SEROTONERGIC NEURONS OF *DROSOPHILA MELANOGASTER* LARVAE

SEROTONERGIC NEURONS OF *DROSOPHILA MELANOGASTER* LARVAE: A STUDY OF THEIR DEVELOPMENT AND FUNCTION

BY

VERÓNICA GABRIELA RODRIGUEZ MONCALVO

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AUTHOR: Verónica Gabriela Rodriguez Moncalvo, *Licenciada* in Biology (University of Buenos Aires, Argentina)

SUPERVISOR: Professor Dr. Ana R. Campos

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ABSTRACT

Drosophila melanogaster is an attractive model organism for the study of numerous fundamental processes including nervous system development and function. This is due to the power of *Drosophila* genetics combined with the high degree of similarity between this organism and vertebrate systems, not only at the molecular level but also at the cellular and behavioural levels.

The first part of my thesis focused on trophic interactions occurring in *Drosophila* larval central nervous system. Specifically, it describes the interaction of serotonin (5-HT)-producing neurons with other three groups of neurons: the larval photoreceptors expressing Rhodopsin 5 (Rh5), the photoreceptor subset expressing Rhodopsin 6 (Rh6), and the larval circadian pacemakers (LNv). I found that both Rh5- and Rh6-expressing fibers contact a 5-HT arborization in the larval optic neuropil, where the 5-HT processes also overlaps with the dendrites of the LNv. The results of my experiments also indicate that the Rh6-expressing terminus is the neural process providing the signal required for the outgrowth of the serotonergic arborization. Furthermore, proper branching of this arborization requires normal Rac function. These findings further support the importance of extrinsic and intrinsic signalling for the assembly of the nervous system.

The remainder of my studies attempted to investigate candidate neurons modulating *Drosophila* larval photobehaviour. Using the larval response to light as a behavioural paradigm and neuronal silencing experiments, my results demonstrate that 5-HT neurons located in the brain regulate the larval photoresponse during development. In

addition, my findings suggest that this modulation occurs at a central level and that is mediated by 5-HT1A_{Dro} receptors. These observations provide new insights into the functions of serotonergic neurons in *Drosophila* as well as how neuromodulators shape neuronal circuit function and ultimately behaviour.

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5-HIAA – 5-hydroxyindole-3-acetic acid

5-HT – 5-hydroxytryptamine (serotonin)

A – abdominal

AC – adenylate cyclase

Ach – acetylcholine

AEL – after egg laying

A/P – anterior-posterior

ato – atonal

BA – biogenic amine

bHLH – basic Helix Loop Helix

BMP – bone morphogenetic protein

BN – Bolwig's nerve

BO – Bolwig's organs

CAM – cell adhesion molecule

Cas – Castor

CCAP – crustacean cardioactive peptide

Cdc42 – cell division cycle 42

CFTR – cystic fibrosis transmembrane conductance regulator

ChaT – choline-acetyltransferase

CNS – central nervous system

CPG – central pattern generator

CRZ – corazonin

CX – central complex

DA – dopamine

DAG – diacylglycerol

DCC – Deleted in colorectal cancer

Ddc – DOPA-decarboxylase

DIAS – dynamic image analysis system

Dl – Delta

DL – dorsolateral

DM – dorsomedial

Dscam-GFP – *Drosophila* Down syndrome adhesion molecule [17.1]-GFP

dSERT – *Drosophila* serotonin transporter

dsRNA – double-stranded RNA

DTRHn (TRH) – neuronal tryptophan hydroxylase (*Drosophila*)

DTPHu – phenylalanine hydroxylase, non-neuronal tryptophan hydroxylase (*Drosophila*)

D/V – dorso-ventral

Eg – Eagle

EGFR – epidermal growth factor receptor

EKO – electrically knock out

elav – embryonic lethal, abnormal vision

EM – electromicroscopy

Eph – Ephrin

EW1 – medial VNC 5-HT neuron (NB7-3)

EW2 – lateral VNC 5-HT neuron (NB7-3)

EW3 –CRZ neuron (NB7-3)

FasII – Fasciclin II

ftz – fushi tarazu

GABA – gamma-aminobutyric acid

GFP – green fluorescent protein

Gl – Glass

GMC – ganglion mother cell

GMR – glass multimer reporter

GPCR – G protein-coupled receptor

gsb – gooseberry

GW – VNC motorneuron (NB7-3)

Hb – Hunchback

hh - hedgehog

hid – head involution defective

hkb – huckebein

IP – inferior protocerebrum

Kr – Krüppel

LON – larval optic nerve

LP – lateral protocerebrum

LNv – small ventral lateral neurons

MAO – monoamine oxidase

MARCM – mosaic analysis with a repressible cell marker

MB – mushroom bodies

MD – multidendritic

MIP – myoinhibiting peptide

msh – muscle segment homeobox

Mtl – Mig-2-like

N – Notch

NB – neuroblast

nkd – naked cuticule

NMDA – N-methyl D-aspartate

NMJ – neuromuscular junction

norpA – no receptor potential

NR – neurogenic region

OA – octopamine

OL – optic lobe

OLP – optic lobe pioneer

OR – Oregon-R

ORK – open rectifier K^+ channel

otd – orthodenticle

PBS – phosphate buffer saline

PBT – PBS + Triton X-100

PDF – pigment dispersing factor

Pdm – POU domain protein

PKA – protein kinase A

PKC – protein kinase C

PLC – phospholipase C

pNR – procephalic neurogenic region

PNS – peripheral nervous system

ptc – patched

Rac – Ras-related C3 botulinum toxin substrate

RI – response index

Rh5 – Rhodopsin 5

Rh6 – Rhodopsin 6

RhoA – Ras homologous member A

Robo – Roundabout

S1-S5 – delamination waves 1-5

sa – spalt

SE – subesophageal

so – sine oculis

SP – anterior medial protocerebrum

svp – seven-up

Syt-GFP – Synaptotagmin1-GFP

T – thoracic

TA – tyramine

tll – tailless

TH – tyrosine hydroxylase

TNT – tetanus toxin light chain

TPH – tryptophan hydroxylase (vertebrates)

TRP – transient receptor potential

TRPL – transient receptor potential like

UAS – upstream activating sequence

VMAT – vesicular monoamine transporter

VNC – ventral nerve cord

vnd – ventral nervous system defective

vNR – ventral neurogenic region

VS – vertical system

VUM – ventral unpaired neurons

Wg – Wingless

Zfh – Zinc-finger/homeodomain

CHAPTER 1: Introduction

1.1. *Drosophila* as a model system

The study of complex biological processes at the molecular, cellular or behavioural level can be readily achieved by using model organisms such as *Drosophila melanogaster*. The fruit fly was introduced into the field of biology research nearly 100 years ago. Starting with Thomas Morgan and his discovery of mutant white-eye males in 1908, followed by the development of chromosomal heredity theory of genetic material, the fly became a useful model system for elucidation of gene structure as well as gene function (reviewed in Burdett and van den Heuvel, 2004; Nichols, 2006). Moreover, the similarity in genetic information, molecular mechanisms and behaviour between fruit flies and mammals, together with the versatile genetic manipulation of *Drosophila* (reviewed in Venken and Bellen, 2005; 2007), have made the fly an excellent model system to elucidate many fundamental processes of relevance to mammals including humans.

1.1.1. *Drosophila* genetics

In the fly, a large number of different genetic tools have been developed and similar approaches have subsequently been implemented in mammalian systems. One of the most powerful of these genetic manipulations is the generation of transgenic animals (Mátés *et al.*, 2007). In this regard, transgenic techniques have been developed that successfully allow for targeted control of gene expression in both a temporal and spatial manner. In *Drosophila*, this can be accomplished by introducing the yeast binary GAL4/UAS system within the genome (Brand and Perrimon, 1993). This genetic tool

involves the cross of parental flies carrying two different transgenes. One carries a transgene containing specific promoters or enhancers fused to the gene of the yeast transcription factor GAL4, whereas the other one carries a gene of interest or target construct fused to the upstream activating sequence (*UAS*) of GAL4. In the progeny of this cross, expression of the gene of interest is activated in a spatial as well as temporal manner, determined by activation of the promoters or enhancers used to drive GAL4 expression and the subsequent binding of GAL4 to its *UAS* element (Brand and Perrimon, 1993). Thus, cells or entire tissues can be labelled if for instance the gene of interest codes for a reporter such as the green fluorescent protein (GFP), or mutant phenotypes can be studied by either introduction of a mutant allele of a given gene or double-stranded RNA (dsRNA) constructs to selectively knockdown gene expression.

1.1.2. Conserved molecular mechanisms and behaviours

In spite of the differences between the number of genes coded in the fruit fly and mammal genomes and the morphological differences between these organisms, increasing evidence indicates that vertebrates and invertebrates share many regulatory networks of transcription factors and signalling molecules, as well as the pathways activated by them (e.g. reviewed in Bier, 2005; Burdett and van den Heuvel, 2004; Giudice, 2001; Hirth and Reichert, 1999). Moreover, these genetic pathways underlie basic developmental processes such as axis polarity, neural development, organogenesis, as well as metabolic processes in both invertebrates and vertebrates (reviewed in Arendt and Nubler-Jung, 1999; Bier, 2005; Nichols, 2006; Thor 1995; Quan and Hassan, 2005).

At the behavioural level, fruit flies exhibit a wide range of complex behaviours, several of which are relevant to higher organisms. These include circadian rhythms, learning and memory, courtship, sleep, aggression, locomotion and even drug abuse (reviewed in Greenspan and Dierick, 2004; Greenspan and van Swinderen, 2004). It should be noted that, for many of these behaviours, the molecular and genetic components were originally identified in *Drosophila* and subsequently in mammalian systems (reviewed in Greenspan and Dierick, 2004; Nichols 2006).

1.2. *Drosophila* larval central nervous system: development and composition

In all higher organisms, the central nervous system (CNS) is composed of many different cell types that generate a complex cellular network. In *Drosophila*, a functional larval CNS necessary to fulfill the requirements of larval behaviour is generated during embryogenesis. As in vertebrates and other invertebrates, the embryonic development of the *Drosophila* larval CNS involves the transformation of a two-dimensional ectodermal sheet of cells into a highly organized three-dimensional structure comprised of a large variety of neurons and glial cells.

Like other developmental processes, CNS formation occurs in stages, beginning with the acquisition of neural fate by a group of embryonic cells and ending with formation and differentiation of different neuronal and glial cell types. Genetic studies in *Drosophila* and vertebrate models indicate that the molecular mechanisms regulating vertebrate and invertebrate neurogenesis are remarkably similar (reviewed in Quan and Hassan, 2005). These developmental steps are regulated by both cell autonomous and

non-autonomous mechanisms. Cell autonomous mechanisms involve the effect of gene activity only in those cells in which gene expression is being modified. On the other hand, a process is referred to as cell non-autonomous if gene activity affects cells other than (or in addition to) cells expressing that particular gene. Regarding non-autonomous pathways, cells may signal one another by lateral signalling and/or inductive signalling. Whereas the former entails signalling between neighbour contacting cells, the latter involves the release of a short-range signalling molecule from one cell, which in turn acts on its corresponding receptor expressed by the other cell (reviewed in Bhat, 1998).

In all species, the CNS originates from the ectodermal neurogenic region (NR). *Drosophila* larval CNS consists of a ventral nerve cord (VNC), which derives from the ventral NR (vNR), and two brain hemispheres, which derive from the procephalic NR (pNR). The VNC is composed of 14 segmental units also known as neuromeres (3 subesophageal (SE) or gnathal, 3 thoracic (T), and 8 abdominal (A)). The last abdominal neuromere (A9) is only rudimentary, and it is usually referred to as 'terminal plexus'. The brain consists of the tritocerebrum, deuterocerebrum and protocerebrum. These brain regions are formed by the intercalary, antennal and ocular/labral supraesophageal segments respectively (Urbach and Technau, 2003a; Younossi-Hartenstein *et al.*, 1996; reviewed in Urbach and Technau, 2003b). Unlike the VNC, whose organization is relatively simple, the structure of the brain is much more complex. Therefore, the study of *Drosophila* CNS development has been primarily focused on neurogenesis in the VNC (reviewed in Technau *et al.*, 2006).

Neurogenesis represents a series of developmental steps beginning with neuroblast (NB) formation. This step comprises determination of neural progenitor cells by neural induction within the neuroectoderm, followed by NB delamination. The next step involves specification of NB identity, determined by unique combinations of gene expression. Lastly, neurogenesis culminates with the elaboration of specific lineages by each NB, giving rise to different neuronal and glial cell types.

1.2.1. Neuroblast formation: neural induction and neuroblast delamination

Neural induction constitutes the initial step in neurogenesis. In *Drosophila*, neurogenesis begins in early gastrula with the generation of specific neural progenitor cells knowns as neuroblasts (NBs). Each ectodermal cell has the alternative of following either a neurogenic or an epidermogenic developmental pathway. It is clear now that the fate specification of these cells depends not only on dorso-ventral (D/V) and anterior-posterior (A/P) positional information but also on cell-cell interactions. There are two main groups of genes involved in the separation of neural progenitor cells from epidermal progenitor cells: the proneural genes and the neurogenic genes (reviewed in Technau *et al.*, 2006). Like in vertebrates, all known proneural genes in *Drosophila* code for transcription factors of the basic Helix Loop Helix (bHLH) family (reviewed in Quan and Hassan, 2005). Expression of these genes is activated in small groups of 4 to 6 neuroepithelial cells called proneural clusters. These clusters are found at specific positions within the neuroectoderm (e.g. Cabrera *et al.*, 1987; Skeath *et al.*, 1992, reviewed in Technau *et al.*, 2006). Shortly after, one cell from each proneural cluster is

singled out and becomes a NB. This is achieved by a mechanism of lateral inhibition, mediated by the neurogenic gene products (reviewed in Technau *et al.*, 2006).

Two of the most studied neurogenic genes involved in this type of cell-cell interaction are *Notch* (*N*) and *Delta* (*Dl*). *N* encodes a transmembrane receptor, whereas *Dl* represents one of *N* ligands (Urban and Technau, 1997; reviewed in Technau *et al.*, 2006). Activation of this receptor in all but one cell in the proneural cluster causes inhibition of proneural gene expression (or function), and the consequent commitment of these cells to a non-neural fate. Therefore, suppression of *N* signalling is necessary for NB formation (Urban and Technau, 1997; reviewed in Technau *et al.*, 2006). Interestingly, requirement of proneural gene expression as well as cell-cell interactions for specification of neural fate has been also shown in vertebrates (reviewed in Arendt and Nübler-Jung, 1999; Nichols, 2006).

NB delamination appears to follow a well-defined and reproducible spatio-temporal pattern (Bossing *et al.*, 1996; Doe, 1992; Schmidt *et al.*, 1997). In some vNR regions, delamination of one daughter cell as early NB occurs after division of neuroectodermal progenitor cells parallel to the ectodermal surface. In other neurogenic areas, NBs delaminate without previous division of the progenitor cells (reviewed in Technau *et al.*, 2006). Several studies have shown that delamination of individual NBs in the VNC occurs in five successive waves (S1-S5) (Bossing *et al.*, 1996; Doe, 1992; Schmidt *et al.*, 1997). NBs delaminate from the ectodermal sheet into the embryo, where the NBs enlarge and form a subepidermal layer. NBs occupy characteristic positions within this array. By stage 11, the entire population of 30 NBs per thoracic and

abdominal hemineuromere has delaminated from the neuroectoderm (Bossing *et al.*, 1996; Broadus *et al.*, 1995; Doe, 1992; Schmidt *et al.*, 1997).

1.2.2. NB specification and NB lineage elaboration

It appears that NBs obtain their unique spatial identity within a hemisegment just before delamination (Udolph *et al.*, 1995; reviewed in Technau *et al.*, 2006). This identity is determined by positional information within the neuroectoderm, in particular with respect to the A/P and D/V axes. Regarding the A/P axis, extensive evidence shows that expression of several segment polarity genes such as *hedgehog* (*hh*) and *gooseberry* (*gsb*) in the proneural clusters play a critical role in the specification of NB identity. In addition, homeobox genes controlling the development of the D/V axis of the ectoderm including *ventral nervous system defective* (*vnd*) and *muscle segment homeobox* (*msh*) have also been shown to be involved in the formation and/or fate specification of proneural clusters and NBs (reviewed in Technau *et al.*, 2006). Thus, NB spatial identity is determined by a specific combination of D/V and A/P patterning genes expressed in the proneural clusters. Furthermore, each NB within a hemineuromere can be uniquely identified not only by the time of delamination and position within the subectodermal layer, but also by the expression pattern of specific molecular markers (Broadus *et al.*, 1995; Doe, 1992; reviewed in Technau *et al.*, 2006).

Following delamination, each NB divides asymmetrically in a stem cell mode to generate a larger NB cell and a smaller ganglion mother cell (GMC), which in turn divides once to produce a pair of post-mitotic daughter cells. These cells differentiate

into either neurons or glial cells. The majority of the NBs generate only neurons, which in turn assume different identities. However, there exist also neural precursor cells called glioblasts, which give rise exclusively to glial cells, as well as neuroglioblasts, progenitor cells that form both glial cells and neurons. Hence, some lineages are exclusively neuronal, some are exclusively glial and some include both neuronal and glial cell types.

Several lines of evidence suggest that in each hemineuromere, each NB gives rise to a unique cell lineage in an invariant temporal sequence (Bossing *et al.*, 1996; Higashima *et al.*, 1996; Novotny *et al.*, 2002; Pearson and Doe, 2003, 2004; Schmid *et al.*, 1999; Schmidt *et al.*, 1997). For instance, a large number of studies indicate that both spatial and temporal identity of NBs contribute to cell diversity within the CNS (reviewed in Pearson and Doe, 2004). It appears that, whereas NB spatial identity determines the type of cells generated (e.g. interneuron, motorneuron or glia), sequential changes in the activation of specific genes (known as temporal identity genes) in each NB determines the birth-order (i.e. early or late) of the cells of a given lineage (reviewed in Pearson and Doe, 2004).

Several reports have now identified at least four transcription factors temporally expressed in a characteristic sequence [Hunchback (Hb) → Krüppel (Kr) → POU domain protein (Pdm) → Castor (Cas)], which provide temporal NB specification during embryonic neurogenesis (Brody and Odenwald, 2000; Grosskortenhaus *et al.*, 2006; Isshiki *et al.*, 2001; Kambadur *et al.*, 1998; Novotny *et al.*, 2002; reviewed in Pearson and Poe, 2004). Thus, a combination of both spatial and temporal identity genes results in different neural cell types in each lineage. Furthermore, it has been proposed that the

interpretation of a particular combination of spatial and temporal cues by cell type identity genes sets off a specific differentiation program (e.g. serotonergic interneuron) (reviewed in Pearson and Doe, 2004).

NBs that are formed in different hemisegments at corresponding positions and times and express the same combination of molecular markers are known as serially homologous NBs. These homologs assume the same or very similar fate, giving rise to almost identical cell lineages (reviewed in Technau *et al.*, 2006). Thus, by the end of embryogenesis, a VNC hemineuromere comprises circa 400 cells, of which ~ 350 are neurons, and the rest corresponds to glial cells (Bossing *et al.*, 1996; Landgraf *et al.*, 1997; Schmid *et al.*, 1999; Schmidt *et al.*, 1996). VNC neurons can be divided into three types: interneurons, extending axons within the CNS to contact other neurons; motorneurons, projecting axons out into the periphery to innervate muscles; and neurosecretory neurons, which send projections either out into the periphery or into the sheath of the CNS to secrete neuropeptides and hormones into tissues and fluids (Campos-Ortega, 1993).

1.2.3. VNC midline

In addition to the progenitor cells deriving from the neuroectoderm, a smaller set of CNS progenitor cells originating from the mesectoderm are also involved in the generation of the VNC. These mesectodermal cells are found between the anlagen of the mesoderm and the neuroectoderm forming a single row on either side (reviewed in Klämbt *et al.*, 1996). When the mesoderm invaginates during gastrulation, both

mesectodermal rows come together along the ventral midline (reviewed in Klämbt *et al.*, 2001; Nambu *et al.*, 1993). The mesectodermal midline progenitors generate five types of clones: one glial and four neuronal lineages (Bossing and Technau, 1994). Thus, each segment or neuromere is formed by two mirror symmetrical hemineuromeres together with the progeny of the ventral midline. It has been shown that the midline cells are important for proper patterning of the ventral ectoderm (Chang *et al.*, 2001; Raz and Shilo, 1993) and mesoderm (Lüer *et al.*, 1997). In addition, they are required for formation of the CNS commissures (Klämbt *et al.*, 1991; Menne and Klämbt, 1994) and for differentiation of lateral neural cells (Kim *et al.*, 2007; Menne *et al.*, 1997).

1.2.4. Development of the brain hemispheres

Formation of the brain hemispheres by neural stem cells occurs in a similar manner to that of the VNC. Moreover, most of the genes involved in generation of the ventral ganglia have also been shown to be important during formation of the brain (Urbach and Technau, 2003a, 2003c; Urbach *et al.*, 2003; reviewed in Technau *et al.*, 2006; Urbach and Technau, 2004). While in the thorax and abdomen and somewhat in the gnathal segments the NBs form a relatively invariant, almost orthogonal subectodermal pattern, the brain presents higher regional NB diversification. This is mainly due to extensive morphogenetic movements that occur in the head anlagen during gastrulation (Younossi-Hartenstein *et al.*, 1996; reviewed in Technau *et al.*, 2006). The orthogonal expression of D/V and A/P genes appears to be less conserved in this region of the CNS (Urbach and Technau, 2003a). As a result, the occurrence of serially

homologous NBs in the brain hemispheres seems to be less evident, particularly in the ocular and labral neuromeres (reviewed in Urbach and Technau, 2003b). It has been suggested that inter-segmental diversity within the procephalic region and between the VNC and the brain may explain structural and functional differences within the brain and between the procephalic and ventral areas of the CNS (reviewed in Technau *et al.*, 2006; Urban and Technau, 2004).

There exist similarities and differences in neurogenesis between the brain and the VNC. For instance, in contrast to what is seen in the VNC, proneural genes in the pNR are expressed in larger ectodermal cell populations (Urbach *et al.*, 2003; Younossi-Hartenstein *et al.*, 1996; reviewed in Urbach and Technau, 2003b). Furthermore, it appears that at least in certain regions of the procephalic neuroectoderm, not one cell but a group of neighbouring cells within a proneural cluster can acquire the NB fate, presumably by reduced lateral inhibition (Urbach *et al.*, 2003; Younossi-Hartenstein *et al.*, 1996). Thus, a higher proportion of neuroectodermal cells are committed to a neural fate.

Similar to what is observed in the vNR, NB formation in the pNR occurs in a stereotypical spatio-temporal pattern from embryonic stage 8 to 11 (Urbach *et al.*, 2003). However, unlike in the vNR, NBs in the pNR do not appear to be generated in waves. Instead, they are constantly added during this embryonic period (Urbach *et al.*, 2003). Besides the two main mechanisms for generation of NBs previously mentioned, a third distinct mode has been observed within certain regions of the pNR. Here, some progenitor cells also divide perpendicular to the ectoderm generating a NB and an

epidermoblast (Urbach *et al.*, 2003). As in truncal hemisegments, each hemineuromere NB expresses a specific set of genes, suggesting that each NB acquires a unique identity (Urbach and Technau, 2003c). Genetic molecular studies have identified circa 100 NBs in each brain hemisphere (Urbach *et al.*, 2003; reviewed in Urbach and Technau, 2004).

1.2.5. Head midline cells

The visual system (optic lobe, larval eye), the stomatogastric nervous system as well as medial parts of the brain are formed by internalization of small populations of protocephalic neuroectodermal cells located along or close to the head midline (Dumstrei *et al.*, 1998; Urbach *et al.*, 2003; Younossi-Hartenstein *et al.*, 1996; reviewed in Urbach and Technau, 2004). Like their counterparts in the mesectoderm of the trunk, these head midline cells stay integrated in the surface ectoderm expressing proneural genes for an extended period of time (Dumstrei *et al.*, 1998; Younossi-Hartenstein *et al.*, 1996; reviewed in Urban and Technau, 2004). Moreover, to form each of the mentioned brain areas, these progenitor cells do not generate NBs by delamination. Instead they invaginate during stage 12-13 as coherent groups generating epithelial vesicles, some of which later on dissociate and are incorporated into the embryonic brain (Dumstrei *et al.*, 1998; Younossi-Hartenstein *et al.*, 1996). Interestingly, these neuroectodermal domains, as well as the truncal mesectoderm, behave similarly to the vertebrate neuroectoderm during the formation of neural tube, as all of them invaginate as coherent groups of cells.

1.3. Larval peripheral nervous system

The *Drosophila* larval peripheral nervous system (PNS) consists of sensory components formed during embryogenesis (Jan and Jan, 1993). Similar to what is observed in the CNS, the PNS in the trunk is segmentally organized (reviewed in Ghysen and Dambly-Chaudière, 1993). Moreover, like in CNS formation, neurogenesis in *Drosophila* PNS is regulated by activation of proneural genes in neural progenitors, N-Dl mediated lateral inhibition and positional information (reviewed in Ghysen and Dambly-Chaudière, 1993; Quan and Hassan, 2005).

Drosophila larvae present different types of sensory organs. These can be classified as mechanosensory, chemosensory (olfactory and gustatory), as well as chordotonal (propioceptive) receptors (Jan and Jan, 1993; reviewed in Lai and Orgogozo, 2004). In addition, there is also a pair of sense organs (known as Bolwig's organs) that connect to the protocerebrum and functions as larval photoreceptors.

1.3.1. Larval visual system

Unlike the complex adult visual system, *Drosophila* larval visual system has a simple stereotypic morphology. In flies, the larval visual system is made of two sets of small photoreceptor organs known as Bolwig's organs (BO). The name derives from the fact that they were first identified by Bolwig (1946) in larvae of *Musca domestica*. In *Drosophila*, each of these organs is composed of 12 cells, whose cell bodies are found in the dorsal pouch epithelium located bilaterally inside the larval head (Green *et al.*, 1993; Steller *et al.*, 1987). Each photoreceptor cell extends an axon, which together fasciculate

to form the Bolwig's nerve (BN, also known as larval optic nerve or LON). The LON extends posteriorly through the optic stalk to the ipsilateral brain hemisphere. There, it turns ventrally and projects through the optic lobe (OL), the target of the adult visual system, towards the larval optic center located in the central brain (Campos *et al.*, 1995).

1.3.1.1. Development of *Drosophila* larval photoreceptors

The OL and the BO derive from the *sine oculis* (*so*)-expressing posterior dorsolateral region of the embryonic procephalic ectoderm, the optic placode (Green et *al.*, 1993; Namba and Minden, 1999). Between stages 12 and 13, *tailless* (*tll*)-expressing cells within the optic placode invaginate as a coherent group, which subsequently detaches from the head ectoderm to form the OL anlagen (Campos *et al.*, 1995; Daniel *et al.*, 1999; Green *et al.*, 1993; Schmucker *et al.*, 1997). In contrast, photoreceptor precursors temporarily remain within the epithelial layer expressing the proneural gene *atonal* (*ato*), and thus defining the BO primordium (Campos *et al.*, 1995; Daniel *et al.*, 1999; Green *et al.*, 1993; Schmucker *et al.*, 1997). Shortly after, 3-4 *ato*-positive founder cells derived from the BO primordium are specified as primary precursors (Daniel *et al.*, 1999; Schmucker *et al.*, 1997; Sprecher *et al.*, 2007; Susuki and Saigo, 2000). Mediated by Spitz-epidermal growth factor receptor (EGFR) signalling, these BO pioneer cells recruit surrounding cells to become secondary precursors (Daniel *et al.*, 1999; Schmucker *et al.*, 1992; Susuki and Saigo, 2000). These cells are then incorporated into the larval eye to generate a complete BO of approximately 12 cells by stage 13-14 (Daniel *et al.*, 1999; Schmucker *et al.*, 1992; Susuki and Saigo, 2000). Around this time, both types of

precursor cells detach from the neuroectoderm, differentiate, and begin forming their axonal projections, maintaining their connection with the OL (Dumstrei *et al.*, 2002; Green *et al.*, 1993; Schmucker *et al.*, 1992, 1997). The transcription factors Glass (Gl) as well as the adhesion molecule Chaoptin are known to be expressed specifically in photoreceptor cells. The former has been shown to be required for their proper development (Krantz and Zipursky, 1990; Moses *et al.*, 1989; Van Vactor *et al.*, 1988).

Contact between the photoreceptor axons and the OL primordium occurs early in development, when both groups of cells are close to each other (Green *et al.*, 1993; Schmuker *et al.*, 1992; Steller *et al.*, 1987). It has been suggested that the growth of the LON is guided by axons of OL pioneer (OLP) cells (Campos *et al.*, 1995; Tix *et al.*, 1989). Moreover, it has been shown that during stage 13 at least one leading axon initiates its growth, followed by the joining of other axons as photoreceptor cells undergo axogenesis and fasciculation (Schmucker *et al.*, 1992, 1997).

First, the LON contacts a group of intermediate target cells (the corner OLP cells) (Campos *et al.*, 1995; Schmucker *et al.*, 1997; Tix *et al.*, 1989). At stage 14, during head involution, the BO begin moving anteriorly away from the optic lobe and the LON elongates, maintaining its contact with the OL primordium (Campos *et al.*, 1995; Holmes *et al.*, 1998; Schmucker *et al.*, 1997). It is has been suggested that BO movement is not only a consequence of this process but may also involve an active migratory process (Holmes *et al.*, 1998). Concomitant with its elongation, the LON connects with a different intermediate target, the central OLP neuron (Campos *et al.*, 1995; Green *et al.*, 1993; Holmes *et al.*, 1998; Schmucker *et al.*, 1992, 1997; Steller *et al.*, 1987; Tix *et al.*, 1989).

Shortly after, the LON is seen projecting toward its final target located within the central brain (Campos *et al.*, 1995; Schmucker *et al.*, 1992, 1997). By stage 16-17, both the BO and the LON have reached their final position (Green *et al.*, 1993; Holmes *et al.*, 1998). At this developmental point, the photoreceptor cells are already found at each side of the pharynx in a pocket of the cephalopharyngeal skeleton, whereas the OL primordium is incorporated to the larval brain (Campos *et al.*, 1995; Green *et al.*, 1993).

By the end of embryogenesis and throughout the entire larval stage, the larval photoreceptors express two types of the photopigment Rhodopsin in a non-overlapping manner. From the 12 photoreceptors, circa 8 cells express the green-absorbing Rhodopsin 6 (Rh6, λ_{max} = 508 nm) and the other 4 photoreceptors express the blue-absorbing Rhodopsin 5 (Rh5, λ_{max} = 437 nm) (Malpel *et al.*, 2002; Sprecher *et al.*, 2007). Interestingly, recent developmental studies indicate that the Rh5 photoreceptors derive from the primary precursor cells, whereas the secondary precursors give rise to the Rh6-expressing cells (Sprecher *et al.*, 2007). Moreover, it appears that differentiation of Rh5 cells requires the transcription factors *spalt* (*sal*) and *orthodenticle* (*otd*), while Rh6 photoreceptor subtype specification requires *seven-up* (*svp*) (Sprecher *et al.*, 2007).

1.3.1.2. Signal transduction and neurotransmitter expression in larval photoreceptors

In contrast to what is observed in vertebrate photoreceptors, stimulation of *Drosophila* photoreceptors by light causes plasma membrane depolarization (reviewed in Hardie, 2001; Hardie and Raghu, 2001). Change in *Drosophila* larval photoreceptor

membrane potential results in activation of the same phototransduction cascade as the one required for visual system function in *Drosophila* adults (Busto *et al.*, 1999; Hassan *et al.*, 2000). Photon absorption by Rhodopsin triggers activation of $G_{\alpha q}$ and phospholipase C (PLC). This causes the production of IP3 as well as diacylglycerol (DAG), and the subsequent activation of two classes of light-sensitive Ca^{2+} channels known as the transient potential receptor (TRP) and the TRP-like (TRPL) channels (reviewed in Hardie, 2001, 2003a; 2003b; 2007; Hardie and Raghu, 2001; Minke and Parnas, 2006). Several studies suggest that it is the DAG branch of the pathway and not that one comprising IP3 that is involved in *Drosophila* phototransduction (Acharya *et al.*, 1997; Chyb *et al.*, 1999; Hardie *et al.*, 2002, 2003; Leung *et al.*, 2008; Raghu *et al.*, 2000a; 2000b; reviewed in Raghu, 2006). Furthermore, accumulating evidence points to DAG and/or its metabolites (e.g. polyunsaturated fatty acids or PUFAs) as the molecules responsible for the opening of TRP/TRPL channels and depolarization of the photoreceptor cell, although the exact gating mechanism remains unknown (e.g. Chyb *et al.*, 1999; Hardie *et al.*, 2003; Leung *et al.*, 2008). Lastly, despite the fact that the nature of the neurotransmitter involved in larval visual system function requires further investigation, immunological studies indicate that the larval photoreceptors express choline-acetyltransferase (ChaT), (Malpel *et al.*, 2002; Yasuyama *et al.*, 1995), hence suggesting acetylcholine (Ach) as the larval photoreceptor neurotransmitter.

1.4. Neural interactions and generation of neuronal networks

Establishment of neuronal connections depends on normal development of neurites (i.e. axons and dendrites) as well as recognition of proper targets. Neurite development is a complex multi-step process involving: i) neurite formation, outhgrowth and guidance, ii) neurite branching and synapse formation, and iii) neurite maintenance and/or stabilization. Both instrinsic and extrinsic mechanisms are believed to regulate neurite morphogenesis as well as precise neuronal connectivity (reviewed in Parrish *et al.*, 2007; Urbanska *et al.*, 2008). Extrinsic or environmental cues include neuronal activity as well as intercellular communication by the action of cell-adhesion molecules and diffusible chemotropic molecules (reviewed in Parrish *et al.*, 2007). Intrinsic molecular mechanisms involve for instance the activity of transcription factors as well as small Rho family GTPases (reviewed in Urbanska *et al.*, 2008; Van Aelst and Cline, 2004).

It is worth mentioning that, unlike in the vertebrate CNS, the cell bodies of insect CNS neurons (as well as secondary NBs and GMCs in the larva) lie in the periphery, forming a cellular cortex. Moreover, insect CNS neurons are uni or monopolar and therefore do not form synapses on the cell bodies but instead they send monopolar neurite projections towards inner neuropils (reviewed in Prokop and Meinertzhagen, 2006). In these 'cell body-free areas', projections further differentiate into dendrites and distal axonal arbors, and synaptic connections are formed (e.g. Landgraf *et al.*, 2003a; Yonoussi-Hartenstein, *et al.*, 2003, 2006; reviewed in Prokop and Meinertzhagen, 2006). In addition, peripheral sensory neurons also project to these neuropils. An ensheathing layer of glial cells surrounds each neuropil, which is thought to create a favourable ionic

environment for synaptic transmission (Yonoussi-Hartenstein, *et al.*, 2003, 2006; reviewed in Prokop and Meinertzhagen, 2006).

