THE CHEMOSENSORY SYSTEM OF
DROSOPHILA LARVAE: NEUROANATOMY
AND BEHAVIOUR

THÈSE
Présentée à la faculté des Sciences de l’Université de Fribourg (Suisse)
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PAR

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Le Châble (Valais, Suisse)

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[Signatures]
Plus il s’abreuvaît à la douce fontaine de la science, plus sa soif grandissait, toujours plus brûlante et impatiente.

— Endless is the search for Truth. (L. Sterne)

A ma famille, un soutien fidèle et indispensable
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Cette thèse porte sur le système chémosensoriel de la larve de *Drosophila melanogaster*, elle s’articule en quatre parties. Le sujet principal fut dirigé sur les aspects organisationnels du système gustatif, afin d’établir la larve comme un système modèle dans l’étude du codage de la sensation du goût. En analysant les projections centrales des afférents gustatifs (1), ainsi que celles de leurs neurones cibles (2), nous avons pu établir qu’il existe différentes sous-régions dans le centre gustatif primaire, selon l’organe d’origine et la réponse fonctionnelle des afférents. Cette étude nous permet également de supposer que les neurones secondaires ont certainement un spectre de réponse plus large que les primaires. Il est intéressant de constater que ces deux caractéristiques sont également présentes chez le système gustatif des mammifères.

Dans les deux autres chapitres, la réponse comportementale des larves en réponse aux odeurs fut examinée. Nous avons tout d’abord étudié l’effet de la pré-exposition à une odeur sur la réponse ultérieure à cette même odeur (3). Des expériences critiques montrent que la réponse peut varier de l’attraction à la répulsion, démontrant que la sensibilité à l’odeur reste intacte, mais que l’animal donne une autre valeur hédonique à celle-ci (contrairement à ce qui était supposé au préalable). Dans un deuxième projet, nous avons examiné la réponse à une odeur en présence d’une autre odeur ambienne, dans le but de déterminer les capacités de discrimination des larves (4). Une approche similaire fut utilisée dans des études sur les rats. Cet exemple montre comment de telles expériences comportementales peuvent avoir des répercussions d’ordre général, soit en soulignant des réserves quant à des problèmes techniques, soit en mettant en lumière des mécanismes moléculaires qui pourraient être similaires dans différentes espèces animales.

En conclusion, les résultats présentés dans cette thèse confirment la place de la *Drosophile* en tant que système modèle de choix dans l’étude du système chémosensoriel et des comportements qui lui sont liés. Ce travail sera sans doute une référence importante pour de futures études dans le domaine.
Abstract

This PhD thesis investigates the larval chemosensory system of *Drosophila melanogaster*, focusing on four different issues. The main work was directed on organisational aspects of the larval taste system and tried to show that the *Drosophila* larva could serve as a gustatory model system of general importance. Analysing both the gustatory projection patterns in the brain (1) and their potential target neurones (2), we were able to demonstrate that different sub-areas exist in the primary taste centre, according to the organ origin and functional response of the neurones, and to propose that second order taste neurones are more broadly tuned than primary ones. Interestingly, those two features are also true for the mammalian system.

The last two chapters deal with the behavioural response of larvae to odours. We first studied the effects of olfactory pre-exposure on subsequent responses to this odorant (3). Critical experiments were able to show that the larval responses can change from attraction to repulsion, an evidence that demonstrates that pre-exposure does not reduce olfactory sensitivity (as interpreted in prior studies), but may rather induce a change in the hedonic value given to the odour. In a second behavioural project, we investigated the response to odorants in the presence of a background olfactory stimulus, trying to elucidate olfactory discriminative abilities (4). Comparison with similar approaches in mammals suggests that behavioural studies in *Drosophila* larvae may have implications of general importance, in underlining specific technical caveats and highlighting cellular and molecular mechanisms that may be similar in different animal species.

In conclusion, the investigations presented in this thesis report may move *Drosophila* ahead as a model system in chemosensation and behaviour. This work may hopefully be used as an important reference for future work in the field.
Chapter 1

Introduction

1.1 Preface

One of the basic questions in neurobiology is to understand how information about the surrounding world is encoded in the brain. The first step in this process is to decipher the molecular features of the perception of sensory information. Taking the example of vision, scientists have described how electromagnetic waves act on light-sensitive proteins, and how these proteins then affect the membrane conductance of the receptor cells, in order to trigger a response. Nowadays, we understand the majority of the cellular events that lead to the transformation of light stimuli into electric signals in the receptor neurones. However, understanding sensory perception not only requires to know how sensory neurones work, but to study how sensory information is processed within the brain. The next step is thus to reveal how the brain deals with sensory information in order to build a comprehensive representation of the outside world. If we take again the example of vision, it has been crucial to study both the neuronal wiring and the responses of neurones in the visual centres, in order to decipher visual information coding. Another question is then to understand the plasticity of the brain, i.e., how previous experience can influence the neural code and further, the behaviours linked to sensory information.

As mentioned above, much is already known about the visual system. In contrast, the chemosensory system is less well understood. How is information about the chemical world coded in our brain? How are chemicals discriminated? How plastic are chemically-driven behaviours? This thesis try to answer such questions using a simple, promising model system: the *Drosophila melanogaster* larva. A sequenced genome, powerful genetic and molecular tools, the availability of simple behavioural tests and the presence of specific genetic tools for behavioural studies are the major advan-
tages of this model organism. Another benefit comes from the high amount of data already available. For instance, the anatomy of the olfactory system has been well described.

In this thesis report, I first present the genetic tools used in this study, then, I follow with a description of the anatomy of the chemosensory system and give information on what is known about neural coding of taste and smell information in *Drosophila* and mammals. The introduction ends with the presentation of what we know about smell and taste discrimination capacities of larvae and adult flies and with a short presentation of the modulation of chemotactic responses by learning in maggots.

The presentation of the results obtained during this thesis is divided in four parts, according to the submitted manuscripts. The major part of this thesis is dedicated to the study of the primary gustatory centre, focusing on taste afferents in the brain (Chapter 2), and on the description of putative second order taste neurones expressing the *hugin* neuropeptide (Chapter 3). Little data was available on the taste system so far; we thus used a neuroanatomical approach using modern biological tools, hoping that our work can be used as a basis for further studies in the field. In the second part of this thesis, we address more complex questions concerning olfactory coding, i.e., about the effects of odour pre-exposure on subsequent chemotaxis (Chapter 4) and about olfactory discrimination in maggots (Chapter 5). In each of these chapters, the relevant questions are introduced, the materials and methods are given, and then the data and the interpretations are presented, all in the form of scientific publications. A final conclusion (Chapter 6) brings an overall view and closes this presentation.

Chapters 2 and 3 represent data submitted as two papers to "J. Comparative Neurology" (recently re-submitted after minor revision), and Chapter 4 is in press in "Animal Behaviour".

### 1.2 *Drosophila* larvae: a model system

**Generalities**

*Drosophila* was introduced as a laboratory animal by Castle at Harvard University in 1901 and soon picked up by Lutz, Loeb, Morgan, and others. Fruit flies are conveniently small, inexpensive, clean, harmless (except for causing occasional allergies), and easy to cultivate. Their generation time lasts only for about 10 days at 25°C, the life cycle includes easily identifiable phases and males are easily distinguishable from females (Ashburner,
1.2. **DROSOPHILA LARVAE: A MODEL SYSTEM**

Furthermore, *Drosophila* has a small number of chromosomes and a convenient chromosomal cytology, which is easily identifiable in polytene chromosomes. All these properties together allowed a systematic analysis of *Drosophila* genetics, and with time, the accumulation of knowledge, the large number of mapped mutations and the rich repertoire of experimental methods, all reinforced the experimental advantages of this popular model system. The recent sequencing of the entire *Drosophila* genome (Adams et al., 2000) was a great progress, which permits bio-informatics studies on a genome wide scale (see next section).

The attribution of the Nobel price to Ed Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus in 1995 definitely established *Drosophila* as a valuable model system for developmental studies. While these scientists were interested in genes controlling development, others used the fly to isolate genes essential for behaviour. This work initiated by Seymour Benzer in the 60’s (for example see Benzer, 1967) led to the identification of different genes crucial for specific behaviours, such as learning and memory (reviewed in McGuire et al., 2005). In the meantime, other people worked on the physiology of the nervous system of *Drosophila*, for instance in the olfactory and gustatory systems (see sections 1.3). The invention of the Gal4/UAS system (Brand and Perrimon, 1993), a powerful genetic tool, made it possible to get into an increasingly more detailed analysis of the different systems. Together with immunostaining and *in situ* hybridisation, mutants and rescue experiments, this permitted to link genes, neural substrate and behaviour (for an exciting example, see Zars et al., 2000). The simplicity of the *Drosophila* adult brain was a great advantage in this work; studying the even simpler larval brain may lead to even greater advances during the next decades, especially in the understanding of chemosensation.

**Genomics: receptor proteins identified**

The Nobel price winner Richard Axel intelligently extended his research on chemosensation from mammals (Buck and Axel, 1991) to the fly. Using bio-informatics tools, his group and others (Clyne et al., 1999; Vosshall et al., 1999; Clyne et al., 2000) scanned the whole fly genome for proteins with seven transmembrane domains; they identified a family of genes with relatively low homology between their members (Vosshall et al., 1999; Scott et al., 2001). This family appeared to comprise two sub-families of genes coding for olfactory and gustatory receptors (ORs and GRs respectively, Fig. 1.1, Robertson et al., 2003). Similar genes were found in other insect...
species. Especially, the atypical gene Or83b, which is involved in receptor localisation, is well conserved (reviewed in Hallem et al., 2006). These discoveries led to an outstanding boost of interest in the field of chemosensation in *Drosophila*. A lot of work focused on the molecular basis of olfactory coding, aided by the detailed knowledge of the neural networks involved. New genetic tools arose, such as olfactory receptor mutants or Gal4 lines whose expressions mimic endogenous Or genes expression. These new tools permitted to link receptor expression, neuronal activity and sensory projections into the brain (reviewed in Hallem et al., 2006, Vosshall and Stocker, forthcoming).

The Gal4/UAS system

Today, the Gal4/UAS system is certainly the most widely used method in neuroanatomical and neurophysiological studies in *Drosophila*. This genetic tool allows the selective activation of any cloned gene of interest in a large variety of tissue- and cell-specific patterns (Brand and Perrimon, 1993). Gal4 encodes a protein that activates transcription in the yeast *Saccharomyces cerevisiae* induced by galactose. It directly binds to a defined site, called "Upstream Activating Sequence" (UAS) and has no target in the fly genome. The tool uses a transposable "P-element" construct that can be inserted in the fly genome at different sites. The P-element contains the Gal4 sequence with either a known promoter (promoter fused Gal4 lines) or a weak promoter that will "trap" enhancers close to the insertion site (enhancer trap line) (Brand and Perrimon, 1993). In both cases, the transcription activator is expressed in a particular spatio-temporal pattern. GAL4 in turn directs transcription of the GAL4-responsive UAS target gene in an identical pattern (Figure 1.2).

This bipartite approach using two separate parental lines, the driver (Gal4) line and the effector (UAS) line, has major advantages. One can, for instance, target the expression of any effector gene in a variety of spatial and temporal fashions by crossing it to distinct Gal4 drivers (Brand and Perrimon, 1993) or target different effector genes in the same cells, by mating a particular Gal4 line to different UAS lines. As an example, one may express different reporters targeted to axons, synapses or cell bodies to get information about the polarity of the cells. This can be accomplished by crossing a variety of Gal4 and UAS lines established in the last decade, which can simply be ordered from public *Drosophila* libraries. Moreover, due to the bipartite system, the level of expression of the effector gene is relatively high, which is very convenient if the promoter of interest drives
Figure 1.1: Phylogenetic tree of gustatory and olfactory receptors. (from Robertson et al., 2003)
little expression (like promoters of Gr genes). New tools in which an effector
gene is placed directly under the control of a promoter are available (Wang
et al., 2004; Melcher and Pankratz, 2005). They permit to visualise different
groups of cells in the same animal. Another great advantage is the invention
of other new tools that can be combined with the Gal4/UAS system (Duffy,
2002), like the Flp-out technique explained in the next section.

Despite the tremendous profit for Drosophila research, the application
of the Gal4/UAS system bears some caveats that one has to be aware of.
First, the level of GAL4 activity is temperature dependent. By altering
the temperature, a wide range of expression levels of the responder can be
achieved. However, this can be turned into an advantage, since it allows for
an increasing flexibility of the system (Duffy, 2002). Second, the insertion
site of the P element can lead to mutation in certain genes; while this is
rare in promoter fused Gal4 lines, it is common in enhancer trap lines (a
lot of mutants were isolated using the enhancer trap technique). Third,
differences in mRNA and protein stability, sensitivity and timing of detec-
tion between different effectors may lead to differences in the pattern and
level of expression of the effector gene (Duffy, 2002). Additionally, it was
shown that the insertion site of the P-element can also have an effect on the
expression pattern of the driver and effector gene (Ito et al., 2003). Nev-
ertheless, although one has to be careful with the interpretation of data, the Gal4/UAS technique and its derivatives are of the most valuable and powerful tools available in biological science.

The FLP-out system

A major restriction of the Gal4 technique is the expression of the activator in a relatively large number of cells. To overcome this problem, different techniques were developed; one of them is the elegant FLP-out system. This tool is based on site-specific DNA recombination. The FLP recombinase is an enzyme native to the 2 micron plasmid of *Saccharomyces cerevisiae*; it alters the arrangement of DNA sequences in very specific way. The FLP recombinase is active at a particular 34 base pair DNA sequence, termed the FRT (FLP recombinase target) sequence. When two of these FRT sites are present in homologous strands, FLP creates double-stranded breaks in the DNA, exchanges the ends of the first FRT with those of the second target sequence, and then reattaches the exchanged strands. This process leads to deletion of the DNA which lies in between (when the FRT sites are in the same direction).

Flies carrying the transgenes UAS> CD2, y+> CD8GFP ('>' representing a FRT sequence) and hs-FLP were produced (FLP recombinase expression is under the control of the promoter of a heat shock protein (Wong et al., 2002)). Excision of the CD2, y+ FLP-out cassette can be triggered by a mild heat shock in random sub-populations of cells, and mosaic animals may be produced, with cells possessing alternatively the initial construct or only the UAS>CD8GFP sequence. In combination with a particular Gal4 line, this technique permits to delimit two sub-populations of GAL4 expressing cells, which are labelled by CD2 or by CD8GFP. This is a particularly efficient way for tracing single neurone projections in the brain.

Testing larval olfaction

In order to correlate olfactory neural activity with behaviour, different paradigms were developed for measuring the response of *Drosophila* larvae to odours. Briefly, larvae are placed in a flat, circular container, together with two odour sources (experimental and control) on opposite sides of the container (see Fig. 1.3A, reviewed in Cobb, 1999). Different measures of the response of larvae were used. When larvae were tested in such an assay *en masse*, i.e., 50 to 100 larvae simultaneously, authors normally waited for 3 to 5 min before counting the numbers of larvae at each side.
CHAPTER 1. INTRODUCTION

of the container and calculating a response index (Monte et al., 1989; Lilly and Carlson, 1990; Cobb, 1999; Heimbeck et al., 1999; Cobb and Domain, 2000). Another protocol using single individuals, calculating, over a period of time, the percent of time passed on each side of the plate or the mean distance to the odour. From this, a response index is defined for each larva, which is averaged over multiple tests (Scherer et al., 2003; Fishilevich et al., 2005). The second technique takes slightly more time, but can be automated (Fishilevich et al., 2005).

![Figure 1.3: Behavioural plate assays. A. For simple olfactory tests, the numbers of larvae less than 30cm away from the odour source and on a similar area on the opposite side are included in the response index calculation. B. In discrimination tests, a supplementary mask or solvent is added on 4 filter papers disposed around the test odour and control positions. Response indices are calculated as for simple tests. C. For taste responses, plates are divided in two halves; a band of 1cm in the middle of the plate is considered as a neutral zone.]

Using these different tests, it was found that larvae were attracted by a majority of odours, even at high concentration (Cobb, 1999), except for a few cases (Cobb and Domain, 2000). The age of larvae influences their behavioural response, and there is also natural variation in the odour response (Cobb and Dannet, 1994). It was recently shown that one pair of functional olfactory neurones is sufficient to trigger attraction (Fishilevich et al., 2005). Moreover, previous experience was shown to influence larval olfactory behaviour (Cobb and Domain, 2000; Scherer et al., 2003; Boyle and Cobb, 2005; Honjo and Furukubo-Tokunaga, 2005).

**Testing larval taste**

The simplicity of olfactory test assays contrasts with the complexity of gustatory assays, although the basic design used is similar: a measure of the attraction or repulsion toward a stimulus (see Fig. 1.3C). However, in the case of gustatory tests, the tastant is mixed with the agar which serves as surface. The difficulty consists in getting a flat agar surface; this is required, since a difference in the surface height can influence larval behaviour. Some studies ignored this problem and poured the two agar types
1.2. DROSOPHILA LARVAE: A MODEL SYSTEM

independently of each other (Rodrigues et al., 1991). In another protocol, authors distributed control and experimental agar before pouring pure agar on it. They then waited for the tastant to diffuse in the surface agar (Lilly and Carlson, 1990). This is not an ideal solution either, because the actual concentration of tastant sensed at the surface cannot be determined and may vary from plate to plate. Moreover, certain large molecules like glucose might even not diffuse at all. A third paradigm by Miyakawa overcame these problems. He placed a spacer along the middle of a Petri dish and poured the different agar types in the two halves. After the agar has solidified, he took out the spacer out and placed larvae in the hole created. The larvae could thus choose between the two sides (Miyakawa, 1982). A caveat still exists here, because it is a one choice test (larvae cannot cross the midline again). Larvae may thus choose their pathway without having sensed the two sides.

Another technique overcomes those caveats. Petri dishes separated in two halves by a plastic bridge are filled with experimental and control agar, then the middle part is filled with one of the two types of agar to get a flat surface (Heimbeck et al., 1999); the tastant appears not to diffuse from one side to the other (J. Colomb, unpublished results). The surface is flat, the concentration of tastant is controlled and larvae can wander around as much as they want. However, the middle part of the plate becomes non-neutral, which might complicate the interpretation of data. A simple way to solve this problem is to randomise the agar poured in the middle part (either experimental or control), carefully choosing the same number of tests for each type of plate.

Using these different paradigms, different questions were asked. Some researchers screened mutants looking for animals defective in their gustatory responses (Rodrigues et al., 1991). They isolated genes responsible for the gustatory system development (Tompkins, 1979; Inamdar et al., 1993). Jenkins and Tompkins (1990) searched for drugs acting on the gustatory system. An interesting study focused on larval discriminative abilities (Miyakawa, 1982), while a more recent one used the Gal4/UAS system to define the neurones responsible for taste recognition (see below, Heimbeck et al., 1999). Despite the number of such studies, little information about the molecular or cellular coding of taste information was collected so far.
The Gal4/UAS tool in behavioural studies

The cellular logic behind information coding can be assessed using the Gal4/UAS technique. Indeed, specific subsets of cells can be manipulated using the appropriate effector gene. For example, synaptic transmission can be blocked using the tetanus toxin gene as an effector. Then, behavioural responses of larvae in which selected neurones are silenced can be assessed and compared to the appropriate control animals: deficits in behaviour can thus be correlated with the activity of specific cells. This approach was used to find evidences showing that the neurones of the terminal organ (TO, see next section) were responsive for taste preference (Heimbeck et al., 1999)

The major limitation of this technique, however, is that very specific Gal4 lines are rare. It is thus very difficult to silence only the cells of interest.

Other effector genes can be used, e.g. to block synaptic transmission in a temperature sensitive manner (UAS-shi<sup>ts</sup>, Kitamoto, 2001), to monitor neural activity (for example UAS-gCam, see Fiala et al., 2002) or to activate neurones using an unrelated stimulus, like light (Lima and Miesenbock, 2005). In yet another technique, a modified version of the mammalian capsaicin receptor is used as an effector gene (Marella et al., 2006); capsaicin thus activates cells that would naturally not respond to this chemical. Unfortunately, this technique has a caveat since, contrarily to earlier beliefs (Marella et al., 2006), flies indeed possess a capsaicin receptor, since they respond to it in certain conditions: given a choice between a sucrose solution or a sucrose solution laced with capsaicin, flies shows a preference for the latter one (Al-Anzi et al., 2006). However, this technique is appropriate to get a rough idea about the responses of the labelled neurones.

1.3 Chemosensory system of *Drosophila* larva

Chemosensory organs

The larval chemosensory apparatus includes three major external sense organs on the head, the dorsal organ (DO), terminal organ (TO), and ventral organ (VO), as well as three internal, pharyngeal organs (Fig. 1.4, Stocker, 1994; Singh and Singh, 1984; Gendre et al., 2004). Each of them consists of several sensilla, a sensillum comprising one to several sensory neurones and three accessory cells, all housed below a common cuticular structure or terminal pore.

