3 Results

3.1 Establishment of the proper protocols (methods)

In this work the role of the MAPK p44/42 and MAPK p38 in different forms of olfactory learning in the honeybee was investigated for the first time. Because manipulating these cascades has not been described in the honeybee before, it was necessary to adjust the technique of inhibition, activation, and detecting of particular proteins in these cascades. It was also necessary to find out the best way of MAPKs treatment, in order to avoid protein denaturation and to conserve actual levels of phosphorylation.

3.1.1 Treatment of the MAPK p44/42 and MAPK p38

With the quantitative determination of protein amount by ELISA and Western blot the question of the best protein treatment arose, how to prevent protein denaturation and fixate protein phosphorylation at its actual level. In a set of experiments two protocols of bee brain treatment before homogenization were compared. The first protocol has already been established in the lab and included an immediate preparation of living bees. Animals were decapitated and their heads mounted into hot wax while still living, so they were able to move its antennae. But since MAPKs can be activated by stimuli, such as a heat shock (Cuenda et al., 1995), another protocol of animal treatment was tested, namely

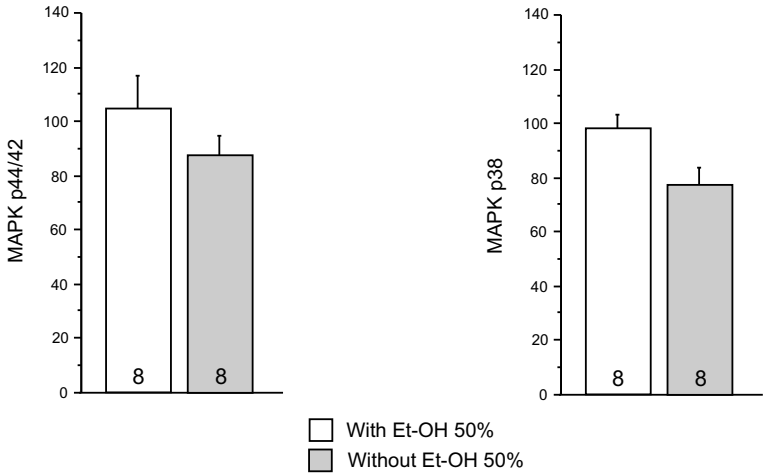

Fig. 3.1: Detectable amount of MAPK p44/42 (left) and MAPK p38 (right) after incubation in 50% ethanol or immediate preparation. Each column represents mean ± SEM values of n animals as indicated by the *numbers* on the bars.

that of incubation in ethanol at -20° for 20-25 min, following decapitation and mounting into hot wax. Three different ethanol concentration were tested: 20%, 50%, and 70%. The best result, i.e. a largest amount of the protein detectable after treatment, was obtained at the concentration 50% for both MAPK p44/42 and MAPK p38 (Fig. 3.1). So the incubation in 50% ethanol was applied for further experiments.

3.1.2 Mammalian antibodies work in the honeybee brain

In a first step it was also necessary to examine, whether mammalian antibodies against different members of the MAPK family can be applied to identify the corresponding proteins in the honeybee brain. Commercially purchased antibodies against MAPK p44/42, phospho-MAPK p44/42, MAPK p38, phospho-MAPK p38, as well as against MEK 1/2 and phospho-MEK 1/2 were tested using Western blotting. Antibodies against MAPK p44/42, MAPK p38 and MEK1/2 recognize the total amount of protein, whereas antibodies against phospho-MAPK p44/42, phospho-MAPK p38 and MEK1/2 detect level of protein only when phospho-

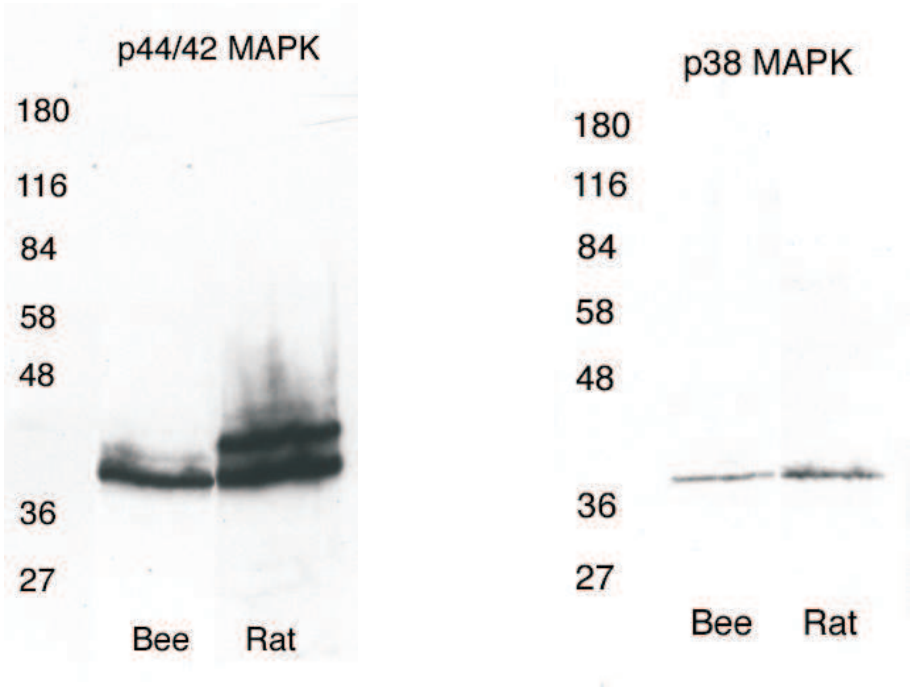

Fig. 3.2: Antibodies against MAPK p44/42 (left) and MAPK p38 (right) recognize corresponding proteins in vertebrates (rat) and in invertebrates (bee). Molecular weight (in kDa) is indicated on the left side of the pictures. Both bee and rat proteins are situated at the same attitude, i.e. they have the same molecular weight (42 and 44 kDa for MAPK p44/42 and 38 kDa for MAPK p38, respectively). Testing of anti-phospho-MAPK p44/42 and anti phospho-MAPK p38 antibodies revealed identical results.

rylated (Boulton and Cobb, 1991). Thus it allows estimation of the relative phosphorylarion level, using the ratio

$$\text{phosphorylation level} = \frac{\text{phospho-MAPK}}{\text{MAPK}},$$

where the phospho-MAPK indicates the level of phosphorylated protein and the MAPK indicates its total level.

The investigation revealed that only the antibodies against MAPK p44/42, phospho-MAPK p44-42, MAPK p38 and phospho-MAPK p38, work in the honeybee (Fig. 3.2). The Western blots of both MEK1/2 and phospho-MEK1/2

Fig. 3.3: The human and honeybee MAPK p44/42 sequences around the phosphorylation site. The latter is marked with a gray frame. Phosphorylation occurs at threonine-202 in human (threonine-159 in the honeybee) and tyrosine-204 in human (tyrosine-161 in the honeybee, respectively), with one glutamate in between for both organisms. It represents the dual specificity of the MAPK p44/42.

contained many smear bands, that revealed either non-specific binding, or ability to bind several antigenes.

