3 Results

3.1 Odors elicit activity patterns in PNs that are equal across animals

Insertion of fura dextran crystals into the lACT in the deuterocerebrum lateral to the alpha lobe led to a selective staining (Fig.2A) of uniglomerular PNs as previously reported (Sachse and Galizia, 2002a). 15 glomeruli were reliably identified in all 135 animals included in this study (Fig.2A,C) using the digital three-dimensional atlas of the honeybee antennal lobe (Galizia et al., 1999a) as a reference (Fig.2C). Honeybee glomeruli are labelled with a number and the name of the antennal nerve tract that innervates them, ranging from T1 to T4, e.g. T1-28 or T3-45 (Flanagan and Mercer, 1989). All glomeruli in this study were from the T1 tract. I have therefore simplified their names by omitting the T1 affix. Subject to the following analysis are the glomeruli 17, 23, 25, 28, 29, 33, 35, 36, 37, 38, 42, 47, 48, 49 and 60, all innervated by the uniglomerular lACT PNs (Abel et al., 2001; Bicker, 1993) Similarly, whenever I talk about 'glomerular activity' in this study, I specifically mean activity of the uniglomerular PNs in each glomerulus, because only these neurons were stained, and therefore only this subpopulation of cells in each glomerulus were measured.

When stimulated with 1-hexanol, 1-octanol and 1-nonanol, glomeruli (PNs) showed the typical spatio-temporal activity patterns previously described (Fig.2B,D) (Sachse et al., 1999; Sachse and Galizia, 2002). In order to compare the response patterns of the three odors in all glomeruli across animals, the average response during the three second odor stimulations are defined as odor response and plotted in Figure 2E. 1-hexanol evoked a strong calcium response in glomerulus 28, and weaker responses in glomerulus 17, 36, 38 and 35, whereas calcium concentration in glomerulus 29 decreased. 1-octanol elicited its strongest response in glomerulus 17 and 28, weakly activated glomerulus 33 and inhibited glomerulus 23, 25 and 29. 1-nonanol evoked a strong response in glomerulus 17, 33 and inhibitions in glomerulus 23 and 25. All responses were consistent across animals as can be seen by the small error bars (Fig.2E).

In summary the response to 1-nonanol was substantially overlapping with that of 1-octanol, while the response to 1-nonanol showed virtually no overlap of activity with 1-hexanol in the 15 observed glomeruli.

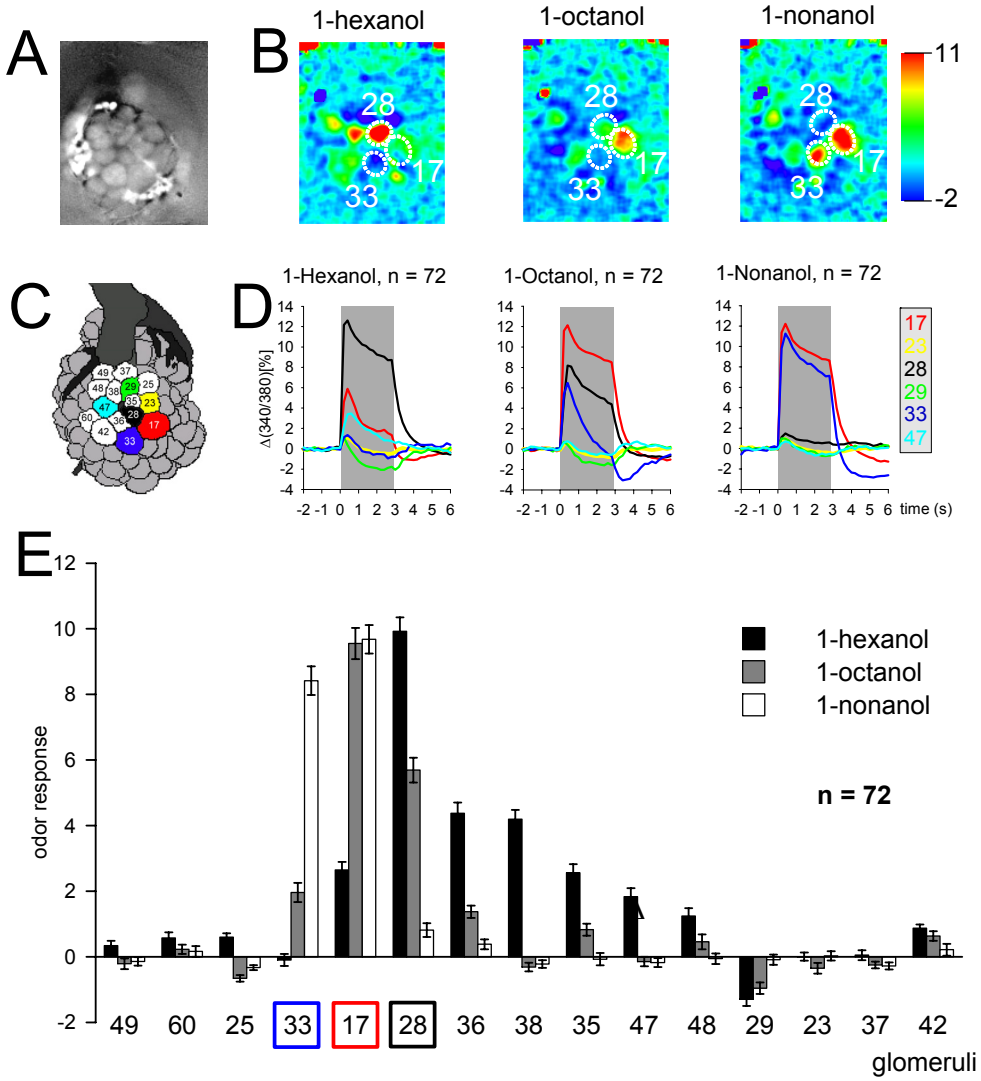

Figure 2

Figure 2: Odor responses in the honeybee AL.
A: Raw fluorescence image of the AL. B: False-color-coded spatial response pattern to 1-hexanol, 1-octanol and 1-nonanol. Glomerulus 17, 28 and 33 are indicated by dotted white circles. Intensities range from -2 (blue) to 11 (red). C: Schematic view of the AL with the 15 identified glomeruli used in this study. E: Time traces of odor responses as the mean of 72 animals. The colors of the time traces correspond to B. Stimulus is marked by a shaded area in the time courses. E: Average odor responses (± SEM, n = 72) of 15 measured glomeruli during the three seconds odor stimulation of 1-hexanol, 1-octanol and 1-nonanol. The three most responsive glomeruli for these odors are indicated by colored squares, glomerulus 17 in red, glomerulus 28 in black and glomerulus 33 in blue. Those three glomeruli are subject to most analysis in the following graphs.

3.2 PN responses to sucrose

PNs did not only respond to odors. In Figure 3 two typical examples of an odor stimulation paired with a sucrose reward are shown, one forward (Fig.3A) and one backward (Fig.3B). Clearly, PNs did respond also to sucrose.

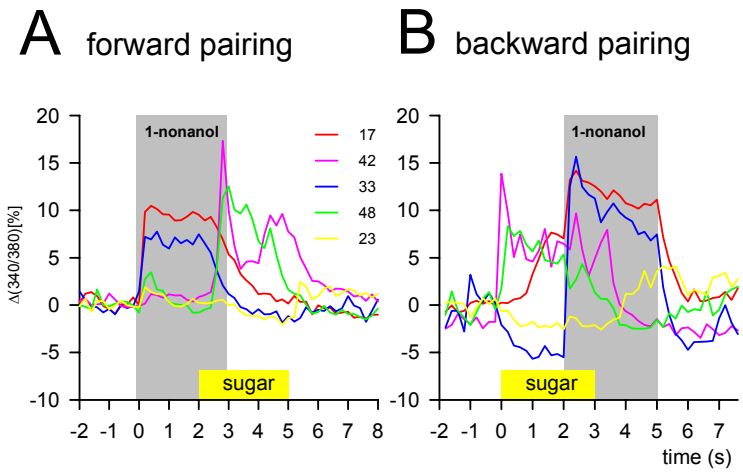

Figure 3: PN responses to sucrose.
Time traces in 5 glomeruli of an individual measurement of a forward pairing (A) and backward pairing (B). The odor-stimulus is marked by a shaded area in the time courses and sucrose is marked by the yellow bar.

I analyzed these responses in more detail by comparing the responses to the following stimuli: using a wooden toothpick, I applied mechanical stimulation, water, 3% and

30% sucrose on the antenna ipsilateral to the stained PNs and 30% sucrose on the proboscis as well as the contralateral antenna. Furthermore, I presented 30% sucrose on the ipsilateral antenna using an Eppendorf pipette.

The PN responses to the tested stimuli were significantly different (*stimuli*, two-way ANOVA, *DF*= 7, *F*= 32.367, *P*< 0.001). The various stimuli did not evoke an evenly distributed activity pattern in the 15 observed glomeruli (*glomerulus*, two-way ANOVA, *DF*= 14, *F*= 6.406, *P*< 0.001) and this significantly interacted with the tested stimuli (*glomerulus* x *stimuli*, two-way ANOVA, *DF*= 98, *F*= 1.740, *P*< 0.001). Responses to the different stimuli were further analyzed in individual glomeruli using Holm-Sidak multiple comparison post-hoc tests.

