2 Materials and Methods

2.1 Methods

2.1.1 Experimental animals

All the experiments were performed on the honeybee *Apis mellifera carnica* adult foragers, caught in the afternoon when leaving the hives for foraging. To avoid genetic homogeneity, bees were caught from different stocks. For each single experiment, which lasted a few days, bees were taken from the same stock, or hive, and at the same time of the day, to avoid differences between control and experimental groups.

Bees were collected one day before the experiment, immobilized by cooling on ice and harnessed in metal tubes with a strip of tape between the head and the thorax, allowing the proboscis and antennae to move freely. In the evening, 18 h before the experiment, all bees were fed to satiation with 30% sucrose water solution, 4-5 drops per bee. Bees were kept overnight in darkness a plastic container at relative air humidity 70% and temperature 20-25° C. Animals were also fed 1 h after retrieval test and every evening to satiation.

Behavioral experiments were performed during the time from May until beginning of October. For *in vitro* experiments animals were collected also during the winter as well, from the artificially climatized flight room. All the experiments were performed at room temperature.

The responsiveness to sucrose solution was tested 15 min before the experiment by applying a single sucrose stimulation to an antenna. Extension of the proboscis triggered by sucrose was monitored and used as a measurement of the sucrose

responsiveness of each bee. Bees which did not react and did not extend the proboscis were left out of experiment.

For the systemic injection of the drug or PBS into the hemolymph of the animal a glass capillary with total volume 20 μl was used. The capillary was inserted into the small hole poked into the thorax, using a metallic curved stick. The injected volume i constituted 0.5 μl. Injection was performed not only 30 min before the olfactory conditioning, as described by Müller and Hildebrandt (1995), but also 24 h before, 30 min, 2h, 4h, and 24 h after conditioning.

For experiments with a pharmacological inhibition of the MAPKK (MEK) activity the synthetic drugs PD 098059, which was shown to significantly affect activity of MAPKK (Dudley et al., 1995; Alessi et al., 1995) and U0126 (Favata et al., 1998) were used. For the inhibition of MAPK p38 cascade SB 203580 (Lee et al., 1999; Thurmond et al., 2001) was applied. In order to evaluate effect of the injection treatment for the animals, bees from the control group were injected with PBS.

2.1.2 Behavioral methods

Habituation and dishabituation

Habituation of the Proboscis Extension Reflex (PER) was performed using 30% sugar solution. Bees were stimulated with a small wooden toothpick on the same antenna once per second. Increasing the number of the stimuli decreases the number of positive responses to the stimulation, until the bee does not extend the proboscis any longer. Such a bee was considered habituated. To show that the bee is still able to respond to the stimulus, the opposite antenna was stimulated with a single sucrose stimulation. The bee extended its proboscis, hence becoming dishabituated. For the statistical the number of applied stimuli required to habituate each single bee was considered. For the statistical comparison of two groups the Mann-Whitney *U*-test was applied.

Sensitization

To sensitize the PER, the bee was first presented with a CS (odor) stimulation, to test the spontaneous reactivity. Then the bee received a single sucrose stimulation to one antenna. Thirty seconds later the odor stimulus was presented on both antennae to test the sensitization. It has been shown (Menzel et al., 1991) that presentation of the sugar excitement increases the possibility of PER in response to the odor stimulus for a few minutes.

For the statistics percentage of animals with positive responses to the presented odor stimulus after sensitization was recorded. Statistical analysis was carried out using the two-sided (bilateral) χ^2-test.

Olfactory conditioning

In the olfactory conditioning of PER a conditioned olfactory stimulus (CS) and an unconditioned stimulus (US) were temporary paired. As a conditioned stimulus carnation oil was used, as an unconditioned stimulus 30% sugar water solution. Carnation oil was dropped onto filter paper and put into a plastic syringe of 20 ml volume with removed piston. To deliver this solution to the bee antennae a clean wooden toothpick was used.

Before conditioning, each bee was placed into the experimental situation in front of an exhauster. The odor (CS) was blown to both antennae for approximately 4 seconds. Two seconds after commencing CS, the sugar solution (US) was applied to both the antennae of the bee, for approximately 2 seconds, so that the total duration of the CS-US paring was approximately 5 seconds (Menzel 1968). In case of multiple trial training the olfactory conditioning procedure was repeated 3 times, with 2 min inter-trial interval.

In the series of experiments with backward conditioning, the CS and US were presented in a backward order, so that CS followed application of US. In some cases only the US was applied.