The neuropil of *Drosophila* VNC is organized into longitudinal columnar dorsal, intermediate, and ventral compartments (reviewed in Prokop and Meinertzhagen, 2006). The ventral compartment receives sensory terminals whereas the dorsal column contains predominantly neurites of motorneurons (e.g. Grueber *et al.*, 2007; Landgraf *et al.*, 1997, 2003a; Landgraf and Thor, 2006; Schrader and Merrit, 2000; reviewed in Prokop and Meinertzhagen, 2006). The intermediate compartment is composed mostly of interneuronal neurites (Landgraf *et al.*, 2003a, 2003b; Vömel and Wegener, 2008; reviewed in Prokop and Meinertzhagen, 2006). In the brain, neuropil compartments are defined by glial septa (Yonoussi-Hartenstein, *et al.*, 2003, 2006). As suggested for some *Drosophila* adult CNS compartments such as the mushroom bodies (MB) and central complex (CX) (reviewed in Heisengerg, 2003; Strauss, 2002), it has been proposed that larval neuropil compartments might represent functional subdivisions of the brain (Yonoussi-Hartenstein, *et al.*, 2003, 2006).

1.4.1. Axonal morphogenesis

Firstly, the processes of axonal growth and pathfinding require cytoskeletal rearrangements, and are believed to be steered by selective adhesion as well as the spatial distribution of attractive and repulsive cues (reviewed in Araújo and Tear, 2003; Luo, 2002; Tessier-Lavigne and Goodman, 1996). These cues can be either gradients of diffusible molecules or cell-associated cues. The latter involves guidepost cells, which

may represent undifferentiated neurons, glial cells or other fibers, displaying surface-bound molecules which mediate signalling upon contact between the growth cone and the substrate. Identification of similar molecules in invertebrates and vertebrates indicate that at least some neurite pathfinding mechanisms have been maintained throughout evolution (reviewed in Dickson, 2002; Zou and Lyuksyutova, 2007). The main diffusible ligand/receptor pairs include members of the Slit/Roundabout (Robo), Semaphorin/Plexin/Neuropilin, and Netrin/DCC families, whereas membrane-bound ligand/receptor pairs involves members of the Semaphorin family, the Ephrins(Eph)/Eph receptors and members of the diverse families of cellular adhesion molecules (CAMs). More recently, several studies have shown that morphogens, growth factors that act in development to specify cell fate, may also possess axon guidance function. These include members of the bone morphogenetic protein (BMP), Hh, and Wingless (Wg/Wnt) families (reviewed in Charron and Tessier-Lavigne, 2005; Zou and Lyuksyutova, 2007).

1.4.2. Morphogenesis of dendrites

As both axons and dendrites constitute neuronal processes, it is reasonable to think that the mechanisms underlying axonal growth, guidance and branching may also be applicable to morphogenesis of dendrites. Indeed, the great majority of environmental cues that regulate axonal morphogenesis have been shown to play analogous roles in dendrites of both invertebrates and vertebrates (i.e. Furrer *et al.*, 2003; Polleaux *et al.*, 1998, 2000; Whitford *et al.*, 2002; reviewed in Grueber and Jan, 2004; Jan and Jan, 2003; Miller and Kaplan, 2003; Urbanska *et al.*, 2008; Van Aelst and Cline, 2004). Moreover,

recent studies mainly in vertebrates indicate that spontaneous electrical activity early in development can affect both axonal pathfinding and dendritic growth before synapse formation. One possible mechanism proposed for such effect is the paracrine action of neurotransmitters (reviewed in Spitzer, 2006).

1.4.3. Small Rho GTPases and neurite development

A common feature regarding both axonal and dendritic formation is that extracellular signals that regulate these events must be interpreted by cell surface molecules to activate downstream signalling pathways, eventually regulating the dynamics of the underlying cytoskeleton (reviewed in Govek *et al.*, 2005; Luo, 2002). Previous studies have shown that members of the Rho family of small GTPases, such as RhoA (Ras homologous member A), Rac (Ras-related C3 botullinum toxin substrate), and Cdc42 (cell division cycle 42) are important regulators of the cytoskeleton dynamics in both vertebrates and invertebrates (reviewed in Govek *et al.*, 2005).

In *Drosophila*, there are 3 Rac-like proteins: Rac1, Rac2, both of which are highly related to the mammalian counterparts Rac1 and Rac2, and Mig-2-like (Mtl), more related to *C. Elegans* Mig-2 (reviewed in de Curtis, 2008). Interestingly, small GTPases have been shown to be involved in many aspects of neuronal morphogenesis including neurite outgrowth and guidance (e.g. Hakeda-Suzuki *et al.*, 2002; Kim *et al.*, 2002; Lee *et al.*, 2003; Ng *et al.*, 2002; Matsuura *et al.*, 2004; reviewed in Govek *et al.*, 2005). At the same time, many extracellular cues/receptors known to be involved in neuronal morphogenesis have been linked directly or indirectly to regulation of small GTPase

activity (e.g. Fan *et al.*, 2003; reviewed in de Curtis, 2008). In accordance with these reports, further observations indicate that small Rho GTPases play a role as mediators of the extrinsic signals involved in neuronal morphogenesis (e.g. Li *et al.*, 2002; Rosso *et al.*, 2005; Sin *et al.*, 2002; Srahna *et al.*, 2006; reviewed in Govek *et al.*, 2005; Urbanzaka *et al.*, 2008).

1.4.4. Target recognition and synapse formation

Neurite growth and guidance to correct regions is followed by recognition of proper targets and synapse formation. Regarding the mechanisms regulating these two latter steps, it is believed that both anterograde and retrograde signals are involved in these processes. Considerable effort has been focused on identifing 'synaptotrophins' that might mediate this trans-synaptic exchange of information. A growing list of mechanisms involved in this process derives from studies in vertebrates and invertebrates, particularly *Drosophila* larval neuromuscular junctions (NMJs) (reviewed in Cline and Haas, 2007; Collins and DiAntonio, 2007). These reports suggest that at least some of the molecules and pathways shown to regulate neurite growth and guidance, especially cell-adhesion molecules and diffusible factors also function as trans-synaptic signals during synapse formation and maturation (reviewed in Cline and Hass, 2007; Collins and DiAntonio, 2007; di Curtis, 2008).

1.4.5. Neurite stabilization and synapse maintenance

The generation of initial connections does not represent the end of the morphological development of neurons. The maturation, function, and stability of neuronal connections are dynamically regulated not only during development but also as a result of neuronal activity and experience. Throughout their life, neurons maintain and refine their connections by growing and pruning neuronal processes, adding and removing synapses, as well as changing synaptic size, shape and function. The maintenance and/or refinement of synaptic connections and the structures that form them (axons and dendrites) are crucial not only for proper wiring of nervous system during development but also for its function at a specific developmental stage of the organism (reviewed in Luo, 2002).

Many of the mechanisms and signalling pathways that regulate neurite outgrowth, guidance and synapse formation during early development are thought to be also involved in this stabilization phase (reviewed in Govek *et al.*, 2005; Luo, 2002; Parrish *et al.*, 2007). For instance, studies in vertebrates show that activation of neurotransmitter receptors is translated into modulation of Rho GTPase activity ultimately affecting dendritic growth (Li *et al.*, 2002; Sin *et al.*, 2002; reviewed in Van Aelst and Cline, 2004; Wong and Ghosh, 2002). In addition, evidence exists indicating that neuronal activity can regulate signalling pathways activated by environmental cues and known to affect dendritic morphology (reviewed in Van Aelst and Cline, 2004; Wong and Ghosh, 2002). Furthermore, several studies suggest that Rho GTPases play crucial roles in spine morphogenesis and maintenance (reviewed in Govek *et al.*, 2005). In *Drosophila*, control

of axonal branching stability by modulation of RhoA signalling pathway in mature MB neurons represents another example (Billuart *et al.*, 2001).

1.5. 'Classical' neurotransmitter systems in *Drosophila*

Drosophila 'classical' neurotransmitters and the molecular mechanisms mediating their function are highly conserved with those of higher organisms. Known fly 'classical' neurotransmitters include γ-aminobutyric acid (GABA), glutamate, Ach, adenosine, dopamine (DA), serotonin (5-hydroxytryptamine, 5-HT), and histamine (HA), all of which signal via both metabotropic and ionotropic receptors. It is important to note that, unlike vertebrates, *Drosophila* does not possess noradrenergic/adrenergic system. Instead, it appears that their corresponding functions in invertebrates including the fruit fly are carried out by the monoamines tyramine (TA) and octopamine (OA) respectively (reviewed in Blenau and Baumann, 2001; Monastirioti, 1999; Nichols, 2006).

5-HT, DA, TA, OA and HA derive from the metabolism of amino acids and therefore are known as biogenic amines (BAs) (Fig. 1.1). BAs have been shown to be important signalling molecules mediating a diverse range of physiological, cellular and behavioural processes (reviewed in Monastirioti, 1999; Nässel, 1996; Restifo and White, 1990). In insects and other invertebrates, BAs may act as neurotransmitters and/or neuromodulators, or they may also be released into the hemolymph and function as neurohormones at peripheral targets (reviewed in Monastirioti, 1999; Nässel, 1996).

BAs in *Drosophila* appears to be expressed in distinct group of neurons (mostly interneurons), which are widely distributed within the CNS in stereotypical pattern. This

broad distribution of BA expression is compatible with the involvement of aminergic neurons in a great variety of processes (e.g. Budnik and White, 1988; Hamasaka and Nässel, 2006; Lundell and Hirsh, 1994; Vallés and White, 1988; Vömel and Wegener, 2008; reviewed in Monastirioti, 1999). Until recently, due to their extensive branching, determining the exact spatial location of aminergic neurite arborizations as well as identifying pre- and post-synpatic partners within the CNS neuropils represented a very difficult task. However, recent advances in molecular genetics together with three-dimensional mapping reconstruction of aminergic neuronal projections have greatly facilitated the study of aminergic neuronal networks (e.g. Landgraf *et al.*, 2003b; Sykes and Condron, 2005; Vömel and Wegener, 2008).

1.5.1. Serotonin: metabolism and neuronal handling

The name serotonin derives from its original isolation and purification by Rapport and colleagues in 1948 as a potent serum vasoconstrictor in vertebrates (sero = serum, tonin = tone) (Rapport *et al.*, 1948a, 1948b; reviewed in Mohammad-Zadeh *et al.*, 2008; Whitaker-Azmitia, 1999). Serotonin is synthesized in two steps by the action of two different enzymes (Fig. 1.1). The first metabolic reaction, which constitutes the limiting-rate step of 5-HT biosynthesis, is carried out by the enzyme tryptophan hydroxylase (known as TPH in mammals and TRH in *Drosophila*). In mammals, two distinct tryptophan hydroxylases have been cloned. One, which is coded by the *Tph1* gene, is found in the peripheral tissues, whereas the other one is coded by the *Tph2* gene and is expressed exclusively in the brain (Walther *et al.*, 2003; Zhang *et al.*, 2004).

Similarly in *Drosophila*, studies have shown the existence of two enzymes capable of hydroxylating tryptophan. In addition to the dual-function enzyme *Drosophila* tryptophan-phenylalanine hydroxylase (DTPH) (Neckameyer and White, 1992), recent studies have shown the presence of a second enzyme in *Drosophila*, DTRH, which presents extensive structural similarity to the mammalian TPH2 (Coleman and Neckameyer, 2005). Neckameyer *et al.* (2007) have shown that DTPH (and referred in this work to as DTPHu) functions *in vivo* as phenylalanine hydroxylase, in addition to its action as peripheral *Drosophila* TPH. On the other hand, DTRH (referred also as to DTRHn) is expressed and has TPH function in *Drosophila* neurons, representing the homolog of TPH2 (Neckameyer *et al.*, 2007). The second step in 5-HT biosynthesis is catalyzed by DOPA-decarboxylase (Ddc), which converts 5-hydroxytryptophan into 5-hydroxytryptamine (5-HT) (reviewed in Monastirioti, 1999).

Once synthesized, this monoamine is stored and concentrated in synaptic vesicles by the action of vesicular monoamine transporters (VMATs) (reviewed in Liu and Edwards, 1997). Similar to mammals, a recent report has demonstrated that *Drosophila* 5-HT neurons express VMATs (dVMATs) (Greer *et al.*, 2005). The primary mechanism for termination of 5-HT signalling is by re-uptake (reviewed in Amara and Kuhar, 1993). 5-HT uptake is performed by the Na^+/Cl^- dependent plasma membrane 5-HT transporter (SERT) (reviewed in Amara and Kuhar, 1993; Mohammad-Zadeh *et al.*, 2008). In *Drosophila*, a 5-HT transporter (dSERT) has been identified (Corey *et al.*, 1994; Demchyshyn *et al.*, 1994), and it is expressed only in 5-HT neurons (Demchyshyn *et al.*, 1994; Novotny *et al.*, 2002). Free cytoplasmic serotonin not stored in vesicles is

metabolized. In vertebrates, the primary route constitutes deamination by the enzyme monoamine oxidase (MAO) in the mitochondrial membrane, generating the inert metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) (reviewed in Mohammad-Zadeh *et al.*, 2008). In insects however, 5-HT and other BAs appear to be inactivated mainly by N-acetylation, O-sulphation or β-alanyl conjugation (reviewed in Sloley, 2004).

1.5.2. *Drosophila* larval 5-HT neurons: development and distribution

Studies performed in different segmented invertebrates reveal that the general distribution of serotonin cells is evolutionary conserved (Lundell and Hirsh, 1994; Nässel, 1996; Schmid *et al.*, 1999; Taghert and Goodman, 1984; Vallés and White, 1986; 1988), suggesting a common developmental pathway. As mentioned before, the development of neuronal lineages in insects including the one giving rise to 5-HT neurons, as well as the genes required for neural specification and differentiation, have been extensively investigated in the thoracic and abdominal segments of the ventral ganglion (e.g. Bossing *et al.*, 1996; Dittrich *et al.*, 1997; Lee and Lundell, 2007; Lundell *et al.*, 1996; Lundell and Hirsh, 1998; Novotny *et al.*, 2002; Schmid *et al.*, 1999; Taghert and Goodman, 1984). Moreover, developmental studies in *Drosophila* indicate that many of the genes involved in neuronal differentiation of VNC 5-HT cells are not expressed in serotonergic neurons located in the brain lobes, suggesting that their development may be differentially regulated (Lundell *et al.*, 1996; Lundell and Hirsh, 1998).

In *Drosophila*, Lundell *et al.* (1996) first demonstrated that the two 5-HT cells located in each VNC hemisegment derive from a particular NB, the NB7-3 (seventh row,

third column), similar to what was previously observed in grasshopper CNS (Taghert and Goodman, 1984). *Drosophila* NB7-3 delaminates during S4-S5 (Bossing *et al.*, 1996; Doe, 1992). It has been shown that this NB can be uniquely identified by co-expression of the patterning genes *engrailed* (*en*) and *huckebein* (*hkb*) (Doe, 1992), shown to be important for normal development of the VNC 5-HT cells (Lundell *et al.*, 1996). Other segment polarity genes involved in NB7-3 formation and development of VNC 5-HT cells are *wg* as well as other members of *wg* signalling pathway including *hh*, *patched* (*ptd*), *naked cuticule* (*nkd*) and *gsb* (Desphande *et al.*, 2001; Matsuzaki and Saigo, 1996; Patel *et al.*, 1989).

Several studies have shown that mitotic divisions of NB7-3 and of the NBs subsequently produced give rise to 3 GMCs (GMC1, GMC2, and GMC3, named according to their birth order), which in turn generate 4 neurons (Fig. 1.3) (Bossing *et al.*, 1996; Dittrich *et al.*, 1997; Higashijima *et al.*, 1996; Isshiki *et al.*, 2001; Karcavich and Doe, 2005; Lee and Lundell, 2007; Lundell and Hirsh, 1998; Lundell *et al.*, 2003; Novotny *et al.*, 2002; Schmid *et al.*, 1999). NB7-3 as well as all its progeny express *eagle* (*eg*), a zinc finger transcription factor that shares homology with steroid receptor superfamily members (Higashijima *et al.*, 1996; Lee and Lundell, 2007; Rothe *et al.*, 1989). Nevertheless, each of the cells of the NB7-3 lineage can be individually distinguished from one another by differential expression of molecular markers.

In addition to NB7-3, only GMC1 and its progeny express Hb (Karcavich and Doe, 2005; Novotny *et al.*, 2002; Lee and Lundell, 2007; Lundell *et al.*, 2003). Division of GMC1 generates the more medial of the two 5-HT cells (EW1), and one motorneuron

(GW), which is distinguished by high expression levels of the Zinc-finger/homeodomain protein Zfh-1(Isshiki *et al.*, 2001; Karcavich and Doe, 2005; Lee and Lundell, 2007; Lundell *et al.*, 2003; Novotny *et al.*, 2002). In contrast, both GMC2 and GMC3 and their progeny express Zfh-2 (Karcavich and Doe, 2005; Lundell *et al.*, 2003; Novotny *et al.*, 2002). GMC2 division produces the more lateral 5-HT neuron (EW2) and a sister cell that undergoes apoptosis mediated by Notch signalling (Karcavich and Doe, 2005; Lundell *et al.*, 2003; Novotny *et al.*, 2002). GMC3 gives rise to the most lateral interneuron (EW3), which has been shown to express the neuropeptide corazonin (CRZ) (Karcavich and Doe, 2005; Lee and Lundell, 2007; Lundell *et al.*, 2003; Novotny *et al.*, 2002). The mechanism by which EW3 is born remains somewhat controversial. Whereas some studies have proposed that GMC3 divides canonically to give rise to both EW3 and a sister cell that undergoes Notch-mediated apoptosis (Lundell *et al.*, 2003; Novotny *et al.*, 2002), there is also evidence that suggests that GMC3 may directly differentiate into EW3 (Karcavich and Doe, 2005). Finally, it appears that NB7-3 undergoes apoptosis after generating GMC3 (Karcavich and Doe, 2005).

Thus, the NB7-3 lineage is composed of 3 interneurons: 2 serotonergic neurons, one of which expresses Zfh-2, and 1 corazonergic neuron. In addition, this NB generates a motorneuron, which expresses Zfh-1 (Bossing *et al.*, 1996; Dittrich *et al.*, 1997; Higashijima *et al.*, 1996; Isshiki *et al.*, 2001; Karcavich and Doe, 2005; Lundell and Hirsh, 1998; Lee and Lundell, 2007; Lundell *et al.*, 2003; Novotny *et al.*, 2002; Schmid *et al.*, 1999). It is worth noting that these molecular markers are not expressed at the same time during development. In the interneurons for instance, it appears that Eg expression

declines at the end of embryogenesis, when neurotransmitter expression begins (Lee and Lundell, 2007; Lundel and Hirsh, 1998; Lundell *et al.*, 2003). Zfh-2 expression in the corazonergic neuron appears to persist into larval stages (Lundell *et al.*, 2003).

Besides differential expression of molecular markers, the interneurons and the motorneuron of the NB7-3 lineage differ in their axonal projection patterns. The motorneuron projects an axon ipsilaterally, which exits the CNS via the intersegmental nerve to innervate the muscles. In contrast, the axonal projections of the interneurons cross the midline to the contralateral side of the VNC via the posterior commissure and bifurcate in both anterior and posterior directions (Bossing *et al.*, 1996; Dittrich *et al.*, 1997; Higashijima *et al.*, 1996; Landgraf *et al.*, 2003b; Schmid *et al.*, 1999).

Besides NB7-3, *eg* has been shown to be expressed in other 3 NBs (NB2-4, NB3-3, and NB6-4) (Higashijima *et al.*, 1996) and transiently in embryonic gonads (Rothe *et al.*, 1989). Interestingly, several studies have demonstrated that *eg* mutants display the correct number of NB7-3 progeny (Dittrich *et al.*, 1997; Higashijima *et al.*, 1996; Lee and Lundell, 2007; Lundell and Hirsh, 1998), but the number of 5-HT-expressing cells in the VNC is reduced and have abnormal projections (Dittrich *et al.*, 1997; Higashijima *et al.*, 1996; Lundell and Hirsh, 1998). These observations indicate that *eg* is required for terminal differentiation of the two more medial interneurons into 5-HT-expressing neurons.

Additional studies aimed at elucidating the mechanisms controlling serotonergic differentiation include those of Couch *et al.* (2004). Re-uptake of released 5-HT by dSERT is crucial for 5-HT neuronal function and represents one of the earliest steps in

neuronal differentiation. The authors demonstrate that expression of dSERT precedes that of 5-HT and occurs right after midline crossing (Couch *et al.*, 2004). In addition, the authors report that both *robo2/3* and *eg* mutants show loss of dSERT activity and/or expression. Furthermore, in *robo2/3* mutants, Eg expression is missing in cells lacking dSERT activity, and many cells do not synthesize 5-HT (Couch *et al.*, 2004). These findings, together with results of rescue experiments suggest that *robo2/3* function in the same genetic pathway as *eg* to positively regulate serotonergic neuronal differentiation (Couch *et al.*, 2004).

The distribution pattern of *Drosophila* larval 5-HT neurons and their projections have been examined relying on immunohistochemical studies (Chen and Condron, 2008; Hamaska and Nässel, 2006; Landgraf *et al.*, 2003b; Lundell and Hirsh, 1994; Mukhopadhyay and Campos, 1995; Vallés and White, 1986; 1988; Vömel and Wegener, 2008). 5-HT-immunoreactive cells can be first seen during late embryogenesis and comprise about 100 neurons (Fig 1.2), most of them corresponding to interneurons (Vallés and White, 1986; 1988; Vömel and Wegener, 2008). During the entire larval stage, their cell number, location, and main projections remain mostly invariant (Chen and Condron, 2008, Vallés and White, 1986; 1988).

In each larval brain lobe, around 13 5-HT-expressing neurons are distributed in four neuronal clusters: 3 anterior medial protocerebrum 1 (SP1) neurons, 4 anterior medial protocerebrum 2 (SP2) neurons, 2 (lateral protecerebrum 1) LP1 neurons, and 3-4 inferior medial protocerebrum (IP) neurons (Fig. 1.2) (Vallés and White, 1986; 1988). In the ventral ganglion of *Drosophila* larva, about 58 neurons form 14 bilaterally

symmetrical clusters, arranged in a reiterated segmental pattern (Fig. 1.2). Nevertheless, strict serial homology between VNC segments is missing. In the SE region, there are 2 cells in each of the first two hemisegments and 3 cells in the third hemisegment. In the T and A region, there is a pair of 5-HT cells in each hemisegment, except for T1 (first thoracic segment) which has 3 cells per hemisegment, and A8 (the last abdominal segment), which present only one neuron per hemisegment (Vallés and White, 1986; 1988; Vömel and Wegener, 2008).

In regards to serotonergic processes, morphological studies show that larval serotonergic neurons send projections and extensive arborizations to many neuropil regions in both the brain and the ventral ganglion (Fig. 1.2) (Chen and Condron, 2008; Hamasaka and Nässel, 2006; Landgraf *et al.*, 2003b; Lundell and Hirsh, 1994; Mukhopadhyay and Campos, 1995; Vallés and White, 1988; Vömel and Wegener, 2008). In the brain, 5-HT-immunoreactive varicosities can be seen covering most of the midbrain, and 3 main transverse 5-HT fiber tracts interconnect both hemispheres (Vallés and White, 1988). In the VNC, 5-HT neurons of a given hemisegment extend ipsilateral projections (Chen and Condron, 2008). In addition, they send neurites to the contralateral hemisegment. There, 5-HT projections bifurcate and form extensive intrasegmental arborizations, innervating the whole neuropil (Chen and Condron, 2008; Lundell and Hirsh, 1994; Sykes and Condron, 2005; Vallés and White, 1986; Vömel and Wegener, 2008). In addition, Vallés and White (1988) observed longitudinal fibers within the VNC. Although obscured by the profuse intrasegmental arborizations, the authors suggested that these longitudinal fibers could represent not only projections deriving from

the brain lobe neurons, but also intersegmental VNC projections (Vallés and White, 1988).

Over the last few years, some of the putative targets and/or synaptic partners of the larval 5-HT neurons have been revealed. For instance, it has been shown that the LON contacts 5-HT processes found in the larval optic center and is required for their development (Mukhopadhyay and Campos, 1995). Other immunohistochemical studies have demonstrated the existence of serotonergic processes in the larval antennal lobe (Python and Stocker, 2002; Roy *et al.*, 2007). In addition, 5-HT projections emerging from the larval brain are found innervating the ring gland, the larval endocrine organ (reviewed in Monastirioti, 1999). Finally, immunohistochemical analyses indicate that 5-HT-expressing peripheral nerves innervate the proventriculus, the midgut and the pharyngeal muscles, constituting also putative targets of the larval 5-HT neurons (Vallés and White, 1988; reviewed in Monastirioti, 1999).

1.5.3. *Drosophila* 5-HT receptors

To date, at least 14 different mammalian 5-HT receptor subtypes have been identified (reviewed in Nichols and Nichols, 2008). On the other hand, only 4 *Drosophila* 5-HT receptor subtypes have been cloned so far. These are the 5-HT1A_{Dro}, 5-HT1B_{Dro}, 5-HT2$_{Dro}$, and 5-HT7$_{Dro}$ receptors, which share considerable sequence similarity with the mammalian 5-HT1A, 5-HT2, and 5-HT7 receptors respectively (reviewed in Blenau and Baumann, 2001; Tierney, 2001). All of them belong to the G protein-coupled receptor (GPCR) superfamily and have different signalling properties

(reviewed in Tierney, 2001). Regarding the expression pattern of *Drosophila* 5-HT receptors, *in situ* hybridization studies have previously shown that all 5-HT_{Dro} receptors are expressed in the embryonic CNS in distinct patterns (Colas *et al.*, 1995; Saudou *et al.*, 1992). Moreover, Northern blot analyses revealed that they are also expressed in larval and adult stages (Colas *et al.*, 1995; Saudou *et al.*, 1992).

1.5.3.1. 5-HT1A_{Dro} receptors

5-HT1A_{Dro} receptors are believed to be orthologs of the mammalian 5-HT1A subtype. They have shown to inhibit adenylate cyclase (AC) as well as activate PLC (Saudou *et al.*, 1992). Regarding their expression pattern, the only information available about 5-HT1A_{Dro} subtype derives from the aforementioned *in situ* studies (Saudou *et al.*, 1992) and a more recent study in adult flies (Yuan *et al.*, 2006). Saudou *et al.* (1992) reported that 5-HT1A_{Dro} expression can be seen during late embryogenesis and is restricted to the CNS. In each abdominal segment this receptor subtype is expressed in a group of 6-8 cells located on the ventral region of the embryonic midline. Based on their position, the authors suggested that these cells might correspond to the ventral unpaired median (VUM) motorneurons (Klämbt *et al.*, 1991; Saudou *et al.*, 1992). In the embryonic thoracic segments, 5-HT1A_{Dro} mRNA was found not only in midline cells but also in cells lateral to them (Saudou *et al.*, 1992). In adults, *in situ* hybridization studies performed by Yuan *et al.* (2006) indicate that 5-HT1A_{Dro} receptors are largely expressed in MB. To date, no data is available regarding specific 5-HT1A_{Dro} expression pattern in the larvae.

1.5.3.2. 5-HT1B$_{Dro}$ receptors

Similar to the 5-HT1A$_{Dro}$ subtype, 5-HT1B$_{Dro}$ receptors are also believed to be orthologs of the 5-HT1A subtype found in mammals, and to inhibit AC as well as activate PLC (Saudou *et al.*, 1992). Interestingly, 5-HT1A$_{Dro}$, and 5-HT1B$_{Dro}$ share 84.3% overall protein sequence homology. Their genes are located within the same region on the right arm of the second chromosome (Saudou *et al.*, 1992). Based on these observations, it has been suggested that these genes most likely derive from a recent gene duplication (Saudou *et al.*, 1992).

In regards to the 5-HT1B$_{Dro}$ subtype expression pattern, Saudou and collaborators observed mRNA expression during late embryogenesis in lateral rows along each side of the VNC midline and low level mRNA expression in VNC midline cells (Saudou *et al.*, 1992). The authors also suggested that the lateral cells might correspond to motorneurons previously identified in that position by Sink and Whitington, (1991) (Saudou *et al.*, 1992). A more recent report has shown 5-HT1B$_{Dro}$ expression in larval and adult CNS (Yuan *et al.*, 2005). In the larva, this receptor is expressed in several regions in both brain hemispheres and VNC, including the OL and the midline. Interestingly, the authors observed expression of 5-HT1B$_{Dro}$ in the LNv (Yuan *et al.*, 2005). In adults, besides 5-HT1B$_{Dro}$ expression in this group of neurons and in the OL, 5-HT1B$_{Dro}$ is mainly expressed in MB, dorsal giant interneurons, pars intercerebralis neurons, and 5-HT neurons in the SE ganglion (Yuan *et al.*, 2005).

1.5.3.3. 5-HT2$_{Dro}$ receptors

The 5-HT2$_{Dro}$ receptor subtype presents ligand binding profiles similar to its mammalian counterparts, the 5HT2 receptor family (Colas *et al.*, 1995). Although the signalling properties of this particular *Drosophila* receptor subtype have not yet been determined, it is feasible that it resembles that of the mammalian 5-HT2 receptors, which primarily activate PLC (reviewed in Tierney, 2001; Nichols and Nichols, 2008).

Regarding 5-HT2$_{Dro}$ expression, *in situ* hybridization studies in early embryos reported that mRNA of this receptor subtype was expressed in the presumptive ectoderm in a segmental pattern, similar to that of the pair-rule gene *fushi tarazu* (*ftz*) (Colas *et al.*, 1995). Later on in embryogenesis as well as in larval stage, the expression of 5-HT2$_{Dro}$ mRNA was restricted to a pair of cells per VNC neuromere (Colas *et al.*, 1995). More recently, Nichols (2007) studied the expression pattern of this receptor by GAL4-mediated β-galactosidase staining. He detected putative 5-HT2$_{Dro}$ expression during early 3rd instar larva in specific brain areas, particularly in medial regions, within commissures connecting the brain hemispheres, and in a small group of cells within the developing OL (Nichols, 2007). Furthermore, this study reported that 5-HT2$_{Dro}$ expression pattern changes during this larval stage and pupal stage (Nichols, 2007). The central brain expression gradually disappears, and a circle-like staining appears at the base of the OL (Nichols, 2007). In the adult, it appears that 5-HT2$_{Dro}$ is highly expressed within the ellipsoid body, the OL, the protocerebrum, in the antennal globe glomeruli, and in gustatory neurons of the SE ganglia (Nichols, 2007).

1.5.3.4. 5-HT7$_{Dro}$ receptors

Like the mammalian 5-HT7 receptors, the 5-HT7$_{Dro}$ receptor subtype is positively coupled to AC (Witz *et al.*, 1990). To date, no data is available regarding the expression pattern of 5-HT7$_{Dro}$ in specific cells or tissues besides the observations made by Saudou and colleagues (1992). These early *in situ* studies in embryos showed a similar 5-HT7$_{Dro}$ mRNA pattern to that of 5-HT1B$_{Dro}$, but the rows of cells in the VNC expressing 5-HT7$_{Dro}$ appear to have a more medial location, possibly also corresponding to motorneurons (Saudou *et al.*, 1992).

1.5.4. Functional roles of 5-HT neurons

In the majority of the animal kingdom, serotonin can act as a neurotransmitter, neuromodulator or neurohormone (reviewed in Weiger, 1997). Accordingly, this BA has been shown to be involved in regulation of a great variety of biological processes in both invertebrates and vertebrates (reviewed in Monastirioti, 1999; Nichols, 2006; Nichols and Nichols, 2008; Weiger, 1997). The broad range of 5-HT roles is most likely due to its widespread distribution as well as the activation of several 5-HT receptor subtypes differentially expressed and coupled to different intracellular signalling pathways (Monastirioti, 1999; Nichols and Nichols, 2008). Interestingly, some 5-HT effects including regulation of aggression, learning and memory, circadian entrainment, and sleep appear to be conserved between flies and mammals (reviewed in Nichols, 2006; Nichols and Nichols, 2008). Furthermore, several studies have demonstrated that the

linkage between 5-HT and drug abuse effects can also be addressed in the fly (reviewed in Nichols, 2006).

In *Drosophila* larva for instance, in addition to a suggested modulatory role in neuroendocrine activity (Vallés and White, 1988; reviewed in Monastirioti, 1999), 5-HT neurons appear to be implicated in regulation of olfactory processing (Neckameyer *et al.*, 2007; Python and Stocker, 2002). Recent studies have also proposed a modulatory effect of 5-HT neurons in larval feeding behaviour (Neckameyer *et al.*, 2007), consistent with a previous observations of 5-HT-expressing fibers innervating pharyngeal muscles, proventriculus and the midgut (Vallés and White, 1988). A developmental study using 3D reconstruction techniques and immunohistochemistry has suggested an autocrine/paracrine autoregulatory function of 5-HT in serotonergic varicosity density in larval VNC neuropils (Sykes and Condron, 2005). The effect of 5-HT appears to be stage-dependent, with 2nd and 3rd instar VNCs being more sensitive than 1st instar larval VNC (Sykes and Condron, 2005).

Several reports indicate that 5-HT increases heart rate at the larval, pupal and adult stage (Dasari and Cooper, 2006; Neckameyer *et al.*, 2007; Nichols, 2006; Zornik *et al.*, 1999). In *Drosophila* adults, 5-HT neurons appear to regulate not only feeding behaviour (Neckameyer *et al.*, 2007) but also insulin signalling and organismal growth (Kaplan *et al.*, 2008), locomotor behaviour (Neckameyer *et al.*, 2007), aggression (Dierick and Greenspan, 2007), and reproductive function (Lee *et al.*, 2001). As well, a recent report has demonstrated that 5-HT neuronal function is critical for place memory formation in flies (Sitaraman, *et al.*, 2008).

In addition, several studies focused on investigating functional aspects of *Drosophila* 5-HT receptors in adult flies have greatly contributed to the elucidation of 5-HT roles as well as the underlying mechanisms. As defined for 5-HT, the requirement of specific 5-HT receptors subtypes in certain biological processes appears to be well conserved between flies and vertebrate systems.

Regarding 5-HT1B$_{Dro}$ for instance, the results of genetic manipulations of this receptor as well as of 5-HT levels have shown a modulatory role of 5-HT in circadian activity (Yuan *et al.*, 2005), similar to what has been previously observed for 5-HT1-like receptors in vertebrates (Horikawa *et al.*, 2000; Smart and Biello, 2001). Like in mammalian systems (Kennaway and Moyer, 1998; Varcoe *et al.*, 2003), *Drosophila* 5-HT2 receptors also appear to mediate 5-HT effects on certain aspects of circadian behaviours in adult flies (Nichols, 2007). Besides, developmental studies in *Drosophila* suggest a role for 5-HT2$_{Dro}$ in germ band extension during gastrulation (Colas *et al.*, 1999a; 1999b; Schaerlinger *et al.*, 2007), consistent with the observations of 5-HT2$_{Dro}$ mRNA expression in the presumptive ectoderm as well as a transient peak of 5-HT synthesis during this developmental time (Colas *et al.*, 1995; 1999b). Interestingly, the requirement of 5-HT and 5-HT2-like receptors in embryonic development has also been demonstrated in vertebrates (reviewed in Nebigil *et al.*, 2001; Nichols and Nichols, 2008).

Lastly, a number of studies have suggested a link between 5-HT and promotion of sleep in mammals and, although still controversial, there is evidence for the involvement of 5-HT1A receptors (reviewed in Jouvet, 1999; Ursin, 2002). In this regard, recent studies in *Drosophila* have demonstrated a sleeping-promoting role for 5-HT1A$_{Dro}$ in

adults, supporting the contention of a positive effect of 5-HT in sleep mediated through 5-HT1A receptors (Yuan *et al.*, 2006).