Additionally, protein sequences were compared in vertebrates and the honeybee, using the sequence data bank (Fig. 3.3). This empirical analysis revealed that the MAPK sequence around the site of phosphorylation is highly homologous in vertebrates (human sequence in the picture) and the honeybee, and the phosphorylation motif itself (TEY) is fully identical.

3.1.3 Inhibition and activation of the MAPK cascade in vitro

For the series of experiments with *in vitro* inhibition and activation of the MAPK cascade it was necessary to establish an inhibitor that would impair phosphorylation of MAPK p44/42 in the honeybee. For inhibition of the MAPK p44/42 cascade an inhibitor of the upstream kinase MEK1/2, PD 098059, was selected. PD 098059 was identified by screening a compound library with a cascade assay measured phosphorylation (Alessi et al., 1995). PD 098059 inhibits MEK1/2 *in vivo* and *in vitro* (Pang et al., 1995; Dudley et al, 1995), which prevents phosphorylation of MAPK p44/42. Thereby it does not affect other kinases, such as PKA or PKC (Dudley et al., 1995; Alessi et al., 1995).

PD 098059 has successfully been used in *in vitro* experiments on *Aplysia* (Crow

Animal group	**Treatment/Incubation time**
1. Control group 1	PBS, 30 min
2. Control group 2	PBS + 20 μM PD 098059, 30 min
3. Activation	PBS + cAMP (10 μM) + ATP (50 μM), 30min
4. Inhibition/Activation	PBS + PD 098059 (20 μM), 10 min; then PBS + cAMP (10 μM) + ATP (50 μM), 20 min

Table 3.1: Activation and inhibition of the MAPK p44/42 *in vitro*. Four groups in the left column indicate four groups of animals; the substances added, as well as incubation time, are shown in the right column.

et al., 1998), and rodents' hippocampal slices (English and Sweatt, 1996; Selcher et al., 2003) as well as in behavioral studies (Blum et al., 1999).

In order to *in vitro* impair the MAPK p44/42 phosphorylation, its previous induction was required. For this purpose mixture of two substances, cAMP and ATP, was applied.

For quantification of the relative protein amount after different treatments, the ELISA-technique was used. Samples of CB-homogenate were prepared as described in "Material and Methods". Each sample was split into four vials which corresponded to four different treatments. The first and the second groups were left as controls, with the difference that into PBS for the second group PD 098059 was added. The samples from the third group were activated by mixture of cAMP and ATP, diluted in PBS. To the samples of the fourth group the inhibitor PD 098059 was added, and the samples were activated by the mixture described above. The amounts and time of incubation are shown in Table 3.1.

Since PD 098059 was diluted in DMSO, equal amounts of DMSO were added into PBS for the first and the third groups. Fig.3.4 **A** shows the basic level of the MAPK p44/42 for all groups of the samples. In Fig. 3.4 **B** the phosphorylated level of MAPK p44/42 is indicated. The third group of samples treated with MAPK p44/42-activating substances cAMP and ATP significantly differs from the other groups. In Fig. 3.4 **C**, where the relative amount of phosphorylated MAPK p44/42 is shown, the third group also differs significantly.

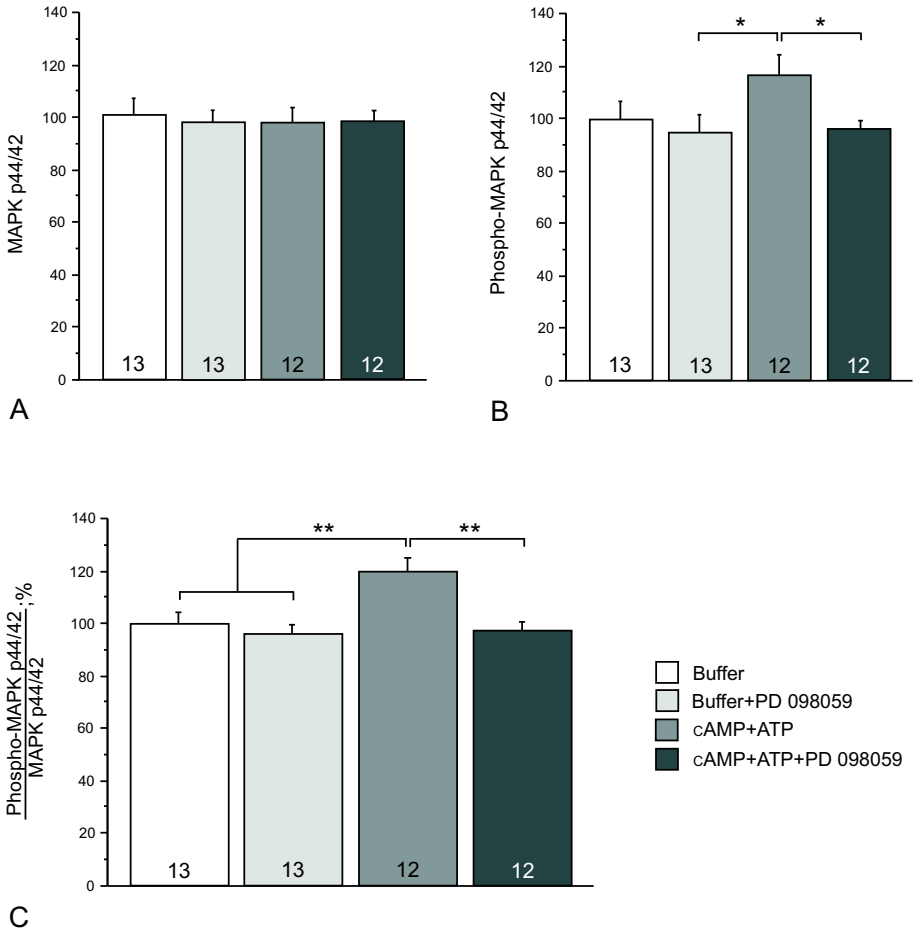

Fig. 3.4: Quantification of the MAPK p44/42 relative phosphorylation level after inhibition and activation *in vitro*. **A**: Basal level of the MAPK p44/42 of all groups of samples **B**: The level of phosphorylated MAPK p44/42 is increased in the group which was incubated with cAMP and ATP **C**: Relative phosphorylation of the MAPK p44/42. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. The group of samples activates with cAMP/ATP mixture significantly differs from the other groups. ANOVA, *p<0.05 and **p<0.005, t-test.

Animal group	**Treatment/Incubation**
1. Control group	PBS, 30 min
2. Activation	PBS + cAMP (10 μM) + ATP (50 μM), 30min
3. Inhibition/Activation	PBS + PD 098059 (20 μM), 10 min; then PBS + cAMP (10 μM) + ATP (50 μM), 20 min

Table 3.2: Activation and inhibition of the MAPK p44/42 *in vitro*. Three groups in the left column indicate for groups of animals; the substances added, as well as incubation time, are shown in the right column.