The DO is composed of the central "dome" and six peripheral sensilla. The dome, whose wall is perforated by thousands of pore tubules, is inner-
vated by the profuse dendritic arbours of 21 olfactory receptor neurones (ORNs). An olfactory function of the 21 neurones innervating the dome is demonstrated by electrophysiological recordings (Oppliger et al., 2000) and ablation studies (Heimbeck et al., 1999). Larvae in which these 21 cells were selectively blocked or ablated by toxin expression became anosmic, suggesting that these neurones are the sole larval ORNs (Fishilevich et al., 2005; Larsson et al., 2004). In analogy to *Musca* (Chu and Axtell, 1971; Chu-Wang and Axtell, 1972a,b), the remaining sensilla of the DO, as well as most of the TO sensilla and one of the five VO sensilla may mostly have a taste function (see Table 1.1, Python and Stocker, 2002). However, these organs very likely include themosensory, mechanosensory or hygrosensory neurones.

![Figure 1.4: Schematic of chemosensory organs in the larval head. Circle size is proportional to the number of cells in the different organs, colours refer to the corresponding peripheral nerves. Red: 21 olfactory receptor neurones. In the CNS, red represents the AL, blue the SOG.](image)

The terminal organ (TO) is located ventrally to the DO and comprises 11 sensilla in the distal group and three in the dorsolateral one, for a total of 33 neurones (28+5). The putative presence of three additional scolopidia (Python and Stocker, 2002) has not been confirmed.

The DO, TO and VO all have their proper ganglion (Fig. 1.4). The ganglion of the DO contains 36-37 sensory neurones (Python and Stocker, 2002). The 21 ORNs among them extend their dendrites as seven triplets into the dome. The dendrites of three other neurones project toward the dorsolateral sensilla of the TO, while the remaining cells innervate the six
## Table 1.1: Sensilla on the larval head and the corresponding numbers of associated neurones. TO: terminal organ.

<table>
<thead>
<tr>
<th>Terminal Organ</th>
<th>sensilla number</th>
<th>neurones per sensilla</th>
<th>TOTAL</th>
<th>gustatory neurones</th>
<th>mechanosensory neurones</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dorsal Organ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dome</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lateral pore receptor</td>
<td>1 x 2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>scolopidium</td>
<td>1 x 1</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>contact. chemorec.</td>
<td>2 x 2</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>unclassified</td>
<td>2 x 2</td>
<td></td>
<td>4</td>
<td>4</td>
<td></td>
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<tr>
<td><strong>TOTAL</strong></td>
<td>1 + 6</td>
<td></td>
<td>21 + 11</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td><strong>Distal TO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>spot</td>
<td>3 x 1</td>
<td></td>
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<td></td>
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<td>1 x 4</td>
<td></td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>11</td>
<td></td>
<td>28</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td><strong>Dorsolateral TO</strong></td>
<td></td>
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peripheral sensilla of the DO. The TO and VO ganglia include 32 and 7 sensory neurones respectively. DO neurones project to the brain via the antennal nerve, while TO and VO ones project together via the maxillary nerve.

Four groups of paired sense organs occur in the larval pharynx (Stocker, 1994; Gendre et al., 2004): The dorsal pharyngeal sense organ (DPS), the dorsal pharyngeal organ (DPO), the posterior pharyngeal sense organ (PPS) and the ventral pharyngeal sense organ (VPS). They possess about 18, 5, 6, and 16 neurones, respectively (Fig. 1.4). They consist of several sensilla each, comprising one to nine sensory neurones (Singh and Singh, 1984; Python and Stocker, 2002; Gendre et al., 2004). Gustatory and mechanosensory function is suggested by the presence of pores or bristles respectively. Three organs project to the brain via the labral nerve and become integrated in the adult system during metamorphosis (DPS, DPO, PPS). In contrast, the VPS, which projects via the labial nerve, disappears during the pupal stage.

Another putative taste organ was reported ventral to the mouth hooks. This labial organ seems to possess three sensilla (Singh and Singh, 1984; Stocker, 1994). On the other hand, the abdominal and thoracic segments also possess poly-innervated organs. They are supposed to have a gustatory function, as induced from their dependence on the *pox neuro* gene (Dambly-Chaudière and Ghysen, 1986; Ghysen et al., 1986; Dambly-Chaudière et al., 1992; Awasaki and Kimura, 2001). In the thoracic segments, the ventral and dorsal "kölbcchen" (sometimes referred to as pits) possess three neurones; in segment A1-7, the small dorsal hair h4 and the lateral papilla p6 have two neurones each; the fused segment A8-A10 was not described in detail but may also contain poly-innervated structures (Ghysen et al., 1986).

### Odorant receptor proteins and neuronal response

Twenty three to 25 odorant receptor proteins (ORs) were found to be expressed in the 21 olfactory receptor neurones (ORNs) of *Drosophila* larvae. Fourteen of these receptors are specific for the larval stage, the 11 others are found both in the larva and in the adult (Couto et al., 2005; Fishilevich et al., 2005; Kreher et al., 2005). Two pairs of ORs are co-expressed in the same neurone: Or33b with Or47a and Or94a with Or94b (Fishilevich et al., 2005). Assessing ligand specificity of the different ORNs yielded similar results, irrespective of the approach used, such as: electrophysiology in an adult ORN expressing only a single larval OR (and not the endogenous OR, Kreher et al., 2005), chemotaxis behaviour of larvae possessing only one
functional ORN (Fishilevich et al., 2005), or electrophysiology of DO neurones (D. Hoare and M. Cobb, communication at the Manchester Maggot Meeting). Remarkably, ORNs located in an adult or a larval sensilla but expressing the same OR presented similar responses. An OR thus appears to define the response properties of the ORN (Dahanukar et al., 2005), and the morphology of the sensilla appears not to have a major impact on the neuronal response. However, specific odorant binding proteins released by non-neuronal cells were shown to be crucial for some specific responses (Xu et al., 2005).

Individual odours are sensed by multiple ORNs and individual ORN respond to many odours (Kreher et al., 2005). Interestingly, comparing the responses of larvae with only one pair of functional neurones with normal larvae, L. Vosshall’s group showed that a combinatorial code was effective (Fishilevich et al., 2005). For instance, responses to propyl butyrate were observed only when two neurones were functional.

Taste receptors

The situation for taste perception is less clear. Different types of proteins seem to be involved: GRs, Ppks and TRPs.

Bio-informatic searches for ORs led to the discovery of related proteins which were supposed to be gustatory receptors (GRs, Fig 1.1). For instance, Gr5a was shown to code for the trehalose receptor (Dahanukar et al., 2001; Ueno et al., 2001; Inomata et al., 2004). Good evidence for the expression of GRs in taste sensilla was provided by the RT-PCR study of Clyne and co-workers. Since expression disappeared in poxn (Clyne et al., 2000), a mutant that lacks chemo-sensilla, GRs seemed to be expressed exclusively in taste organs. In situ hybridisation in the adult fly successfully demonstrated the expression of six GRs in taste sensilla (Gr66a, 47a, 32a, '98A1', 28be and 33a) and of three GRs in olfactory sensilla (Gr21a, 10a, 63a). However, in situ did not work for other GRs (probably because of low expression of these genes) and was not tested for larvae (Scott et al., 2001). A different approach was thus chosen, taking advantage of the Gal4/UAS system.

Fusing the putative promoter region of Gr genes to the Gal4 gene, different Gal4 lines were produced (Dunipace et al., 2001; Scott et al., 2001). In these lines, Gal4 expression should theoretically mimic Gr expression. However, this approach may lead to unspecific expression, since the construct may lack important regulatory elements. Gal4 expression in these lines was shown to be dependent on the poxn gene; it is often (but not always) limited
1.3. CHEMOSENSORY SYSTEM OF DROSOPHILA LARVA

to taste organs (Dunipace et al., 2001). Since the expression of the Gal4 lines was not tested in combination with in situ hybridisation (Scott et al., 2001), firm conclusions about the genuine GR expressions cannot be drawn. However, the high amount of Gr-Gal4 line expression data available suggests that, in the adult, the majority of tested GRs are co-expressed with GR66a in neurones responsive to bitter compounds (Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006). In contrast, GR5a-Gal4 showed expression in another set of GRNs, which appeared to be responsive to sugars (Thorne et al., 2004; Wang et al., 2004).

Genes of the Pickpocket family (Ppk) are good candidates for salt receptors. In situ hybridisation and promoter-fused Ppk-Gal4 line demonstrated that some of these genes are expressed in the TO, DO and thoracic chemosensory cells (Liu et al., 2003a). In the same study, using dominant negative forms of the genes and RNAi constructs, it was proposed that Ppk11 and Ppk 19 were involved in the response toward NaCl at attractive concentrations.

Transient receptor proteins (TRPs) and TRP-like proteins are additional candidates of gustatory receptors. These proteins were shown to be responsible for the perception of noxious chemicals as well as temperature sensation in mammals (Caterina et al., 1997); the related painless gene in Drosophila was shown to be expressed in the TO (Tracey et al., 2003; Al-Anzi et al., 2006). A variety of different proteins could thus be involved in taste sensation. However, GRs seem to be the only ones specific for this role and thus to be expressed exclusively in GRNs.

Chemosensory systems’ wiring

Remarkably, the functional architecture of the olfactory system is similar in adult Drosophila and in the mouse: neurones expressing a given OR extend axons that converge in the primary olfactory centre, to form spatially discrete synapses with second order projection neurones (PNs, Fig. 1.5). These synapses are organised into spherical substructures called glomeruli, which consist of ORN terminals, PN dendrites, and arborisations of a network of local inhibitory interneurones (LIs). Individual glomeruli therefore collect input of ORNs expressing a given OR gene (reviewed in Keller and Vosshall, 2003). Convergent wiring of ORNs expressing the same OR, which therefore respond to the same odorants, may provide a coding basis for the brain, which may further translate patterns of glomerular activity into perception of a stimulus.

The larval olfactory system was shown to have a similar basic design
as its adult counterpart and thus as the mouse, but without cellular redundancy (Fig. 1.5, Ramaekers et al., 2005): twenty one ORNs confront essentially similar numbers of larval antennal lobe (AL) glomeruli. Some processing of odour information occurs certainly in the AL, via the LIs, whose individual arborisations cover the entire AL. Information is then transferred to higher brain centres via not many more than 21 PNs. They establish recurrent synapses in the AL and stereotypical axon terminals in one or two of about 34 glomerular structures in the calyx of the mushroom bodies (MBs), as well as in the lateral horn (Masuda-Nakagawa et al., 2005; Ramaekers et al., 2005). There are two types of third order neurones of the MBs. The majority of embryonic born neurones appear to arborise in one or two calyx glomeruli (Ramaekers et al., 2005), while larval born neurones arborise in about six of them (Masuda-Nakagawa et al., 2005). In brief, there is a simple 1:1:1:1:1 relationship among ORNs, AL glomeruli, PNs and maybe even calyx glomeruli and therefore, an absence of convergent and divergent connectivity in the AL (Fig. 1.5, Ramaekers et al., 2005).

Very little is known about the wiring of the larval gustatory system, apart from a basic description of selected gustatory afferents (Scott et al., 2001). More information is available about the adult primary taste centre. In adults, taste information is indeed sent to multiple target areas in the
sub-oesophageal ganglion, which does not show any obvious glomerular organisation (Stocker, 1994; Rajashekhar and Singh, 1994, but see Shanbhag and Singh, 1992). A distinction between projections from maxillary palp and pharyngeal sensilla was observed using cobalt filling of afferents (Stocker and Schorderet, 1981); this difference was recently confirmed using genetic labelling of neurones (Wang et al., 2004). Pharyngeal sensilla were shown to have bilateral projections (Stocker and Schorderet, 1981), while the majority of labellar projections are unilateral (Nayak and Singh, 1985). However, bitter responsive labellar neurones were recently shown to project bilaterally (Thorne et al., 2004).

If little is known about afferent projection patterns, nearly no information is available about second order neurones in insects. In *Drosophila*, a recent study reported about putative second order taste neurones, expressing a neuropeptide named *hugin*. These neurones appeared to control feeding behaviour in adults (Melcher and Pankratz, 2005). Interestingly, there seemed to be a similar number of such neurones in the larva and the adult. A detailed description of their morphology is presented in the chapter 3. In addition, motor neurones appeared to arborise in the anterior part of the SOG. An overlap with taste afferent was hypothesised (Rajashekhar and Singh, 1994).

In the grey flesh fly, local interneurones activity was recorded (Mitchell and Itagaki, 1992). In the honey bee, the VUMX1 neurone was shown to receive appetitive taste information in the SOG, and to mediate sugar reward learning in the AL and the MB (Menzel, 2001). Another group of about 100 neurones were shown to connect the SOG with the calyces of the MBs (Schröter and Menzel, 2003). These neurones were supposed to transmit taste information to this learning centre, suggesting that bees can use taste information as conditioned stimuli. Still, relatively little information is available about gustatory centres in the insect brains.

## 1.4 Chemosensory coding

### Olfactory coding

The stereotypical organisation of virtually all levels of the olfactory system (Fig. 1.5) is highly suggestive of a mechanism of odour coding that employs spatial patterns of glomerular activation to represent olfactory stimuli, but direct proof of this model is lacking. Recent efforts of optically recording olfactory glomeruli confirmed that the glomerulus is a functional unit in the olfactory system (Fiala et al., 2002; Ng et al., 2002; Galizia
and Menzel, 2000). These investigators showed, first, that a given odorant activates a reproducible subset of glomeruli, which is invariant between different individuals; second, that with increasing concentration, additional glomeruli are recruited into the activity pattern. On the other hand, electrophysiological experiments in the zebrafish carried out by Laurent and co-workers (Friedrich and Laurent, 2001) led to an alternative hypothesis of odour coding, which did not rely solely on the hypothesis of glomerular encoding, but rather favoured a temporal model. In this model, odour stimulation induces stimulus-specific alterations in the synchrony of local field potentials. It was proposed that these temporal parameters were central to the process of representing odorants, especially fine differences between those odours that are structurally similar and are likely to produce overlapping patterns of activation.

Experiments in the honeybee (Sachse and Galizia, 2002) that examined information flow in the AL suggested that inhibitory LIs play a major role in modulating the output of glomerular activity. The inhibitory network filters and processes the olfactory information that arrives in the glomeruli from the ORNs and produces a coherent stimulus-specific output. How is olfactory information from the AL represented at higher levels in the brain? PN tracing studies in *Drosophila* (Wong et al., 2002; Marin et al., 2002) suggested that PNs having dendrites in the same AL glomerulus also have similar patterns of axonal terminals in the lateral horn. Moreover, these patterns appeared strongly conserved between different individuals. The spatio-temporal coding of the AL thus appears to be conserved in higher brain centres, although convergence of different olfactory units (ORN-glomeruli) occurs in these regions.

Despite the efforts invested in the elucidation of olfactory coding, many questions remain open. For instance, where in the brain is the combinatorial code "decoded": can we find neurones firing only in response to one odour? How does the brain decide whether two olfactory stimuli are different or not?

**Gustatory coding**

How taste information is processed by the nervous system remains largely unanswered, not only because of a scarcity of data, but also because different approaches yielded conflicting results (reviewed in Meyerhof, 2005). In the periphery of the mammalian system, using genetic tools, it was proposed that bitter, sour, sweet and umami taste qualities should be perceived by four different sets of taste receptor cells (TRCs), each express-
1.5 Complex behaviours linked to chemo-sensation

Discriminative abilities

How can we test whether an animal can discriminate between two chemicals $A$ and $B$? Different protocols were developed. The first one involves a learning paradigm (Guerrieri et al., 2005): odour $A$ is trained (for example rewarded) while odour $B$ is tested: if $B$ does not trigger the conditioned response, one can conclude that $B$ is discriminated from $A$. A second test is based on a masking strategy: the response to odour $A$ is tested in the presence of a background concentration of odour $B$. A third assay is called cross-adaptation test: animals are placed in presence of odour $A$ and therefore adapt to it. Subsequently, the response to odour $B$ is tested.
Interpretation of the learning paradigm is made difficult because of the generalisation principle (learning can be generalised between two discriminated but similar chemicals (Ghirlanda and Enquist, 2003)) and by findings which showed that learning may alter the odour code (Faber et al., 1999, but see Peele et al., 2006). Nevertheless, this technique was able to show that discrimination in bees depends on odorant similarities, i.e., the relative carbon chain length and the presence of specific functional groups (alcohol, aldehyde, esters,...). Moreover, generalisation was shown to be often asymmetric (learning A generalises to B, but B does not generalise to A, Guerrieri et al., 2005. Also, discrimination of odorants was better with high concentration of dissimilar -but not similar- odorants (Wright and Smith, 2004). If we expect odour similarities and concentrations to be variables predicting discriminative abilities (because they influence the firing pattern of receptor neurones and therefore the neuronal representation of the odours), the asymmetry is surprising. It appeared to be odorant dependent (certain odours generalise more easily than others). Whether this effect is due to odour coding (for instance due to asymmetric inhibition from LIs, Sachse and Galizia, 2002) or to the learning procedure has still to be determined.

The masking approach has the advantage of being direct, quick and simple. It avoids the problems linked with learning, but unfortunately has its own caveats. First, the absence of a response cannot be directly interpreted as an absence of discrimination, since the animal may just not respond to A because it feels comfortable (or in danger) in the presence of B. Indeed, such effects of performance in a specific context was reported in Drosophila larvae (Gerber and Hendel, 2006). Second, information represented by the absence of firing of specific glomeruli would certainly be lost: if the neural image of A is included but different from the one of B, it will not produce any responses. Asymmetrical results are thus to be expected in this paradigm. Third, the model predicts that adaptation to odour B may be necessary in order to respond to odour A, i.e., an absence of response can be linked to problems of sensory adaptation rather than to problems of discrimination (Kelliher et al., 2003). This technique, which was rarely used in olfactory tests, was elegantly applied by Miyakawa (1982) in order to look for taste discrimination: he showed that Drosophila larvae were able to distinguish between different sugars and different salts.

The third paradigm was conducted with Drosophila larvae (Cobb and Domain, 2000; Boyle and Cobb, 2005; Wuttke and Tompkins, 2000), and recently also with adult flies (Chandra and Singh, 2005). It appeared to combine the disadvantages of the first two. It actually is a learning paradigm
with all the afore mentioned problems and since it is thought to act at the periphery (although this is still in debate (Wilson, 2000)), asymmetries in the results are expected. Indeed, this paradigm was shown to lead to asymmetric results and to be very difficult to interpret (Cobb and Domain, 2000; Boyle and Cobb, 2005, see chapter 4). However, authors showed that odour similarities can also predict olfactory discrimination, as tested in this paradigm.

In conclusion, discriminative abilities were so far assessed by different approaches: a learning approach for olfaction, mainly using honeybees as a model system, and a masking approach for taste using *Drosophila*. Results are still sparse but already helped in understanding of chemosensory coding, both in insects and in mammals.

**Olfactory learning in *Drosophila* larvae**

Similar to adults, *Drosophila* larvae use chemosensory cues in order to orient themselves in their environment, for example to locate the optimal food source. Their behavioural response to odorants is not genetically fixed, but can be modified by experience. We already mentioned that pre-exposure to an odorant lead to a subsequent change in their response toward that odorant. In addition, if the odorant is presented concomitantly with a reward or punishment, larvae exhibit associative learning. Indeed, electroshock as a negative unconditioned stimulus (US) was reported to induce associative learning (Tully et al., 1994); but these results were not reproduced. On the other hand, using gustatory cues as US proved to be effective in changing hedonic values of odours (Scherer et al., 2003; Honjo and Furukubo-Tokunaga, 2005), as well as changing responses toward white light (Gerber et al., 2004). Remarkably, only positive reinforcement was effective in prior tests (Hendel et al., 2005), although recent work showed that this effect is dependent on the environmental context during the test (Gerber and Hendel, 2006): larvae apparently use the learned information only when facing a challenging contextual situation. Interestingly, olfactory learning in *Drosophila* larvae appears to share similarities with adult learning: it depends on the output of MBs during retrieval but not acquisition, on the *amnesiac* gene and *dCREB* (Honjo and Furukubo-Tokunaga, 2005), as well as on the *synapsin* gene (Michels et al., 2005).
1.6 This thesis

In order to dissect the cellular events which are crucial for chemosensory-driven behaviours, we need a complete picture of the underlying neural substrate as well as a good description of the tested behaviours. Accordingly, a major part of this thesis was devoted to the description of the yet puzzling gustatory system: A map of the primary taste centre in *Drosophila* larvae was established (Chapter 2), and a search for second order taste neurones was conducted (Chapter 3). Independently, the behavioural effects of simple odour exposure were investigated, a study which yielded surprising results (Chapter 4). Finally, we developed a paradigm for testing olfactory discrimination and we investigated the effect of NO in this behaviour (Chapter 5).
Chapter 2

Architecture of the primary taste centre of
*Drosophila melanogaster* larvae

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2.1 Abstract

A simple nervous system combined with stereotypic behavioural responses to tastants, together with powerful genetic and molecular tools, have turned *Drosophila* larvae into a very promising model for studying gustatory coding. Using the Gal4/UAS system and confocal microscopy for visualizing gustatory afferents, we provide a description of the primary taste centre in the larval central nervous system. Essentially, gustatory receptor neurons target different areas of the suboesophageal ganglion (SOG), depending on their segmental and sensory organ origin. We define two major and two smaller subregions in the SOG; one of the major areas is a target of pharyngeal sensilla, the other one receives inputs from both internal and external sensilla. In addition to such spatial organization of the taste centre, our data suggest that aversive and attractive stimuli might be processed in the anterior and posterior part of the SOG, respectively. Our results also suggest less co-expression of gustatory receptors than proposed in prior studies. Finally, dendritic projections of putative second order taste neurons seem to cover large areas of the SOG and may thus receive multiple gustatory inputs. The corresponding broad sensitivity of secondary taste neurons is reminiscent of the situation in mammals.
2.2 Introduction

The primary goal of chemosensory neurobiology is to understand how information about the chemical environment is encoded by the nervous system. *Drosophila* is being intensely used as a model system for deciphering the olfactory code (Keller and Voshall, 2003; Dahanukar et al., 2005; Jefferis, 2005; Rutzler and Zwiebel, 2005; Hallem et al., 2006). The recent discovery of a family of gustatory receptors (GRs) in the fly (Clyne et al., 2000; Scott et al., 2001) has also boosted interest in the study of taste. The simple nervous system of *Drosophila* larvae, in combination with powerful genetic and molecular tools may be of great advantage for studying gustatory coding principles. Moreover, larvae exhibit interesting behaviours in response to tastants. They can discriminate between different salts and different sugars (Miyakawa, 1982) and are able to use gustatory information as reward (Scherer et al., 2003; Gerber et al., 2004).