1.6. *Drosophila* neuropeptide systems

Besides 'classical' neurotransmitters, the fly CNS also produces a large number of neuropeptides (Baggerman *et al.*, 2005; Park *et al.*, 2008; Santos *et al.*, 2007; reviewed in Nässel, 2002). They derive from larger precursor proteins, the prepropeptides. Several immuncytochemical and *in situ* studies in insects indicate that neuropeptides are expressed not only in interneurons but also in neurosecretory or endocrine cells as well as motorneurons (e.g. Park *et al.*, 2008; reviewed in Homberg, 2002; Nässel, 2002). Furthermore, neuropeptides are found widely spread within the insect CNS, presenting unique stereotypical distribution patterns (Park *et al.*, 2008; Santos, *et al.*, 2007; reviewed in Nässel 2002; Nässel and Homberg 2006). In some cases, they are found co-localizing with other neuropeptides as well as 'classical' neurotransmitters, including GABA and BAs (reviewed in Nässel and Holmberg, 2006).

Neuropeptides have been shown to be involved in a variety of biological processes in insects, including development, metabolism, reproduction, and circadian rhythms (reviewed in Nässel, 2002; Nässel and Homberg, 2006). They can be released as neurohormones into the circulation or locally within the CNS. In this latter case, they may act in a paracrine manner or affect specific synapses. Neuromodulation function can be served by both circulating peptide and peptide released within the CNS. So far, the actions of all insect neuropeptides known to date (except for those of insulin-like

peptides) are mediated by GPCRs (reviewed in Nässel, 2002; Nässel and Homberg, 2006). Similar to BAs, the identity of input and output targets of peptidergic neurons in *Drosophila* are largely unknown. Interestingly, it has been shown in *Drosophila* that some peptidergic neurons express functional 'classical' neurotransmitter receptors, including those mediating BA signalling (Hamaska *et al.*, 2005; 2007; Vömel and Wegener, 2007; Wegener *et al.*, 2004; Yuan *et al.*, 2005).

1.6.1. Larval small ventral lateral neurons

The clock system represents one of the most extensively studied neuropeptide systems in *Drosophila* (reviewed in Helfrich-Förster, 2005). In the fly, the master circadian clock that regulates behavioural rhythmicity including locomotor activity has been localized to bilateral sets of clock gene-expressing neurons in the brain (Kaneko and Hall, 2000; reviewed in Helfrich-Forster, 2005). In particular, a ventral group of 4 small lateral neurons (LNv) present in the larval brain has been characterized in detail (Kaneko and Hall, 2000; reviewed in Helfrich-Forster 2005). These cells express the pigment dispersing factor (PDF) peptide from the beginning of larval life through to the adult stage (Helfrich-Forster, 1995; 1997). PDF has been shown to be the main circadian output factor released by the LNv (Helfrich-Forster, 1995; Park *et al.*, 2000). The cell bodies of the LNv are located laterally, at the anterior margin of the developing optic medulla of the OL (Helfrich-Forster, 1997; Kaneko and Hall, 2000; Malpel *et al.*, 2002; reviewed in Helfrich-Forster, 2005), whereas their dendritic arborizations are found in the larval optic neuropil of the central brain (Hamasaka and Nässel, 2006; Helfrich-Forster,

1997; Kaneko and Hall, 2000; Malpel *et al.*, 2002). Axonal projections deriving from these neurons are observed in the dorsal region of the brain hemispheres (Hamasaka and Nässel, 2006; Helfrich-Forster, 1997; Kaneko and Hall, 2000; Malpel *et al.*, 2002).

Until recently, the cellular targets of the LNv had not been studied in detail. Although it remains to be further investigated, a group of dorsal clock neurons (DN1) has been proposed as post-synaptic candidates of the LNv (Kaneko and Hall, 2000; Hamasaka and Nässel, 2006; Hamasaka *et al.*, 2007). Regarding their putative pre-synaptic partners, it has been reported that the LON interacts with the dendritic tree of this group of PDF-expressing clock cells in the larval optic neuropil (Malpel *et al.*, 2002). This is in accordance with the findings of nicotinic-mediated stimulation of these peptidergic neurons in culture (Wegener *et al.*, 2004). This anatomical interaction can be first seen in late embryogenesis and it is maintained throughout the entire larval stage (Malpel *et al.*, 2002). Interestingly, the development of the LNv dendritic processes depends on the presence of the LON (Malpel *et al.*, 2002), similarly to what was previously shown for the larval optic center 5HT arborization (Mukhopadhyay and Campos, 1995). More recent findings indicate that these neurons also receive inhibitory GABAergic and glutamatergic inputs (Hamasaka *et al.*, 2005; 2007).

1.6.2. Larval corazonergic neurons

The undecapeptide CRZ was initially isolated from the corpora cardiaca of the cockroach *Periplaneta americana* as potent cardioactive peptide (Veenstra, 1989). Subsequent studies showed that this peptide is involved in other physiological functions

including induction of dark cuticular pigmentation in several orthopteran species (Hua *et al.*, 2000; Tanaka, 2000; Tawfik *et al.*, 1999). In addition, it has been shown that in the moth *Manduca sexta*, CRZ plays a role in ecdysis (Kim *et al.*, 2004).

The CRZ-encoding gene was cloned in *Drosophila* by Veenstra (1994). CRZ expression and the distribution pattern of corazonergic neurons in the fly have been recently studied (Choi *et al.*, 2005; 2006; 2008; Johnson *et al.*, 2005; Landgraf *et al.*, 2003b; Lee *et al.*, 2008; Santos *et al.*, 2007). In 3rd instar larvae, CRZ in the brain hemispheres is detected in three pairs of dorsolateral (DL) neurons as well as a pair of dorsomedial (DM) neurons (Choi *et al.*, 2005; Lee *et al.*, 2008). In the VNC, CRZ is found in eight pairs of symmetrically positioned neurons, lateral to 5-HT neurons (Choi *et al.*, 2005; Landraf *et al.*, 2003b; Lee *et al.*, 2008; Santos *et al.*, 2007).

To date, the functions of this neuropeptide in *Drosophila* are largely unknown. Nevertheless, based on expression patterns in the larvae, it has been suggested that CRZ may act as a neurohormone and also as neuromodulator within the CNS. In adults, it may be associated with regulation of biological rhythms (Choi *et al.*, 2005) and trehalose metabolism (Lee *et al.*, 2008). Interestingly, a recent report have demonstrated that the CRZ gene is transcriptionally regulated by two different mechanisms, one present in the DM neurons and the other one in the DL and the VNC neuronal groups (Choi *et al.*, 2008). It has been proposed that this difference may reflect unique functions for these two neuronal subsets (Choi *et al.*, 2008).

1.7. Central pattern generating circuits and rhythmic behaviours

The formation of precisely organized neuronal networks allows neurons to rapidly and accurately communicate with their targets, integrating and responding to sensory and internal stimuli with an appropriate range of behavioural outputs. The activation of distinct neuronal circuits gives rise to the diverse functions controlled by the nervous system, from cognition to movement.

Rhythmic behaviours such as breathing, locomotion and feeding require the activity of specialized neuronal circuits called central pattern generators (CPGs) (reviewed in Grillner, 2006; Marder and Buchner 2001, Marder *et al.*, 2005). Thus, in order to generate a variety of motor behaviours, the nervous system of a given organism is equipped with a number of pre-assembled networks that form unit CPGs, available for differential activation (reviewed in Grillner, 2006; Marder *et al.*, 2005). Furthermore, it is believed that complex behavioural responses requiring for instance the spatiotemporal coordination of muscles located in different segments (i.e. multisegmented movements) or the coordination of different sets of movements (e.g. breathing and running), involve coordination of multiple CPGs (Marder *et al.*, 2005). In addition, the activity of CPGs may be subject to adaptation by its interaction with sensory information as well as neuromodulators, such that motor patterns are adjusted according to not only specific environmental conditions but also developmental changes (reviewed in Grillner, 2006; Marder and Buchner 2001, Marder *et al.*, 2005).

1.7.1. *Drosophila* larval locomotion

Drosophila larval crawling is a stereotypic motor behaviour that comprises rhythmic waves of forward peristalsis alternated with brief occasional episodes of head swinging, turning and backward locomotion (Berrigan and Pepin, 1995; Pereanu *et al.*, 2007; Suster *et al.*, 2003; Wang *et al.*, 1997; 2002). Many behavioural and electrophysiological studies have demonstrated that *Drosophila* larval locomotion is a rhythmic behaviour controlled by the activity of CPGs (Barclay *et al.*, 2002; Fox *et al.*, 2006; Saraswati *et al.*, 2004; Song *et al.*, 2007; Suster and Bate, 2002; Suster *et al.*, 2004; Wang *et al.*, 1997; 2002).

Neurons of the larval circuit controlling *Drosophila* larval crawling have not yet been identified. Previous findings have suggested that the CPGs controlling this behaviour are located within the thoracic and abdominal segments of the VNC (Cattaert and Birman, 2001). Moreover, the results of *in vitro* studies also suggest that *Drosophila* locomotor CPGs are activated by descending cholinergic inputs localized within the larval brain (Cattaert and Birman, 2001). These findings appear to be in agreement with those obtained in larval crawling of *Manduca sexta* (Johnston and Levine, 1996; Johnston, *et al.*, 1999). Comparably, vertebrate CPGs controlling locomotion are thought to be localized within the spinal cord and to be activated by a command center located in the midbrain (reviewed in Grillner, 2006). In addition, pharmacological evidence indicates that central NMDA or NMDA-like receptors are involved in the control of *Drosophila* larval locomotion (Cattaert and Birman, 2001).

Interestingly, several behavioural studies have also demonstrated the importance of the brain in *Drosophila* locomotor control. For instance, different studies have shown that mutations in the CX cause locomotor defects (e.g. Poeck *et al.*, 2008; reviewed in Strauss, 2002). Moreover, recent findings by Pereanu *et al.* (2007) show that mutants that lack the protocerebrum exhibit alterations in larval movement and that bilateral severing of the brain hemispheres resulted in the absence of peristaltic activity. These observations further support the idea that brain structures are necessary to activate and maintain larval CPG activity controlling peristaltic movement (Pereanu *et al.*, 2007).

1.7.2. Sensory inputs and their impact on CPG activity

Perturbations can occur during the performance of a movement. Therefore, the sensory input produced by such a change may influence CPG activity to respond accordingly. It is generally believed that CPG outputs generate the basic motor oscillation, whereas sensory input modulate or coordinate this core rhythm. Alternatively, rhythmic patterns may be achieved by use of sensory feedback and the loss of this sensory information disrupts the behaviour controlled by CPGs (reviewed in Dickinson *et al.*, 2000; Marder and Bucher, 2001). Most likely, to what extent sensory input play or not a crucial role on generation of motor patterns depends not only on the nature of the sensory feedback but also on the behaviour and organism considered.

In *Drosophila*, accumulating evidence has provided some clues as to how and what kind of sensory information may affect the activity of the CPGs controlling larval locomotion. For example, a previous study suggested that, although the CPGs have the

ability to assemble and function in the absence of sensory inputs, the latter are necessary for generation of normal motor patterns (Suster and Bate, 2002). Nevertheless, it has been shown more recently that multidendritic (MD) sensory neurons are indeed essential for generation of rhythmic locomotor patterns of *Drosophila* larva, and that disruption of these sensory inputs causes arrest of larval crawling (Song *et al.*, 2007). MD neurons are found tiling the internal wall of the body muscles. Although their function in *Drosophila* larvae is currently unknown, it has been suggested that they may act as propioceptive receptors sensing body wall stretching (Song *et al.*, 2007). One possible explanation for the discrepancy of the results mentioned above is that in the former study (Suster and Bate, 2002), not all sensory inputs were completely affected. In fact, the larval mutants studied by Suster and Bate (2002) possessed still a significant number of MD neurons, and the strength of the driver used to silence all sensory inputs was shown not to be as efficient as the one used by Song and colleagues (2007). Thus, the results of Song *et al.* (2007) suggest that likely both central and sensory contributions are important for the generation of *Drosophila* larval locomotor behaviour.

1.7.2.1. Visual input and modulation of locomotion

It is widely known that visual information plays an important role in locomotor control in many organisms. Behavioural optomotor responses in flying insects such as fruit flies constitute an example of how sensory information, particularly visual processing, may shape motor behaviour (e.g. Duistermars *et al.*, 2007; Strauss *et al.*, 1997; Tammero *et al.*, 2004; reviewed in Frye and Dickinson, 2004). In *Drosophila*

adults for instance, the thoracic flight motor circuits are driven by sensory inputs, including those in charge of visual processing. It is known that visual information can induce steering manouvers such as saccades, rapid stereotyped turns that fruit flies perform to modify flight direction (reviewed in Frye and Dickinson, 2004).

Drosophila larval photobehaviour constitutes another example revealing the importance of visual information in locomotor control. *Drosophila* larvae in the foraging stage show a marked aversion to light (Sawin-McCormack *et al.*, 1995). Many different assays have been developed and used to study larval photobehaviour. These include population paradigms such as the plate assay (Iyengar *et al.*, 1999; Lilly and Carlson, 1990) as well as individual assays such as the checker assay (Hassan *et al.*, 2000) and the ON/OFF assay (Busto *et al.*, 1999; Hassan *et al.*, 2005; Scantlebury *et al.*, 2007). Individual assays have been shown to be particularly effective at measuring the effect of visual input on larval locomotion. Behavioural analyses of wild type and visual-defective mutants using these paradigms indicate that the light stimulus indeed modulates larval movement (Busto *et al.*, 1999; Hassan *et al.*, 2000, 2005; Scantlebury *et al.*, 2007). Furthermore, these studies demonstrate that modulation of larval locomotion by light is characterized by changes in different locomotor parameters including reduced distance travelled and speed, as well as increased direction change and headswinging (Busto *et al.*, 1999; Hassan *et al.*, 2000, 2005; Scantlebury *et al.*, 2007).

1.7.3. Neuromodulation of motor networks

It is widely believed that neuromodulators including BAs are capable of regulating CPG activity (reviewed in Dickinson, 2006). Modulation of the motor pattern produced by a given circuit can be achieved at different levels within the neuronal network in charge of such output. For instance, modulators can exert their effect at the level of the sensory inputs, on the CPG per se, and/or on the motor neurons directly (reviewed in Marder and Calabrese, 1996). Moreover, modulation could derive from neurons that are part of the CPG (often referred to as intrinsic neuromodulation) or from sources outside the network (extrinsic neuromodulation) (reviewed in Katz, 1998; Katz and Frost, 1996). At the same time, the mechanism of modulation can involve changes in intrinsic membrane properties of the CPG neurons, modification of synaptic strength or a combination of both. Hence, the functional significance of CPG neuromodulation is the production of many different outputs, ultimately enabling behavioural flexibility (reviewed in Dickinson, 2006).

A large body of evidence indicates that neuromodulatory inputs may cause short-term changes on CPG activity for adaptation to environmental needs or produce long-lasting effects to maintain network integrity (e.g. Thoby-Brisson and Simmers, 1998; 2002; reviewed in Dickinson, 2006; Marder and Calabrese, 1996; Marder *et al.*, 2005). Furthermore, neuromodulators have been shown to play a crucial role in developmental tuning of neuronal circuit function, particularly important for ontogenetic plasticity (reviewed in Fenelon *et al.*, 1998; Rauscent *et al.*, 2006). In this regard, some studies have demonstrated that progressive acquisition of neuromodulatory effects on a given

target network can be correlated with the axonal ingrowth of descending inputs (reviewed in McLean *et al.*, 2000). In other cases, it is possible that descending neurons reach their target early in development but they acquire their modulatory substances gradually or even change them throughout developmental growth of the organism, as suggested for the stomatogastric ganglion circuit in lobsters (e.g. Le Feuvre *et al.*, 2001; reviewed in Marder *et al.*, 2005).

1.7.3.1. Regulation of locomotion by neuromodulators

One neuromodulator that appears to be involved in regulating most vertebrate locomotor networks is 5-HT (reviewed in Grillner, 2006; Schmidt and Jordan, 2000). For instance, developmental studies in *Xenopus* have shown a correlation between the ingrowth of descending 5-HT fibers into the tadpole spinal cord and the maturation of swimming, suggesting 5-HT-mediated modulation of motor output in a stage-dependent fashion (reviewed in McLean *et al.*, 2000).

Regarding *Drosophila*, physiological, genetic and behavioural studies have begun to unravel the role and identity of modulators regulating larval locomotion. For instance, physiological studies using semi-intact 3rd instar larval preparations have addressed the role of BAs in modulation of sensory-CNS-motor networks (Dasari and Cooper, 2004). The authors demonstrated that 5-HT, in addition to OA and DA, was able to modulate the motor activity pattern, although the site of action for these effects (i.e. on sensory neurons, interneurons and/or motorneurons) could not be addressed with these preparations (Dasari and Cooper, 2004). Additionally, behavioural analysis of mutant

larvae with altered levels of OA and TA has shown that these BAs have opposite effects on locomotor behaviour and that a correct balance between them is required for normal larval crawling (Saraswati *et al.*, 2004). Moreover, a more recent report indicates that these two BAs are important for coordination and modulation of larval locomotor patterns, exerting their effect at a central level (Fox *et al.*, 2006). Lastly, behavioural studies performed in constant light have shown that silencing of subsets of aminergic neurons including those expressing 5-HT and DA causes increased larval turning, further suggesting a role for these BAs in regulation of larval locomotion (Suster *et al.*, 2003).

1.8. Thesis objectives and overview

It is widely known that the biogenic amine serotonin is an important signalling molecule in the CNS of both vertebrates and invertebrates. The distribution pattern of 5-HT neurons and their projections in *Drosophila* have been extensively studied (Chen and Condron, 2008; Hamaska and Nässel, 2006; Landgraf *et al.*, 2003b; Lundell and Hirsh, 1994; Mukhopadhyay and Campos, 1995; Vallés and White, 1986; 1988; Vömel and Wegener, 2008). Developmental studies have focused mainly on VNC 5-HT neuronal lineage analysis and on identifying genes required for differentiation of VNC serotonergic neurons (Bossing *et al.*, 1996; Dittrich *et al.*, 1997; Higashijima *et al.*, 1996; Isshiki *et al.*, 2001; Karcavich and Doe, 2005; Lee and Lundell, 2007; Lundell *et al.*, 1996; Lundell and Hirsh, 1998; Lundell *et al.*, 2003; Novotny *et al.*, 2002; Schmid *et al.*, 1999). Nevertheless, information is missing regarding 5-HT neurite development during larval stage and the underlying signalling pathways involved in this process. Moreover,

although it has been shown that 5-HT neurons are widely distributed in *Drosophila* larval CNS, their neuronal interactions and synaptic partners and/or targets are largely unknown.

The majority of functional studies performed in *Drosophila* have focused on examining the role that 5-HT cells play in the adult fly (Dierick and Greenspan, 2007; Lee *et al.*, 2001; Neckameyer *et al.*, 2007; Sitaraman, *et al.*, 2008; Yuan *et al.*, 2005; 2006), whereas little is known about their function in the larva (e.g. Neckameyer *et al.*, 2007). This is also true for *Drosophila* 5-HT receptors, as functional aspects of these receptors at the larval stage have not previously been described. Furthermore, detailed studies of the expression patterns of the different 5-HT_{Dro} receptor subtypes are also missing. Thus, it is clear that additional functional studies of *Drosophila* 5-HT neurons and 5-HT_{Dro} receptors need to be done in order to unravel the processes regulated by serotonin in *Drosophila* larva.

Previous reports have shown that the larval optic neuropil, the region of the brain where the larval photoreceptor termini are found, is also invaded by serotonergic projections (Mukhopadhyay and Campos, 1995), as well as by dendrites of the LNv (Malpel *et al.*, 2002). Furthermore, results from these studies indicate that the LON appear to contact both groups of neurons and that its presence is required for the development of their arbors (Malpel *et al.*, 2002; Mukhopadhyay and Campos, 1995). However, it has never been investigated before whether both photoreceptor subsets (i.e. Rh5 and Rh6) and/or the brain PDF-expressing cells interact with the larval optic neuropil

5-HT arborization, and which of these neurons may be providing a trophic signal required for the development of this 5-HT arbor.

The branching of this 5-HT neurite increases during larval stage (Mukhopadhyay and Campos, 1995), within the same developmental window that the larval response to light appears to decrease (Sawin-McCormack *et al.*, 1995). In addition, behavioural analysis of larval locomotion in constant light conditions shows that synaptic inactivation of a neuronal subgroup that includes the 5-HT cells increases larval turning (Suster *et al.*, 2003). Together, these observations raised the intriguing possibility of 5-HT mediated-neuromodulation of larval photobehaviour.

These previous findings laid the ground for my Ph.D. research. My thesis work addressed two short-term objectives. The first objective involved a detailed analysis of the interactions of 5-HT neurons with the larval photoreceptors and the LNv. The signalling mechanism underlying the development of the 5-HT arborization found in the larval optic neuropil was examined. The second objective was to probe candidate neurons for a role in the developmental modulation of the larval photobehaviour. Here, I demonstrate that 5-HT neurons are involved in the regulation of the larval response to light during development.

1.8.1. Genetic dissection of trophic interactions in the larval optic neuropil of *Drosophila melanogaster* (Rodriguez Moncalvo and Campos, 2005)

Bearing in mind previous observations of Malpel *et al.*, (2002) and Mukhopadhyay and Campos (1995), I proceeded to examine the interaction in the larval

optic center among the neural processes of the 5-HT neurons, LNv and larval photoreceptors. To this end, I used the GAL4/UAS system (Brand and Perrimon, 1993) and immunohistochemical techniques to perform labelling, ablation as well as neuronal silencing experiments in a cell-specific manner. The results of these experiments allowed us to conclude that both Rh5- and Rh6-expressing fibers contact the larval optic neuropil 5-HT projections, which is also found overlapping with the dendritic tree of the LNv. Furthermore, the Rh6 photoreceptors are required for the development of the serotonergic arborization, and partial suppression of their synaptic activity by targeted expression of tetanus toxin light chain (TNT) inhibits normal branching of the 5-HT processes. Lastly, our results indicate that Rac function is also required for proper development of the serotonergic arborization.

1.8.2. Role of serotonergic neurons in the modulation of *Drosophila* larval response to light

In order to examine whether 5-HT neurons and other subsets of neurons may play a modulatory role in photobehaviour during larval development, we utilized the larval response to light in the ON/OFF assay as a behavioural paradigm. In addition, we took advantage once again of the GAL4/UAS system and used this genetic tool to manipulate neuronal activity. Expression of TNT and other neuronal silencers specifically in serotonergic neurons causes an increase in the response to light from late 2nd instar to late 3rd instar larval stage. *TRHn* mutant larvae also display elevated photoresponse. Surprisingly, the results of the present study indicate that this modulation appears to occur

at a central level rather than at the level of the photosensory input. Finally, our findings suggest that the modulatory effect of 5-HT in larval photobehaviour is mediated by 5-HT1A_{Dro} receptors.

Figure 1.1. Pathways for biosynthesis of the BAs serotonin, dopamine, tyramine, and octopamine. Tryptophan is the substrate for the first reaction in serotonin synthesis. The amino acid tyrosine is the starting point for production of dopamine as well as tyramine and octopamine. It has been suggested that DADH may synthesize tyramine from dopamine (dotted arrow). DADH: dopamine dehydroxylase; DDC: DOPA decarboxylase; TβH: tyramine β-hydroxylase; TDC: tyrosine decarboxylase; TH: tyrosine hydroxylase; TRH: tryptophan hydroxylase. (Modified and Reprinted from PLoS ONE, 3(3), Vömel M and Wegener C, Neuroarchitecture of aminergic systems in the larval ventral ganglion of *Drosophila melanogaster*, e1848).

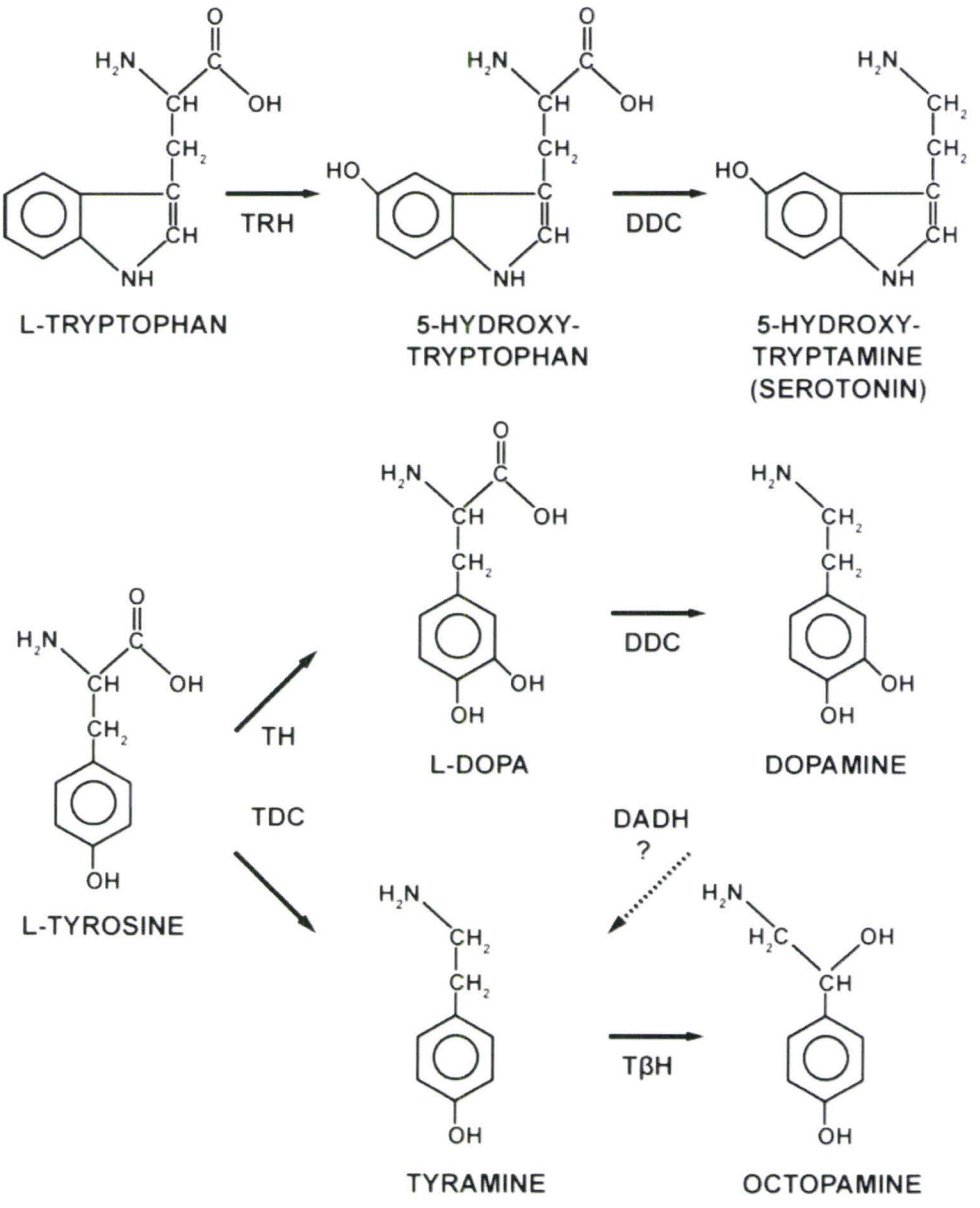

L-TRYPTOPHAN
TRH
5-HYDROXY-
TRYPTOPHAN
DDC
5-HYDROXY-
TRYPTAMINE
(SEROTONIN)
L-TYROSINE
TH
TDC
L-DOPA
DDC
DOPAMINE
DADH
?
TYRAMINE
TβH
OCTOPAMINE

Figure 1.2. The serotonergic system of *Drosophila* larva. Schematic diagram depicting the approximate position of 5-HT-expressing neuronal cell bodies as well as their main projections and 5-HT-immunoreactive neuropils (stippled areas). The neuronal clusters in the brain hemispheres consist of IP, LP1, SP1, and SP2. In the ventral ganglion, 5-HT neurons are found forming clusters of bilateral pairs: 3 subesophageal (SE1-SE3), 3 thoracic (T1-T3) and 8 abdominal (A1-A8). (Reprinted from Journal of Comparative Neurology, 268(3), Vallés AM and White K, Serotonin-containing neurons in *Drosophila melanogaster*: development and distribution, 414-28, Copyright (1988), with permission from John Wiley & Sons, Inc.).

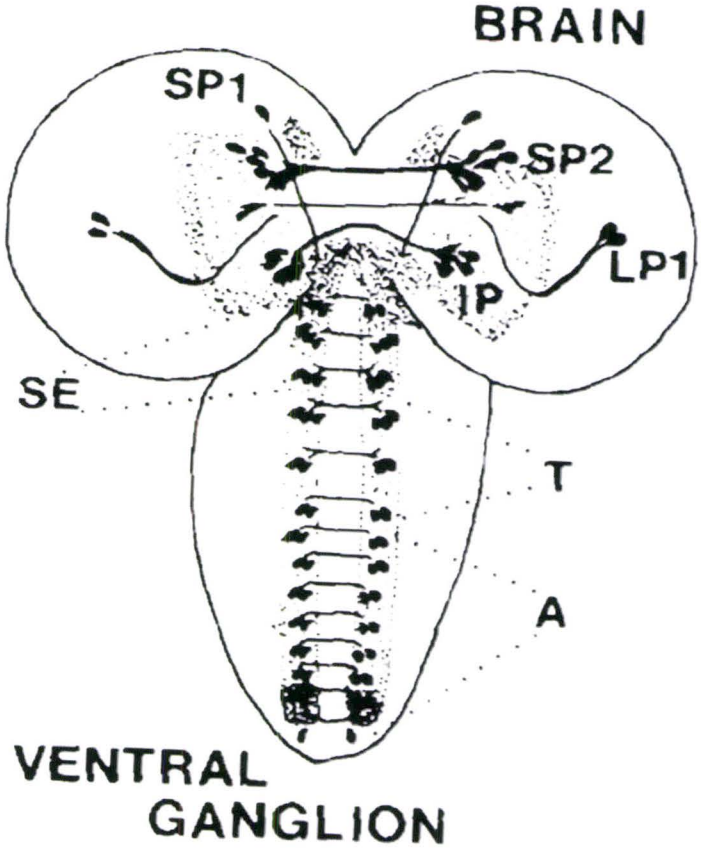
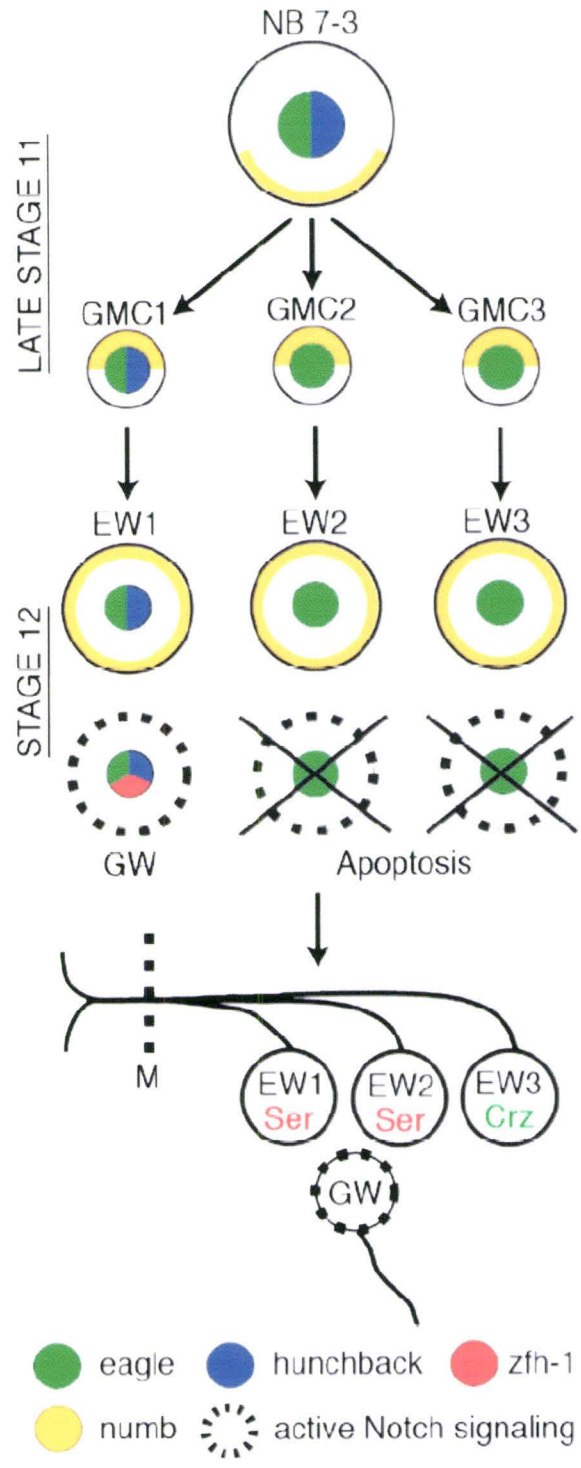

BRAIN
SP1
SP2
LP1
IP
SE
T
A
VENTRAL
GANGLION

Figure 1.3. Development and molecular marker expression of the NB 7-3 lineage. Three GMCs are derived from NB7-3, which delaminates during late stage 11. During stage 12, Numb, an inhibitor of Notch signalling, is asymmetrically distributed during GMC divisions generating 3 cells (EW1-3) in which this protein is present, and 3 sister cells (GW and 2 apoptotic cells) that do not receive Numb. Besides NB7-3, only GMC1 and its progeny express the transcription factor Hunchback. GMC1 produces 2 cells: the more medial 5-HT neuron (EW1) and a motorneuron (GW). In this cell, non-canonical Notch signalling is required for Zfh-1 expression. GMC2 generates the more lateral 5-HT cell (EW2) and a cell that undergoes canonical Notch-mediated apoptosis. Division of GMC3 produces a corazonergic neuron (EW3) and a sister cell that also goes undergoes apoptosis mediated by canonical Notch signalling. Alternatively, GMC3 may directly differentiate into EW3. At the end of embryogenesis (stage 17), EW1-3 start expressing their corresponding neurotransmitters, whereas Eagle expression decreases. Eagle levels in GW declines earlier, by stage 16. All three interneurons send axonal projections across the midline to the contralateral side of the VNC, whereas GW projects an ipsilateral axon. (Reprinted from Molecular and Cellular Neuroscience, 36(1), Lee Hk and Lundell MJ, Differentiation of the *Drosophila* serotonergic lineage depends on the regulation of Zfh-1 by Notch and Eagle, 47-58, Copyright (2007), with permission from Elsevier).


NB 7-3
LATE STAGE 11
GMC1
GMC2
GMC3
EW1
EW2
EW3
STAGE 12
GW
Apoptosis
M
EW1
Ser
EW2
Ser
EW3
Crz
GW
eagle
hunchback
zfh-1
numb
active Notch signaling

CHAPTER 2: Materials and Methods

2.1. Fly Stocks

All *Drosophila melanogaster* stocks were raised at 23-25°C in standard medium containing inactivated yeast, sucrose and agar. A mix of propionic and phosphoric acid as well as 10% tegosept in ethanol were added to the medium upon cooling to prevent mold growth.

For cell ablation and neuronal silencing experiments, cellular labelling, as well as down- or up-regulation studies, we employed the conventional GAL4/UAS system (Brand and Perrimon, 1993). The different GAL4 and UAS lines used in this study are listed in Table 1 and 2 respectively. In addition, the strain Oregon-R (OR) was used as wild type control. Mutant stocks of different genes and other lines also utilized in the present work are described in Table 3.