In the retention tests 2, 24, 48, 72, 96 hours after conditioning the CS was presented alone, and the animals responding with or without PER were calculated

for each group.

The data were analyzed using the ANOVA-test for repeated measurements. If appropriate, a χ^2-test was used as a *post hoc* test.

2.1.3 Immunohistochemistry

Bees were cooled down on ice and then decapitated. Bee brains were dissected and fixed in PBS containing 4% formaldehyde for 2h at 4 C°. The tissue was dehydrated in increasing grades of ethanol (50-100%), terminating in xylene and subsequently incubated for infiltration in paraplast (Sigma) and embedded in the latter for sectioning. The sections (10μm) were mounted on poly-D-lysine coated slides. After rehydration, starting with xylene followed by decreasing grades of ethanol (100-50%), the slides were washed two times (10 min each) with PBS-Tx (PBS, containing 0.1% Triton X-100). Prior to antibody application the slides were blocked against non-specific binding with blocking solution (PBS-Tx containing 1% BSA) for 1 h at RT. The antibodies diluted 1:500 in blocking solution were applied to the sections and incubated for 12 h at 4 C°. Then the section were washed (three times 15 min each) in PBS-Tx, and incubated for 2 h at RT with biotinylated anti-rabbit IgG diluted 1:2000 in blocking solution. After three 10-min washes, the slides were incubated with streptavidine-alkaline phosphatase complex diluted 1:2000 in blocking solution for 1 h at RT. The color reaction was developed in 20 ml 0.1 M^{-1} TRIS-buffer, pH 8.8, containing 0.1 M^{-1} NaCl, 1 M^{-1} $MgCl_2$, 1 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate and 0.5 mg NBT (nitro blue tetrazolium). After termination of the staining process, sections were dehydrated and mounted with Entelan.

2.1.4 Western blot

Western blotting was used for two series of experiments: for comparison of the MAPK p44/42 and MAPK p38 from rat and bee brains, and for finding substances which would be able to activate MAPK as well as to block its activity in the honeybee brain.

Preparation of the bee and rat brain homogenate samples for comparative analysis

Bee To extract bee brains for comparative analysis with rat brain by Western blotting, bees were immobilized on ice and their heads were cut off with a razor blade. The heads were mounted into hot wax using a soldering gun. The heads were opened frontally by a flat cut. The glands, tracheae and ocelli were gently removed with fine tweezers. The brain was placed into a glass-glass douncer for homogenization in the Western Blot Buffer.

Four bee brains were homogenized in the same douncer in 300 μl of Western Blot Buffer. After homogenizing the tissue, the samples were placed into a vial and Loading Buffer was added in a ratio of 1:5. The total volume in the vial was 360 μl.

Rat A piece of frozen rat brain of weight 0.0254 g was thawed and homogenized in 500 μl of the Western Blot Buffer. The homogenization was performed in a glass-glass douncer. To further decrease the protein concentration, 50 μl of rat brain homogenate was taken and diluted in 150 μl of the Western Blot Buffer. 300 μl of the brain material was afterwards taken and put into a plastic vial of total volume 1.5 ml. The Loading Buffer was added into each vial in a ratio of 1:5 (60 μl of the buffer was added), so that the total volume in each vial reached 360 μl.

Both the bee and rat samples were heated in a steam bath for 3 min. Next, the probes were loaded onto stacking gel for electrophoresis, 30 μl per a slot alternating bee and rat samples. Pre-stained marker was loaded beside the samples.

Electrophoresis In the Western blotting procedure the proteins from the brain samples (for the preparation of the samples see above) were electrophoretically resolved on 12% SDS-polyacrylamide gels in the running buffer at 25 mA for each gel during approximately 40 min. Samples from differentially treated groups alternated. To determine molecular weights, the pre-stained SDS molecular weight standard mixture ("pre-stained marker") from Sigma was used.

Blotting After a successful electrophoresis, gels and filter paper were placed into a cuvette filled with the blotting buffer for impregnation. The following transmission of the proteins from the SDS-gel to a nitrocellulose membrane was performed in the Fast Blot Chamber proceeded at the current force 0.4 mA, voltage 22 V and duration 40 min for one gel. For two and more simultaneously blotted gels the current force needed to be increased at further 0.4 mA for each additional gel. Before starting the blotting, the membrane, gel and filter paper were carefully placed into the Fast Blot Chamber in the following order: 3 layers of filter paper, nitrocellulose membrane, gel, 3 layer of filter paper. To allow blotting, the filter paper, nitrocellulose membrane and gel might be imbued with running buffer.

