4 Discussion

The goal of this work was to describe role of the MAPK p44/42 and MAPK p38 in olfactory learning in the honeybee *Apis Mellifera*. For the first time it was shown that inhibition of the MAPK p44/42 within a time window of 30 min before and 120 min after conditioning impairs the formation of LTM. Forward multiple trial training led to increasing in phosphorylation level of the MAPK p44/42. Since immunohistological study revealed presence of MAPK p44/42 in both the ALs and MBs, both neuropiles implicated in olfactory learning, MAPK p44/42 activity was determined in both of these tissues.

The increased activation, or increased level of phosphorylation, of MAPK p44/42 was observed both in antennal lobes and mushroom bodies 40 min after training, and only in mushroom bodies 120 min after training. This suggests that in MBs may take place long-lasting processes, whereas ALs activity is requires during short-term processes. The were no changes in the MAPK p38 activity observed, which suggests that MAPK p38 is not implicated in olfactory learning in honeybees.

4.1 Tools to investigate the MAPK cascade in vivo

The most critical problem in this work was the evaluation of techniques that allowed measurement and manipulation of MAPK activity *in vivo*. While induced changes *in vivo* in MAPK activity could be determined using both phospho-dependent and phospho-independent antibodies, which have been successfully

used both in *Aplysia* (Sharma et al., 2003b) and rodents (Atkins et al., 1998; Blum et al., 1999), the MEK 1/2 inhibitor PD 098059 was applied for blocking experiments.

PD 098059 was identified by screening a compound library with a cascade assay that measured phosphorylation of myelin basic protein (MBP) in the presence of GST-MEK1 and GST-MAPK fusion proteins. In this assay, the MAPK-catalized phosphorylation of MBP was dependent on activation by MEK (Dudley et al., 1995; Alessi et al., 1995). PD 098059 was shown to block the activity of MEK and, thus to prevent activation of MAPK. Is has also been shown that PD 098059 crosses cell membranes (Dudley et al., 1995) and thus can be applied both for *in vitro* and *in vivo* experiments.

The inhibitory activity of PD 098059 is reversible (Dudley et al., 1995) and acts in a cytostatic, rather than cytotoxic, manner. This also corresponds to the findings in behavioral tests: injection of PD 098059 24 h before conditioning did not affect MAPK cascade.

Several other studies demonstrated that PD 098059 inhibits MEK1/2 *in vivo* and *in vitro* (Pang et al., 1995; Dudley et al, 1995) without affecting PKA, PKC, or JNK (Dudley et al., 1995; Alessi et al., 1995). Indeed, the experiments with activation and inhibition of the MAPK p44/42 cascade *in vitro* demonstrated that this inhibitor very selectively blocks MEK1/2 and thus affects further phosphorylation of MAPK p44/42 (Fig.3.4 and Fig.3.5). PD 098059 has successfully been used in *in vitro* experiments (Crow et al., 1998), hippocampal slices (English and Sweatt, 1996; Selcher et al., 2003) as well as in behavioral studies (Atkins et al., 1998; Blum et al., 1999).

4.2 Involvement of the MAPK cascade in non-associative learning

Habituation

The experiments with inhibition of the MAPK p44/42 activation revealed that it does not affect habituation of the PER in the honeybee (Fig.3.8). The dishabituation was also not affected. It suggests that habituation is not mediated by MAPK p44/42 signalling pathway in the honeybee. This findings correspond to the findings in rat hippocampus. It has been found that habituation to an open field in an aversive behavioral task did not alter phosphorylation level of hippocampal MAPK p44/42 in rats (Alonso et al., 2003).

Sensitization

In the honeybee, sensitization is an increase, or amplification, of a PER due to a preceding excitement. If the bee receives a stimulus (e.g. sugar water) on an antenna, so it increases its responsiveness to the following presentation of a novel stimulus (e.g. odor).

Synaptic facilitation of sensory-motor (SN-MN) synapses in *Aplysia* has been used as a cellular model of behavioral sensitization. This synaptic facilitation exists in at least three mechanistically distinct temporal phases: short-term facilitation (STF), which lasts <30 min, intermediate-term facilitation (ITF), lasting >90 min, and long-term facilitation, lasting >24 h (Sutton and Carew, 2000). A single pulse of 5-HT, which is released during sensitization training (Marinesco and Carew, 2002), produces STF, whereas five pulses produce two temporally distinct phases: ITF and LTF. Similarly, a single tail shock leads to STM (lasting <30 min) for sensitization, whereas five repeated shocks produce both ITM (lasting > 90 min) and LTM (lasting >24 h) (Sutton et al., 2001).

MAPK p44/42 plays different roles in these temporally distinct facilitation or

memory phases. In the experiments with 5-HT and tail shocks, application of a single stimulus or five stimuli was used. A single pulse of 5-HT or a single tail shock induce STF or STM, respectively. In the experiments with single stimulation (5-HT and a tail shock), Sharma and colleagues (2003) demonstrated that MAPK p44/42 activity is required neither for STF nor for STM for sensitization in *Aplysia*. These findings are in agreement with results of sensitization experiments in the honeybee, where the inhibition of MAPK 44/42 activation did not effect sensitization after a single sucrose stimulation (Fig.3.9).