While olfactory coding relies on a multitude of combinatorial patterns of activity in olfactory glomeruli (Keller and Voshall, 2003), taste information appears to be assigned to a small number of categories already in the periphery (Scott, 2004). Gustatory signals thus seem to be coded by activating groups of sensory cells (‘labelled line’ coding). However, finer discrimination within a category is possible (Caicedo and Roper, 2001; Glendinning et al., 2002; Ishimoto and Tanimura, 2004) and in the mammalian gustatory system, central neuronal responses become broadly tuned to multiple categories (Sato and Beidler, 1997). In insects, only few data on central gustatory processing has been collected (Mitchell et al., 1999). These data suggest that taste information in the brain depends on the pattern of activity across neurons (‘across-fiber’ coding) (Smith and St John, 1999; Lemon and Smith, 2006; Stapleton et al., 2006). Remarkably, a recent review that both model are too static to account for the high modulation of gustatory responses and that new models will be needed in the future (Jones et al., 2006).

Taste sensilla in adults of *Drosophila* and other insects usually contain four gustatory receptor neurons (GRNs), which were shown to respond to either water, sugar, or low or high salt concentrations (Ishimoto and Tanimura, 2004). Deterrent compounds (sensed as bitter by humans) appear to activate a subset of salt responding cells, exhibiting some selectivity (Meunier et al., 2003). Sweet and deterrent compounds seem to be recognised by a family of seven transmembrane GRs, which are related to odorant receptors (Scott et al., 2001; Robertson et al., 2003; reviewed in Hallem et al., 2006). For instance, GR5a was shown to be the receptor respon-
2.2. INTRODUCTION

sive for trehalose perception (Dahanukar et al., 2001; Ueno et al., 2001; Inomata et al., 2004). Another GR member, GR68a, appears to be essential for pheromone perception (Bray and Amrein, 2003). More surprising, GR21a is expressed in CO$_2$ responsive olfactory cells in the antenna (Suh et al., 2004) and was recently suggested to be expressed in CO$_2$ responsive cells in larvae as well (Faucher et al., 2006). In contrast, salt responses are not mediated by GRs, but by ionic channels apparently encoded by the pickpocket gene family (Ppk, Liu et al., 2003a). Finally, proteins of the transient receptor potential (TRP) family were recently shown to be involved in chemical perception (Al-Anzi et al., 2006), apart from their role in pain and temperature sensation (Tracey et al., 2003).

The expression pattern of the GR genes in adults was essentially assessed indirectly, via Gal4 reporter gene expression (Dunipace et al., 2001; Scott et al., 2001; Liu et al., 2003a; Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006). Although reporter expression patterns may not accurately reflect GR expression patterns, the collected data suggested that many GRNs cells coexpress multiple receptors. For instance, the majority of GRs seemed to be expressed in Gr66a expressing cells, which mediate aversive behaviour (Thorne et al., 2004; Wang et al., 2004). Moreover, the majority of sugar responsive cells appeared to coexpress at least three different sugar receptors (Ishimoto and Tanimura, 2004). However, as a principle, putative sugar GRs and bitter GRs seem to be expressed in different subsets of GRNs, allowing initiation of distinct behavioural responses, i.e., attraction versus repulsion.

The central projection patterns of adult GRNs established by reporter gene expression (Thorne et al., 2004; Wang et al., 2004) correlate well with previous reports (Stocker and Schorderet, 1981; Nayak and Singh, 1985; Shanbhag and Singh, 1992). First, projections from different gustatory organs such as legs, labellum and pharyngeal organs segregate in separate regions in the suboesophageal ganglion (SOG). Second, a distinction between the projection of GRNs sensitive to attractive and aversive cues was reported. Interestingly, recent work showed that water responsive cells exhibit similar projections as sweet responsive neurons (Inoshita and Tanimura, 2006). Moreover, labellar GRNs mediating aversive behaviour appear to project bilaterally, whereas those responsible for attractive behaviour remain ipsilateral (Thorne et al., 2004; Wang et al., 2004). Bilateral projections are also typical for pharyngeal sensilla (Stocker and Schorderet, 1981; Rajashekar and Singh, 1994).

The larval taste system is less well documented. It also comprises both external and internal sense organs, most of which are multimodal (Stocker,
1994). External organs include the terminal organ (TO; with taste, stretch, touch and thermoreceptive neurons), the ventral organ (VO; with taste and touch receptors), and the dorsal organ (DO) (Singh and Singh, 1984; Stocker, 1994; Python and Stocker, 2002). The DO is the unique larval olfactory organ (Fishilevich et al., 2005), which in addition contains putative mechanoreceptive and taste sensilla (Stocker, 1994). Other external putative taste organs may occur in thoracic and abdominal segments (Dambly-Chaudière and Ghysen, 1986). Gustatory identity is suggested from the presence of polyinnervation and their dependence on the taste sensillum-specific popn gene (Dambly-Chaudière et al., 1992). Internal organs comprise the dorsal, ventral and posterior pharyngeal sense organs (DPS, VPS and PPS, respectively) and the dorsal pharyngeal organ (DPO) (Gendre et al., 2004). The axons deriving from the DO as well as three axons from the TO project to the brain via the antennal nerve, while the other TO neurons and all of the VO neurons pass to the SOG through the maxillary nerve. Fibres from the DPS, DPO, PPS and the VPS travel via the labral and labial nerves, respectively (Python and Stocker (2002), see also Fig. 1.4, P. 11).

Using the Gal4-based approach in combination with the Flp-out strategy (Wong et al., 2002)), we investigated the connectivity of the primary larval gustatory centre in the SOG. Essentially, we compared the morphology of GRNs belonging to different organs or having potentially different response profiles. Our data suggest a similar but not identical organization in the larval taste centre compared to its adult counterpart. Apart from information about primary taste neurons, we also collected examples of putative second order taste neurons. Other interesting candidate taste interneurons are described extensively in chapter 3.

### 2.3 Materials and Methods

**Fly strains**

*Drosophila* stocks were raised on standard cornmeal medium at room temperature; CantonS (CS) was used as a wild type control strain. Transgenic P[GAL4] lines were kindly provided by K. Scott (Gr2a, 21a, 22f, 28be, 32a, 47a, 66a; (Scott et al., 2001; Wang et al., 2004)), H. Amrein (Gr5a, 8a, 22b, 22e, 22f, 28be, 32a, 59b, 59e, 59f, 64a, 64e, 66a; (Dunipace et al., 2001; Thorne et al., 2004)) and M.J. Welsh (PPK 6, 10, 11, 12, 19; (Liu et al., 2003a)). As a reporter line, we used UAS-mCD8:GFP (Lee and Luo, 1999). Larvae for the clonal anal-
2.3. MATERIALS AND METHODS

Analysis were obtained by crossing males of a GAL4 line with virgins hs-FLP;CyO/Sp;UAS>y+ CD2>CD8;GFP (Wong et al., 2002). For simultaneous labelling of different receptors, we crossed the males mentioned before with virgins of the genotype w-;UASCD2;Gr66a-I-GFP or w-; SP/Cyo; Gr66a-I-GFP, UASCD2. We obtained those flies by combining the Gr66a-I-GFP/TM6 stock (kindly provided by K. Scott; Wang et al., 2004) and the CyO/UAS-CD2 and UASCD2 (III) stocks (Bloomington stock centre). For behavioural studies we crossed UAS-gCcaMP56; UAS-VR1E600K; Tm2/Tm6 (kindly provided by R. Axel; Marella et al., 2006) virgins to Gr66a-Gal4 or CS males (from H. Amrein) or males of the same UAS line to virgins GH86 or CS.

Clone induction

FLP recombinase was induced by placing tubes containing larvae 1-2 days after egg laying (AEL) in a water bath maintained at 37°C during 1 hr (Gr-Gal4 clones) or 50 min (MJ94 clones).

Immunofluorescence

Antibody staining was adapted from an earlier protocol (Ramaekers et al., 2005). Briefly, young third instar larvae (72-96h AEL) were predissected in phosphate buffer (PB; 0.1M, pH= 7.2). The brains attached to the body wall were fixed for 20 min in PB containing 3.7% formaldehyde, and subsequently rinsed in PBT (0.3% Triton X-100 in PB). They were further dissected and placed for 2 hrs in PBT in 5% goat serum (NGS) at room temperature for blocking. Subsequently, they were incubated with a cocktail of primary antibodies overnight at 4°C. Primary antibodies included anti-ChAT (dilution 1:500) from P. Salvaterra (Beckman Research Institute, City of Hope, Duarte, CA), anti-GFP (Molecular Probes), anti-CD2 (1:100; Serotec GmbH, Düsseldorf, Germany) and nc82 (1:20) from A. Hofbauer (University of Regensburg, Germany). After several rinses in PBT, samples were incubated overnight in PBT-NGS with the secondary antibodies (anti-rabbit Alexa 488-conjugated and anti-mouse Cy3-conjugated, diluted 1:200; Molecular Probes). After several rinses, brains were mounted in Vectashield (Vector Labs), with nail polish used as spacer. The CNS was mounted with the ventral nerve cord on top.
CHAPTER 2. PRIMARY TASTE CENTRE

Image Acquisition and Processing

Images of the peripheral nervous system were taken by using a fluorescence microscope (Leica DM R) equipped with a CCD camera. Stacks of confocal images at 0.93µm focal plane spacing were collected with a Biorad MRC 1024 confocal microscope and Laser Sharp image-collection software. Images were then processed with Image J freeware (http://rsb.info.nih.gov/ij/index.html), curves (input to output options) were readjusted for each colour independently but always on the whole picture. The intensity of unspecific background staining was lowered using the "dust and scratches" filter in Adobe Photoshop for Macintosh 7.0 software.

Behavioural tests

Behavioural tests were performed using a modified previous protocol (Lilly and Carlson, 1990). Petri dishes separated in two halves with a plastic bridge (Greiner 635102) were filled first on both sides with 24ml of agar, in order to minimise the required amount of tastant. After drying the agar, either 7ml of 1.5% agarose were poured on the control (C) side, or agarose mixed with tastant on the stimulus (S) side, respectively. The second halves of the plates were poured 5-10 min later with the other type of agar. The central region was simultaneously filled with the second agar type, in order to get a flat surface. The plates were then dried for 1 hr. Plates are thus divided into two halves containing agar alone and agar with tastant, respectively, the middle portion containing either type of agar in a random manner. To avoid any bias toward the type of agar in the centre region and the sequence of pouring, in 50% of the cases the C side, and in the other 50% the S side was poured first.

Three days old larvae (AEL; reared at 25° in a 12/12 light dark cycle) were collected using sugar solution and then rinsed in tap water. About 50 larvae were distributed along the separating plastic bridge and allowed to freely move on the entire plate. After 15 min in total darkness, a photo of the dish was taken and larvae were counted. A response index was calculated:

\[ RI = \frac{Ns - Nc}{Ns + Nc} \]

Ns and Nc referring to the numbers of larvae present on test and control areas, respectively. Animals found at a distance of less than 0.5cm from each side of the bridge were discarded. Multiple tests were done in parallel; half of the plates were turned by 180° to compensate for any other
2.4 Results

In this paper, we present a description of the sensory projections in the primary taste centre of *Drosophila* larvae. Our observations allowed us to delimit the target region in the SOG dealing with gustatory information, and furthermore to define sub-areas that may be associated with different taste properties. We made use of the genetic tools provided by this species as well as the known taste organs morphology (Fig. 2.1A-C), studying the expression patterns of different Gal4 reporter lines, namely (i) twenty lines driven by different Gr promoters (for some Gr promoters also multiple lines), lines whose adult expression was previously described (Dunipace et al., 2001; Scott et al., 2001; Bray and Amrein, 2003; Thorne et al., 2004; Wang et al., 2004), (ii) five Ppk-Gal4 lines, including three that were already described (Liu et al., 2003a), and (iii) the enhancer trap lines GH86 (Heimbeck et al., 1999) and MJ94 (Gendre et al., 2004) that were both shown to be expressed in GRNs.

Using the UAS-CD8GFP reporter line, we visualised the expression of these lines in the peripheral and central nervous system (Fig. 2.2). The widespread expression pattern of certain Gal4 lines was dissected by tagging single cells via the Flp-out technique (Wong et al., 2002). In such preparations, one or a few of the Gal4 expressing cells are labelled by UAS-CD8GFP, whereas the rest of the Gal4 expressing cells are labelled by UAS-CD2 (Fig. 2.3A-D, L-O). Furthermore, using CD2 as a reporter, we investigated the expression of different Gal4 lines in the background of Gr66a-GFP. Studying coexpression allowed us to compare the location and morphology of sensory terminals deriving from different sensory neurons (Fig. 2.3E-K). Finally, we describe a putative second order gustatory neuron labelled in the GH146 Gal4 line (Fig. 2.3Q-S). Our results are schematized in Figs. 2.1 and 2.4.

Peripheral expression

The *Drosophila* larva comprises about 90 pairs of GRNs, located in different sensory organs in the head and on the body wall (Table 5.1). The major chemosensory organs in the larval head are the DO, TO and VO, as well as four additional organs along the pharynx (Fig. 2.1A,B cf. Singh and Singh, 1984; Python and Stocker, 2002). All of these organs contain
Figure 2.1: Diagrams of larval head chemosensory organs (A-C), their nerves (A) and their central target areas (D-F, horizontal views). A: Projections in the MN (blue) derive from the TO and VO, those in the LrN (green) from the DPS, DPO and PPS, those in the LbN (dark green) from the VPS, and those in the AN (red) from the DO. Note an atypical DO neuron whose dendrites extend in the TO (TODO). Projections from thoracic organs are shown in magenta. SOG entry points of the nerves were deduced from confocal images at horizontal orientation. B: Ventral view (Nomarski optics) of the DO with its prominent olfactory "dome" (red arrowhead), the TO (blue arrowhead), the VO (blue arrow) and the mouth hooks (asterisk). C: Close-up of TO showing two "dorsolateral" sensilla (red arrowheads) and five "distal" sensilla (blue arrowheads). D: Four major chemosensory target areas (1-4) can be distinguished. An "atypical" projection from Gr21a-Gal4 labelled TO neurons stays apart from these areas (asterisk). See text for further details. The sense organs providing input for each of these areas are given on the right. E-F: Projections of Gr2a-Gal4 and Gr66a-GFP labelled neurons, respectively, as deduced from figs. 2.2 and 2.3 . Black lines in D-F represent the neuropile borders established by anti-ChAT labeling (cf. Fig. 2.2); the antennal lobe is show in light red.
multiple sensilla. Many of the sensilla, e.g. those of the TO, can be distinguished by their cuticular protrusions (Fig. 2.1C). This enabled us to identify in many cases the type of sensillum to which the labelled neurons belonged (Fig. 2.2, insets).

Of the 20 Gr-Gal4 lines tested, five showed expression neither in larvae nor in adults and five additional lines were expressed only in the adult taste system (Table 2.1). However, the ten remaining Gr-Gal4 lines showed expression in both the larval and adult gustatory system (Table 2.1). Three of the five Ppk lines used also were expressed in the larval taste system (Table 2.1), but in contrast to a previous report (Liu et al., 2003a), we did not detect expression of Ppk19 in taste organs. The number of labelled GRNs in the Gal4 lines studied varied from 1 to 18 pairs (Table 2.1). GH86 showed expression in more than 30 GRNs (Table 2.1; Fig. 2.4), in some ORNs and in certain non-neuronal cells, but lacked expression in central neurons (Heimbeck et al., 1999), and MJ94 showed expression in many if not all sensory neurons apparently including the entire set of GRNs but again no expression in central neurons (Gendre et al., 2004).

With the exception of Gr32a-Gal4, the expression patterns of the Gal4 lines were consistent from animal to animal (although the level of expression varied to a certain extent). In addition, different inserts of the same Gr-Gal4 construct revealed similar patterns in the majority of cases. Exceptions were Gr22b-Gal4 and Gr66a-Gal4; in the former, only one of the two strains (B7) labelled VPS neurons (Fig. 2.2G and data not shown). For Gr66a-Gal4, one line (from K. Scott) showed less expression than the other one (from H. Amrein), the latter being similar to the Gr66a-GFP line (compare Fig. 2.2B, C). Analogous pattern differences between these two lines were previously reported in adult flies (Wang et al., 2004). None of the lines studied displayed asymmetrical expression patterns. However, small differences in the level of expression were sometimes noticed between neurons on the left and right side (Fig. 2.3F and data not shown) and for particular cell types. For example, staining was weaker for the TO and VO neurons than for other neurons in the Gr2a-Gal4 line (Fig. 2.1B).

In Gr-Gal4 lines, expression was rare outside the gustatory system and was completely absent from the olfactory organ. Remarkably, Gr21a-Gal4 which labels olfactory neurons in the adult antenna (Suh et al., 2004) showed expression in one TO neuron, but not in olfactory neurons of the DO (Fig. 2.2F). In contrast, Gr68a-Gal4 labelled non-neuronal cells of the TO, in addition to its expression in two VPS neurons (data not shown). Similarly, Gr22e-Gal4 labelled cells belonging to the TO, VPS, DPS and DPO; these cells lacked axons and should thus be non-neuronal (Fig. 2.2J).
### Table 2.1: Number of cells labelled per taste sensilla in the different lines used.

<table>
<thead>
<tr>
<th>Lines</th>
<th>TO</th>
<th>DO</th>
<th>VO</th>
<th>DPS</th>
<th>DPO</th>
<th>PPS</th>
<th>VPS</th>
<th>Additional expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr66a-GFP</td>
<td>4</td>
<td>1</td>
<td></td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Gr66a* (Scott’s line)</td>
<td>2(1)</td>
<td>1(0)</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr28be*</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr22b</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Gr22e</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>+ multidendritic,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ non neuronal TO</td>
</tr>
<tr>
<td>Gr68a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>+ non neuronal TO</td>
</tr>
<tr>
<td>Ppk12</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ppk6**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr21a*</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr2a*</td>
<td>1</td>
<td>2</td>
<td>1(0)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>+ 3 ventral pits</td>
</tr>
<tr>
<td>Gr59f</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ppk11</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr59b</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr32a*</td>
<td>1?</td>
<td>1?</td>
<td>0?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Total labelled** | 7-13 | 3 | 1 | 8 | 2 | 4 | 4 | Total: 32-38
**N° of taste cells** | 23 | 11 | 4 | 18 | 5 | 6 | 16 | Total: 90

*not expressed: Gr8a, Gr10a, Gr22a, Gr22f, Gr63a, Ppk10, Ppk19**

*expressed only in adults: Gr5a, Gr47a, Gr59e, Gr64a, Gr64e*

*Table 2.1: Number of cells labelled per taste sensilla in the different lines used. Total does not enclose GH86 labelled cells and takes coexpression into account. Lines in italics show expression in a subset of cells labelled by Gr66a-GFP (Ppk11, Gr59b and Gr32a coexpression was not assessed). Gr59b show weak expression and Gr32a expression was not consistent from brains to brains. Numbers in brackets represent data reported in prior studies. *: lines described in Scott et al. (2001). **: lines described in Liu et al. (2003a). Total number of taste cells in sensilla were deducted from Python and Stocker (2002) and Gendre et al. (2004). Numbers of cells labelled by GH86 were deduced from Heimbeck et al. (1999).*
Gr22e-Gal4 showed also expression in multidendritic neurons in head, thoracic and abdominal segments (data not shown). As expected from the ionic channel nature of Ppk proteins, and as reported previously (Liu et al., 2003a), Ppk-Gal4 lines showed expression in various body parts apart from the gustatory system. For instance, Ppk11-Gal4, that labelled three TO neurons, showed also strong expression in the tracheal system, obscuring neuronal patterns in the CNS (data not shown).

