Appendix I

Introduction

Calcium indicators exhibit a change in fluorescence intensity upon binding of Ca^{2+} ions. Calcium binding in Cameleon2.1 is mediated by calmodulin (Figure 2), which is a ubiquitous Ca^{2+} binding protein within cells. Thus two concerns arise when an additional Ca^{2+} binding protein is genetically introduced into cells. First of all, there might be an interaction between the Cameleon2.1 calmodulin and endogenous calmodulin or calmodulin dependent enzymes. Miyawaki and co-workers (1999) were able to show in hippocampal neurons from Sprague-Dawley rat embryos that this was not the case. However, they pointed out that an increase in Ca^{2+} buffering within the cells expressing Cameleon2.1 is unavoidable, which is the second concern to be considered. There is scarcely a physiological reaction within the brain that is not regulated, directly or indirectly, by calcium ions (Augustine et al., 2003). Thus introduction of a constitutively expressed Ca^{2+} binding protein such as Cameleon2.1 might change the properties of the cells expressing this sensor. In particular as in the experimental flies employed in this study the expression of Cameleon2.1 begins during development, namely with the onset of OR gene expression between 54 and 60 hours after puparium formation (APF) (Clyne et al., 1999), and Ca^{2+} is known to play a role in (neuronal) development (Augustine et al., 2003). Therefore I recorded electroantennograms (EAGs) from antennae of the flies of the same genotype as employed in the Ca^{2+} imaging experiments and compared them to EAGs of wildtype flies.

In *Drosophila* the electroantennogram (EAG) measures the potential difference between the inner haemolymph and the cuticle surface of the antenna (Ignell and Hansson, 2005). It is thought to reflect the summed receptor potentials of responding ORNs in the vicinity of the recording electrode. Thus EAGs are only an indirect measure of receptor activation but they can be used to detect changes in the peripheral neurons' olfactory responses of mutants when compared to appropriate controls (de Bruyne, 2003). Among others, EAGs have been used in *Drosophila* to characterize the response spectrum of Or43a (Stortkuhl and Kettler, 2001), the scutoid

mutation (Dubin et al., 1995) and the acj6 mutation (Ayer, Jr. and Carlson, 1991). In these studies changes in the olfactory responses were reflected in EAG amplitude changes. Thus, the EAG seemed to be a good measure for a detection of differences in ORN responses between experimental and wild type flies.

Materials and Methods

Preparation of flies

Flies studied were three to five days old females. The flies tested were those employed for the calcium imaging experiments (i.e. F1-progeny of crosses between Or22a-Gal4 flies (Vosshall et al., 2000) and Cameleon2.1 flies (Fiala et al., 2002)). Flies were blown into 10µl pipette tips with the aid of an aspirator. The pipette tip was cut just above the fly's head and pressed into plasticine with the fly head-up in order to push its head out of the opening (Figure A.1). Care was taken not to push out the fly's head too far such that the aristae were still stuck in the pipette tip thereby reducing possible movement.

Electroantennogram set-up

Flies were placed under a stereo microscope (WPI, Sarasota, USA). Recording electrodes were pulled with a micro pipet puller (David Kopf Instruments, Tujunga, USA) and filled with 0.015M potassium chloride solution. The saline made electrical contact with a high impedance 10x pre-amplifier (Syntech, Hilversum, Netherlands) via an Ag/AgCl wire. The output was led into an analog-digital converter that sampled the voltage at 25 Hz. The signals were viewed on a PC (Dell, Frankfurt, Germany) using EAG software (Syntech, Hilversum, Netherlands). The recording and reference electrode were mounted on two manual micromanipulators (WPI, Sarasota, USA). The reference electrode was inserted into the head just above the mouthparts where it came into contact with the haemolymph. The recording electrode was positioned at the proximal medial region of the third antennal segment where ORNs expressing Or22a are located (Figure A.1) (Bhalerao et al., 2003;Dobritsa et al., 2003;Vosshall et al., 2000).