An opposite effect of inhibition of the MAPK activation was observed in the experiments with multiple stimulation. It has been shown that five pulses of 5-HT activate MAPK p44/42 (Michael et al., 1998) and induce its translocation to the sensory (presynaptic) neuron nucleus (Martin et al., 1997), and that inhibition of the MAPK p44/42 blocks the induction of LTF in cultured SN-MN synapses. Thus, activation of MAPK p44/42 plays a crucial role in the induction of LTF.

As it has been shown previously, five pulses of 5-HT cause not only LTF but also ITM, lasting up to 3 h at the SN-MN synapses (Mauelshagen et al., 1996; Sutton and Carew, 2000) and five tail shocks induce ITM in the same time domain (Sutton et al., 2000), which led to sustained activation of MAPK p44/42 in the tail SN-MN synapses. Using an inhibitor of MEK1/2, Sharma and collegues (2003) showed that MAPK p44/42 activity is required for ITF, in addition to LTF. They also examined role of MAPK p44/42 for ITM and LTM for sensitization in *Aplysia*. Their results demonstrated the requirement of MAPK activation for induction of ITM and LTM for sensitization.

4.3 Localization of the MAPK p44/42 and MAPK p38 in the bee brain

In this work the presence of MAPK p44/42 in the ALs and MBs of the honeybee was shown. MAPK p38 was expressed only in ALs.

Both the neuropiles are extremely important for olfactory learning in the honeybee (Menzel and Müller, 1996) and *Drosophila* (Heisenberg et al., 1985). Local cooling of selected parts of the brain induced retrograde amnestic effects, which depend on the time interval between the single trial learning and the site of cooling. Thus, retrograde amnesia can be induced if the ALs are locally chilled within a minute after single-trial conditioning. Cooling of the MBs, in particular their calyx region, within 5-7 min after conditioning also leads to retrograde amnesia. No amnestic effect was observed after chilling the lateral protocerebral lobe (lateral horn) (Menzel and Müller, 1996; Menzel, 2001). In *Drosophila*, flies with chemically ablated MBs or with structural MB mutations are defective in associative learning (Heisenberg et al., 1985; de Belle and Heisenberg, 1994); and the gene products of the biochemical memory mutants *dunce*, *rutabaga*, and *leonardo* are enriched in the MBs (Davis, 1993b).

The gene products of *dunce* and *rutabaga* involved in memory formation are mainly expressed in mushroom bodies (Nighorn et al., 1991; Han et al., 1992). Other studies have pointed out the role of mushroom bodies in olfactory learning in *Drosophila*. Thus, Heisenberg and team showed that the defects in olfactory learning occur due to mutations, in which the structure of mushroom bodies is altered, or due to chemical knock-out of neuroblasts important for mushroom bodies development (Heisenberg et al., 1985; deBelle and Heisenberg, 1994).

MAPK p44/42 is localized in the honeybee brain both in the synaptical regions and in the cells somata, which corresponds to the previous results that have been published for MAPK p44/42 localization in the rat hippocampus, a structure associated with associative learning (Fiore et al., 1993). Such a localization suggests that MAPK p44/42 may phosphorylate both nuclear and cytoplasmatic targets which are involved in associative learning in the honeybee.

4.4 Involvement of the MAPK cascades in associative learning and memory formation

It has been shown in *Aplysia*, that not only long-term facilitation but also long-term depression (LTD) is mediated by a member of MAPK family, the MAPK p38. Activation of this kinase facilitates LTD and blocks LTF, and inhibition of MAPK p38 blocks LTD and enhances LTF, respectively (Guan et al., 2003).

In vertebrates, the MAPKs are expressed abundantly in the CNS, especially in areas that are thought to be involved in learning and memory such as the hippocampus and cerebellum (Lee et al., 2000; Zhen X. et al., 2001). Clinical and experimental studies have shown that certain types of memory (including declarative memory in humans and spatial memory in animals) are dependent on the hippocampus and possibly the entorhinal, peririhnal and parahippocampal cortices (Morris et al., 1982). Hippocampal lesions and pharmacological interventions in rodents have been shown to cause poor performances in several spatial memory tasks (Morris et al., 1982; Moser et al., 1993).

