Chapter II

Global inhibition and glomerulus specific connections shape the output of the antennal lobe in *Drosophila*

Summary

In order to understand how odor information is represented and processed in the antennal lobe (AL) of *Drosophila melanogaster*, we have optically recorded glomerular calcium responses to single odors and odor mixtures from olfactory sensory neurons (OSN) and projection neurons (PN). Odor mixtures offer a good tool to analyze odor processing because experimental results can be tested against clear predictions. At the level of the OSNs, the representation of odor mixtures can almost always be predicted from the response patterns of the components. PN responses to mixtures, however, show clear evidences of mixture suppression. Furthermore, application of picrotoxin (PTX), an antagonist of $GABA_A$ receptors, enhances odor responses, influences their temporal course and eliminates mixture suppression at the PN level. Our results can be best explained by postulating the existence of two local networks in the fly AL: a network with glomerulus specific connections, probably including excitatory and inhibitory components, and a PTX sensitive, inhibitory global network that acts equally on all glomeruli with proportional strength to the global AL input.

Introduction

Animals live in a complex olfactory world, and it is the task of the olfactory system to detect and classify thousands of odors that appear in nature in different combinations and concentrations. A fundamental question is how the olfactory system maintains its coding capacity over a wide range of stimulus complexities. We have addressed this question by analyzing the responses to single odors and odor mixtures at different processing stages in the AL of *Drosophila*.

In *Drosophila* each OSN expresses one (in a few cases two) class-specific odor receptor protein (OR), and all OSNs expressing the same OR innervate one or few of ~50 glomeruli (Laissue et al., 1999; Vosshall, 2000; Fishilevich and Vosshall, 2005). Glomeruli are the

functional units of the AL, where ~1300 OSNs (Stocker, 1994) converge onto ~150 PNs (Stocker, 2001) and an estimated number of ~100 local neurons (LN) (Stocker et al., 1997; Ng et al., 2002). The convergence ratio between OSNs and PNs in the AL glomeruli is in the range of 30:1 (Stocker, 1994). Most PNs innervate only one glomerulus (Wong et al., 2002; Marin et al., 2002). Although most inhibitory (GABAergic) and excitatory (cholinergic) LNs described so far have broad arborizations and innervate all or most glomeruli (Stocker et al., 1990; Ng et al., 2002; Shang et al., 2007; Okada, personal communication), some LNs with arborizations in few glomeruli have been found (Wilson and Laurent, 2005). It has also been shown that the identity and concentration of an odor is encoded in the combination of activated OSNs over time (de Bruyne et al., 2001; Hallem et al., 2004) and that OSN activity is not simple relayed to the PNs in the AL glomeruli, but is modified by input from the LN network (Wilson et al., 2004; Wilson and Laurent, 2005; Shang et al., 2007).

There is a long tradition in the study of behavioral responses to odor mixtures both in vertebrates (Laing and Francis, 1989; Wiltrout et al., 2003; Kay et al., 2003; Cometto-Muniz et al., 2005; Kay et al., 2005) and invertebrates (Derby et al., 1996; Smith, 1998; Deisig et al., 2003), but only in recent years the technical advances have allowed a physiological approach to this topic. Many studies in vertebrates have focused on the coding of odor mixtures at the peripheral level (Duchamp-Viret et al., 2003; Oka et al., 2004), at the level of the output of the olfactory bulb (Giraudet et al., 2002; Tabor et al., 2004; Lin et al., 2005) and also at the level of the olfactory cortex (Lei et al., 2006; Zou and Buck, 2006). Studies in invertebrates, on the other hand, have focused mostly on the peripheral aspects of odor coding (Akers and Getz, 1993; Derby, 2000; Deisig et al., 2006; Carlsson et al., 2007), and not much is known about what happens with odor mixture information in the AL network. Moreover, previous studies have focused either on the input or the output of the AL/OB (Sun et al., 1993; Giraudet et al., 2002; Heinbockel et al., 2004; Deisig et al., 2006; Broome et al., 2006). However, an analysis of the input and output representations of odor mixtures (Tabor et al., 2004), is necessary in order to understand how the cellular network within the OB/AL processes olfactory activity.

The stereotypic organization of the olfactory system of *Drosophila*, its relative simplicity and the availability of genetic tools offer an ideal model to analyze the coding of odor mixtures, because it allows the possibility of measuring odor evoked calcium responses at the input and output level of the AL network in the same identified glomeruli across many animals. We have thus direct access to the odor-information fed into the network (OSN input) and to the result of processing within the network (PN output), which allows us to draw conclusions

about the mechanisms involved in the processing of odor information. Furthermore, the use of pharmacological tools provides information about the role of specific network components in odor processing.
We present evidences showing that global inhibition and glomerulus specific modulation are involved in the shaping of PN responses in the fly AL.

Materials and Methods

Flies

Adult female *Drosophila melanogaster* (age: 1-3 weeks) were used. Flies were reared in standard medium (100ml contained: 91.8 ml water, 11.8 g sugar beet syrup, 1.08 g yeast, 0.406 g agar, 9.4 g cornmeal, 0.24 ml propionic acid), and kept in incubators at 25°C, 50-60% relative humidity with a 12h :12h light : darkness regime.
All experimental flies contained the P[UAS:G-CaMP 1.3] insertion in the first chromosome (crossed from y*w*P[UAS:G-CaMP];CyO/Sp;+, flies provided by Jing Wang, New York), and a promoter:Gal4 insertion to direct expression of the calcium sensor to the desired neuron population. Mass labeling of OSNs was achieved with flies of the following genotype: *yw*P[UAS:G-CaMP];P[OR83b:Gal4] (provided by Leslie Vosshall, New York). This line drives expression to 60% of all OSNs (Larsson et al., 2004).
OSNs innervating glomerulus DM2 were measured in *yw*P[UAS:G-CaMP];+;P[Or22a:Gal4]/TM2 flies. OSNs innervating glomeruli DM3 and DM5 were of genotype *yw*P[UAS:G-CaMP]/P[Or33b:Gal4];CyO/+; P[UAS:G-CaMP]/+ or *yw*P[UAS:G-CaMP]/P[Or33b:Gal4];Sp/+; P[UAS:G-CaMP]/+ (new crosses were set up every week and F1 flies were used). The second copy of G-CaMP was required to enhance the fluorescence. No differences between the two genotypes (CyO vs. Sp) were found. The parental lines *yw*P[UAS:G-CaMP];CyO/Sp; P[UAS:G-CaMP] as well as P[Or33b:Gal4] and +;+;P[Or22a:Gal4] were provided by Leslie Vosshall (New York). PN flies were of the following genotype: *yw*P[UAS:G-CaMP];P[GH146:Gal4]/CyO (stable line crossed from +; P[GH146:Gal4] flies provided by Reinhard Stocker, Fribourg). These flies express G-CaMP in 80-90 PNs.

Fly preparation

Flies were prepared as previously described (Pelz et al., 2006). Briefly, flies were immobilized on ice for ten minutes and then fixed to a Plexiglas stage by the head and neck, with thorax and abdomen hanging (Fig. 2.1A). Legs and abdomen were fixed to the stage with n-eicosane (Sigma Aldrich, Germany) to reduce movement. Antennae were pulled forward using fine wire (Rediohm-800, HP Reid Inc, USA) in order to allow visual access to the antennal lobes. Then, a thin polyethylene foil supported by a plastic coverslip was sealed to the head with two-component silicon (KwikSil, WPI, Germany) and a hole was cut to expose the cuticle. After placing a drop of Ringer´s saline (in mM: 130 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 36 Saccharose, 5 Hepes, pH 7,3 (Estes et al., 1996)) on top of the head, we cut a window in the cuticle. Glands and airbags were removed, and saline was exchanged several times. Flies were now ready for imaging.

Imaging

Images were acquired with a CCD camera (TILL Photonics, Germany) mounted on a fluorescence microscope (Olympus BX51WI, Japan) equipped with a 20x water dip objective (NA 0.95, X LUM Plan FI, Olympus, Japan). Excitation light of 465 nm was produced with a monochromator (Polychrome II, TILL Photonics, Germany). A 490 nm dichroic mirror was used together with a 506-547 nm band pass emission filter. Binned image size was 153 x 106 pixels on chip, corresponding to 244.8µm x 169.6µm at the preparation. We varied the exposure time between 70 and 120 ms to adjust for different basal fluorescence values across preparations. Eight-second films were recorded with an acquisition rate of 4 Hz. Odors were applied 3 s into each measurement. Inter-stimulus interval (ISI) was 2 minutes.

Stimulation

For the experiments shown in Figures 2.1, 2.2 and 2.6 a constant air stream generated with a vibrating armature air compressor (Reischle Thomas, Switzerland) and regulated to a flow of 240 ml/min with a rotameter (Analyt, Germany) was split into six independent channels. In

each of these channels the air could be directed either through an empty 20 ml glass vial or through a glass vial containing 5 ml of odor dilution in mineral oil (10^{-2} v/v) or mineral oil alone. Glass vials were sealed with aluminum ring caps with a silicon-Teflon septum.

The switch between air and odor was performed by simultaneously activating two pinch valves (two way solenoid pinch valves, Farmington Engineering, USA), one placed before the odor/air vial and the other one after the odor/air vial. Two valves for each channel were required to avoid contamination by passive odor diffusion. After the second set of valves all odor channels were plugged to a six-to-one Teflon manifold with a small mixing chamber. The output of the manifold was directed to the fly antennae. In order to achieve the same concentration for the single odors and the odors in the binary mixture, two channels were simultaneously open during all stimulations (1-hexanol + mineral oil, 2-heptanone + mineral oil or 1-hexanol+2-heptanone). This means that beside the dilution in mineral oil, odors were further diluted in the air (1/6). An air exhaust was placed behind the preparation to rapidly eliminate the odor molecules from the fly environment.