These findings demonstrate that the activation of the MAPK p44/42 cascade can be induced *in vitro* by application of 10 μM cAMP accompanied by 50 μM ATP and blocked by addition of an inhibitor PD 098059, at concentration 20 μM.

To demonstrate on the Western blot the effect of inhibiting and activating treatments, CB-homogenate of each individual bee brain was prepared as described in "Materials and Method". Samples of each bee brain were split into three groups: control; activated; inhibited and activated. As with the previous experiment, the first group of samples was left as a control, the second received the activating mixture of cAMP and ATP diluted in PBS, and the samples of the third group were consequently blocked by PD 098059 and activated by cAMP and ATP. The amounts and the time of incubation are indicated in Table 3.2. As with the previous experiment, PD 098059 was diluted in DMSO and equal amounts of DMSO were added to the PBS for PD 098059 non-treated groups. As can be seen in Fig. 3.5, phosphorylation of the MAPK p44/42 is affected by using PD 098059. The impairment of induced MAPK p44/42 phosphorylation is indicated by a weak staining of the corresponding band ("Pp"-band under "I"-group). The following treatment with cAMP and ATP does not lead to phosphorylation, whereas the same sample, treated with cAMP and ATP without adding PD098059, demonstrates a rather elevated level of MAPK p44/42 phosphorylation. This can be concluded from the stronger staining of the corresponding band ("Pp"-band in the "A"-group).

For inhibition of the MAPK p38 cascade SB 203580 was selected. It has been

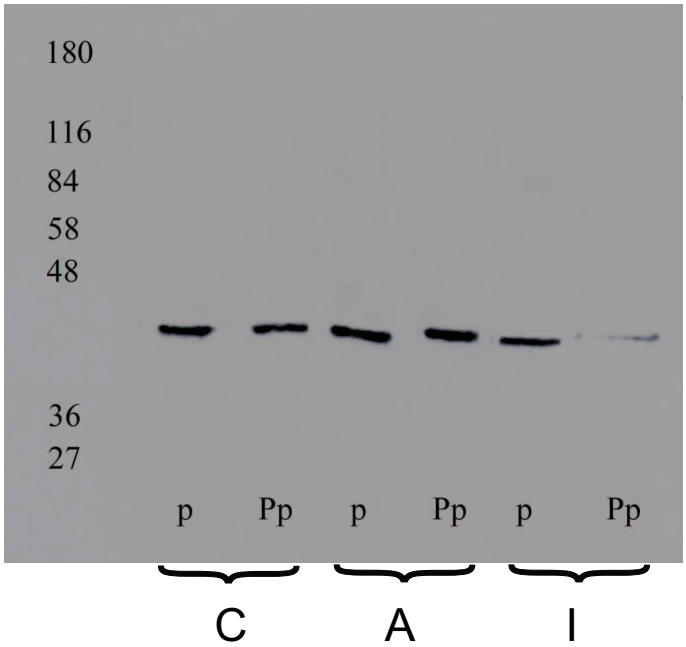

Fig. 3.5: Activation and inhibition of the MAPK p44/42 *in vitro*. The numbers on the left side of the picture show the molecular weight in kDa. The black bars indicate the non-phosphorylated (p) and phosphorylated (Pp) MAPK p44/42 in three groups: control (C), activated (A), and inhibited and then activated (I). Weak staining of the band which corresponds to the phosphorylated MAPK p44/42 in the "I"-group indicates efficacy of the inhibition.

widely used in *in vitro* (Cuenda et al., 1995; Ward et al., 1997) and *in vivo* (Zhen et al., 2001) experiments. However, the activation of MAPK p38 was hard to induce in the laboratory context. All stress stimuli tested (stress pheromones, nicotine, osmotic shock) did not cause significant increase of MAPK p38 phosphorylation.

3.2 Localization of the MAPK p44/42 and MAPK p38 in the honeybee brain

Immunohistochemical detection of MAPK p44/42, phospho-MAPK p44/42, MAPK p38 and phopho-MAPK p38 was performed by using antibodies against MAPK