Blocking In order to bind the free sites remained on the nitrocellulose membrane after blotting, the membranes were incubated in a Blocking Buffer (0.5% BSA in PBS) with 2-3 drops of Tween. For the procedure of blocking membranes were placed into small glass containers with blocking buffer, which were gently shaken on the mixer during 1.5 h at RT.

Primary antibody Immediately after blocking antibodies against either MAPK p44/42, phospho-MAPK p44/42, MAPK p38, or phospho-MAPK p38 were applied to the membrane. These antibodies were diluted 1:2000 in the Blocking Buffer plus Tween. Since the samples from different groups of animals alternated, the membrane was cut into strips using a razor blade. Thus, each stripe carried either one sample or pre-stained marker (for details see below). The membrane stripes were incubated in these primary antibody solutions for 12 h at +4° C with gentle mixing.

Secondary antibody Next morning the membrane strips were carefully washed (three times for 5 min each) in PBS, and secondary antibodies (peroxidase-conjugated anti-rabbit), diluted 1:10000 in the Blocking Buffer, were applied to the membrane for 1-1.5 h at RT. After incubation with the peroxidase the

membrane strips were again washed (three times for 5 min each) in PBS, and put together onto two film stripes reconstructing the original order. After this the reconstructed membrane was placed into a small plastic cuvette containing chemiluminescence reagents solution for 3 min. Immediately after this the membrane was covered with two polyethylene foils (above and below the membrane), to protect the X-ray film from moisture. The membrane was exposed to a Kodak X-ray film three times: for 20 s, for 60 s and for 5 min. Films were developed and fixed in special photo-reagents until the films became transparent. Then the films were washed with plenty of water and dried by warm air. The film with the best image resolution was taken for the further consideration.

Preparation of the bee brain samples for in vitro activation of the MAPK by Western Blot

Bee heads were mounted into wax and opened as described above. After removing the glands, tracheae and ocelli, the optical lobes (OL) were dissected from the central brain (CB) by two longitudinal cuts. CB was then homogenized in 500 μl PBS in a glass-glass douncer during 20 s. The CB-homogenate from each bee was split into three plastic vials which corresponded to three different treatments afterwards. The first group of samples were incubated in PBS on ice for 30 min. The activating mixture was added to the second group of samples for 30 min. The samples of the third group were incubated in PD 098059 solution for 10 min, then activated for 20 min by the same mixture as the samples from the second group. Since PD 098059 was diluted in DMSO, equal amount of DMSO was added to PBS for PD 098059-non-treated groups.

After these treatments the 10 μl of Loading Buffer was added into each sample group, and they were immediately loaded onto the gels for electrophoresis.

2.1.5 Quantitative determination of the relative protein amount by ELISA (Enzyme Linked Immuno-Sorbent Assay)

For ELISA-analysis Falcon pro-bind 96-well polystyrene plates were used. Before coating the plates with samples, all wells of each ELISA-plate were filled with 50 μl of the ELISA buffer.

Preparation of the central brain, mushroom bodies and antennal lobes samples for ELISA

For *in vitro* experiments, bees were immobilized on ice and their heads were opened by a flat cut as described above.

Animals for conditioning experiments underwent head opening by cutting a small square window in the head capsule one day before the experiment. At different time points (depending on particular experiment, 15, 40, 120 and 240 min) after conditioning, animals were put into 50% ethanol and incubated for 25-30 min at -20° C. This was done in order to terminate the processes of MAPK phosphorylation and to prevent dephosphorylation. After that the bees were decapitated and their heads were mounted into hot wax and opened. In both cases, the glands, tracheae and ocelli were removed.

For *in vitro* experiments the CBs, including mushroom bodies, antennal lobes and protocerebrum, were dissected from the optical lobes and homogenized in 1000 μl of PBS and transferred into plastic vials.

For the conditioning experiments either CBs or antennal lobes (ALs) and mushroom bodies (MBs), separated from each other by a horizontal cut (Fig. 2.1), were homogenized in ELISA buffer (a CB in 1000 μl; ALs in 500 μl and MB also in 500 μl). The AL and MB samples contained also a part of protocerebrum (due to cutting). Then samples were placed onto ELISA-plates.

