2 Methods

2.1 Preparation

Adult foraging bees, *Apis mellifera carnica*, were caught at the hive in the afternoon one day before the experiments, cooled for anaesthesia and fixed in a recording stage (Galizia et al., 1997). Animals were experimentally naïve, but may have experienced odors during foraging flights. They were fed with sucrose water (30%) until satiation in the evening. Over night animals were kept at 20°C in a dark humidified box. On the day of the experiment the bees were prepared as follows: a window was cut posterior to the antennae in the head capsule and glands and trachea were carefully removed to allow visual access to the brain. A glass electrode was coated with crystals of fura-dextran (potassium salt, 10.000 MW, Invitrogen, Germany), dissolved in 3% bovine serum albumin solution, and injected into the right deuterocerebrum dorsolateral to the alpha-lobe, aiming for the l-ACT. The dye was left to travel along the tract for 4-8 hours. Before the experiments the antennae were fixed with dental wax (Kerr, Sybron Dental Specialities, USA). Animals were immobilized by waxing the thorax and abdomen to the recording chamber with bee wax (Deiberit 502, Dr. Boehme & Schoeps, Dental GmbH, Germany). The brain was rinsed with Ringer solution (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7, 500 mosmol; all chemicals from Sigma) to remove extracellular dye. To achieve stable imaging experiments, strong movements of the brain needed to be prevented. Therefore, a second hole was cut ventrally to the antennae to expose the strong muscles connected to the oesophagus. The compact structure of muscles, esophagus, and supporting chitin was lifted and kept under tension (Mauelshagen, 1993). Thus, stabilization was accomplished without damaging the brain. However, this preparation impairs the proboscis extension reflex. To allow for sugar presentation on the proboscis during experiments the proboscis was manually extended and placed on a small glass capillary on the recording chamber. Following the preparation procedure the bees were left to recover for at least 30 minutes.

2.2 Imaging

Imaging was done using the T.I.L.L. Photonics imaging system Polychrome IV (Germany). Monochromatic excitation light alternated between 340 and 380nm. Fluorescence was detected at a sampling rate of 5 Hz through a 20x, 0.95 W Olympus objective, an Omega 505 DRLPXR dichroic mirror and a 515 nm LP filter with an Imago-QE CCD camera (1376 x 1040 pixel, 8x binned on chip to 172x130µm) mounted on a fluorescence microscope (Olympus BX-50WI). The spatial resolution was 2.4 µm² per pixel.

Odors were diluted in mineral oil, 1-hexanol [10^{-2}], 1-octanol [10^{-3}] and 1-nonanol [10^{-3}]. 5 µl of the odor solution were applied onto a 1 cm^2 piece of filter paper and placed in a plastic syringe. Using a computer controlled olfactometer (Galizia et al., 1997) odors were injected into a continuous air stream which was directed to the antennae. The duration of odor stimulation was 3 seconds, for detailed odor protocol see descriptions of individual experiments.

2.3 Data analysis

All data were analyzed using custom software written in IDL (Research systems, CO). Before calcium signals were analyzed, the raw data of the individual experiments were firstly manually corrected for movement within each measurement and secondly for shifts in between measurements of each animal. We corrected the data for scattered light to avoid artificial correlations between glomeruli which was noticeable before correction. The difference between the smoothed image and the observed image was taken as a correction factor, i.e. we subtracted this difference from the original image. We therefore calculated $F_i^{'} = F_i - \left[sm(F_i) - F_i\right] = 3 * F_i - sm(F_i)$. The images F' are the scattered-light corrected images. Note that this procedure does not change the measured values in areas where there is no spatial contrast: in these areas, the low-passed filtered image has the same value as the original image, and consequently $F' = F$.

For calcium concentration data we calculated the ratio 340/380 nm multiplied by factor 100. Background fluorescence was determined by an average of 5 frames obtained before stimulation and was subtracted from every frame of a measurement, thereby setting measurements to 0 just before stimulus onset. The resulting values are labelled Δ(340/380)[%].

The glomerular structure was visible in the fura ratio images, which allowed us to identify the glomeruli on the basis of their morphological borderlines using the digital atlas of the AL as a reference (Galizia et al., 1999). This method has already been described in detail (Galizia et al., 1999;Sachse et al., 1999). For time courses squares of 9x9 pixels (always well within the glomerulus chosen) were placed onto the centre of each identified glomerulus, their values were averaged, and the time courses were plotted against time.