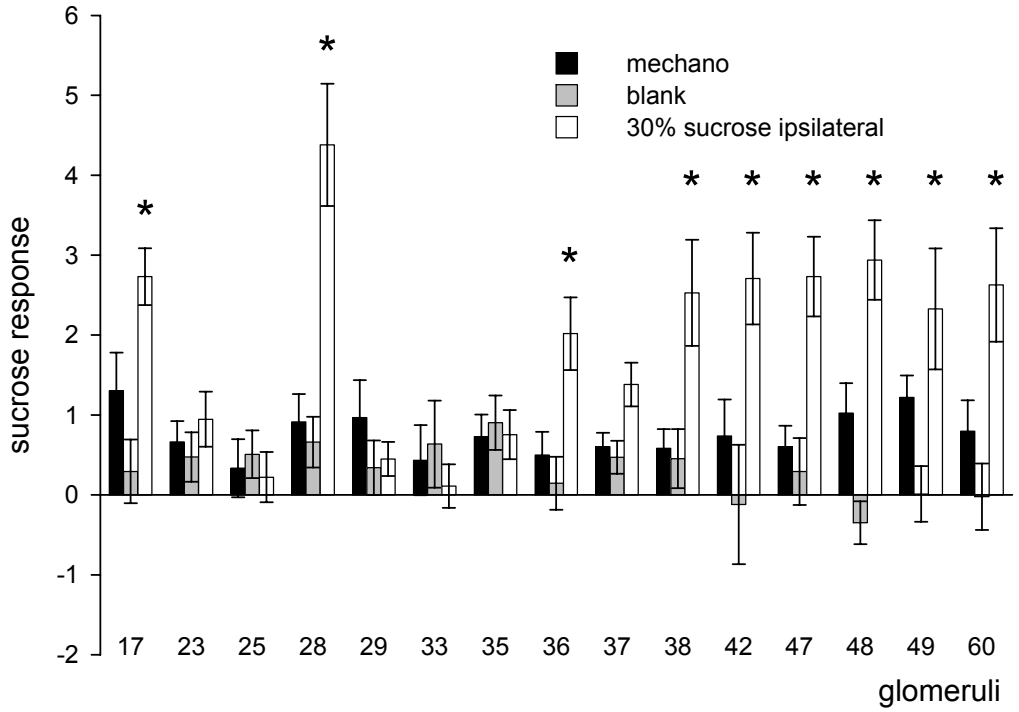

Figure 4: PN responses to 30% sucrose applied with a soaked toothpick
Mean sucrose responses (± standard error of mean, n = 9) of all 15 observed glomeruli to sucrose stimulation and mechanical stimulation of the ipsilateral antennae to the stained PNs. Responses are compared with a blank trial without stimulation. Statistically different responses are indicated by asterisks (* *P< 0.05,* Holm-Sidak, post-hoc multiple comparison method).

Touching the antenna with a wooden toothpick soaked in 30% sucrose, evoked an activity pattern which was different from mechanical stimulation and blank trials without stimulation (Fig.4). These responses were equal across animals. PN responses in glomerulus 17, 28, 36, 42, 47, 48, 49 and 60 were significantly different from mechanical stimulation and no stimulation (Holm-Sidak, all $P< 0.05$). PNs responded only to sucrose when stimulated on the antenna ipsilateral to the stained PNs. Responses to mechanical stimulation, stimulation of the contralateral antenna or the proboscis were not different from blank trials without stimulation (Holm-Sidak, all $P> 0.05$; Fig.5).

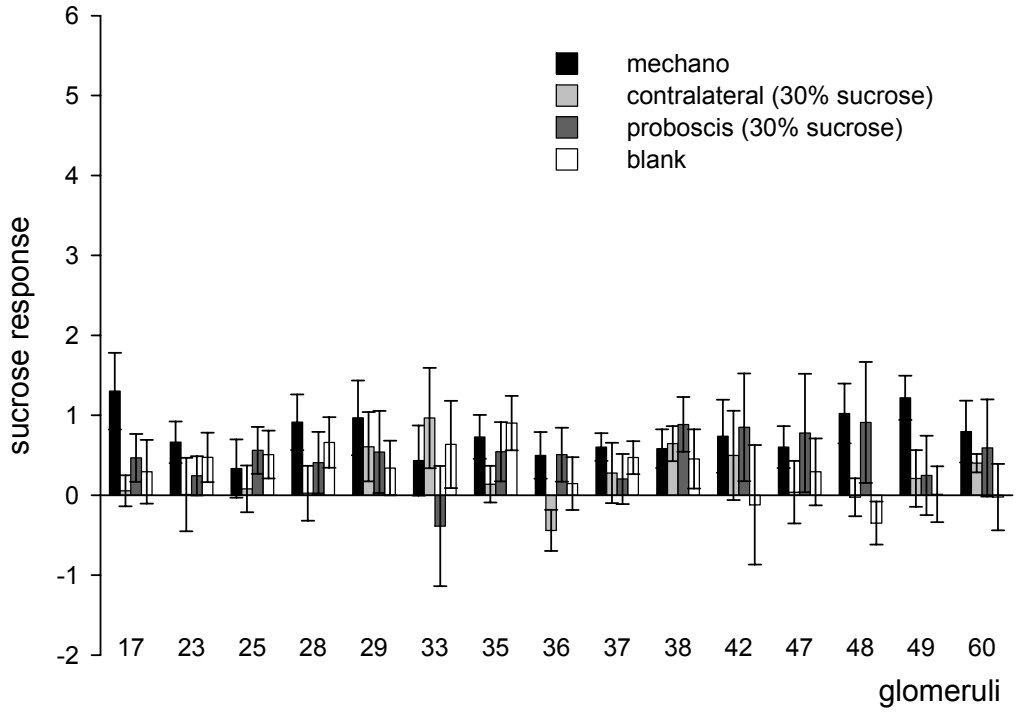

Figure 5: PNs do not respond to mechanical stimulation of the antennae, nor to stimulation with 30% sucrose on a toothpick of the proboscis or the contralateral antenna.
Mean sucrose responses (± standard error of mean, n = 9) of all 15 observed glomeruli. Responses are compared with a blank trial without stimulation. Responses are not statistically different (all *P> 0.05,* Holm-Sidak, post-hoc multiple comparison method).

Stimulation with a wooden toothpick soaked in either, water, 3% sucrose or 30% sucrose were not distinguishable except for one glomerulus (Holm-Sidak, all $P > 0.05$; Fig.6).

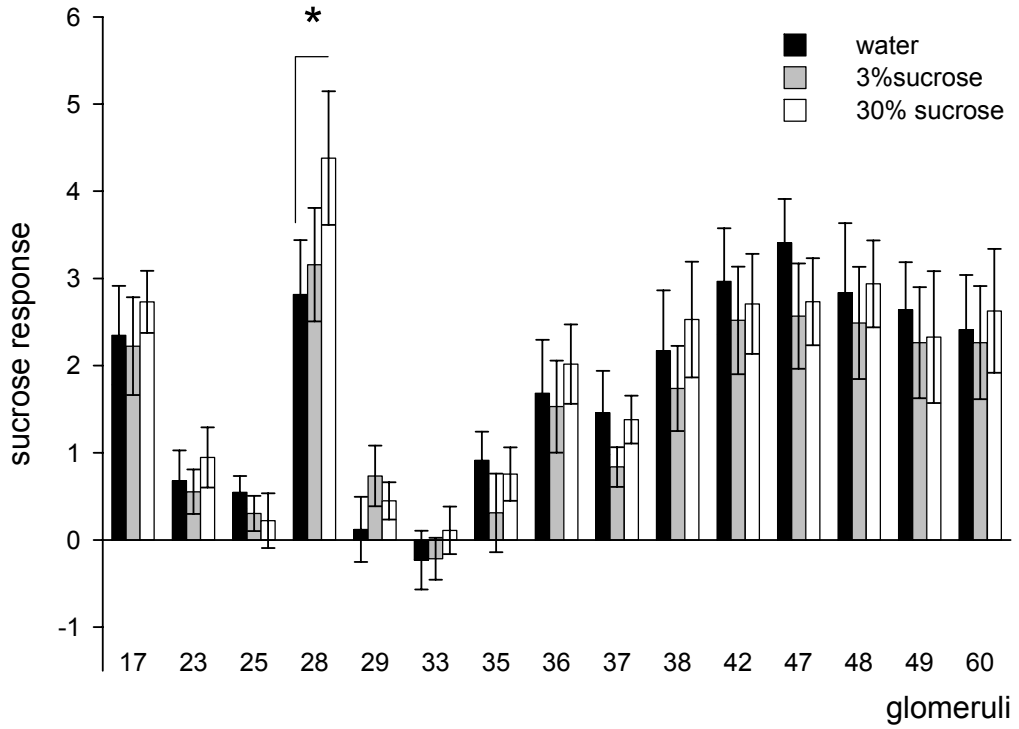

Figure 6: PN responses to water, 3% and 30% sucrose applied on a wooden toothpick.
Mean sucrose responses (± standard error of mean, n = 9) of all 15 observed glomeruli to water, 3%, 30% sucrose stimulation of the ipsilateral antenna to the stained PNs. Statistically different responses are indicated by asterisks (* $P < 0.05$, Holm-Sidak, post-hoc multiple comparison method).

Comparison of the application method of 30% sucrose between a soaked wooden toothpick and a clean Eppendorf pipette, revealed a fundamental difference between the two (Fig.7). The PN sugar responses in glomerulus 28 in case of a soaked toothpick was more than 4 times higher in glomerulus 28 (Holm-Sidak $P < 0.001$) and slightly lower in glomerulus 33 ($P < 0.032$). Actually, the response of PNs in glomerulus 28 to 30% sucrose applied by the pipette was not even different from

mechanical nor blank trials (Holmes-Sidak, P> 0.45, compare Fig.5). PN responses in all other glomeruli were not significantly different.

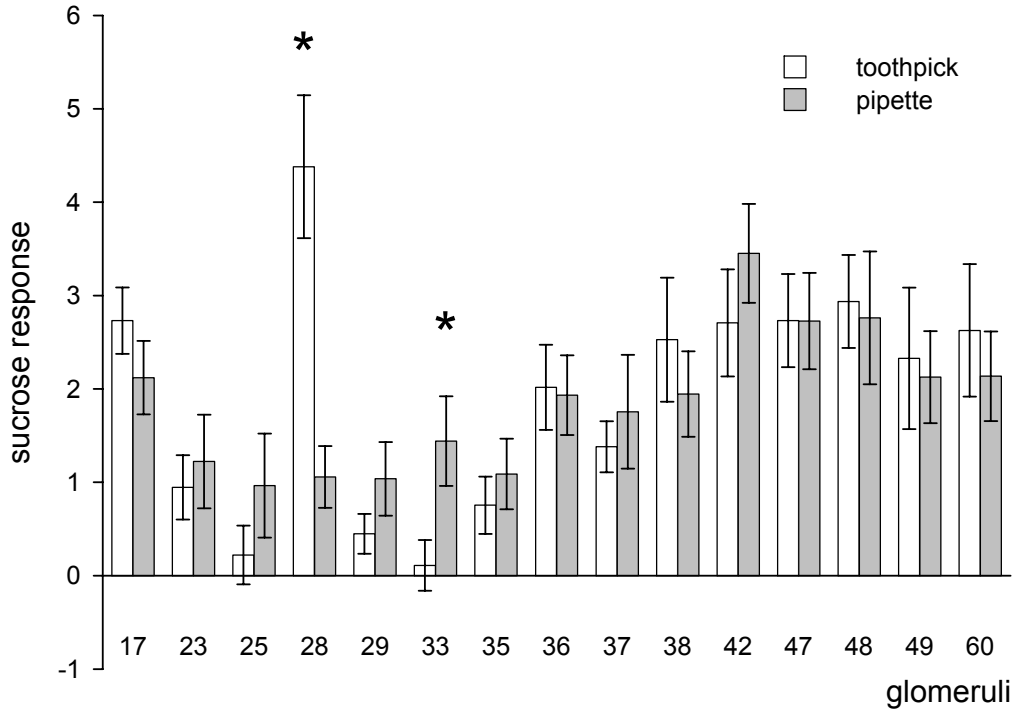

Figure 7: PN responses to 30% sucrose depend on the application method
Mean sucrose responses (± standard error of mean, n = 9) of all 15 observed glomeruli to sucrose stimulation and mechanical stimulation of the ipsilateral antenna to the stained PNs. Responses are compared with a blank trial without stimulation. Statistically different responses are indicated by asterisks (* *P< 0.05,* Holm-Sidak, post-hoc multiple comparison method).

