Material&Methods

Animal preparation & staining

Preparation and staining were done as described in (Sachse and Galizia, 2002). Foraging honeybees were caught upon leaving their hives and anesthetized by cooling them for five minutes on ice. Then they were transferred into Plexiglas stages and immobilized with bee wax (Deiberit 502, Dr Boehme&Schoeps, Dental GmbH, Germany). For dye injection, a window was cut into their head capsule and the glands covering the brain were removed. Glass tip electrodes were coated with Fura-2 dextran (potassium salt, 10.000 MW, Invitrogen, Germany) dissolved in a 4% solution of BSA in Ringer (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7, 500 mosmol; all chemicals from Sigma). For a retrograde mass fill of PNs, the electrodes were introduced into the lACT, laterally to the alpha lobe. Afterwards the cut out cuticle was returned in place and bees were fed a 30% sucrose solution until satiated. During the following 8 hours, the duration resulting in optimal staining, the bees were kept in a moist place. For the final preparation the antennae were fixed with dental wax (Kerr, Sybron Dental Specialities, USA) and the trachea covering the AL were removed. To minimize movement artifacts, the esophagus was removed, too.

Imaging and odor stimulation

Imaging was done at a 20x magnification (Olympus XLum PlanFl 20x/0.95W) using an upright microscope (Olympus BX50WI, both Olympus, Germany) and a CCD based Till Photonics imaging system (TillPhotonics, Germany). Monochromatic excitation light was sent through a dicroic filter (410 nm) at 340 and 380 nm. Light emission was low pass filtered at 440 nm. For each measurement, 40 double images were taken at a frequency of 5Hz. Recordings were binned to a pixel size of 2µm.

We tested 16 aliphatic hydrocarbons, varying in functional group (primary and secondary alcohols, aldehydes and ketones) and carbon chain length (C6-C9) at 4 different concentrations (diluted in mineral oil, from 10-1 to 10-4). For each odor and concentration, 5ml were kept in a rubber sealed 10 ml glass vial. For odor stimulation, a

PAL system (CTC Analytics) sampled 2 ml of odor saturated headspace from the vials and injected them into a constant stream of clear air (1.5 m/s). Stimulus duration was 2 seconds and measurements were separated by a 2.5 minute inter-trial interval. Odors were measured from low to high odor concentration. Before and after each concentration series, a control block consisting of one measurement of pure air, 1-nonanol and pure mineral oil each was measured. Measurements were conducted as long as the animal was responsive to the control odor 1-nonanol. In general 2-3 concentration series could be obtained per animal.

Data analysis

All data was analyzed using custom software written in IDL (Research systems, CO). Data was corrected for scattered light using the following formula (Sachse and Galizia, 2002): $F_i^{'} = F_i - \left[sm(F_i) - F_i\right] = 5 * F_i - sm(F_i)$.

Where F' are the scattered-light corrected images, F_i the images before correction and $sm(F_i,width=40\mu m)$ the image smoothed with a boxcar average of 40 µm. Subsequently, we calculated the ratio 340/380 nm and subtracted the background fluorescence (the average fluorescence during the first second of each measurement), thereby setting measurements to 0 just before stimulus onset. Before calcium signals were further analyzed, the individual experiments were firstly corrected for movement within each measurement and secondly for shifts in between measurements of each animal. For glomerulus identification, we calculated images representing the degree of correlation between neighbouring pixels. As glomeruli have been shown to be functional units (Wachowiak *et al.*, 2004), data points stemming from the same glomerulus are highly correlated over time, while they are uncorrelated when stemming from different glomeruli. In these correlation images, the glomeruli were nicely visible and were identified according to their morphological borderlines using the digital atlas of the AL as a reference (Galizia *et al.*, 1999a). For time courses squares of 7x7 pixels (always well within the glomerulus chosen) were placed onto the centre of each identified glomerulus, their values were averaged, and the time courses were plotted against time. The resulting values are labelled Δ(340/380)[%].

For false-colour display only, the maximal value after stimulus onset was calculated and the mean value one second before stimulus onset was subtracted. The resulting image was median and Gaussian filtered (3x3 pixels kernel size each).

All statistical analysis was done in R. The linear discrimant analysis (LDA), principal component analysis (PCA) and the correlation between our data and the behavioural generalization matrix were done on averaged glomerular responses. For each of the 16 different odors the mean responses of 14 glomeruli were averaged across animals. The result was one 16x14 matrix for each of the four concentrations and each frame measured. The linear discrimant analysis was carried out using the lda function from the MASS package in R (Venables and Ripley, 2005). For the correlations between our data and behaviour, we used a behavioural generalization matrix published by *Guerreri et al.* (Guerrieri *et al.*, 2005) which is based on the same odors. For each time point measured, we calculated the Euclidean distances between the glomerular representations of all odors and then compared them to the behavioural generalization matrix, calculating their Pearson's product moment coefficient. The same was done for a subset of the odors, namely the primary and secondary alcohols. The significance of correlation values is hard to interpret. The standard statistical procedure for testing correlation values (Zars t-test) compares them against the assumption that they are zero, irrespective of their actual size. During stimulation, all correlations between our data and the behavioural data did differ from zero in a highly significant way ($p < 0.001$). But as PNs exhibit spontaneous activity also in the absence of odorants, some correlation values were significant already before odor onset. We therefore computed a Monte Carlo analysis. We randomly permuted our physiological distance matrices before calculating the correlations. This was done 100.000 times for each time point and concentration and the result was a symmetric distribution of 100.000 correlation values with a mean of zero. The minimal and maximal values of these distributions were approximately -0.4 and 0.4 when calculating the correlations for all odors and -0.7 and 0.7 when calculating the correlations only for the primary and secondary alcohols. Correlation values greater than the maxima resulting from the Monte Carlo analysis were considered as significant.