2.2. Histology, immunohistochemistry and imaging

Larval brains with the eye-antenna attached to them were dissected in 1X Phosphate Buffered Saline (PBS), and fixed in 4% paraformaldehyde (pH = 7.2 - 7.4) for 30-45 minutes at room temperature. This was followed by 3 washes in 1X PBS and 5-8 washes using 1X PBS with 0.5% Triton X-100 (0.5% PBT). Specimens were incubated in a blocking solution made of 10% goat serum in 0.5% PBT for 1 hour at room temperature. Afterwards, brains were incubated with the appropriate primary antisera in 0.5% PBT with 10% goat serum at 4°C overnight. In order to label the photoreceptor cells, we used the mouse monoclonal 24B10 antibody (1:100), which recognizes the glycoprotein Chaoptin expressed specifically on the photoreceptor-cell plasma membrane

(Van Vactor *et al.*, 1988; Zipursky *et al.*, 1984). 5-HT neurons were visualized using rabbit anti-serotonin (1:200) (Protos Biotech Corp., NY). Also, in certain cases mouse anti β-galactosidase antibody was used (1:100) (Promega Corp., Madison). The following day, specimens were rinsed thoroughly with 0.5% PBT for 4 hours with changes every 20 minutes, and then blocked again as described above. Next, the brains were incubated with the appropriate secondary antibodies following the same procedure as with the primary antisera. The secondary antibodies used were Alexa 488 or 594-conjugated goat anti-mouse IgG (1:200) (Molecular Probes Inc., Eugene, OR) and Texas Red-conjugated goat anti-rabbit IgG (1:200) (Jackson InmunoResearch Laboratories, Inc., West Grove, PA). Finally, specimens where washed with 0.5% PBT for 4 hours and mounted in 70% glycerol in PBS.

The larval brains were viewed in a Nikon Eclipse ∈800 microscope. Confocal images were obtained with either a Bio-Rad Radiance MRC 600 Krypton/Argon laser confocal microscope using the LaserSharp software or a Zeiss confocal microscope using LSM510 software. All images represented projections made of z-stack sections, obtained by sequential or multi-track scanning. The number of sections and section thickness varied depending on the sample between 3-20 and 0.5-1 μm respectively. The contrast and brightness of the confocal images were adjusted using Adobe Photoshop 5.0 software for Macintosh. In cell ablation and neuronal silencing experiments, analysis of all specimens was performed blind at first.

2.3. Harvest and synchronization of larvae used in all assays

Adult flies as well as egg and larval collections used in behavioural experiments were kept at 25°C in constant darkness. The protocol carried out for larval harvesting was similar to the one previously used in our laboratory (Busto *et al.*, 1999; Hassan *et al.*, 2000). Briefly, 4-7 day old parental flies were allowed to mate and lay eggs overnight in fly houses containing food plates (60 mm x 15 mm, Fisher Scientific, Houston, Tx) supplemented with vitamin A (Jamieson Laboratory, β carotene, 1.25g/L). The next day, following a 2 hour pre-collection done to discard any stored eggs in the female ovopositors, a 1 hour-egg collection was performed. At 20-22 hours after egg laying (AEL), all hatched larvae were removed from the collection plate under a dissection microscope. After incubating the remainder of the eggs for a period of 2 hours, approximately 30-40 newly hatched larvae were collected and transferred to a fresh food plate and allowed to grow until 70, 89, 96, 115 and 120 hours AEL.

2.4. Verification of larval stages

Besides performing synchronized larval collections and timing their development at 25°C, several behavioural and anatomical characteristics of the larvae were used to confirm the expected larval stage. Some of the anatomical features that can be used to distinguish the different larval stages are the shape of their mouth hooks and the number of teeth, as well as the morphology of the anterior spiracles (Asbushner, 1989). Therefore, these characteristics were checked after every larva was tested. In addition, especially to tell apart foraging from wandering 3rd instar larvae, study of their behaviours

such as digging into the food or wandering on the lid of the plate, reversion of spiracles and emptiness of the guts were performed. In this last case, empty guts were verified by disappearance of blue-colored food (Maroni and Stamey, 1983). For this purpose, food colouring solution (0.05% bromophenol, Sigma) was dissolved in the regular fly medium. Egg collection and larval growth were conducted in the coloured-food plate following the harvesting protocol mentioned above. Animals were removed from their plates at early wandering stage (115 h AEL) and rinsed with distilled water to remove any excess of food from their bodies. Verification of minimal residual blue staining remaining at the posterior tip of larval guts, characteristic of wandering stage, was performed under a Nikon SMZ1500 light microscope. When necessary, specimens were also photographed.

2.5. Photobehavioural assay and data collection

Photobehavioural assays were conducted at 25°C using the ON/OFF assay previously utilized in our laboratory (Busto *et al.*, 1999; Hassan *et al.*, 2005; Scantlebury *et al.*, 2007). During pre-test and test conditions, larvae were manipulated using a moist paintbrush under a red safelight (20 W incandescent lamp with a Kodak GBX-2 filter), the same employed for studies of *Drosophila* circadian locomotor behaviour in free running conditions ('constant darkness', Sehgal *et al.*, 1992). It has been reported that *Drosophila* does not react to light of wavelengths above 650 nm (Ashburner, 1989). Moreover, previous larval photobehaviour assays performed in our laboratory using only the red safelight as light source have demonstrated that *Drosophila* larvae do not respond under these light conditions (Busto, M.Sc thesis, 1998).

Previous to the beginning of the photobehavioural assay, single larvae were removed from the food plate and carefully rinsed with distilled water to eliminate any excess of food. Afterwards, each larva was placed for 1 minute on a pre-test plastic petri dish (100 mm x 15 mm) containing 15 ml of 1% agar. This pre-test plate was used to allow the larva to familiarize with the surface. To start the assay, individual larvae then were placed in the center of a test agar plate and subjected to alternate 10 second-pulses of light and dark for a total of at least 40 seconds (for wandering larvae) or 60 seconds (for foraging larvae) or until reaching a total test time of 120 seconds. A serial device MacIO microcontroller (MacBrick, Netherlands) and a relay unit (AZ696) were used to obtain a 10-second periodicity of the light stimulus.

For quantitative analysis of larval photobehaviour, we used a semi-automatic tracking system previously used in our laboratory (Busto *et al.*, 1999; Hassan *et al.*, 2005). This system utilizes a custom macro program written in NIH-Image 1.62f processing software and ran by a Macintosh computer (PowerMac 9500/200). The semi-automatic system involves stylus/tablet-based tracking of larval movement. The tablet used is an Intuos tablet (Wacom technology Co. WA). To standardize the tracking methodology during the assay, the stylus, which is connected to an ADB port, was always placed on the tablet at a position corresponding with the tail end of the larva (seen on the computer screen). As well, the stylus is used to start and end the assay by pressing either its lower or upper button respectively.

During the ON/OFF assay using the semi-automatic system, testing of larval photobehaviour is conducted using a cool white bulb (20W Cool White, Philips in a

Rapid Star mechanism; Thomas Lighting). Larval behaviour is visualized on a computer screen using a Fujinon TVZ zoom lens (Fuji Optical Co) attached to a CCD TV camera (Elvo Mfg Co., TSE 272S). The computer macro not only controls through the relay unit when the light turns on and off, but also registers the pulse number and distance travelled in each pulse in real time. This information can be seen through the assay in a separate window. Once the assay is finalized, the software automatically calculates a response index, RI = [(total distance traveled in the dark period-total distance travelled in the light period)/total distance travelled in both the periods], which is displayed in the same window as the other data. All data deriving from behavioural studies and represented as RIs in figures is depicted as means ± S.E.M.

When a more detailed qualitative analysis of larval photobehaviour in the ON/OFF assay was required, we used a more advanced tracking software known as Dynamic Image Analysis System (DIAS, 3.2; Solltech, Inc., Iowa, USA). This system has been recently used in our laboratory for a kinematic description of larval locomotion during the ON/OFF assay (Scantlebury *et al.*, 2007). Larva handling during pre-test and test conditions was similar to the way performed in the ON/OFF assay using the semi-automatic system. However, in this case larvae were tested in the assay for only a minute.

To analyze larval photobehaviour in the ON/OFF assay using the DIAS system a similar but separate computer-light set up was utilized. In this case, the light source used for the assay is a cool white bulb (15W, Cool White, Sylvania). In addition, larval behaviour is captured via a macro video zoom lens (18-108, F2.5 MVZL, OPTEM

International, Fairport, NY, USA) attached to a monochrome digital firewire camera (PL-A641, PixeLINK, Ottawa, Canada), and digitally recorded at 2 frames per second (fps) using Pixelink Capture Software ran by a Macintosh G4/733 MHz workstation. Once the movies were recorded, they were analyzed using DIAS to characterize the dynamics of larval locomotion during the assay. For the purpose of generating larval movement paths, larval perimeters were generated using the 'Auto Trace DIC' function within the DIAS software. The software also automatically calculated the center position or centroid of each larva by taking in account the x, y coordinates of pixels in each larval perimeter. In order to represent the path taken by the larva, centroid tracks for each of them were created by plotting the succession of centroid movement through the assay. As well, perimeter stacks were generated according to serial changes in the x, y coordinates of the larval outline.

2.6. Locomotory behavioural assay in constant darkness

The larval photoresponse as measured in the ON/OFF assay depends on larval locomotion. Therefore, as a control, movement of all larvae used in this study was also examined in constant dark to verify that basic aspects of locomotion were not affected by the genetic background of the larvae. Thus, each larva tested in the ON/OFF assay was also subjected to a 30 second-locomotory test using a similar manipulation protocol to the one mentioned above, but in this case under constant safe-light conditions. For quantitative analysis, the assay was performed using the semi-automatic system. Data is shown when required as mean number of pixels travelled in 30 seconds ($\overline{X}$) ± S.E.M.

When needed, the DIAS software was used for a qualitative description of larval locomotion. In this case, larval centroid tracks and perimeter stacks were generated as described before.

2.7. Touch sensitivity assay

The touch sensitivity test was performed as mentioned previously in Caldwell *et al.*, 2003 with minor modifications. To avoid bias, this experiment was performed blind. During this assay, general handling of early foraging 3rd instar larvae was the same as during the photobehavioural assay. In this case, larval behaviour was observed under a dissection microscope illuminated by a red filter-adapted light source to ensure stimulus-free conditions ('constant darkness'). At the beginning of this assay, single larvae were placed on a non-nutritive agar plate identical to the ones used in the photobehavioural assay and allowed them to initiate linear movement. Then, each subject was gently touched with an eyelash on its anterior segments during free-run locomotion. Each larva was touched four times with an interval of 10-15 seconds between strokes. To quantify larval responsiveness to the stimulus, scores 0 to 4 were assigned to the different responses observed (Fig. 2.1). A score of 0 was given in those cases where larvae did not respond to the stimulus, whereas a full stop or hesitation was scored as 1. Larvae that retracted briefly but resumed their forward movement were scored as 2. In those cases in which larvae withdrew their anterior segments followed by a turn away from the stimulus with an angle < 90°, their responses were scored as 3. Finally, when larvae retracted and turned away from the stimulus with an angle > 90°, their behaviour was scored as 4. The

values obtained for each larva were added, and therefore individual larval scores ranged from 0 to 16. Data is shown as mean score for each group ($\overline{X}$) ± S.E.M.

2.8. Statistical analysis

For statistical analysis, either Minitab 10.5 Xtra for Macintosh or Minitab 13.1 software for PC was used. The statistical tests employed in the analysis of data included one-way analysis of variances (ANOVAs), and Tukey's-pairwise comparisons. Normality test on the residuals of the ANOVAs were conducted using the Rootogram test as well as the Ryan Joiner test (W-test). Verification of equal variances of the samples was performed by the F-test or Bartlett's test. In all statistical tests performed, the level of significance (α) was 0.05.

Figure 2.1. Stereotypical larval responses to touch and scores assigned to each observed mechanoresponse. After allowing individual larva to begin free forward crawling, each of them was gently touched four times with an interval of 10-15 seconds between strokes. In order to quantify larval responsiveness to the mechanical stimulus, scores 0 to 4 were assigned to the different responses observed. In those cases where larvae did not respond to the stimulus, a score of 0 was assigned. A score of 1 was given when larvae fully stopped or hesitated. Larvae that retracted briefly but continued forward locomotion were scored as 2. When larvae withdrew their anterior segments and turned away from the stimulus with an angle < 90°, their behaviour was scored as 3. Larvae that retracted and turned away from the stimulus with an angle > 90°, were scored as 4. (Reprinted from Neuron, 12(6), Kernan M, Cowan D, and Zuker C, Genetic dissection of mechanosensory transduction: mechanoreception-defective mutations of *Drosophila*, 1195-1206, Copyright (1994), with permission from Elsevier).

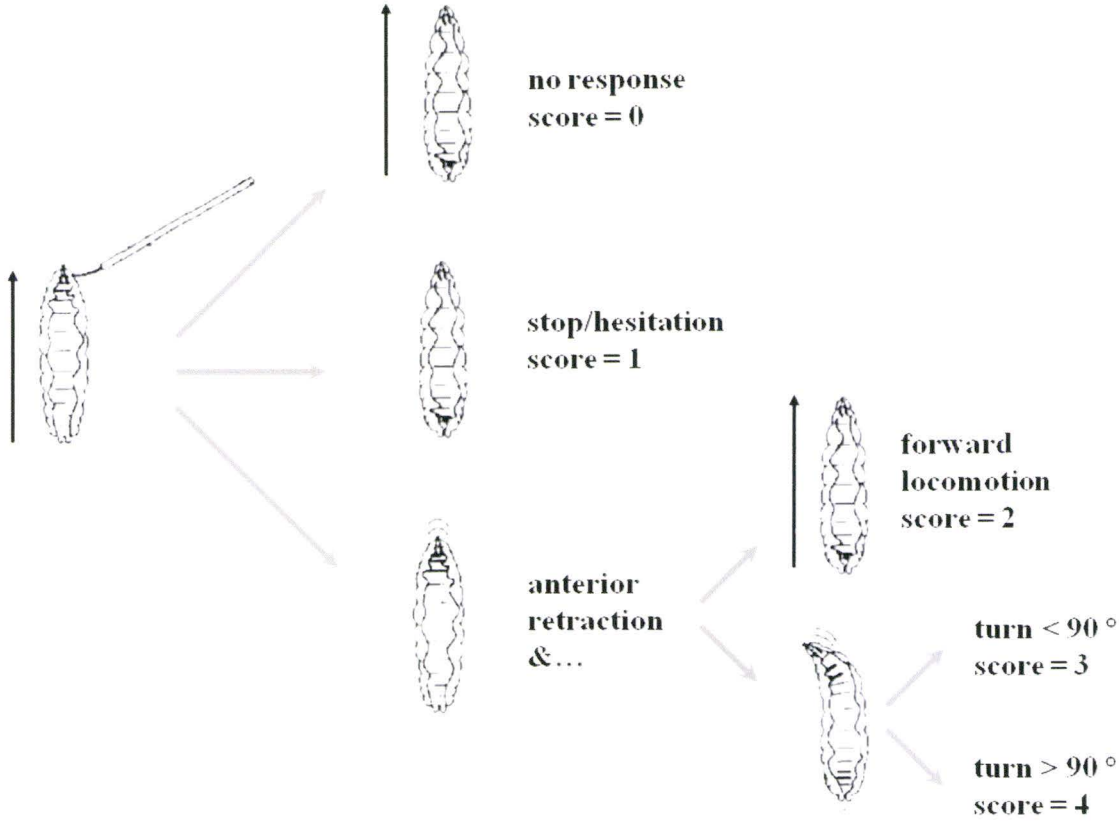

no response
score = 0
stop/hesitation
score = 1
anterior
retraction
&…
forward
locomotion
score = 2
turn < 90 °
score = 3
turn > 90 °
score = 4

Table 1. GAL4 lines used in this study

Strain	**Gene promoter/ regulatory region controlling *GAL4* expression**	**Larval expressing-tissues or cells**	**Reference or Source**
w;GMR-GAL4	*glass multimer reporter* (*GMR*)	all larval photoreceptors	Bloomington Stock Center (Indiana University, IN) stock # 1104
w;;Rh5-GAL4	*rhodopsin 5* (*Rh5*)	Rh5 larval photoreceptors	Claude Desplan (New York University, New York, NY)
w;;Rh6-GAL4	*rhodopsin 6* (*Rh6*)	Rh6 larval photoreceptors	Claude Desplan (New York University, New York, NY)
yw;PDF-GAL4	*pigment dispersing factor* (*PDF*)	brain small ventral lateral neurons (LNv) and 6-8 VNC neurons	Paul Taghert (Washington University School of Medicine, St. Louis, MO)
w;;Ddc-GAL4	*DOPA-decarboxylase* (*Ddc*)	serotonergic, dopaminergic and corazonergic neurons	Jay Hirsch (University of Virginia, Charlottesville, VA)
w;;TH-GAL4	*tyrosine hydroxylase* (*TH*)	dopaminergic neurons	Jay Hirsch (University of Virginia, Charlottesville, VA)
w;;CRZ-GAL4	*corazonin (CRZ)*	corazonergic neurons	Youn-Jeong Choi (University of Tennessee, Knoxville)
w;TRH-GAL4	*tryptophan hydroxylase* (*TRH*)	serotonergic neurons	Barry Condron (University of Virginia Medical School, Charlottesville, VA)

Table 1. Contd.

Strain	**Gene promoter/ regulatory region controlling *GAL4* expression**	**Larval expressing-tissues or cells**	**Reference or Source**
w;;pBac{GAL4D, EYFP}5-HT2^{PL00052}	*5-HT2$_{Dro}$*	many regions in the CNS (cell type not specified)	Bloomington Stock Center (Indiana University, IN) stock # 19367
w;elav-GAL4	*embryonic lethal, abnormal vision* (*elav*)	all post-mitotic neurons	Bloomington Stock Center (Indiana University, IN) stock # 8765

Table 2. UAS lines used in this study

Strain	Gene fused to *UAS*	Funcion of the gene	Reference or Source
yw;UAS-hid	*head involution defective (hid)*	cell death	Grether *et al.*, 1995
yw;;UAS-rpr	*reaper (rpr)*	cell death	White *et al.*, 1996
yw;UAS-mCD8:GFP	*mouse cluster differentiation 8 (mCD8) - green fluorescent protein (GFP)*	green fluorescent labelling of cellular membrane	Bloomington Stock Center (Indiana University, IN) stock # 5130 & 5137
yw;UAS-tau-lacZ/Cyo	*microtubule-associated protein (tau) - β-galactosidase (lacZ)*	visualization of cell body and neural projections	Bloomington Stock Center (Indiana University, IN) stock # 5148
w;UAS-TNT-E	*tetanus neurotoxin light chain (TNT)* (E, weak active form)	cleavage of synaptobrevin - inhibition of synaptic vesicle docking	Sweeney *et al.*, 1995
w;UAS-TNT-G	*TNT* (G, strong active form)	cleavage of synaptobrevin - inhibition of synaptic vesicle docking	Sweeney *et al.*, 1995
w;UAS-TNT-VIF	*TNT* (VIF, inactive form)	no function (control)	Sweeney *et al.*, 1995
yw;UAS-EKO	*Electrically Knock Out (EKO)* (modified Shaker K^+ channel)	attenuation of neuronal electrical activity	White *et al.*, 2001
w;Kr^{If-1}/CyO; UAS-ORK1Δ-C1/TM6C, Sb^1	*Open Rectifier K^+ channel 1 (ORK1)* (modified)	attenuation of neuronal electrical activity	Bloomington Stock Center (Indiana University, IN) stock # 8928

Table 2. Contd.

Strain	Gene fused to *UAS*	Funcion of the gene	Reference or Source
yw;UAS-ORK1Δ-NC1	*ORK1*- Non-conducting (control)	normal neuronal electrical activity	Bloomington Stock Center (Indiana University, IN) stock # 6587
w;UAS-5-HT1A$_{Dro}$	*5-HT1A$_{Dro}$*	5-HT signalling	Julian Dow (University of Glasgow, Glasgow)
w;;UAS-5-HT1B$_{Dro}$/TM3,Ser	*5-HT1B$_{Dro}$*	5-HT signalling	Amita Sehgal (University of Pennsylvania, Philadelphia)
yw;UAS-5-HT1B$_{Dro}$RNAi/Cyo	*5-HT1B$_{Dro}$ RNAi*	down-regulation of 5-HT1B$_{Dro}$ receptor expression	Amita Sehgal (University of Pennsylvania, Philadelphia)
w;UAS-5-HT1B$_{Dro}$	*5-HT1B$_{Dro}$*	5-HT signalling	Amita Sehgal (University of Pennsylvania, Philadelphia)
w;UAS-5-HT2$_{Dro}$	*5-HT2$_{Dro}$*	5-HT signalling	Luc Maroteaux (Université de Strasbourg, Illkirch)
w;UAS-5-HT7$_{Dro}$	*5-HT7$_{Dro}$*	5-HT signalling	Julian Dow (University of Glasgow, Glasgow)
w;;UAS-slit	*slit*	signalling for neurite development/guidance and cell migration	Roger Jacobs (McMaster University, Ontario)
w;;UAS-TRH	*TRH*	5-HT biosynthesis	Dierick and Greenspan, 2007

Table 3. Other stocks used in this study

Strain	Gene affected	Characteristics/Phenotype	Reference or Source
w;GMR-hid	*glass (gl)*	ablation of larval photoreceptors and CNS *gl*-expressing cells by expression of *hid*	Grether *et al.*, 1995
Rac2$^{\Delta}$ ry	*Rac2*	null mutant (deletion) homozygous viable disruption of Rac signalling	Bloomington Stock Center (Indiana University, IN) stock # 6675
yw; Rac1^{J11} FRT2A/TM6B	*Rac1*	null mutant (point mutation) embryonic lethal disruption of Rac signalling	Bloomington Stock Center (Indiana University, IN) stock # 6674
yw;Rac1^{J11} Rac2$^{\Delta}$ FRT2A/TM6B	*Rac1* and *Rac2*	null mutant (point mutation, deletion) embryonic lethal disruption of Rac signalling	Bloomington Stock Center (Indiana University, IN) stock # 6677
w;; pBac{PB} CG9122^{c01440} (pBacTRH)	*tryptophan hydroxylase (TRH)*	strong hypomorph mutant (insertional mutation) homozygous viable reduced 5-HT synthesis	Bloomington Stock Center (Indiana University, IN) stock # 10531
eg^{P289}	*eagle (eg)*	null mutant (P-element insertion) homozygous viable reduced number of 5-HT neurons	Marta Lundell (University of Texas, San Antonio, TX)
w;;eg^{18B}/TM3,Sb	*eagle (eg)*	null mutant (deletion) homozygous lethal reduced number of 5-HT neurons	Marta Lundell (University of Texas, San Antonio, TX)
w;;eg^{mz360}	*eagle (eg)*	hypomorphic mutant (P-element insertion) homozygous viable reduced number of 5-HT neurons	Marta Lundell (University of Texas, San Antonio, TX)

CHAPTER 3:

Genetic dissection of trophic interactions in the larval optic neuropil of *Drosophila melanogaster*

Verónica G. Rodriguez Moncalvo and Ana Regina Campos

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Contribution to this research

Verónica G. Rodriguez Moncalvo performed all of the immunohistochemical and behavioural assays presented in this paper. In addition, she also made all the figures that appear in this work. Both Verónica G. Rodriguez Moncalvo and Ana R. Campos worked together in the writing of this manuscript.

3.1. Abstract

The larval visual system of *Drosophila melanogaster* consists of two bilateral clusters of 12 photoreceptors, which express Rhodopsin 5 and 6 (Rh5 and Rh6) in a non-overlapping manner. These neurons send their axons in a fascicle, the larval optic nerve (LON), which terminates in the larval optic neuropil. The LON is required for the development of a serotonergic arborization originating in the central brain and for the development of the dendritic tree of the circadian pacemakers, the small ventral lateral neurons (LNv) (Malpel *et al.*, 2002; Mukhopadhyay and Campos, 1995). Here, we show that both Rh5- and Rh6-expressing fibers overlap equally with the 5-HT arborization and that it, in turn, also contacts the dendritic tree of the LNv. The experiments described here aimed at determining whether Rh5-or Rh6-expressing fibers, as well as the LNv, influence the development of this serotonergic arborization. We conclude that Rh6-expressing fibers play a unique role in providing a signal required for the outgrowth and branching of the serotonergic arborization. Moreover, the innervation of the larval optic neuropil by the 5-HT arborization depends on intact Rac function. A possible role for these serotonergic processes in modulating the larval circadian rhythmicity and photoreceptor function is discussed.

Keywords: rhodopsin, photobehaviour, *Drosophila,* serotonin, larva, mutant, photoreceptors, pacemaker

3.2. Acknowledgements

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3.3. Introduction

Assembly of neural circuits requires the orchestration of multiple intrinsic and extrinsic signals (for reviews see Grueber and Jan, 2004; Jan and Jan, 2003; McAllister, 2000). For instance, the role of neurotrophins in dendrite development and maintenance is well established (Huang and Reichardt, 2001; McAllister, 2001; McAllister *et al.*, 1995). Recently, receptor-ligand systems initially identified on the basis of their function in axon guidance have been added to the growing list of extrinsic cues that modulate dendrite development (Furrer *et al.*, 2003; Polleux *et al.*, 2000; Whitford *et al.*, 2002). Furthermore, the role of afferent neurotransmission in dendritic development is also well established and conserved in many organisms (Li *et al.*, 2002; Rajan and Cline, 1998; Rajan *et al.*, 1999; Sin *et al.*, 2002; Wong and Ghosh, 2002).

Although the underlying mechanisms by which extrinsic factors exert their effects on dendritic and axonal growth remain largely unknown, accumulating evidence in different model systems have revealed links between some of these factors and the activation of Rho GTPases (Fan *et al.*, 2003; Hu *et al.*, 2001; Li *et al.*, 2002; Sin *et al.*, 2002; Yamashita *et al.*, 1999). Moreover, these studies support the notion that members of the Rho family of GTPases function as key integrators of extrinsic and intrinsic cues that regulate the underlying dendritic and axonal cytoskeleton.

This report examines the interaction among three groups of neural processes in the *Drosophila melanogaster* larval optic neuropil. The central nervous system of *Drosophila* like in many other insects follows a typical organization in which the somata

of neurons and glia form an outer layer surrounding the inner neuropil where axons and dendrites are segregated (Campos *et al.*, 1995; Nassif *et al.*, 2003). By the end of embryogenesis, the larval optic nerve (LON) terminates within the optic lobe anlagen in the larval optic neuropil area. The larval visual system of *Drosophila* consists of a pair of bilateral visual organs also known as Bolwig's organs, situated just anterior to the cephalopharyngeal skeleton (Green *et al.*, 1993; Steller *et al.*, 1987). Each of these visual organs is made up of 12 photoreceptor cells that differentiate during stage 13 of embryogenesis (Campos *et al.*, 1995; Green *et al.*, 1993) and are divided in 2 subsets: ~ 4 cells expressing the blue-absorbing opsin Rh5 (λ_{max} = 437 nm) and ~ 8 cells expressing the green-absorbing opsin Rh6 (λ_{max} = 508 nm) (Malpel *et al.*, 2002).

It has been previously shown that the LON overlaps with a serotonergic arborization that originates from cell bodies located in the central brain (Mukhopadhyay and Campos, 1995). More recently, it has been reported that the LON terminus overlaps with the dendritic arborization of a subset of circadian pacemaker neurons, the small ventral lateral neurons (LNv) (Malpel *et al.*, 2002). Interestingly, and similar to what was previously shown for the serotonergic arborization, the development of the LNv dendritic arbor is dependent on contact with the LON (Malpel *et al.*, 2002; Mukhopadhyay and Campos, 1995).

Here, we show that both Rh5- and Rh6-expressing fibers overlap with the 5-HT arborization, which in turn, also contacts the dendritic tree of the LNv. The results of cell ablation experiments indicate that the presence of Rh6-expressing fibers is necessary for the development of the serotonergic arborization. Moreover, suppression of synaptic

activity by targeted expression of tetanus toxin light chain (TNT) in Rh6-expressing fibers prevents the full development of this 5-HT arborization suggesting that this is at least in part an activity-dependent process. Finally, our results implicate Rac signalling in the development of the serotonergic arborization.

3.4. Results

3.4.1. Both the Rh5-and the Rh6-expressing fibers overlap with the larval optic neuropil 5-HT arborization

The LON is formed by two groups of axons distinguished by the non-overlapping expression of Rh5 and Rh6 (Malpel *et al.*, 2002). In late third instar larvae the LON is found intimately associated with a 5-HT arborization in the larval optic center (Fig. 3.1A-C) and (Mukhopadhyay and Campos, 1995). However, whether both sets of photoreceptor cells are involved in this contact was not known. In order to address this question, brains dissected from wandering third instar larvae in which the Rh5-specific photoreceptors neurons express mCD8-GFP under the regulation of the *Rh5* promoter (*yw;UAS-mCD8-GFP;Rh5-GAL4*, N = 18) were labelled with 5-HT antibody (Fig. 3.1D-F). Similarly, aged specimens dissected from strains in which mCD8-GFP was specifically expressed in Rh6 neurons were labelled with 5-HT (*w;UAS-mCD8-GFP;Rh6-GAL4*, N = 18) (Fig. 3.1G-I). At the level of resolution afforded by the confocal microscope, while variable from specimen to specimen, the termini of either subset of photoreceptor axons did not display any distinguishing characteristics. Likewise, the degree of overlap between both subset of photoreceptor axons and the 5-HT arborization in the larval optic neuropil was apparently the same (Fig. 3.1F and I).

3.4.2. The 5-HT arborization is intimately associated with the dendritic tree of the LNv in the larval optic neuropil

It has been previously reported that the larval LNv contact both, the Rh5- and the Rh6-expressing fibers and that this interaction with the LON provides trophic support for the development of the LNv dendritic tree (Malpel *et al.*, 2002). We asked whether the LNv would also contact the serotonergic arborization in the larval optic center. To that end, larval brains in which the LNv were labelled by virtue of the targeted expression of mCD8-GFP (*yw;PDF-GAL4,UAS-mCD8-GFP*, N = 11) were treated with 5-HT antibody (Fig. 3.2). As shown in Fig. 3.2C, the 5-HT arborization was found in intimate association with the dendritic tree of the larval LNv (arrowhead).

3.4.3. The development of the serotonergic arborization does not depend upon the presence of the Rh5-expressing fibers or the larval LNv

In order to determine whether the Rh5-expressing fibers influence the development of the 5-HT arborization, we investigated the impact of absence of Rh5 fibers on the integrity of the serotonergic arborization as seen by anti 5-HT immunolabelling. For this purpose, the Rh5-expressing photoreceptors were ablated by targeted expression of the cell death gene *hid* under the regulation of the *Rh5-GAL4* driver. In this and all subsequent experiments cell ablation was assessed by the concomitant expression of the mCD8-GFP reporter or by photoreceptor-specific protein Chaoptin staining (Zipursky *et al.*, 1984). As shown in Fig. 3.3B, no obvious defect in the 5-HT arborization (arrowhead) is observed when the Rh5-expressing photoreceptors

are absent (*yw;UAS-mCD8-GFP/UAS-hid;Rh5-GAL4/+*, N = 31), suggesting that this subset of photoreceptor cells is not required for the proper innervation of the larval optic neuropil by the serotonergic arborization. A similar result was observed when the LNv were ablated due to targeted expression of *rpr* death gene by *PDF-GAL4*. In these specimens the serotonergic arborization is indistinguishable from that of wild type samples suggesting that it develops normally in the absence of the LNv (Fig. 3.3D, arrowhead) (*UAS-mCD8-GFP,PDF-GAL4/UAS-rpr*, N = 14).

3.4.4. Rh6-expressing fibers are required for the development of the serotonergic arborization

In order to examine whether the Rh6-expressing fibers are required for normal development of the serotonergic arborization, the Rh6 cells and their axons were ablated by targeted expression of *hid* driven by the *Rh6-GAL4* element. No 5-HT labelling was detected in the larval optic neuropil area when the Rh6 fibers were absent (Fig. 3.4 compare A to C) (*UAS-mCD8-GFP/UAS-hid;Rh6-GAL4/+*, N = 26). These results are indistinguishable from those obtained by the complete absence of the LON in *glass* mutants as previously reported by Mukhopadhyay and Campos (1995) or due to the expression of *hid* under the *glass multimer reporter (GMR)* promoter (Fig. 3.4B, N = 16). Thus, these observations suggest that, among the larval photoreceptors, the presence of the Rh6-expressing fibers is specifically required for the development of the serotonergic arborization.

3.4.5. Simultaneous ablation of Rh5 photoreceptors and LNv does not disrupt the development of the 5-HT arborization

The results presented above do not address whether Rh6 projection is sufficient for the development of the 5-HT arborization in the larval optic neuropil. In order to assess this, one would need to eliminate all other neurons known to project to the larval optic neuropil except the Rh6 photoreceptor cells. While the identity of all neurons that project to the larval optic center is not known, two other types of neurons, namely the Rh5 photoreceptors and the LNv, have been shown not to be required individually for the presence of 5-HT labelling in the larval optic center. Given that there are only 3-4 Rh5 axons and a similar number of LNv axons, it is possible that reduction of a putative trophic support provided by either Rh5 or LNv alone is not sufficient to impact the development of the 5-HT arborization. In order to address this question and to determine whether the requirement of Rh6 projection for the development of the 5-HT arborization in the larval optic neuropil is a feature unique of these neurons we ablated Rh5 photoreceptors and LNv simultaneously by the targeted expression of *rpr* as described above. No obvious defect in the 5-HT arborization is observed when both the LNv and the Rh5-expressing fibers are absent (arrowhead in Fig. 3.5B) (*PDF-GAL4,UAS-mCD8-GFP/UAS-rpr;Rh5-GAL4/+*, N = 16). Thus, these results further confirm that the larval LNv and Rh5-expressing fibers are not required for the normal development of the 5-HT arborization.

3.4.6. Absence of 5-HT arborization in LON ablated larvae is due to reduced branching and not lack of 5-HT expression

Disruption in the development of the serotonergic arborization as a consequence of afferent ablation is inferred by the absence of 5-HT immunolabelling in the larval optic neuropil. As such these results do not distinguish between reduction in 5-HT synthesis and/or transport, or impaired branching of these neurons. In order to distinguish between these alternatives we sought to visualize these neurons and their projections by targeting the expression of GFP using a *GAL4* driver regulated by the *DOPA-decarboxylase* (*Ddc*) gene regulatory region (Li *et al.*, 2000). In these larvae, serotonergic as well as dopaminergic neurons and their projections can be visualized by virtue of GFP expression driven by the *Ddc* gene promoter. The GFP expressing projection that corresponds to the serotonergic arborization in the larval optic neuropil can be identified due to its stereotypic position relative to other landmarks and its intimate association with the terminus of the larval optic nerve (Fig. 3.6A-C). Ablation of larval photoreceptors by expression of the cell death gene *hid* in these larvae, appears to impair the branching of the 5-HT arborization as seen by the absence of GFP as well as 5-HT labelling (Fig. 3.6D-F). These results demonstrate that the LON provides a putative trophic signal required for the branching of this arborization rather than for the expression and localization of 5-HT.