An intriguing question was whether the observed responses to sucrose reflect an input of the modulatory reward pathway. If it does, the response during a forward pairing would be expected to be different from the summed responses of separate stimulations with an odor and sucrose. This effect should be most prominent in the PNs responding to the odor. I calculated the mean response during a forward pairing, including the 5 second time interval with both, the odor and sucrose stimulation. During the similar time interval the responses to odor and sugar alone were derived. As can be seen in Figure 8 the response during a reinforcement trial of an odor was

not different from the summed response to the individual components, odor and sucrose (t-test, for all glomeruli $P > 0.05$).

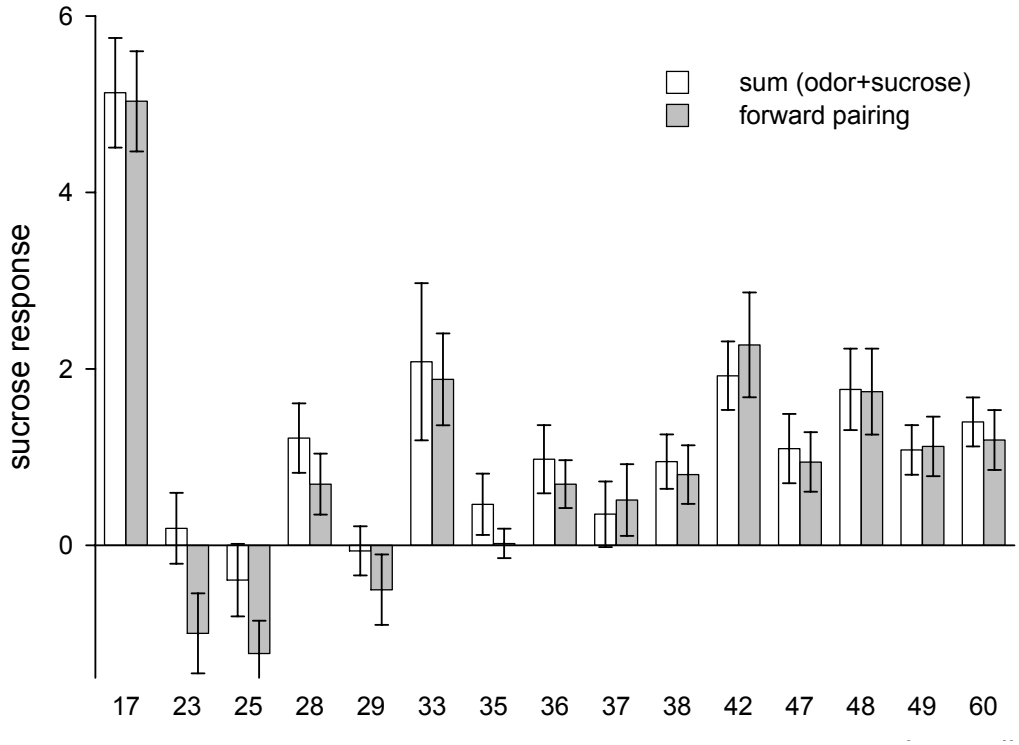

Figure 8: PN odor responses are not modulated during reinforcement with sucrose.
Mean compound responses to odor and sucrose stimulation during reinforcement (± standard error of mean, n = 13) compared with the summed response to separate stimulations with the individual components, odor and sucrose. Responses in all 15 observed glomeruli are not significantly different (all $P > 0.05$, t-tests for individual glomeruli).

3.3 Repeated odor stimulation does not change the odor's representation (set 1)

All 54 animals of set 1 received two pre-test 1-nonanol presentations. In Figure 9 average PN odor responses in glomerulus 17 and 33 across animals were similar for the two pre-tests. The same held true for all 15 observed glomeruli (RM two-way ANOVA, Holm-Sidak multiple comparisons, smallest $P = 0.059$ for glomerulus 17, all other $P > 0.1$). The second stimulus was defined as the pre-test for the following analysis.

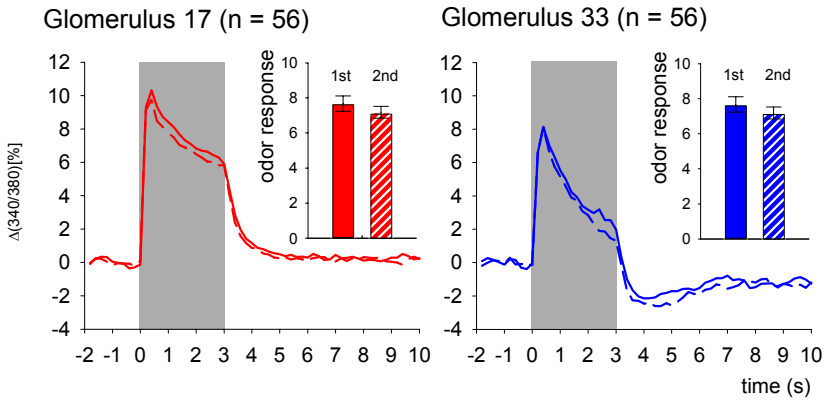

Figure 9: PN odor responses are similar in the two pre-tests.
Average calcium responses in two identified glomeruli, 17 and 33, to the first and second 1-nonanol stimulation (mean time trace of all 54 animals of set 1). The odor-stimulus is marked by a shaded area. ITI = 2min. Inset shows average odor responses (± SEM, n = 54) during stimulation.

It is necessary to know the response variability to repeated odor stimulation alone before investigating the possibly more complex effects of reinforcers such as a sucrose reward. Six successive 1-nonanol stimulations (1N¯group, Fig.10A) elicited similar PN odor responses in all glomeruli. During the half an hour of the experiment, responses remained unchanged in both magnitude and shape (see responses to glomerulus 17 and 33 in Fig.10B)

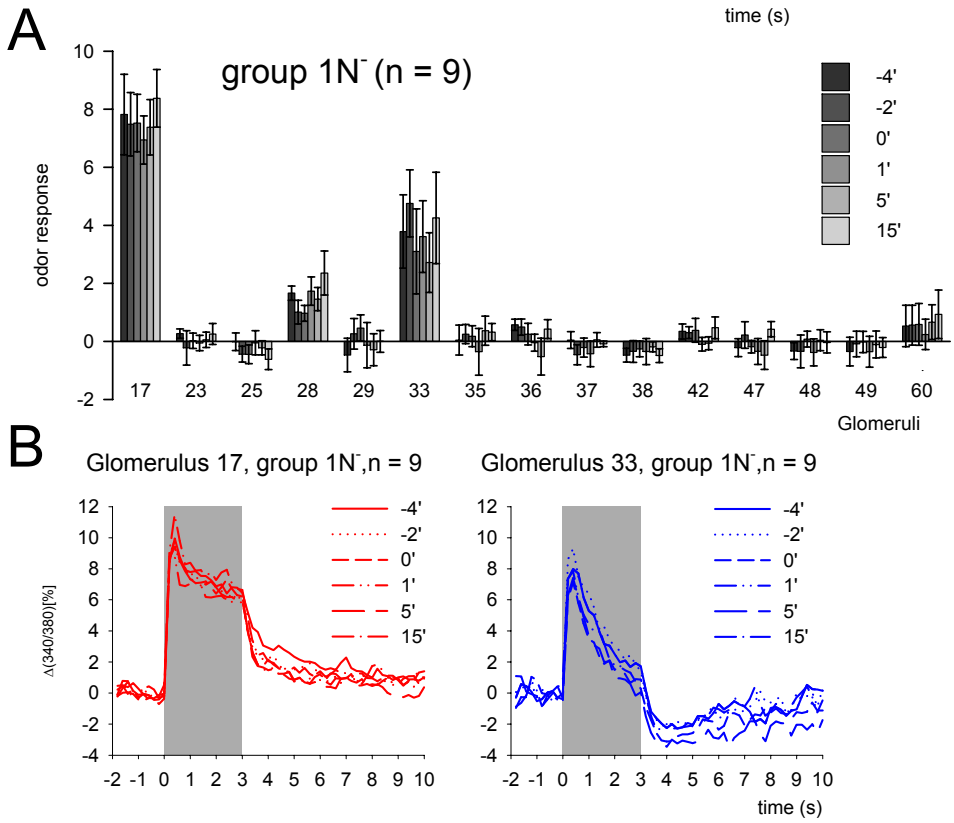

Figure 10: Unrewarded odor responses are stable over time.
A: group 1N⁻, average responses (± SEM, n = 9) of 15 identified glomeruli to six consecutive 1-nonanol stimulations. B: Corresponding average time traces exemplified for glomeruli 17 and 33. The odor-stimulus is marked by a shaded area.

3.5 Absolute conditioning does not change the representation of a learned odor

All stimulus protocols of set 1, like successive odor stimulation, single and multiple trial conditioning, backward and forward pairing (Fig.11, exemplified for glomerulus 17 and 33), had no different effect on the glomerular PN odor responses at different points in time (no significant interaction between *group* and *time*, RM two-way ANOVA, lowest *P* for glomerulus 17: *DF*= 16, *F*= 1.614, *P*= 0.067, in all other cases *P*> 0.3).