The average calcium response of a particular glomerulus during stimulation was defined as 'response'. For example, the mean response during a three seconds odor stimulation yielded the 'odor response'.

For false-colour display, for each pixel the mean value during the three second stimulation was calculated and the mean value during one second before stimulus onset was subtracted. The resulting image was median filtered in space, 3x3 pixels for every pixel.

Statistical analysis was performed using Sigmastat (SPSS Inc; Chicago,IL,USA).

2.4 Responses to sucrose water

In 9 experimentally naïve animals we investigated the responses to sucrose water. One hour before preparation for imaging, all animals received a 30% sucrose stimulation on the antennae. Only animals which responded with a PER were subject of the following experiment. Sucrose was diluted in water to a concentration of 3% and 30%. Sucrose, water and mechanical stimuli were applied with a wooden toothpick, which is an established method for PER conditioning (Guerrieri et al., 2005b). Stimuli were always applied to the antenna ipsilateral to the stained PNs

unless otherwise indicated. All animals received two 1-nonanol stimulations (as above) to check for normal odor responses to determine successful PN staining. Thereafter, they received the following stimulus protocol:

- mechanical stimulation
- water
- 3% sucrose
- 30% sucrose
- 30% sucrose on the proboscis (only n = 4)
- 30% sucrose on the antennae *contralateral* to the stained PNs.

Stimulus duration was always one second. Stimuli were applied manually using a micromanipulator. Stimulation was guided by a computer controlled auditory signal.

In a second set of 13 animals, the stimulation method differed. Instead of applying the sucrose with a wooden toothpick, a 10µl Eppendorf pipette was used. However, in those animals only the responses to 30% sucrose were determined. Stimulus duration was 3 seconds.

The mean response of the first second during stimulation was defined as the response and analyzed in all observed glomeruli. For statistical analysis we compared the responses with a two-way ANOVA, with *glomerulus* and *stimulus* as factors. In case of significant differences, Holm-Sidak multiple comparison post-hoc tests were used.

2.5 Absolute conditioning

We investigated the effects of reinforcement on the neural representation of the odor 1-nonanol in the following experiments. The experiment consisted of three sequential parts: pre-test, conditioning and test (Rescorla, 1988). Throughout the experiment the duration of odor stimulation was always 3 seconds. We determined the ‘naïve’ response to 1-nonanol in the pre-test phase during which all animals received two presentations of 1-nonanol with an interstimulus interval of 2 minutes. During conditioning the sucrose reward was applied for 3 seconds to the ipsilateral antennae

of the imaged AL and the proboscis. Sucrose stimulation was achieved by delivering a drop of 30% aqueous sucrose solution to the antennae and the proboscis, using a 10µl Eppendorf pipette with standard tips.
In case of a forward pairing, the odor preceded the reinforcement in a backward pairing the other way round, with an overlap of 1 second.
During the test phase all animals were presented with 1-nonanol after 1, 5 and 15 minutes following the conditioning.
54 animals were assigned to 5 groups being subjected to the different conditioning protocols:

Logic of naming the groups :
The symbol indicates whether 1-nonanol was unrewarded (-), forward paired with sugar water (+) or backward paired with sugar water (bw). The number preceding the symbol represents the repetition of conditioning trials.

1. 1- 1-trial unrewarded 1-nonanol, n = 9
2. 1bw 1-trial backward conditioning, n = 14
3. 1+ 1-trial forward conditioning, n = 9
4. 3bw 3-trial backward conditioning with an ITI interval of 2 minutes , n =11
5. 3+ 3-trial forward conditioning with an ITI of 2 minutes, n = 11.

Odor responses (mean calcium response during odor stimulation) in every glomerulus were analyzed with a two-way repeated measures (RM) ANOVA, with the different *groups* as one factor and the pre-test- and test-responses at different points in *time* as the repeated factor. Multiple comparisons were performed using the Holms-Sidak post-hoc test.