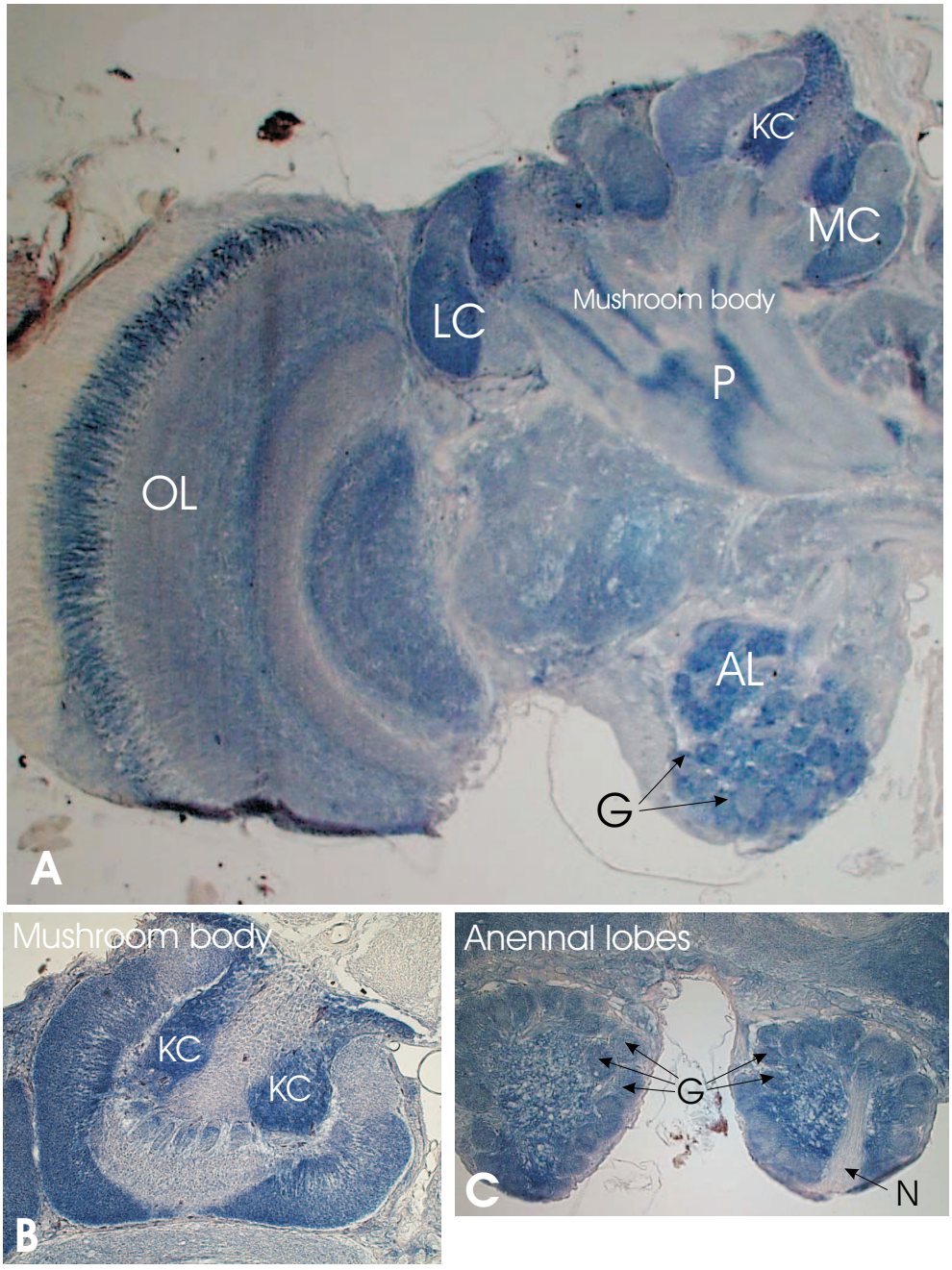

Fig. 3.6: Immunolocalization of the phosphorylated MAPK p44/42 in the bee brain. **A:** The central brain and optical lobes (OL). The strongest staining is observed in the mushroom bodies (MB) regions such as Kenyon cells (KC), lateral and medial calyces (LC and MC, respectively), pedunculus (P) and in the antennal lobes (AL). **B:** MB with stained Kenyon cells and calyces. **C:** Staining in the ALs. Glomeruli (G) and a neuronal tract (N) are indicated by arrows.

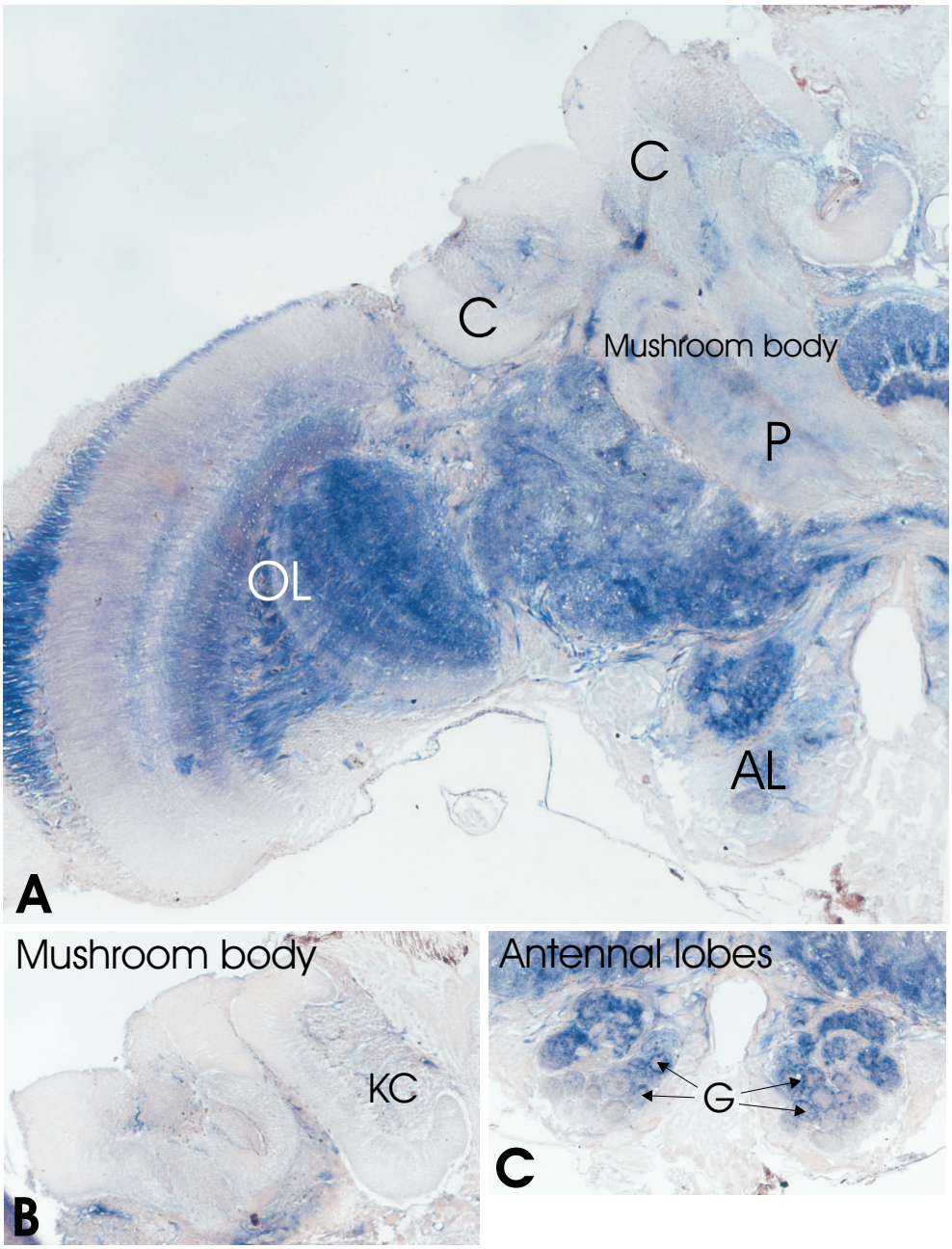

Fig. 3.7: Immunolocalization of the MAPK p38 in the bee brain. **A:** The central brain and optical lobes (OL). The strongest staining is observed in the antennal lobes (AL). **B:** Immunoreactivity in the MBs (C, calyx; P, pedunculus) is barely detectable. **C:** Staining in the ALs. Glomeruli (G) are indicated by arrows.

p44/42, phospho-MAPK 44/42, MAPK p38 and phospho-MAPK p38. As can be seen from Fig. 3.6**A**, phosphorylated MAPK p44/42 is predominantly localized in the mushroom bodies, antennal lobes, and optical lobes.

In both ALs and MBs, staining displays a clearly compartmentalized pattern. Thus, in the MBs, phospho-MAPK p44/42 is expressed in the Kenyon cells somata (Fig. 3.6 **A** and **B**), lateral and medial calyces, and pedunculus. In the ALs, the labeling is concentrated in the glomeruli (Fig. 3.6 **A** and **C**), which are the areas of synaptic connection between sensory neurons, local interneurons, and output interneurons which project to the MBs. In Fig. 3.6**C** a neuronal tract can be seen. The distribution of non-phosphorylated MAPK p44/42 is rather homogenous within the brain and the staining is considerably more weak.

MAPK p38 is predominantly expressed in the ALs, as shown in Fig. 3.7 **A** and **C**, some glomeruli are stained. In the MBs, the immunoreactivity is barely detectable (Fig. 3.7**B**). The immunihistostaining of phosphorylated MAPK p38 is rather weak in all brain compartments and therefore not shown.