For the experiments in Figures 2.3 to 2.5 a main air flow (1l/min) was directed to the fly through a glass tube (~5 mm diameter). A secondary air stream was added through a lateral hole in the glass tube (150 ml/min). The secondary air stream was added to the main air stream with a 6:1 Teflon manifold which collected the double outputs of 3 magnetic valves (Lee, USA). Each magnetic valve had two positions. In the closed position air was guided to the manifold through an empty Pasteur pipette and in the open position through a Pasteur pipette containing two round filter papers (∅ = 1 cm) loaded with 20µl of odor dilution or mineral oil. All odors were diluted 1/100 in mineral oil. Odor presentation lasted 1 sec. Odor mixtures were achieved by simultaneous switching of 2 or 3 valves. Odor-loaded Pasteur pipettes were disconnected from the valves immediately after each block of stimulations (3 single odors + 4 mixtures) and clean air was flushed through the valves for 1 minute to avoid contamination. Filter papers were replaced after each experiment.

The following odors were used in the second set of experiments: 1-butanol, isopentyl acetate, propionic acid, 2-heptanone and 1-hexanol. The following binary and ternary mixtures were tested: 1-butanol:isopentyl acetate; 1-butanol:propionic acid; propionic acid:isopentyl acetate; 1-butanol:pentyl acetate:propionic acid; 1-hexanol:2-heptanone, 1-hexanol:propionic acid; 2-heptanone:propionic acid and 1-hexanol:2-heptanone:propionic acid.

Odors were acquired from Sigma-Aldrich, and were of the highest purity available. New odor dilutions were prepared every week for the first experiment set, and once every month for the second set. Odor presentation lasted 1 s. Odors were presented in a pseudo randomized

sequence across animals. In all cases odor mixtures were performed in such a way that the concentration of each component was the same during the single odor presentation and the presentation in the mixture. Thus, the total amount of odor molecules was higher during mixture presentation.

Pinch valves and magnetic valves were activated by a 12 V pulse given by a VC6 controller unit (Perfusion Valve System, Warner Instruments, USA). Stimulation was controlled electronically with custom written software (Olfastim, Alexander Galkin) which sent TTL pulses to the corresponding channels through a USB digital-analog converter (Mem-Pio, BMC Messsysteme GmbH, Germany).

Odor concentration was in the lower part of the dynamic range of the concentration-response curve for all odors (data not shown).

Pharmacology

A stock solution of Picrotoxin (Sigma) was prepared with DMSO (100 mM) and kept protected from light at -20°C. Before each experiment, the stock solution was further diluted in Ringer's to achieve a final concentration of 5 µM. In each stimulation block the two single odors and their mixture were presented with an ISI of 2 minutes. After this block was repeated twice, the Ringer solution covering the brain was exchanged with a solution containing PTX. After two minutes the stimulation block was presented twice. After treatment, the PTX solution was removed by suction and the brain was flushed with Ringer's several times. The stimulation block was presented again twice after washing. The odor sequence within each stimulation block was changed in a pseudo randomized way within and between experiments.

The concentration of PTX was set to 5 µM because concentrations of 10µM and higher induced spontaneous calcium waves in the AL (data not shown), which made it impossible to analyze the data. Application of DMSO 5×10^{-5} % (v/v) alone produced no changes in response amplitude or temporal dynamic.

Data analysis

Flies with reliable calcium responses throughout the protocol and no or negligible responses to mineral oil were considered for data analysis. Data were processed with custom made

routines written in IDL (Research Systems Inc., USA). First, films were corrected for movement artifacts using anatomical landmarks. A bleach correction was performed by fitting a logarithmic function of the form $F(x) = a \times e^{(bx)} + c$ to the average light intensity in the AL over time. Frames covering 4.5 s after stimulus onset were excluded from the bleach correction calculation. After bleach correction the relative calcium change was calculated for each frame as $\Delta F/F\ (\%) = (F_i\text{-}F_0)/F_0 \times 100$, where F_i is the absolute fluorescence of the *i*th frame and F_0 the average fluorescence of the 5 frames before stimulus onset (background fluorescence).

Color coded images were generated by subtracting the average fluorescence of 4 frames during stimulation minus the average fluorescence of the 4 frames before stimulus. A Gaussian filter was applied to these images (kernel = 3 pixels) to reduce noise.

In order to identify the glomeruli, a set of odors was presented at a concentration that only elicits responses in one or few known classes of OSNs (information extracted from (de Bruyne et al., 2001; Hallem et al., 2004; Fishilevich and Vosshall, 2005; Couto et al., 2005). Glomerulus identification was also performed based on anatomical cues. Some glomeruli could be reliably identified in all flies, but not assigned to a glomerulus in the *Drosophila* antennal lobe atlas (Laissue et al., 1999) due to the lack of spatial resolution. The following landmark odors and concentrations were used for glomerulus identification: ethyl-hexanoate 10^{-6} (v/v) for **DM2**; pentyl acetate 10^{-6} (v/v) for **DM3**, E2-hexenal 10^{-4} (v/v) for **DL5** and ethyl-3-hydroxybutyrate 10^{-6} (v/v) for **DM5**. VA1lm was identified due to its location at the point where the antennal nerve enters the AL. Glomerulus **X1** was characterized by its response to 1-butanol and 1-hexanol, and could correspond to glomeruli DA2 or DA4 due to its position. Glomerulus **X2** was activated by 1-butanol and was located next to glomerulus DM5. This glomerulus could correspond to VM2 due to its position. Glomerulus **X3** was characterized by its negative response to propionic acid in the OSNs and the vicinity to a glomerulus activated by 1-butanol (X1).

For time traces, the average light intensity of a 7×7 pixel square for each glomerulus was calculated. The response amplitude was calculated as the average of 4 frames during stimulation minus the average of 4 frames before stimulation.

The time to maximum was calculated as the time (in ms) from stimulus onset to the maximum of the calcium response (Fig. 2.6).

Statistical analysis

Further analysis was performed with Excel (Microsoft Office 2003), R (http://www.R-project.org), SigmaStat (SPSS Inc., USA) and Statistica (Statsoft).

For each glomerulus and mixture, the lower bound was calculated as the response to the strongest component. The upper bound was the response to a 2-fold higher concentration of the same odor. A single tailed Wilcoxon Signed Rank Test ($\alpha = 0.025$) comparing the measured response to the mixture and the lower or upper bound was performed for each glomerulus (in the second set of experiments only the lower bound was available and tested). In OSNs, some glomeruli showed negative responses to some odors. Since it is not clear how negative responses are evoked, all odor mixtures that evoked negative responses and/or contained components that evoked negative responses were excluded from the analysis (see Figs. 2.3CD and 2.4B).

In the pharmacological experiment, a Two-Way RM ANOVA was performed for each glomerulus. Planned comparisons between the response to the mixture and the lower bound in each treatment phase (odor effect, levels: 2-heptanone, 1-hexanol and mixture) and between the responses to each odor under the different treatments (treatment effect, levels: Ringer and PTX) were performed using contrasts for the Least Square Difference Means (General Linear Model Statistica). A Two-Way RM ANOVA was also performed to compare the time to reach maximum for the different odors and treatments. In all cases the first presentation of each stimulus under control conditions (Ringer) was compared with the second presentation during the PTX treatment (see above) for each odor. Since not all animals gave reliable responses after PTX treatment, the wash phase of the experiment was left out of the statistical analysis.

The box-plot in Figure 2.5A contains the following information: the whiskers correspond to $\pm 1.5 \times$ IQR (interquartile range). Values below $-1.5 \times$ IQR or above $1.5 \times$ IQR are considered outliers (circles). The hinges correspond to the Q_{25} and Q_{75} (1^{st} and 3^{rd} quartiles). The line in the middle of the box shows the median of the distribution. The notches represent $\pm 1.58 \times$ IQR/sqrt(N), and give a rough idea of the 95% confidence interval for the difference between two medians (in cases of similar N) (McGill et al., 1978). The width of the boxes is proportional to number of observations.

Results

In order to understand the way in which the AL network of *Drosophila* processes olfactory information, we analyzed the glomerular responses to a series of monomolecular odorants and their binary and ternary mixtures. Odor evoked calcium changes were measured in the OSN and PN.

The null hypothesis

As a first step in the analysis of mixture representation and processing it is necessary to establish what would be the expected response to an odor mixture in the absence of interactions between its components. Since the response profile of OSNs in *Drosophila* is determined by the OR they express (Hallem et al., 2004) and each glomerulus receives input from only one class of OSNs, we can base the predictions about the expected outcome in the case of no interaction on a model of two ligands binding a single receptor (Tabor et al., 2004). If component A is the best ligand in a mixture of A and B, and the concentrations of A and B are within the dynamic range of the concentration-response relationships for that receptor, the stimulation with AB should result in one of the following cases: (1) If A activates the receptor and B does not, the response to the mixture (R[AB]) should correspond to the response to A (R[A]); (2) if A and B activate the receptor with equal intensity, so that AB would be comparable with AA, R[AB] should be as high as R[AA]; (3) finally, if A and B activate the receptor, but are not equally potent as ligands, R[AB] should lie between R[A] and R[AA]. Therefore, if the measured response to the mixture R[AB] is either lower than R[A], or higher than R[AA], then the components of the mixture must "interact" at some level. We define the first case (R[AB]<R[A]) as "mixture suppression" and the second case (R[AB]>R[AA]) as "mixture synergism". In the case of the olfactory system, mixture suppression or synergism can arise at the receptor level (e.g. when B is a competitive antagonist of A), or at the level of the neural network.

Representation of single odors and binary mixtures

We first analyzed the response patterns of OSNs and PNs in flies stimulated with 1-hexanol, 2-heptanone and their binary mixture. Color-coded pictures of the odor evoked calcium

responses are shown in Figure 2.1B. Odor responses were reliable across animals, with 2-heptanone activating glomeruli DM2 and DM3 and 1-hexanol activating glomeruli DM5 and X1 (Fig. 2.1BC). A two-fold increase in odor concentration induced responses of higher amplitude. Other glomeruli were active only in some animals, or could not be unambiguously identified. Responses followed a monophasic time course: calcium concentration increased rapidly with stimulus onset and decreased after reaching a maximum at or briefly after stimulus offset.

The glomerular response pattern to the binary mixture qualitatively corresponded to the combined response patterns to the single odors: all glomeruli present in the response pattern of either component were also active upon stimulation with the mixture, and no new glomeruli were recruited (Fig. 2.1BC). No qualitative differences were found in the time course of the responses between the single components and the mixture (Fig. 2.1C).

