., 2001) but also stress (Raingeaud et al., 1995; Wang et al., 1998) and apoptosis (Kummer et al., 1997; Kanamoto et al., 2000) in mammalian cells.

MAPK p44/42, or ERK, pathway

A module that mediates inputs from growth and neurotrophic factors, cytokines and Ca^{2+} influx is the MAPK p44/42, or ERK cascade.

The term MAPK is used to refer to the entire superfamily of signalling cascades and also specifies the prototype MAPK as the extracellular-regulated kinase (ERK). Thus, the two ERK MAPK isoforms, MAPK p44 and MAPK p42, are referred to as ERK1 and ERK2, respectively (Davis et al., 1993a; Sweatt, 2001).

These three kinases represent a functional core of a cascade (Fig. 1.2). The first kinase in this particular cascade is the MAP kinase kinase kinase (MAPKKK, or Raf-1 and B-Raf in this ERK-cascade) which receives the inputs from GTFases or other protein kinases that are connected to G-protein-coupled receptors or cell surface receptors. The MAPKKK activates the second, a MAP kinase kinase (MAPKK, or MEK), by serine/threonine phosphorylation. MAPKKs (MEKs) are dual specificity kinases which in turn activate a MAP kinase (MAPK p44 and MAPK p42) by phosphorylating both the threonine and tyrosine residues (Fig. 1.2). The exact phosphorylation site is Thr-Glu-Tyr (TEY). The MAPK p44/42 are present in the cytoplasm of quiescent cells. Phosphorylated MAPK p44/42 can translocate into the nucleus where they induce gene expression by activating specific transcriptional factors either directly or indirectly (Wang et al., 2004).

MAPK p38 pathway

p38 mitogen-activated protein kinase (MAPK p38) was originally identified as a serine/threonine kinase activated by stimulation of monocytes with bacterial lipopolysaccharide (LPS), and was later shown to regulate LPS-induced produc-

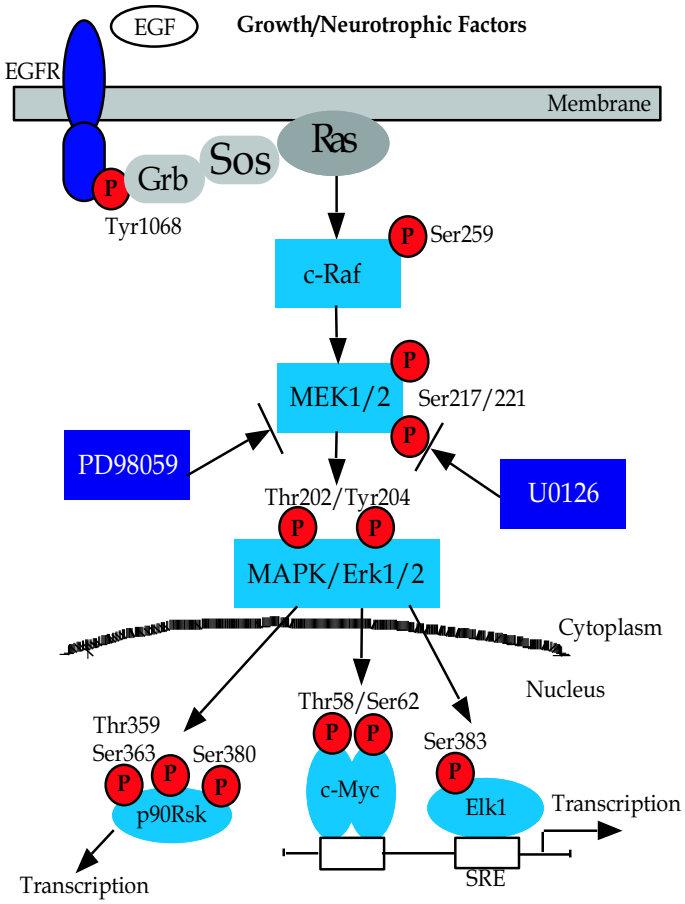

Fig. 1.2: Schematic organization of the MAPK p44/42, or ERK, cascade.

tion of the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) (Lee et al., 1994).