In the rat, activation of MAPK p44/42 in hippocampus was reported to be essential for long-term spatial memory as measured in the water maze (Blum et al., 1999) and in memory consolidation for fear conditioning (Atkins et al., 1998). The pharmacological antagonism of MAPKs was also shown to impair spatial learning (Blum et al., 1999; Selcher et al., 1999) and fear conditioning in the rat (Atkins e al., 1998) and taste-aversion learning in the mouse (Swank, 2000). Some evidence suggest that LTM may involve a specific and differential activation of the different families of MAPKs. For example, Berman et al. (1998) showed that rats which had developed a long-term memory of a specific taste demonstrated a differential activation of MAPK p44/42 and JNK but not MAPK p38 in the insular cortex when exposed to a novel taste. Blum and colleagues demonstrated that the inhibition of the MAPK p44/42 cascade in dorsal hippocampi (CA1/CA2 subfields) did not impair acquisition, but blocked the formation of long-term spatial memory. In contrast, intrahippocampal infusion of a specific inhibitor of

the p38 MAPK, did not interfere with memory storage (Blum et al., 1999). It has also been published, that in young rats, infuson of the MEK 1/2 inhibitor PD 098059 into olfactory bulbs prevented the aversive olfactory learning (Zhang et al., 2003).

These studies suggested that, depending on the learning task and model system used, various subfamilies of MAPKs may become involved in long-term memory formation. Thus, investigating associative learning in rabbits (by classical eye-blink response, where US was an air puff and CS was a tone delivered from a speaker), Zhen and colleagues (2001) found that MAPK p44/42 and MAPK p38 are differentially activated in dorsal hippocampus and in cerebellar vermis during acquisition. They showed that both the MAPK p44/42 and MAPK p38 were significantly elevated in cerebellar vermis at the time of 180 min after the third conditioned session, whereas only MAPK p44/42 was significantly elevated in the dorsal hippocampus. Thereby the activation of MAPK p44/42 was only seen in animals demonstrating associative learning. Animals, receiving explicitly unpaired presentations of stimuli failed to demonstrate any acquisition of CS-elicited responses and also failed to demonstrate any activation of MAPK p44/42 in the dorsal hippocampus or MAPK p44/42 and MAPK p38 in the vermal cerebellum. However, in contrast with the findings from Zhen et al. (2001), MAPK p38 is not involved in the honeybee olfactory learning.

Although there has not been much published about involvement of the MAPK p38 in associative aversive learning, the difference in MAPK p38 involvement in associative learning in rabbits and the honeybee may result from application of different USs. In the eye-blink conditioning Zhen et al. (2001) used an air puff delivered to the eye of the animal, which elicited a defensive reaction – blinking – and thus could be considered as a stressful stimulus. It is a well-known fact that MAPK p38 is activated by different stress factors (New and Han; 1998). In the olfactory conditioning in the honeybee, an appetitive stimulus – sucrose – was applied. It does not have any stress component and thus can be considered rather as a positive reward.

Not only are MAPK p44/42 and MAPK p38 differentially implicated in different types of tasks but it is also possible to distinguish between two isoforms, MAPK p44 and MAPK p42. Thus, several studies investigated involvement of MAPK p44 and MAPK p42 in LTP, a physiological model for memory. Different LTP-inducing stimulations cause activation of different MAPKs. It has been found that in the LTP in mice, the MAPK p44 and MAPK p42 isoforms are also differentially activated. Selcher et al. (2001) analyzed hippocampal slices of mice, lacking the ERK1 (MAPK p44) isoform. To induce LTP in the area CA1, two 1-sec 100-Hz tetani (high frequency stimulation, HFS), separated by 20 s, were applied. They found no difference between MAPK p44 knockout mice and littermate wild-type controls in either posttetanic potentiation or LTP. This lack of phenotype was not caused by any compensatory change in ERK2 (MAPK p42) protein levels or enhanced MAPK p42 activation in ERK1 knockout mice. Their findings demonstrate that the activation of the MAPK p44 is not required for LTP induced by HTF in mice. In behavioral tests in mice, the fear conditioning and passive avoidance task, as it has been found by Selcher and colleagues (2003), the MAPK p44 isoform is not necessary for amygdala-dependent emotional learning, whereas the MAPK p42 is.

It has also been demonstrated that MAPK p42, but not MAPK p44, is activated in area CA1 of the rat hippocampus in response to LTP-inducing HFS (English and Sweatt 1996; Wang et al., 2001), whereas in the nicotine-induced LTP both the MAPK p44 and MAPK p42 were activated in the area CA1 of the rat hippocampus (Wang et al., 2001).

In agreement with results described above (spatial memory in rats, Blum et al., 1999; fear conditioning, Atkins et al., 1998; exposure to a novel taste in rats, Berman et al., 1998), in the honeybee the MAPK p44/42 is activated after multiple forward trial training. Due to application of ELISA-technique for determination of MAPK activity, it was not distinguished in the present work between MAPK p44 and MAPK 42. Inhibition of the MAPK p44/42 cascade shortly before and after conditioning impairs LTM formation, whereas inhibition of the

MAPK p38 does not affect LTM. These findings suggest that only the MAPK p44/42 and not the MAPK p38 is involved in associative olfactory learning in the honeybee.

4.5 MAPK p44/42 activation is necessary for the formation of associative LTM in the honeybee

4.5.1 Time window of learning induced MAPK p44/42 activation

In the honeybee, MAPK p44/42 was activated by olfactory learning in both the ALs and MBs. Measurements reveal a clear difference between ALs and MBs in the duration of this learning induced activation of MAPK p44/42.