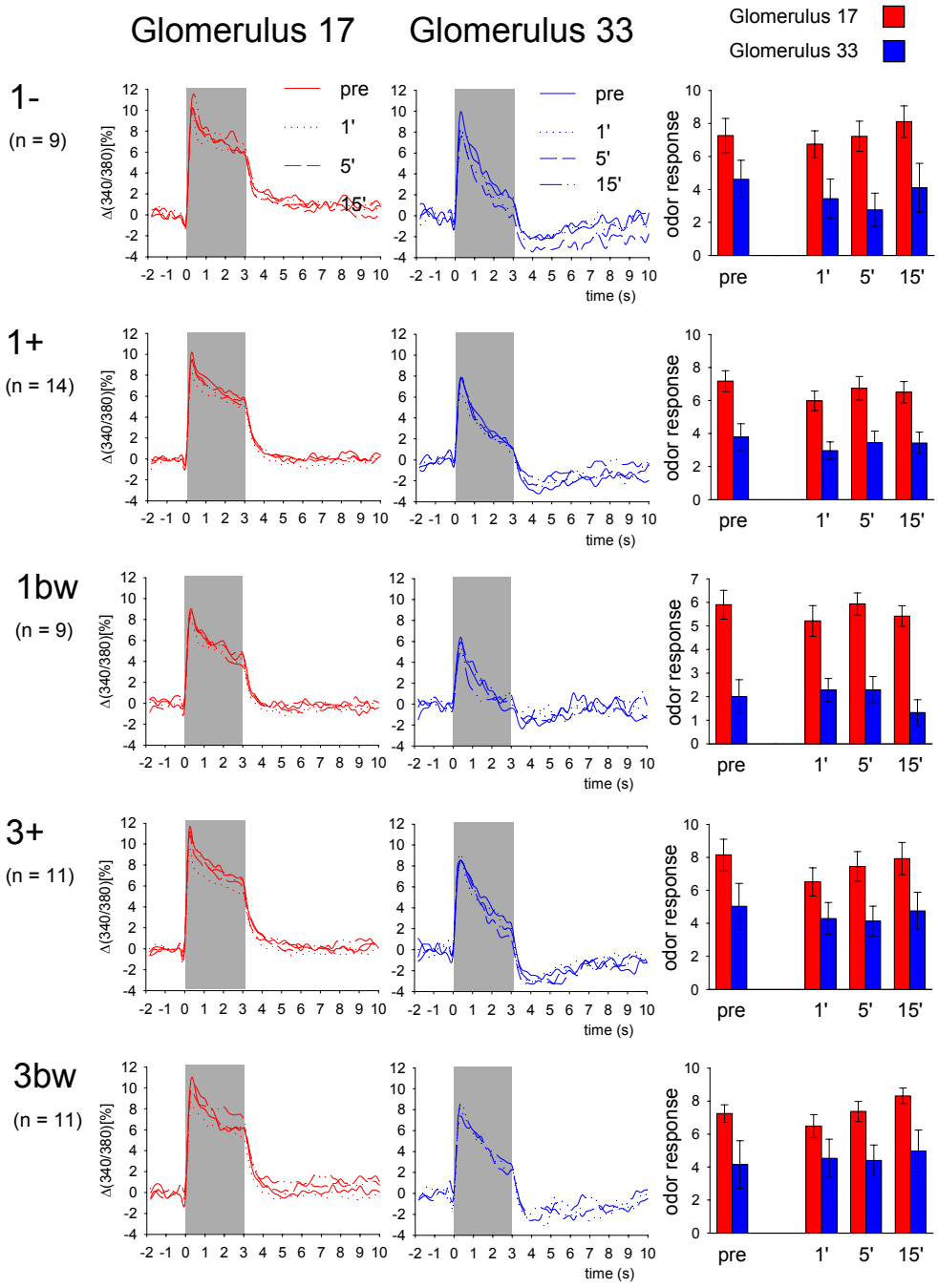

Figure 11: Neural odor representation remains stable following sucrose reinforcement.
Mean PN response traces of glomerulus 17 and 33 to 1-nonanol in the pre-test compared with tests at 1, 5 and 15 minutes following conditioning. *left:* the third column shows the average odor responses of glomerulus 17 and 33 (± SEM, number of animals for each group are given in the graph).

This indicated that absolute learning does not change the neural representation of the learned odor in lACT projection neurons. While there were no learning related changes, the possibility of changes remained, that were independent of the different paradigms and common across all groups. Pooling all animals, I found that differences of the odor responses were significantly different in some cases (RM two-way ANOVA: glomerulus 17: D = 4, F= 9.267; P= 0.001, glomerulus 36: DF= 4, F= 16.816, P= 0.001, glomerulus 38: DF= 4, F= 14.216, P= 0.013, glomerulus 42: DF= 4, F= 16.714, = 0.001). Multiple-comparisons revealed the following. Test responses to 1-nonanol at 1 minute were attenuated in glomerulus 17 (Holmes-Sidak, P = 0.001) and the inhibition in glomerulus 38 was reduced (P= 0.024). At 15 minutes, test odor responses in glomerulus 38 showed less inhibition (P= 0.001), whereas glomeruli 36 (P= 0.015) and 42 (P= 0.03) were slightly increased. All other glomeruli remained unchanged compared to the pre-test at all time points.

3.6 The sequence of the CS-US pairing does not influence the PN responses during conditioning

Unlike single trial conditioning, three trial forward pairing leads to a prolonged elevation of PKA activity which is necessary for inducing long term olfactory memory (Müller, 2000). Three trial backward conditioning, however, does not lead to prolonged PKA elevation. Therefore, I investigated the responses to the CS-US pairing during multiple trial conditioning. When comparing forward (group 3+) with backward conditioning (group 3bw; Fig.12), it was not possible to compare the CS only, because of the overlap with the US. For example, when taking the first second of the CS stimulation, the backward would already contain the US (group 3bw), while during the last second the forward group would contain the US (group 3+). I therefore analyzed the combined response to CS and US during the conditioning trial (see Methods). Compound responses to the CS and US did not differ between the two paradigms (smallest P for glomerulus 48 with DF= 1, F= 1.972, P = 0.176). There was no difference between the successive *trials* (smallest P for glomerulus 25 with

DF= 2 , *F*= 1.399, *P* = 0.259) and no significant interaction between the *paradigms* and *trials* (smallest *P* for glomerulus 29 with *DF*= 2, *F*= 1.441, *P*= 0.295).

However, there is one exception: Glomerulus 60 shows a difference in *trial* (*DF*= 2, *F*= 3.355, *P*= 0.045) and a Holmes-Sidak multiple comparison revealed that trial 2 is different from both, trial 1 and 3 (*P* = 0.03). In both groups the second trial is slightly reduced. Glomerulus 60 neither shows a pronounced response to 1-nonanol nor sucrose stimulation. Furthermore, this result could not be reproduced in a following experiment, including 3 trial and even 5 trial absolute conditioning (data not shown). I therefore regard this result as biologically irrelevant.

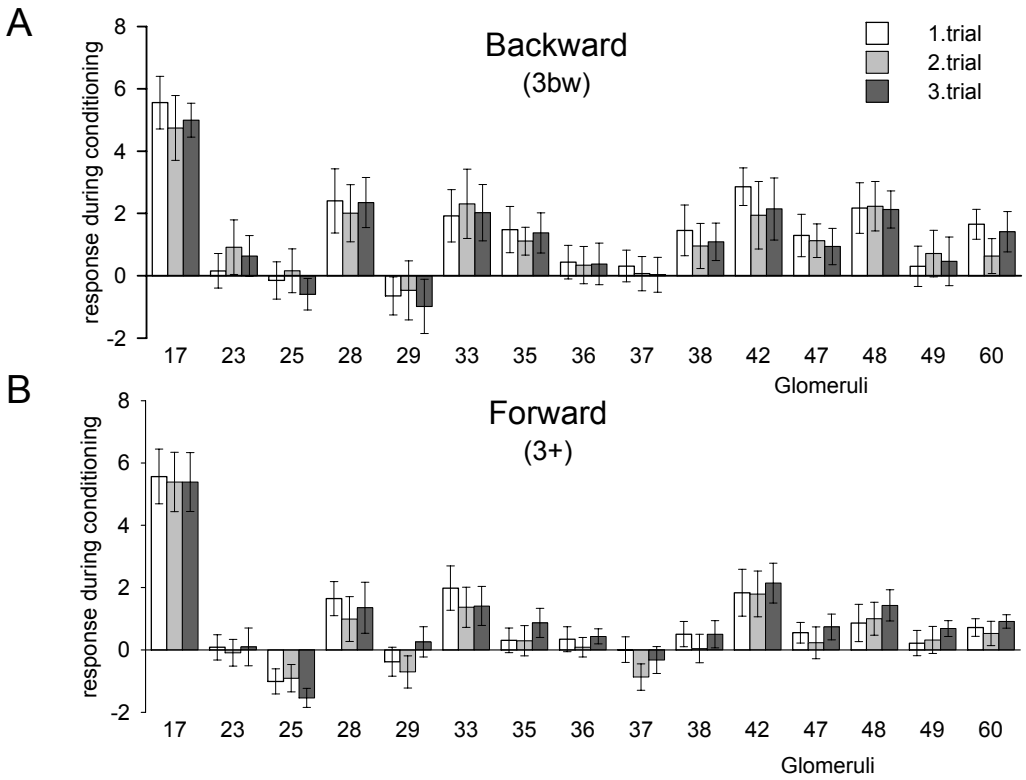

Figure 12: Responses are stable during conditioning.
The compound response to 1-nonanol and sucrose is not modulated during multiple trial conditioning. A: Average compound responses (± SEM, n = 11) during 1st, 2nd and 3rd trial of backward conditioning of 1-nonanol (group 3bw). B: Similarly for forward conditioning (group 3+ with n = 11).

3.7 Stable odor responses in PNs in the AL throughout absolute and differential appetitive conditioning (set 2)

Having established that olfactory conditioning does not change the representation of the conditioned odor, I wondered whether other odors might be modified by learning. To this end, again, the naïve odor responses were determined. In the pre-test phase, all animals received three stimulations of 1-nonanol, 1-hexanol and 1-octanol in a randomized order before and following the conditioning. The three pre-test odor responses in an individual animal were almost completely overlapping, as can bee seen in Figure 13.