3.3 Inhibition of the MAPK p44/42 phosphorylation does not impair non-associative learning

3.3.1 Habituation

For investigation of MAPK involvement into the non-associative forms of learning, MAPK p44/42 activity was inhibited during habituation and sensitization (see below). Bees received an injection of either PBS (control) or PD 098059 30 min before habituation of PER. The number of stimuli necessary for completed habituation was counted. Only the left antennae were stimulated. After habituation was completed, bees were dishabituated by a single stimulation of the right antenna. Fig. 3.8 shows that the inhibition of the MAPK p44/42 has no effect on habituation. Despite the large animal number, these groups do not differ from

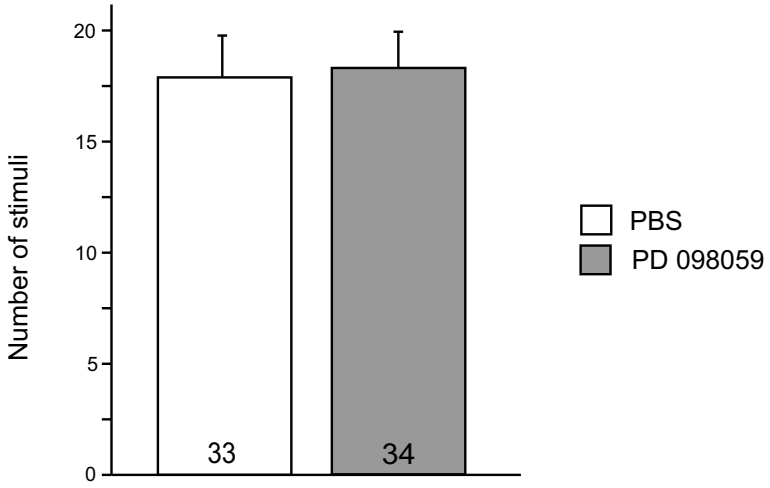

Fig. 3.8: Effect of MAPK p44/42 cascade inhibition on habituation. 30 min after injection of PD 098059. The vertical axis shows the number of stimuli necessary to attain habituation of the PER. Each column represents mean ± SEM values of n animals as indicated by the *numbers* on the bars. The groups do not differ significantly. p>0.05, Mann-Whitney U test.

each other significantly. Dishabituation in both the groups was also unaffected.

3.3.2 Sensitization

Sensitization is another form of non-associative learning and expressed by an increased facilitation to release a PER. In the experiment bees received a single sugar stimulation to an antenna and 30 min later they received an odor stimulus. Inhibition of the MAPK p44/42 activity 30 min before stimulation did not impair sensitization, see Fig. 3.9. The percentage of the PD 098059-treated animals which showed PER after the sensitization does not differ from the animals treated with PBS.

Since neither habituation nor sensitization are impaired by MAPK p44/42 activity inhibition, it can be supposed that MAPK p44/42 is not implicated in the non-associative form of learning in the honeybee.

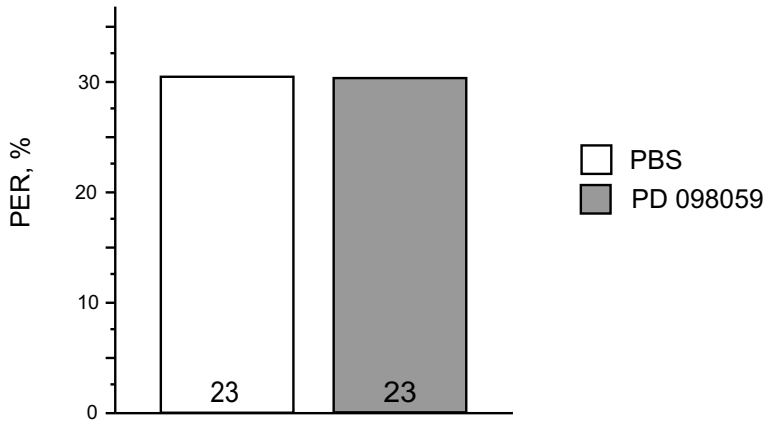

Fig. 3.9: Effect of inhibition of MAPK p44/42 activity on sensitization 30 minutes after injection of PD 098059. The data represent PER % of n animals as indicated in the columns. The PBS- and PD 098059-injected groups do not differ significantly. p=0.00, χ^2-test.

3.4 Quantification of the relative phosphorylation level of MAPK p44/42 and MAPK p38 at different time points after associative olfactory learning

In order to define whether MAPK p44/42 and MAPK p38 are involved into associative olfactory learning and, if so, to identify the time window of their activation resulting from olfactory conditioning, the levels of relative MAPK p44/42 and MAPK p38 phosphorylation at different time points after learning was quantified using ELISA.

In this series of experiments, bees were conditioned either multiple (three pairings CS-US) or received only the US three times. Before analyzing the phosphorylation level, it was necessary to prove that the treatment does not cause alteration of the MAPKs total level and the latter does not change during the experiment. Brain samples of the animals were analyzed at 15, 40, 120 and 240 min after conditioning or presenting the US. Fig. 3.10 and Fig. 3.11 demonstrate

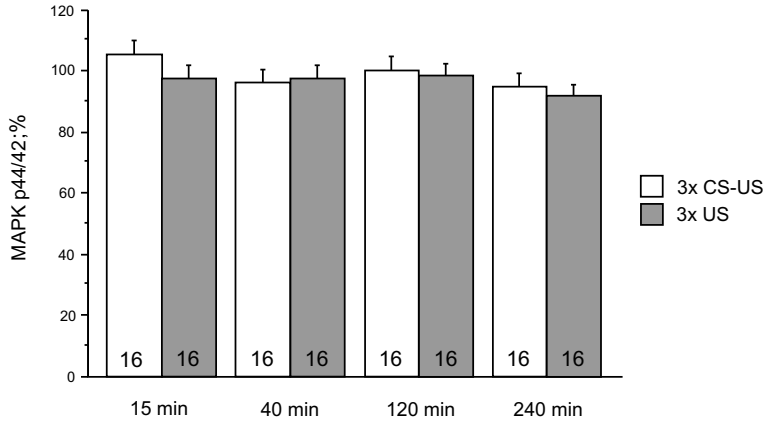

Fig. 3.10: Relative amount of the total MAPK p44/42 at the time points 15, 40, 120 and 240 min after conditioning. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. The differences are not significant. ANOVA, p>0.05, t-test.