MAPK p44/42 activation in the honeybee brain has a transient character, which corresponds to the previous studies on LBA in the rats (LTM, Schafe et al., 2000), in the CA1 hippocampal area (LTP, English and Sweatt, 1996; Selcher et al., 2001), and during LTF in *Aplysia* (Sharma et al., 2003a).

Studies in *Aplysia* LTF demonstrated that after five tail shocks (duration 1.5 s each, with ITI 10 min) MAPK p44/42 is significantly activated in the SNs 1 h after stimulation. Three hours after the last shock the difference was no longer statistically significant. Similarly with tail shocks, MAPK p44/42 was also activated in the SNs after five repeated 5-HT pulses (5 min each with ITI 15 min). A significant difference in the MAPK p44/42 activation was observed immediately after training and lasted until 1 h. Three hours after stimulation the difference was no longer significant. For the 5-HT experiments the relative MAPK p44/42 phosphorylation was measured at 0, 1, 3 and 20 h after the end of stimulation; for experiments with tail shocks at 0, 1, 3 and 22 h after stimulation (Sharma et al., 2003a).

The findings at the time point of 1 h after stimulation is in agreement with

Object animal	**Training paradigm used**	**Time of MAPK activation after multiple stimulations**	**Sites of MAPK activation**	**Selected links**
Aplysia	LTF	60 min	SN	Sharma et al., 2003
Rat	LTP, HFS	2-60 min	Hippocampus, CA1	English and Sweatt, 1996, Wang et al., 2001
	LTP, TFS	2-15 min	Hippocampus	Giovannini et al., 2001
	Spacial learning (Morris Water Maze)	5 - 30 min	dorsal hippocampi CA1/CA2	Blum et al., 1999
	Fear conditioning: cued and contextual contextual	60-120 min 60 min	Hippocampus	Atkins et al., 1998
Honeybee	olfactory learning	40 min 40 - 120 min	AL MB	present study

Table 4.1: Time of MAPK p44/42 activation in different organisms after different stimulation paradigms.

findings in the honeybee, where the MAPK p44/42 was activated 40 min after training in both ALs and MBs and lasted until 120 min in the MBs. An "immediate" activation of the MAPKp44/42 in *Aplysia* after the end of the last serotonin stimulus may result from a quite long duration of the stimulus (5 min) and quite long inter-trial interval (15 min). By the end of the last 5-HT stimulus, the total time of the stimulation ran up to 85 min. In the honeybee, the ITI was only 2 min long and the stimulus itself was presented for several seconds. Both in the honeybee and *Aplysia*, the MAPK p44/42 phosphorylation was not changed after a single stimulus (for *Aplysia*: Michael et al., 1998).

The time of MAPK p44/42 activation was also investigated on LTP in rat hippocampal slices. Interestingly, that depending on LTP-inducing stimulation used, MAPK p44/42 was activated at different time points. Thus, it has been shown that the HFS (two trains of 100 Hz 1-1.5 s each with intertrain interval of 20 s), applied three times with the ITI of 10 min induces MAPK p42 activation at 20 min after tetanization (Wang et al., 2001, tested at 20 min). With respect to the intertrial interval, these findings correspond to that in the honeybee. However, there are some contradictory data obtained by English and Sweatt (1996). They showed that the MAPK p42 activation is increased at 2 min after multiple HFS and returns to the baseline by 1 h. In both cases, the MAPK p44 was not activated.

Beside the HFS-induced LTP, Wang and colleagues examined LTP induced by nicotine (35 min stimulation). They showed that the level of phosphorylation of both MAPK isoforms p44 and p42 was significantly increased, comparing to control, at 20 min after beginning of the nicotine stimulation (Wang et al., 2001). However, they made tests only at 20 min after stimulation, what does not deliver a full picture of the dynamics of the MAPK p44/42 activation by nicotine.

Another kind of stimulation that can elicit LTP is theta-frequency stimulation (TFS, 5 Hz); it mimicks a hippocampal theta-rhythm and therefore is physiologically relevant. Giovannini et al (2001) performed experiments with LTP induced by TFS paired with β-adrenergic receptor activator isoproterenol (ISO), a proto-

col that may be particularly relevant to normal patterns of hippocampal activity during learning. They tested the level of phosphorylated MAPK p42 at 2, 15, and 60 min after the end of TFS-ISO stimulation and found it increased at both 2 and 15 min. MAPK p44 was not activated at any time points tested.

Determination of the time window of MAPKs activation after training has also been performed *in vivo*. Series of experiments on the lateral and basal nuclei of the amygdala (LBA) revealed the transient significant activation of MAPK p44/42 in these areas after multiple trial Pavlovian fear conditioning. Fear conditioning resulted in significant increase in phospho-MAPK immunoreactivity for both MAPK p42 and MAPK p44 at 60 min after training but not at other time points tested (15, 30, 180 min after training) (Schafe et al., 2000). No significant differences were detected for the total level of the MAPK p42 and MAPK p44.