In order to analyze the existence of mixture interactions we measured the amplitude of the response to each component and the mixture, and defined the lower and upper bound of the no-interaction interval for each glomerulus (see Materials and Methods).

For OSNs, the amplitude of the response to the mixture was within the no-interaction interval in all glomeruli (Fig. 2.1D). In contrast, PN responses to the mixture were significantly lower than the lower bound in glomeruli DM2 and DM5 (Wilcoxon Signed Rank Test, $p<10^{-4}$, N=22), and were within the no interaction interval for X1 and DM3 ($p>0.1$).

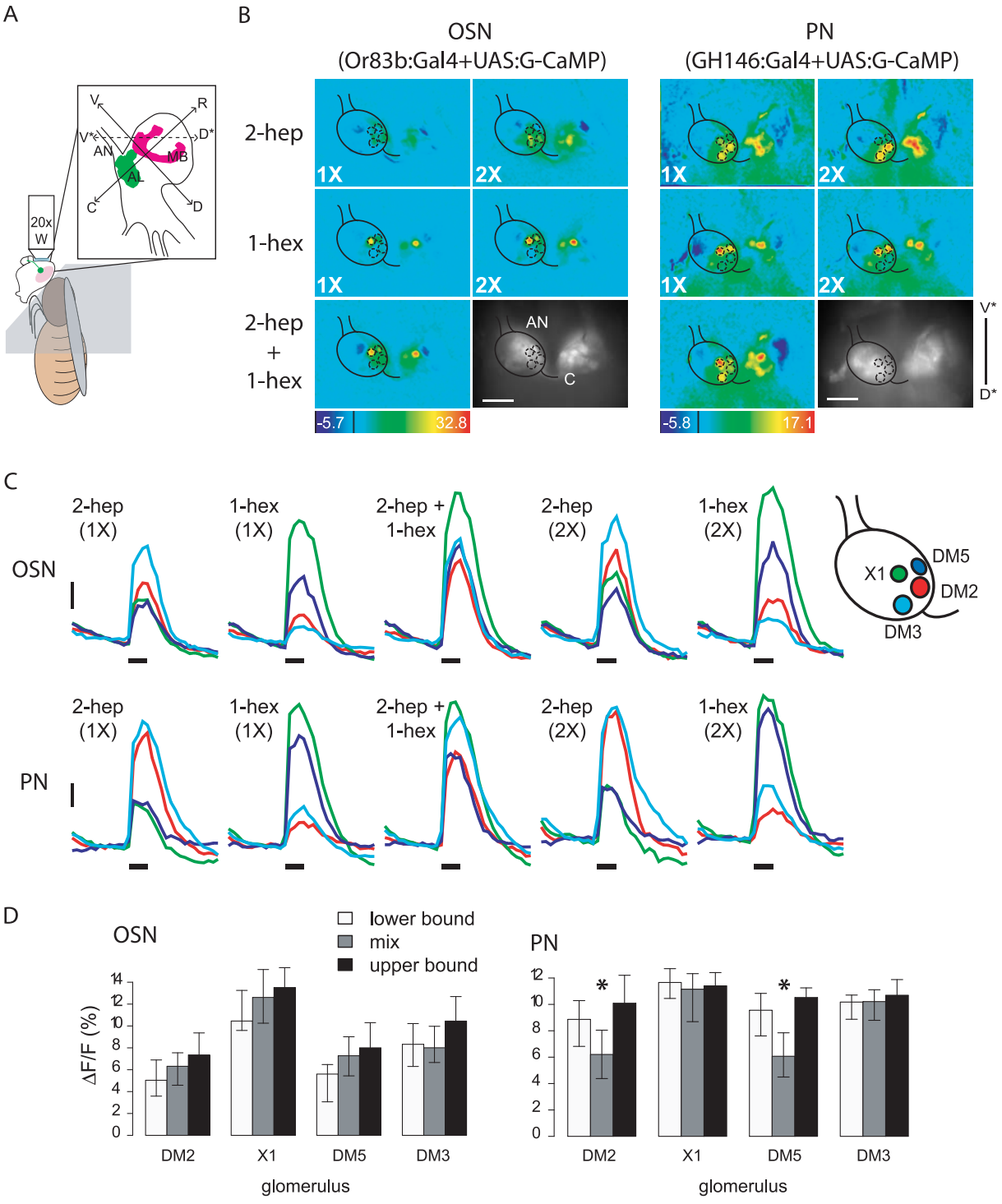

Figure 2.1 - Representation of odor mixtures in the AL

(A) Schematic (lateral) view of the preparation. The antennae are pulled forward to allow optical access to the antennal lobes. The frontal view of the antennal lobes obtained with this preparation is tilted with respect to the neural axis. Inset: AN: antennal nerve; AL: antennal lobe; MB: mushroom body; V* and D* stand for the ventral and dorsal directions tilted with respect to the neural axis. **(B)** Raw fluorescence pictures and color-coded odor responses for one fly for the OSNs (left) and one fly for the PNs (right). 1-hexanol and 2-heptanone were presented alone [in one- (1X) and two-fold (2X) concentration], and together as mixture. The mask overlaying the left ALs shows the position of the measured glomeruli. The numbers in the color scale indicate the maximum and minimum ΔF/F (%) values used for scaling. AN: Antennal nerve; C: commissure. Scale bar = 50 µm. **(C)** Median time traces of the responses to 2-heptanone (1X and 2X), 1-hexanol (1X and 2X) and the mixture

measured in glomeruli DM2, DM3, DM5 and X1 (see Materials and Methods for details on glomerulus identification, see scheme for approximate glomerulus localization). The time of odor stimulation (1 s) is indicated by the black bar below each trace group. The scaling bar indicates 2.5% ΔF/F for the OSNs and 2% ΔF/F for the PNs. N=23 animals for the OSNs and 22 for the PNs. **(D)** Median responses to the best odor in 1X concentration (lower bound), the mixture and the best odor in 2X concentration (upper bound) in each analyzed glomerulus. 2-heptanone was the best odor for glomeruli DM2 and DM3; 1-hexanol was the best odor for glomeruli DM5 and X1. Error bars indicate the Q_{25} and Q_{75}. Asterisks indicate the two cases where the response to the mixture was significantly lower than the lower bound ($p< 0.05$, Wilcoxon´s Test for paired samples, $N_{\text{lower bound OSN}}=23$, $N_{\text{upper bound OSN}}=22$, $N_{\text{lower bound PN}}=22$, $N_{\text{upper bound PN}}=17$).

To exclude the possibility that mixture interactions in OSNs might have been masked by light scattering from other glomeruli, we repeated the experiment in flies where the expression of G-CaMP was restricted to single glomeruli. Expression in glomeruli DM2, and DM5 and DM3 was driven by the promoter regions of OR22a and OR33b respectively (Fishilevich and Vosshall, 2005). Color-coded responses and morphological views are shown in Figure 2.2A. For glomeruli DM2 and DM3, no mixture interaction was found at the single glomerulus level: the response to the binary mixture was between the lower and upper bound (Fig. 2.2BC). In glomerulus DM5, on the other hand, responses to the mixture were below the lower bound (Wilcoxon Signed Rank Test, $p<0.001$, N=16). Summing up, we found mixture suppression in glomerulus DM5 at the level of the OSNs. This could result from interactions at the receptor-ligand level or they could be mediated by the AL network, e.g. through presynaptic inhibitory input onto OSN axons. Mixture suppression was still present in the PNs in this glomerulus. In glomerulus DM2 mixture interactions only occurred in PNs but not in OSNs (Figs. 2.1D and 2.2C) indicating an effect of the AL network. There was no interaction in glomeruli X1 and DM3 in any of the tested neuron populations.

Since the measured response to the mixture was in all cases below the upper bound of the no-interaction interval, we conclude that no mixture synergism takes place in the fly AL for the mixture of 1-hexanol and 2-heptanone.