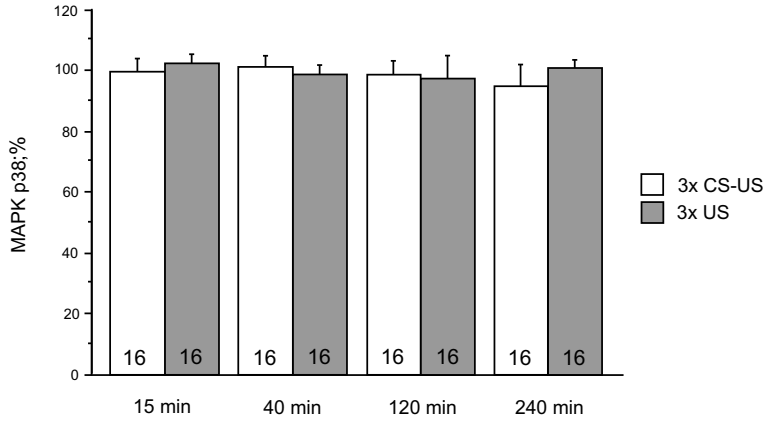

Fig. 3.11: Relative amount of the total MAPK p38 at the time points 15, 40, 120 and 240 min after conditioning. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. The differences are not significant. ANOVA, p>0.05, t-test.

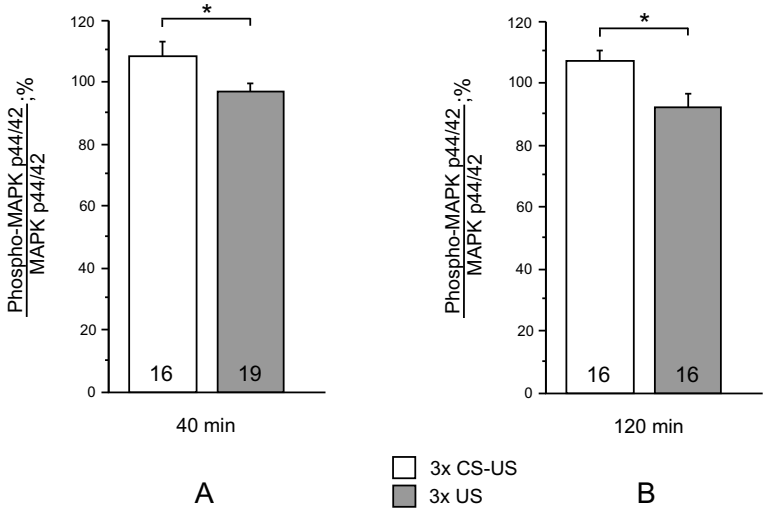

Fig. 3.12: Relative phosphorylation of MAPK p44/42 in the central brain of the honeybee 40 (**A**) and 120 (**B**) minutes after stimulation. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. *p<0.05, t-test.

the total amount of MAPK p44/42 and MAPK p38, respectively, at these time points.

In a next experiment bee were conditioned analogically to the previous experiment (3x CS-US vs. 3x US) and relative phosphorylation of MAPKs was analyzed at the same time points: 15, 40, 120 and 240 min after training. For MAPK p44/42 a significant difference between 3x CS-US and 3x US conditioned animals was observed after 40 min and lasted until 120 min after learning (Fig. 3.12). Therefore further experiments were performed at one or both of these time points.

In the next step the question was whether ALs or MBs contribute to an increased observable MAPK p44/42 activity level. So I measured the relative phosphorylation level in these brain parts separately from each other. The results show, that at 40 min after learning an increased level of MAPK p44/42 phosphorylation exists both in ALs and MBs (Fig. 3.13), whereas at 120 min it is observed only in MBs (Fig. 3.14).

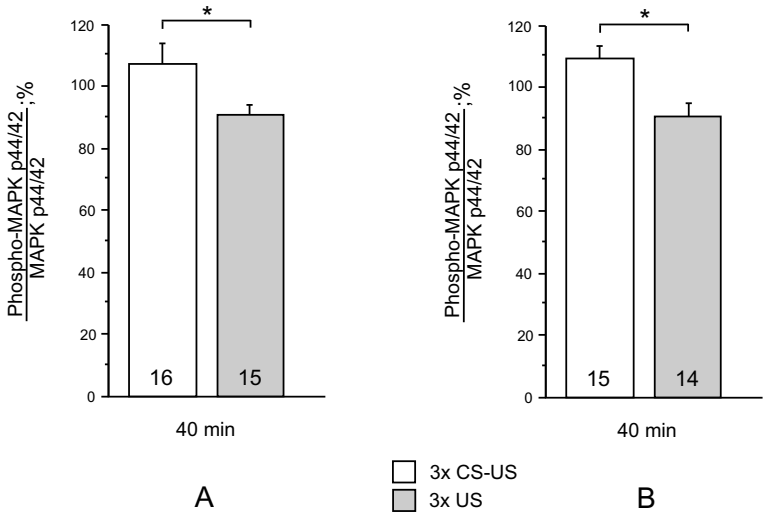

Fig. 3.13: Relative phosphorylation of MAPK p44/42 in the antennal lobes (**A**) and mushroom bodies (**B**) of the honeybee 40 minutes after stimulation. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. *p<0.05, t-test.

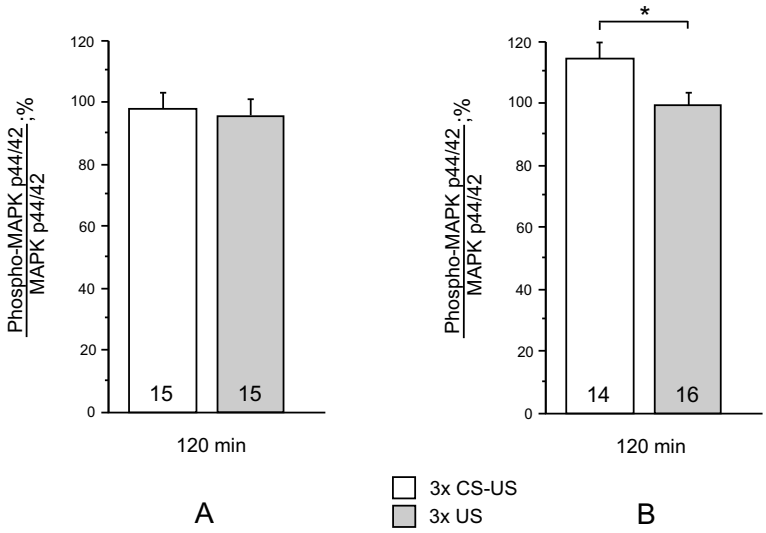

Fig. 3.14: Relative phosphorylation of MAPK p44/42 in the antennal lobes (**A**) and mushroom bodies (**B**) of the honeybee 120 minutes after stimulation. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. *p<0.05, t-test.

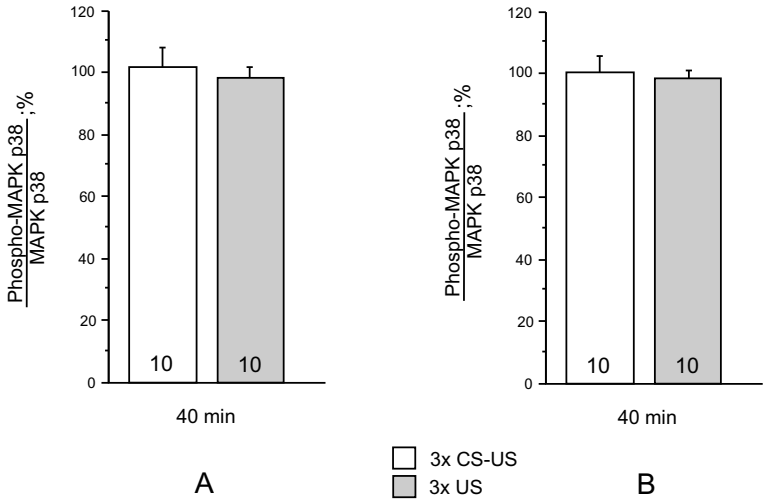

Fig. 3.15: Relative Phosphorylation of MAPK p38 in the antennal lobes (**A**) and mushroom bodies (**B**) of the honeybee 40 minutes after stimulation. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. p>0.05, t-test.