Atkins and colleagues (1998) carried out experiments with contextual and cued-contextual fear conditioning of rats. For the contextual experiments the animal was placed into an experimental chamber with a grid floor and 5 foot shocks with ITI 2-3 min were applied, in between trials the animal was removed from the chamber. For the cued/contextual task the foot shock was followed by a tone, three pairings were performed with ITI of 2 min.

With both fear conditioning protocols, the MAPK p42 was activated in hippocampus 1 h after training. No significant change in MAPK p44 phosphorylation was observed with the contextual protocol; however, an increase was observed with the cued-contextual protocol at this (1 h) time point. After cued-contextual conditioning, both MAPK p44 and MAPK p42 were activated 1 h after learning and activation of only MAPK p42 lasted until 120 min after training. Level of MAPK p44 and MAPK p42 phosphorylation was tested 0, 1, 3, and 20 h after conditioning (Atkins et al., 1998). The total protein kinase levels remained constant. These findings are in agreement with results obtained from honeybee. The coincidence of time courses of the MAPK p44/42 activation may result from similar learning protocols used (three trials with ITI of 2 min, *in vivo* training).

A slight difference in the time course of MAPK p44/42 activation between that

observed after LTP, where MAPK p42 was activated at time point 2 min after stimulation (English and Sweatt, 1996), and results from associative learning in the honeybee and rats (rat: Atkins et al., 1998) may indicate that the kinetics *in vivo* during learning are different from those in *in vitro* LTP. The temporal sequence of the biochemical events underlying learning and LTP may be the same, yet shifted during associative learning. Alternatively, the protein kinase activation that occurs with associative training may result from mechanisms distinct from those recruited in LTP.

Since in the present work MAPK p44 and MAPK p42 in the honeybee were not distinguished between, these two isoforms may differently contribute to the LTM in honeybee. It could explain the difference in time window of MAPK p44/42 activation in the distinct brain areas, ALs and MBs. According to the findings in LTP (English and Sweatt, 1996; Wang et al., 2001) and fear conditioning (Atkins et al., 1998; Giovannini et al., 2003), the MAPK p42 may play a greater role for LTM formation in the honeybee. Since it has been found in behavioral conditioning that MAPK p44 and MAPK p42 both are activated 1 h after learning (Atkins et al., 1998; Schafe et al., 2000), and only MAPK p42 remains activated by 2 h after learning (Atkins et al, 1998), MAPK p44 can be activated/presented in both the AL and MBs 40 min up to 1 h after learning, and MAPK p42 can be activated/presented only in the MBs, where the activation was observed 2 h after learning.

Other evidence to suggest that the MAPK p42 might play a greater role for LTM in honeybee comes from the investigation of the total MAPK p42 and MAPK p44 amounts in rat hippocampus. It has been reported by Kanterewicz et al. (2000) that the amount of total MAPK p44 in rat hippocampus appears to be considerably less than MAPK p42. Another possible explanation would be that in ALs and MBs the MAPK p44/42 is activated by two distinct mechanisms, thus resulting in different time courses of MAPK p44/42 phosphorylation in the ALs and MBs.

4.5.2 Possible mechanisms of MAPK p44/42 activation. Interaction with other signalling cascades

The differences in the honeybee MAPK p44/42 activation time course in the AL and the MB suggest that there might be different mechanisms of MAPK p44/42 activation, which are characterized by distinct activation kinetics.

Indeed, it has been demonstrated that MAPK p44/42 cascade can be activated via PKA (Vossler et al., 1997) and PKC (Crow et al., 2001), two cascades reported to mediate formation of particular memory phases in the honeybee (Grünbaum and Müller, 1998; Müller, 2000; Thomas, 2002). However, it has been reported that PKA can also inhibit the activation of MAPK p44/42 (Sweatt, 2001). Thus, PKA regulates the MAPK p44/42 cascade both by inhibiting and activating it, supposedly via distinct pathways. Two ways of MAPK p44/42 activation have been characterized: Ras/Raf-1/MEK/ERK (Ras/Raf-1) and Rap-1/B-Raf/MEK/ERK (Rap-1/B-Raf), Fig.4.1 (Anderson et al., 1990; Bos, 1998).

Both MEK upstreams regulators, Ras and Raf-1, have been shown to be involved in in learning and memory (Brambilla et al., 1997; Kawasaki et al., 1998; Dhaka et al., 2003). However, while Raf-1 is ubiquitously expressed, B-Raf displays highest level of expression in neural tissue (Barnier et al., 1995). B-Raf has been identified as a major MEK activator, even in cells where its expression was barely detectable by Western blotting analysis (Catling et al., 1994; Reuter et al., 1995; Mikula et al., 2001). B-Raf displays consistently higher affinity for MEK1 and MEK2 than Raf-1 (Papin et al., 1996; Papin et al., 1998) and is more efficient in phosphorylating MEK1/2 (Pritchard et al., 1995; Marais et al., 1997). These findings suggest that the Rap-1/B-Raf pathway might be the major way of MAPK p44/42 activation during neuronal processes and processes of learning.