For the *in vitro* experiments each bee brain homogenate was divided into four groups. Each group was treated separately until loading onto ELISA plates (see

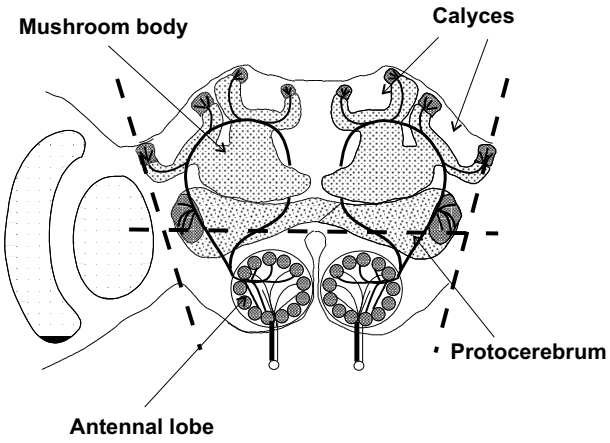

Fig. 2.1: Schematic image of the bee brain. The main parts involved in learning processes are marked. The dashed lines indicate the cuts made during preparation.

"Results").

Coating All ELISA-plates were pre-filled with 50 μl of the ELISA buffer.

For quantitative determination using ELISA, the plates were coated with the samples from the bee brain, where they bound to high-bonding surface of the well. The samples were spread in the first column, one sample per well. To allow analysis the same sample with two antibodies (against MAPK and phospho-MAPK), this distribution was repeated in the seventh column of the plate. Thus, each sample existed in two wells. The volume of added sample constituted 50 μl per a well and the total volume in the well implemented 100μl. Then, within the row of wells, content of each sample was well mixed and diluted using a multi-channel pipette. The dilution series was carried out, with each sample in five different concentrations and the sixth and the twelfth columns were left blank (concentration step "0"). Thus, the dilution factor was 1:2. The rest remained in the pipette after the fifth and the eleventh columns was thrown away. Thus, the volume of content in each well was again 50 μl. To allow better coating, 100 μl of the ELISA buffer was added into each well. The total amount was 150 μl per well. After this treatment the ELISA-plates were incubated in a fridge at +4° C for 1

h. Within this time the protein molecules bound to the special protein-binding sites of the microtiter on the surface of the wells.

Blocking After binding of the antigene to the wells, the liquid content of the wells was removed and the plate was consequently incubated in the Blocking Buffer (5% BSA in PBA, 300 μl into each well) for 1.5 hours, gently shaking at RT or +4° C. The incubation in buffer containing BSA was performed in order to block the remaining binding sites of the microtiter.

Primary antibody After 1.5 h the Blocking Buffer was removed and the primary antibodies either against MAPK p44/42 and phospho-MAPK p44/42 or against MAPK p38 and phospho-MAPK p38 diluted 1:2000 in the Blocking Buffer, were applied. Each sample was analyzed with both phospho- and non-phospho-antibodies. The plates were incubated at +4° C during 12 h with constant shaking.

Secondary antibody Next morning the plates were washed with PBS (three times for 5 min each), and the plates were incubated for 1 h with the biotinylated secondary antibody (anti-rabbit (IgG), diluted 1:4000 in the Blocking Buffer, 100 μl per well). Subsequently the plates were again washed with PBS (3 times each 5 min) and then incubated for 40-45 minutes in avidin-coupled, conjugated with alkaline phosphatase (ExtrAvidin), diluted 1:10 000 in the blocking buffer, 100 μl per well.

Staining After following final washes in PBS (three times for 5 min each) the content of the wells was stained by addition of the substrate ortho-nitro-phenyl-phosphate (ONPP), diluted in RXN buffer, 7 mg in 20 ml of 1mM RxN buffer. 200 μl was added to each well.

To quantify the amount of antigen, the substrate conversion by phosphatase was measured with an ELISA-reader at the wave length 405 nm versus 620 nm background.

Data analysis The optical density values of the dilution series of one brain resulted in a linear function which slope reflected the concentration of the antigen. To compare measurements between different ELISA plates, each plate was coated with samples representing all treatment groups within particular experiment. Because only measurements performed on the same ELISA plate can be directly compared and only the relative protein amounts are detectable using this method, either eight CB-samples or four MB- and four AL-samples were measured on one plate using two different antibodies. The slopes measured on each plate were normalized to the mean value of the measurements of the control group of brain samples, resulting in a mean value of 1 ±SEM. Four to sixteen ELISA plates were measured per experiment, and the normalized values were pooled. Since the values in each measuring group were normally distributed, the unpaired *t*-test was used to proof the significance.