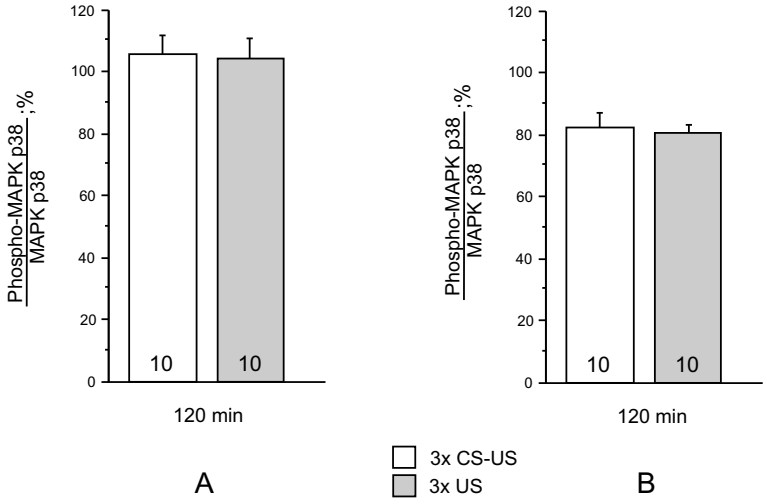

Fig. 3.16: Relative phosphorylation of MAPK p38 in the antennal lobes (**A**) and mushroom bodies (**B**) of the honeybee 120 minutes after stimulation. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. p>0.05, t-test.

Despite the continued level of the MAPK p38 in the central brain, ALs and MBs were analyzed separately. The change in MAPK p38 relative phosphorylation level might have been so slight and fine that analyzing ALs and MBs together might have given no evidence. Another possibility might have been that a decrease in the phosphorylation state in one of the brain area abolishes an increase in another. However, the results demonstrated neither an increase nor a decrease in relative phosphorylation state of the MAPK p38 (Fig. 3.15 and Fig. 3.16).

These findings suggest, that only MAPK p44/42, and not MAPK p38, is mediated by associative olfactory learning in the honeybee.

3.5 Comparison between forward-, backward-conditioned and naive animals

Since MAPK p38 is not mediated by associative olfactory learning in the honeybee, the following experiments were dedicated to the further investigation of the MAPK p44/42 and its involvement into the olfactory learning. The findings above arose the question of where the enhanced level of relative MAPK p44/42 phosphorylation, resulting from olfactory conditioning, comes from. One possibility would be that the phosphorylation level in conditioned bees is rally enhanced compared to the phosphorylation level in naive animals. On the other hand, the difference between 3x CS-US vs. 3x US conditioned groups might result from a decrease of MAPK p44/42 phosphorylation in the 3x US-stimulated bees.

To answer this question forward (3x CS-US), backward (3x US-CS), and naive bees were compared. Additionally a group of 1x CS-US conditioned animals was tested, to check if a single pairing would result in an increased activity of MAPK.

Relative phosphorylation was measured in MBs 40 minutes after training. Mushroom bodies were chosen due to more stable significant difference between groups (see previous experiment). The relative amount of MAPK p44/42 phosphorylation was measured by ELISA. In Fig. 3.17 four groups of animals are

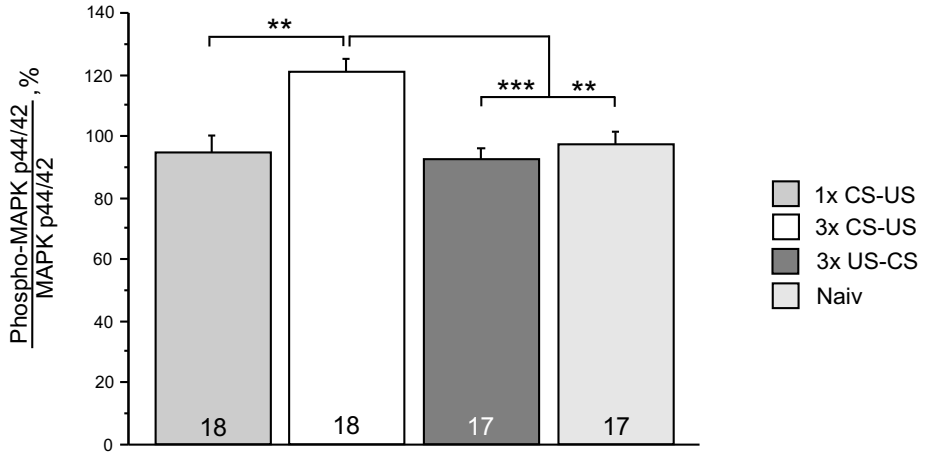

Fig. 3.17: Relative phosphorylation of MAPK p44/42 in the mushroom bodies of the honeybee 40 minutes after stimulation. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. **p<0.005, ***p<0.0005, t-test.

shown. The group of multiple forward conditioned animals differs from the other three (multiple backward conditioned, single trial forward conditioned and naive animals) significantly, the difference is app. 20%.

These findings clearly demonstrate that the increased phosphorylation and thus the activity of MAPK p44/42 is a result of multiple forward conditioning.

3.6 Inhibition of the MAPK p44/42 activation 30 min before conditioning impairs MAPK p44/42 phosphorylation

To show that inhibition of upstream kinase MAPKK (MEK1/2) activity during conditioning affects the phosphorylation of MAPK p44/42 afterwards, bees were injected with PBS (control) and PD 098059 30 min before training. Then each group of animals were conditioned either 3x CS-US or 3x US-CS, so there were four groups (Fig. 3.18). Relative phosphorylation of MAPK p44/42 was measured