Using CD2 as a reporter, we also studied the expression of different Gal4 lines in the background of Gr66a-GFP, a line which labels a relatively large proportion of GRNs (18 pairs: one fifth of GRNs; Fig. 2.4). As expected form the situation in the adult (Thorne et al., 2004; Wang et al., 2004), Gr28be-, Gr22b- and Gr22e-Gal4 lines showed expression in a subset of Gr66a-GFP positive GRNs (Table 2.1). More surprising, Gr68a-Gal4 and even Ppk12-Gal4 showed coexpression with Gr66a-GFP (Fig. 2.3K). In contrast, Gr21a-Gal4, Gr2a-Gal4, Gr59f-Gal4 and Ppk6-Gal4 showed no overlap with Gr66a-GFP expression (Table 2.1). For another three Gal4 lines, coexpression with Gr66a-GFP was not checked. Together, these seven lines stained at least 11 and at most 17 GRNs that are not included in the Gr66a-GFP pattern (depending on their relative coexpression in TO neurons, see Table 2.1). About one third of GRNs were thus labelled by the Gr-and Ppk-Gal4 lines studied (29-35, from the total of 90). Interestingly, the enhancer trap line GH86 was expressed in Gr66a-GFP negative cells (Fig. 2.3I-J, periphery not shown), suggesting that the GH86 pattern covers another third of GRNs (Table 2.1, Fig. 2.4).

Central projections of GRNs

First of all, we used the neuropile marker anti-choline-acetyltransferase (ChAT; Fig. 2.2A1) in order to define landmarks for limiting the SOG; this allowed us to accurately map the projections of the different GRNs labelled by different Gal4 lines (Fig. 2.2). In the cases of Gr2a-Gal4 and Gr66a-Gal4 that labelled neurons from multiple organs (Fig. 2.2A-B), we used the Flp-out technique (Fig. 2.3A-D) to associate each terminal projection with its proper sensory organ (Fig. 2.1E-F). Then, studying the projections of different Gal4 lines or of the GH86 line in the background of Gr66a-GFP allowed us also to assess the spatial relations of two sets of projections (Fig. 2.3E-J). Finally, in order to see if the GRNs not expressed in those different Gal4 lines could show other types of projections, we also generated Flp-out clones in MJ94, which is not only expressed in all GRNs but also in sensory neurons of other modalities (Fig. 2.3L-O). Because the afferent
CHAPTER 2. PRIMARY TASTE CENTRE

Figure 2.2:
Fig. 2.2 Expression patterns of Gr-Gal4 and Ppk-Gal4 lines, visualized by UAS-CD8GFP reporter labelling (or direct GFP expression in Gr66a-GFP: C). The panels show the sensory projection patterns in the SOG (stained by anti-GFP; green) in the background of anti-ChAT staining (magenta); insets (except A1/A3) refer to the corresponding GFP expression patterns in the periphery (expression in additional sensory organs is indicated). The SOG is shown horizontally, with anterior on top. The Z stacks of the confocal images (comprising 22-28 µm) include the entire depth of the projections (except in H, see below). Insets in A1/A3 display the 5 ventral most sections of the Z stack (allowing better identification of antennal lobe and antennal nerve), insets in B,D,E and F refer to the TO and its sensilla, and the remaining insets show the entire larval head with the labelled sensory organs (asterisks: mouth hooks). Names of lines shown in italics are coexpressed with Gr66a-GFP. Refer also to text and to Fig. 2.1, especially for description of "regular" projections and areas localisation. Scale bars: 50 µm.

A1,2,3. Anti-ChAT staining, Gr2a pattern and merged image, respectively. (A1) Stronger anti-ChAT staining occurs posterior to MN and LbN entries (see also B,D). (A2) Weakly labelled terminals of MN afferents (TO & VO neurons, arrows) are adjacent to those of LrN afferents (DPS neurons, arrowheads). Small signs in inset refer to dendrites, large ones to cell bodies. (A3) Projections from ventral pits and non-olfactory DO neurons both target area 3 (white arrows). DPS terminals are located at the anterior SOG neuropile border (area 4, white arrowhead; cf. Fig. 2.1E).

B. Gr66a-Gal4 (from K. Scott). "Regular" MN and LrN projections in area 2, note the absence of staining at the midline in area 1 (arrowhead). Additional expression in an "atypical" neuron of a dorsolateral TO sensillum (TODO neuron: magenta arrowhead) shows its projection through the AN and its terminals in area 4 (arrow).

C. Gr66a-GFP (used in coexpression studies) shows more widespread pattern in pharyngeal sensilla than Gr66a-Gal4 (see also Fig. 2.3K). The projection patterns are similar as in B, although fibers deriving from the LrN cross the midline (arrowhead). Projections of VPS neurons were too weakly stained to be detected.

D,E. MN projections from TO neurons labelled by Gr28be (D) and Gr59f (E) are similar, except that the latter extend more laterally (arrowhead).

F. Distinct projection of another TO neuron revealed by Gr21a.

G. Gr22b shows "regular" LbN projections ending close to LrN terminals but only in area 2.

H. Overlapping terminals of "regular" LbN and MN projections in area 2, labelled by Ppk6. The overlap (arrows) is best visible in a single optical section (inset 2). Optical sections comprising the most dorsal part of the LbN nerve projection are missing (arrowheads).

I. Overlapping "regular" LrN and MN projections in area 2, labelled by Ppk12. Widespread expression of Ppk12 may hide expression in other GRNs, which is suggested by the multifiber appearance of terminals in area 1.

J. Gr22e labels many neural and non-neuronal cells. Detailed analysis of the periphery (not shown) suggests expression in the sense organs given in the inset. In addition to projections similar to C, extra projections in the ventral nerve cord (arrow) deriving very likely from multidendritic neurons are stained.
Figure 2.3:
2.4. RESULTS

Figure 2.3. Clonal analysis (A-D) and double labelling studies (E-K) of different Gr-Gal4 and Ppk-Gal4 lines. Flp-out analysis of the MJ94 line (L-O) and dissection of the putative second order taste neuron labelled by GH146 (Q-S). Z confocal stacks (depth 20-28µm; 35µm in Q-R) of sensory projections labelled with anti-GFP (green) and on top of (in magenta): anti-CD2 (A-K,P,R-S) or the neuropile markers anti-ChAT (L-O) or nc82 (Q). Horizontal views, anterior on top. Scale bars: 50µm, if not otherwise indicated.

A-D. Clonal analysis of Gr2a and Gr66a permits to link the different projections with their organ of origin (cf. Figs. 2.1E-F, 2.2A-B). Neurons that underwent Flp-out are labelled by GFP, non-flipped neurons by CD2. A. DO neurons targeting area 3. B. In addition to the DO and ventral pit projections projecting in area 3 (magenta arrowhead), this multiple clone also show that: the terminals of a VO neuron (white arrowhead) are in close proximity to those of a TO neuron (labelled by CD2; magenta arrow) in area 2 (i), and DPS projections in area 4 (green arrows) are ventral to the TO projections endings (green arrow), as shown in selected optical sections of the squared region (insets), although some overlap remains (white arrow). C. The TODO neuron (magenta arrowhead) terminates near the "regular" TO projection in area 4 (white arrowhead). D. The terminals of a single TO neuron (white arrowhead) as well as those of a DPS neuron (arrow) overlap with CD2 labelled pharyngeal projections in area 2.

E-K: Gr66a-GFP expression (green; cf. Fig. 2.2C) related to the expression of other Gal4 lines visualized by CD2 (in magenta; cf. Figs. 2.2A,E-J). E. Terminals of TO and VO neurons labelled by Gr2a are in close proximity with those of a Gr66a-GFP labelled TO neuron in area 2 (white arrowheads); best illustrated by a 150º turn (inset). A Gr2a-labelled DPS neuron (magenta arrow) projects slightly more mediially than the TODO neuron labelled by Gr66a in area 4 (green arrowhead, see Fig. 2.1D). F-G. Projections of a Gr59f-labelled TO neuron (F) and of TO and VPS neurons labelled by Ppk6-Gal4 (G), are in close proximity to the Gr66a-GFP projections in area 2 (arrow). H. In contrast, the TO projection labelled by Gr21a extends more posteriorly than the Gr66a-GFP projections. I-J. The projections labelled by the GH86 enhancer trap line overlap with those of Gr66a-GFP in the lateral part of area 2 (arrows), although being slightly more posterior, especially in area 1. K. Peripheral expression of Gr66a-GFP and Ppk12 (cf. Fig. 2.2B, I). Higher magnification (insets) shows GFP expression in CD2 labelled cells (magenta arrowheads). Because CD2 is membrane-tagged while GFP is cytosolic, few white areas are visible.

L-O. Flp-out clones generated in the MJ94 line. Projections crucial for our interpretation are marked (see text). L. Two afferents of unknown organ origin carried by the LrN project to the SOG (arrow, area 2) and to the ventral nerve cord (arrowhead), respectively. M. The projection of a head multidendritic neuron (arrow) enters via the MN and extends to the ventral nerve cord. N. Two neurons, deriving very likely from the TO, display a projection pattern (arrows) posterior to area 2, similar to a Gr21a-Gal4 labelled neuron (cf. Fig. 2.2F), whereas a DPS neuron forms a terminal arborisation similar to the one labelled by Gr2a in area 4 (arrowhead, cf. Fig. 2.2A). O. An afferent from the LrN, perhaps deriving from the DPS, projects to the area 1 but does not extend further contralaterally (arrow).

P. Flp-out clones of Or30a-Gal4, which shows extra expression in a pair of obviously non-olfactory DPS neurons, which exhibit a bilateral projection (arrow; arrowhead: nerve entry).

Q-S. The GH146 line labels a putative gustatory interneuron that projects to the lateral horn and the mushroom bodies. Arrowheads and arrows indicate dendritic and azonal portions, respectively; asterisks indicate cell bodies. Olfactory projection neurons (green signs) and the putative gustatory second order neurones (white signs) are stained by CD8 (green). Q. Clone of GH146 on top of nc82 labeling. Apart from two olfactory PNs (one in each brain hemisphere), the right putative gustatory interneuron is labelled and shows arborisation in the contralateral SOG (arrowhead) R-S. An olfactory projection neuron (green signs) and a putative gustatory interneuron (white signs) on top of the entire GH146 pattern (in magenta). R. The right brain half is shown (midline stippled). S. Higher magnification of the output region. Both types of neurons project to the mushroom body calyx (stippled contour) and to the lateral horn, terminating close to each other in both areas.
Figure 2.4: Diagram showing proven and suggested patterns of coexpression of the different Gal4 lines used, as well as the proportion of GRNs labelled. Evidence of coexpression was obtained by double labelling and visualisation of the periphery (cf. Fig. 3S). Square size corresponds to the numbers of labelled GRNs (i.e. Gr28be representing one pair of GRNs). From the total of 90 GRNs, one fifth is labelled with Gr66a (red) and one third with GH86 (blue). Coexpression of the 7 GRNs labelled by Ppk11, Gr32a, and Gr59b with other lines was not assessed (blue squares and question mark). Lines labelled in green are not coexpressed with Gr66a, but they may or may not be coexpressed with GH86 (green squares). The TO neuron labelled by Ppk6 might be identical to the one labelled by Gr2a (?), but not those labelled by Gr59f and Gr21a, since the Gr21a neuron had particular brain projection and the dendrite of Gr59f extended to a different TO sensillum. The black part represents the unlabelled GRNs, which were studied in MJ94 clones. For simplicity, coexpression between lines that are coexpressed with Gr66a-GFP is not shown (except for the known coexpression of Gr68a, Gr22b and Gr66a in two VPS neurons), as well as extra expression in non-GRNs.
projection patterns appeared to depend mainly on their peripheral origin, the following description of taste afferents is grouped according to the different peripheral nerves, starting with the most stereotyped ones. We numbered projections areas from medial to lateral (Fig. 2.1D) irrespective of sequence of appearance in this description.

Maxillary nerve (MN)

MN projections derived from GRNs in the TO and VO (Fig. 2.1A) and remained on the ipsilateral side of the SOG throughout. All of the projections analyzed showed similar morphologies (Figs. 2.2A-E,H-J; 2.3B,E-G,I-J), except for one TO neuron labelled by Gr21a-Gal4 (see below). "Regular" projections are best described in Gal4 lines showing expression only in one TO neuron (Fig. 2.2D-E). After entering the SOG quite posteriorly, the MN afferents passed horizontally toward the midline. Upon arriving in the neuropile proper (about 1/3 from lateral to medial), they turned anteriorly and slightly dorsally. In the middle of the SOG neuropile (from posterior to anterior), they bent laterally and ventrally again, establishing terminal extensions, in a target region that we called area 2 (Fig. 2.1D). The exceptional projection of the TO neuron labelled by Gr21a-Gal4 turned sooner anteriorly and terminated more posteriorly than the regular TO neurons (Figs. 2.2F; 2.3H).

Labral nerve (LrN)

LrN projections which entered the SOG on its anterior part, comprised GRNs from the DPS, DPO and PPS (Fig. 2.1A). We found both ipsilateral (Figs. 2.2A-B, 2.3O) and bilateral projections (Fig. 2.3P); the latter seemed to encompass the majority of labelled projections (Fig. 2.2C, G, I-J). Most of the LrN afferents followed those from the MN into area 2 (Fig. 2.2I) and generally continued to the midline region into a terminal area 1 (Fig. 2.1D, but see Fig. 2.2B). In the majority of cases they then seemed to target the contralateral area 2 (Fig. 2.3P, but see Fig. 2.3O). An exception was one DPS fibre labelled by Gr2a-Gal4 (Figs. 2.2A2; 2.3B, see also Fig. 2.3N), which produced an ovoid-shaped cluster of terminals at the neuropile border of the SOG lateral and ventral to area 2 (Fig. 2.3B, insets), inside area 4 (Fig. 2.1D).
Labial nerve (LbN)

LbN projections derive from GRNs in the VPS (Fig. 2.1A); they remain ipsilateral. Fibres enter the brain more posteriorly than those from the MN (Fig. 2.2H) and then travel at the midline, more dorsally than all other projections (Fig. 2.2H). They finally join and get intermingled with pharyngeal (Fig. 2.2G) and TO projections (Fig. 2.2H) in area 2 (Fig. 2.2H, inset).

Antennal (AN) and thoracic nerves

Only few Gal4 lines showed expression in GRNs associated with the AN or thoracic nerves. Hence, whether the observed projection patterns are the common ones for these neurons or rather exceptional cases remains unknown. Specifically, Gr2a-Gal4 labels DO and thoracic neurons (Fig. 2.2A), whereas Gr66a-Gal4 labels one of the three neurons associated with the DO but extending their dendrite in a TO sensillum (referred to as TODO neuron, Fig. 2.2B-C). AN projections comprise both DO and TODO neurons (Fig. 2.1A), which establish distinct but always ipsilateral terminals. Two DO neurons labelled by Gr2a-Gal4 (Figs. 2.2A; 2.3A-B) followed olfactory receptor axons in the AN, but continued in posterior direction instead of passing into the antennal lobe. They ended in a small cluster at the neuropile border in area 3 (Fig. 2.1D, E), posterior to area 2 and area 4. The TODO neuron labelled by Gr66a-Gal4, in contrast to the DO neurons mentioned before, turned medially after its entrance from the antennal nerve and ended in an ovoid target area at the SOG neuropile border (Figs. 2.1D, F, 2.2B), just lateral to the exceptional DPS projection in area 4 (Fig. 2.3C-E). Thoracic projections from ventral pits organs established ipsilateral terminals in the ventral nerve cord and travelled further anteriorly to end adjacent to, but not intermingled with, DO projections (Figs. 2.1E; 2.3A).

Projections from other sensory modalities

Our data provide also some information about afferents mediating other modalities than taste, such as smell, touch, stretch, temperature or humidity (Stocker, 1994; Liu et al., 2003b). For example, olfactory receptor neurons projecting to the larval antennal lobe (Fishilevich et al., 2005; Ramaekers et al., 2005, see Fig. 2.2A, inset) are labelled by both MJ94 and GH86 enhancer trap lines (Fig. 2.3I). Moreover, Gr22e-Gal4 demonstrates that multidendritic neurons - thought to be pain receptors (Tracey et al.,
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Furthermore, as shown in clones of MJ94, which labels sensory neurons of different modalities, some afferents carried by the maxillary and labial nerve have their target areas in the ventral nerve cord (Fig. 2.3E-G). These results suggest that the SOG might be devoted principally to taste processing (see discussion).

Functional testing

The central projections of GH86 and Gr66a-GFP labelled GRNs were essentially similar, adjacent to each other along their path. However, the target area of GH86 appeared to be slightly more posterior, although overlap can be found in the lateral portion of the SOG (Fig. 2.3I-J). To check whether the two sets of GRNs corresponded to functionally different categories, we took advantage of the Gal4/UAS system, in order to drive expression of a modified version of the mammalian capsaicin receptor (Marella et al., 2006). This technique allows one to artificially activate GRNs by capsaicin, which normally does not drive any behavioural response in chemotaxis assays (but see discussion). Thus, testing larval behaviour toward capsaicin permits one to assess the basic function of these cells. Our data show that control larvae (CS, heterozygous GH86, GR66a-Gal4 and UAS-CapsR) were not reacting to capsaicin in our tests (Fig. 2.5). However, the expression of the capsaicin receptor in Gr66a-Gal4 cells induced aversion toward capsaicin, suggesting that these cells normally respond to aversive stimuli. In contrast, the expression of the receptor in GH86-positive cells did not lead to either attraction or repulsion, suggesting that functionally different cells are labelled in this line (Fig. 2.5, see discussion). From this, we conclude that the sets of neurons expressed by Gr66a-Gal4 and GH86, which show distinct but overlapping projections in the SOG, are functionally distinct.

Second order taste neuron

To search for putative second order gustatory neurons, we studied the expression patterns of a number of Gal4 enhancer trap lines that show labelling in the SOG. A candidate gustatory interneuron was identified in the GH146-Gal4 line, which is known for its expression in olfactory projection neurons (Stocker et al., 1997; Heimbeck et al., 2001). This novel neuron showed contralateral projections in the SOG (Fig. 2.3Q). Expressing the presynaptic reporter synaptobrevin-GFP did not label these projections,
CHAPTER 2. PRIMARY TASTE CENTRE

Figure 2.5: Behavioural responses of larvae of different genotypes toward capsaicin. Positive scores indicate attraction, negative ones repulsion. Lines, boxes and whiskers indicate respectively median, quartiles and extreme values, excluding outliers (dots: data out of 1.5 times interquartile range from the quartiles) A. Double heterozygous Gr66a-Gal4/UASCapsR larvae avoided capsaicin ($p<0.01$), whereas controls did not respond at all. B. Double heterozygous GH86/UASCapsR larvae did not respond to capsaicin, similar to control larvae.

suggesting that they might be postsynaptic (data not shown). Another process of this neuron - perhaps representing an output connection - extended via a lateral path to the lateral protocerebrum and further to the mushroom body calyx (Fig. 2.5R-S). In both regions, arborisations were present, partially overlapping with the terminals of olfactory projection neurons. Other good candidates of gustatory interneurons are the hugin expressing neurons (Melcher and Pankratz, 2005); a detailed study of their anatomy is provided in the chapter 3.

2.5 Discussion

By using Gr-Gal4, Ppk-Gal4 and enhancer trap lines, in combination with the Flp-out technique, we extended previous work about the projections of GRNs in the CNS of the *Drosophila* larva (Scott et al., 2001). We described about one third of the estimated 90 larval GRN projections individually, we studied another third more globally using the GH86 line (Fig. 2.4) and we visualized many of the remaining GRN projections with the MJ94-Gal4 line. We thus believe that our interpretations were based on a rather complete and detailed description of the afferent morphologies. We observed that the sites of afferent terminals in the SOG are correlated primarily with their nerve provenance, and therefore with their sensory organ of origin. This allowed us to delimit sub-areas in the SOG, corresponding to the origin of afferents (Fig. 2.1D). However, we also found indications for a subtle functional division of the SOG. Furthermore, studying the patterns of Gal4 lines together with Gr66a-GFP expression provided evidence about
GRs co-expression and possible overlap of the terminals of different GRNs. Finally, observations about putative second order taste neurons enabled us to hypothesise about the principles of gustatory coding in *Drosophila* which may be analogous to the current mammalian models.

**SOG architecture**

By comparing the site of terminal projections in the SOG with their peripheral origin, which we assessed by identifying cell bodies and dendrites, we established that neurons projecting through the same nerve show essentially similar terminal patterns (Fig. 2.1D). In contrast, we were not able to recognize consistent differences in the projections of Gr66a-GFP and those of Gr-Gal4 or Ppk-Gal4 lines if their afferents travelled in the same nerve. An exception is Gr21a-Gal4 (see below). We showed that the majority of these projections are close to each other (Fig. 2.3B,D), this suggests that there is little spatial segregation between projections from neurons expressing different receptors. However, we do not know whether the selection of patterns visualized is biased to some extent by the Gal4 lines available (see below). Yet, the projection patterns relate mainly to the nerve taken by a particular afferent and, accordingly, to the segmental origin of the GRN. This is reminiscent of the gustatory projections in larval *Manduca sexta*, which stay in the neuromeres corresponding to the segmental origin of the organs (Kent and Hildebrand, 1987).

However, three exceptions were found. First, GRN afferents travelling via the AN show at least two different kinds of terminals: While neurons deriving form non-olfactory sensilla of the DO terminate in *area 3*, TODO neurons target *area 4*. Second, a TO neuron labelled by Gr21a-Gal4 projects more posteriorly than the remaining TO neurons. Closer inspection revealed that these terminals remain outside the antennal lobe, in contrast to a previous report (Scott et al., 2001). Since this neuron was shown to respond to CO$_2$ (Faucher et al., 2006), this particular target region may be devoted to the sensation of this chemical. Third, projections from pharyngeal sensilla do not belong to one homogeneous class: we found projections covering only the ipsilateral *area 2*, projections ending in *area 1* and projections showing bilateral terminals. Moreover, certain DPS neurons terminate in *area 4*. The functional implications and the developmental constraints leading to these differences remain to be investigated.