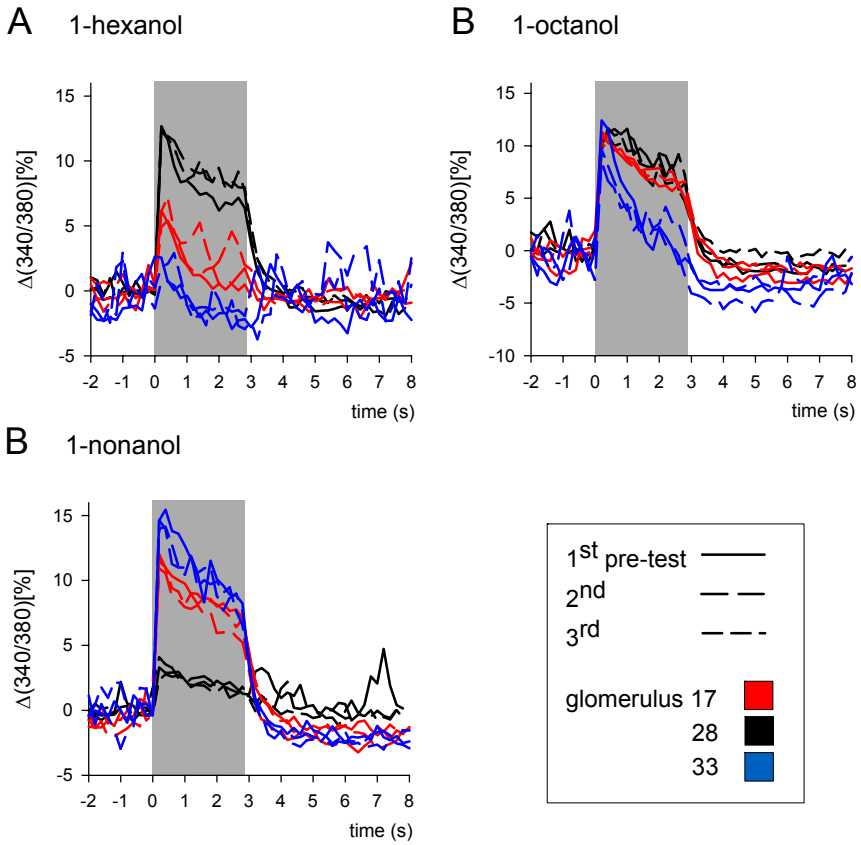

Figure 13: PN odor responses during the pre-test phase of an individual animal. Shown are the response traces of glomeruli 17, 28 and 33 of bee 041123br. Stimulus is marked by a shaded area in the time courses. A: 1-hexanol. B: 1-octanol. C: 1-nonanol.

Statistical analysis of the three pre-test and test odor responses to each odor revealed no significant difference within pre-test (Fig. 14) and within test odor responses, thereby legitimating averaging (ANOVA for all odor responses in each glomerulus, *DF*= 2, smallest *P*= 0.079). For quantification, therefore, the average of the three responses preceding the treatment was defined as the 'pre-test odor response' for every odor and likewise for the 'test odor response'.

There was no significant effect of the various conditioning paradigms (*group*) on any of the test odor responses in comparison with the pre-test (*time*) (i.e. the CS+, the CS- or the neutral odor) (no significant interaction *group* x *time*, RM two-way ANOVA, smallest *P* with *DF* = 6, *F*= 2.152, *P* = 0.059, all other *P*-values above 0.1). Due to the lack of significant interaction between *group* and *time* multiple statistical comparisons between pre-test and test responses within groups are redundant.

In more detail, the different paradigms gave the following results. Corroborating the results of the first set of experiments, the odor response to the CS did not even change after five trials of absolute conditioning (group $5N^+$; Fig. 15). Mean time traces as well as the odor responses were equal in pre-test and test odor responses (exemplified for glomerulus 17, 28 and 33). Also, the neutral odors, given only before and after conditioning, were unaffected. Unfortunately I did not include any group receiving a similar but unrewarded odor sequence. Group $5N^+$ was included into our experiments to compare possible effects of absolute conditioning with those of differential conditioning.

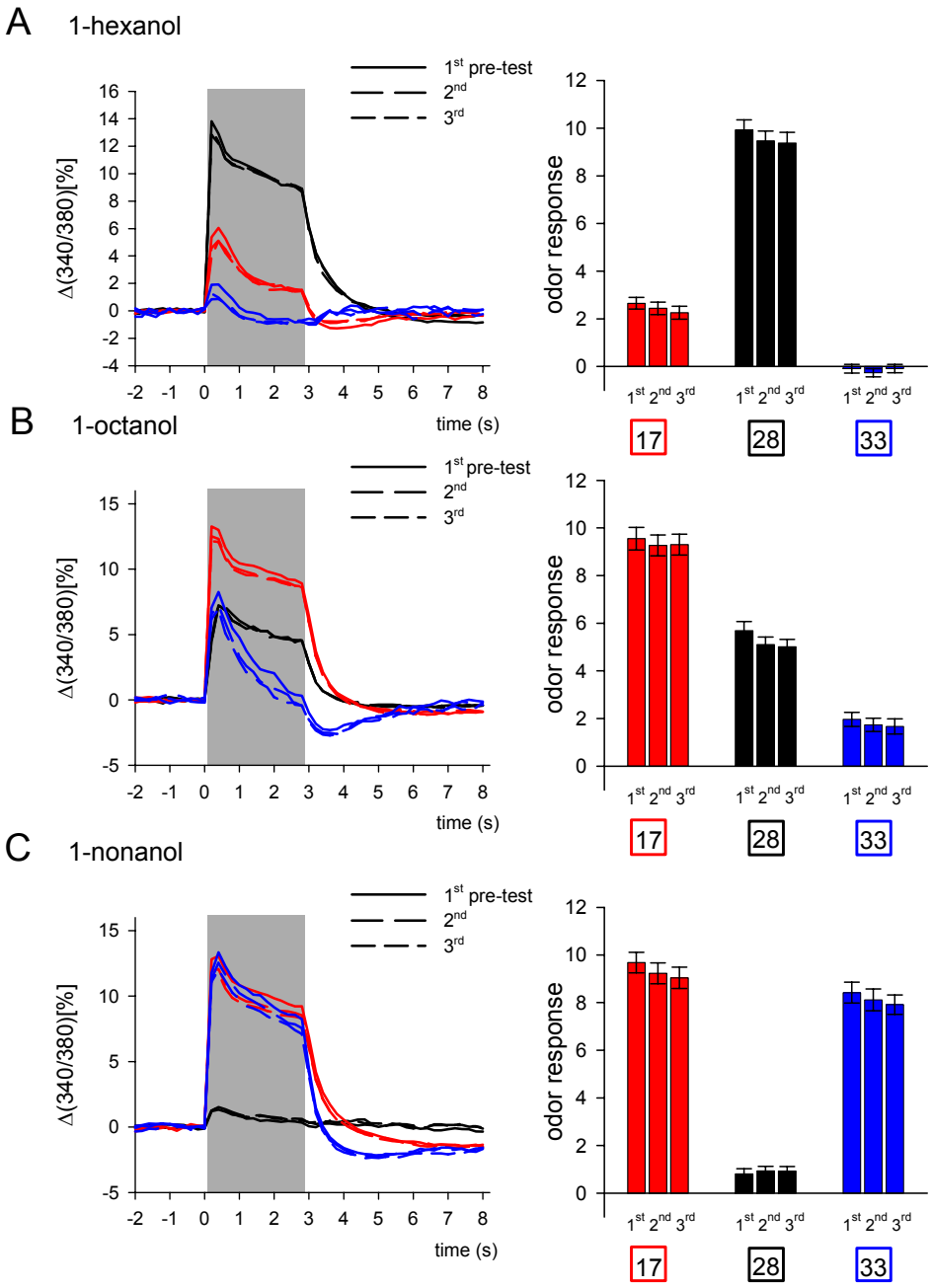

Figure 14: PN odor responses in the three pre-test are not statistically different. Shown are the mean response traces of glomeruli 17, 28 and 33 on the *left* and the corresponding mean odor responses (± standard error of mean, n = 72) on the *right*. Stimulus is marked by a shaded area in the time courses. A: 1-hexanol. B: 1-octanol. C: 1-nonanol.

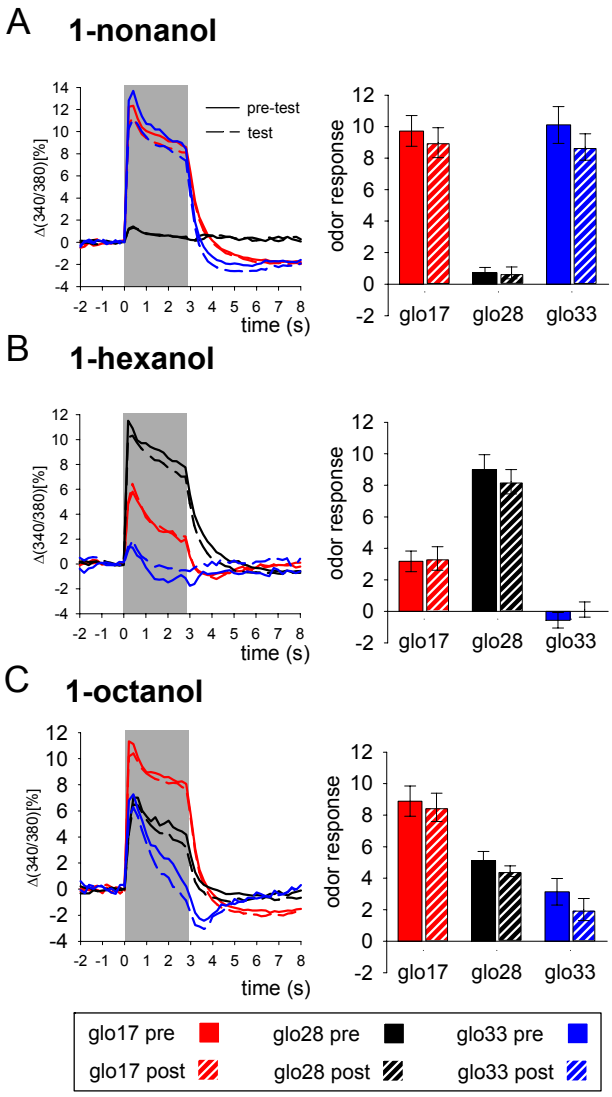

Figure 15: Absolute conditioning to 1-nonanol does not change the responses to other odors.
A: Group $5N^{+}$, the average response traces of the identified glomeruli 17, 28 and 33 to 1-nonanol are shown on the left. Solid lines indicate the pre-test response and the dotted line the test response subsequent conditioning. The respective mean odor responses (± standard error of mean, n = 15) are plotted on the right. B: Similarly for the responses to 1-hexanol and C: 1-octanol.