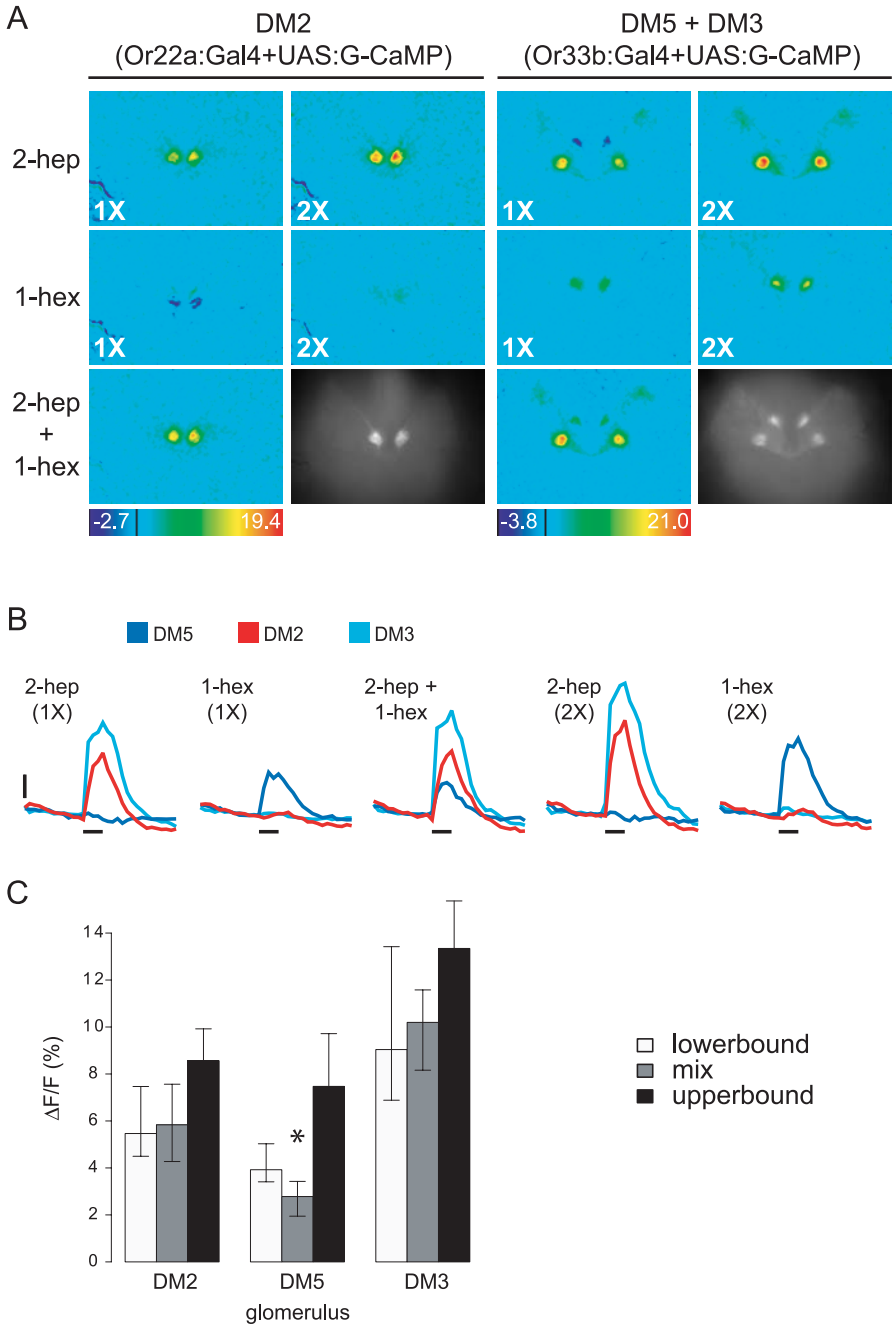

Figure 2.2 - Mixture suppression at the OSN level

(A) Raw fluorescence pictures and color coded pictures of the responses to 1-hexanol, 2-heptanone and the binary mixture in flies where single classes of OSNs were labeled. Or22a:Gal4 was used to label OSNs innervating DM2 (left, N=14). Or33b:Gal4 was used to label OSNs innervating DM3 and DM5 (right, N=16). **(B)** Median time traces of the responses to 2-heptanone (1X and 2X), 1-hexanol (1X and 2X) and the mixture measured in glomeruli DM2, DM3 and DM5. The time of odor stimulation (1 s) is indicated by the black line below each trace group. The scaling bar indicates 2.5% ΔF/F. N=14 for the DM2 and 16 for the DM3 and DM5. **(C)** Median responses to the best odor in one-fold concentration (lower bound), the mixture and the best odor in two-fold concentration (upper bound) in each glomerulus. Error bars indicate the Q_{25} and Q_{75}. Asterisks indicate the case where the response to the mixture was significantly lower than the lower bound ($p < 0.05$, Wilcoxon´s Test for paired samples). 2-heptanone was the best odor for DM2 and DM3 and 1-hexanol for DM5.

A glomerulus specific network alters the representation of single odors and mixtures in PNs

Are mixture interactions odor-dependent? Do they increase with increasing complexity of the mixture? To answer these questions we tested an additional set of odors and its binary and ternary mixtures. Since there were no cases of mixture synergism in the first set of experiments, odors were measured only at one concentration and the data were tested for mixture suppression only. Two sets of three odors were tested: the first set included 1-hexanol (X), 2-heptanone (N) and propionic acid (P), and the second set included isopentyl acetate (I), 1-butanol (B) and propionic acid. Eight glomeruli (DM2, DM3, DM5, DL5, VA1lm, X1, X2 and X3. See Materials and Methods) responded reliably to at least one of the tested odors and were used for subsequent analysis. The spatial representation of B, I, P, all binary mixtures and the ternary mixture in OSNs and PNs is shown in Figure 2.3AB. The response amplitudes for each single odor and the mixtures are shown in Figure 2.3C.

Interestingly the best odor for a given mixture in a given glomerulus was not always the same for OSNs and PNs. In glomerulus DM2, for example, responses to 1-butanol were higher than responses to isopentyl acetate in the OSNs, but lower in the PNs (Fig. 2.3C, see also Table S1 in the Appendix). These differences in the relative response magnitudes for different odors within a glomerulus is a further evidence that, even during stimulation with monomolecular odors, PN responses are shaped by the AL network, in such a way that the gain for each glomerulus is regulated in an odor specific way.

We defined interaction index as the difference between the lower bound and the measured response to the mixture. Positive interaction indices significantly higher than zero indicate mixture suppression. Interaction indices not significantly different from zero correspond to the cases where the response to mixture is not different from the lower bound, and might reflect weak mixture suppression (Duchamp-Viret et al., 2003). Negative interaction indices significantly lower than zero indicate absence of mixture suppression. The interaction indices for all mixtures in OSNs and PNs are shown in Figure 2.3D.

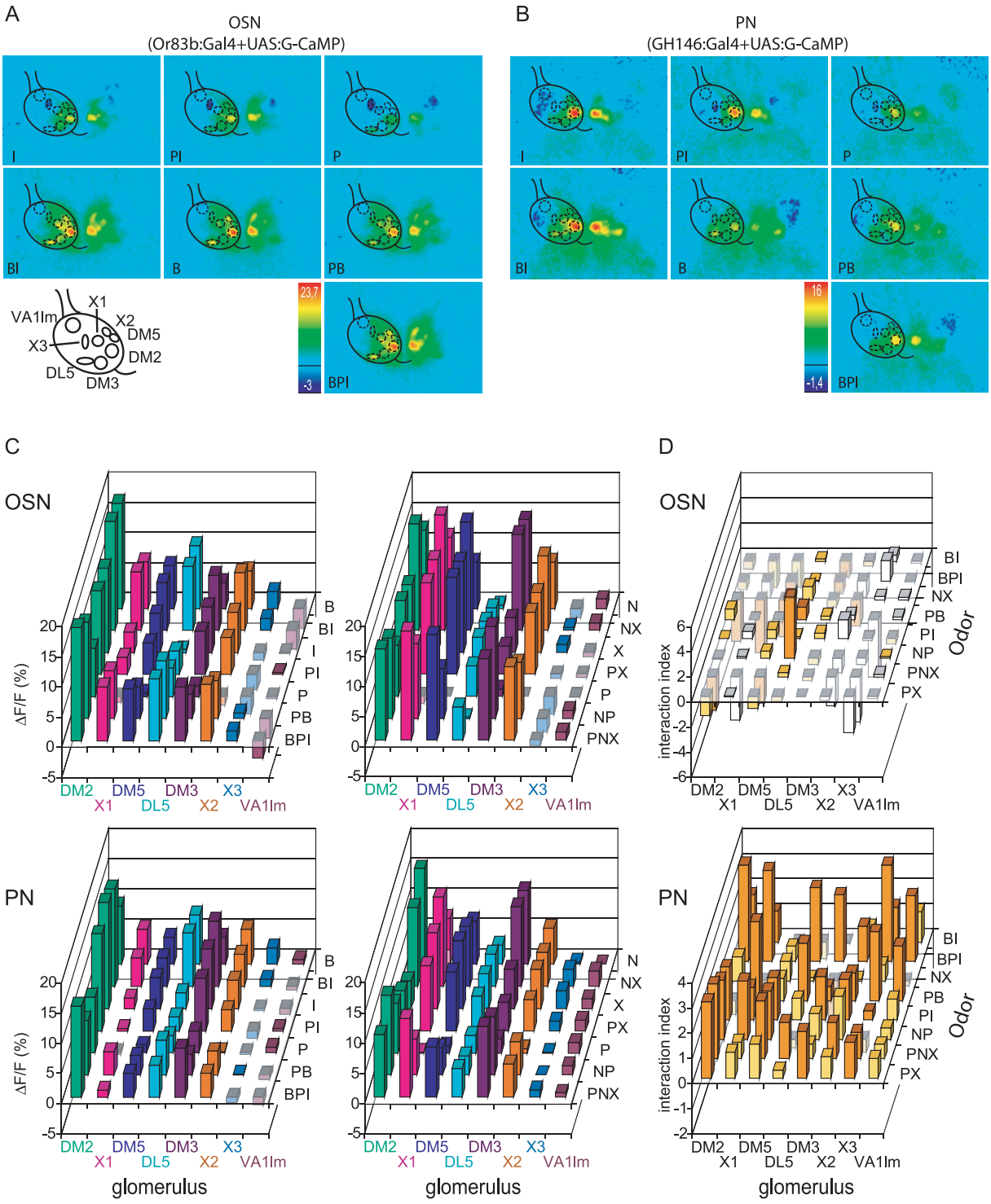

Figure 2.3 - Mixture interactions in OSNs and PNs

(A) Color coded pictures of OSN responses to 1-butanol (B), isopentyl acetate (I), propionic acid (P), all binary mixtures (BI, PI, PB) and the ternary mixture (BPI) in a single fly. The mask over the left AL shows the position of the eight glomeruli included in the analysis. **(B)** Color coded pictures of PN responses to 1-butanol (B), isopentyl acetate (I), propionic acid (P), all binary mixtures (BI, PI, PB) and the ternary mixture (BPI) in a single fly. The mask over the left AL shows the position of the eight glomeruli taken into account for the analysis. **(C)** Amplitude of the median responses to 1-butanol (B), isopentyl acetate (I), propionic acid (P), all binary and the ternary mixture (BI, PI, PB and BPI) (left) and to 1-hexanol (X), 2-heptanone (N), propionic acid (P); all binary mixtures (NX, NP, PX) and the ternary mixture (PNX) (right). Note that some odors elicit negative responses in some glomeruli. Upper

row: OSN (N=9), lower row: PN (N=10-15). **(D)** The interaction index (lower bound – response to the mixture, where the lower bound is the response to the best component for the corresponding mixture) for OSNs and PNs. Orange bars indicate cases where the lower bound was significantly lower or higher than the response to the mixture (Wilcoxon Signed Rank Test, $p<0.05$). Yellow bars indicate cases where no significant difference was found between the lower bound and the mixture (Wilcoxon Signed Rank Test, $p>0.05$). White bars indicate cases where at least one of the components evoked a negative response. These cases had to be excluded from the analysis because they do not comply with the assumption that all components of the mixture bind to the same binding site of the OR, and thus our no-interaction hypothesis is not valid. Significant interaction indices > 0 indicate cases of mixture suppression. Significant interaction indices < 0 indicate cases where mixture suppression can be excluded. Note that mixture synergism was not tested in this experiment. Upper panel: OSN, lower panel: PN.