Based on Gr2a-Gal4 projections, Scott and co-workers (Scott et al., 2001) already reported discrete terminal regions for gustatory projections in the larval CNS. Our description allowed us to define four major target
regions in the SOG (Fig. 2.1D). A midline area 1 receives inputs exclusively from pharyngeal organs (DPS, DPO, PPS). A larger, more lateral area 2 is defined by the convergence of inputs from both internal sensilla (including VPS) and external sensilla (TO, VO). A small posterior area 3 appears to be the target of non-olfactory DO neurons and ventral pit neurons. Because all evidence about area 3 projections was collected in Gr2a-Gal4, it remains indeed possible that this area is related to a particular chemical rather than to particular sensilla. An anterior lateral area 4, adjacent to the antennal lobe, accommodates the terminals of one or a few DPS neurons and of a TODO neuron. Finally, the GRN labelled by Gr21a-Gal4 has its own, specific target region posterior to area 2. As in the adult, the SOG can thus be divided in different regions that are targets of different organs. However, unlike adults (Stocker and Schorderet, 1981; Nayak and Singh, 1985; Shanbhag and Singh, 1992; Thorne et al., 2004; Wang et al., 2004), the projections from internal and external organs do not segregate but remain intermingled.

Interestingly, most of the pharyngeal projections are bilateral, similar to the situation in the adult (Stocker and Schorderet, 1981; Rajashekhar and Singh, 1994). Terminals are sometimes restricted to area 1, but often extend to the contralateral area 2. In contrast, GRNs belonging to non-pharyngeal sensilla generally establish exclusive ipsilateral projections. An example are Gr66a-Gal4 labelled TO projections. This is very striking because adult Gr66a-Gal4 projections from the labial palp are clearly bilateral (Thorne et al., 2004). The reason of this disparity is unknown. It might be correlated with different functions of the two sensory organs. For maggots that live in a semi-liquid environment, bitter taste information from the TO may help to navigate up or down a gradient, a behaviour that may use laterality information. In contrast, Gr66a-Gal4 positive labellar GRNs may participate in food rejection, which very likely does not require spatial information. Compatible with such an interpretation, adult Gr66a-positive leg GRN projections remain ipsilateral, suggesting that they might be involved in chemotaxis.

SOG and non-gustatory cues

In the blowfly, mechanosensory neurons were thought to target a specific region of the SOG (Edgecomb and Murdock, 1992). A similar segregation of the targets of mechanosensory neurons and GRNs is known from leg taste sensilla in Drosophila (see for instance Murphey et al., 1989). Our data show that multidendritic neurons that may be involved in pain sen-
2.5. DISCUSSION

...sation (Tracey et al., 2003), project to the ventral nerve cord. Strikingly, we observed that some afferents from the MN or LrN also terminate in the ventral nerve cord. This suggests that certain non-gustatory neurons associated with taste sensilla may have their targets outside the SOG. However, further studies will be required to clarify the sensory modalities involved and to answer whether the SOG receives direct mechanosensory input or not.

Evidence that temperature sensation may be encoded in the SOG is more compelling. TO neurons were shown to respond to heat (Liu et al., 2003a). Moreover, cells labelled by the GH86 line are necessary for thermotaxis (Liu et al., 2003a). It is thus likely that some of the TO neurons labelled by GH86, which we observed to project entirely into the SOG (Fig. 2.3I-J), are thermosensitive. Interestingly, a link between taste and temperature sensitivity was described at the molecular level. For instance, the mammalian capsaicin receptor appears to respond to heat, taste and probably painful stimuli (Caterina et al., 1997). Similarly, in *Drosophila*, the painless gene was shown to be involved in moderate thermal sensation, pain sensation (Tracey et al., 2003) and in the perception of iso thiocyanate, a bitter tasting chemical (Al-Anzi et al., 2006). Further evidence for links between taste, temperature and pain sensation is provided by the expression of painless in the TO (data not shown, Tracey et al., 2003) and by the expected expression of the taste receptor Gr22e in pain sensitive multidendritic neurons, as suggested by the Gr22e-Gal4 pattern.

Functional subdivisions?

Anatomical and functional evidence has shown that adult GRNs project to different target areas in the SOG, according to their receptivity to bitter or sweet compounds (Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006). In these studies, Gal4 lines directed by the promoters of the trehalose receptor Gr5a and of the bitter receptor Gr66a served as marker lines. As already mentioned, we did not find any obvious difference when comparing the projection patterns of different Gr-Gal4 and Ppk-Gal4 lines, with the exception of Gr21a-Gal4. However, since none of the three Gr5a-Gal4 lines that we tested showed any expression in larvae, our failure of detecting two distinct projection patterns might merely indicate that we had selected only bitter- and salt-sensitive GRNs, which together would project in a region different from the sugar-sensitive neurons. Indeed, GRNs labelled by GH86 which were suggested to mediate chemotaxis toward sugars (Heimbeck et al., 1999), appear to project more posteriorly than the majority of
the GRNs labelled by Gr66a-GFP.

In order to correlate these subtle differences in projection with functional responses, we tested the basic sensitivity of the GRNs labelled by Gr66a-Gal4 or by GH86 via the UAS-capsaicin receptor approach (Marella et al., 2006). Thus, we measured chemotaxis toward capsaicin of larvae expressing a modified version of the mammalian capsaicin receptor in these two sets of cells (Fig. 2.5). Wild type and other control larvae were not responding to this chemical in this assay, although there is evidence that adults may sense it (Al-Anzi et al., 2006). Larvae expressing the receptor via the Gr66a-Gal4 line provided by H. Amrein (which shows a similar expression pattern as Gr66a-GFP) were repelled by capsaicin. This suggests that normally these neurons respond to aversive stimuli. In contrast, driving expression of the capsaicin receptor with GH86 did neither elicit aversion nor attraction. Several explanations might account for this result. First, expression could have been too low to trigger responses in the neurons. However, this is not very likely because the levels of GFP expression driven by GH86 and Gr66a-Gal4 appeared similar. Second, natural sensation of capsaicin could prevent larvae from showing attractive behaviour in this test, although it does not drive avoidance. This also seems unlikely since capsaicin does not prevent, but strengthen the attraction of adult flies toward sucrose (Al-Anzi et al., 2006). As a third possibility, we rather believe that expressing the capsaicin receptor in GH86 activates functionally different neurons mediating attractive and aversive stimuli, respectively, which may lead to indifferent behaviour. Taken together with a previous tetanus toxin expression study (Heimbeck et al., 1999), our data suggest that a subset of GH86 labelled neurons may respond to palatable stimuli like sugars.

The primary larval taste centre might thus well be divided into an anterior part which is labelled by Gr66a-Gal4 and responds to aversive stimuli, and a posterior part which is represented by subpopulation of GH86 neurons and responds to attractive cues. This difference, if it in fact exists, remains much more subtle than in the adult system where sweet and bitter responsive neurons have clearly segregated central projections (Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006).

Hints about GRs expression

In the transgenic lines used, the expression of Gal4 is controlled by the putative promoter region of GR or Ppk genes (about 1kb in length). Conclusions from the reporter patterns about native gene expression must thus be
taken with caution. Indeed, a number of observations, such as expression in non-neuronal cells in certain lines, pattern differences between different insertion lines (e.g. in Gr22b or Gr66a), the unexpected expression of Gr68a-Gal4 (a receptor proposed to mediate pheromone perception (Bray and Amrein, 2003)) in VPS neurons, or differences with prior reports (Table 2.1), doubt whether these lines accurately reflect gene expression. Nevertheless, our data provide good evidence for co-expression of multiple taste receptors per cell (Fig. 2.4), in contrast to a prior report based on fewer lines (Scott et al., 2001). Interestingly, our results suggest co-expression of Gr66a and Ppk 12 (Fig. 2.3K), which would fit with observations that bitter responsive cells in the adult also respond to salt at high concentration (Meunier et al., 2003).

The four lines Gr2a-Gal4, Gr59f-Gal4, Gr21a-Gal4 and Ppk6-Gal4, which were not co-expressed with Gr66a-GFP, might thus be expressed in neurons responding to different, but perhaps also repulsive, tastants. In contrast to studies in the adult suggesting that the majority of GRs are co-expressed with Gr66a (Thorne et al., 2004; Wang et al., 2004), we found an overlap of only 60%. This discrepancy may be due to a difference between adult and larval systems; alternatively, the data in the adult were incomplete. Indeed, Gr21a was not taken into account in adult studies because it showed expression in olfactory neurons. Also, possible co-expression of Gr2a-Gal4, Gr59f-Gal4 and Ppk-Gal4 with Gr66a-GFP was not checked.

Second order neurons

An interesting candidate taste interneuron revealed by the GH146 enhancer trap line arborizes in the SOG and extends a process via the lateral horn in the mushroom body calyx, a brain region involved in larval olfactory learning (Honjo and Furukubo-Tokunaga, 2005). The putative dendritic arborisation in the SOG extends bilaterally and is relatively large (3-7 µm). Hence it may receive inputs from different GRNs, suggesting that it is broadly tuned. Interneurons that possibly link taste inputs with the mushroom bodies were also described in the honeybee (Schröter and Menzel, 2003). From their morphology and lack of biogenic amine expression, these neurons were thought to send taste information straight to the mushroom body, rather than having a modulatory function. Modulatory information from taste inputs is thought to be mediated by unpaired median cells both in bees (Hammer and Menzel, 1995) and in Drosophila (Sinakevitch and Strausfeld, 2006). Taste information was shown to be used as a reward or punishment by larvae (Scherer et al., 2003; Gerber and Hendel, 2006). In
this context it would be interesting to know whether larvae can use taste information also as a conditioned stimulus, i.e., if their response toward taste stimuli may be modulated by experience.

A cluster of 20 putative second order taste neurons, the *hugin* neurons, which express the *hugin* neuropeptide, is presented in the chapter 3. Interestingly, arborizations of these neurons in the SOG are ipsilateral in *area 2* and bilateral in the *area 1*, covering the entire areas. This is consistent with afferent projections, supporting direct connectivity between GRNs and the *hugin* cells. Moreover, it suggests that laterality information encoded by the afferents may be sent to the second order taste neurons and thus to higher brain centres.

**Concluding remarks**

Previous studies on insect taste centres focused on adults of the flies *Drosophila* (Stocker, 1994; Thorne et al., 2004; Wang et al., 2004), *Phormia regina* (Yetman and Pollack, 1986; Edgecomb and Murdock, 1992) and *Neobellieria bullata* (Mitchell and Itagaki, 1992) and of mosquitoes (Ignell and Hansson, 2005). In all of these species, central taste projections were shown to be governed primarily by their organ of origin. However, when tested, sensory receptivity also proved to be crucial: attractive and aversive information being segregated. Similar, albeit limited, information was collected from larvae of *Manduca sexta* (Kent and Hildebrand, 1987) and *Drosophila* (Scott et al., 2001). Using the genetic tools available in *Drosophila*, we studied the larval gustatory afferents and provide a detailed description of the primary taste centre of larvae. We show that sub-areas of the SOG are associated with different organs, similar to the situation in the adult. In contrast, functional divisions of this neuropile were more difficult to assess. The putative second order taste neurons described so far, all exhibit extensive projections in the SOG and may therefore receive inputs from different GRNs. We thus postulate that secondary gustatory neurons may be more broadly tuned than primary ones, similar to the mammalian system (Smith and St John, 1999; Stapleton et al., 2006). This apparent conservation in taste processing is reminiscent of the similarities between the mammalian and insect olfactory systems. If functional studies confirm our anatomical data, *Drosophila* larvae may become an interesting and powerful model for studying how animals decode gustatory information.
Chapter 3

Genetic dissection of a neural circuit underlying feeding behaviour in *Drosophila*: distinct classes of *hugin* expressing neurones

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3.1 Abstract

The *hugin* gene of *Drosophila* encodes a neuropeptide with homology to mammalian neuromedin U. The *hugin* expressing neurons are exclusively localized to the suboesophageal ganglion of the central nervous system and modulate feeding behaviour in response to nutrient signals. These neurons send projections to the protocerebrum, the ventral nerve cord, the ring gland and the pharynx, and may receive synaptic input from gustatory sense organs. In this study, we have investigated the morphology of the *hugin* neurons at a single cell level using clonal analysis. We show that single cells project to only one of the four major targets. In addition, different *hugin* cells overlap in their projection to a specific brain region lateral to the foramen of the oesophagus, which could be a novel site of neuropeptide release for feeding regulation. Our study reveals unexpected complexity in the morphology of individual *hugin* neurons, which has functional implication for how they coordinate feeding behaviour and growth.
CHAPTER 3. DISTINCT CLASSES OF HUGIN NEURONES

3.2 Introduction

Feeding is one of the most conserved activities of animals. Although animals have evolved a wide spectrum of feeding behaviours in terms of food preferences and foraging strategies, there is a fundamental need to regulate food intake relative to growth, reproductive and metabolic needs. Since the groundbreaking work on the cloning of the gene encoding the hormone leptin, much progress has been made in mammals in analysing the role of various neuropeptides in food intake and energy balance regulation. These have come mostly from knock-out experiments and subsequent physiological analysis of feeding behaviour and metabolic consequences. However, the neural circuits in the brain that mediate the activities of these genes, and how these circuits function under various nutrient conditions and experiences, remain largely unknown.

*Drosophila* provides a genetically accessible system for studying the neural circuits that control innate behaviour such as feeding and mating. We recently identified a group of neurons in the *Drosophila* central nervous system (CNS), named *hugin* neurons, that modulates feeding behaviour in response to nutrient signals. We also provided evidence that *hugin* is a *Drosophila* homolog of the mammalian gene encoding the neuropeptide neuromedin U, which has been shown to regulate food intake and body weight regulation in rodents. These observations suggested that *hugin* and neuromedin U may be part of a conserved neural pathway for regulating feeding behaviour and metabolism.

In the *Drosophila* larva, *hugin* is expressed in 20 cells of the suboesophageal ganglion (SOG). The *hugin* neurons send projections to the protocerebrum, the ventral nerve cord, the central neuroendocrine organ (known as the ring gland) and the pharynx. Furthermore, arborizations of the *hugin* neurons in the SOG lie in close proximity with axon terminals of specific gustatory sensory neurons, leading to the proposal that *hugin* neurons may represent second order interneurons that mediate taste information. The projection pattern of the *hugin* neurons also raised the issue of the target specificity of individual *hugin* neurons. In this study, we use genetically produced clones to analyse, at the single cell level, the morphology of individual neurons of the *hugin* neural cluster in the *Drosophila* larva. Our results indicate that single neurons project to single targets. Furthermore, the single cell data revealed complexities in the morphology of individual *hugin* neurons which were not apparent from studying the entire neuronal cluster. This has led to the identification of a novel region bordering the oesophageal foramen and the SOG that could be involved in
feeding regulation.

3.3 Material and methods

Clonal Analysis

Flies harboring hugS3-Gal4 was crossed with those carrying the Flp out construct [yw hsFLP; Sp/ CyO;UAS>CD2y+>CD8-GFP], a gift of Barry Dickson (IMP, Vienna). A 24 hour egg collection was heat shocked for 2 hours at 37°C. The larval brains were prepared at late third instar.

Histochemistry and Fluorescence Microscopy

Immunofluorescent stainings were done essentially as described in (Melcher and Pankratz, 2005). Images were taken using a Zeiss (Oberkochen, Germany) LSM 510 META in transmission mode or a LEICA TCS SP2 (Wetzlar, Germany). Primary antibodies (α-βGal [Cappel], or α-GFP [Abcam, Cambridge, United Kingdom], used at 1 : 1000) were applied over night at 4°C and secondary fluorescent antibodies (Cy2-coupled α-rabbit and Cy3-coupled α-mouse diluted at 1 : 200 [Jackson Immunoresearch, West Grove, Pennsylvania, United States]) were applied for 1 hour at room temperature. Samples were mounted in Mowiol and analysed using a Zeiss LSM 510 META in confocal multitracking mode, generating optical 1- to 1.5-µm sections (using a Zeiss 40x/1.2W C-Apochromat lens) or 2.5-µm sections (using a Zeiss 25x/0.8Imm Plan-Neofluar lens). For direct detection of YFP fluorescence, larval brains of appropriate genotype were dissected in chilled Drosophila Ringer’s solution on ice, and mounted without fixation in PBS. Other antibodies used for immunofluorescence were 22C10 diluted 1 : 100 (Developmental Studies Hybridoma Bank, Iowa City, Iowa, United States), α-elav diluted 1 : 300 (Developmental Studies Hybridoma Bank), and nc82 (gift of Erich Buchner, Wuerzburg) diluted 1 : 50 as well as Alexa488-coupled α-mouse antibodies diluted 1 : 200 (Molecular Probes, Eugene, Oregon, United States) and Cy3-coupled α-rabbit antibodies diluted 1 : 200 (Jackson Immunoresearch, West Grove, Pennsylvania, United States). Nuclear counterstaining was performed using Draq5 (Biostatus Ltd., Leicestershire, United Kingdom), diluted 1 : 1000 together with secondary antibodies.

For stainings with a CD2 background, the procedure was adapted from the protocol of Heimbeck et al. (1999). Briefly, one hour heat shocked (in a water bath) young third instar larvae (72-96h AEL) were predissected
in phosphate buffer (PB; 0.1M, \(pH = 7.2\)). The brains attached to the body wall were fixed for 20 min in PB containing 3.7% formaldehyde and subsequently rinsed in PBT (0.3% Triton X-100 in PB). They were further dissected and placed for 2 hrs in PBT in 5% goat serum (NGS) at room temperature for blocking. Subsequently, they were incubated with a cocktail of primary antibodies overnight at 4°C. Primary antibodies included \(\alpha\)-ChAT (dilution 1 : 500) from P. Salvaterra (Beckman Research Institute, City of Hope, Duarte, CA), \(\alpha\)-GFP (Molecular Probes), \(\alpha\)-CD2 (1 : 100; Serotec GmbH, Düsseldorf, Germany) and nc82 (1 : 20) from A. Hofbauer (Universiy of Regensburg, Germany). After several rinses in PBT, samples were incubated overnight in PBT-NGS with the secondary antibodies (\(\alpha\)-rabbit Alexa 488-conjugated and \(\alpha\)-mouse Cy3-conjugated, diluted 1 : 200; Molecular Probes). After several rinses, brains were mounted in Vectashield (Vector Labs), with nail polish used as spacer. The CNS was mounted with the ventral nerve cord on top. Images of the periphery were taken by using a fluorescence microscope (Leica DM R) equipped with a CCD camera. Stacks of confocal images at 0.93\(\mu\)m focal plane spacing were collected with a Biorad MRC 1024 confocal microscope and Laser Sharp image-collection software. Images were then processed with Image J freeware (http://rsb.info.nih.gov/ij/index.html), curves (input to output options) were readjusted for each color independently but always on the whole picture. The intensity of unspecific background staining was lowered using the "dust and scratches" filter in Adobe Photoshop for Macintosh 7.0 software.

### 3.4 Results

The hugin gene is expressed exclusively in 20 neurons in the SOG of the Drosophila larva, where they project to four distinct targets: the protocerebrum, the ventral nerve cord, the ring gland, and the pharynx (Figure 3.1A-D; Melcher and Pankratz, 2005). hugin encodes a prepropeptide that can give rise to several potential neuropeptides (Meng et al., 2002). The cleavage pattern is conserved in insects and mammals (Figure 3.1E), and one of the peptides, PK2, is homologous to the mammalian NmU8 (Melcher et al., 2006). In order to determine the morphology of individual hugin neurons, we used the Flp out technique (Wong et al., 2002) to generate single cells marked with a fluorescent genetic marker. We describe below the details of each target.
Figure 3.1: hugin neural cluster and gene structure. Size bars represent 50µm. A. Schematic drawing of the CNS of a 3rd instar Drosophila larva. Antennal lobe (AL), foramen of the oesophagus (F), mushroom body (MB), ring gland (RG), suboesophageal ganglion (SOG), ventral nerve cord (VNC). B. In situ hybridisation showing hugin gene expression. The hugin positive cells are located in the SOG (arrow). C. Immunohistochemical staining against GFP expressed under the control of a hugin promoter. The four major targets are shown: protocerebrum (PC), VNC, RG and pharynx (PH). D. Lateral view of the CNS (marked by broken lines) of a living larva expressing YFP under the control of a hugin promoter. Projection leaving the CNS towards the PH is marked by a star. E. Homology of the hugin prepropeptide to Anopheles hugin homolog and human NmU prepropeptide based on the cleavage pattern.
CHAPTER 3. DISTINCT CLASSES OF HUGIN NEURONES

Projections to the protocerebrum

Single clones of hugin cells were obtained that show projections to the ipsilateral protocerebrum (Figure 3.2A-I). Different representative clones are shown to illustrate the variations in the morphology of single cells. These cells also show arborization ventro-lateral to the foramen of the oesophagus in a region that is innervated by gustatory receptor neurons expressing Gr66a (Figure 3.2K,L). We have previously observed connections between the left and right protocerebrum (Melcher and Pankratz (2005); Fig 3.2H), and interestingly, we have obtained clones where projections branch onto both hemispheres (Fig 3.2E). Although we have not observed any cells that only go contralaterally, this is not proof against the existence of such cells. We also obtained clones in which four cells on one side of the CNS all project to the ipsilateral protocerebrum (Figure 3.2I). This implies that the protocerebrum is innervated by at least eight cells.