In neural cells (Dugan et al., 1999), as well as in cells originating from the neural crest (Frodin et al., 1994) and melanocytes (Englaro et al., 1995), cAMP has a positive effect on MAPK p44/42 activation. The role of B-Raf in this process has

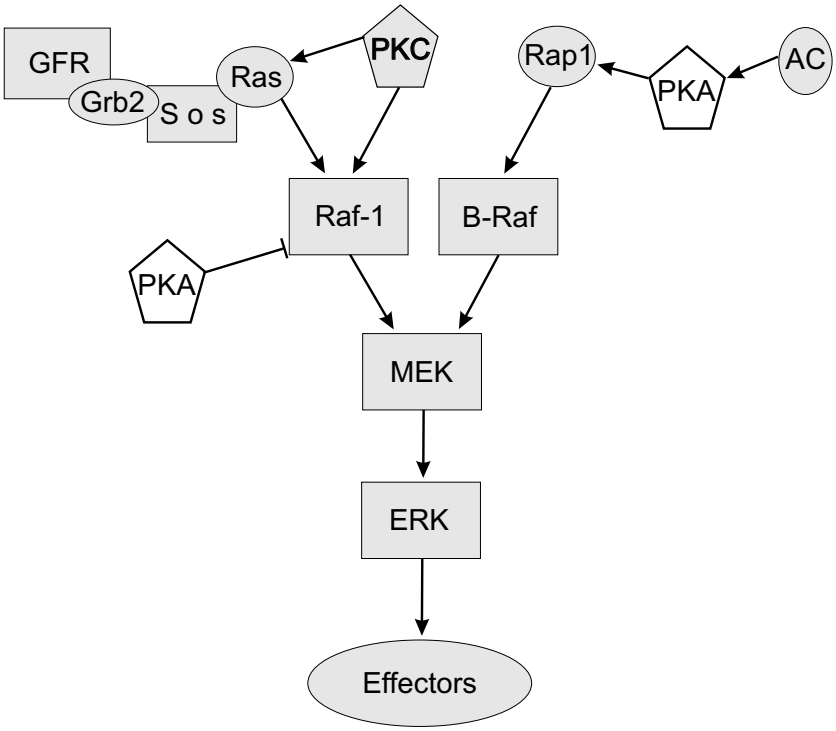

Fig. 4.1: Two pathes of MAPK p44/42 (ERK) activation: Ras/Raf/MEK/ERK pathway and Rap1/B-Raf/MEK/ERK pathway. The Ras/Raf pathway is inhibited by PKA, whereas the Rap/B-Raf activated. PKC has a positive effect on the Ras/Raf-1 pathway. Both Ras and Raf-1 can be activated trough the PKC (Sweatt, 2001).

been clearly established: B-Raf is expressed in all these cell types, its activity is stimulated by cAMP (Vossler et al., 1997, Dugan et al., Busca et al., 2000) and a dominant negative mutant of the kinase inhibits cAMP-induced ERK activation (Busca et al., 2000). In addition, PKA does not display a direct inhibitory effect on B-Raf activity such as that observed for Raf-1.

In the honeybee learning, the fast component of PKA activity is observed 1-2 min after either a single US or a pairing CS-US, or a backward pairing US-CS (Müller, 2000). If the multiple conditioning takes place, the activity of PKA in the AL is prolonged up to 3 min (Müller, 2000) and contributes to LTM formation. The learning-induced activation of PKC in the AL is observed at 1 h after olfactory

conditioning and lasts up to 3 days (Grünbaum and Müller, 1998; Thomas, 2002). The activation of MAPK p44/42 was observed at 40 after learning at earliest (15, 40, 120, and 240 min after conditioning were tested).

Taken together, these findings suggest that the upregulation through PKA or/and PKC alone is not sufficient to trigger MAPK p44/42 activation. There might be another signalling cascade which would also interfere with activation of MAPK p44/42 cascade. Indeed, it has been shown in other systems, such as *Aplysia* and rats, that other pathways, such as cGMP-dependent protein kinase (PKG), Ca^{2+}-calmodulin-dependent protein kinase I and II (CaMKI and CaMK II) are involved in neuronal plasticity and LTM consolidation (Liu et al., 2003; Sung et al., 2004; Schmitt et al., 2005). In *Aplysia*, PKG was shown to regulate long-term hyperexcitability (LTH). In the soma, apPKG phosphorylates apMAPK p44/42, resulting in its entry into the nucleus. Studies using recombinant proteins *in vivo* and *in vitro* indicate that apPKG can directly phosphorylate the threonine moiety in the TEY activation site of apMAPK when the -Y- site contains a phosphate (Sung et al., 2004). In rats, PKG is involved into LTP in the visual cortex (Liu et al., 2003) and in the hippocampus (Monfort et al., 2002). Implication of PKG into passive avoidance learning in chicks was shown (Edwards et al., 2002).