The interaction profile was different in OSNs and PNs (Fig. 2.3D and 2.4B). While only two cases of mixture suppression were found in OSNs (both in glomerulus DL5), every glomerulus showed suppression for at least two mixtures in the PNs (Fig. 2.3D). Some examples of the interaction dissimilarities between OSNs and PNs are shown in Figure 2.4A.

In OSNs, we found negative responses to one component of the mixture and positive or no responses to the other component in some glomeruli. For example, in glomerulus X3, the response to propionic acid was negative but the response to 1-butanol was positive (Fig. 2.3C). These cases, as well as cases where the responses to the mixture and both components were negative were excluded from the analysis (see Materials and Methods).

The differences between OSNs and PNs in their interaction profiles were statistically tested with a Two-Way ANOVA for each glomerulus. The interaction between the factors (neuron type vs. odor mixture) was significant in all glomeruli except X2. Post hoc paired comparisons show that the differences between OSNs and PNs are odor and glomerulus dependent. Therefore, at least part of these interactions must be mediated by a network that is not uniform across the AL. We then sought to describe this network in more detail.

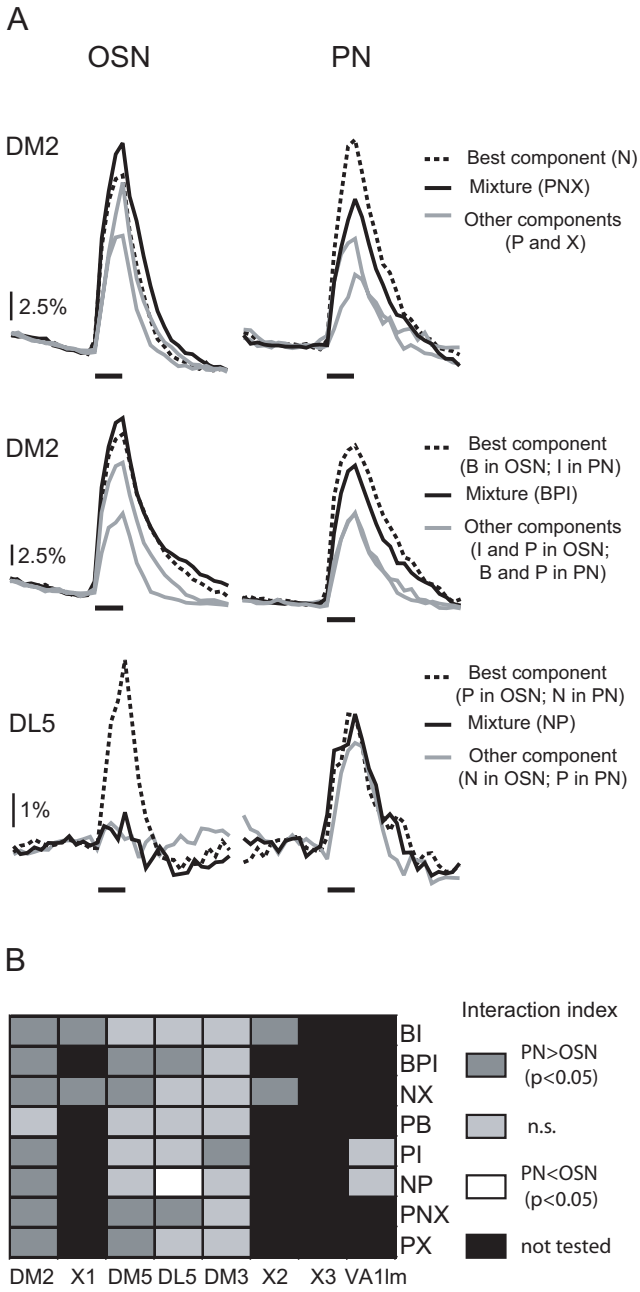

Figure 2.4 - OSNs and PNs show different mixture interaction profiles

(A) Median traces of the responses to the components and mixtures in three exemplary cases. **Top:** responses to 1-hexanol (X), propionic acid (P), 2-heptanone (N - best odor) and their ternary mixture (PNX) in glomerulus DM2. The interaction index was significantly < 0 in OSNs and significantly > 0 in PNs. **Middle:** responses to 1-butanol (B - best odor in the OSNs), isopentyl acetate (I, best odor in the PNs), propionic acid (P) and their ternary mixture (BPI) in glomerulus DM2. The interaction index was not significantly different from 0 for the OSNs, and significantly > 0 for the PNs. Note that the best component for this mixture was B in OSNs and I in PNs. **Bottom:** responses to propionic acid (P), 1-heptanone (N) and their binary mixture (NP). The interaction index was significantly > 0 for the OSNs and not significantly different from 0 for the PNs. Note that the best component was P in OSNs and N in PNs. Median traces from 9 flies for OSNs and 10 for PNs. **(B)** Comparison of the interaction profiles between OSNs and PNs. A Two-Way ANOVA with the factors neuron type (levels: OSN and PN) and odor mixture (levels: BI, PI, PB, NX, NP, PX, BPI and PNX) was performed for each glomerulus, and followed by a Pair-wise Multiple comparison (Holm-Sidak method). Dark grey cells indicate significant differences between the interaction indices of OSNs and PNs, where interaction index in PNs was higher than interaction index in OSNs. Light grey cells correspond to no significant differences

between PNs and OSNs. The white cell corresponds to the only case where the interaction index was significantly lower in PNs than in OSNs (lower traces in Figure 4A). Some cased had to be excluded due to the negative responses to some components of the mixtures in the OSNs (black cells). N_{OSN}= 9. N_{PN}= 10-15.

Across all glomeruli, the interaction index was higher for the binary mixtures than for the ternary mixtures, while at the PN level, the interaction index was higher for ternary mixtures than for binary mixtures (Fig. 2.5A). Since the addition of a component to the mixture almost always implies an increase in the input to the AL, an increase in mixture suppression with increasing mixture complexity could involve a global inhibitory network. However, since no significant correlation was found between the global input to the AL (measured as the cumulative OSN activity over all glomeruli) and the global interaction index in the PNs (measured as the cumulative interaction index over all glomeruli) (Fig. 2.5B) (Spearman Rank Order Correlation, $p>0.4$), mixture suppression cannot be mediated only by a global network.
In order to investigate whether glomerulus specific connections could explain the observed mixture interactions, we analyzed the correlation between the input (OSN response to the different mixtures) and the interaction index in the PNs for all glomerulus pairs. The result of this analysis is the matrix of correlation coefficients shown in Table 2.1. Positive correlation coefficients indicate that the interaction index in a glomerulus *i* increases with increasing OSN activity in a glomerulus *j* and suggest the existence of inhibitory connections between the two glomeruli.

OSN input

PN interaction index	DM2	X1	DM5	DL5	DM3	X2	X3	VA1lm
DM2	-0.17	0.12	0.38	0.10	-0.02	0.14	-0.14	0.29
X1	0.21	-0.79	-0.69	0.33	-0.48	-0.79	0.19	-0.52
DM5	-0.76	0.17	0.12	-0.67	0.10	-0.10	-0.88	0.74
DL5	0.21	-0.36	-0.10	0.26	-0.50	-0.24	0.05	-0.19
DM3	-0.29	-0.36	-0.07	0.10	-0.43	-0.36	-0.26	0.00
X2	-0.43	-0.38	-0.31	-0.41	-0.02	-0.60	-0.69	0.43
X3	0.48	-0.21	-0.10	0.83	-0.60	0.05	0.74	-0.71
VA1lm	0.48	-0.76	-0.62	0.83	-0.71	-0.52	0.55	-0.81

Table 2.1 - Correlation between input and interaction index in single glomeruli

Each cell shows the correlation coefficient calculated with the Spearman Rank Order Correlation between the response in the OSNs and the interaction index in the PNs for all pairs of glomeruli. Grey cells shows cases where the correlation was significant ($p<0.05$). Correlation coefficients > 0 indicate that the mixture suppression in the PNs (measured as positive interaction index values) increased with increased input. Correlation coefficients < 0 indicate that the mixture suppression decreased with increased input. The former cases could be explained with inhibitory connections between the glomeruli. The latter cases could be explained with excitatory connections between the glomeruli (See Figure 2.5C).

For example, increased activity in glomerulus DL5 increases mixture suppression in glomerulus X3 (represented as a gray arrow from DL5 to X3 in Fig. 2.5C). Negative correlation coefficients indicate that mixture suppression in a glomerulus *i* decreases with increasing OSN activity in a glomerulus *j*, and suggest the existence of excitatory connections between the two glomeruli; for example, input activity in glomerulus X3 is correlated with a decrease in mixture suppression in glomerulus DM5 (represented with a black arrow in Fig. 2.5C). These effects might be mediated by direct or indirect connections between the glomeruli, through which the input to one glomerulus influences the output of another glomerulus. The model in Figure 2.5C, based on the correlation coefficients shown in Table 2.1, shows that in most cases increased input to one glomerulus correlated with a decrease of the mixture suppression in other glomeruli. Such glomeruli might be connected through excitatory LNs and or a disinhibitory circuit of inhibitory LNs. Some inhibitory effects were found, which would point to the existence of glomerulus specific inhibitory connections (see Discussion).