Projections to the ventral nerve cord

Single hugin cells also project down the ventral nerve cord (Figure 3.3A-D). The morphology of these neurons is striking. In addition to a long process travelling down contralaterally along the lateral neuropil border of the ventral cord, there are four shorter fibers projecting up and down just left and right of the midline (Figure 3.3A-C). The long projections that extend down the lateral side of the ventral nerve cord branch out at the tip (Figure 3.3D); the precise targets are not known. The two fibers that project in anterior direction pass along each side of the foramen and end at the medial part of the protocerebrum. We have previously shown that there are 20 hugin neurons, of which there are four pharyngeal and four ring gland neurons (Melcher and Pankratz, 2005). In view of the above data suggesting the existence of eight protocerebral neurons, it is likely that the number of ventral cord neurons is four. Accordingly, CD2 staining was detected in the lateral projection of the ventral cord (Fig. 3.3C), which is unilateral: Two neurons per brain half thus appear to project in the ventral cord.

Projections to the ring gland

Single hugin cells projecting to the contralateral side of the ring gland were also observed (Figure 3.3E-I). In addition, these cells are characterized by an ipsilateral process which stops lateral to the oesophageal foramen. The projection length observed can vary (e.g. compare Figure 3.3F with 3.3G).
3.4. RESULTS

Figure 3.2: Projections to the protocerebrum. Flip out clones of hugin cells stained against GFP (green), either without additional staining (A,B), with α-elav background staining (C-E), or on top of non flipped out hugin cells stained against CD2 (magenta) (F-I). Overlap of projections from Gr66a expressing gustatory receptor neurons and hugin neurons (J-L). Size bars represent 20µm (C,D,F-L) or 50µm (A,B,E). A-D. Single cells projecting to the ipsilateral side of the protocerebrum (arrow: cell bodies). E. Single cell projecting to both sides of the protocerebrum. F-G. Single cells projecting to the ipsilateral hemisphere. H. Two cells projecting to the right hemisphere; note the thin connection between the hemispheres (star). I. Four cells projecting to the left side of the protocerebrum, one cell projecting to the right side, one cell projecting to the pharynx. J-K. GR66a neuron projections (green) overlap the arborizations of hugin cells (magenta) lateral to the oesophageal foramen. L. Single confocal stacks (four representative numbered stacks) from Z-projections in K.
Figure 3.3: Projections to the ventral nerve cord and to the ring gland. Size bars represent 20µm (C,D, F-H) or 50µm (A,B,E). A-B. Single hugin cells (green) projecting to the ventral nerve cord (VNC) (arrows: cell bodies). C. Single cell projecting to the VNC (green); non flipped out hugin cells are shown in magenta. D. Magnification of terminal projections of hugin cells at the the posterior end of the VNC. E-G. Single cells projecting to the ring gland (RG) (arrows: cell bodies). The star in F marks the point of entry into the RG. H. Single cell projecting to the RG (green) in the context of the other hugin cells (magenta). The position of the antennal lobes (AL) is outlined for orientation.
3.4. RESULTS

We consistently observe fibers projecting to the border of the antennal lobe and the SOG (Figure 3.3H); however, the processes sometimes extend further dorsally (Figure 3.3G). This may be due to thinning of fibers in the more dorsal regions. In the ring gland, the **hugin** cells establish dense arborizations on the side ipsilateral to the entering fiber, and weaker arborizations after crossing to the other side (Figure 3.3F). Possible target cells in the ring gland could be the corpora cardiaca (Siegmund and Korge, 2001).

**Projections to the pharynx**

The fourth class of **hugin** neurons project to the anterior pharynx, close to the cephalopharyngeal skeleton (Figure 3.4A-C). The projections leave the SOG, make a U-turn, and end at the anterior part of the dorsal pharyngeal muscles. Whether the pharyngeal neurons in fact innervate the muscles is not known. Projections can be seen that cross the midline and those that do not, but since the pharyngeal neurons are located close to the midline, this distinction is sometimes difficult (Figure 3.4D,E). In addition, these neurons have short projections along each side of the foramen (Figure 3.4E-G).

**The **hugin** gustatory circuit and the olfactory system**

The close intermingling of axon endings of gustatory receptor neurons and arborizations of **hugin** neurons in the SOG suggested that **hugin** neurons could act as gustatory interneurons (Melcher and Pankratz, 2005). One of the questions raised from the earlier study was to which classes of **hugin** neurons these arborizations belonged. Current analysis demonstrates that these arborizations derive from neurons that project to the protocerebrum (see Figure 3.2J-L). As gustatory responses are often functionally interconnected with olfactory responses, we investigated the morphological relationship between the protocerebral **hugin** neurons and the antennal lobe, the first relay centre for olfactory signaling. First, we asked whether the arborizations of the protocerebral neurons overlapped with the antennal lobe. Protocerebrum-specific **hugin** clones in nc82 neuropil background staining indicated that the arborizations lie just at the border of the antennal lobe but do not intermingle with it (Figure 3.5A). This is supported by clones in the adult, where the antennal lobes are significantly larger relative to the SOG (Figure 3.5B). We next analysed the projections to the larval protocerebrum with respect to the mushroom body calyx, a secondary olfactory
CHAPTER 3. DISTINCT CLASSES OF HUGIN NEURONES

Figure 3.4: Projection to the pharynx. Size bars represent 20\(\mu\)m (D,E,F) or 50\(\mu\)m (C,G). A. 2-photon-microscope image of the head region of a living 3rd instar larva expressing YFP under the control of a hugin promoter. The head is orientated to the left. The fiber extending to the PH is marked by an arrow. Star marks the ending of the projection. The strong autofluorescence below the star is from the cephalopharyngeal skeleton. The boxed area is magnified in B. B. Pharynx region of living larva; arrows point to the projection terminals. C. 2-photon-microscope image of a living larva. The picture is taken from a ventral view. The CNS is marked by white line; projections to the pharynx (PH) are marked by arrows. The U-turn made by the projection is marked by a star. Projections to the protocerebrum (PC) and the ventral nerve cord (VNC) are also visible. D-E. Single hugin cells (arrows) with a process (star) leaving the CNS towards the PH. F. Cells projecting to the PH (star) and VNC, both shown in green, on top of non-flipped out hugin cells (magenta). G. Two cells (arrows) with projections leaving the CNS.
relay centre. Consistent with our earlier results, the *hugin* projections lie dorsal to the mushroom body calyx (Fig 3.5C-F). Thus, at the morphological level, we do not see an overlap of *hugin* neurons with the central olfactory pathway. However, since *hugin* encodes a secreted peptide, an influence on the olfactory system cannot be excluded.

**hugin** neuronal architecture at the foramen-SOG boundary

The spherical arborization of the protocerebral *hugin* neurons lie in a region lateral to the foramen that border the SOG. Other classes of *hugin* neurons do not show such spherical arborization, but they all show projections into a region directly juxtaposed to the foramen and the SOG. The architecture of the *hugin* neurons within this region indicates that the projections lie in close proximity to each other (Figure 3.5G-J). The ventral nerve cord neurons (Figure 3.5K-M), the ring gland neurons (Figure 3.5N,O) and the pharyngeal neurons (Figure 3.5P-R) all have processes that extend just lateral to the foramen. For the ventral cord neuron, there is also a small arborization at the bottom end of the foramen, i.e. at the border to the SOG (Figure 3.5L-M). For the pharyngeal neuron, two additional spiked projections can be seen extending dorsally and ventrally in a similar region (Figure 3.5Q-R). Thus, in addition to having specific targets outside the SOG, the different *hugin* neurons have distinct but overlapping projections within or near the SOG. These observations suggest that the region bordering the lateral foramen and the SOG may have a special role in mediating *hugin* neuronal function.

3.5 Discussion

Single *hugin* neurons project to only one of the four major targets

Understanding how the brain controls behaviour requires a thorough knowledge of the underlying neural circuitry. Although many behaviour have been studied and numerous genes required for specific behaviour have been identified, the details of the underlying neural circuits are far less understood. The *hugin* neuronal circuit provides an opportunity to dissect, at a single cell resolution, the connectivity patterns of a cluster of neurons that modulate feeding behaviour.
Figure 3.5: Position of hugin projections relative to the olfactory system and to the foramen. Size bars represent 20µm (C) or 50µm (A,D,G,K,P). A. Single hugin cell (green) in the larval CNS projecting to the protocerebrum. The arrow marks the larval antennal lobe (AL), which is stained by the synaptic marker nc82 (red). B. Adult brain with three hugin cells projecting to the protocerebrum; antennal lobes (AL) are marked by nc82 (magenta). C-D. Adjacent stacks of left hemisphere of a larval brain; hugin cells are stained in green. The neuropil including the mushroom body calyx (CX) is marked by ChAT (magenta). E-F. The region in D marked by white rectangle, showing the terminals of the protocerebral hugin (green) relative to the calyx (magenta). The panel F is rotated by 100°. G-J. 3-D reconstruction of 14 hugin cells, rotated at different angles. The cell bodies are located on the ventral side of the SOG. All projections initially proceed dorsally towards the foramen before extending to their final targets. The star in H denotes the arborization located lateral to the foramen. Directional arrows are included in figures showing a lateral view of the hugin neurons: anterior (A), posterior (P), dorsal (D), ventral (V). K-M. 3-D reconstruction of a single cell projecting to the ventral nerve cord. The numbers are for orientation during image turning. The small arborization at the bottom end of the foramen is marked by a star. Arrows mark the cell bodies. N-O. 3-D reconstruction of a single cell projecting to the ring gland (see Figure 3.3F). Stars mark the short processes that project laterally to the foramen. P-R. 3-D reconstruction of a single cell projecting to the pharynx. The numbers are for orientation during image turning. In addition to the two short projections running on each side of the foramen (numbers 1 and 2), there are processes (star) extending dorsally and ventrally. Q and R are magnifications of P.
3.5. DISCUSSION

Figure 3.6: Summary of different hugin neural projection classes. The grey shaded area represents the SOG. A. Schematic drawing of the locations of the hugin cell bodies; only those in the left hemisphere are shown. B. Summary of the four hugin cell projection targets. C. Schematic drawing of a single cell projecting to the PC. D. Schematic drawing of a single cell projecting to the VNC. E. Schematic drawing of a single cell projecting to the RG. F. Schematic drawing of a single cell projecting to the PH. Note for all classes the additional projections to a region ventro-lateral to the foramen, near the SOG.
Our analysis revealed that the 20 cells of the *hugin* cluster consists of four classes whose cell bodies are arranged fairly symmetrically straddling the midline: eight projecting to the protocerebrum, and four each to the ventral nerve cord, to the ring gland and to the anterior pharynx (Figure 3.6). Within a given neuronal class, it is further possible that individual neurons have distinct ipsi- or contra-lateral projections.

**Different *hugin* neurons share overlapping projections at the foramen-SOG border**

In addition to having unique projection targets, the *hugin* neurons also possess additional, partially overlapping patterns of projections in a region ventro-lateral to the foramen. These findings further demonstrate the necessity for a single cell analysis, since it has revealed insights in the pattern which would have gone undetected due to overlapping patterns from other neurons. The class of *hugin* neurons projecting to the protocerebrum have wide arborizations at the doral border of the SOG. These arborizations intermingle with the terminals of the gustatory receptor neurons labeled by the Gr66a-GFP line. The fact that these arborizations belong to the *hugin* neurons that project to the protocerebrum is consistent with the view that *hugin* may act to relay gustatory information to higher brain centres (Melcher and Pankratz, 2005). The protocerebral neurons also seem to project both ipsilaterally and contralaterally. This is interesting in view of the directionality of the incoming projections of the GR expressing neurons, i.e. whether gustatory sensory signals become relayed ipsi- or contra-laterally to their initial reception side. Previous studies in the adult (Wang et al., 2004; Thorne et al., 2004) have shown, for example, that Gr66a neurons project both ipsi- and contralaterally from the labellum to the SOG, whereas Gr5a neurons project only ipsilaterally (Thorne et al., 2004). One possibility is that ipsilateral and contralateral Gr66a projections become relayed by differently projecting protocerebral *hugin* neurons (For additional discussion, see Chapter 2).

The classes of *hugin* neurons that project to the ventral nerve cord, ring gland, and pharyngeal muscles also have additional projections that run lateral to the foramen. The ventral nerve cord neurons project along both sides of the foramen, ending near the top of the protocerebrum. The ring gland neurons have short projections on the ipsilateral side (i.e., opposite from the projection that goes to the ring gland), while the pharyngeal neurons send out short projections on both sides of the foramen. The ventral nerve cord and the pharyngeal neurons also have short processes that
project dorsally and ventrally near the foramen. Thus, all four cell types, in addition to their projections outside the SOG, have projections that terminate at or near the border between the foramen and SOG. This region may thus have a special function in integrating the activities of the different *hugin* neurons. Since the region is very close to the foramen, i.e., the canal in the CNS through which the oesophagus passes, it is possible that *hugin* neuropeptides are secreted directly onto the oesophagus at this site. In this regard, a specific set of *hugin* neurons innervate the ring gland, the major neuroendocrine organ of *Drosophila* larva which is also innervated by the insulin-producing cells (Brogiolo et al., 2001; Rulifson et al., 2002). It is thus possible that the ring gland is used for global control of growth and metabolism, whereas the newly defined region targeted by *hugin* neurons is utilized for local control of feeding. Alternatively, this region could represent the tritocerebrum. This brain structure has not been precisely defined anatomically in the *Drosophila* larva, but based on embryonic studies (Hirth et al., 2001) the larval protocerebrum could be located in a region ventro-lateral to the foramen. In either case, it would be interesting to see if other neuropeptide producing neurons in the SOG also project to this region.

**Neural circuit for integration of gustatory and metabolic signals**

We have analysed a neuronal cluster whose members all express a common gene, *hugin*, but which can be divided into four different classes, each having a specific morphology and projection pattern. Two of these classes are confined to the CNS whereas the other two project to peripheral targets as well. The protocerebral neuron may mediate gustatory information, while the targets of ring gland and pharyngeal neurons are clearly relevant for feeding behaviour, growth and metabolism. These structural considerations suggest that the *hugin* neural circuit might function in integrating external sensory and internal metabolic information to regulate feeding and growth.
Chapter 4

Complex behaviour change after odour pre-exposure in *Drosophila* larva

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4.1 Abstract

A variety of odorants attract *Drosophila* larvae, although this behaviour can be modulated by experience. For instance, larvae pre-exposed to an attractive odorant may subsequently display less attraction toward the same compound. In previous reports, this phenomenon was interpreted as a drop in olfactory sensitivity, due to sensory adaptation. However, we present here data that do not agree with such an explanation. Our results rather suggest that olfactory pre-exposure induces a change in the hedonic value of the odour. Although we did not succeed in elucidating the exact nature of the underlying mechanism, we can reject a decrease or increase in sensitivity or an association of the odour with the absence of food as interpretations of the observed changes in behaviour. In addition to question previous interpretations of odour pre-exposure effects, this study stresses the complexity of *Drosophila* larval behaviour.
CHAPTER 4. ODOR PRE-EXPOSURE EFFECTS

4.2 Introduction

Both vertebrates and invertebrates exhibit surprisingly efficient behaviour in response to biologically relevant olfactory signals. They are able to extract odours related to food, dangerous conditions or mates from a highly complex chemical environment. Accordingly, the sensitivity to background odours is subject to modification, acting mainly through the mechanism of olfactory adaptation. Also, the actual meaning of an odour is not rigidly programmed, but depends on its context and may change over time. Therefore, animals are susceptible to adapt their olfactory-driven behaviours notably by olfactory learning. These behavioural modifications, albeit well defined in human psychophysiological assays, are difficult to identify in experiments involving animal models.

Olfactory adaptation is defined by psychophysiologists as a reduction of sensitivity to an odour after repeated or prolonged exposure to that same odour (Dalton, 2000). This definition comprises both olfactory adaptation and habituation as defined by Bernhard and van der Kooy (2000), and gives no indication about its cellular basis (sensory adaptation (Zufall and Leinders-Zufall, 2000) or central habituation (Wilson, 2000)). Different properties of olfactory adaptation behaviour were highlighted. For instance, the degree of adaptation was shown (i) to depend on the intensity of the odorant during pre-exposure, and (ii) to be odorant-specific. Indeed, odorant specificity was used to test discriminative ability in *Drosophila*: a decrease in the response to an odour B after pre-exposure to an odour A was interpreted as an incomplete discrimination of the two odours (Cobb and Domain, 2000; Boyle and Cobb, 2005).

Olfactory learning has been studied intensively, in particular in the context of classical conditioning in both vertebrates and invertebrates, using many different approaches (reviewed in Milner et al., 1998; Davis, 2005)). For instance, in *Drosophila* larvae, olfactory or visual cues (CS; conditioned stimulus) become more attractive after association with a pleasant gustatory stimulus (US; unconditioned stimulus) (Scherer et al., 2003; Gerber et al., 2004; Honjo and Furukubo-Tokunaga, 2005). In some cases, the new behaviour resulting from the association between a CS and a US can be elicited by another stimulus, CS’, similar to the CS. This phenomenon, called generalisation, was used to measure similarity between different and often discriminated stimuli (Ghirlanda and Enquist, 2003; Wright and Smith, 2004; Guerrieri et al., 2005).

The processes of adaptation and learning (i.e. the loss of sensitivity and a change in hedonic value, respectively) are theoretically well distinct, but
are empirically difficult to separate. For instance, olfactory adaptation is commonly tested by comparing olfactory responses of animals pre-exposed to the odorant with control animals. A lower response is interpreted as a reduced sensitivity to the odour, reflecting olfactory adaptation. However, this lower response could also indicate that the animal values the odour as less positive. Such an effect was demonstrated in a *C. elegans* study where pre-exposure to an odorant in the absence of food, a protocol previously thought to lead to olfactory adaptation (Colbert and Bargmann, 1995), was shown to lead to olfactory associative learning (Nuttley et al., 2002). In this situation, the absence of food acts as a negative US associated with the odorant, leading to a decrease of the chemotactic response towards that odorant.

*Drosophila* has been used for decades to decode the neural and genetic basis of behaviour. Since the olfactory system of larvae is organized similarly to the adult one despite its limited number of odorant receptor neurons (Kreher et al., 2005; Ramaekers et al., 2005; Fishilevich et al., 2005), the fruitfly maggot appears to be a promising model system to study olfactory processing. Evidence for the presence of olfactory associative learning in *Drosophila* larvae were reported (Scherer et al., 2003; Hendel et al., 2005; Honjo and Furukubo-Tokunaga, 2005). On the other hand, Cobb and co-workers (Cobb and Domain, 2000; Boyle and Cobb, 2005) used olfactory adaptation of larvae in order to test olfactory discrimination and, accordingly, proposed models of peripheral olfactory coding. Wuttke and Tompkins (2000) tested larvae mutant for *trp*, a gene encoding a calcium channel whose expression is required during development for olfactory adaptation in the adult (Störtkuhl et al., 1999). They observed no effect of *trp* loss-of-function in their experimental set-up. However, the authors supposed that only olfactory adaptation was modifying larval behaviour, and did not test for the presence of different forms of learning. Using a protocol modified from Cobb and Domain (2000), we provide here evidence that in some cases, larvae pre-exposed to an odorant can still sense it, although they are no longer attracted. In contrast to what was demonstrated in *C. elegans*, we show that the changes in behaviour do not rely on the presence or absence of food. While the exact mechanisms underlying the observed behavioural changes remain unknown, the data allow us to question previous interpretations of behavioural modifications after odour pre-exposure.
4.3 Material and methods

Fly care

_Drosophila_ stocks were raised on standard cornmeal medium at 25° and in a 12/12 light dark cycle. CantonS (CS, kindly provided by T. Preat) was used as a wild type control strain.

Odorants

Butanol (Fluka cat. 19420), hexanol (Fluka cat. 52828), nonanol (Fluka cat. 74278) ethyl caproate (Aldrich cat. 14.896-2) and ethyl acetate (Merck cat. 109623.1000) were used, all highest purity grade. Odorants were displayed on filter paper disks of 10 mm diameter (Schleicher and Schuell cat. 589/2).

Dilutions of odorant were made in water. Since chemicals were soaked on wet filter paper, even pure odorants were actually diluted in water; we can thus assume that vapour concentration is directly proportional to the corresponding volume of pure soaked odorant (Cometto-Muniz et al., 2003).

Behavioural tests

Experiments were performed using agar plates consisting of Petri dishes of 85 mm diameter without ergot (Greiner cat. 632180) covered with 2.5% Select Agar (Invitrogen cat. 30391-023). Sugar and dry yeast plates were plated covered with 1% Select Agar containing 0.5% autolyzed yeast (DIFCO cat. 0229-17-6) and 7.5% sugar (from local grocery store). Yeast plates were produced by covering the surface of the standard agar plates with fresh baking yeast (from local grocery store) soaked with distilled water. Tests were performed on young third instar larvae (75±3 hr after egg laying). As no difference was seen between tests performed in the morning or afternoon, all data were pooled. Control and experimental groups were always tested in parallel, using larvae from the same culture bottle.