When 1-nonanol was conditioned differentially against another odor, this also did not induce any changes in the odor responses of any of the odors involved. Test-responses to the CS+, CS- and neutral odor in the differentially conditioned animals (groups $5N^{+}O^{-}$ and $5N^{+}H^{-}$) were not different to those which received the same odor sequence but without rewarding 1-nonanol (groups $5N^{-}O^{-}$ and $5N^{-}H^{-}$). Even if animals had to differentiate between odors that activate highly overlapping glomerular patterns in the AL (exemplified for glomerulus 17, 28 and 33, Fig.16), 1-nonanol and 1-octanol (group $5N^{+}O^{-}$), odor responses to all CS+, CS- and neutral odor remained stable compared to the control group $5N^{-}O^{-}$.

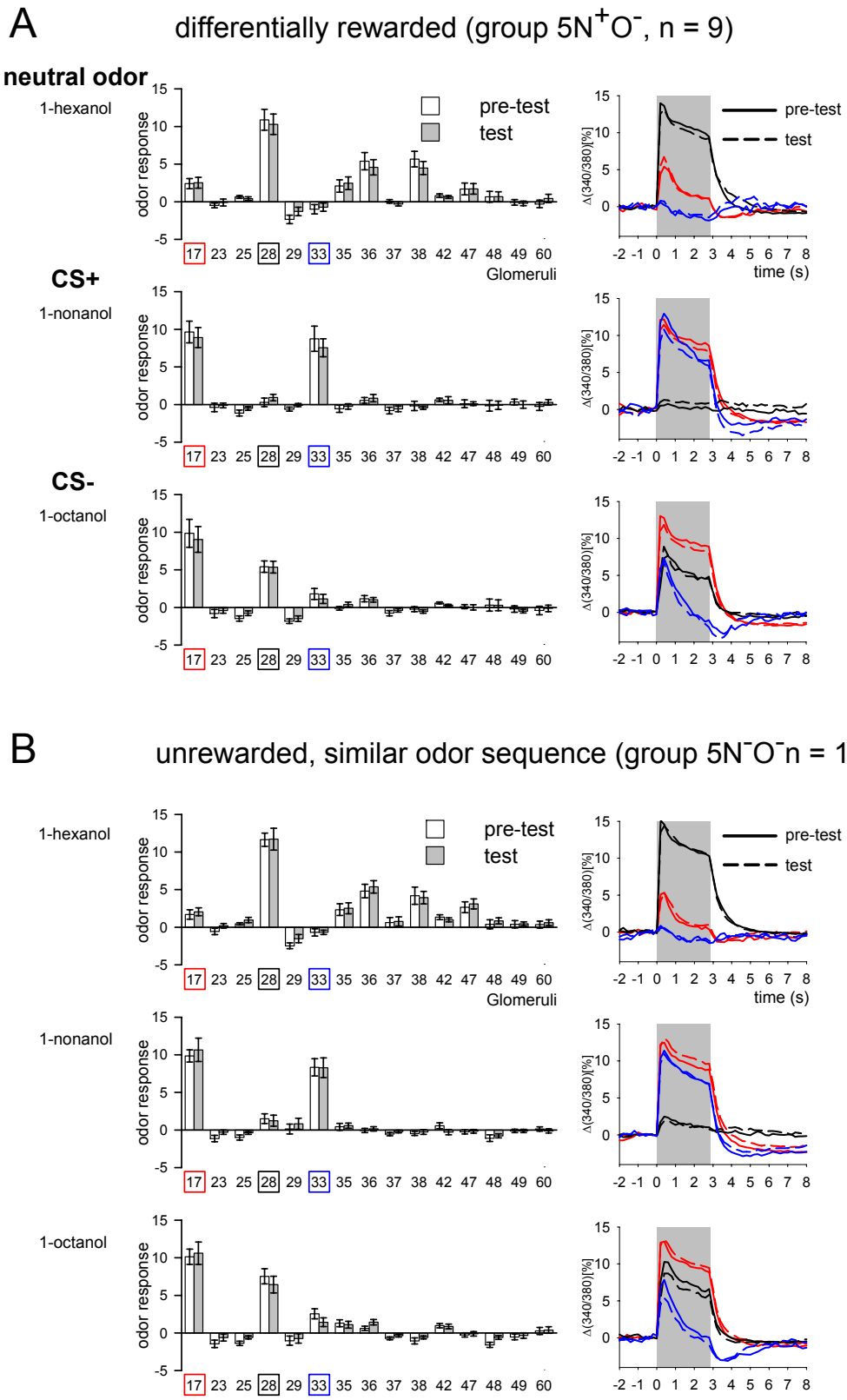
A
differentially rewarded (group 5N+O-, n = 9)
neutral odor
1-hexanol
CS+
1-nonanol
CS-
1-octanol
odor response
pre-test
test
17 23 25 28 29 33 35 36 37 38 42 47 48 49 60
Glomeruli
Δ(340/380)[%]
time (s)
B
unrewarded, similar odor sequence (group 5N-O-n = 10)
1-hexanol
1-nonanol
1-octanol

Figure 16: Differential conditioning does not change odor responses.
A: Shown are the results for differentially rewarded group $5N^+O^-$, *first row*: neutral odor 1-hexanol, *second row:* (CS+) 1-nonanol and *third row:* (CS-) 1-octanol.
(left): Mean odor response (± SEM) before (white) and after (grey) differential conditioning for all glomeruli, *right*: corresponding average time courses, exemplified for glomerulus 17, 28 and 33. The odor-stimulus is marked by a shaded area in the time courses. Time-scale is set to 0 with stimulus on-set.
B: Similarly for group $5N^-O^-$, animals were unrewarded but received the similar odor sequence as $5N^+O^-$ above.

From behavioral studies it is well established that the acquisition in differential conditioning shows an interesting dynamic. After the first reinforcement of the CS+, the response to the CS- is enhanced, although it was never paired with the US, indicating that animals generalize between CS+ and CS-, but as differential training progresses, the response to the CS- returns to, or falls below, the spontaneous response level (Bitterman et al., 1983). For quantification, I took the first second of the odor response to avoid overlap with the sucrose US. The overlapping odor responses to the repeated trials of glomerulus 17, 28 and 33 are shown for group $5N^+O^-$ and $5N^-O^-$ in Figure 17. The higher variability of the time traces for the CS+ after the 2nd second during stimulation compared to the unrewarded control group is due to the response to the US. The physiology of the measured PNs did not correlate with the behavioral phenomena as the different paradigms had no significant effect on odor signals in different trials for neither CS+ nor CS- (RM two-way ANOVA, DF = 4, all $F < 2.168$, all $P > 0.083$). Furthermore, the time traces of each of the 15 observed glomeruli in response to the CS+ and the CS- did not change in overall shape.

However, in both training protocols, responses in the different trials in glomerulus 33 were significantly different ($DF = 4$, $F < 4.553$, $P = 0.003$). The response to 1-octanol showed a significant run-down with both, trial 1 and 2 being different from trial 4 and 5 (Holmes-Sidak multiple comparisons, all P-values $P < 0.023$).

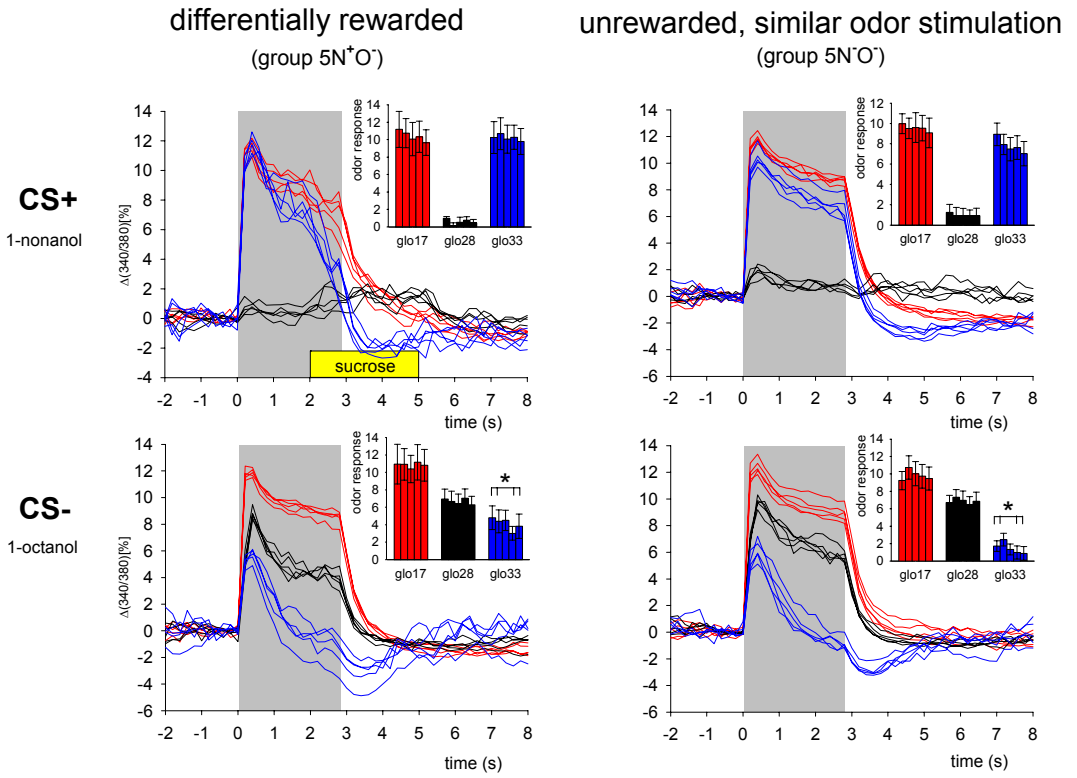

Figure 17: During differential conditioning odor responses remain unmodulated. Shown are the odor responses during the first second of odor stimulation of a five trial differential conditioning to 1-nonanol as the CS+ (upper row) and 1-octanol as the CS- (lower row). On the left are the results for the conditioned animals and on the right for the control animals, which were unrewarded but received a similar odor sequence. Mean response traces of glomeruli 17, 28 and 33 are presented in parallel with the mean odor responses during odor stimulation (± standard error of mean, n = 8). The odor-stimulus is marked by a shaded area in the time courses. Time-scale is set to 0 with stimulus on-set.