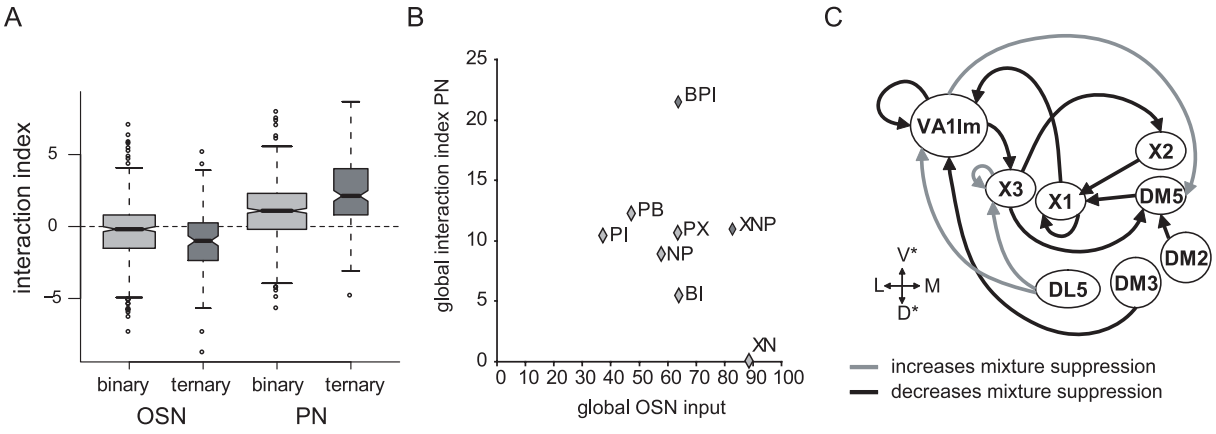

Figure 2.5 - Glomerulus specific interactions shape mixture responses in PNs

(A) Box plot of the interaction index for binary and ternary mixtures in OSNs and PNs pooled over all mixtures, glomeruli and animals. The width of the boxes is proportional to the number of observations (N: binary OSN = 432, 9 animals, ternary OSN = 144, 9 animals, binary PN = 558, 15 animals, ternary PN = 186, 15 animals). (See materials and methods for details on the box-plot). **(B)** Relationship between the global input to the AL during mixture stimulation (cumulative OSN responses over all glomeruli), and the global interaction index in the PNs (cumulative PN interaction indices over all glomeruli). No significant correlation could be found between the two variables (Spearman Rank Order Correlation, $p>0.4$). Light grey symbols correspond to binary mixtures, dark grey to ternary mixtures. **(C)** Putative connectivity map reflecting the correlation analysis between the input to single glomeruli (OSN) and the interaction index in the PNs of single glomeruli (See Table 2.1). Black arrows indicate cases where the activity in glomerulus A positively affects the activity in glomerulus B (increased input to A correlates with **decreased** mixture suppression in B). Grey arrows indicate cases where the activity of glomerulus B negatively affects the activity in glomerulus B (increased input to A correlates with **increased** mixture suppression in B). Glomeruli are arranged according to their position in the AL.

Pharmacological manipulation of mixture interactions

The data provided so far show that inhibitory interactions among glomeruli (e.g. mixture suppression) influence odor responses in PNs. A large population of inhibitory interneurons is GABAergic. As in mammals, fast GABAergic inhibition in insects is mediated by picrotoxin-sensitive $GABA_A$ receptors. We therefore analyzed PN response patterns induced by 1-hexanol, 2-heptanone and their mixture before and during application of picrotoxin (PTX) (Fig. 2.6).

Application of PTX (5μM) did not qualitatively alter the population of activated glomeruli for the single odors or for the mixture: all glomeruli active in the absence of PTX (i.e. DM2, DM3, DM5 and X1) were also active during drug application and no additional glomeruli were recruited (Fig. 2.6A).

However, PTX significantly increased the responses in the strongest glomeruli for each odor, i.e. for 2-heptanone in glomeruli DM2 and DM3 (Two-way repeated measurement ANOVA and planned comparisons, $p<0.001$ and $p<0.05$, respectively, N=15), and for 1-hexanol in glomeruli DM5 and X1 ($p<0.05$, N=15) (Fig. 2.6B). The amplitudes of the responses evoked by the mixture were significantly increased in all measured glomeruli ($p<0.001$ for DM2, $p<10^{-4}$ for X1, $p<10^{-6}$ for DM5 and $p<10^{-4}$ for DM3).

PTX treatment did not only change the response amplitude but also its time course, unveiling the existence of a phasic component in odor responses (in the first 500 ms after stimulus onset), which is blocked by inhibitory input under control conditions (Fig. 2.6C). Accordingly, the time to reach the maximum of the responses was shifted from ~1000 ms to ~500 ms after stimulus onset during PTX application (Fig. 2.6CD, Two-Way RM ANOVA, $p<0.001$, N=15). The shift in the time to reach the maximum of the response was independent of the glomerulus and the odor (interaction treatment x odor: $p> 0.05$ for all glomeruli).

Responses to the mixture before PTX application corresponded to the expected response calculated from the components' responses. Thus, unlike the animals in the first experiment (see Fig. 2.1D), these animals did not show significant mixture suppression in any glomerulus. During PTX application, however, responses to the mixture were significantly higher than the lower bound in all measured glomeruli (Fig. 2.6C; Two-way repeated measurement ANOVA, N=15, $p<0.05$ for DM2, $p<10^{-4}$ for X1, $p<0.005$ for DM5 and $p<0.005$ for DM3). This result shows that the magnitude of the inhibitory input blocked by PTX was higher during stimulation with a mixture than during stimulation with the single

components. Thus, PTX treatment revealed a mixture suppression in all glomeruli that was invisible under control conditions. The fact that all glomeruli show a PTX sensitive inhibition and that the inhibition was stronger when more glomeruli were activated (single odor vs. mixture) is in agreement with the existence of a global inhibitory network which receives input in all or most glomeruli and makes output onto all or most glomeruli. A global inhibitory network, however, cannot explain the effects found in the first two experiments, where mixture suppression was glomerulus specific and odor dependent. Thus, at least two different local networks must be independently shaping the PN responses in the fly AL: a PTX sensitive global network and a glomerulus specific network.

Figure 2.6 - PTX modulates PN responses →

(A) Color-coded odor responses before (left) and during (right) application of PTX 5µM. The position of the analyzed glomeruli is indicated by the mask over the left AL. The numbers in the color scale indicate the minimum and maximum ΔF/F (%) used for scaling. **(B)** Mean response amplitude in glomeruli X1, DM5, DM3 and DM2 before (ringer), during (PTX) and after treatment (wash). The asterisks indicate a significant difference between the lower bound and the response to the mixture. The letters indicate a significant difference between the treatments (Ringer vs. PTX) (in all cases $p<0.05$) within each odor stimulus (1-hexanol, 2-heptanone and mixture) (Two-way RM ANOVA and planned comparisons). The data corresponding to the wash were not included in the statistical test. Error bars indicate the SD of the mean (N=15). **(C)** Effect of PTX on the time course of odor responses: Median responses to 1-heptanone, 1-hexanol and the mixture in glomeruli DM5 and DM2 before (ringer, grey line) and during application of PTX 5µM (black line) (N=15). The traces corresponding to the wash were excluded for clarity. The time of odor presentation (1 s) is indicated by the black bar under each trace. The scaling bar indicates 2.5% ΔF/F. Notice that beside the increase in response amplitude, the time to the maximum of the response is shifted to an earlier time point during PTX application. **(D)** Time to maximum (mean ± SD) of the odor response measured for 2-heptanone, 1-hexanol and the mixture in glomeruli DM2, DM3, DM5 and X1. A Two-way RM measurement ANOVA indicated no significant difference in the time to maximum between the odors (1-hexanol, 2-heptanone and mixture), and a significant difference in the time to maximum between the treatments (Ringer and PTX, $p<0.05$). No significant interaction was found between the two factors (odors and treatment). Each glomerulus was tested separately. As shown in C, the time to maximum is significantly longer before PTX application than during PTX application. This effect could be "washed out" to a certain degree (not tested).

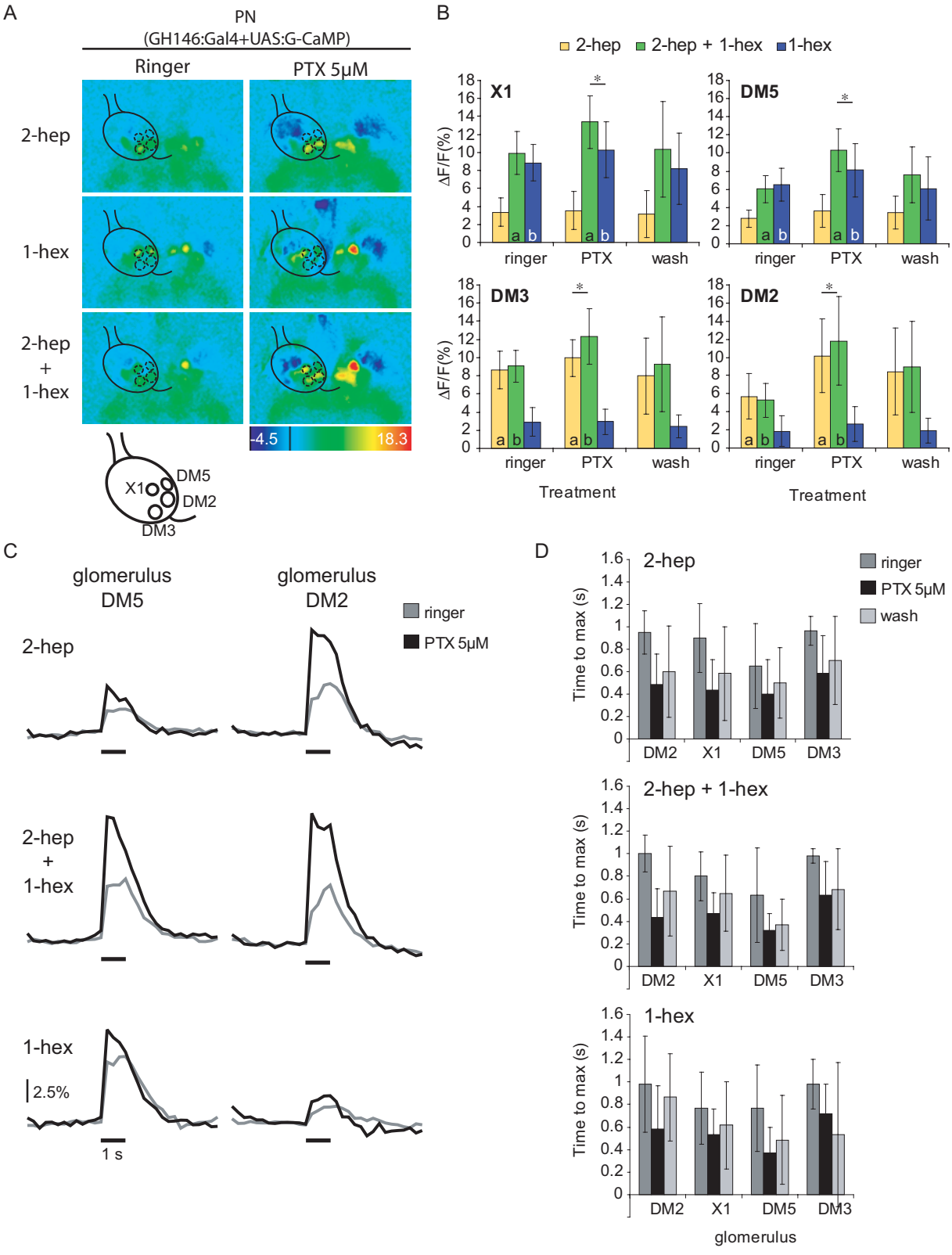

Figure 2.6 - PTX modulates PN responses

(see Page 60)

Discussion

We have analyzed the role of the AL network in odor processing using two approaches. First, we investigated the existence and sources of across-glomeruli interaction by comparing the responses to odorants and their mixtures. Second, we pharmacologically manipulated the AL network with a $GABA_A$ antagonist (PTX) and compared odor and mixture responses before and during drug application.