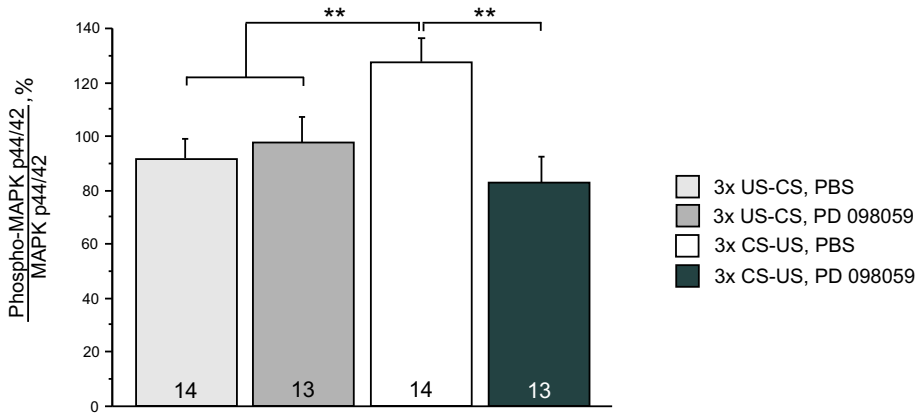

Fig. 3.18: Inhibition of the MAPKK p44/42 (MEK1/2) 30 min before conditioning. Relative phosphorylation at 40 min after multiple forward and backward training is shown. The 3x CS-US PBS-injected group differs from the 3x US-CS PBS-injected, 3x CS-US PD 098059-injected and 3x US-CS PD 098059-injected groups. Each column represents mean ± SEM values of n measurements as indicated by the *numbers* on the bars. $^{**}p<0.005$, t-test.

in MBs 40 min after conditioning using ELISA.

The results of this experiment demonstrate the impairment of the MAPK p44/42 phosphorylation in the PD 098059-injected forward conditioned group. Even though bees injected with PD 098059 were also conditioned by forward multiple trials, they do not indicate an increased level of MAPK p44/42 activity. The PBS-injected and forward conditioned group differs significantly from and the other groups tested.

3.7 Inhibition of the MAPK p44/42 phosphorylation before, during and after associative learning

To investigate the MAPK p44/42 contribution to the LTM formation and for a definition of the MAPK p44/42 activation character on the behavioral level, multiple conditioned bees were observed during four days after training. To prove that this kinase is involved in LTM formation, the MAPK p44/42 cascade was

blocked at different time points before, during and after conditioning. As in the other experiments, PD 098059 was chosen as an inhibitor of MAPK p44/42 activation. Bees were treated as described in "Materials and Methods". Injections were performed 24 h and 30 min before conditioning, 30 min, 2 h, 4 h and 24 h after conditioning. PD 098059 has a transient character of action (Dudley et al., 1995), so the injection 24 h before conditioning does not block the MAPK p44/42-cascade any longer. To check the acquisition and LTM formation, the animals were tested by giving them a single CS at time points 2 h, 24 h, 48 h, 72 h and 96 h after learning. Fig. 3.19 demonstrates that inhibition of the MAPK p44/42 activity only during and shortly after olfactory conditioning leads to LTM impairment at day 2-4 after conditioning, whereas the difference between PBS- and PD 098059-injected groups at the third and fours days is more significant then at the second day after conditioning. These findings demonstrate that activation of MAPK p44/42 cascade during and shortly after learning is required for LTM formation in the honeybee.

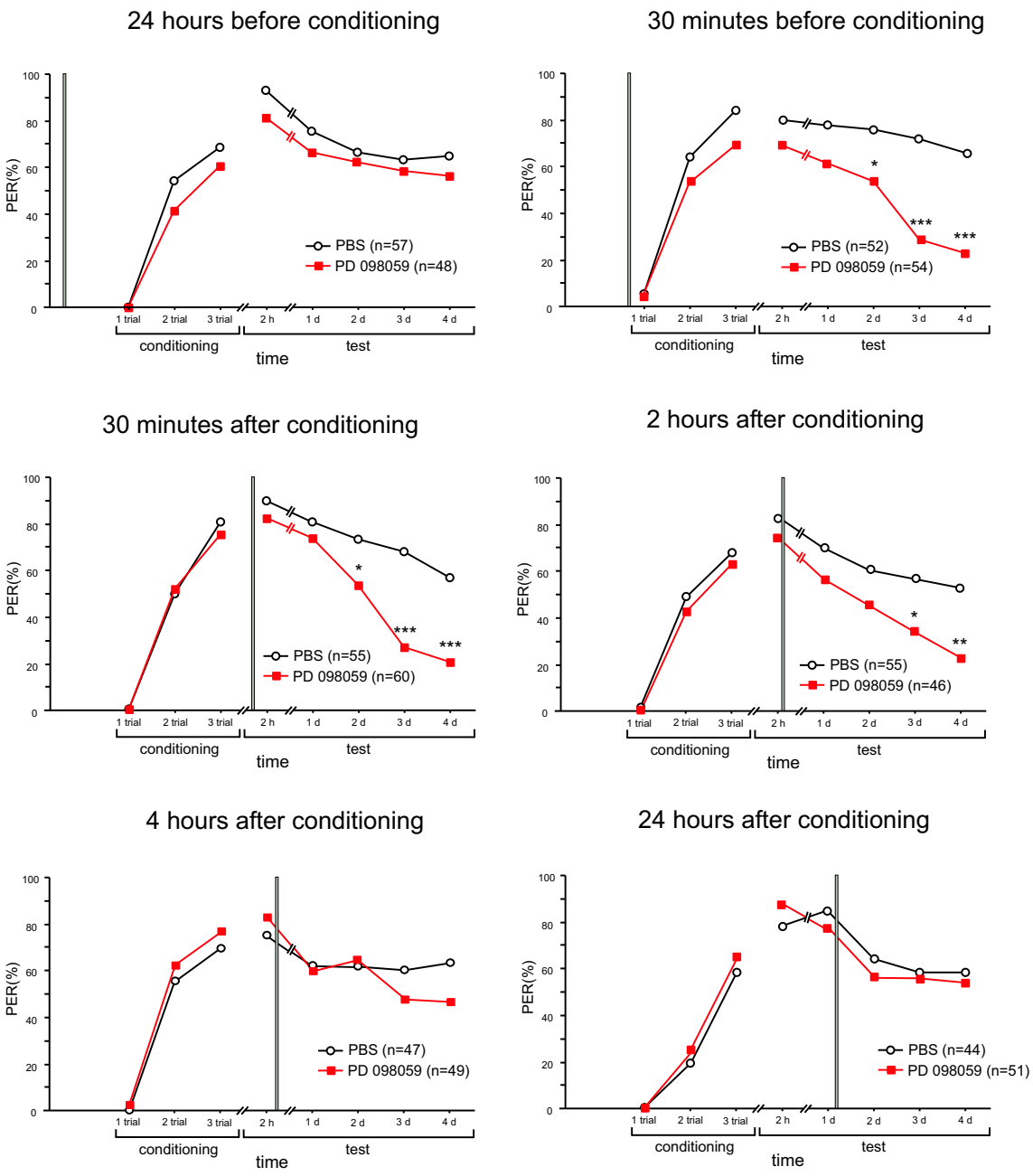

Fig. 3.19: Inhibition of MAPK p44/42 activation at different time points before or after conditioning. On the horizontal axis duration of the experiment is shown: conditioning 1-3 trials with ITI 2 min; test of acquisition 2 h after conditioning and memory retention test up to for days after conditioning. Each data point represents results from n animals. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$; χ^2-test.