2.2 Materials

2.2.1 Buffers and solutions

Name	Amount	Units	**Content**
PBS	137	mM	NaCl
	2.7	mM	KCl
	10.1	mM	Na_2HPO_4
	1.8	mM	KH_2PO_4
Blocking Buffer	0.5	%	BSA in PBS
ELISA buffer			PBS
	1	M	UREA
	1	mM	EGTA
	1	mM	EDTA
RxN-buffer	0.1	M	Tris-HCl, pH 8,7
	1	mM	$MgCl^{2+}$
Staining solution for ELISA	1	mM	ONPP (Ortho-Nitrophenyl-Phosphat) in RxN-buffer
Staining solution for Immunohistochemistry	1	mg	NBT (Nitroblue-Tetrazolium)
	2	mg	BCIP (5-Bromo-4-Chloro-3-Indolyl-phosphat) prediluted in 20 μl DMSO in
	10	ml	RxN-buffer
Western Blot Buffer	50	mM	Tris, pH 7.8
	1	mM	EDTA
	1	mM	EGTA
Loading Buffer	0.5	M	Tris-HCl, pH 6.8
	5	%	SDS
	5	%	Mercaptoethanol
	20	%	glycerin
	4	%	bromphenolblue
Running Buffer for SDS-gel	2.5	mM	Tris
	19.2	mM	glycin
	0.1	%	SDS

Resolving gel	10	%	acrylamide
	0.3	M	bisacrylamide
	0.375	M	Tris-HCl, pH 8.3
	0.1	%	SDS
	0.05	%	ammoniumpersulfate
	0.25	%	TEMED
Stacking gel	4	%	acrylamide
	0.12	%	bisacrylamide
	0.166	M	Tris-HCl, pH 6.8
	0.1	%	SDS
	0.05	%	ammoniumpersulfate
	0.25	%	TEMED
Blotting Buffer	20	%	Methanol
	50	mM	Tris
	40	mM	glycin
	0.14	M	SDS
Prestained marker	0.5	mg	myosin
	0.5	mg	β-galactosidase
	0.5	mg	phosphorylase B
	25	mg	BSA
	25	mg	albumin
	5	mg	carboxy anhydrase

2.2.2 Chemicals

Name	Obtained from
BSA (Bovine albumin)	AppliChem
Bisindolylmaleimide 1	Calbiochem
Hydrochloride	Calbiochem
D (+)-saccharose	AppliChem
Carnation oil	Drug store
UREA	Merck
Inhibitor PD 098059	Cell Signalling Technology
Inhibitor SB 203580	Cell Signalling Technology
cAMP	Signa
ATP	Sigma
DMSO (Dimethyl sulfoxide)	Sigma
NBT	Sigma
BCIP	Sigma

2.2.3 Antibodies and enzymes

Name and dilution	Obtained from
MAPK p44/42 antibody 1:2000 in blocking puffer	Cell Signalling Technology (New England Biolabs)
Phospho-MAPK p44/42 antibody 1:2000 in blocking puffer	Cell Signalling Technology (New England Biolabs)
MAPK p38 antibody 1:2000 in blocking puffer	Cell Signalling Technology (New England Biolabs)
Phospho-MAPK p38 antibody 1:2000 in blocking puffer	Cell Signalling Technology (New England Biolabs)
Anti-rabbit IgG, biotin conjugate 1:4000 in blocking puffer	Sigma (Deisenhofen)
Avidin-Phosphatase 1:10000 in blocking puffer	Sigma (Deisenhofen)
Anti-rabbit IgG-Peroxidase 1:10000 in blocking puffer	Sigma (Deisenhofen)

2.2.4 Consumable supplies

Name	Obtained from
X-Ray film X-ARS	Kodak (Rochester)
Rf developer Agfa G 150	Siemens (Berlin)
Rf fixer Agfa G 350	Siemens (Berlin)
Nitrocellulose membrane Optitran BA-S 83	Schleicher und Schuell (Basel)
Whatman filter paper	Schleicher und Schuell (Basel)
5μl glass capillary	Selzer (Waglhäusel)
ELISA-plates	Falcon (Becton Dickinson)
Eppendorf vials 50 and 1500 μl	Eppendorf (Hamburg)
Syringe	Roth (Karlsruhe)
Pipette tips (diverse)	Roth (Karlsruhe)

2.2.5 Devices

Name	Obtained from
Binocular	Wild(Heerbrugg)
Light sourse KL 1500 LCD	Zeiss (Jena)
ELISA-reader 400 ATX with curve-fit software	STL Labinstruments
Electrophorese chamber	self-made
Fast blot chamber Trans-Blot SD	BioRad (München)
Glass-glass douncer	Roth (Karlsruhe)
Slide-dryer MEDAX	MEDAX Nagel KG (Kiel)
Microtome 1512	Leitz (Germany)