Larvae were washed from the food with 17% sucrose solution. After three rinses in tap water, about 50 larvae were put in a Petri dish for 5 to 15 min. They were then transferred to a pre-exposure plate that contained either an odorant (Pre-exposed Group) or water (Control Group) spread on four 10mm filter paper disks evenly spaced along the edge of the plate. The amount of odorant indicated in the text for the pre-exposure plates
relates to the total, i.e., 10µl corresponds to 4x2.5µl. Larvae were pre-
exposed during 10 min in a shut off incubator, under a fume hood and
subsequently transferred in a clean agar plate for a rest period of 10 min
under the fume hood in presence of light. This procedure, 10 minutes pre-
exposure and 10 minutes of rest, was performed either 1 or 3 times.

The tests were performed as described previously (Heimbeck et al.,
1999). Briefly, larvae were placed in the middle of an agar plate contain-
ing a pair of filter paper disks on opposite sides, soaked respectively with
odorant and water (Fig. 1.3A). The odorant was put randomly to the left
or the right side of the plate. The test plates were then placed under a
cardboard cache, in a fume hood. After 5 min, a picture of each test plate
was taken and larvae were subsequently counted. A response index (RI)
was calculated:

\[ RI = \frac{Ns - Nc}{Ns + Nc} \]

Ns represents the number of animals at a distance \( d \leq 30 \text{mm} \) from the
odour source. Nc is the number of larvae found inside an identical sur-
face on the opposite side. Positive and negative RIs reflect attraction and
avoidance, respectively, and \( RI = 0 \) indicates indifferent behaviour. Data
presented in the same graph were always from experiments done in parallel.

Statistics

For group comparison, we used the arcsines transformation of the propor-
tion of larvae moving to the odorant

\[ A = A \sin \sqrt{P}, \quad \text{where } P = \frac{Ns}{Ns + Nc} = \frac{RI + 1}{2} \]

The value \( (A) \) was the dependent variable of a univariate test, weighted
by the total number of choosers \( (T = Ns + Nc) \); in cases where the odorant
side (on the left or right side of the plate) had an effect, this information
was added as a fixed factor. For comparison between more than two groups,
a tukey post hoc test was performed. Difference between two groups was
always confirmed by a Mann-Whitney U test (non-parametric).

Significant difference from \( RI = 0 \) was assessed using a one sample T
test. The statistical tests were performed and plots were generated using
computerized programs (SPSS for Macintosh, v.11).
4.4 Results

pre-exposure of larvae to an odorant does not exclusively lead to olfactory adaptation.

In order to measure olfactory adaptation, we used a protocol modified from Cobb and Domain (2000). Briefly, the behavioural effect of odor pre-exposure was tested by comparing the olfactory response of larvae pre-exposed to an odorant with the response of larvae pre-exposed to water. We measured this response by calculating the proportion of larvae moving towards or away from an odorant (chemotaxis); the results are depicted as a Response Index ($RI$) ranging from $-1$ (total repulsion) to $+1$ (total attraction). Whereas Cobb and Domain pre-exposed the larvae for 1 hour, we used a 10 min pre-exposure period and we allowed the larvae to rest for another 10 min before testing them (for details, see Materials and Methods).

Figure 4.1A shows that control larvae were attracted by ethyl acetate (1µl) whereas larvae pre-exposed during 10 min to 4µl of this compound presented $RIs$ that were reduced by 85%, to a level not significantly different from 0 ($p > 0.2$). In previous studies, comparable results were interpreted as olfactory adaptation (Störtkuhl et al., 1999; Cobb and Domain, 2000; Wuttke and Tompkins, 2000). However, an alternative hypothesis was not addressed in these studies, i.e., that the larvae may have associated the odorant with an unidentified negative US. In order to discriminate between these two interpretations, we performed two sets of experiments.

If associative learning indeed occurred, repeating the pre-exposure to the odorant should increase the strength of the association between the odorant (that corresponds to the CS) and the unidentified negative US (Rescorla and Wagner, 1972). Hence, negative $RIs$ are to be expected. In contrast, if the changes were due to olfactory adaptation only, further pre-exposure cycles would lead to a $RI$ closer to 0. Our results showed that after three cycles of pre-exposure, larvae indeed avoided the odour ($p < 0.001$) whereas control larvae were still normally attracted (Figure 4.1A). In order to demonstrate that this effect was not specific to ethyl acetate, we tested a second, dissimilar odorant, butanol (2.5µl). Control larvae were strongly attracted, while larvae pre-exposed one or three times to 10µl butanol were repelled (Figure 4.1B). This result argues against a pure adaptation hypothesis.

In the second set of experiments, we tested the effect of pre-exposure to two repelling odorants: nonanol (Cobb and Domain, 2000) at a concentra-
Figure 4.1: Olfactory responses of larvae pre-exposed to water (black bars) or to an odorant (white bars) during 10 min or 3x10 min, as indicated. The concentrations applied for pre-exposure are 4 times higher than those used for the tests. A. Letters represent different groups of data significantly different from each other with p < 0.01. One or three cycles of water exposure have no effect on the response to ethyl acetate. After 1 cycle of pre-exposure to 4µl of ethyl acetate, larvae do not respond anymore to 1µl of this odorant (RI not significantly different from 0). However, after 3 cycles of pre-exposure, larvae are repelled by the odorant (negative score, significantly different from 0). B. One cycle of pre-exposure to 10µl of butanol is sufficient to trigger negative chemotaxis toward 2.5µl of this chemical. After 3 cycles, the score is not significantly different. C-D. 10 min pre-exposure to 0.5µl ethyl caproate or 2.5µl nonanol does not affect the subsequent responses to these repellents (note high sample size for nonanol: n = 22). Error bars show means ± 1.0 standard error of the mean; n is the number of independent tests involving about 50 larvae each.
CHAPTER 4. ODOR PRE-EXPOSURE EFFECTS

tion of 2.5µl and ethyl caproate at 0.5µl that we found to be strongly repulsive for larvae (A.Ramaekers, unpublished observation). If pre-exposure was associated to olfactory adaptation, RIs would be expected to be closer to 0 as compared to the controls presenting negative RIs. On the other hand, if pre-exposure lead to an association between the repelling odorant (CS) and a negative US, one should observe an increase of the avoidance, i.e., more negative RIs as compared to the controls. Our results revealed that with both odorants, the RIs of control and pre-exposed larvae were indistinguishable, indicating that pre-exposure had no effect on larval olfactory responses to these odorants (Figure 4.1C and D). While these results are difficult to interpret, they indicated that, in the case of these repelling odorants, no evidence for the presence of olfactory adaptation nor olfactory learning could be demonstrated.

Behavioural changes: effects of stimulus strength and stimulus specificity

The reduction of olfactory attraction due to adaptation is dependent both on stimulus strength and stimulus specificity (Dalton, 2000). Nevertheless, such dependence is also a characteristic of other learning mechanisms, such as classical conditioning (Ghirlanda and Enquist, 2003; Wright and Smith, 2004). In previous studies (Störtkuhl et al., 1999; Cobb and Domain, 2000; Wuttke and Tompkins, 2000; Fletcher and Wilson, 2002), authors interpreted such observations as evidence for the presence of adaptation in their experimental set-up. In order to question this interpretation, we tested whether those characteristics were also applying to our protocol, although it does not solely involve adaptation.

In Figure 4.2A, the response of larvae toward 2.5µl of butanol after pre-exposure to increasing concentrations of this odorant is plotted. A negative correlation between the proportion of larvae approaching the odorant and the concentration of odorant during the pre-exposure had been found (covariate analysis, $F_{1-29} = 63.7, p < 0.001$). When pre-exposed to 10µl of butanol, larvae subsequently avoided the odorant, whereas they showed a null RI when pre-exposed to smaller concentrations of this compound. Therefore, similarly to the findings of previous studies, the proportion of larvae that chose or avoided the odorant side appeared to depend on the odorant concentration during pre-exposure.

In order to investigate the role of stimulus specificity on the behavioural change, we tested the response toward butanol (2.5µl) after pre-exposure to different odorants (Figure 4.2B). pre-exposure to ethyl acetate had no
4.4. RESULTS

Figure 4.2: Olfactory responses toward butanol (2.5µl, A and B) and ethyl acetate (sC) after 10 min pre-exposure to different quantities of the same odorant, (A, C) or different odors or water (B). Letters represent data significantly different with $p < 0.05$, "bc" means that the difference between this group is not significantly different from either b or c, but different from a. A. 1.6µl of butanol during pre-exposure is sufficient to change behaviour significantly from the control condition, but the difference is higher after pre-exposure with 4µl or 10µl. B. Effect of pre-exposure to ethyl acetate 4µl, hexanol 4µl and butanol 10µl. The difference between groups pre-exposed to water and ethyl acetate are close to (but do not reach) significance ($p = 0.59$). C. Dose-response curve to ethyl acetate after pre-exposure to 4µl of this odorant or to water ($n \geq 8$). Note that 0 on the abscissa represents the same condition as in Figure 4.1A left, demonstrating that we obtained a similar result in this replicated experiment. For error bars and $n$, see legend of Fig. 4.1. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$).
or little effect on the attraction towards butanol ($p = 0.059$), suggesting that the drop in $RI$ following pre-exposure was indeed stimulus-specific. In contrast, the response towards butanol of larvae pre-exposed to another aliphatic alcohol, hexanol, was lower than the $RI$ of control larvae pre-exposed to water ($p < 0.001$), and higher than the $RI$ of larvae pre-exposed to butanol ($p = 0.026$). Therefore, we found that the behavioural changes related to odorant pre-exposure were also characterized by a certain stimulus-specificity in our set-up.

**No visible increase in olfactory sensitivity**

Studies on human olfaction showed that in some cases, odorant exposure can increase olfactory sensitivity (Dalton, 1996), and that this effect may be due to peripheral mechanisms (Yee and Wysocki, 2001). Assuming that higher concentrations of odorant could become aversive, such an effect could explain our results, as proposed by Boyle and Cobb (2005). We therefore investigated this hypothesis, by testing the responses of larvae towards different concentrations of ethyl acetate, after pre-exposure to $4\mu l$ of either this compound or water (Figure 4.2C). We choose to test responses toward ethyl acetate because this odorant appears to be repulsive at high concentration. A sensitivity increase effect would shift the dose response curve to the left following the dashed line drawn in Figure 4.2C, moving the detection threshold toward a lower concentration. Our data do not fit with this prediction. We rather observed that the response of pre-exposed larvae followed the curve of the control larvae, albeit with a lower amplitude. In particular, it appeared that the detection threshold keeps stable. Thus, our results strongly argue against the presence of an effect on sensitivity.

**The decrease of the olfactory responses following odorant pre-exposure is not modified by the addition of food or water.**

The observed odour avoidance behaviour of pre-exposed larvae suggests that associative learning might be involved in our experimental set-up. In an attempt to test this hypothesis, we tried to identify the negative US possibly causing the drop of olfactory responses after pre-exposure. Indeed, in a similar paradigm, it was previously demonstrated that *C. elegans* associate odorants with the absence of food that acts as a negative US (Nuttley et al., 2002). To determine whether *Drosophila* larvae form similar associations, we pre-exposed larvae in agar plates containing food.
4.4. RESULTS

Because learning performance can drop with a context change between learning and test phases (Haney and Lukowiak, 2001; Law et al., 2004), pre-exposure and tests were made in the same type of plates.

![Graph](image)

**Figure 4.3:** Olfactory responses to butanol after pre-exposure to water (black bars) or butanol (white bars), in different types of plates (pre-exposure and tests were performed in the same type of plates; see Materials and Methods). A. The presence of sugar and dry yeast in the agar neither changed the behaviour of control nor of pre-exposed larvae. B. Comparison of experiments on normal plates or in the presence of fresh yeast, with pre-exposure during 10 min or 3x10 min, as indicated. The presence of fresh yeast on the agar reduces absolute RI values of both control and pre-exposed larvae in the same range (significant differences are indicated by asterisks: * p < 0.05, ** p < 0.01). The responses of larvae pre-exposed 3x10 min on yeast plates are significantly different from 0. See text for further details.

The addition of sucrose and dry yeast to the agar plates did not affect larval behaviour (Figure 4.3A). However, we could not exclude that the food was non-available to the animals in this set-up. We thus used agar plates spread with fresh baking yeast in the next experiment. Such ‘fresh yeast plates’ were shown to drive larval foraging behaviour (Pereira et al., 1995). We indeed observed that larvae tended to cluster on the yeast (data not shown), indicating that they recognized it as a food source. Figure 4.3B shows that the responses of all groups to butanol were closer to 0 in yeast plates. A straightforward explanation could be that odour or taste of the yeast distracted larvae from responding to butanol during the test having no effect during pre-exposure. The fact that the differences in the absolute RI values between normal and yeast plates were similar for control and pre-exposed larvae (Figure 4.3B, and data not shown) argues in favour of this hypothesis. Moreover, after three cycles of exposure to butanol in yeast plates, larvae were actively repelled by butanol (p < 0.01), suggesting that they still learn in the presence of food. Thus, the absence of food cannot be a major US, since its suppression does not eliminate the behavioural modification.
Additional experiments in which the amount of water was increased in the plates did not affect behaviour (data not shown), suggesting that dryness does not represent a negative US either.

4.5 Discussion

Some odours that do not trigger chemotaxis may still be sensed by larvae.

Similar to previous studies (Cobb and Domain, 2000; Wuttke and Tompkins, 2000; Boyle and Cobb, 2005; see also Michels et al., 2005) we observed that larvae modify their response to an odorant after pre-exposure to the same compound. We also confirmed that this modification is stimulus-specific and dependent upon the stimulus strength during pre-exposure. In particular, the positive (attractive) response to ethyl acetate dropped almost to 0 after 1 pre-exposure cycle. Olfactory adaptation was previously considered as the psychological basis of such observation. In this work, we questioned this interpretation. In particular, by increasing the number of pre-exposure cycles from 1 to 3, we found that the larval response to ethyl acetate was converted from attraction to avoidance. In addition, in the case of butanol, one pre-exposure cycle was sufficient to induce this inversion of behaviour. Interestingly, this phenomenon had already been measured with octanol (Cobb and Domain, 2000), and was further investigated recently (Boyle and Cobb, 2005). Those results suggest that the difference between the responses to ethyl acetate measured after 1 and 3 pre-exposure cycles was quantitative rather than qualitative. The $RI$ close to 0 measured after one pre-exposure cycle to ethyl acetate could correspond to a partial inversion of the olfactory behaviour rather than a decrease in sensitivity, as was proposed in previous studies.

However, our observations could still be explained exclusively on the basis of adaptation. As proposed by Cobb and Domain (2000), an odorant would elicit responses in both "attraction" and "repulsion" receptor neurons (mediating attractive or repulsive behaviour, respectively). The latter would adapt more slowly, being the only cells that remain firing after pre-exposure. Consequently, a partial loss of sensitivity would lead to repulsion. This model predicts that with further exposure, the response becomes extinct. In contrast to this prediction, we observed that additional periods of pre-exposure do not reduce the avoidance but on the contrary tend to lead to more negative $RI$s. Therefore, we propose that the change from attraction to repulsion after several cycles of pre-exposure might be due to
mechanisms different from adaptation.

Previous reports also based their interpretation in terms of olfactory adaptation on the demonstration that the behavioural modifications depended on stimulus intensity and stimulus-specificity, which are typical properties of olfactory adaptation (Cobb and Domain, 2000; Dalton, 2000; Wuttke and Tompkins, 2000). However, our study questions this assumption, since both properties also apply to our protocol that we demonstrated to be largely independent from adaptation mechanisms. Hence, other learning processes are characterized both by a dependence on stimulus concentration and stimulus-specificity. This is for instance the case for classical conditioning (Wright and Smith, 2004).

**Hypotheses to be ruled out**

At first, we considered whether non-associative mechanisms such as sensitisation could be involved in the observed behavioural modifications. If sensitisation was induced by pre-exposure to the odorant, one should observe a subsequent response increment as compared to the control response, without affecting sensitivity. This hypothesis is obviously not compatible with our results since pre-exposure led to a decrease or even a shift towards avoidance of the olfactory responses.

An alternative hypothesis presented by Boyle and Cobb (2005) predicts that pre-exposure would increase the sensitivity to the odorant. In this case, a concentration $c$ of the odorant would be perceived like if a concentration $d > c$ had been presented (Dalton, 1996), and the animals would behave accordingly: the dose-response curve would be shifted toward lower concentrations (see dashed line in figure 4.2C). Since, like for adults, attractive odorants might become repulsive at high concentrations (Figure 4.2C, see also Boyle and Cobb (2005)), one could predict that following pre-exposure, a given concentration eliciting attractive response in the control conditions, would become repulsive. However, the shift to lower concentrations of the dose-response curve following pre-exposure predicted by this model was not confirmed by our results. Moreover, Boyle and Cobb strikingly observed no increase in the attractiveness of any odour following prestimulation with the same or a different odorant (Boyle and Cobb, 2005); their data therefore do not fit with this hypothesis either.

Finally, we tested the hypothesis of a classical conditioning. If larvae indeed associated odorants with the absence of food, as do nematodes (Nuttle and Kamin, 2002), addition of yeast to the pre-exposure plates should have reduced or abolished the behavioural modification (Annau and Kamin, 2005).
1961). In contrast, our experiments revealed that addition of food did not eliminate the repulsion effect (Figure 4.3). Thus, we conclude that the absence of food did not act as a US.

Dryness could be ruled out as an US as well, since the addition of water in the pre-exposure agar plate had no effect on the behavioural modifications. According to another report (Dukas, 1999), the manipulation of larvae per se may play the role of a negative US. However, in our protocol, larvae were also manipulated before being in contact with the odorant. Consequently, the odor could not be a good predictor of this putative US and should not become associated with it (Rescorla and Wagner, 1972).

Boyle and Cobb (2005) obtained similar results as we did, although in their set-up larvae were prevented from having direct (gustatory) contact with the odorant; this contact thus appears not to be required for the behavioural change. However, a deleterious effect of high odorant concentrations could still act as the US. Assuming that the noxious effect depends on odorant concentration, smaller doses of odorant should be correlated with a weaker US. Hence, this hypothesis predicts that pre-exposure to decreasing concentrations of odorant should result in smaller behavioural changes. This is indeed what we observed in the case of butanol (Figure 4.2A).

**Fixed hedonic value for repellent odorants**

The fact that the response to nonanol and ethyl-caproate does not change after pre-exposure is striking. We assume that these odorants may already possess an innate and fixed hedonic value that could not be modified by our set-up. Interestingly, when Honjo and Furukubo-Tokunaga tried to associate an odour (CS) with sucrose (US), they found that this association was possible only when using certain odorants and not others (Honjo and Furukubo-Tokunaga, 2005). These data show that larvae can react differently to olfactory associative learning, depending on the odorant chosen as the CS. A similar effect could explain our result with aversive odours.

**Concluding remarks**

We have shown that olfactory adaptation is not sufficient to explain the behavioural modifications provoked by pre-exposure to an odorant. However, we were not able to shed light on the exact nature of the mechanisms involved. Our data does not fit with an increase in olfactory sensitivity, but rather tend to indicate that associative learning processes could play a central role. However, identification of the negative US involved would
be required in order to drive any firm conclusion. A possible candidate US may be the odorant per se that would be toxic at high concentrations. Alternatively, different mechanisms could act in parallel, or other non-associative learning mechanisms are conceivable (like disinterest of larvae for a previously experienced odorant).

*Drosophila* larvae are often considered as continuous feeders that exhibit a very limited behavioural repertoire. In particular, their olfactory system, characterized by no more than 21 receptor neurons, is thought to be rudimentary. In contrast, this study and others (Scherer et al., 2003; Gerber et al., 2004; Honjo and Furukubo-Tokunaga, 2005; Gerber and Hen-del, 2006) stress the complexity and plasticity of larval olfactory-driven behaviour that could mirror the importance of odour perception for larval survival. Together with evidence demonstrating adult-like connectivity in the larval olfactory system (Ramaekers et al., 2005), they suggest that olfactory cues may be much more crucial for larval survival than previously assumed.
Chapter 5

A role for NO in olfactory discrimination?

5.1 Introduction

Olfactory systems play crucial roles in insect survival and reproductive success, mediating responses to food, mates, predators and oviposition sites. Insects therefore possess a sensitive olfactory system that can detect and discriminate among a diverse array of chemicals. Recent progress in determining the chemical specificities and functional properties of the olfactory receptor proteins (Kreher et al., 2005; Fishilevich et al., 2005) provided insight into the mechanisms underlying odour coding in insects, and especially in *Drosophila melanogaster*. However, the central processes involved in olfactory coding are less well understood, although the architecture of the system was well described (Stocker, 2001; Keller and Vosshall, 2003). Especially, the cellular events underlying odour discrimination were only marginally investigated.

Imaging experiments in the honeybee (Sachse and Galizia, 2002) that examined information flow in the AL suggested that inhibitory local interneurons (LIs) play a major role in modulating the output of glomerular activity. Accordingly, imaging and patch clamp studies in the fruit fly antennal lobe (Ng et al., 2002; Wilson et al., 2004) suggested that the inhibitory network filters and processes the olfactory information that arrives at the glomeruli from the ORNs, thus producing a coherent stimulus-specific output. However, determining how odours are encoded in the brain requires to correlate behavioural outputs linked to olfactory inputs with synaptic activity in the brain. In practice, this cannot be achieved without measuring discriminative abilities of animals.