In order to assess the existence of mixture interactions we have chosen a simple and conservative hypothesis. For each glomerulus we have defined the response to the most potent odor as the lower bound of the no-interaction interval, and the response to a 2-fold higher concentration of the same odor as the upper bound. We argue that all responses that lie within this range can be fully predicted from the components' responses, while responses that fall outside this interval represent cases of mixture interaction (modified from Tabor et al., 2004). This is a conservative approach, which reduces the number of false positives, but might underestimate the occurrence of mixture interactions. For example, when both odors elicit a response but the response to the mixture matches the lower bound of our estimation (i.e. the response to the strongest component), there is a weak mixture suppression (Duchamp-Viret et al., 2003), but these cases would be ignored in our conservative analysis.

Glomerulus specific interactions shape AL output

OSN responses to mixtures could mostly be predicted from the components' responses, in agreement with previous findings (Tabor et al., 2004; Deisig et al., 2006; Carlsson et al., 2007). There were rare cases of mixture suppression in OSNs (e.g. the responses to the mixture of 1-hexanol and 2-heptanone in glomerulus DM5, Fig. 2.2). Different mechanisms could underlie mixture interactions in OSNs: (1) different odors might have different diffusion times in the fluid that surrounds the OSNs (Laing, 1987), (2) mixture components might interact at the molecular level due to their receptor binding properties (Oka et al., 2004) or (3) they might activate antagonistic or synergistic second messenger cascades (Duchamp-Viret et al., 2003). (4) Inhibitory input from the AL/OB network can reduce depolarization in OSN axons, thus reducing glomerular calcium signals of OSNs. (Distler and Boeckh, 1997; Wachowiak et al., 2002; Wachowiak et al., 2005; McGann et al., 2005). Further experiments are required to understand these mixture interactions at the periphery.

In contrast, we found strong mixture interactions in PNs (Figs. 2.1, 2.3 and 2.4), showing that these are mediated by the AL network. We did not find any case of mixture synergism. Inhibitory interactions (mixture suppression) were not globally distributed, but limited to certain glomeruli, depending on the mixture tested. We calculated the correlation between the input strength (OSN response) and the mixture interaction at the PN level (PN interaction index) for all glomeruli pairs. The result is a matrix of proposed between and within glomeruli effects (Table 2.1). Activity in some glomeruli enhanced mixture suppression in others or themselves (cases with positive correlation coefficient, e.g. glomerulus DL5 onto VA1lm, X3 onto itself). Activity in some glomeruli reduced mixture suppression in others or themselves (cases with negative correlation coefficients, e.g. glomerulus DM2 onto glomerulus DM5, VA1lm onto itself) (Fig. 2.5C). Odor and glomerulus specific mixture suppression might result from the interplay between global inhibitory and glomerulus specific lateral excitatory input (Olsen et al., 2007) (see below).

Fast GABA neurotransmission is involved in constitutive global inhibition and mixture suppression

Previous studies have shown that GABA is one of the neurotransmitters involved in odor processing in the insect AL (Sachse and Galizia, 2002; Wilson and Laurent, 2005). In our experiments, PTX application increased the amplitude of the odor responses, modified their time courses and revealed mixture suppression.

PTX had its strongest effect during the first 500 ms of the odor response, suggesting that $GABA_A$ receptors mediate an inhibitory input that suppresses the phasic component of odor responses. This result is consistent with previous pharmacological studies in *Drosophila* showing that PTX application only affects the early phase of the odor response, while blockers of metabotropic GABA receptors affect a later phase of the response (Wilson and Laurent, 2005). LNs which respond to odors with short bouts of action potentials are known from electrophysiological recordings (Wilson et al., 2004). It is possible that PTX blocks the inhibition of these LNs onto PNs or OSNs axons.

The inhibitory network that is affected by PTX treatment is likely to be a homogeneous, global network because the effect of PTX increased with stronger input to the AL. We thus conclude that a PTX sensitive global inhibitory network, which receives input in all or most glomeruli and gives output in all or most glomeruli, constitutively suppresses odor responses

in the AL. Although we cannot exclude that glomerulus specific effects are also mediated by fast GABA neurotransmission, our results provide no evidences in this direction.

Glomerular PN response patterns reflect the integrated input from OSNs, GABAergic multiglomerular LNs and putative glomerulus specific excitatory and/or inhibitory LNs. Since OSNs show almost no mixture suppression, mixture suppression in the PNs must reflect the integrated input from LN network. For example, global inhibitory input could suppress responses in all glomeruli (see Fig. 2.6) while glomerulus specific excitatory input enhances responses only in a subgroup of glomeruli, thus creating a glomerulus specific interaction profile.

What is the neuronal substrate of mixture interactions? In flies both GABAergic and cholinergic multiglomerular LNs have been described (Ng et al., 2002; Shang et al., 2007) (Stocker, 1994). Multiglomerular GABAergic LNs could fulfill the task of the global inhibitory network. Furthermore, it has been proposed that mutltiglomerular cholinergic LNs could be involved in broadening the response profile of PNs (Shang et al., 2007), and that broadening of PN response profiles occurs in a glomerulus dependent manner (Olsen et al., 2007). LNs which innervate only a few glomeruli could be involved in such glomerulus-specific interactions. Alternatively, it is conceivable that multiglomerular LNs with widespread arborization possess electrical sub-compartments and/or that their receptors and synapses are asymmetrically distributed along the branches, thus acting as functionally asymmetrical neurons in spite of their homogeneous morphology. Heterogeneous LNs, with dense innervation in one glomerulus and weak innervation in others have been described in honeybees (Fonta et al., 1993). It has been proposed that these neurons could be involved in sharpening of PN response profiles (Sachse and Galizia, 2002; Galan et al., 2004; Linster et al., 2005).

Taken together, we found evidences for several functional interaction modes in the *Drosophila* AL. Some interactions occur within olfactory glomeruli (see recurrent connections in Fig. 2.5C). Other interactions are global and sensitive to PTX, and thus likely to be mediated by $GABA_A$ receptors. This network might mediate a global gain control or be involved in action potential synchronization (Laurent, 1996; Christensen et al., 2003; Sachse and Galizia, 2006). Finally, glomerulus-specific interactions were found, which could be mediated by inhibitory (Wilson and Laurent, 2005), and excitatory connections (Shang et al., 2007). Their role could be to decorrelate across-glomeruli patterns in order to increase the coding capacity of the system (Wilson et al., 2004; Linster et al., 2005).