3.4.7. Suppression of synaptic activity in the Rh6-expressing fibers disrupts the branching of the 5-HT arborization

In an attempt to investigate whether synaptic activity of Rh6-expressing fibers may influence the development of the 5-HT arborization, a weak tetanus-toxin light chain allele (*TNT-E*) or a strong tetanus-toxin light chain allele (*TNT-G*) was expressed under the control of *Rh6-GAL4* driver. The *TNT* gene product cleaves synaptobrevin thereby inhibiting synaptic vesicle docking (Sweeney *et al.*, 1995). In CNS specimens dissected from larvae in which Rh6 photoreceptors expressed *TNT-E*, a blind analysis revealed that 37.9% of the lobes displayed a notable alteration in the branching of the 5-HT arborization (N = 56, data not shown). A more penetrant phenotype was observed when these cells expressed the stronger *TNT-G* allele. In these specimens, 79.3% of the lobes displayed a similar reduction of the 5-HT branching as seen when the less active form of *TNT* was expressed (Fig. 3.7C, N = 30). Comparable results were obtained when *TNT-E* or *TNT-G* were expressed under the control of general photoreceptor driver *GMR-GAL4*. In these specimens, 39.7% of the *GMR-GAL4xUAS-TNT-E* (N = 26; data not shown) and 72.7% of the *GMR-GAL4xUAS-TNT-G* lobes (Fig. 3.7D; N = 22) showed such 5-HT branching disruption. In all these cases, the larval photoreceptors developed normally as determined by the pattern of photoreceptor-specific protein Chaoptin staining (Zipursky *et al.*, 1984) (Fig. 3.7, insets).

In order to dissect the role of electrical versus synaptic activity in the development of the serotonergic arborization, we expressed a genetically modified Shaker K^+ channel (the EKO channel) under the control of the *Rh6-GAL4* element. This K^+ channel

attenuates electrical activity by being activated at potentials close to E_k and by remaining open (White *et al.*, 2001). Confocal micrographs of third instar larval brains labelled with 5-HT antibody did not reveal any impact on the development of the 5-HT arborization due to the expression of the EKO channels in the Rh6 cells (N = 27; data not shown). A similar result was observed when the EKO channel was expressed in all larval photoreceptor cells through the use of the *GMR-GAL4* driver (N = 14; data not shown). It is possible that partial suppression of excitability was achieved by expression of these modified channels in these cells. However, behavioural assays carried out with third instar *GMR-GAL4/UAS-EKO* larvae revealed a significant reduction in the response to light compared to controls and as revealed by their mean RIs (*GMR-GAL4/UAS-EKO*, N = 14, RI = 0.11; *UAS-EKO/+*, N = 13, RI = 0.40; OR, N = 16, RI = 0.34; ANOVA: $F_{(2,40)} = 41.51$, $p < 0.001$). Taken together, these results suggest that the innervation of the larval optic neuropil by the 5-HT arborization does not depend on evoked synaptic activity of the Rh6-expressing fibers. Moreover, these results support the notion that spontaneous synaptic activity is sufficient to induce the branching of this serotonergic arborization.

3.4.8. Rac signalling is required for the branching of the 5-HT arborization

While the identity of the LON-derived signal is not known yet, results obtained in other model systems point to Rac GTPases as possible integrators in the activity-dependent development of the serotonergic arborization. Therefore, we investigated the integrity of this projection in larvae with reduced *Rac* function. To this end we took

advantage of a mutant chromosome carrying null mutations in two *Rac* genes (*Rac1* and *Rac2*) present in the *Drosophila* genome (Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002). Heterozygotes carrying at least one wild type copy of either one of the *Rac* genes survive until after the third instar larval stage. Moreover, organisms homozygous for just the *Rac2* null allele are viable. The level of reduction in Rac signalling afforded by these heteroallelic combinations did not cause any major developmental defect in the third instar larval brain as seen by the normal overall 5-HT staining (data not shown). Similarly, the larval photoreceptors developed appropriately as determined by the pattern of photoreceptor-specific protein Chaoptin staining (Zipursky *et al.*, 1984) (Fig. 3.8, insets).

Therefore we reasoned that the residual *Rac* function provided by one wild-type copy of *Rac1* or *Rac2* was sufficient for most of the basic developmental processes required for the assembly of the larval circuitry that takes place earlier during embryogenesis. However, it was possible that inductive processes, such as that which takes place in the larval optic neuropil, may require Rac signalling which is above the level of that provided by the heteroallelic combinations as described before.

The integrity of the 5-HT arborization and the degree of overlap with the LON was analyzed in blind experiments. Figure 3.8 depicts representative confocal micrographs of these specimens. Development of the 5-HT arborization was markedly reduced in 83% of CNSs dissected from larvae carrying only one functional copy of either *Rac1* (N = 42) or *Rac2* (N = 22) (Fig. 3.8 B, C). While the degree of reduction of the 5-HT arborization was similar in all mutant combinations analysed, the penetrance of

this phenotype varied considerably. Marked disruption in the 5-HT arborization was seen in 37% of the *Rac2 /Rac2* CNSs (data not shown). Interestingly, the reduction of the 5-HT arborization caused by reduced Rac function is similar to that found in larvae in which synaptic transmission was suppressed by the targeted expression of *TNT* (Fig. 3.7) and less than that observed when the entire LON was ablated (Fig. 3.4).

The overall integrity of the 5-HT system and the visual system morphology suggest that reduction of Rac function caused by these heteroallelic combinations does not have a pleiotropic effect on the nervous system development that would explain the disruption of the 5-HT arborization. Thus, although the present results do not provide a direct link between the LON-derived signal and Rac activation, they strongly suggest that a Rac-dependent signalling pathway is involved in the transduction of the signal provided by the LON for the development of this arborization.

3.5. Discussion

The dendritic arbor of the LNv, the termini of Rh5 and Rh6 photoreceptors overlap equally with the serotonergic arborization in the larval optic neuropil (Fig 3.1 and 3.2). Ablation of two of these groups of neurons, the 3-4 Rh5 photoreceptors and the 4 LNv did not disrupt the development of the 5-HT arborization (Fig. 3.3 and 3.5). However, ablation of Rh6-expressing fibers that originate from circa 8 retinular neurons had a drastic effect on the development of this 5-HT arborization (Fig. 3.4C). These observations are consistent with those found in *glass* (*gl*) mutants (Mukhopadhyay and Campos, 1995) and in *GMR-hid* larval brains (Fig. 3.4B) in which the LON is absent, suggesting that the Rh6 photoreceptors are uniquely required for the development of this serotonergic arborization.

The role of afferent activity in the development of postsynaptic partners is well documented in various systems (Miller and Kaplan, 2003; Sin *et al.*, 2002; Van Aelst and Cline, 2004; Wong and Ghosh, 2002). Consistent with these observations is the finding that disruption of synaptic activity of the Rh6 fibers by targeted expression of tetanus-toxin light chain ("weak", TNT-E or "strong", TNT-G) caused reduction in the branching of the 5-HT arborization in the majority of specimens (Fig. 3.7D). Targeted expression of TNT completely eliminates evoked synaptic transmission and decreases spontaneous synaptic vesicle release by about 50% (Deitcher *et al.*, 1998; Sweeney *et al.*, 1995). Attenuation of electrical activity due to the expression, in Rh6 cells or in all photoreceptors, of the modified K^+ channel EKO did not have any effect on the

development of the 5-HT arborization. It is possible that only partial suppression of excitability was achieved in these larvae, similar to what was observed for adult photoreceptors (White *et al.*, 2001). However, behavioural assays carried out with third instar *GMR-GAL4/UAS-EKO* larvae revealed a significant reduction of the response to light compared to wild type larvae suggesting that evoked potentials had been significantly suppressed (data not shown). These observations are consistent with the finding that maintenance of larvae in the dark throughout development does not alter the morphology of the 5-HT projection in the larval optic neuropil (M. Mukhopadhyay and A.R. Campos, personal communication). Similarly, larvae carrying mutations in the *no receptor potential* (*norpA*) gene encoding the light-activated PLC required for phototransduction did not reveal any disruption in the development of the 5-HT arborization (data not shown).

Taken together, these results suggest that proper branching of the 5-HT arborization in the larval optic neuropil does not depend on evoked synaptic activity of the Rh6-expressing fibers but may rely on spontaneous neurotransmitter release. Alternatively, expression of TNT disrupts this process independently of its effects on synaptic physiology. A requirement for synaptobrevin function for the proper expression of the neural cell adhesion molecule Fasciclin II (FasII) has been reported in *Drosophila* (Baines *et al.*, 2002; Hiesinger *et al.*, 1999). Consistent with these observations is the demonstration that manipulation in the level of FasII expression mimics some aspects of the phenotypic consequences of synaptic suppression due to expression of TNT (Baines *et al.*, 2002).

Thus, the partial disruption of the 5-HT arborization by targeted expression of TNT on the LON afferents, by comparison to the complete disruption observed when Rh6 photoreceptors are ablated, may be explained by either partial suppression of spontaneous neurotransmitter release or by a synaptic vesicle release-independent effect such as modification of FasII expression. Alternatively, Rh6 fibers may provide an activity-independent trophic support that potentiates and/or maintains activity-dependent processes, similar to what is found in other systems. For example, in the developing cortex Sema3A, neurotrophins and Slit interact to specify the basic morphology of cortical neurons. As development proceeds, the control of further growth and branching is shifted to activity-dependent mechanisms that rely on global and local increases of intracellular calcium (reviewed by Wong and Ghosh, 2002).

Our observations suggest a role for *Rac1* and/or *Rac2* signalling in the transduction of the signal provided by the LON (Fig. 3.8). These results are consistent with previous studies suggesting a requirement for the Rho family of small GTPases, notably RhoA, Rac, and Cdc42 in neuronal morphogenesis. For instance, Hakeda-Suzuki *et al.* (2002) and Ng *et al.* (2002) have shown that *Drosophila* MB neurons mutant for *Rac1* and *Rac2* present defects in axon growth, guidance and branching. Interestingly, Rac has also been shown to be important for dendritic branching stability and morphogenesis of dendritic spines (reviewed in Govek *et al.*, 2005; Luo, 2002; Van Aelst and Cline, 2004). For example, Lee *et al.* (2003) have shown that *Rac1* mutant *Drosophila* DA neurons developed fewer dendritic branches than wild type neurons in the third instar larval stage. Our observations are unique in *Drosophila* in that they suggest a

role for Rac signalling in activity dependent neuronal morphogenesis. Alternatively, Rac signalling may be required for the synaptobrevin-dependent developmental process discussed above and reported previously (Baines *et al.*, 2002; Hiesinger *et al.*, 1999).

Drosophila adults that have been reared in complete darkness since embryogenesis still display rhythmic behaviour albeit not in synchrony with other individuals in the population (e.g. Sehgal *et al.*, 1992). These observations support the notion that a circadian clock is assembled during embryogenesis and is functional as the larva hatches. Synchronization of the circadian clock or its entrainment can be achieved by light treatment as early as the end of the first instar indicating that, in addition, photic input pathways are in place by the end of embryogenesis which are capable of resetting the pacemaker neurons (Malpel *et al.*, 2004; Sehgal *et al.*, 1992). Recent reports demonstrate a role for the larval visual system as a photic input pathway in entrainment during larval development (Malpel *et al.*, 2004). Whether all or a subset of the 12 larval photoreceptors found in each of the two larval eyes are equally involved in this process has not yet been addressed. Similarly, it is not known whether the larval visual system functions as an entrainment input pathway equally throughout larval development. Relevant to these questions are our previous observations that indicate that Rh6-expressing photoreceptors are not involved in the basal response to light measured in our behavioural assay (Hassan *et al.*, 2005). The results reported here demonstrate that these same photoreceptors (Rh6) are uniquely required for the induction of a 5-HT arborization that innervates the larval optic neuropil and that, it in turn, overlaps with the dendritic arborization of the main larval pacemaker neurons, the LNv. Thus, it is possible that the

larval photoreceptor function, as a circadian input pathway, is modulated by their interaction with the 5-HT arborization that takes place during the third instar larval stage.

Several reports demonstrate the presence of circadian rhythms in the visual system of insects. Of note are the extensive analysis of Meinertzhagen and colleagues that established the existence in *Musca domestica* and *Drosophila melanogaster* adults, of circadian oscillation in the number of synapses between the outer photoreceptors (R1-R6) and the first order lamina interneurons L1 and L2, and in the diameter of L1 and L2 axons (Pyza and Meinertzhagen, 1993; 1995; 1999). These neuroanatomical changes are believed to be regulated by the neuromodulators 5-HT and pigment dispersing factor (PDF) present in two sets of neurons that innervate the optic lobe neuropiles of adult flies (Chen *et al.*, 1999; Meinertzhagen and Pyza, 1996; Pyza and Meinertzhagen, 1996). This conclusion is supported by the observation that in the *Caliphora* compound eyes, the circadian rhythmicity of the light evoked response measured in electro retinograms (ERG) is affected by injection of 5-HT and PDF (Chen *et al.*, 1999). A recent report detailing circadian oscillation in the larval response to light (Mazzoni *et al.*, 2005) supports the hypothesis that the 5-HT arborization described here may be modulating larval visual system function.

Alternatively, the fact that 5-HT processes innervating the larval optic neuropil are found overlapping with the dendritic arborization of the LNv may suggest a direct modulation of the *Drosophila* larval pacemaker neurons. It has been shown that serotonin, in addition to its function in behaviour, also plays a role in modulating circadian locomotor activity and heart rate in insects. For instance, it has reported that

injection of the specific neurotoxin 5,7-DHT, which causes selective degeneration of serotonergic neurons, modified the level of locomotor activity and period of circadian rhythmicity in the blowfly (Cymborowski, 2003). Furthermore, it has been demonstrated that serotonin increases heart rate in *Drosophila* (Johnson *et al.*, 1997; Johnson *et al.*, 2002), supporting the idea of a direct modulatory effect of this neurotransmitter on pacemaker cells.

Previous developmental analysis of the 5-HT arborization has shown that the contact between the LON and the serotonin process in the larval optic center occurs during late second-early third instar larval stage (Mukhopadhyay and Campos, 1995). After that, the 5-HT processes undergo further branching. These observations suggest that this serotonergic arborization may have a role in the function of the larval visual system during the third instar larval stage. Interestingly, it is towards the end of this stage that the larva becomes progressively less photophobic, attaining photo neutrality just before pupariation (Sawin-McCormack *et al.*, 1995). Moreover, it has been shown that 5-HT is able to modulate the voltage dependency of K^+ channels in *Drosophila* adult photoreceptors (Hevers and Hardie, 1995; Kauranen and Weckstrom, 2004). Hence, it is possible that the innervation of the larval optic center by this 5-HT arborization plays a role in the modulation of the photobehaviour that occurs during the foraging-wandering transition (Sawin-McCormack *et al.*, 1995).

Figure 3.1. The termini of Rh5 and Rh6 expressing photoreceptors overlap with a 5-HT arborization in the larval optic neuropil. A-C, Low magnification confocal micrographs of a wild type wandering third instar larval hemisphere immunolabelled with anti-5-HT detected by Texas Red-conjugated secondary (red in A and in all subsequent panels and figures) and 24B10 monoclonal antibody detected by Alexa 488-conjugated secondary (B, green), showing the relationship between the LP1 serotonergic cell bodies (arrow in A), the serotonergic arborization in the larval optic center (arrowhead in A), the LON (arrowhead in B) and the developing adult retinal projection (arrow in B). C, Merge of A and B. The box represents the area studied in all panels and subsequent figures. D-I, High magnification confocal micrographs of GFP expression in Rh5 or Rh6 specific photoreceptors by targeted expression of the *UAS-mCD8-GFP* construct using either the *Rh5-GAL4* or *Rh6-GAL4* drivers (D-F, *UAS-mCD8-GFP;Rh5-GAL4*; G-I, *UAS-mCD8-GFP;Rh6-GAL4*). D, The LP1 cell bodies lie near the 5-HT arborization in the larval optic neuropil. E, The termini of the Rh5-expressing photoreceptor axons in the same region. F, Merge of D and E showing the overlapping between the 5-HT arborization and the Rh5 photoreceptors termini (arrowhead). The inset shows a higher magnification of this region. G-I, Same as in D-F but showing the overlap of the Rh6 photoreceptor termini with the 5-HT arborization in the larval optic neuropil (arrowhead in I). Scale bar in A represents 40 μm and is valid for B and C. Scale bar in D and G represents 10 μm and is valid for E, F, H and I.

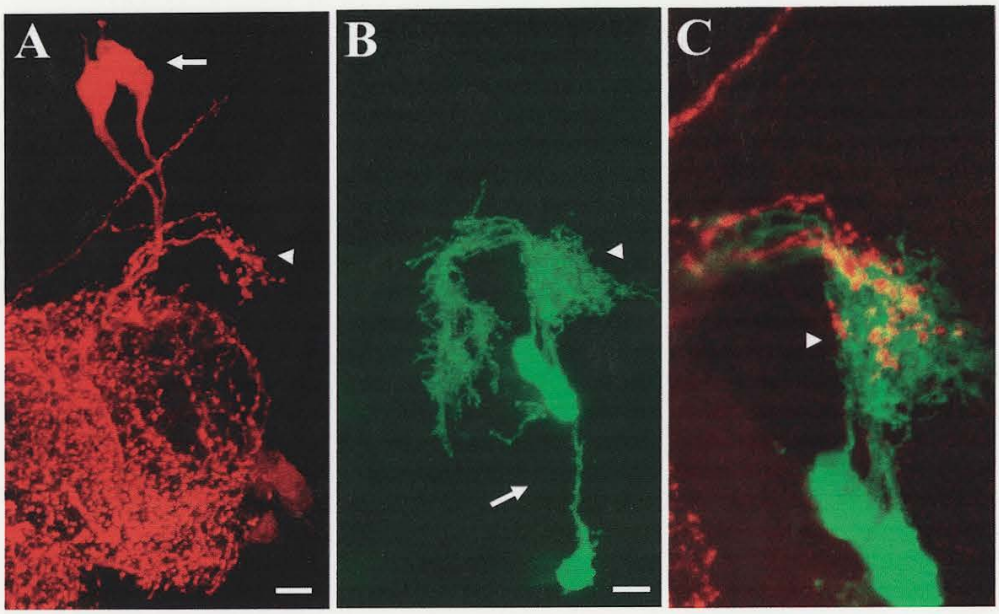
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Figure 3.2. The dendritic arborization of the LNv overlaps with the serotonergic innervation of the larval optic neuropil. Confocal micrographs of brains dissected from wandering third instar larvae (*PDF-GAL4,UAS-mCD8-GFP*) in which GFP (green) expression is targeted to the main circadian pacemakers, the LNv, and labelled with anti 5-HT antibody (red) and detected as mentioned before. A, LP1 serotonergic cells (arrow) and the larval optic neuropil 5-HT arborization (arrowhead). B, LNv (arrow) and their dendritic tree (arrowhead) in the larval optic center. C, Higher magnification merge of panels A and B, showing the overlap between the LNv dendrites and the 5-HT arborization. Scale bars: 10 μm.

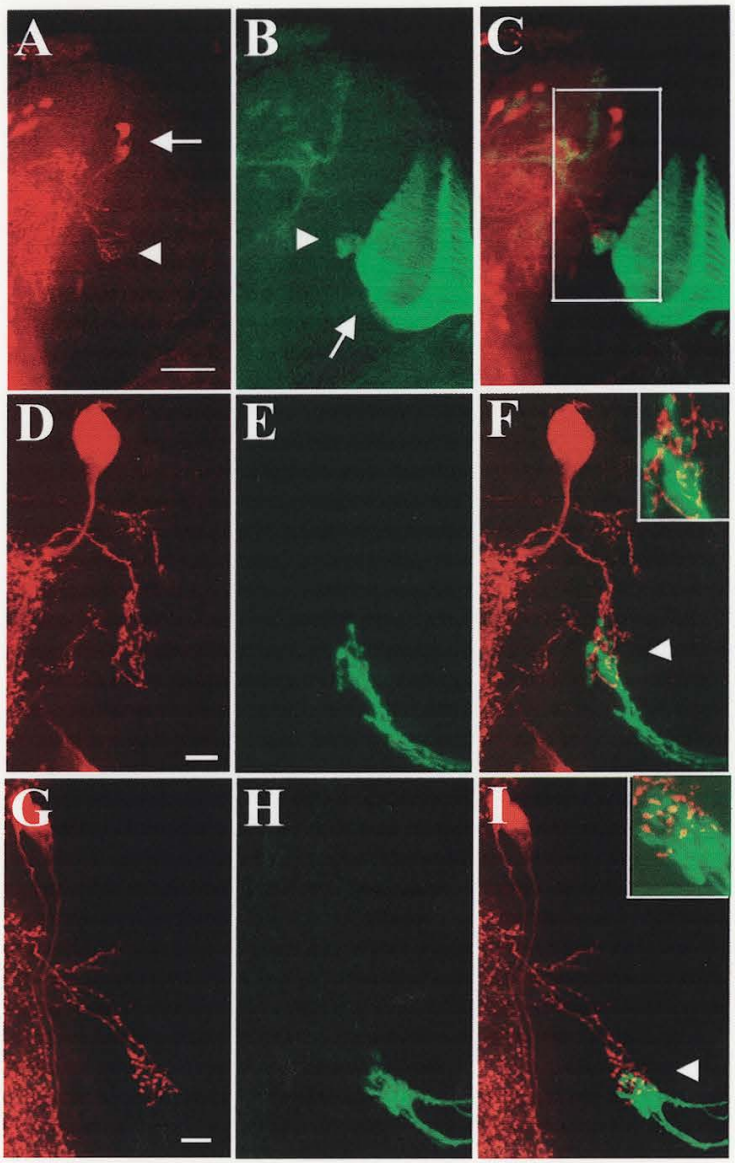
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Figure 3.3. Ablation of the Rh5-specific photoreceptors or LNv does not affect the development of the 5-HT arborization. The Rh5 photoreceptors or the LNv were ablated by targeted expression of the dead genes hid or rpr respectively and the dissected brains were labeled with 5-HT antibody as described before. In all specimens ablation was nearly complete as determined by the absence of expression of GFP in the targeted cells. A, Parental strain (*UAS-mCD8-GFP/+;Rh5-GAL4/+*) showing the stereotypical location of the 5-HT arborization (arrowhead). B, *UAS-mCD8-GFP/UAS-hid;Rh5-GAL4/+*. Ablation of Rh5 photoreceptors has no apparent effect in the development of the 5-HT arborization (arrowhead). C, Parental strain (*PDF-GAL4,UAS-mCD8-GFP/+*) showing the stereotypical location of the 5-HT arborization (arrowhead). D, *PDF-GAL4,UAS-mCD8-GFP/UAS-rpr*. Similar to what is observed for the Rh5 photoreceptors, the LNv are not required for the development of the 5-HT arborization (arrowhead). Scale bars: 10 μm.

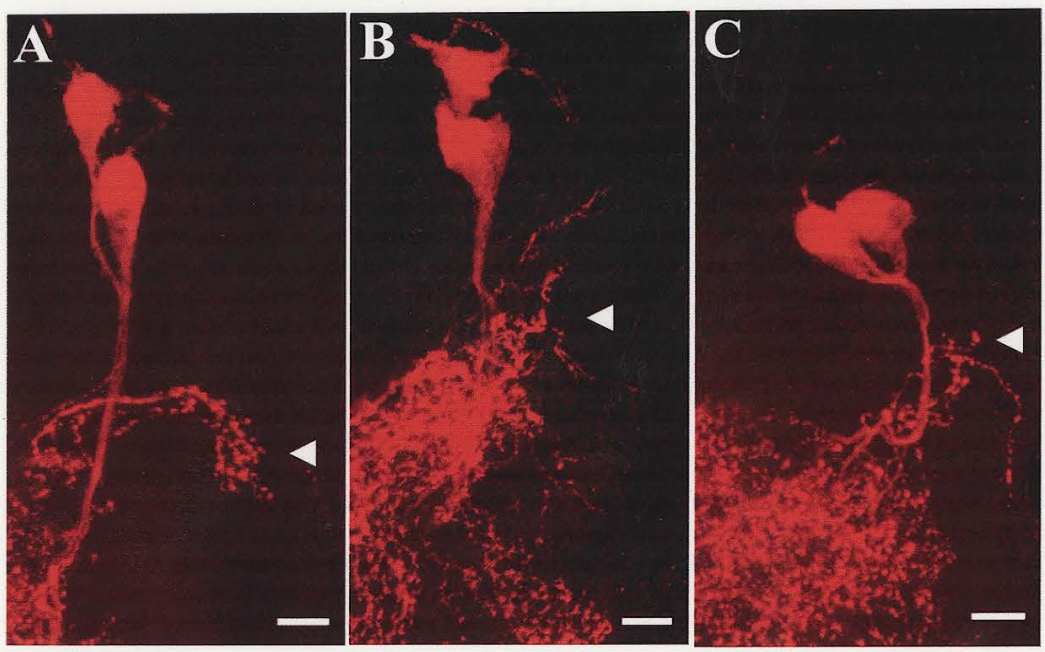
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Figure 3.4. The Rh6 photoreceptors are required for the proper development of the 5-HT arborization. A, Wild type parental control (*UAS-mCD8-GFP/+;Rh6-GAL4/+*) showing the stereotypical 5-HT innervation of the larval optic neuropil (arrowhead). B, *GMR-hid*. Ablation of the larval eye by the targeted expression of the cell death gene *hid* in all photoreceptors causes a significant reduction in the 5-HT arborization (arrowhead). C, *UAS-mCD8-GFP/UAS-hid;Rh6-GAL4/+*. Ablation of all Rh6 photoreceptors by targeted expression of hid in these cells causes a similar reduction in the serotonergic arborization (arrowhead). Scale bars: 10 μm.

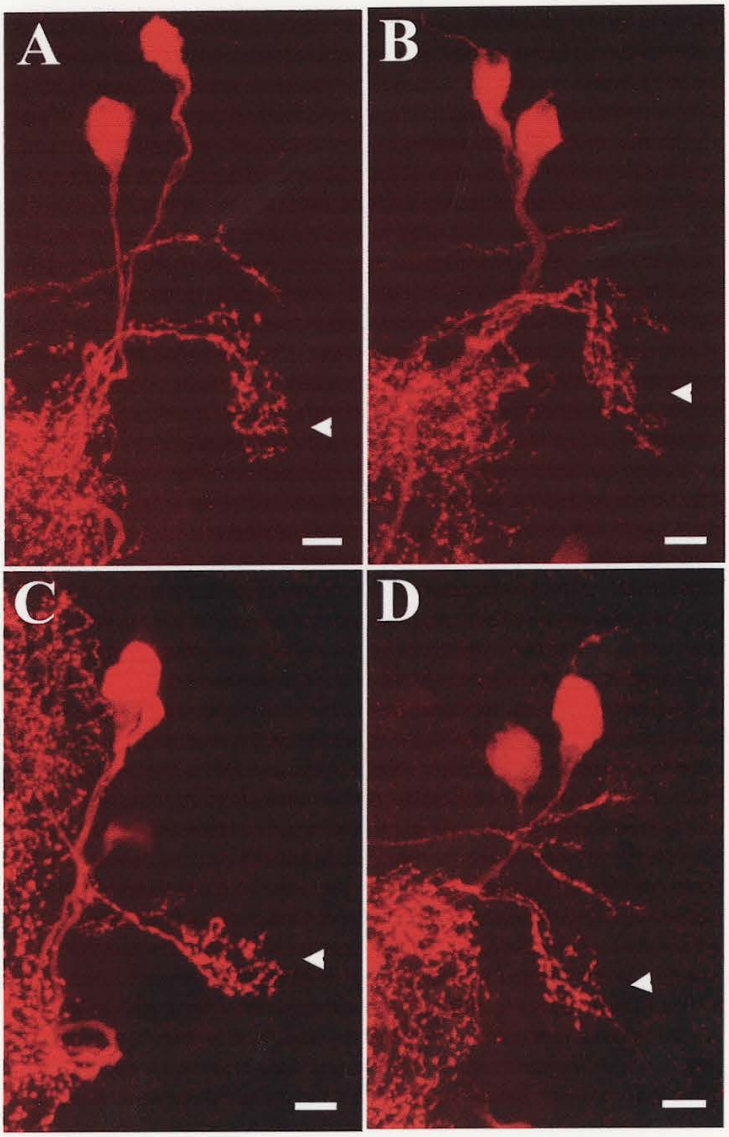
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Figure 3.5. Normal development of the larval optic neuropil 5-HT arborization in the absence of both the LNv and the Rh5-expressing fibers. Both A and B panels depict confocal micrographs of third instar larval brains labelled with anti 5-HT antibody and detected as before (red). A, Brain dissected from parental strain (*PDF-GAL4,UAS-mCD8-GFP/+;Rh5-GAL4/+*) showing the stereotypical location of the 5-HT arborization (arrowhead). B, *PDF-GAL4,UAS-mCD8-GFP/UAS-rpr;Rh5-GAL4/+*. The development of the 5-HT arborization is normal (arrowhead) in the absence of both the LNv and the Rh5 photoreceptors. Scale bars: 10 μm.

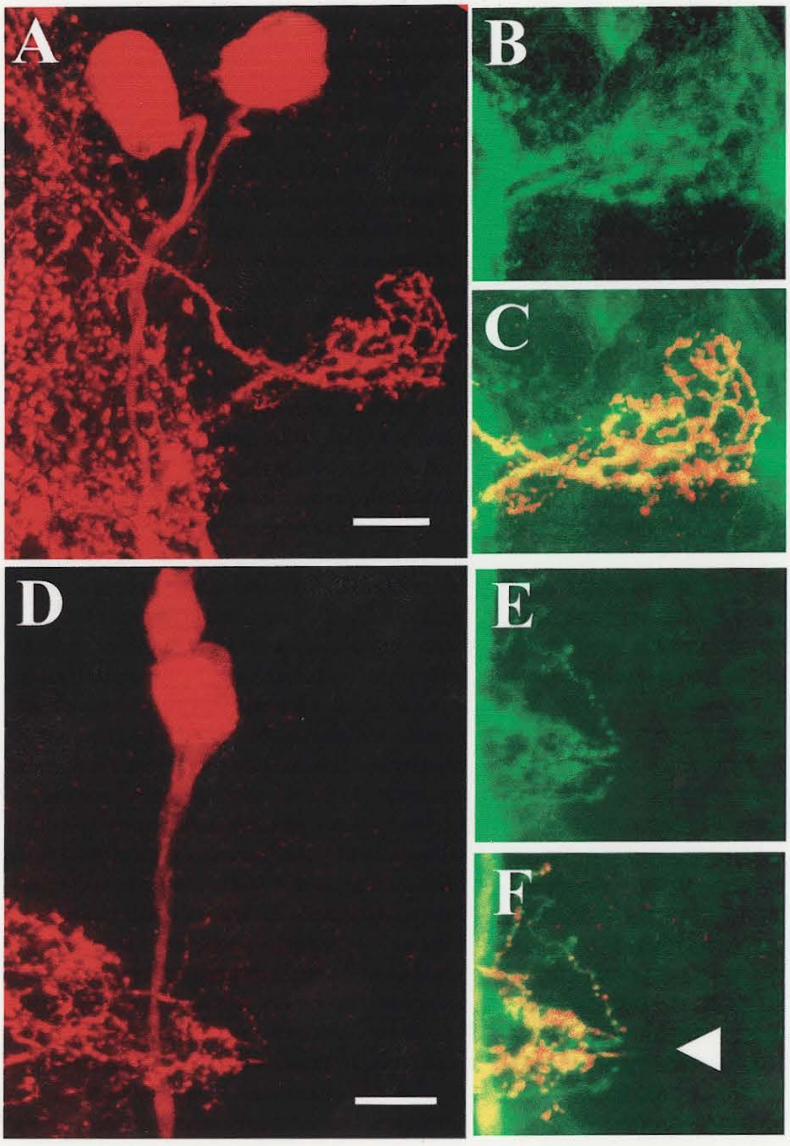
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Figure 3.6. Absence of 5-HT arborization in LON ablated larvae is due to reduced branching and not lack of 5-HT expression. In order to visualize the development of the serotonergic arborization independently from the expression of 5-HT, a *Ddc-GAL4* driver was used to target GFP expression to these neurons. Panels A-C depict the wild type parental control specimen (*Ddc-GAL4,UAS-mCD8-GFP*) showing the expected 5-HT arborization in the larval optic neuropil (A) and Ddc-regulated GFP expression in the same structure (B and C). Ablation of all photoreceptors in these flies by introducing the *GMR-hid* construct (*GMR-hid;Ddc-GAL4,UAS-mCD8-GFP*) dramatically reduces this arborization as seen by the lack of Ddc driven GFP expression (D) as well as 5-HT staining (E and F) (arrowhead). Scale bars: 10 μm.

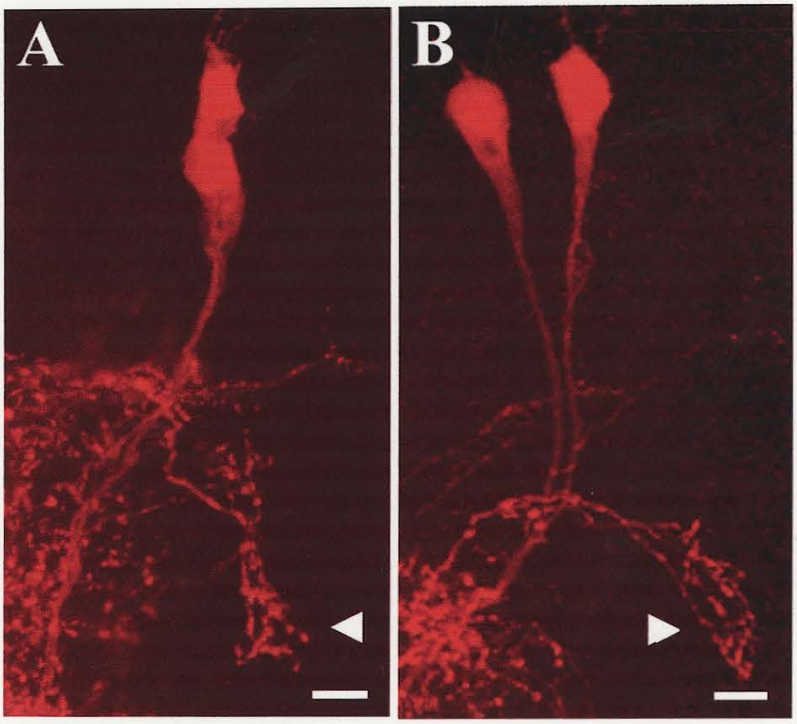
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Figure 3.7. Suppression of synaptic activity in the Rh6-expressing fibers disrupts the branching of the 5-HT arborization. In order to determine whether the induction of the 5-HT arborization by the larval Rh6 photoreceptor axons is an activity dependent process, tetanus toxin light chain (TNT) that suppresses synaptic activity was expressed in all larval photoreceptors or in the Rh6 photoreceptors only. The morphology of the Rh6 photoreceptor termini or the LON terminus was largely normal as evaluated by the concomitant expression of GFP or 24B10 monoclonal antibody staining detected by Alexa 488-conjugated secondary (green) respectively (insets). A, Wild type parental control *GMR-GAL4/+*. B, Control in which an inactive form of TNT is expressed in all photoreceptors (*GMR-GAL4/UAS-TNT-VIF*). C, Expression of TNT-G in all photoreceptors (*GMR-GAL4/UAS-TNT-G*) reduces the extent of 5-HT arborization (arrowhead). D, A similar phenotype is seen (arrowhead) when expression of TNT-G is restricted to Rh6 photoreceptors only (*UAS-mCD8-GFP/UAS-TNT-G;Rh6-GAL4/+*). Scale bars: 10 μm.