3.8 Olfactory responses in a multidimensional coding space

Having shown that odor-responses in individual glomeruli were not modified by learning, I asked whether there might still be an across glomeruli modification, that would not be apparent in an analysis based on individual glomeruli. If a considerable but individually different number of glomeruli change their odor response due to learning only slightly, it might have been overlooked by our analysis. Therefore, I considered the integrated response of all glomeruli and analyzed putative changes between pre-test and test odor responses. I achieved this by taking a multidimensional perspective. In a three dimensional space, spun by the three most responsive glomeruli, an odor response is represented by a loop (Fig.18A). At the maximum of the response, the traces of the different odor responses exhibited the greatest distance to each other. Once the stimulation ceased the traces returned to a common origin.
The maximum of the odor response can be viewed as a three dimensional odor-vector. In Figure 18B the odor-vectors to 1-hexanol, 1-octanol and 1-nonanol are shown. The Euclidian distance (d_{ij}) between two odor-vectors (i and j) can easily be calculated with the following:

$d_{ij} = \sqrt{\sum_{k=1}^{n} (x_{ik} - x_{jk})^2}$, $x_{j,k}$ = odor response, n = dimensions (glomeruli).

The formula used for this operation is applicable to n-dimensions. The observed 15 glomeruli constitute the 15 dimensions of the odor response vectors. Odor distances varied slightly among groups. However, using statistics I found that neither differential conditioning, absolute conditioning nor successive odor repetitions had any significant effect on the distances between odors comparing pre-test and test odor responses (two-way ANOVA, *DF*= 6, *F*= 1.379, *P*= 0.237). The results for groups $5N^+$, $5N^+O^-$ and $5N^-O^-$ are shown in Figure 18C.

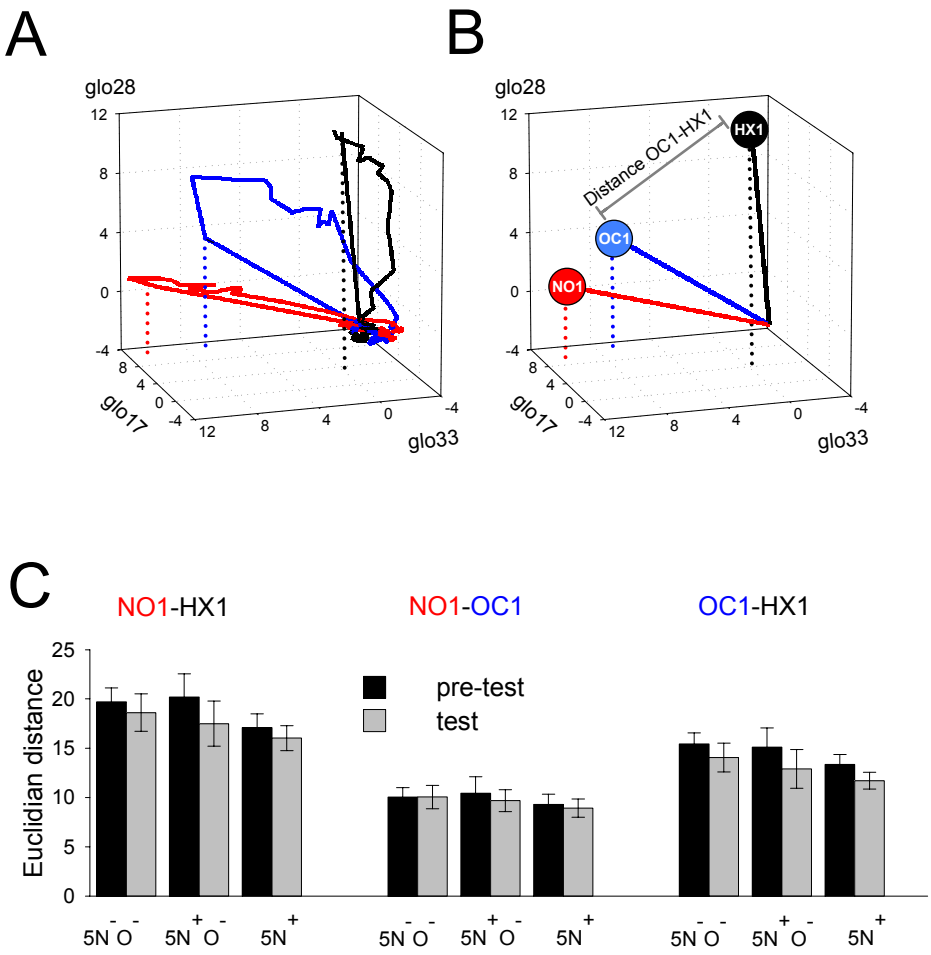

Figure 18: Odor responses in a multidimensional coding space.
Response trajectory to 1-nonanol (red), 1-octanol (blue) and 1-hexanol (black) in a three dimensional space spun by glomeruli 17, 28 and 33. Dashed line shows response after 200ms. B: Schematic drawing of the odor response vectors to the three alcohols. C: Euclidian distances between the three odor vectors, comparing them between pre-test and test responses. Shown are the results for the groups $5N^+O^-$, $5N^+$ and $5N^-O^-$.

3.9 Comparing effects in individual animals

As yet, I neglected the possibility that plasticity of odor responses might be different in each animal. All analyses so far addressed the question of whether olfactory learning leads to changes in odor coding that are consistent across animals. However, this leaves open the possibility that plasticity of odor responses might be different in each animal. For example, learning an odor could lead to more glomeruli being activated by that odor, but if these were different among animals no significant effect could be found across animals. Therefore, I was concerned to accomplish an integrative analysis of individually different changes.

According to non-parametric statistics, I defined that a glomerulus gave an increased response to an odor after learning only when each of the three test responses were higher than each of the three pre-test responses, and vice-versa for decrease.

I found that several glomeruli had either increased or decreased their response, as shown in detail for group $5N^{+}O^{-}$ in Figure 19. But there were no obvious differences between the CS+ and the CS-. To increase the power of this analysis, I combined the two differentially trained groups $5N^{+}O^{-}$ and $5N^{+}H^{-}$, similarly the respective unrewarded control groups $5N^{-}O^{-}$ and $5N^{-}H^{-}$ (Fig.19B). However, the average number of increased glomeruli for the CS+, CS- and neutral odor is not different between trained and unrewarded animals.

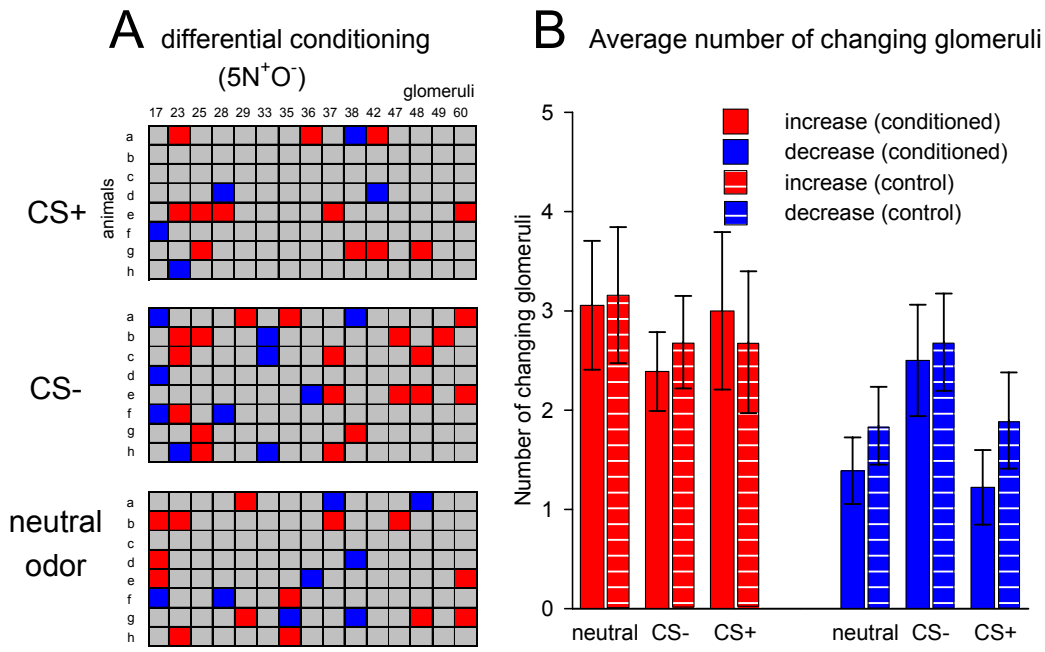

Figure 19: Occasional response changes are not related to associative learning.
A: Shown are the results of the comparison between pre-test and test odor response to the CS+, CS- and the neutral odor in the group $5N^{+}O^{-}$. Increase are depicted in red (all three pre-tests < all three tests), decreases (all three pre-tests > all three tests) in blue for all 15 glomeruli in all 8 animals. Grey indicates no change. B: Summarizes these results for the differentially conditioned animals (group $5N^{+}O^{-}$ and $5N^{+}H^{-}$ combined, n = 19) and compares the results with the respective control groups (group $5N^{-}O^{-}$ and $5N^{-}H^{-}$ combined, n = 19). Depicted is the average number of glomeruli (± standard error of mean) showing a change in response to CS+, CS- and the neutral odor. Increase in red, and decrease in blue. Results of the control group are striated.