2.6 Sequential stimulation with three odors and differential conditioning

Again, the experiment was divided into three parts: pre-test, conditioning and test. Both, the pre-test and test phase consisted of three blocks with three odor stimulations (1-hexanol, 1-octanol and 1-nonanol). Within each block each odor was presented at a different position in the stimulation order. For example three blocks could be ABC, CAB, BCA, note that A was at the first, the second and the third position in the three blocks. This pseudo randomized order was chosen to prevent any effect of a certain odor sequence. It allowed us to average the three pre-tests to yield one pre-test response to every odor which could then be compared with a test response obtained similarly. Throughout the whole experiment stimulus duration was 3 seconds and the interstimulus interval 1 minute. In case of a reinforcement the odor presentation overlapped for 1 second with the 3 second sucrose stimulation of the antennae and the proboscis (stimulation as for absolute conditioning).

The pre-tests and tests were similar between all 72 animals, which enabled us to compare differences induced by the 7 different conditioning paradigms:

Logic of naming the groups :
The number denotes the repetition of conditioning trials. Conditioned odors are indicated by capital letters: 1-hexanol (H), 1-octanol (O) and 1-nonanol (N). The elevated symbol indicates whether odors were reinforced (+) or remained unrewarded (-). Thus, $5N^{+}O^{-}$ represents a 5 trial differential conditioning, with 1-nonanol reinforced and 1-octanol unrewarded.

1. $3N^{+}$ 3 trial absolute conditioning with an intertrial interval of 2 minutes, as the CS- is absent. n = 10
2. $3N^{+}O^{-}$ 3 trial differential conditioning, with 1-nonanol as the CS+ and 1-octanol as the CS-, n = 9
3. $5N^{+}$ 5 trial absolute conditioning with an intertribal interval of 2 minutes, as the CS- is absent. n = 15

4. $5N^{+}O^{-}$ 5 trial differential conditioning, with 1-nonanol as the CS+ and 1-octanol as the CS-, n =9
5. $5N^{+}H^{-}$ 5 trial differential conditioning, with 1-nonanol as the CS+ and 1-hexanol as the CS-, n = 10
6. $5N^{-}O^{-}$ 5 trial sequential odor stimulation with the same order as in $5N^{+}O^{-}$ but without rewarding any odor. n =10
7. $5N^{-}H^{-}$ 5 trial sequential odor stimulation with the same order as in $5N^{+}H^{-}$ but without rewarding any odor. n =9.

5 minutes subsequent the conditioning protocols the animals were subjected to the tests as described above.
Odor responses to the three odors in every glomerulus were analyzed with a two-way RM ANOVA, with the different *groups* as one factor and pre-test and test-responses as the repeated factor *time*. Multiple comparisons were performed using the Holms-Sidak post-hoc test.

All experimental protocols are summarized in Fig.1. The main purpose of this study was to investigate the effects of associative and non-associative learning paradigms on odor responses. To be able to compare the effects among the different groups and relate possible conditioning specific modulations of odor responses to particular glomeruli and their interactions, 1-nonanol was always the reinforced odor (CS). In one part of the experiments (set 1) we investigated changes of the responses to 1-nonanol due to repeated stimulations, and pairing of this odor with a sucrose reward (absolute conditioning) with special focus on different points in time after the treatment. Thereafter, we included 1-hexanol and 1-octanol into the stimulation protocols (set 2) allowing us to examine the effects of absolute conditioning of 1-nonanol on these odor responses and the effect of a more complex learning paradigm, like differential conditioning.