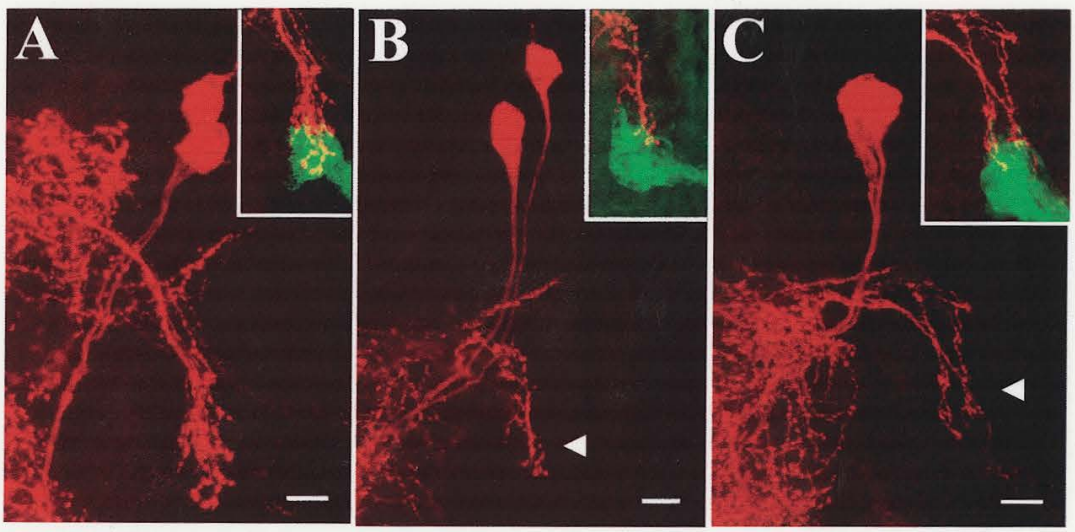
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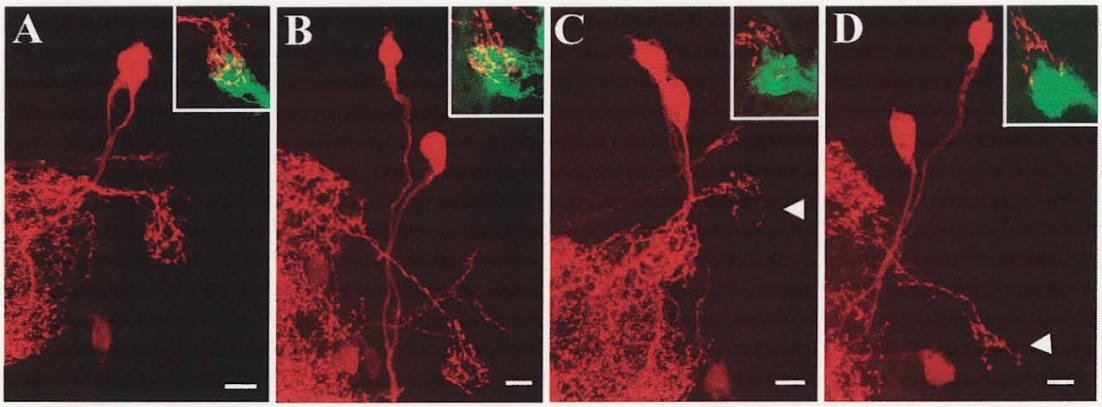
Figure 3.8. Induction of 5-HT arborization by the larval optic nerve depends on Rac signalling. Brains from larvae in which Rac function was reduced by mutations in either one of the two Rac genes were dissected and processed for 5-HT labelling as before. The morphology of the larval optic nerve was normal as visualized using the photoreceptor specific 24B10 monoclonal antibody (green). The following panels depict representative confocal micrographs. A, Wild type control OR. B, *Rac1 Rac2/Rac2* mutant brain. C, *Rac1 Rac2/Rac1* mutant brain. As seen in B and C (arrowheads) both mutants show a reduction in the branching of the serotonergic arborization. The insets in B and C show a higher magnification of the 5-HT arborization as its reduced overlap with the LON. Scale bars: 10 μm.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CHAPTER 4:

Role of serotonergic neurons in the modulation of *Drosophila* larval response to light

Verónica G. Rodriguez Moncalvo and Ana Regina Campos

(Manuscript ready to be submitted)

Contribution to this research

Verónica G. Rodriguez Moncalvo has carried out all of the immunohistochemical and behavioural studies as well as the assembly of all figures presented in this work. Verónica G. Rodriguez Moncalvo and Ana R. Campos co-wrote this manuscript.

4.1. Abstract

Serotonin (5-hydroxytryptamine, 5-HT) can function as a neurotransmitter, neuromodulator or neurohormone, regulating several biological processes in both invertebrates and vertebrates. In *Drosophila*, about 100 5-HT neurons innervate different larval CNS domains, including the larval optic center, where they contact the larval photoreceptors (Mukhopadhyay and Campos, 1995; Rodriguez Moncalvo and Campos, 2005). Behavioural studies performed previously in our laboratory have shown that the *Drosophila* larval photoresponse is characterized by changes in several locomotor parameters, demonstrating that the larval response to light can be utilized as a model to study control of locomotion (Busto *et al.*, 1999; Hassan *et al.*, 2000). Here we have used the larval response to light as a behavioural paradigm to assess the role of 5-HT neurons in the developmental regulation of larval behaviour. Our results indicate that serotonergic cells located in the brain hemispheres contribute to the decrease in the photophobic response observed during the later stages of larval development. Furthermore, our results suggest that this 5-HT modulatory effect occurs at a central level and may be mediated by 5-HT1A$_{Dro}$ receptors.

Keywords: *Drosophila*, larva, photobehaviour, serotonin, modulation, locomotion

4.2. Acknowledgements

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4.3. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an evolutionarily conserved neurotransmitter/neuromodulator that regulates numerous behaviours in both invertebrates and vertebrates (reviewed in Weiger, 1997). In *Drosophila*, 5-HT neurons have been implicated in regulation of reproductive function (Lee *et al.*, 2001), circadian rhythms (Yuan *et al.*, 2005; Nichols, 2007), sleep (Yuan *et al.*, 2006), heart rate (Dasari and Cooper, 2006; Neckameyer *et al.*, 2007; Nichols, 2006; Zornik *et al.*, 1999), feeding (Neckameyer *et al.*, 2007), olfaction (Neckameyer *et al.*, 2007; Python and Stocker, 2002), fly aggression (Dierick and Greenspan, 2007) and place memory (Sitaraman *et al.*, 2008).

The spectrum of 5-HT functions is consistent with its wide distribution and ability to activate several 5-HT receptor subtypes, which in turn are coupled to different signalling pathways (reviewed in Monastirioti, 1999; Nichols and Nichols, 2008). In *Drosophila* four GPCR 5-HT receptors have been identified: 5-HT1A_{Dro}, 5-HT1B_{Dro}, 5-HT2$_{Dro}$, and 5-HT7$_{Dro}$ (reviewed in Tierney, 2001). 5-HT1A_{Dro} and 5-HT1B_{Dro} lead to inhibition of adenylate cyclase and activation of phospholipase C, whereas 5-HT7$_{Dro}$ appears to activate adenylate cyclase (reviewed in Tierney, 2001). *In situ* hybridization studies indicate that all *Drosophila* 5-HT receptors are expressed in the embryonic CNS (Colas *et al.*, 1995; Saudou *et al.*, 1992). 5-HT1A_{Dro}, 5-HT1B_{Dro} and 5-HT2$_{Dro}$ receptor subtypes are expressed in adult CNS, while 5-HT1B_{Dro} and 5-HT2$_{Dro}$ are detected also in 3rd instar larval CNS (Nichols, 2007; Yuan *et al.*, 2005; 2006).

Drosophila larval locomotion consists of rhythmic peristaltic waves interrupted by episodes of pause, turning and head swinging, and occasionally backward crawling (Berrigan and Pepin, 1995; Green *et al.*, 1983; Wang *et al.*, 1997). As other rhythmic behaviours, larval locomotion is generated by activity of central pattern generators (CPG) and may be modified by neuromodulators such as serotonin as well as by sensory information (reviewed in Marder and Bucher, 2001; Marder *et al.*, 2005).

Drosophila larvae spend most of their time inside the food substrate. During this period (foraging stage), they are repelled by light (Sawin-McCormack *et al.*, 1995). The larval photophobic response is characterized by modifications in different motor parameters such as increased pausing, head swinging and direction change as well as reduced linear speed (Busto *et al.*, 1999; Hassan *et al.*, 2000; Scantlebury *et al.*, 2007). This behaviour is down-regulated during development, achieving near photoneutrality during wandering stage, when the larva leaves the food searching for a proper site to undergo metamorphosis (Sawin-McCormack *et al.*, 1995).

Drosophila larval photoreceptors consist of two bilateral clusters of 12 photoreceptors (Steller *et al.*, 1987). Their axons form the larval optic nerve (LON), which projects toward the optic neuropil in the brain (Green *et al.*, 1993; Campos *et al.*, 1995). These cells express either Rhodopsin 5 (Rh5) or 6 (Rh6) (Malpel *et al.*, 2002; Sprecher *et al.*, 2007) but only Rh5-expressing cells are required for the larval response to light detected in our assays (Hassan *et al.*, 2005).

Circa 100 5-HT neurons project toward different regions of the *Drosophila* larval CNS, including the larval optic neuropil, where they contact the LON (Hamasaka and

Nässel, 2006; Mukhopadhyay and Campos, 1995; Rodriguez Moncalvo and Campos, 2005; Vallés and White, 1988; Vömel and Wegener, 2008). Here, we report that 5-HT neurons in the brain hemispheres modulate the response to light during larval development, possibly through activation of 5-HT1A_{Dro} receptors.

4.4. Results

4.4.1. Silencing of DOPA-decarboxylase (Ddc) neurons increases the response to light throughout larval development

In order to identify neurons that provide neuromodulatory input for the developmental regulation of the larval behaviour, we used genetic tools to suppress synaptic transmission in candidate neurons and study the impact on the larval response to light at different times during development. To that end, we employed the GAL4/UAS system (Brand and Perrimon, 1993). In this approach, specific enhancers or promoters are used to regulate the expression of the yeast transcription factor GAL4. A gene of interest, such as different neuronal silencers or dsRNA constructs, is placed under the control of the GAL4-responsive upstream activating sequence (*UAS*), thereby, allowing their expression in a tissue-specific manner (Brand and Perrimon, 1993).

We began by expressing tetanus toxin light chain (TNT) in *Ddc* neurons by means of the *Ddc-GAL4* driver (Li *et al.*, 2000). Ddc catalyzes the last step in the synthesis of both serotonin and dopamine, and thus it is found in both 5-HT and dopaminergic neurons (reviewed in Monastirioti, 1999). TNT cleaves the vesicle-associated protein synaptobrevin and its targeted expression disrupts evoked neurotransmitter release and decreases spontaneous release by ~ 50% (Sweeney *et al.*, 1995). The behavioural paradigm used was the ON/OFF assay, which has been previously used in our laboratory (Busto *et al.*, 1999; Hassan *et al.*, 2005; Scantlebury *et al.*, 2007). In this assay, a single larva placed on a non-nutritive agar surface is subjected to intermittent 10 second-pulses

of light. Thus, response to light can be measured by changes in different locomotory parameters such as distance travelled, head swinging behaviour and change of direction that occurs when the larva is exposed to a light pulse (Busto *et al.*, 1999; Hassan *et al.*, 2005; Scantlebury *et al.*, 2007). Here we measured the response to light using the difference in distance travelled during the dark and light pulses and reflected in a response index (RI) (where RI = [(total distance travelled in the dark period - total distance travelled in the light period)/total distance travelled in both the periods]).

We found that larvae expressing active TNT (TNT-G) in Ddc neurons (henceforth referred to as *Ddc:TNT-G* larvae) show a significant increase in their response to light throughout larval development when compared to control larvae expressing inactive TNT (TNT-VIF) (referred to as *Ddc:TNT-VIF* larvae) (Fig. 4.1). It is worth noting that during wandering stage, wild type larvae display nearly photoneutral behaviour (Sawin-McCormack *et al.*, 1995). Nevertheless, inactivation of Ddc-expressing neurons elicits a robust response to light during this larval stage.

Perimeter stacks and centroid tracks of representative 3rd instar foraging *Ddc:TNT-G* larvae generated using Dynamic Imaging Analysis Software (DIAS) showed that the high response to light of these larvae is characterized by increased pausing, head swinging behaviour and backward movement, as well as by sharp changes in path direction (Fig. 4.2A). In contrast, control *Ddc:TNT-VIF* larvae when exposed to the light pulse rarely perform backward locomotion, and present limited head swinging behaviour and change of direction (Fig. 4.2B). As expected, analysis of crawling patterns of wandering control *Ddc:TNT-VIF* larvae showed that, during the light ON pulses, these

larvae exhibit mostly linear movement (Fig. 4.2D). In contrast, wandering *Ddc:TNT-G* larvae still respond to light (Fig. 4.2C).

In order to determine whether the silencing of Ddc neurons caused a developmental delay that would explain the elevated larval response to light all genotypes were tested for developmental timing (see material and methods). The presence of specific morphological characteristics including the shape of their mouth hooks, the number of teeth, and the morphology of the anterior spiracles as well as behavioural characteristics such as crawling outside the food and emptying of the gut that occurs in the wandering larval stage demonstrated that suppression of Ddc neuronal activity did not change the timing of larval molts or the onset of the transition from foraging to wandering (data not shown and Fig. A1). In addition, we did not observe differences in pupation time between groups (data not shown).

It has been suggested that TNT expression may cause other phenotypes independent from its role as neuronal silencer (Hiesinger *et al.*, 1999; Baines *et al.*, 2002). Therefore, we carried out similar experiments using genetically modified Shaker and open rectifier K^+ channels, *UAS-EKO* and *UAS-ORK1Δ-C* respectively, previously used to suppress neuronal excitability (Nitabach *et al.*, 2002; White *et al.*, 2001; e.g. Ishimoto *et al.*, 2005; Luan *et al.*, 2006). Larvae expressing *UAS-EKO* and *UAS-ORK1Δ-C* constructs in Ddc neurons show an increase in their response to light from late 2nd to late 3rd instar stage, similar to that displayed by *Ddc:TNT-G* larvae (Fig. A2 & A3). We conclude that the function of Ddc expressing neurons is required for the regulation of the response to light during larval development.

4.4.2. 5-HT neuronal function is required for proper modulation of the larval response to light

In order to determine the relative contribution of serotonergic and dopaminergic neurons to the increase in the response to light observed in *Ddc:TNT-G* larvae, we took advantage of *GAL4* driver constructs expressed exclusively in both neuron types (Chen and Condron, 2008; Friggi-Grelin *et al.*, 2003).

Tyrosine hydroxylase (TH) performs the rate-limiting step in dopamine biosynthesis and is expressed specifically in dopaminergic cells (Lundell and Hirsh, 1994; data not shown). Tryptophan hydroxylase (TRH, known as TPH in mammals) catalyzes the biosynthesis of 5-hydroxytryptophan from the amino acid tryptophan and constitutes the rate-limiting step in 5-HT production. *Drosophila* has two enzymes able to synthesize 5-HT: neuronal tryptophan hydroxylase (DTRHn, referred here as to TRH), whose expression pattern in the CNS matches that of 5-HT (Bao *et al.*, 2008; Neckameyer *et al.*, 2007; Fig. A4), and phenylalanine hydroxylase (DTPHu), that functions as a non-neuronal or peripheral tryptophan hydroxylase (Coleman and Neckameyer, 2004; 2005; Neckameyer *et al.*, 2007).

Targeted expression of active TNT using the *TH-GAL4* driver did not cause any change in larval photobehaviour (Fig. 4.3), demonstrating that increase in the response to light seen in *Ddc:TNT-G* larva is apparently not due to inactivation of dopaminergic neurons. In contrast, expression of TNT-G in serotonergic neurons only *(TRH:TNT-G)* caused a marked increase in the response to light of early foraging and wandering 3rd instar larvae relative to that of control larvae (*TRH:TNT-VIF*) (Fig. 4.3). Of note, the

level of this increase is comparable to that displayed by *Ddc:TNT-G* larvae, suggesting that in these larvae the increase in the response to light is due mainly to the suppression of serotonergic neurons.

Strong hypomorph or putative null mutants for the *TRH* locus (and referred to as *pBacTRH*) are viable and show diminished 5-HT staining in the CNS (Neckameyer *et al.*, 2007). Consistent with our previous observations, *pBacTRH* mutant larvae present an increase in their photoresponse when compared to that of heterozygous parental control larvae (Fig. A5), further confirming a role for serotonergic function in the modulation of the larval response to light.

It has been reported that a third group of cells, the CRZ neurons, express Ddc during 3rd instar stage and thus may contribute to the phenotype of *Ddc:TNT-G* (Landgraf *et al.*, 2003b; Sykes and Condron, 2005; Vömel and Wegener, 2008). In order to evaluate the contribution of CRZ neuronal function to the regulation of larval response to light we used *CRZ-GAL4* driver (Choi *et al.*, 2006) to target the expression of TNT-G. *CRZ:TNT-G* larvae showed a small but significant increase in the response to light when compared to control *CRZ:TNT-VIF* larvae in both foraging and wandering 3rd instar stages (Fig. 4.3).

Taken together, these findings demonstrate that 5-HT neurons but not dopaminergic neurons are involved in developmental regulation of the larval response to light. Furthermore, our results indicate that corazonergic neurons may also contribute in part to the modulation of this larval behaviour during development.

4.4.3. Silencing of 5-HT neurons does not disrupt larval locomotion

Locomotion represents a task-relevant behaviour for the measurement of the larval response to light in the ON/OFF assay. Thus, it is important to determine whether inactivation of serotonergic neurons has an impact on locomotion in general. To that end, we measured the distance travelled by early foraging 3rd instar *TRH:TNT* larvae in constant dark during 30 seconds using the semi-automatic tracking system. Behavioural analysis showed that *TRH:TNT-G* and *TRH:TNT-VIF* larvae move equally well. Furthermore, no difference was found between the distance travelled by *TRH:TNT-G* and *TRH:TNT-VIF* larvae (*UAS-TNT-G/TRH-GAL4*, N=27, $\overline{X}$ = 259.74 ± 4.79 pixels; *UAS-TNT-VIF/TRH-GAL4*, N=26, $\overline{X}$ = 266.08 ± 6.87 pixels; ANOVA: $F_{(1,51)} = 0.58$, $p = 0.45$). In addition, we used DIAS to evaluate the pattern of locomotion of early foraging 3rd instar *Ddc-TNT* larvae in constant darkness. Representative perimeter stacks of *Ddc:TNT-G* larva, similar to that of control *Ddc:TNT-VIF* larva, shows a regular linear arrangement of larval outlines (Fig. A6). Thus, these observations indicate that inactivation of serotonergic neurons does not disrupt the overall pattern of larval crawling.

4.4.4. Silencing of 5-HT neurons does not increase the response to mechanical stimuli

It is possible that silencing of the 5-HT neurons causes increased response to other external stimuli besides light. Kernan and collaborators (1994) have shown that wild-type 3rd instar larvae present a stereotypical response to touch. A stroke with the tip of an

eyelash across the anterior segments of the larva during linear locomotion causes a discrete set of responses. These responses range from withdrawing from the stimulus and turning away from it to no response at all.

We used a modified version of the touch sensitivity assay (Caldwell *et al.*, 2003) to determine whether inactivation of serotonergic neurons also affect the larval response to mechanostimulation. Individual 3rd instar foraging *TRH:TNT* larvae were touched four times during free crawling and the different responses observed were scored using the criteria of Caldwell *et al.* (2003) and shown in Fig. 2.1 (see also materials and methods). The scores for each individual larva were added and used to calculate the mean touch response of each larval group ($\overline{X}$). Our observations indicate that *TRH:TNT-G* larvae show a small but significant reduction in mechanosensitivity when compared with *TRH:TNT-VIF* larvae (*UAS-TNT-G/TRH-GAL4*, N = 20, $\overline{X}$ = 5.95 ± 0.37, *UAS-TNT-VIF/TRH-GAL4*, N=20, $\overline{X}$ = 7.2 ± 0.35; ANOVA: $F_{1,38}$ = 5.93, $p < 0.05$). Hence, these results demonstrate that synaptic silencing of 5-HT neurons do not cause an overall increase in the response to external stimuli.

4.4.5. Modulation of the response to light requires 5-HT neurons located in the brain hemispheres

A total of 52 serotonergic neurons are found in the VNC in a segmental pattern, forming 14 bilaterally symmetrical clusters: 3 in the subesophageal region, 3 in the thoracic segments and 8 in the abdominal segments (Vallés and White, 1986, 1988; Vömel and Wegener, 2008). 5-HT projections in each segment, bifurcate ipsilaterally as

well as contralaterally, innervating the entire neuropil (Chen and Condron, 2008; Landgraf *et al.*, 2003b; Sykes and Condron, 2005; Vömel and Wegener, 2008). Thus, it is possible that modulation of larval photoresponse is carried out by 5-HT neurons located in the VNC.

The zinc-finger transcription factor Eg is required for differentiation of the VNC 5-HT neurons but not for those located in the brain hemispheres (Dittrich *et al.*, 1997; Higashijima *et al.*, 1996; Lundell and Hirsh, 1998). *eg* mutants carrying different alleles display different degree of disruption of VNC 5-HT neurons and the distribution of affected cells appears to be random (Lundell and Hirsh, 1998; our observations). For instance, larvae homozygous for the eg^{P289} hypomorphic allele show severe reduction in the number of 5-HT neurons in both the abdominal and thoracic segments, and to a lesser extent, in the subesophageal segments (Lundell and Hirsh, 1998, Fig. 4.4; Table 1). In contrast, larvae carrying the heteroallelic *eg-GAL4*/eg^{18B} combination present an overall less drastic reduction in the number of 5-HT-expressing cells of the VNC (Fig. 4.4; Table 1).

Here we used *eg* mutations to evaluate the relative requirement for the larval response to light of serotonergic neurons located in the brain hemispheres versus those located in the VNC. The response to light of these mutants during both foraging and wandering 3rd instar stages is indistinguishable from those of parental control larvae (Fig. 4.5), demonstrating that the 5-HT neurons located in the VNC are not required for regulation of larval photobehaviour. Together, these observations point to the 5-HT

neurons located in the brain hemispheres as being critical for modulation of the larval response to light.

4.4.6. 5-HT-mediated modulation of larval photobehaviour does not occur at the photoreceptor level

In *Drosophila*, approximately 13 5-HT neurons can be seen projecting and arborizing in each brain hemisphere, innervating many different areas of the supraesophageal ganglion including the larval optic neuropil where it overlaps with the photoreceptor termini (Vallés and White, 1988; Mukhopadhyay and Campos, 1995; Rodriguez Moncalvo and Campos, 2005). The progressive increase in the innervation of the larval optic neuropil by 5-HT fibers from late 2nd instar to late 3rd instar larval stage coincides with the developmental down regulation of the larval response to light (Mukhopadhyay and Campos, 1995; Sawin-McCormack *et al.*, 1995; Fig. 4.6 and 4.7), suggesting that 5-HT neurons may be exerting their effect at the photoreceptor level.

We have previously reported that ablation of Rh6-specific photoreceptors prevents the appearance of 5HT arborization in the larval optic neuropil (Rodriguez Moncalvo and Campos, 2005). Similarly over-expression of Slit in either all photoreceptors or in the Rh6 subset suppresses branching of the 5-HT processes in the larval optic neuropil (Fig. A7). Thus, in order to establish whether innervation of the larval optic neuropil by 5-HT neurons is required for the dramatic down-regulation of larval response to light seen at the onset of wandering behaviour we tested the response to light of 3rd instar larvae in which the development of the optic neuropil 5-HT arborization was disrupted (*UAS-hid;Rh6-*

GAL4/+ and *GMR-GAL4/+;UAS-slit/+*). The results shown in Fig. 4.8 show that lack of 5-HT innervation of the larval optic neuropil does not cause any significant disruption in the larval response to light as measured in the ON/OFF assay. Hence, we conclude that 5-HT-mediated regulation of larval photobehaviour does not occur at the photoreceptor level.

4.4.7. 5-HT1A$_{Dro}$ is a candidate 5-HT receptor mediating modulation of the larval photoresponse

In *Drosophila* four 5-HT receptors have been identified so far (5-HT1A$_{Dro}$, 5-HT1B$_{Dro}$, 5-HT2$_{Dro}$, and 5-HT7$_{Dro}$). Limited expression data suggest that all receptors are expressed in the CNS throughout *Drosophila* development (Colas *et al.*, 1995; Nichols, 2007; Saudou *et al.*, 1992; Yuan *et al.*, 2005; 2006). Mutations are only available for 5-HT1A$_{Dro}$ and 5-HT2$_{Dro}$ genes (Nichols, 2007; Yuan *et al.*, 2006).

Knowledge of the 5-HT receptor involved in the down-regulation of the larval response to light will aid the identification of neurons critical for the developmental modulation of the larval response to light. Here we used a combination of up- and down-regulation approaches in an attempt to identify receptor candidate/s involved in this phenomenon. For up-regulation studies, we took advantage of *UAS* constructs available for all receptors identified to date (Colas *et al.*, 1999b; Kerr *et al.*, 2004; Yuan *et al.*, 2005; 2006). Down-regulation was limitedly achieved by targeted expression of a dsRNA construct available for 5-HT1B$_{Dro}$ (Yuan *et al.*, 2005), and a hypomorphic mutation in the 5-HT2$_{Dro}$ gene (*5-HT2*^{PL00052} allele, Nichols, 2007). Although 5-HT1A$_{Dro}$

loss-of-function mutant larvae are viable, we were not able to test these larvae as they displayed a developmental delay phenotype of variable penetrance (data not shown). Pan-neural expression of all *UAS* constructs was achieved by using the *elav-GAL4* driver.

Based on the results obtained so far we reasoned that increased 5-HT signalling achieved by up regulation of 5-HT receptors (5-HT1A_{Dro}, 5-HT1B_{Dro}, 5-HT2_{Dro}, and 5-HT7_{Dro}) might reduce the larval response to light in the 3rd instar foraging larva. In contrast, if down-regulation of 5-HT signalling by either expression of specific dsRNA constructs (5-HT1B_{Dro}) or a single gene mutation (*5-HT2*^{PL00052}) caused an increase in the response to light this would be likely more noticeable in the 3rd instar wandering larvae, which normally hardly respond to the light stimulus in the ON/OFF assay.

Forced expression of 5HT1A_{Dro} receptors, using the pan-neuronal driver *elav-GAL4* but not of any other 5-HT receptor subtypes, caused a significant decrease in the response to light of foraging 3rd instar larvae (Fig. 4.9). On the other hand, wandering larvae homozygous mutant for the *5-HT2*$_{Dro}$ gene (*5-HT2*^{PL00052}) showed the characteristic low response to light when compared with parental controls (data not shown). Similarly, targeted pan-neural expression of the dsRNA construct for the 5-HT1B_{Dro} receptor did not affect the response to light of wandering 3rd instar larvae (data not shown). Taken together, these observations point to 5-HT1A_{Dro} receptor subtype as a candidate receptor involved in the 5-HT-mediated modulation of larval response to light.

4.5. Discussion

All organisms possess a finite number of neuronal networks. Therefore, neurons and circuits must be multifunctional to provide individuals with a variety of behavioural outputs necessary to adapt to environmental and developmental changes. In this regard, neuromodulation constitutes a powerful way to modify the function of an existing circuit without altering the 'hard-wiring' of such network (reviewed in Birmingham and Tauck, 2003).

Little is known about the function of 5-HT neurons in *Drosophila* larvae. Like in other organisms including the adult fly, their broad distribution in the nervous system suggests multiple roles for these cells. Here, we report on the role of serotonergic neurons in the developmental modulation of *Drosophila* larval response to light, a paradigm to study regulation of locomotion.

4.5.1. 5-HT neurons play a role in the modulation of the larval response to light

Larvae in which 5-HT neuronal activity has been suppressed by expression of TNT-G (Fig. 4.1-4.3) and other neuronal silencers (Fig. A2 and A3) present an increased response to light as well as partial suppression in the down regulation of the response to light that occurs with the onset of wandering behaviour. Furthermore, our results demonstrate that inactivation of these neurons does not result in a generalized disruption in the larval response to external stimuli.

Behavioural analysis of *TRH* mutant larvae further supports a neuromodulatory role for 5-HT neurons in the developmental regulation of the larval photobehaviour (Fig. A5). Of note is that the increased response observed in *pBacTRH* mutants does not appear to be as high as that obtained after silencing of 5-HT neurons by TNT expression. In spite of carrying a null allele of the *TRH* gene, *pBacTRH* larvae show only decreased 5-HT expression in the CNS (Neckameyer *et al.*, 2007). It has been suggested that this is due, perhaps, to the re-uptake of circulating 5-HT synthesized peripherally by DTPHu (Neckameyer *et al.*, 2007). Thus, it is possible that, in these mutants, small amounts of 5-HT are still released from serotonergic neurons.

It has been reported that neuronal 5-HT regulates larval feeding (Neckameyer *et al.*, 2007) and body size in adult flies (Kaplan *et al.*, 2008). Nevertheless, decreased 5-HT levels or release does not appear to affect larval growth, as the size of *Ddc:TNT-G*, *TRH:TNT-G* or *TRH* mutant larvae is within the range of wild type controls (data not shown). These observations are consistent with our conclusion that silencing of the 5-HT neurons did not cause a developmental delay.

As motor performance is crucial for analysis of photobehaviour in our assay, it is important to consider the impact of diminished 5-HT synthesis or release on this task-relevant behaviour. Previous observations indicate that the locomotion of *TRH* mutant larvae is normal as measured by the number of body wall contractions (Neckameyer *et al.*, 2007). Our results agree with those of Neckameyer and collaborators (2007), as *Ddc:TNT-G*, *TRH:TNT-G* as well as *TRH* mutant larvae showed normal locomotion in constant dark (Fig. A6 and data not shown).

The fact that the response to light of *Ddc:TNT-G* and *TRH:TNT-G* larvae are similar does not mean that the only Ddc-expressing neurons involved in developmental modulation of larval photobehaviour are the serotonergic neurons. Indeed, our findings suggest that CRZ neurons may contribute in part to this regulation (Fig. 4.3). Moreover, differences in the strength of the promoter must be taken into consideration when comparing the relative contribution of different neuronal groups after targeted silencing using different *GAL4* drivers. Thus, at this point in time we cannot establish how much less than 5-HT neurons the CRZ neurons contribute to the modulation of the larval response to light.

It has recently been shown that, during 3rd instar larval stage, a fourth group of neurons immunoreactive for crustacean cardioactive peptide (CCAP) and myoinhibiting peptide (MIP) located in the ventral cord are also detected by using the *Ddc-GAL4* construct (Vömel and Wegener, 2008). Therefore, we cannot exclude the possibility that these Ddc cells may also play a modulatory role in the developmental regulation of larval photobehaviour.

4.5.2. Modulation of the larval photoresponse requires 5-HT neurons located in the brain hemispheres

Mutations in the *eg* gene affect serotonergic neurons located in the subesophageal, thoracic and abdominal segments of the VNC but not those 5-HT neurons located in the brain lobes (Dittrich *et al.*, 1997; Lee and Lundell, 2007; Lundell and Hirsh, 1998; Fig. 4.4). The remaining VNC 5-HT neurons often show severe pathfinding defects (Dittrich

et al., 1997; Lundell and Hirsh, 1998). *eg* mutant larvae respond to light as heterozygous control larvae in the foraging stage and show the expected reduction in this response as they reach the wandering stage, demonstrating that 5-HT neurons located in the VNC are not required in this process (Fig 4.5).

It has been shown that CRZ neurons located in the VNC also express *eg* during 3rd instar stage (Karcavich and Doe, 2005; Landgraf *et al.*, 2003b; Lee and Lundell, 2007; Lundell *et al.*, 2003; Novotny *et al.*, 2002; Sykes and Condron, 2005). It is yet to be established whether VNC CRZ neurons are also affected in *eg* mutants. If so, our results suggest that this subset of CRZ cells may not be involved in the modulation of larval photobehaviour.

The development of the 5-HT processes in the larval optic neuropil and their contact with the LON coincides with the gradual decrease in the larval aversion to light suggesting that 5-HT neurons may be modulating this larval behaviour at the photoreceptor level (Mukhopadhyay and Campos, 1995; Sawin-McCormack *et al.*, 1995; Fig. 4.6 and 4.7). However, absence of the 5-HT arborization or the disruption of its branching did not impact the response to light of either foraging or wandering larvae (Fig. 4.8).

Neuromodulators may regulate rhythmic motor behaviours by acting at different levels within a specific neuronal circuit, that is, at the sensory and/or central level (reviewed in Marder *et al.*, 2005). The latter may involve modulation within the CPG or at the level of the motorneurons (reviewed in Marder and Bucher, 2001; Marder *et al.*, 2005). The CPG controlling *Drosophila* larval locomotion is thought to be located in the

VNC (Cattaert and Birman, 2001). Our results indicate that 5-HT neuromodulatory effect occurs either at a central level other than the CPGs (e.g. higher order interneurons) located in the brain or directly on the CPGs by descending inputs from the brain hemispheres. Regarding the latter possibility, early immunohistochemical studies have suggested the existence of 5-HT longitudinal fibers deriving from the brain lobe neurons (Vallés and White, 1988).

4.5.3. 5-HT1A$_{Dro}$ receptors may be involved in 5-HT-mediated modulation of larval photobehaviour

Our results suggest that 5-HT1A$_{Dro}$ receptors may play a role in the developmental modulation of the larval response to light (Fig. 4.9). These investigations represent the first step towards the identification of the 5-HT receptors mediating this phenomenon. Additional experiments are required for the identification of the target cells on which larval 5-HT neurons act to modulate the larval response to light.

Figure 4.1. Larvae expressing active TNT in Ddc neurons present increased response to light. Photobehavioural responses of larvae expressing either active (TNT-G) or inactive (TNT-VIF, control) in Ddc-expressing cells measured in the ON/OFF assay at different developmental stages. RIs were obtained using the semi-automatic tracking system. Compared to what is observed in control *UAS-TNT-VIF/+;Ddc-GAL4/+* larvae, *UAS-TNT-G/+;Ddc-GAL4/+* larvae showed increased response to light during late 2nd (~70 h AEL), 3rd instar foraging (89 & 96 h AEL) and wandering (115 & 120 h AEL) larval stages. (late 2nd instar: *UAS-TNT-G/+;Ddc-GAL4/+*, N=11, RI=0.52; *UAS-TNT-VIF/+;Ddc-GAL4/+*, N=12, RI=0.29; ANOVA: $F_{(1,21)} = 21.53$, $p < 0.001$; early foraging 3rd instar: *UAS-TNT-G/+;Ddc-GAL4/+*, N=35, RI=0.50;*UAS-TNT-VIF/+;Ddc-GAL4/+*, N=23, RI=0.28; ANOVA: $F_{(1,56)} = 62.79$, $p < 0.001$; late foraging 3rd instar: *UAS-TNT-G/+;Ddc-GAL4/+*, N=15, RI=0,51; *UAS-TNT-VIF/+;Ddc-GAL4/+*, N=14, RI=0.28; ANOVA: $F_{(1,27)} = 30.92$, $p < 0.001$; early wandering 3rd instar *UAS-TNT-G/+;Ddc-GAL4/+*, N=24, RI=0.31;*UAS-TNT-VIF/+;Ddc-GAL4/+*, N=23, RI=0.14; ANOVA: $F_{(1,45)} = 23.38$, $p < 0.001$; late wandering 3rd instar *UAS-TNT-G/+;Ddc-GAL4/+*, N=27, RI=0.33;*UAS-TNT-VIF/+;Ddc-GAL4/+*, N=24, RI=0.13; ANOVA: $F_{(1,49)} = 43.36$, $p < 0.001$).