3.10 Plasticity of PN odor responses in the AL

I stated above that the statistical analysis revealed changes of odor responses that were group independent. Even if changes in individual odor responses are not related to learning, they still represent a change that might jeopardize reliable odor identification by the animal. I therefore sought to understand the logic of these learning independent odor-response changes. To this end, I pooled all data across conditioning paradigms, and renamed pre-test and test odor responses as early and late odor responses respectively, for clarity. The early and late odor responses are depicted in Figure 20 for all odors and all glomeruli. A considerable number of glomerular odor responses did change from early to late responses (detailed statistics in Fig.20). For all odors, inhibitory responses always decrease comparing early with late odor responses (e.g. glomerulus 23 to hexanol: early = -0.657 ± 0.08, late = 0.0177 ± 0.08, DF= 1, F= 5.122, $p<0.001$). The strongest responding glomerulus only decreased for 1-hexanol (glomerulus 28: early = 10.209 ± 0.159, late = 9.671 ± 0.159, DF=1, F= 5.727, p= 0.020). However, this is a relative change of only 5% compared to for example 23% in glomerulus 38 (early = 4.755 ± 0.132, late = 3.644 ± 0.132, DF=1, F= 35.278, $p<0.001$). Whereas in most cases the change is either a decrease in excitation or inhibition, in some cases the odor response even increased, for example the odor response to 1-octanol in glomerulus 36 (early = 0.588 ± 0.0789, late = 1.111 ± 0.0789, DF=1, F= 21.984, $p<0.001$).

These results are supported by the analysis of the percentage of animals which show either a decrease or an increase in a particular glomerular odor (according to the analysis of individually different changes, pie plots in Fig.20). For example in almost 50% of the animals, the responses in glomerulus 23 increased. This is in line with the significant decrease in inhibition mentioned above.

In Figure 21 the average time traces of early and late odor responses to 1-nonanol are shown in comparison with individual responses to show that the small changes are stimulus correlated and not statistical artefacts.

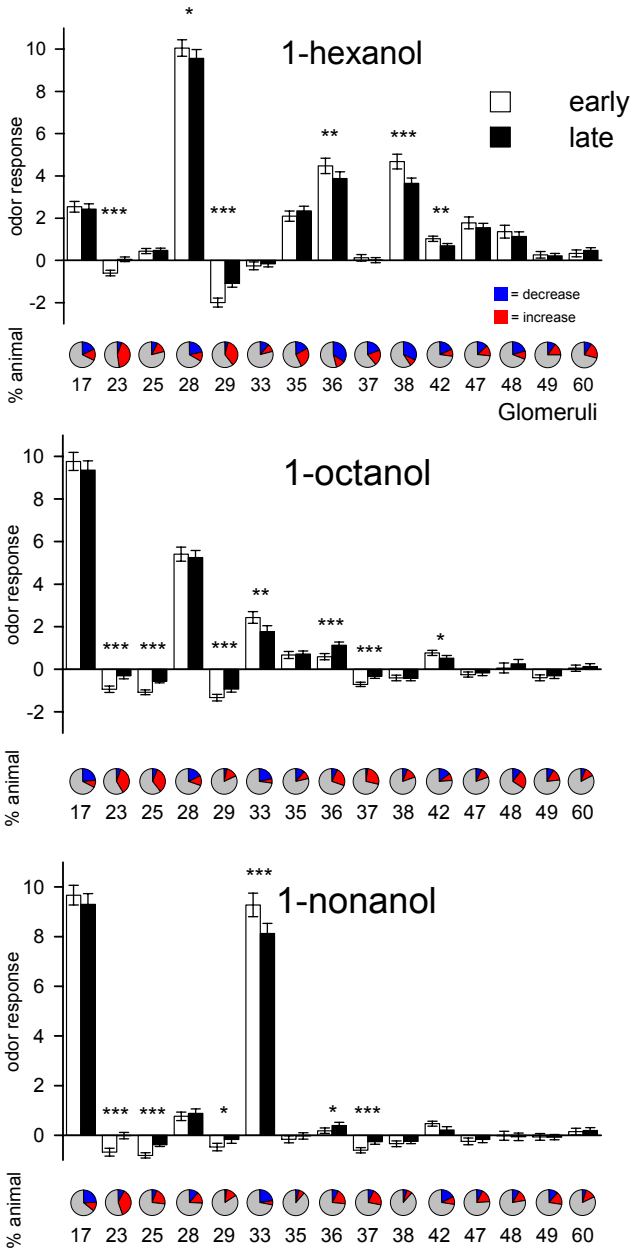

Figure 20: Group independent changes of PN odor responses.
Mean odor responses (± standard error of mean, n = 74) of all animals are shown for 1-hexanol, 1-octanol and 1-nonanol in all 15 glomeruli. Pre-test and test odor responses are now termed early and late odor responses to clarify that these results do not correspond to any specific treatment. Statistically different responses are indicated by asterisks (RM two-way ANOVA, *P<0.05. **P<0.01, ***P<0.001). Below the bar chart, for every glomerulus the percentage of animals showing an increase (red, all three pre-tests < all three tests) or decrease (blue, all three pre-tests > all three tests) is depicted in a pie chart.

1-nonanol

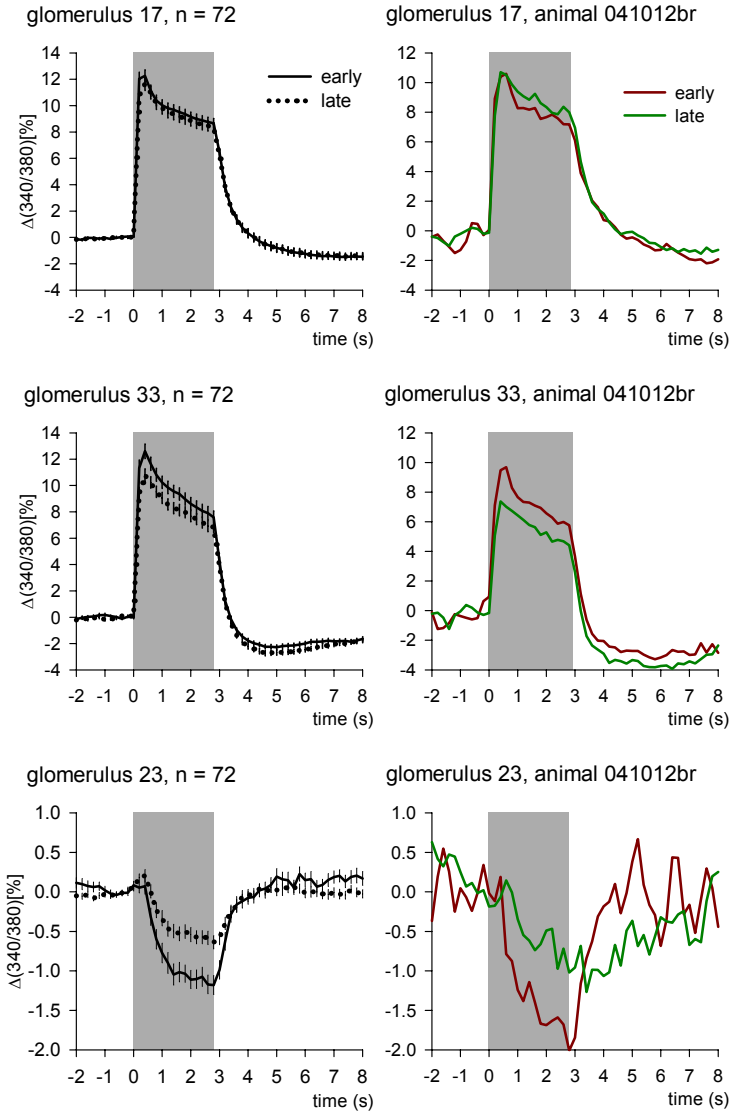

Figure 21

Figure 21: Early and late odor responses – time traces.
Shown are the early and late odor responses to 1-nonanol. *Left*: mean response traces of glomeruli 17, 23 and 33 (± standard error of mean, n = 72) in comparison with early and late responses of an individual bee (*right*).

3.11 The role of lACT PNs in PER to odors and sucrose

All physiological measurements of this study were done on PNs of the lACT tract, which are but a subpopulation of all PNs. I therefore sought to investigate the role that these PNs play in odor-coding and odor driven behavior in behavioral experiments. I was not able to observe PER conditioning during imaging as movement of the animal resulted in signal artefacts. Thus, for a behavioral analysis, animals were prepared and PNs stained similarly to the imaging experiment, but with the difference of leaving the muscles responsible for PER intact and the proboscis free to move.

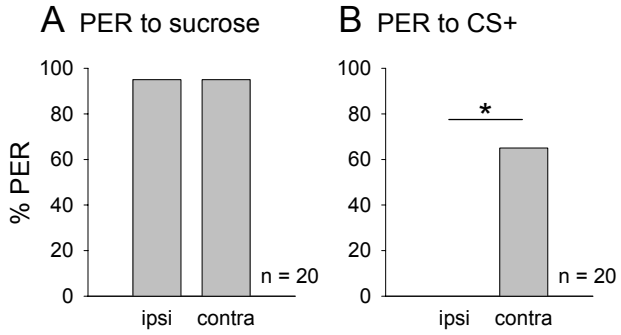

Figure 22: The lACT-tract is necessary for odor recognition in response to the odor CS.
A: Shown are the percentage of animals which responded to a sucrose stimulation on either the ipsi- or the contralateral antenna to the stained PNs. B: Shown are the percentage animals which responded to a learned odor on either the ipsi- or the contralateral antenna to the stained PNs (* $P<0.001$, Mann-Whitney).

Inserting a dye crystal into the lACT disrupts the tract by cutting it. Nevertheless, all animals responded to the sucrose with a PER, no matter whether the stimulus was

applied to the antenna ipsilateral or contralateral to the stained PNs (Fig.21A). Stimulation of the proboscis also led to a robust response. This shows that the US pathway across the antennal lobe had remained intact also on the side were the lACT was damaged by dye injection. Subsequent three trial absolute conditioning to 1-nonanol, 65% of the animals responded with a PER to the CS 1-nonanol, showing that the animals learned. However, when the CS was presented unilaterally on only the antenna ipsilateral to the stained PNs, the animals did not respond with a PER (Fig.21B), indicating that the intact lACT is necessary for odor recognition. Unilateral stimulation of the intact side did elicit a PER, indicating that the unilateral stimulation itself did not cause the unresponsiveness.