MAPK p38 has also been implicated in some of the signalling pathways induced by these proinflammatory cytokines (Marshal et al., 1998; New and Han, 1998). Overproduction of these cytokines has been implicated in wide variety of diseases with an inflammatory component, such as rheumatoid arthritis, endotoxic shock, inflammatory bowel disease, and osteoporosis (Redman et al., 2001). A few works pointed out the role of MAPK p38 in neuronal plasticity (Guan et al., 2003) and formation of LTM (Zhen et al., 2001).

In mammals, all MAPK p38 isoforms identified can be activated by osmolarity

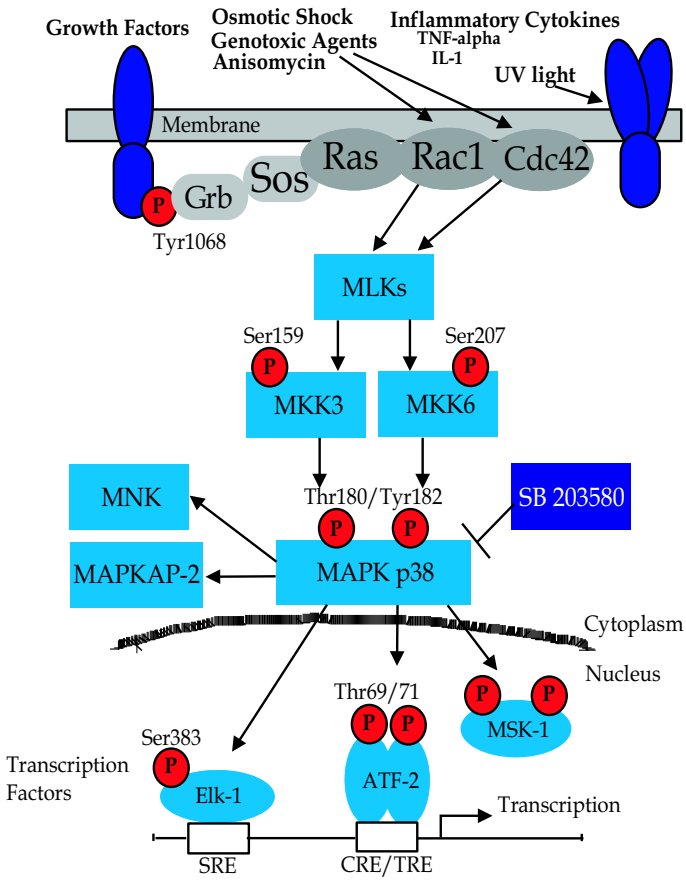

Fig. 1.3: Schematic organization of the MAPK p38 cascade.

changes in a cell environment and by a variety of other stress signals from the extracellular environment, such as UV irradiation and oxidation (Raingeaud et al., 1995). Besides the interleukins and TNFα, MAPK p38 is also activated by other stressful stimuli, such as translational inhibitor anisomycin (Meier et al., 1996), arsenite, H_2O_2 (Raingeaud et al., 1995), and heat shock (Cuenda et al., 1995). Thrombin (Guay et al., 1997) and glutamate (Kawasaki et al., 1997) can also activate MAPK p38 pathway.

Since the role for MAPK p38 in associative learning in rabbits has been well documented (Zhen et al., 2001), the role of MAPK p38 in associative learning in the honeybee must also be described.

1.9 The goal of this work

Since involvement of MAPK p44/42 and MAPK p38 in different forms of neuronal plasticity and learning has been well documented in other organisms, such as *Aplysia*, *Drosophila*, and rodents, the role of these signalling cascades in the honeybee associative learning must be investigated.

Previous studies revealed contribution of AMP-dependent kinase (PKA) and protein kinase C (PKC) in associative learning in the honeybee. Since these second-messenger pathways can interact with the MAPK cascade, it also suggests the possible role of MAPKs in the honeybee learning.

The questions to be investigated in this work are:

- How can the role of MAPKs in learning be investigated?
- In which honeybee brain areas are MAPKs localized?
- What is the role of MAPKs in associative and non-associative forms of learning?
- Do MAPK p44/42 and MAPK 38 differ in their involvement in associative olfactory learning in the honeybee?
- What are time dynamics of MAPK activation after conditioning?