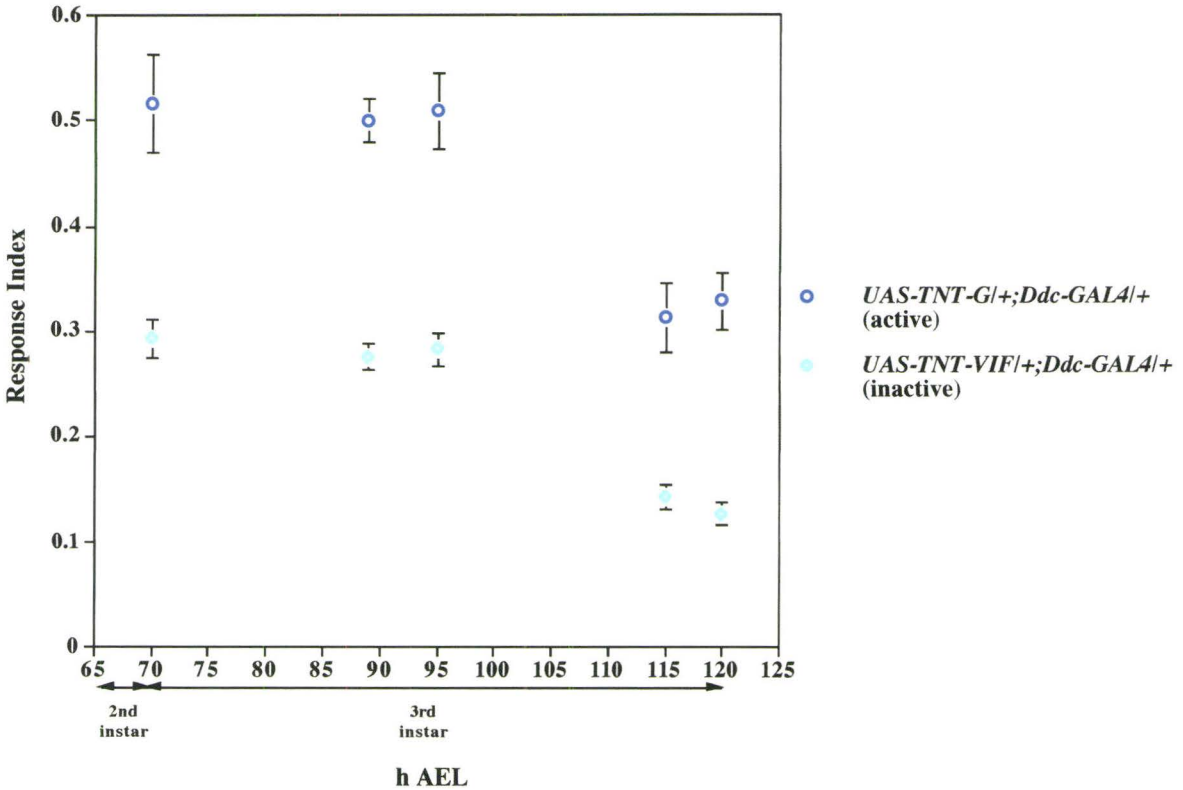
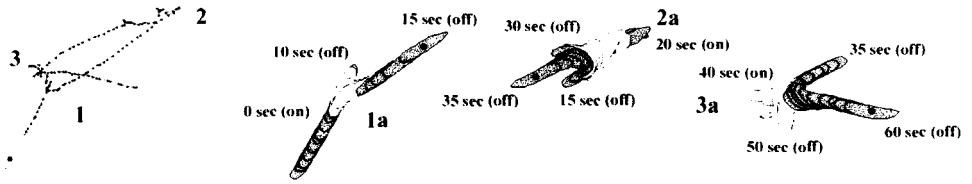

Response Index
0.6
0.5
0.4
0.3
0.2
0.1
0
65
70
75
80
85
90
95
100
105
110
115
120
125
2nd instar
3rd instar
h AEL
UAS-TNT-G/+;Ddc-GAL4/+ (active)
UAS-TNT-VIF/+;Ddc-GAL4/+ (inactive)

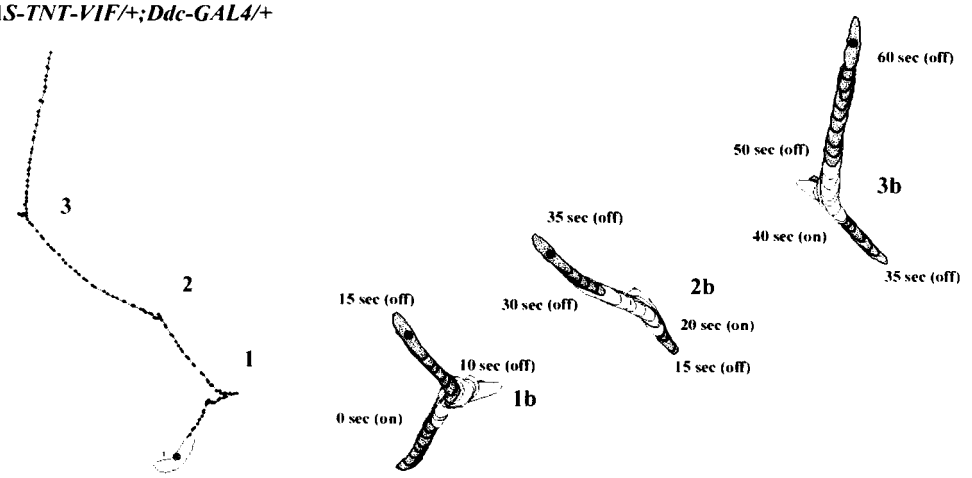
Figure 4.2. Representative locomotor patterns during the ON/OFF assay of 3rd instar larvae expressing TNT in the Ddc neurons. Centroid stacks (A-D) and perimeter stacks (1-3) were generated using DIAS. A,C, *UAS-TNT-G/+;Ddc-GAL4/+* (*Ddc:TN-TG*) larvae. B,D, *UAS-TNT-VIF/+;Ddc-GAL4/+* (*Ddc:TNT-VIF*, control) larvae. In each case, panel 1 represents 5 seconds (sec) prior the beginning of the assay and the first 15 sec of the assay, panel 2 depicts the following 20 sec, and panel 3 represents the last 25 sec of the assay. Behaviour recorded during the light (ON) pulses is shown as white larval images, whereas behaviour in the dark (OFF) pulses is shown as shade larval images. During foraging stage, analysis of centroid paths reveals a higher degree of centroid clustering and of irregular centroid arrangement in *Ddc:TNT-G* larvae (A) compared with those in *Ddc:TNT-VIF* larvae (B). These suggest that *Ddc:TNT-G* larval performance in the assay involves longer pausing time and less linear movement. Analysis of perimeter stacks further demonstrates these observations. During the light (ON) pulses, foraging *Ddc:TNT-G* larvae exhibited backward motion and increased pausing, as well as a more pronounced head swinging behaviour and change of direction when compared with those of *Ddc:TNT-VIF* larvae (compare 1a-3a with 1b-3b). Although not as pronounced, similar differences can be observed during wandering stage (C and D). In this stage, *Ddc:TNT-G* larvae still respond to light (C, 1c-3c), whereas *Ddc:TNT-VIF* larvae rarely modify their linear movement (D, 1d-3d).

early foraging 3rd

A *UAS-TNT-G/+;Ddc-GAL4/+*

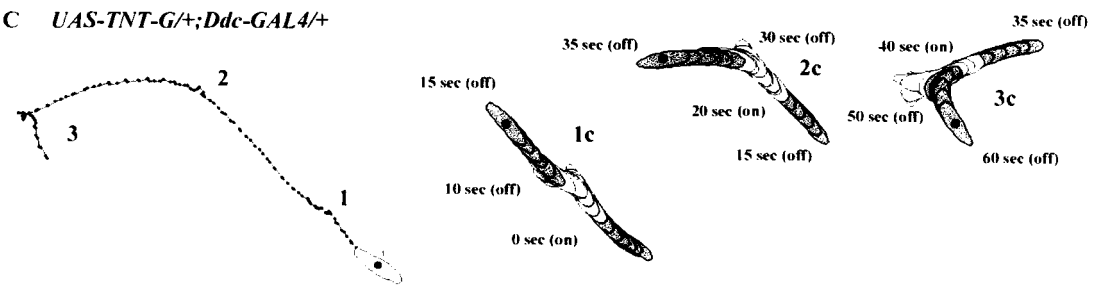


B *UAS-TNT-VIF/+;Ddc-GAL4/+*



early wandering 3rd

C *UAS-TNT-G/+;Ddc-GAL4/+*



D *UAS-TNT-VIF/+;Ddc-GAL4/+*

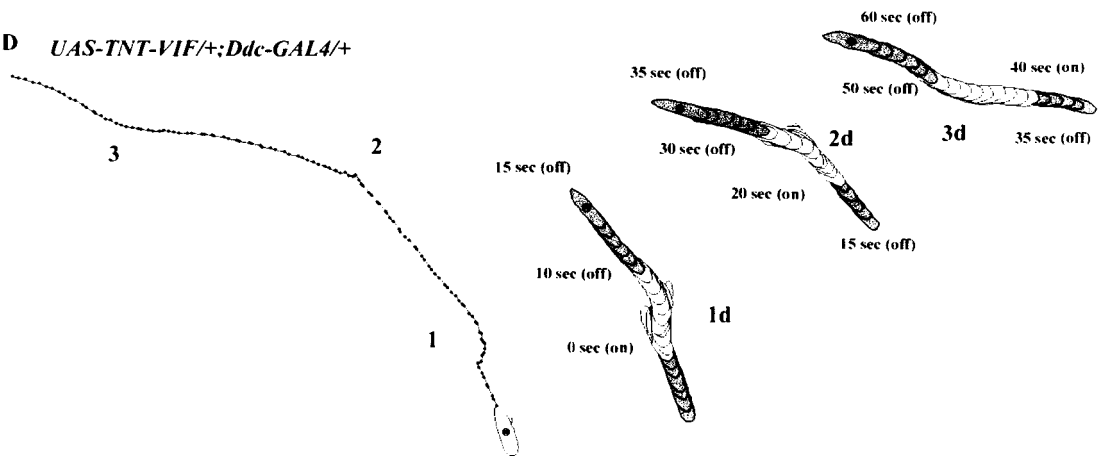
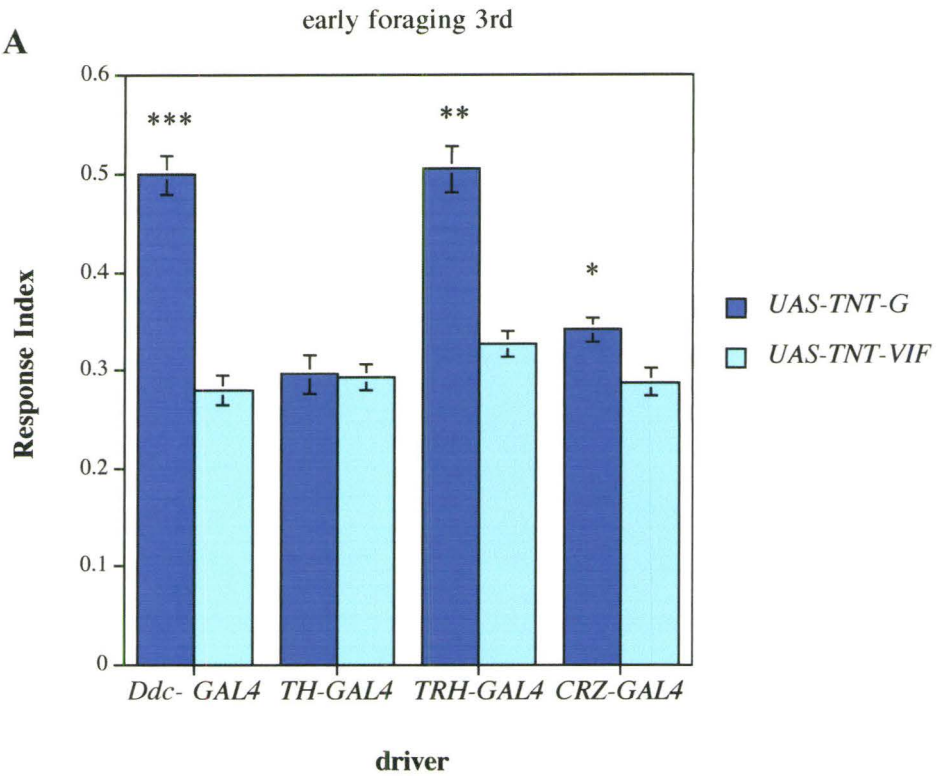


Figure 4.3. Photoresponse in the ON/OFF assay of 3rd instar larvae expressing TNT in different subsets of Ddc neurons. A, foraging stage. B, wandering stage. Expression of TNTG under control of *TH-GAL4* did not affect the larval response to light during 3rd instar, suggesting that dopaminergic neurons do not contribute to the increase in the response to light observed in *Ddc:TNT-G* larvae (early foraging 3rd instar: *UAS-TNT-G/+;TH-GAL4/+*, N=17, RI=0.30; *UAS-TNT-VIF/+;TH-GAL4/+*, N=22, RI=0.29; ANOVA: $F_{(1,37)} = 0.02$, p=0.89; early wandering 3rd instar *UAS-TNT-G/+;TH-GAL4/+*, N=30, RI=0.09; *UAS-TNT-VIF/+;TH-GAL4/+*, N=25, RI=0.09; ANOVA: $F_{(1,53)} = 0.01$, p =0.91). On the other hand, CRZ neurons appear to slightly contribute to the down-regulation of the larval response to light during foraging as well as wandering stage. (early foraging 3rd instar: *UAS-TNT-G/+;CRZ-GAL4/+*, N=20, RI=0.34; *UAS-TNT-VIF/+;CRZ-GAL4/+*, N=15, RI=0.29; ANOVA: $F_{(1,33)} = 8.34$, $p<0.05$; early wandering 3rd instar *UAS-TNT-G/+;CRZ-GAL4/+*, N=22, RI=0.17; *UAS-TNT-VIF/+;CRZ-GAL4/+*, N=25, RI=0.12; ANOVA: $F_{(1,45)} = 4.59$, $p <0.05$). Interestingly, *TRH-TNT-G* and *TRH-TNT-VIF* larvae present similar RI values to those observed in *Ddc-TNTG* and *Ddc-TNT-VIF* larvae (early foraging 3rd instar: *UAS-TNT-G/TRH-GAL4*, N=21, RI=0.50; *UAS-TNT-VIF/TRH-GAL4*, N=21, RI=0.33; ANOVA: $F_{(1,40)} = 44.4$, $p<0.01$; early wandering 3rd instar *UAS-TNT-G/TRH-GAL4*, N=41, RI=0.29; *UAS-TNT-VIF/TRH-GAL4/+*, N=27, RI=0.13; ANOVA: $F_{(1,66)} = 68.31$, $p <0.01$). *** $p<0.001$, ** $p<0.01$, *$p<0.05$.


A
early foraging 3rd
Response Index
0.6
0.5
0.4
0.3
0.2
0.1
0
***
**
*
Ddc- GAL4
TH-GAL4
TRH-GAL4
CRZ-GAL4
driver
UAS-TNT-G
UAS-TNT-VIF

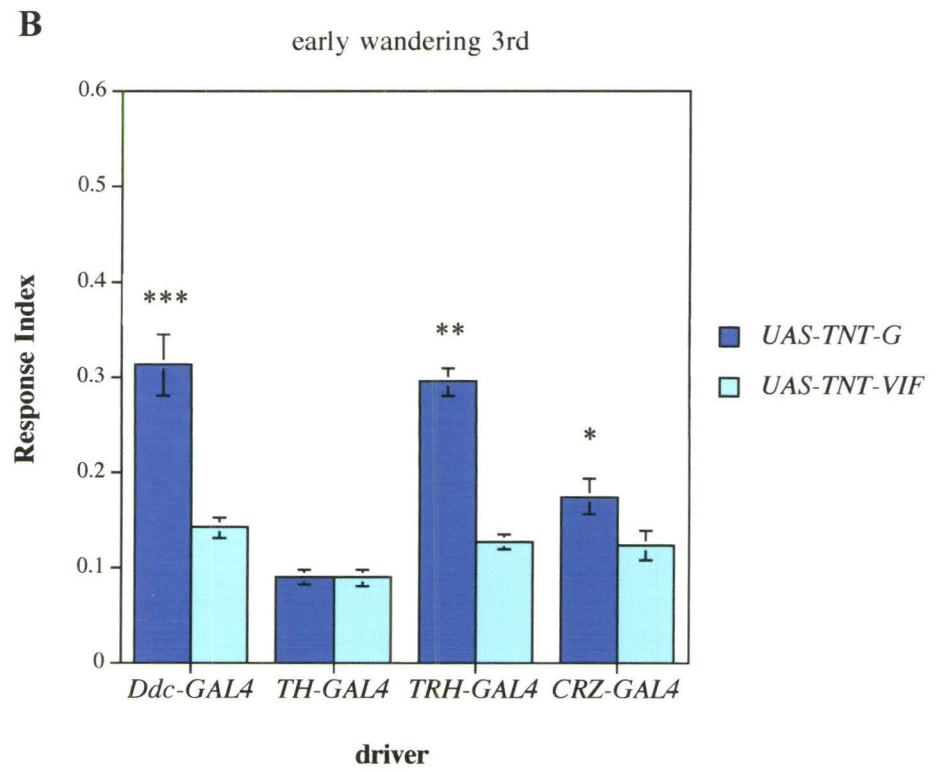

B
early wandering 3rd
Response Index
0.6
0.5
0.4
0.3
0.2
0.1
0
***
**
*
Ddc-GAL4
TH-GAL4
TRH-GAL4
CRZ-GAL4
driver
UAS-TNT-G
UAS-TNT-VIF

Figure 4.4. *eagle* mutant larvae present reduced number of 5-HT-expressing neurons in the VNC. A-F, Confocal micrographs of 3rd instar wandering wild type OR, eg^{P289} and eg^{18B}/*eg-GAL4* mutant brains stained with 5-HT antibody and detected by Texas Red-conjugated secondary. B, D, and F represent the insets of A, C, and E respectively. 5-HT immunolabelling reveals a decreased number of 5-HT neurons in the abdominal, thoracic, and subesophageal segments in the CNSs of both eg^{P289} and eg^{18B}/*eg-GAL4* mutants. Note how this phenotype is much more severe in eg^{P289} mutants than in eg^{18B}/*eg-GAL4* mutants (See also Table 1). Scale bars in A,C and E represent 50 μm, whereas in B, D, and F scale bars represent 10 μm.

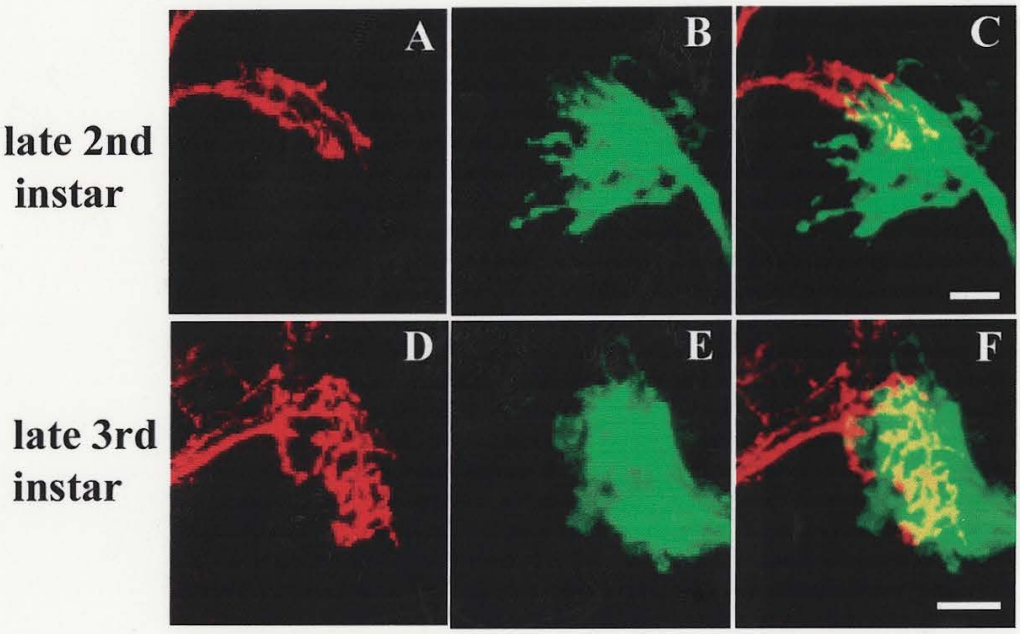

late 2nd instar
late 3rd instar
A
B
C
D
E
F

Figure 4.5. 3rd instar *eagle* mutants show normal response to light. Photoresponse of foraging and wandering 3rd instar eg^{P289} and eg^{18B}/*eg-GAL4* mutant larvae as well as control larvae during the ON/OFF assay. As measured by their RIs, eg^{P289} and eg^{18B}/*eg-GAL4* larvae show normal photoneutral response when compared with heterozygous parental control larvae in the same stage (early foraging 3rd instar: eg^{P289}, N=21, RI=0.317; eg^{P289}/+, N=26, RI=0.328; ANOVA: $F_{(1,45)} = 0.34$, p= 0.561; early wandering 3rd instar eg^{P289}, N=41, RI=0.06; eg^{P289}/+, N=25, RI=0.063; ANOVA: $F_{(1,64)} = 0.10$, p =0.747; early foraging 3rd instar: eg^{18B}/*eg-GAL4*, N=19, RI=0.3283; eg^{18B} /+, N=21, RI=0.3113; ANOVA: $F_{(1,38)} = 0.93$, p= 0.34; early wandering 3rd instar eg^{18B}/*eg-GAL4*, N=26, RI=0.094; eg^{18B} /+, N=21, RI=0.064; ANOVA: $F_{(1,45)} = 3.43$, p =0.06).

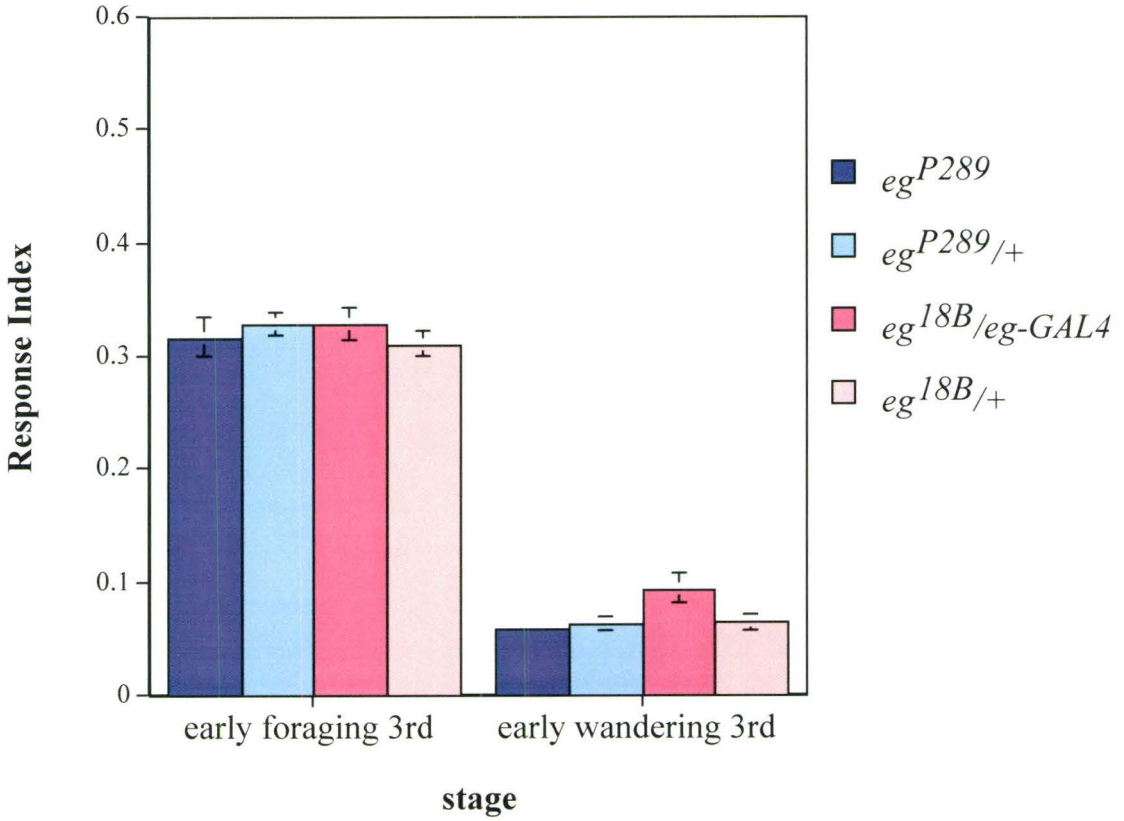

0.6
0.5
0.4
0.3
0.2
0.1
0
Response Index
early foraging 3rd
early wandering 3rd
stage
eg^{P289}
$eg^{P289/+}$
$eg^{18B/eg\text{-}GAL4}$
$eg^{18B/+}$

Figure 4.6. Branching of the larval optic neuropil 5-HT arborization at different points in larval development. (A-C) Wild type (OR) late 2nd instar larval brain (~70 h AEL). (D-F) Wild type (OR) 3rd instar wandering larval brain (~120 h AEL). (A,D) 5-HT arborization immunolabelled with anti-5-HT and detected as before. (B,E) larval photoreceptors stained with 24B10 monoclonal antibody and detected by Alexa 488-conjugated secondary. (C) Merge of A and B. (F) Merge of D and E. The branching of the 5-HT arborization increases during development up to the level seen in D (compare A and D). Scale bars: 10 μm.

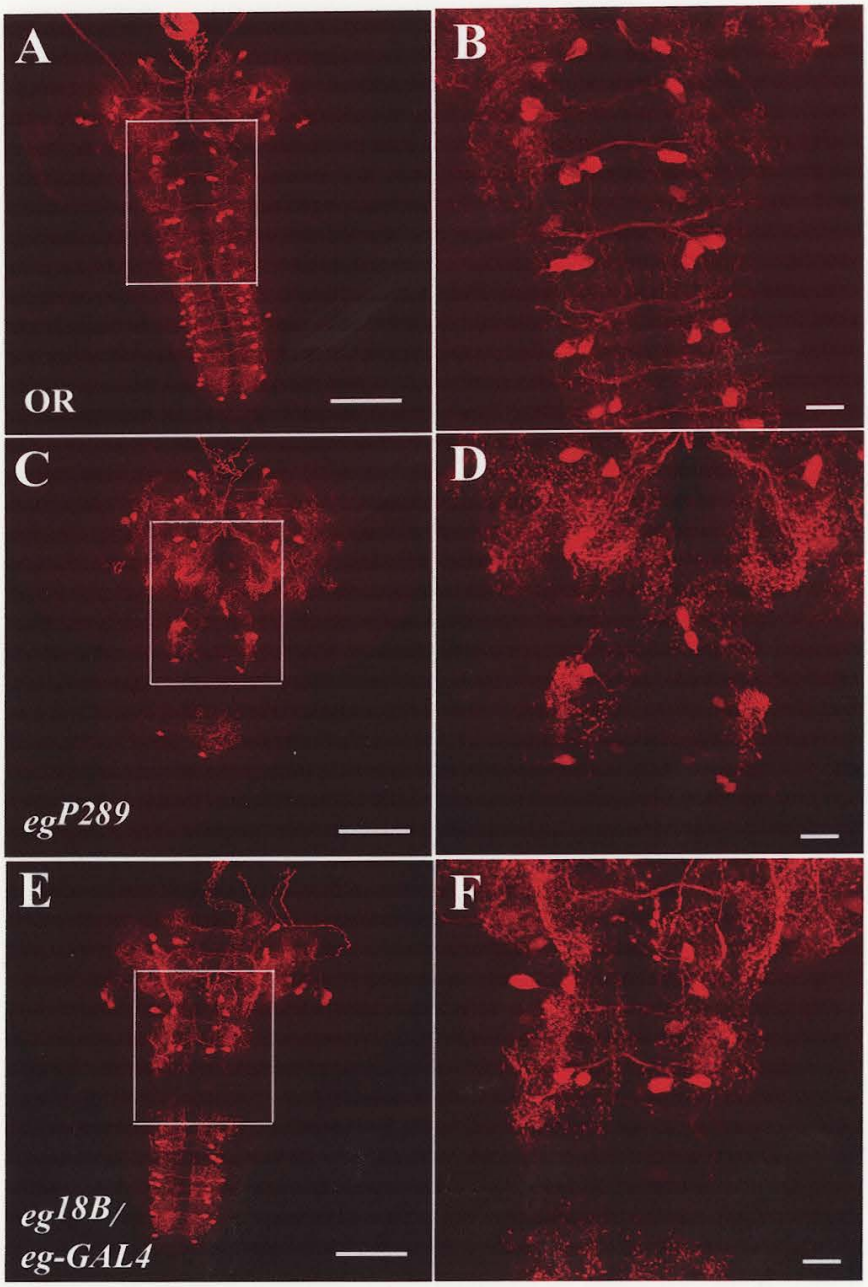

A
B
OR
C
D
egP289
E
F
eg18B/
eg-GAL4

Figure 4.7. Behavioural response in the ON/OFF assay of wild type Oregon-R (OR) larvae tested at different times during development. The response to light was measured as Response Index (RIs) using the semi-automatic tracking system. The larval photophobic response decreases during development, reaching low levels at the wandering stage. 70 h AEL = late 2nd foraging stage, 89 h AEL = early 3rd foraging stage, 96 h AEL = late 3rd foraging stage, 115 h AEL = early 3rd wandering stage, 120 h AEL = late 3rd wandering stage.

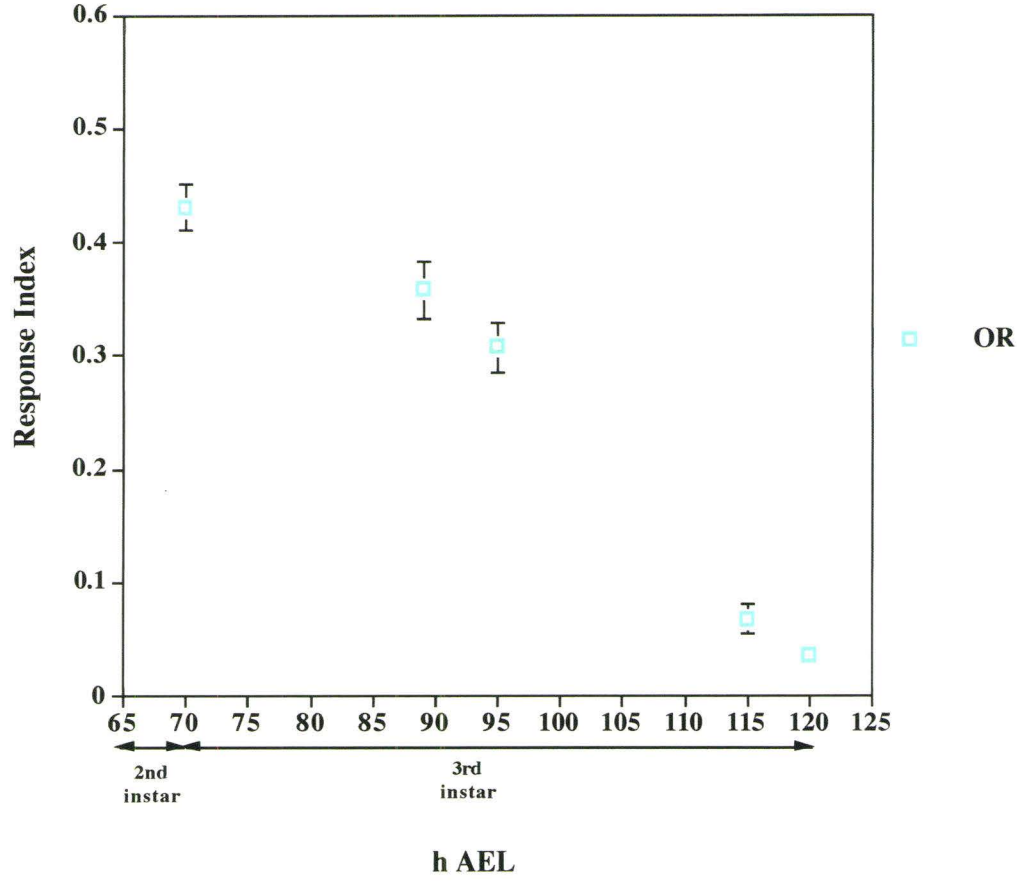
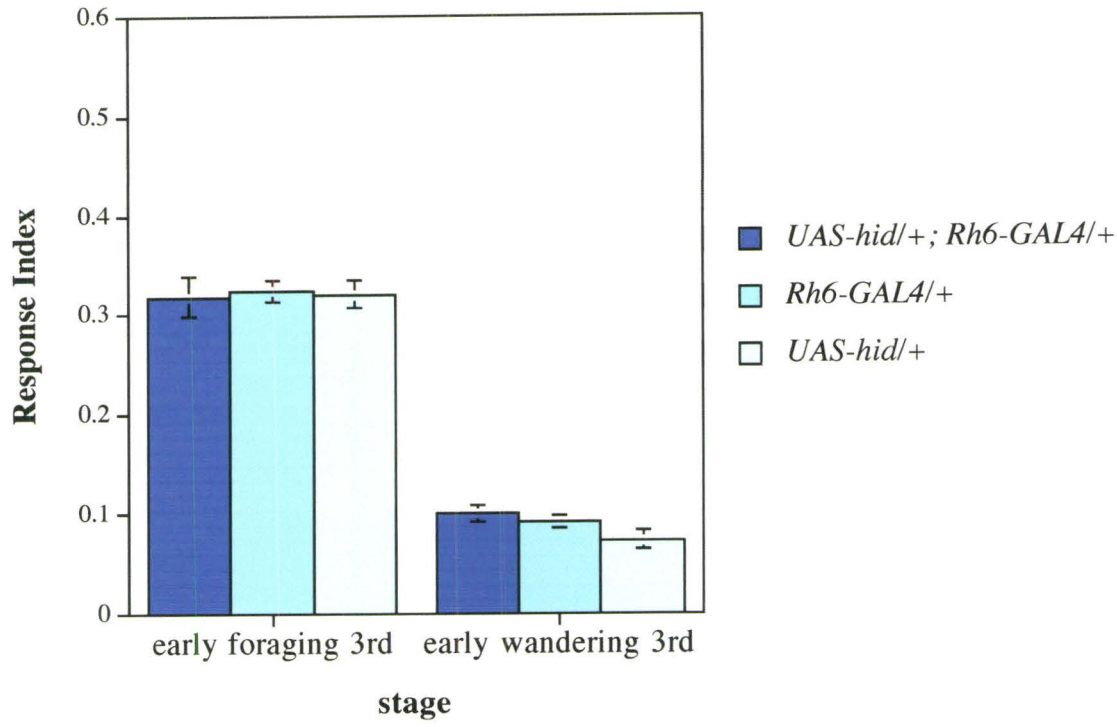

0.6
0.5
0.4
0.3
0.2
0.1
0
Response Index
65
70
75
80
85
90
95
100
105
110
115
120
125
2nd instar
3rd instar
h AEL
OR

Figure 4.8. Disruption in the development of the larval optic neuropil 5-HT arborization does not cause any effect in larval photobehaviour. A, Photobehaviour of *UAS-hid/+;Rh6-GAL4/+* larvae, as well as of *UAS-hid/+* and Rh6-GAL4/+ parental control larvae. B, Behavioural response of 3rd instar *GMR-GAL4/+;UAS-slit* larvae and parental control *GMR-GAL4/+* and *UAS-slit/+* larvae. In both cases, RIs were obtained in the ON/OFF assay using the semi-automatic tracking system. In accordance to what was previously reported (Hassan *et al.*, 2005), 3rd instar foraging larvae in which the Rh6 photoreceptors do not develop as a result of targeted expression of *hid* show normal photoresponse (*UAS-hid/+;Rh6-GAL4/+*, N=12, RI=0.32; *Rh6-GAL4/+*, N=18, RI=0.32; *UAS-hid/+*, N=17, RI=0.32; ANOVA: $F_{(2,44)} = 0.06$, $p = 0.95$). Similarly, no significant differences were found among groups at early wandering 3rd instar stage (*UAS-hid/+;Rh6-GAL4/+*, N=22, RI=0.1; *Rh6-GAL4/+*, N=24, RI=0.09; *UAS-hid/+*, N=21, RI=0.07; ANOVA: $F_{(2,64)} = 2.94$, $p = 0.06$). In the case of Slit expression, the RIs for the strains are significantly different at early foraging 3rd instar stage (ANOVA $F_{(2,68)} = 5.77, p < 0.05$). Nevertheless, *post hoc* analysis of paired mean comparisons revealed that expression of Slit under control of *GMR-GAL4* caused a small decrease in the larval response to light when compared to the photoresponse of *GMR-GAL4/+* larvae but not to that of *UAS-slit/+* larvae (*GMR-GAL4/+;UAS-slit/+*, N=20, RI=0.33; *GMR-GAL4/+*, N=31, RI=0.38; *UAS-slit/+*, N=20, RI=0.34). No differences were found at early wandering stage between *GMR-GAL4/+;UAS-slit/+* larvae and parental controls (*GMR-GAL4/+;UAS-slit/+*, N=20, RI=0.06; *GMR-GAL4/+*, N=13, RI=0.07; *UAS-slit/+;* N=20, RI=0.07; ANOVA: $F_{(2,50)} = 0.23$, $p = 0.80$). Taking in account that the larval optic

neuropil 5-HT arborization is either missing in *UAS-hid/+;Rh6-GAL4/+* larvae (Rodriguez Moncalvo and Campos, 2005) or underdeveloped in *GMR-GAL4/+;UAS-slit/+* larvae (Fig. A7), these behavioural results suggest that these 5-HT processes do not participate in the developmental modulation of the response to light.