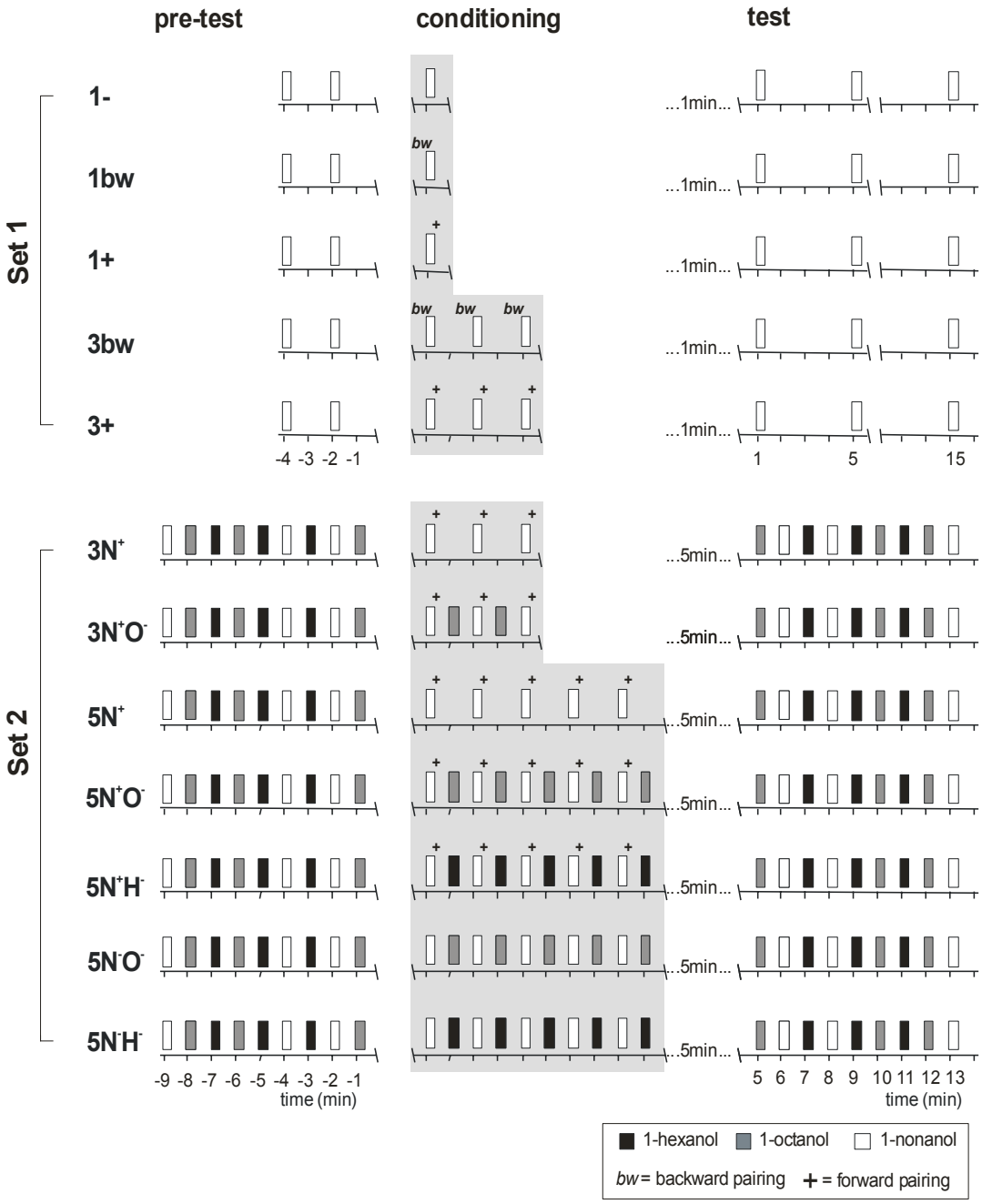

Figure 1: Logic of naming the groups and experimental design
The number denotes the repetition of conditioning trials. Conditioned odors are indicated by capital letters: 1-hexanol (H), 1-octanol (O) and 1-nonanol (N). The elevated symbol indicates whether odors were reinforced (+) or remained unrewarded (-). Thus, $5N^{+}O^{-}$ represents a 5 trial differential conditioning, with 1-nonanol reinforced and 1-octanol unrewarded.

2.7 Behavior

The animals were prepared almost exactly as in the imaging experiments. The only difference was that the oesophagus and the muscles around the proboscis were left intact which ensured the PER. At first, animals were tested for a PER to sucrose stimulation of each individual antenna, the ipsilateral and the contralateral antenna to the stained lACT PNs. Thereafter, animals were left alone for 30 minutes before starting with the learning experiment to avoid any effects due to sensitization (Menzel et al., 1991).

Odor stimulation with 1-nonanol was again 3 seconds, similar to the absolute conditioning experiments. Interstimulus interval was 2 minutes throughout pre-test and conditioning. All animals received two pre-test odor stimulations. Thereafter the animals were conditioned by a 3 trial absolute conditioning as described above. 5 minutes subsequent conditioning the animals were tested for PER upon 1-nonanol stimulation of the ipsi-, the contralateral antenna to the stained lACT PNs and of both antennae. The order of stimulation was randomized between animals. Stimulation of only one antenna was achieved by covering one antenna with a sealed plastic tube. Finally, all animals were examined for stained lACT PNs under a fluorescent microscope.