A requirement for the CaMK-kinase (CaMKK) pathway upstream of ERK (MAPK p44/42) in LTP induction has been demonstrated by Schnmitt et al. (2005). They found that both the pharmacological inhibitor of CaMKK, and the dominant-negative CaMKI (dnCaMKI), a downstream target of CaMKK, blocked neuronal NMDA receptor-dependent MAPK p44/42 activation. Involvement of CaMKII in long-term changes of synaptic excitability (Soderling and Derkach, 2000) and consolidation of LTM (Micheau and Riedel, 1999; Suenaga at al., 2004) has also been demonstrated.

However, contribution of signalling cascades, such as PKG and CaMKs, to the honeybee associative learning remains to be established.

4.5.3 Potential targets of the MAPK p44/42 that could be involved in associative learning in the honeybee

LTM formation is generally dependent on protein synthesis (Davis and Squire, 1984) and a role for MAPK p44/42 in LTM has been demonstrated in a number of different learning paradigms in invertebrates and vertebrates (Bailey et al., 1997; Martin et al., 1997; Atkins et al., 1998; Blum et al., 1999; Sharma et al., 2003 Selcher et al., 1999; Selcher et al., 2003; Zhang et al., 2003; Ahi et all., 2004).

The potential downstream candidates regulated by MAPK p44/42 during learning are numerous and diverse. Since the MAPKs are localized both in dendrites and cell bodies (Fiore et al., 1993), they can regulate not only various transcriptional factors, such as Mblk-1 (Park et al., 2003), p90Rsk-1 and Elk-1 (Davis et al., 2000; Sananbenesi et al., 2002), or CREB (Bozon et al., 2003; Ahi et al., 2004) and its downstream effectors C/EBP (Alberini et al., 1994) and AF (Bartsch et al., 2000), but also vehicle proteins such as synapsin (Jovanovic et al., 1996), cytosceletal elements such as MAP2 (Brugg and Matus, 1991), second messenger systems such as cytosolic phospholipase A_2 (Lin et al., 1993) and voltage-gated ion channels such as Kv4.2 (Adams et al., 2000).

Kv4.2 channel belongs to the family of voltage-gated K^+ channels of the A-type and is presented in rat hippocampus (Adams et al., 2000). Kv4.2 is phosphorylated by MAPK during LTP induction (Morozov et al., 2003), which leads to increased excitability and membrane depolarization of neurons, which in turn increases the magnitude of the calcium influx and the probability of triggering LTP and LTM formation. It has been shown that not only MAPK but also PKA, PKC and CaMKII phosphorylate the Kv4.2 (Anderson et al., 1999). Although in the honeybee this channel has not been described yet, another channel of this family has been characterized in the Kenyon cells of the honeybee (Pelz et al., 1999; B. Grünewald, personal communication). Due to the spatial interference with MAPK p44/42 localization in the honeybee MBs, this channel could be one of the potential targets of MAPK p44/42 in the honeybee brain.

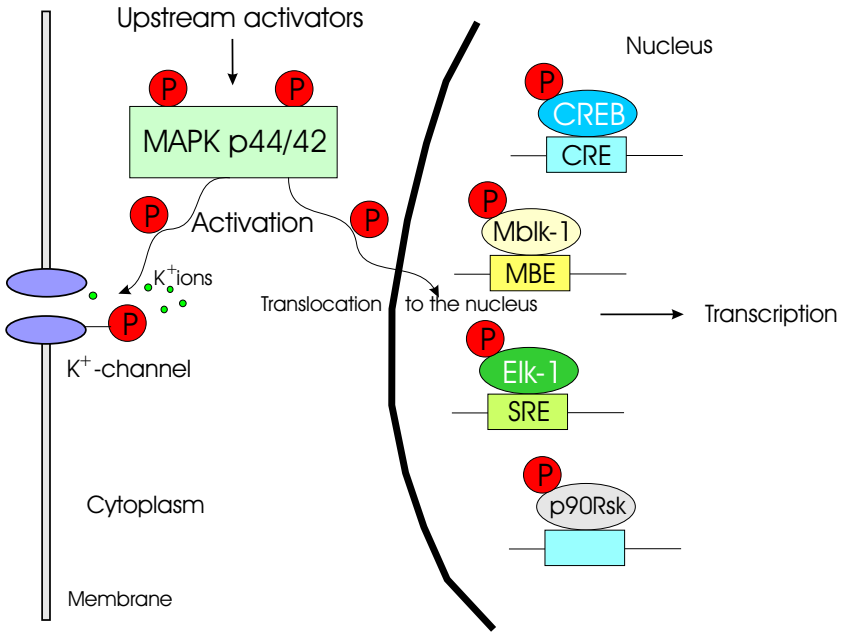

Fig. 4.2: Some potential cytoplasmatic and nuclear targets of MAPK p44/42 activation, which could be implicated in associative learning in the honeybee.

Synapsin is a synaptic vesicle protein and "marker" for synaptic activity. Involvement of synapsin in associative learning in *Drosophila* (Michels et al., 2005) and in LTP in rat (Sato et al., 2000) has been demonstrated. There were no direct comparison between honeybee learning and synapsin activity, but it was shown by Malun et al. (2001) that the various types of MB ablation differentially affected the amounts of synapsin, expressed in the central brain. Since the MB are involved in associative learning in the honeybee it can not be excluded that synapsin also interferes with LTM formation in the honeybee.