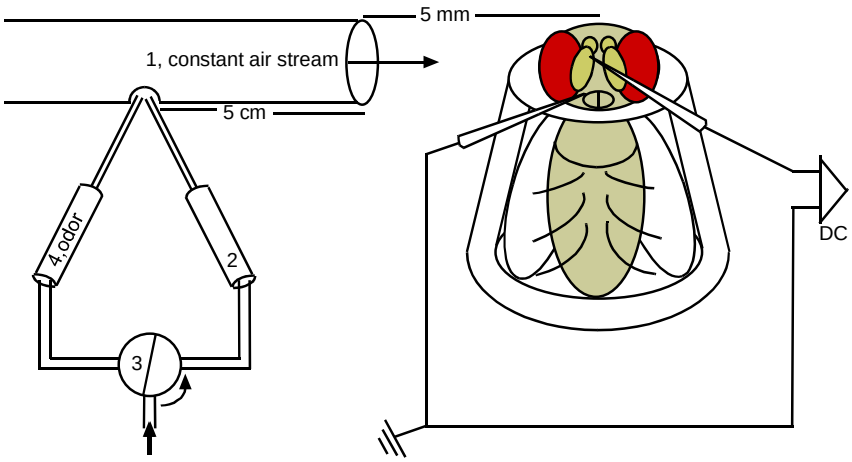

Figure A.1 Experimental setup for recordings of electroantennograms. The left side shows the stimulus application system. The main constant air stream (1) was supplied by a gas bottle containing synthetic air at 1l/min via a glass tube. A minor constant air stream (2) was supplied by the same gas bottle at 60ml/min via a syringe which was inserted into the main constant air stream. During odor stimulation the minor air stream was redirected by a computer controlled solenoid valve (3) to a second syringe which was equipped with an odor-laden filter paper (4). The end of the constant airstream was positioned at a distance of 5cm from the fly's head. The right side shows the fly prepared for EAG recordings. The recording electrode was positioned in the proximal-medial region of the antenna; the reference electrode was inserted just above the mouthparts.

Odor stimulation

Odors were dissolved at 10^{-2} [vol/vol] in mineral oil and delivered via a system similar to the one described by de Bruyne et al (1999) (Figure A.1). A glass tube supplying a continuous synthetic air-stream (1l/min) was positioned at a distance of 5mm from the fly's head. Two syringes were placed into the glass tube at 5cm distance from its opening, one of them empty providing a second continuous air-stream (60ml/min), the other one filled with a piece of filter paper laden with 20µl of odor dilution. During stimulus application the continuous air-stream was redirected into the odor-laden syringe for 1 second thus avoiding a change in airflow.

The odors tested were chosen for two different reasons. Ethyl acetate, methyl salicylate, and benzaldehyde are commonly used in EAG recordings (Ayer, Jr. and Carlson, 1991;Dubin et al., 1995;Martin et al., 2001). Thus, their response characteristics are quite well known and served as a control for correct positioning of the recording electrode and as a first control of whether the gross physiology of the flies was normal. The remaining odors were chosen because they elicited responses in ORNs expressing Or22a and thus would enable the detection of a Cameleon2.1

influence on the odor responses in the experimental flies. Those odors were pentyl acetate , 3-methyl-1-butanol (because of the specific response type (I) elicited by this odor in Or22a ORNs), ethyl propionate , ethyl-2-methyl-butananoate and methyl hexanoate (odors activating Or22a) as well as the diluent mineral oil.

Data analysis and results

Presentation of an odor resulted in a deflection of the DC signal thus indicating a depolarization. The EAG recordings were analyzed by determining the maximum amplitude of the odor evoked response. Responses to a particular odor were averaged across animals for the experimental and the control flies respectively. A three-way ANOVA with odor, individual and genotype as factors where individual was nested in genotype showed a main effect of individual ($df = 36$; $F = 8.33$, $p < 0.0001$) and a main effect of odors ($df = 8$; $F = 255.83$; $p < 0.0001$) but no effect of genotype ($df = 1$; $F = 0.39$; $p = 0.532$) as shown in Figure A.2. Thus, the odor-evoked responses of Or22a ORNs additionally expressing Cameleon2.1 are not different from those of wildtype Or22a ORNs.

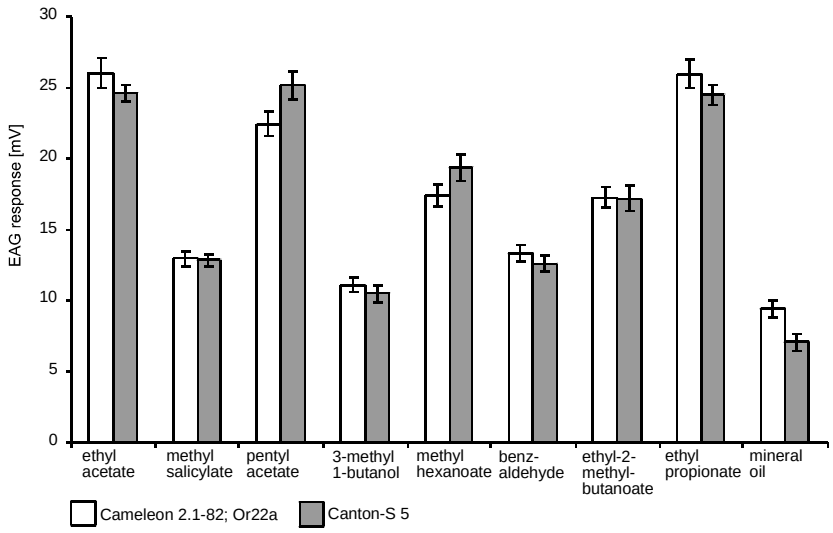

Figure A.2 Electroantennogram (EAG) recordings from experimental and wild-type flies. Bars represent mean of response amplitudes upon odor presentation (experimental, i.e. Cameleon 2.1; Or22a flies n = 18; control, i.e. Canton S5 flies n = 20). Error bars present SEM. There was no statistically significant difference between experimental and control flies (three-way nested ANOVA, factors: odor, individual and genotype, individual nested in genotype; main effect of individual $df = 36$; $F = 8.33$, $p < 0.0001$; main effect of odors $df = 8$; $F = 255.83$; $p < 0.0001$; no effect of genotype $df = 1$; $F = 0.39$; $p = 0.532$).