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Appendix

		OSN				PN				
	glo	mix		b.o.	lowerbound	mix		b.o.	lowerbound	
BI	DM2	18.01	[13.48 ; 19.95]	B	17.40	[14.30 ; 19.10]	16.18	[14.40 ; 18.30]	I	17.59	[15.45 ; 19.46]
	X1	9.73	[7.18 ; 11.25]	B	7.71	[5.47 ; 10.06]	4.76	[1.54 ; 6.82]	B	5.91	[3.68 ; 7.39]
	DM5	7.94	[4.83 ; 10.42]	B	7.10	[4.51 ; 8.17]	4.83	[4.28 ; 6.36]	I	5.21	[3.83 ; 6.08]
	DL5	10.58	[7.16 ; 13.47]	B	10.33	[7.19 ; 16.51]	8.91	[7.46 ; 9.66]	B	8.19	[6.88 ; 10.49]
	DM3	9.13	[7.85 ; 10.34]	I	7.59	[6.52 ; 9.25]	10.87	[8.90 ; 11.35]	I	9.95	[8.09 ; 12.92]
	X2	9.61	[5.42 ; 11.24]	B	6.17	[5.32 ; 8.54]	5.40	[4.02 ; 6.18]	B	5.87	[4.43 ; 6.72]
	X3	1.94	[1.18 ; 3.31]	B	2.78	[1.59 ; 4.58]	1.18	[-0.67 ; 1.88]	B	2.76	[2.24 ; 3.14]
	VA1lm	-3.12	[-3.98 ; -2.06]	B	-3.31	[-4.19 ; -1.09]	-0.51	[-1.08 ; 0.93]	B	0.82	[-0.95 ; 1.86]
NX	DM2	17.68	[11.83 ; 18.04]	N	13.01	[10.39 ; 13.95]	13.03	[11.80 ; 17.32]	N	15.79	[12.11 ; 17.74]
	X1	19.11	[16.54 ; 22.79]	X	15.35	[13.52 ; 17.70]	14.77	[12.59 ; 17.78]	X	12.53	[10.62 ; 16.6]
	DM5	18.01	[16.69 ; 19.47]	X	15.18	[13.75 ; 15.90]	9.97	[7.91 ; 12.15]	X	11.25	[9.40 ; 12.32]
	DL5	3.74	[3.27 ; 7.66]	X	5.14	[3.74 ; 7.14]	5.30	[3.93 ; 6.65]	X	5.69	[4.78 ; 7.14]
	DM3	15.87	[13.70 ; 17.42]	N	14.71	[13.12 ; 16.75]	13.12	[10.37 ; 13.87]	N	12.15	[8.37 ; 13.4]
	X2	12.39	[8.48 ; 14.03]	X	9.96	[7.72 ; 10.78]	5.59	[5.26 ; 7.11]	X	6.52	[5.63 ; 7.7]
	X3	0.51	[-0.27 ; 2.35]	X	1.31	[0.60 ; 1.43]	2.81	[1.97 ; 3.40]	X	2.92	[2.25 ; 4.73]
	VA1lm	1.24	[-0.56 ; 1.73]	N	1.49	[1.46 ; 1.73]	2.58	[-0.43 ; 3.88]	X	1.69	[0.49 ; 3.6]
PB	DM2	15.94	[12.31 ; 18.51]	B	17.40	[14.36 ; 19.1]	9.05	[7.59 ; 11.43]	B	9.70	[7.92 ; 10.69]
	X1	8.00	[5.02 ; 9.79]	B	7.71	[5.47 ; 10.06]	3.92	[0.77 ; 4.66]	B	5.91	[3.69 ; 7.39]
	DM5	5.28	[5.14 ; 7.24]	B	7.10	[4.51 ; 8.17]	3.20	[2.70 ; 3.78]	B	4.55	[2.57 ; 6.09]
	DL5	9.60	[5.99 ; 14.66]	B	10.33	[7.19 ; 16.51]	5.83	[4.51 ; 7.53]	B	8.19	[6.89 ; 10.49]
	DM3	4.12	[2.24 ; 4.60]	B	4.33	[2.93 ; 5.75]	3.39	[2.63 ; 5.24]	B	4.49	[2.78 ; 6.49]
	X2	6.38	[5.06 ; 7.71]	B	6.17	[5.32 ; 8.54]	3.94	[3.4 ; 6.87]	B	5.87	[4.43 ; 6.72]
	X3	0.99	[-0.10 ; 2.51]	P	-2.85	[-4.78 ; -2.19]	0.20	[-1.06 ; 0.94]	B	2.76	[2.24 ; 3.14]
	VA1lm	-3.14	[-3.48 ; -1.39]	B	-3.31	[-4.19 ; -1.09]	-0.73	[-1.93 ; 0.09]	P	0.99	[-0.30 ; 1.65]
PI	DM2	13.85	[12.27 ; 16.70]	I	13.97	[9.46 ; 15.25]	16.00	[13.27 ; 17.32]	I	17.59	[15.45 ; 19.46]
	X1	2.86	[1.63 ; 3.55]	I	3.38	[2.12 ; 3.82]	0.37	[-0.30 ; 1.60]	I	1.80	[0.37 ; 3.22]
	DM5	5.30	[4.11 ; 6.08]	I	6.44	[2.65 ; 7.25]	3.60	[2.75 ; 4.77]	I	5.21	[3.83 ; 6.07]
	DL5	3.98	[2.65 ; 4.65]	P	4.67	[2.85 ; 4.94]	2.33	[1.20 ; 3.80]	P	2.92	[1.92 ; 4.05]
	DM3	7.24	[6.89 ; 7.8]	I	7.59	[6.52 ; 9.25]	8.73	[7.27 ; 11.00]	I	9.95	[8.09 ; 12.92]
	X2	6.10	[5.02 ; 7.03]	I	6.69	[2.43 ; 7.28]	3.50	[2.87 ; 3.93]	I	4.72	[3.85 ; 6.07]
	X3	-2.47	[-4.19 ; -1.91]	P	-2.85	[-4.78 ; -2.19]	-1.26	[-1.93 ; -0.16]	I	-0.37	[-0.81 ; 0.55]
	VA1lm	0.34	[-0.83 ; 0.76]	P	-0.35	[-1.00 ; 0.04]	0.45	[-0.26 ; 0.94]	P	0.99	[-0.30 ; 1.65]
PN	DM2	13.38	[8.89 ; 14.00]	N	13.01	[10.39 ; 13.95]	13.20	[11.19 ; 15.35]	N	15.79	[12.11 ; 17.74]
	X1	12.43	[8.94 ; 12.92]	N	11.83	[9.38 ; 13.39]	5.94	[4.29 ; 6.78]	N	7.54	[5.47 ; 8.69]
	DM5	8.25	[6.05 ; 8.53]	N	7.71	[6.90 ; 8.14]	4.54	[2.92 ; 7.09]	N	7.15	[4.79 ; 8.22]
	DL5	0.19	[-0.31 ; 1.63]	P	5.04	[2.88 ; 5.17]	3.18	[2.15 ; 4.29]	N	3.15	[2.40 ; 4.70]
	DM3	15.31	[11.67 ; 15.74]	N	14.71	[13.12 ; 16.75]	9.69	[9.25 ; 11.86]	N	12.15	[8.37 ; 13.40]
	X2	9.25	[7.16 ; 10.06]	N	7.60	[6.10 ; 8.61]	5.54	[3.80 ; 6.13]	N	6.03	[4.60 ; 7.05]
	X3	-2.33	[-5.04 ; -1.60]	P	-2.89	[-4.14 ; -2.44]	0.05	[-0.58 ; 1.28]	P	0.07	[-1.76 ; 0.21]
	VA1lm	1.30	[0.95 ; 2.00]	N	1.49	[1.46 ; 1.73]	1.49	[0.06 ; 2.41]	P	1.70	[0.81 ; 2.62]
PX	DM2	12.27	[9.54 ; 14.45]	X	11.29	[8.70 ; 12.41]	6.63	[4.34 ; 8.40]	P	8.20	[6.63 ; 10.18]
	X1	15.26	[12.49 ; 18.92]	X	15.35	[13.52 ; 17.7]	10.75	[9.99 ; 15.65]	X	12.53	[10.62 ; 16.60]
	DM5	16.08	[13.29 ; 17.04]	X	15.18	[13.75 ; 15.9]	9.69	[8.08 ; 10.25]	X	11.25	[9.40 ; 12.32]
	DL5	6.14	[5.25 ; 8.01]	X	5.14	[3.74 ; 7.14]	5.76	[4.44 ; 7.27]	X	5.69	[4.78 ; 7.14]
	DM3	4.73	[2.60 ; 5.72]	X	5.71	[4.44 ; 6.56]	4.62	[3.03 ; 6.80]	X	6.56	[5.05 ; 8.07]
	X2	10.30	[7.66 ; 10.64]	X	9.96	[7.72 ; 10.78]	5.11	[4.74 ; 6.29]	X	6.52	[5.63 ; 7.70]
	X3	-0.70	[-1.63 ; 0.29]	P	-2.89	[-4.14 ; -2.44]	1.67	[1.04 ; 2.00]	X	2.92	[2.25 ; 4.73]
	VA1lm	-0.57	[-1.96 ; 0.09]	X	-0.71	[-2.27 ; -0.38]	1.76	[-0.80 ; 2.40]	X	1.69	[0.49 ; 3.60]
BPI	DM2	18.80	[14.09 ; 19.75]	B	17.40	[14.30 ; 19.10]	15.13	[12.52 ; 15.91]	I	17.59	[15.45 ; 19.46]
	X1	8.94	[7.47 ; 11.17]	B	7.71	[5.47 ; 10.06]	1.14	[0.58 ; 2.66]	B	5.91	[3.68 ; 7.39]
	DM5	8.66	[5.56 ; 10.34]	B	7.10	[4.51 ; 8.17]	3.94	[3.58 ; 4.93]	I	5.21	[3.83 ; 6.07]
	DL5	10.32	[7.36 ; 13.90]	B	10.33	[7.19 ; 16.51]	5.27	[3.62 ; 6.59]	B	8.19	[6.88 ; 10.49]
	DM3	8.93	[7.78 ; 9.18]	I	7.59	[6.52 ; 9.25]	8.11	[6.34 ; 8.69]	I	9.95	[8.09 ; 12.92]
	X2	9.32	[4.91 ; 12.73]	B	6.17	[5.32 ; 8.54]	3.96	[3.37 ; 4.90]	B	5.87	[4.43 ; 6.72]
	X3	1.60	[-0.55 ; 1.81]	B	2.78	[1.59 ; 4.58]	-0.94	[-1.51 ; -0.56]	B	2.76	[2.24 ; 3.14]
	VA1lm	-2.81	[-4.11 ; -0.78]	B	-3.31	[-4.19 ; -1.09]	-1.27	[-2.34 ; -0.51]	B	0.82	[-0.95 ; 1.86]
PNX	DM2	15.19	[13.05 ; 17.60]	N	13.01	[10.39 ; 13.95]	10.30	[9.68 ; 14.23]	N	15.79	[12.11 ; 17.74]
	X1	18.19	[14.93 ; 21.30]	X	15.35	[13.52 ; 17.70]	12.98	[9.60 ; 15.89]	X	12.53	[10.62 ; 16.60]
	DM5	17.51	[15.24 ; 18.57]	X	15.18	[13.75 ; 15.9]	8.33	[4.52 ; 9.86]	X	11.25	[9.40 ; 12.32]
	DL5	5.53	[3.35 ; 6.31]	X	5.14	[3.74 ; 7.14]	4.70	[2.16 ; 6.13]	X	5.69	[4.78 ; 7.14]
	DM3	13.60	[12.39 ; 16.37]	N	14.71	[13.12 ; 16.75]	11.68	[9.17 ; 12.94]	N	12.15	[8.37 ; 13.40]
	X2	12.23	[8.05 ; 13.69]	X	9.96	[7.72 ; 10.78]	5.52	[4.69 ; 7.30]	X	6.52	[5.63 ; 7.70]
	X3	-0.88	[-2.43 ; 1.67]	P	-2.89	[-4.14 ; -2.44]	1.22	[0.11 ; 2.47]	X	2.92	[2.25 ; 4.73]
	VA1lm	1.40	[-0.77 ; 1.98]	N	1.49	[1.46 ; 1.73]	0.69	[-0.01 ; 2.12]	X	1.69	[0.49 ; 3.60]

Table S1 - Responses to the mixtures and lower bound of the no-interaction interval

Median and interquartile interval (median [Q_{25};Q_{75}]) for all mixture responses and the corresponding lower bound values. b.o.: best odor. Values are expressed as $\Delta F/F$ (%) and are the median values over all measured animals (N= 9 for OSN and 10-15 for PN). Grey cells: response to the mixture significantly different from the lower bound (Wilcoxon Signed Rank Test, p>0.05)