A series of experiments with contextual fear conditioning revealed that activation of both the transcription factors Elk-1 (ternary complex factor) and p90Rsk-1 (ribosomal S6 kinase-1) by MAPK p44/42 is specific for associative learning in rats (Sananbenesi et al., 2002 and 2003; Ahi et al., 2004). An increased phosphorylation level of p90Rsk-1 was observed after cued-contextual fear conditioning in the CA3 hippocampal area and also in the dentate gyrus of rats (Sananbenesi

et.al., 2002). Davis et al. (2000) have demonstrated the activation of the Elk-1 after induction of LTP in the dentate gyrus of rats. The Elk-1 is assembled on the serum response element (SRE), a DNA sequence motif present within the upstream regulatory region of immediate early genes, including *zif268* which is strongly upregulated in LTP (Bozon et al., 2003). Inhibition of the MAPK p44/42 activity blocks Elk-1 activation and thus SRE-regulated gene expression (Davis et al., 2000). Although the Elk-1 and p90Rsk-1 seem to be potential downstream effectors of MAPK p44/42 in the honeybee, these transcription factors have not been described in this animal so far.

A transcription factor termed Mblk-1 is a mushroom body-selective transcription factor. Mblk-1 is preferentially expressed in the large-type Kenyon cells of the honeybee brain (Takeuchi et al., 2001; Park et al., 2003) and functions in the MB neural circuits in the honeybee brain (Park et al., 2003). The binding site of Mblk-1 is termed MBE (Mblk-1-binding element). Park and team (2003) showed that Mblk-1 transactivates promoters containing MBEs and can be directly phosphorylated by Ras/MAPK pathway. MAPK p44/42 phosphorylates the Mblk-1 on the Ser-444 (recognition/phosphorylation sequence for the MAPK p44/42 are amino acids positions 442-445).

Also PKA and CaMKII can phosphorylate the Mblk-1 *in vitro* (Park et al., 2003). Since the Mblk-1 is localized in MB, a prominent structure in the honeybee brain involved in associative learning, and since it is activated via protein kinase cascades implicated in learning and memory formation, the Mblk-1 transcription factor might be also implicated in processes of learning in the honeybee. However the role of Mblk-1 in the honeybee associative learning has not been yet established.

Another potential substrate of MAPK p44/42, involved in learning processes, is the transcriptional factor CREB (4.2). Among the transcription factors involved in neuronal plasticity, learning and memory, CREB is the best characterized. It has been demonstrated by Zhang et al. (2003) that inhibition of MAPK p44/42 activity during associative learning can also block phosphorylation of the CREB in

rats. Some other studies pointed out the enhanced level of CREB phosphorylation in rat hippocampus after fear conditioning (Ahi et al., 2004) and LTP (Impey et al., 1998).

Interestingly, two isoforms of *Aplysia* CREB, CREB-1 and CREB-2, have an antagonistic effect on long lasting processes. For LTF to take place, not only activation of CREB-1 is required, but also the removal of the repressive action of CREB-2 (Bartsch et al., 1995).

Experiments on *Aplysia* revealed that injection of phosphorylated form of CREB-1 by itself could initiate the long-term memory process (Bartsch et al., 1998). CREB-2, in contrast, is a memory-suppressor gene which represses transcription mediated by CREB-1 (Bartsch et al., 1995; Michael et al., 1998). Injection of CREB-2 antibodies into *Aplysia* sensory neurons allowed LTF to be induced by a single 5-min 5-HT application (Bartsch et al., 1995). It has been demonstrated by Michael et al. (1998), that CREB-2 is phosphorylated by MAPK p44/42 followed repeated exposure to 5-HT; this in turn relieve its ability to inhibit long-lasting processes. Interestingly, that CREB-2 can also be phosphorylated by MAPK p38 (Guan et al., 2003).

CREB contains the basic domain/leucine zipper motifs and binds as dimers to cAMP-respons element (CRE) (De Cesare and Sassone-Corsi, 2000). CREB has been shown to activate C/EBP and AF, a set of immediate response genes, which in turn activate downstream genes, such as elongation factor 1α (EF1α). This chain of events gives 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) that converts short-term to long-term processes and may represent the mechanism underlying the long-term memory (Bailey and Chen, 1988). Similarly with the Mblk-1, it has been demonstrated that CREB transcriptional activity is also stimulated via PKA and CaMK pathways (Ahi et al., 2004).

In the honeybee, eight CREB-isoforms have been characterized (Eisenhardt et al., 2003). It has also been shown that injection of CREB antibodies impairs formation of LTM in the honeybee (Froese et al., 2005; D. Eisenhardt, personal

communication).

For better understanding the mechanisms underlying LTM formation in the honeybee, the role of various downstream effectors of MAPK p44/42 in LTM remains to be elucidated.