Pharmacological manipulations that affected both the local field po-
potential oscillation and the modulation of PN output by LIs caused honeybees to lose their ability of discriminating between closely related odorants, although leaving the discrimination of more divergent stimuli unaffected (Stopfer et al., 1997). However, such studies stop short of demonstrating that glomerular activity patterns are the salient information that the animal uses to encode the odour. *Drosophila* provides a unique system, whose powerful genetic tools may allow to understand how the olfactory circuitry serves to generate and organise these complex behaviours.

One of the potential candidate molecules in discrimination studies was nitric oxide (NO, Hosler et al., 2000). NO is a membrane-permeant signalling molecule synthesised by a single Ca$^{2+}$/calmodulin dependent nitric oxide synthase in *Drosophila* (Regulski and Tully, 1995). Although found to act through other cascades, NO is mostly known to take effect by activating a soluble guanylyl cyclase (sGC), leading to the formation of cyclic GMP (cGMP), a cellular mechanism applying to both vertebrate and invertebrate nervous systems (Muller, 1994; Regulski and Tully, 1995; Davies, 2000). It is an unconventional neurotransmitter as it diffuses across membranes to neighbouring cells, instead of being targeted through synaptic vesicle release. The distribution of NO-producing and NO-responsive (sGC positive) cells suggests that NO may act both as a retrograde synaptic messenger and as an intracellular messenger (Bicker et al., 1996; Wildemann and Bicker, 1999; Murata et al., 2006). In the olfactory system, NO was hypothesised to mediate communication between ORNs and PNs in *Manduca sexta* (Nighorn et al., 1998) and other insects (Ott and Elphick, 2002), as well as being involved in LI oscillations in the mollusc *Limax maximus* (Gelperin et al., 2000).

The role of NO in olfactory processing was assessed, but its action on olfactory learning in the honeybee (Muller, 1996; Hosler et al., 2000) and the cricket (Matsumoto et al., 2006), made olfactory discrimination tests difficult to interpret. We focused our study on *Drosophila* larva, because this system combines simplicity and relevance as it contains every standard element of olfactory systems, though in minimal number (Kreher et al., 2005; Ramaekers et al., 2005, see Fig. 1.5, P. 16). We modified a paradigm from V. Rodrigues (Rodrigues, 1980) in order to test olfactory discrimination independently of learning effects. We used a pharmacological approach that permitted us to propose that NO signalling plays an role in olfactory coding. However, this hypothesis still requires to be confirmed by a genetic approach.
5.2 Material and methods

Fly care

*Drosophila* stocks were raised on standard cornmeal medium at $25^\circ$ and in a 12/12 light dark cycle. CantonS (CS, kindly provided by T. Preat) was used as a wild type control strain.

Olfactory tests

Experiments were performed using Petri dishes of 85 mm diameter without ergot (Greiner cat. 632180), covered with 2.5% Select Agar (Invitrogen cat. 30391-023). Tests were performed on young third instar larvae ($75\pm3$ hr after egg laying). As no difference was seen between tests performed in the morning or afternoon, all data were pooled. Control and experimental groups were always tested in parallel.

Larvae were washed from the food with 17% sucrose solution. After three rinses in tap water, larvae were tested, as described previously (Heimbeck et al., 1999). Briefly, larvae were placed in the middle of an agar plate containing a pair of filter paper disks on opposite sides, soaked respectively with odorant and water (Fig. 1.3A, P. 8). The odorant was put randomly to the left or the right side of the plate. The test plates were then placed under a cardboard cache, in a fume hood. After 5 min, a picture of each test plate was taken and larvae were subsequently counted. A response index ($RI$) was calculated:

$$RI = \frac{Ns - Nc}{Ns + Nc}$$

$Ns$ represents the number of animals at a distance $d \leq 30$mm from the odour source. $Nc$ is the number of larvae found inside an identical surface on the opposite side. Positive and negative $RIs$ reflect attraction and avoidance, respectively, and $RI = 0$ indicates indifferent behaviour. Data presented in the same graph were always from experiments done in parallel.

Masking tests

Masking tests were performed similarly as simple olfactory tests, except that the masking or control odorant was previously added on four filter papers distributed on each side of the test and control ones as shown in Fig. 1.3B, P. 8.
CHAPTER 5. NO AND OLFACTORY DISCRIMINATION

Statistics

For group comparison, we used the arcsines transformation of the proportion of larvae moving to the odorant

$$A = A \sin \sqrt{P}, \quad \text{where } P = \frac{Ns}{Ns + Nc} = \frac{RI + 1}{2}$$

The value $A$ was the dependent variable of a univariate test, weighted by the total number of choosers ($T = Ns + Nc$); in cases where the odorant side (on the left or right side of the plate) had an effect, this information was added as a fixed factor. For comparison between more than two groups, a Tukey post hoc test was performed. Difference between two groups was always confirmed by a Mann-Whitney U test (non-parametric).

Significant difference from $RI = 0$ was assessed using a one sample $T$ test. The statistical tests were performed and plots were generated using computerised programs (SPSS for Macintosh, v.11).

5.3 Results and work in progress

Dose-response curves

We tested the response of wild type (CS) larvae to various odorants at decreasing concentration by dilution in paraffin oil. The dose-response curves typically followed a sigmoid shape (Fig. 5.1) starting from indifferent behaviour at low concentrations to positive scores that reached a plateau. However, negative scores were found for hexyl acetate. Ethanol, ethyl caproate and acetoin drove nearly no response and were not further used. Some variability in maximal $RI$s was found between different experimental days, but the basic shape of the curves remained unchanged. In particular, the response threshold concentrations were similar (data not shown). At high concentration, the responses dropped again for specific odorants (Fig. 5.1 isoamyl acetate, ethyl acetate, see also Fig. 4.2C, on P. 73). This may be due to a saturation of the odorant in the Petri dishes or a toxic effect of the chemical at high concentrations. Indeed, in the case of isoamyl acetate, the proportion of larvae staying in the middle portion of the plate was largely increased at high concentration, suggesting an intoxication effect (data not shown).
Figure 5.1: Olfactory responses of CS larvae toward different dilutions of various odorants. The abscissa shows the $-\log(\text{concentration})$, with 0 representing 1µl of pure odorant. Points refer to the mean, error bars to the standard error of the mean. The lines represent the fitted regression curves; the black ones include all data, the dotted ones do not include the points indicated on top of each graph. Arrows point to the concentrations used in further studies.
CHAPTER 5. NO AND OLFACTORY DISCRIMINATION

Masking tests

We chose to use the early plateau as a concentration for the test odour, i.e., the lowest concentration which induced a maximal $RI$. We then decided to use the mask odours at the lowest concentration that exhibited total masking of himself. To determine it, we tested different mask odour concentrations for hexanol (Fig. 5.2). On the basis of this experiment, we decided to use the mask odorants at the same concentration as when used as a test odour (Fig. 5.1, arrows). We then tested different combinations of odorants, comparing the response to the test odorants in presence of the solvent (paraffin oil) or the mask odours. Note that paraffin oil masking has no effect on $RI$ or may even increase the $RI$ toward cyclohexanol ($RI = 0.44 \pm 0.06$ and $0.58 \pm 0.06$, respectively, $p > 0.83, n = 8$).

![Figure 5.2: Olfactory responses toward hexanol (1µl) in the presence of decreasing concentration of hexanol as a mask odorant (abscissa). Using the same quantity of odorant as mask and test led to a total masking effect.](image)

We tested the responses of different odorants masked either by themselves, by isoamylacetate, cyclohexanol, and ethyl acetate (Fig. 5.3A), as well as the response to hexanol masked by ethyl acetate and pentanol, and the response to ethyl acetate masked by hexanol and pentanol (Fig. 5.3B). The control responses (white bars) correspond to the responses seen in Fig. 5.1, apart from heptanol, which led to negative $RI$s in this new round of tests. Remarkably, the response to the odorant masked by himself was not always 0 (see response to hexyl acetate); in such cases, no conclusion about the mask effect could be drawn.

The variability of the responses was high, and sometimes even a high number of tests was not sufficient to get statistically different responses.
5.3. RESULTS AND WORK IN PROGRESS

(see Fig 5.3, heptanol graph). However, the graphs show that three different cases can be found: either the mask had no effect on the response, or it had the same effect than the auto-mask (for example, cyclohexanol masked perfectly 3-methyl-1-butanol), or it had an intermediate effect, leading to a response significantly different from both the control and the automask scores (see, for instance, the ethyl acetate mask effect on the response to ethyl-3-hydroxybutanol).

![Discrimination tests](image)

Figure 5.3: Discrimination tests. The odour used as test is indicated on top of the graphs. Stars indicate combinations used in further studies A. Three masking odours were used as indicated with the colour code. Numbers of independent tests are indicated below the graphs. Lettering denotes groups statistically different from each other, with \( p < 0.05 \), "AB" means that the difference between this group is not significantly different from either A or B. B. reciprocal masking tests with hexanol, ethyl acetate, and pentanol. Numbers of independent tests are indicated.

L-NAME effects

Previous studies on NO function used L-NAME, an arginine agonist, as an effective NO synthase inhibitor. This drug is expected to be effective within an hour if administrated locally by injection (Muller, 1996), but can be conveniently fed to larvae (Foley and O’Farrell, 2003). In our experiment, we fed larvae with food containing L-NAME during 24 hours before the
experiment, in order to ensure that the drug had reached the tissues by
the time of the tests.

We chose three combinations of odorants, for which we expected to
see an effect of L-NAME administration (Fig. 5.3, stars). We tested the
responses to these odorants –in presence or absence of mask odours– of
larvae previously fed with the drug or without its application (Fig. 5.4).
Two different effects were found. In some cases, NO inhibition appeared to
reduce the response toward the odorant (Fig. 5.4 A-B). In another case,
it did not affect the simple response, but rather affected discriminative
abilities (Fig. 5.4 C). This latter effect might apply as well for the two
first cases, but may be non-significant because of the effect on the normal
response.

Unfortunately, we were not able to find why L-NAME affected nor-
mal responses only in certain circumstances. Indeed, tests with the same
odour (ethyl acetate, Fig. 5.4 A,C) performed on the same day (data not
shown) showed these differences to some extent. From this, it appeared
that internal variability and sampling differences may cause variation be-
tween different experiments. Still, NO inhibition seems to be able to affect
both olfactory responses and olfactory discrimination. However, the actual
mechanism involved remains to be uncovered (see Discussion and Outlook).

5.4 Discussion and outlooks

Accuracy of the masking test

The high variability in olfactory behavioural responses is a caveat to the
study of olfactory processing. The efforts invested in controlling variables
appeared to be effective for simple olfactory choice tests. An exception were
the responses to heptanol which were actually different between two ex-
periments, shifting from attraction to aversion. In contrast, masking tests
appeared to be less consistent, especially in the cases of absence of or par-
tial masking. For instance, cyclohexanol appeared to partially mask the
response to ethyl-acetate in some cases, but to have no effect in others.
This might be due to the concentration of the mask odorant that we chose:
the early plateau concentration in the dose-response curve might be close
to the critical concentration that induces an effect. Using a higher concen-
tration may reduce the variability, but it may in parallel limit the possi-
bility to artificially induce failing in this behavioural challenge. Indeed, a
more precise study using masks at varying concentration (Rodrigues, 1980)
tended to show that a higher concentration of mask may lead to stronger
5.4. DISCUSSION AND OUTLOOKS

Figure 5.4: Olfactory responses after NO inhibition by L-NAME. A-B. Responses to ethyl acetate (A.) and to 3-methyl-1-butanol (B.), masked (grey) or not (white) by cyclohexanol or ethyl-acetate, respectively, in control and L-NAME fed larvae. The presence of the mask and of the drug both have a significant effect on the RI. However, no "mask x drug" effect was detected, meaning that the presence of the drug did not significantly induce lower discrimination abilities for these odorants. C: Responses to ethyl acetate masked (grey) or not (white) by pentanol of control and L-NAME fed larvae. L-NAME had no effect on the response to ethyl acetate per se, but reduced the response in the presence of the mask pentanol (p < 0.05).
behavioural effects, although a ten-fold increase induced relatively small changes.

Despite these problems of variability, we believe that careful experiments made in parallel can yield reliable results. Especially, the similar effects of hexanol and pentanol, two chemically similar molecules, tend to argue for the credibility of these tests.

A role of NO?

Feeding of the NO inhibitor L-NAME to larvae appeared to both reduce their normal response and their discriminative abilities. NO may therefore be involved in different aspects of the olfactory system. For example, administration of L-NAME was shown to affect axon pathfinding and cell proliferation (Kuzin et al., 1996; Bicker, 2005). An action of L-NAME on developmental processes is thus to be expected. Since the age of the larva was shown to affect their chemotaxis (Cobb, 1999), a developmental effect may well explain the behavioural differences we see.

On the other hand, NO was postulated as a second messenger in GRNs (Murata et al., 2006). NADPHd staining is normally related to NO synthase activity. Its presence in ORN cell bodies (data not shown; Bicker, 2001) suggests that NO may also act as a second messenger in ORNs. This would well explain the differences in response to unmasked odorants. NO was also hypothesised to mediate communication between ORNs and PNs (Nighorn et al., 1998; Ott and Elphick, 2002). This modulation may be important for shaping responses of PNs, which may be necessary for fine discrimination of odours.

Different effects of L-NAME on olfactory behaviour may thus be proposed, and they may all be effective, acting concomitantly. In order to look more closely at these hypotheses, a better timing control of NO synthase inhibition is necessary.

To improve control on NO synthase inhibition, we propose to use flies bearing a dominant negative form of this protein under the control of a heat shock promoter. Simply by using different heat shock protocols, we may not only confirm our results, but also elucidate if L-NAME exerts development effect on the olfactory system or not.
Chapter 6

Conclusions

In this thesis, we used new behavioural, molecular and genetic tools in order to investigate the anatomy and function of the chemosensory system in a simple model organism: the *Drosophila melanogaster* larva. Taking advantage of different Gal4 and UAS lines from different labs, as well as by modifying existing behavioural paradigms and drug application protocols, we studied the taste system and behaviours of larvae in response to odours.

This work shows that the design of the taste system is similar but not identical to its adult counterpart. We found subtle functional divisions in the primary taste centre, by studying taste afferents (chapter 2). These findings were confirmed by the dissection of the putative dendritic arborisation of *hugin* neurones (chapter 3). The basic organisational principles of the taste system seems to be in accord with the mammalian design. Primary taste neurones may well be assigned to specific taste modalities (although subtle differences in response profiles may be expected) whereas central neurones may be more broadly tuned. The coding strategies of the two systems may thus be similar. This is reminiscent of the correspondence between the olfactory systems in the two animal phyla.

In the second part of this thesis, we showed that olfactory pre-exposure does not lead to sensory adaptation, but rather to a learning effect (chapter 4). This may be of interest in further learning experiments using this model system. On the other hand, the bases of olfactory discrimination were investigated using a masking test (chapter 5). Preliminary results suggest that NO signalling may be implicated in olfactory coding.

The complexity and variability of larval behaviours is clearly related to larval survival. As an increasing number of studies similar to the present one suggest, the idea of the larva as a mere feeding machine with a very restricted behavioural repertoire is slowly vanishing. As a striking example of behavioural complexity, larvae were shown to have surprisingly good
learning and memory skills (for a review, see Gerber and Stocker, in press). Therefore, apart from being an attractive model because of its simple nervous system and the powerful tools available in this species, *Drosophila* larvae will have to be considered more and more as animals with a sophisticated behavioural repertoire. These behaviours have to cope with many more tasks than just food ingestion. Larval survival involves an entire set of strategies and decisions in a changing and challenging environment, which obviously requires behavioural complexity. Hence, what the *Drosophila* larval model offers is to approach complex behaviours in a simple nervous system.


BIBLIOGRAPHY


## Abbreviations

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<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>AN</td>
<td>Antennal Nerve</td>
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<tr>
<td>ChAT</td>
<td>choline acetyl-transferase</td>
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<tr>
<td>DO</td>
<td>Dorsal Organ</td>
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<tr>
<td>DPO</td>
<td>Dorsal Pharyngeal Organ</td>
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<tr>
<td>DPS</td>
<td>Dorsal Pharyngeal Sense Organ</td>
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<tr>
<td>FRT</td>
<td>Flp Recombinase Target</td>
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<tr>
<td>GR</td>
<td>Gustatory Receptor</td>
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<td>Gr-Gal4</td>
<td>Gal4 line controlled by a GR promoter</td>
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<tr>
<td>GRN</td>
<td>Gustatory Receptor Neuron</td>
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<td>LAL</td>
<td>Larval Antennal Lobe</td>
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<td>LbN</td>
<td>Labial Nerve</td>
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<tr>
<td>LrN</td>
<td>Labral Nerve</td>
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<tr>
<td>LI</td>
<td>Local Interneuron</td>
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<tr>
<td>MB</td>
<td>Mushroom Bodies</td>
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<td>MN</td>
<td>Maxillary Nerve</td>
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<tr>
<td>Ppk</td>
<td>Pickpocket (gene family)</td>
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<tr>
<td>OR</td>
<td>Odorant Receptor</td>
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<tr>
<td>ORN</td>
<td>Olfactory Receptor Neuron</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>PN</td>
<td>Projection Neuron</td>
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<tr>
<td>PPS</td>
<td>Posterior Pharyngeal Sense Organ</td>
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<tr>
<td>RI</td>
<td>Response Index</td>
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<tr>
<td>SOG</td>
<td>Suboesophageal ganglion</td>
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Birth: 9 April 1979
Single
Swiss

**Education**

2006 PhD in Biology
Université of Fribourg (CH). (exam scheduled in november)
PhD dissertation: “The chemosensory system of *Drosophila* larvae: neuroanatomy and behaviour”
Thesis director: Prof. R.F Stocker

2002 Diploma in Biology
University of Fribourg (CH)
Subject: 'Anatomical and functional studies of the chemosensory system of the *Drosophila* larva suggest the presence of a gustatory target area in the antennal lobe?'

1998 “Maturité fédérale type C” (scientific subsection), in the 'Collège de la royale Abbaye de St. Maurice'

**Overview**

Numerous presentations in different regional and international meetings are hints of the quality of the relatively abundant, and soon published, studies accomplished during my PhD thesis and diploma work. On the other hand, teaching experience and meeting organization, as well as involvement in the theatre group of the university, show my interest in academic life.

**Teaching experience**

2006 Fluorescence and Confocal Microscopy (lecture course to master student, 3 hours)

2003–2006 To study behavior. (course during the practical course, 2 hours)

2003-2005 An introduction to learning and memory and their molecular mechanisms. (2 hours, part of the lecture course 'Developmental and Neurogenetic' given by R.F. Stocker).

**Supervision**

2005 Co-supervisor of the diploma work of Claire Huguenin (with Dr. A. Ramaekers) on the role of NO in olfactory discrimination in *Drosophila* larvae.
Organization of conferences
2004 Co-organizer of the PhD meeting in Cerniat. (30 participants).

Other tasks
Creation of the R.F. Stocker’s lab website.
Cashier of and actor in the theater group 'les apostrophes' in 2002-2005.
Co-director in the theater group "les apostrophes" in 2006

Languages
French (mother tongue), English (fluent) and German (good knowledge), Spanish (basics).

Computer skills
Publishing: TeX, Photoshop, Illustrator, Image J, HTML, Office
Publications and unpublished work


3 Julien Colomb*, Rüdiger Bader*, Bettina Pankratz, Anne Schröck, Reinhard F. Stocker, and Michael J. Pankratz. “Genetic dissection of a neural circuit underlying feeding behavior in *Drosophila*: distinct morphology of single *hugin* expressing neurons”. (Submitted). * the two authors participated equally to this work.


Meetings presentation

2006 Sub-regions in the primary taste center of *Drosophila* larvae. ORAL PRESENTATION at the 11th European Drosophila neurobiology Conference, Leuven, BE

2005 Neuroarchitecture of the larval gustatory system. POSTER at the CSH neurobiology of Drosophila meeting, USA,(NY)


2004 Different sensory projections define subregions in the first gustatory center of *Drosophila* larval brain. POSTER at the "Neurofly" meeting in Neuchâtel

2004 Neuroanatomical studies in *Drosophila melanogaster*: the gustatory system. ORAL PRESENTATION at the PhD meeting in Cerniat, Switzerland.

2004 Food-independent learning after olfactory exposure in agar plate in *Drosophila melanogaster* larvae. POSTER at the PhD meeting in Cerniat.

2004 Learning by odor exposure in agar plate. ORAL PRESENTATION at the behavioural neurobiology of *Drosophila* larvae meeting in Würzburg, Germany.
2002  Functional studies of the chemosensory system of the*Drosophila* larva using a new tool: the GAL4 / UAS-shi system.
POSTER at the neurofly in Dijon, France.

2002  Functional studies of the chemosensory system of the*Drosophila* larva using a new tool: the GAL4 / UAS-shi system.
ORAL PRESENTATION at the Swiss Drosophila meeting in Basel.
Acknowledgements

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I want also to thank Dr. Bertram Gerber, who taught me the rigour needed in scientific experiments, both for those published by others and for those I was planning to do. I entered the world of science by the door of behavioural experiments, with his wonderful help. I’d like to thank also my biology and philosophy teachers in St. Maurice school: they gave me the taste for biological studies (thanks Prof. Vionnet) and the rigour in thinking and a great introduction to the hypothetico-deductive model (thanks Prof. Pignat).

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