A


0.6
0.5
0.4
0.3
0.2
0.1
0
Response Index
early foraging 3rd
early wandering 3rd
stage
UAS-hid/+; Rh6-GAL4/+
Rh6-GAL4/+
UAS-hid/+

B

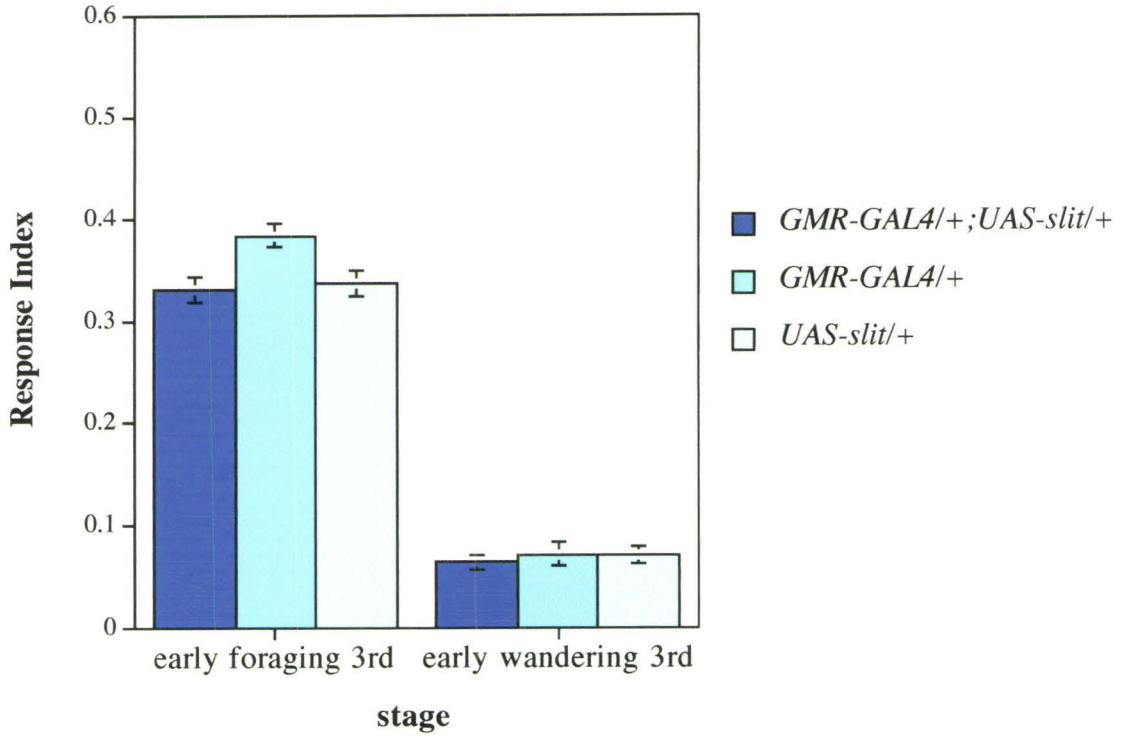

0.6
0.5
0.4
0.3
0.2
0.1
0
Response Index
early foraging 3rd
early wandering 3rd
stage
GMR-GAL4/+;UAS-slit/+
GMR-GAL4/+
UAS-slit/+

Figure 4.9. 3rd instar foraging larvae over-expressing 5-HT1A$_{Dro}$ receptors in all neurons show decreased larval response to light. Targeted individual overexpression of 5-HT1A$_{Dro}$, 5-HT1B$_{Dro}$, 5-HT2$_{Dro}$, and 5-HT7$_{Dro}$ receptors in all post-mitotic neurons was carried out by using the panneuronal driver *elav-GAL4*. Interestingly, foraging 3rd instar larvae expressing 5-HT1A$_{Dro}$ receptors but not any of the other 5-HT receptor subtypes in the nervous system showed significantly reduced response to light when compared with both parental control larvae (*elav-GAL4/UAS-5HT1A$_{Dro}$*, N = 27, RI= 0.19; *elav-GAL4/+*, N=20, RI= 0.33; *UAS-5HT1A$_{Dro}$ /+*, N = 30, RI= 0.36; ANOVA: $F_{(2,74)}$= 34.61, $p<0.001$). These findings suggest that 5-HT1A$_{Dro}$ receptors may mediate central modulatory effect of 5-HT on larval photobehaviour. *** $p<0.001$.

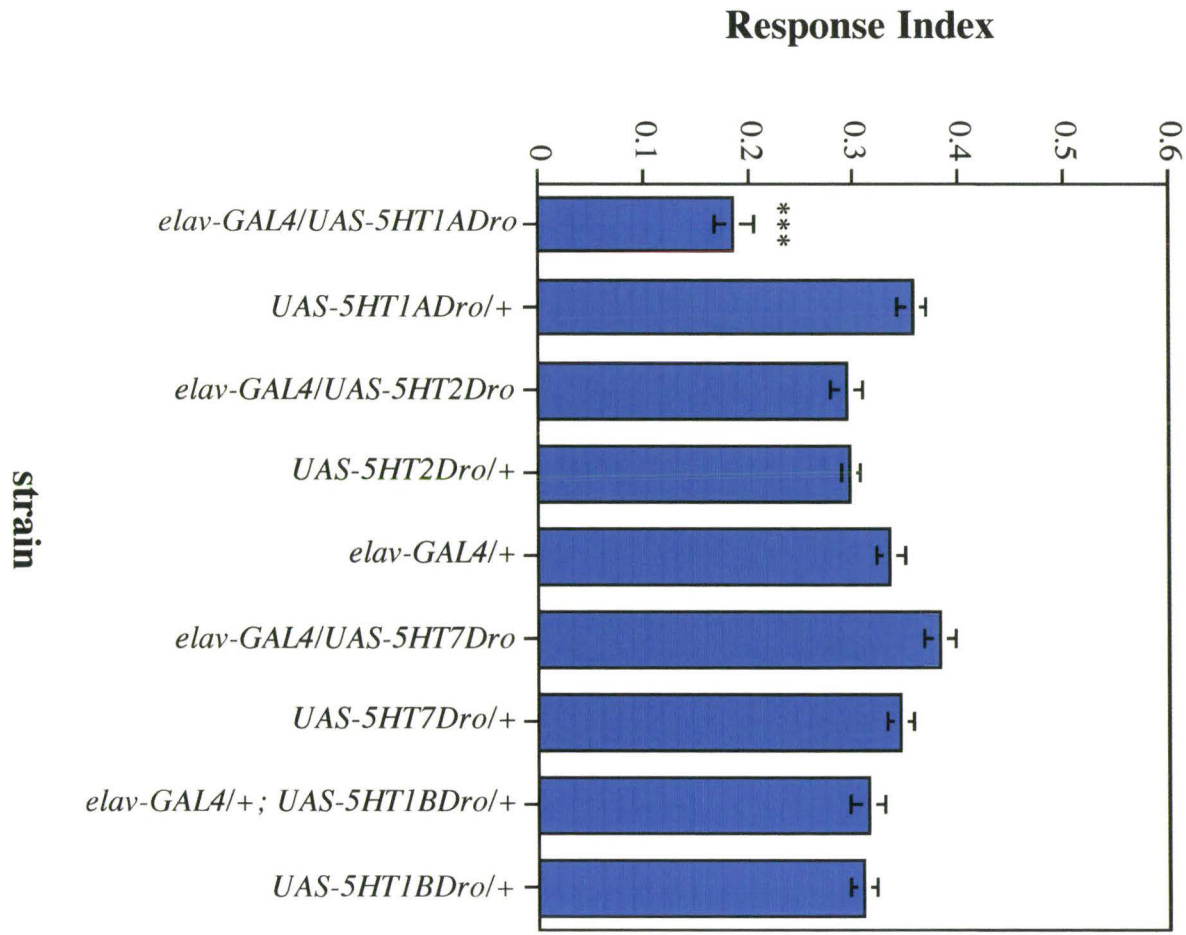

Response Index
0
0.1
0.2
0.3
0.4
0.5
0.6
***
elav-GAL4/UAS-5HT1ADro
UAS-5HT1ADro/+
elav-GAL4/UAS-5HT2Dro
UAS-5HT2Dro/+
elav-GAL4/+
elav-GAL4/UAS-5HT7Dro
UAS-5HT7Dro/+
elav-GAL4/+; UAS-5HT1BDro/+
UAS-5HT1BDro/+
strain

Table 4.1. Number of 5-HT neurons present in different segments of the VNC of 3rd instar wandering wild type OR larvae, eg^{P289} mutant larvae, and heteroallelic eg^{18B}/*eg-GAL4* mutant larvae. Data is shown as mean ± S.E.M for each group and as percentages relative to the number found in VNC of OR larvae.

	No. of 5-HT cells present		
	subesophageal segments (SE1-SE3)	**thoracic segments (T1-T3)**	**abdominal segments (A1-A8)**
OR (n = 20)	14 ± 0 (100 %)	14 ± 0 (100 %)	30 ± 0 (100 %)
***eg*^{P289} (n = 16)**	6.88 ± 0.79 (49.11 %)	1.06 ± 0.36 (7.59 %)	2.44 ± 0.35 (8.13 %)
***eg*^{18B}/ *eg-GAL4* (n = 19)**	7.42 ± 0.54 (62.95 %)	3.79 ± 0.48 (32.14 %)	21.68 ± 0.64 (72.28 %)

CHAPTER 5: Discussion

5.1. Conclusions

In any metazoan organism, normal CNS function depends on proper development of its individual cellular components as well as their correct organization into neural networks. In addition to intrinsic genetic programs, complex interactions between these components are crucial for precise CNS development and circuit assembly. These neural networks constitute the cellular basis by which the nervous system receives, processes and integrates sensory information. Furthermore, precise regulation of circuit activity provides a given organism with the capability to generate specific behavioural outputs for adaptation to any environmental or developmental changes.

My thesis research was aimed at examining the development of 5-HT neurons in *Drosophila* larva. More specifically, my experiments focused on unravelling 5-HT neuronal interactions in the larval optic neuropil as well as possible mechanisms underlying serotonergic innervation of this CNS area. In addition, and taking advantage of a behavioural paradigm previously used in this laboratory, I pursued functional studies in order to gain deeper insights into the regulation of *Drosophila* larval photobehaviour during development. For this purpose, I investigated the possible involvement in this phenomenon of different neuronal subtypes including the serotonergic neurons.

5.1.1. Cell-cell interactions in the *Drosophila* larval neuropil and 5-HT arborization development

It has been previously shown that, in the larval optic neuropil, the LON contacts both the 5-HT processes and the dendritic tree of the LNv (Malpel *et al.*, 2002; Mukhopadhyay and Campos, 1995). I report that both subsets of larval photoreceptors, the Rh5- and Rh6-expressing cells overlap with the serotonergic arborization (Rodriguez Moncalvo and Campos, 2005). Furthermore, in agreement with observations published soon after by Hamasaka and Nässel (2006), my results indicate that the larval optic neuropil 5-HT projections also interact with the dendritic arborization of the brain PDF neurons (Rodriguez Moncalvo and Campos, 2005).

It has been shown that the presence of the LON is necessary for normal morphogenesis of both the LNv dendritic arbor and the larval optic center 5-HT processes (Malpel *et al.*, 2002; Mukhopadhyay and Campos, 1995). However, whether both photoreceptor subsets and/or the brain PDF neurons were necessary for proper 5-HT arborization development had not been formerly determined. My findings indicate that only the Rh6 photoreceptor subset is required for the development and/or maintenance of the 5-HT arbor (Rodriguez Moncalvo and Campos, 2005).

As previously mentioned, signals of an intrinsic and/or extrinsic nature regulate neurite morphogenesis. Furthermore, the development of any given neurite may be influenced by more than one intrinsic and/or extrinsic factor, exerting their morphogenetic role at the same developmental time or in a sequential fashion (reviewed in Friauf and Lohmann, 1999; Van Aelst and Cline, 2004; Wong and Gosh, 2002).

Studies in vertebrate and invertebrate systems suggest that, before synapse formation, early neurite morphogenesis and guidance is driven mainly by intrinsic genetic programs as well as activity-independent environmental cues, and that neuronal activity becomes important only at later stages, once neuronal connections are formed and mature (reviewed in Goodman and Shatz, 1993; Libersat, 2005; Wong and Gosh, 2002). However, increasing evidence indicates that spontaneous activity may play essential roles also early in neurite outgrowth, pathfinding and target recognition, suggesting that neuronal activity is likely required at all developmental stages (reviewed in Friauf and Lohman, 1999; Spitzer, 2006). Because neuronal connections are not yet formed at early stages, one possible mechanism involved is the paracrine action of neurotransmitters.

The importance of intrinsic and extrinsic factors on neurite development and architecture appears to be weighted differently depending on the type of neuron, brain area and animal studied (reviewed in Cline, 2001; Libersat and Duch, 2004; McAllister, 2000; Wong and Gosh; 2002). For instance, it is reasonable to think that in peripheral sensory neurons whose dendrites are not postsynaptic to axonal input, dendritic fields are specified by strict genetic control. Several studies in *Drosophila* indicate that this seems to be the case at least for MD neurons (Gao *et al.*, 1999; 2000; Gao and Bogert, 2003; Grueber and Jan, 2004). On the other hand, one could expect a relatively significant contribution from external cues such as released signals from afferent inputs or synaptic targets to the development of motorneuronal and interneuronal neurites. Examples in both vertebrates (Rajan and Cline, 1998) and invertebrates (Kent and Levine, 1993; Mizrahi and Libersat, 2002; Scott *et al.*, 2003) support this idea.

In many developing systems (e.g. Meinertzhagen *et al.*, 2000), contact between synaptic partners correlates temporally with maturation of neurites. The contact of the 5-HT arborization and the LON is first seen at late 2nd instar, followed by further branching of the 5-HT processes during 3rd instar stage (Mukhopadhyay and Campos, 1995; this study). These observations, in conjunction with the findings that the presence of the LON (or Rh6 fibers) is required for normal 5-HT branching, indicates that an extrinsic signal deriving presumably from the Rh6-expressing cells is involved in the development of the larval optic neuropil 5-HT arborization. This hypothesis is strengthened by the results of my TNT expression experiments, which suggest that a vesicle released-molecule derived from the Rh6-expressing cells is required for 5-HT arbor morphogenesis and/or maintenance.

This extrinsic signalling mechanism could represent an activity-dependent or -independent process, or a combination of both. Although my present findings do not discriminate between activity-dependent and -independent mechanisms, it appears at least that evoked neurotransmitter release from the Rh6 cells is not required for proper branching of the 5-HT arborization. On the other hand, a quantitative correlation between partial disruption of 5-HT arbor development and partial suppression of spontaneous synaptic vesicle release by targeted TNT expression in the Rh6 photoreceptors suggest that spontaneous neuronal activity may be involved in this developmental process. This hypothesis is further supported by the fact that the optic neuropil 5-HT projection develops normally in both larvae reared in constant dark throughout development and *norpA* mutant larvae in which larval photoreceptors are present but are not functional. At

this point in time however, a synaptic activity-independent effect of TNT expression such as modification in FasII levels cannot be ruled out.

Interestingly, comparable observations regarding a role for spontaneous but not sensory-evoked neuronal activity in neurite morphogenesis has been proposed in *Drosophila* optic lobe vertical system (VS). VS1 neurons are interneurons that present highly stereotyped dendritic branching (Scott *et al.*, 2002). Scott and collaborators have shown that, whereas dark-reared and visually experienced flies possess VS1 neurons with similar dendritic and axonal morphology, *GMR-hid* flies lacking photoreceptors present VS1 cells with reduced dendritic branching complexity and overall length (Scott *et al.*, 2003). Thus, the authors suggest that photoreceptors spontaneous activity may be responsible for normal development of higher order visual processing VS dendrites (Scott *et al.*, 2003).

Previous studies indicate that Slit and Netrin signalling are involved in the development of normal VNC 5-HT neuronal projections (Couch *et al.*, 2004). Thus, in an attempt to further investigate the nature of the putative extrinsic trophic signal released by the Rh6 photoreceptor subset and required for proper development of the 5-HT arborization, we manipulated the expression levels of these two diffusible molecules as well as Wg. Over-expression of the secreted protein Slit (Fig. A7) but not others diffusible molecules such as Netrins (A and B) or Wg (data not shown) in all or only the Rh6 photoreceptors during larval development causes a similar 5-HT underbranching phenotype to the one observed after targeted TNT expression. Interestingly, Slit has been shown to be expressed in *Drosophila* adult visual system, where it appears to be required

for proper projection patterns of visual centers (Tayler *et al.*, 2004). Despite the fact that my preliminary findings do not address whether larval photoreceptors in normal conditions indeed secrete Slit, these results suggest that this diffusible molecule may constitute an extrinsic signal required for proper development and branching of the 5-HT arborization. In this scenario, this activity-independent Slit-mediated mechanism might be acting alone or in conjunction with photoreceptor spontaneous activity to regulate normal 5-HT arbor morphogenesis.

It is worth noting that, compared with the complete ablation of the 5-HT arbor observed when the Rh6 cells are absent, the outgrowth of the main 5-HT branches appears to be unaffected by expression of TNT or Slit in the Rh6 photoreceptors. As mentioned above in the case of TNT expression, this could be due to partial disruption of spontaneous activity of the Rh6 photoreceptor subset. Thus, this activity-dependent process might be indeed sufficient to promote initial outhgrowth, branching and maintenance of the 5-HT arborization. Alternatively, the LON itself and/or other LON-dependent optic neuropil afferents might provide a different trophic signal (e.g. diffusible or cell-bound molecule) required for the initial growth of these 5-HT projections. Photoreceptor spontaneous activity could take control over or potentiate the effect of this initial trophic signal to regulate further growth of the serotonergic processes.

It is now widely accepted that the effect of neuronal activity in neurite development is mediated by Ca^{2+}-induced signalling (reviewed in Chen and Ghosh, 2005). Furthermore, it is believed that neuronal activity-induced Ca^{2+} signalling exerts its effects on neurite morphogenesis by activation of multiple signalling pathways, mediated

by different signal transducers including Rho family GTPases (reviewed in Chen and Gosh, 2005). These signalling mediators have been shown to affect neurite outgrowth, guidance and branching by regulating its cytoskeleton organization (reviewed in de Curtis, 2008; Govek *et al.*, 2005; Luo, 2002). My present findings indicate that the small GTPase Rac is required for normal development and/or branching of the larval optic neuropil arborization. Furthermore, as it has been previously proposed by studies in vertebrates (e.g. Li *et al.*, 2002; Sin *et al.*, 2002), and recently in *Drosophila* (Rosso *et al.*, 2005; Srahna *et al.*, 2006) my results suggest a possible mediatory role for Rho GTPases in the effects of neuronal activity on *Drosophila* arbor morphogenesis.

The idea of multiple synaptic contacts in *Drosophila* CNS is not surprising. In fact, it is known that, unlike what occurs in the vertebrate brain, in most *Drosophila* central synapses a single presynaptic release site connects to two or more postsynaptic elements (reviewed in Prokop and Meinertzhagen, 2006). This phenomenon has been extensively studied in the adult visual system (e.g. Fröhlich and Meinertzhagen, 1983; Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001; Nicol and Meinertzhagen, 1982; Takemura *et al.*, 2008). One possible explanation for this structural wiring arrangement is that, unlike in vertebrates where network complexity is achieved by increasing the number of neurons, *Drosophila* synaptic divergence and circuit complexity are attained by increasing the number of components participating in each neuronal synapse (Prokop and Meinertzhagen, 2006).

Several lines of evidence suggest that the two larval photoreceptor subsets may perform different functions during larval stage. The basic function of the larval visual

system is the perception of the light stimulus. In this regard, we have previously demonstrated that only the Rh5, but not the Rh6 photoreceptors, are required for normal larval photoresponse (Hassan *et al.*, 2005). Additionally, it has been shown that the larval visual system is partially responsible for circadian entrainment during larval stage (Malpel *et al.*, 2004). Until now, it was believed that the Rh6 photoreceptors were the subset retained through metamorphosis to form part of the H-B extraretinal eyelet, an adult structure implicated in circadian rhythmicity (Helfrich- Förster *et al.*, 2002; Malpel *et al.*, 2002). Nevertheless, it has been recently shown that in fact the 8 larval Rh6 photoreceptors degenerate during prepupation, whereas the Rh5 subset remains through metamorphosis and switches from Rh5 to Rh6 expression, constituting indeed the larval photoreceptor subtype becoming the adult extraretinal eyelet (Spretcher and Desplan, 2008). Furthermore, Spretcher and Desplan (2008) propose that the Rh5 photoreceptors support both visual system functions while Rh6 cells appear to play exclusively a circadian input role during larval stages. Finally, my studies showing the requirement of the Rh6 photoreceptors for proper development of the larval optic neuropil 5-HT arborization suggest a trophic function for this group of photoreceptors (Rodriguez Moncalvo and Campos, 2005).

It has been previously shown that 5-HT is involved in regulation of different aspects of circadian behaviours in *Drosophila* adults including entrainment of the circadian clock (e.g. Nichols, 2006; Yuan *et al.*, 2005; 2006). Thus, taking into consideration the anatomical findings mentioned above, it is possible that 5-HT neurons in the larva indirectly regulate circadian entrainment through their interaction with the

larval photoreceptors. Alternatively, 5-HT neurons may play a role in circadian rhythm regulation exerting their effect directly on the pacemaker neurons. Spatial association of serotonergic systems and clock cells has been previously reported in both vertebrates (e.g. Pickard and Rea, 1997) and *Drosophila* adults (Hamasaka and Nässel, 2006; Yuan *et al.*, 2005). Consistent with the results published by Hamasaka and Nässel (2006), my observations indicate that the larval optic neuropil 5-HT projections also interact with the PDF dendritic arborization (Rodriguez Moncalvo and Campos, 2005). Interestingly, Yuan *et al.* (2005) reported the presence of 5-HT1B_{Dro} receptor expression in larval LNv. Furthermore, Ca^{2+} imaging studies demonstrated that 5-HT decreases intracellular Ca^{2+} in dissociated LNv (Hamasaka and Nässel, 2006). Anatomical data also suggests a putative interaction between the output terminations of the LNv and 5-HT processes in the larval dorsal protocerebrum (data not shown; Hamasaka and Nässel, 2006). Thus, in addition to the possibility that *Drosophila* larval 5-HT neurons might play a role in circadian entrainment by affecting larval photoreceptor function, increasing evidence suggests that these cells might be involved in regulation of circadian rhythmicity by acting directly on the brain PDF-expressing neurons.

5.1.2. Modulation of the larval response to light by serotonergic neurons

Previous studies have shown that modification of serotonin levels regulate light response in adult flies (Yuan *et al.*, 2005). Here, I report that disruption of synaptic activity of larval 5-HT neurons and presumably of 5-HT release as well as reduced 5-HT synthesis increases the larval response to light during both foraging and wandering larval

stages. These results strongly suggest that 5-HT neurons play a role in developmental modulation of larval photobehaviour. Furthermore, my observations indicate that this regulation appears to occur at a central level, most likely involving serotonergic cells located within the brain hemispheres, and to be mediated by 5-HT1A_{Dro} receptors.

Several studies have demonstrated that *Drosophila* larval locomotion, like other rhythmic movements, is controlled by CPGs. Furthermore, it is widely accepted to date that sensory as well as neuromodulatory inputs constitute an important component of locomotion control. Particularly, previous experiments conducted in the laboratory of Dr. Campos have shown that the larval response to light can be used as a model to study regulation of larval crawling by the light stimulus. Here, I presently propose that 5-HT neurons play a role as neuromodulatory input for developmental regulation of the larval photoresponse.

Serotonin has been shown to act as neuromodulator of neuronal circuits including CPGs in many different vertebrate as well as invertebrate systems (e.g. reviewed in Grillner, 2006; Katz, 1998; Schmidt and Jordan, 2000). As other neuromodulators, 5-HT may exert its modulatory effect acting as a neurohormone, at a specific synapse or in a paracrine fashion. Presumably, TNT expression could interfere with any of these forms of 5-HT actions. Nevertheless, given that most of CNS 5-HT neurons represent interneurons (Vallés and White, 1988), it is reasonable to think that most likely the effect of serotonin is occurring at a synaptic or paracrine level.

As mentioned before, neuromodulation of a given network may be intrinsic or extrinsic to that particular circuit (reviewed in Katz, 1998; Katz and Frost, 1996).

Regarding 5-HT-mediated modulation of the larval response to light, two pieces of evidence suggest that the modulatory effect of serotonergic neurons in larval photoresponse is extrinsic to the larval locomotor CPGs. Firstly, previous findings indicate that the CPGs controlling larval crawling are located in the VNC (Cattaert and Birman, 2001). Nevertheless, my present results demonstrate that 5-HT neurons of the ventral ganglion are not involved in developmental modulation of larval photobehaviour. Thus, it appears that the 5-HT neurons responsible for this effect are located within the brain hemispheres and therefore most likely they do not form part of the CPGs controlling larval locomotion.

Neuromodulators may regulate CPG output by acting at any location within the locomotory circuit, i.e. at the sensory input level, the CPG itself, the CPG-motor synapse or at the motorneuron-muscle level (reviewed in Marder and Calabrese, 1996). It is important to note that, although the cell bodies of the 5-HT neurons involved in modulation of the larval photoresponse are presumably localized in the brain lobes, their modulatory projections could potentially exert their function at any level within the circuit controlling this behaviour. Although this issue remains to be further investigated, previous physiological studies have shown that 3rd instar NMJs of muscle 6 are insensitive to 5-HT application (Sparks *et al.*, 2004). If this is true in all muscles, these results suggest that 5-HT-mediated modulation of larval photoresponse most likely does not take place at the motorneuron level. Yet, my present findings indicate that this regulation does not appear to occur at the photoreceptor level either. In addition, the modulatory effect of 5-HT neurons on larval photobehaviour could be direct or indirect.

For instance, serotonin might be acting at an inhibitory synapse to enhance presynaptic release of an inhibitory transmitter, as shown in other systems (reviewed in Weiger, 1997).

It is widely accepted that activation of GPCRs and consequently of second messenger pathways constitutes typically the mechanism underlying neuromodulation (reviewed in Birmingham and Tauck, 2003). In this regard, most 5-HT receptor subtypes in vertebrates and all the ones cloned until now in *Drosophila* have been identified as GPCRs (reviewed in Nichols and Nichols, 2008; Tierney, 2001), making serotonin an excellent candidate for neuromodulatory actions on neural circuits. Although the involvement of other *Drosophila* 5-HT receptor subtypes cannot be totally ruled out, my findings suggest that 5-HT1A$_{Dro}$ receptors take part in 5-HT-mediated modulation of larval photobehaviour. In vertebrates, 5-HT1A is expressed in many areas of the brain (reviewed in Pucadyil *et al.*, 2005). It is known that the main downstream signalling event triggered by binding of 5-HT to these receptors involves inhibition of AC (reviewed in Pucadyil *et al.*, 2005; Raymond *et al.*, 2001). For instance, it has been shown recently that serotonergic inhibition of the excitability of stellate and pyramidal neurons in the superficial layers of the entorhinal cortex occurs by means of activation of 5-HT1A receptors (Deng *et al.*, 2007). This causes activation of Gαi3 which in turn leads to down-regulation of protein kinase A (PKA) activity and disinhibition of two-pore domain K^+ (K2P) channels. Consequently, the increase in K2P channel activity produces neuronal hyperpolarization and modulation of neuronal excitability (Deng *et al.*, 2007). Thus, it is

possible that a similar signalling mechanism to the one observed in the entorhinal cortex is responsible for 5- HT1A$_{Dro}$-mediated modulation of *Drosophila* larval photobehaviour.

Apart from this primary signal transduction pathway, it is known that activation of vertebrate 5-HT1A receptors causes also activation of G-protein $\beta\gamma$-subunits, initiating additional signal transduction pathways. These include activation of PLC and protein kinase C (PKC), and regulation of inwardly rectified K^+ channels, Ca^{2+} channels, and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels (reviewed in Pucadyil *et al.*, 2005; Raymond *et al.*, 2001). In fact, early studies have shown that 5-HT1A$_{Dro}$ also activates PLC (Saudou *et al.*, 1992). Hence, one additional or alternative possibility is that 5-HT1A$_{Dro}$-mediated effect on the larval photoresponse occurs via activation of G$\beta\gamma$-subunits and PLC, similar to what is observed in vertebrates.

The nervous system of any organism must be capable of accommodating additional behavioural demands as a result of changes that occur continuously not only in the environment but also throughout the ontogeny or development of the individual. Different means by which the nervous system acquires such plasticity involve in many cases the assembly of new neural networks, reconfiguration of the connectivity of pre-existing circuits, and/or modifications in the synaptic or electrical properties of the elements that form such networks (reviewed in Rauscent, 2006).

As in other holometabolous insects, development of the adult fly requires a massive remodelling of the larval nervous system during metamorphosis to fulfill the requirements for the control of adult behaviour. Regarding phototactic behaviour, *Drosophila* undergoes a metamorphic transition during which a complete transformation

occurs from a crawling photophobic larva to a flying adult that is attracted to light. During most of larval life, *Drosophila* individuals show aversion to light and remain buried inside the substrate. Nevertheless, at the end of 3rd instar stage, larvae become photoneutral and begin to crawl out of the substrate in search for a proper place to undergo pupation. It is reasonable to think that, at this point in larval development, *Drosophila* nervous system would require to 'turn off' the larval photophobic behaviour in order to allow the individual to adjust to this new behavioural demand. Hence, taking in account my present results, it is quite tempting to speculate that 5-HT-mediated modulation may constitute one, but most likely not the only means by which this photobehavioural transition is achieved. Lastly, modulation of motor response to light stimuli by 5-HT neurons might represent a common mechanism. Interestingly, 5-HT has been previously implicated in modification of phototactic behaviour in other invertebrates such as crustaceans (Helluy and Holmes, 1990; Helluy and Thomas, 2003; Maynard *et al.*, 1996; McPhee and Wilkens, 1989; Tain *et al.*, 2006, 2007) and molluscs (Crow and Forrester, 1986; 1991; Schuman and Clark, 1994).

5.2. Perspective and future directions

In the last few years, our knowledge regarding 5-HT neuronal development and function in both vertebrates and invertebrates has been significantly broadened. Nevertheless, many questions remain to be addressed. In *Drosophila* for instance, although a considerable amount of work has revealed many of the genes required for specification and differentiation of 5-HT neurons of the VNC, those responsible for

determining 5-HT cell fate in the brain hemispheres await identification. In regards to neurite growth and synapse formation, for the most part it remains to be investigated to what extent dendritic and axonal pathfinding, target selection and synaptic development of *Drosophila* 5-HT neurons are determined by either intrinsic or extrinsic mechanisms as well as the identity of those molecules involved. Furthermore, future investigations of synaptic partners of these neurons as well as a better characterization of the spatial distribution of 5-HT receptors, together with functional studies, would help to uncover new roles for these cells. In particular, it would be of great interest to further explore the development of the 5-HT processes found in the larval optic neuropil as well as the mechanisms underlying 5-HT-mediated modulation of the larval photobehaviour.

5.2.1. Further studies regarding the development of the larval optic neuropil 5-HT arborization

Consistent with other studies (Hamasaka and Nässel, 2006; Mukhopadhyay and Campos, 1995), I have found that serotonergic processes overlap with the LON as well as the PDF-expressing dendritic tree in the larval optic center (Rodriguez Moncalvo and Campos, 2005). Whereas the identity of the two latter neuronal groups is known, it remains unclear which of the 5-HT neurons innervate this specific CNS neuropil. It appears that these 5-HT processes correspond to axons deriving from two sets of serotonergic neurons: 1) a pair of neurons projecting from the contralateral brain hemisphere and 2) the LP1 neurons (Hamasaka and Nässel, 2006). Nevertheless, the extensive number of 5-HT projections and arborizations found innervating the larval

brain lobes obscures the origin of these particular CNS 5-HT processes. Thus, in order to verify that the above mentioned 5-HT cells are indeed the ones projecting to the larval optic center one could employ mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo, 1999). The use of this method would allow for GFP labelling of individual or small sets of 5-HT neurons that, together with 5-HT immunostaining, will help to trace the larval optic center processes back to their corresponding cell bodies.

The morphology of the larval optic neuropil 5-HT arborization suggests that this projection may be a putative release site (i.e. an axonal arbor). However, whether it indeed represents an input or output terminal remains elusive. Although electron-microscopy (EM) examinations still constitute the most accurate and complete approach to analyze synaptic circuitry, the availability of *Drosophila* genetic tools have begun to provide new methods to dissect the pre- and post-synaptic compartments that form synaptic circuits. Different molecular markers have been shown to be valuable in revealing putative pre- and post-synaptic terminals of different group of neurons. For instance, ectopic expression of the *Drosophila* Down syndrome adhesion molecule [17.1]-GFP (Dscam-GFP) fusion protein has been shown to localize to post-synaptic compartments but not to axons or pre-synaptic sites (Wang *et al.*, 2004). Recently, Dscam-GFP has been recently used as dendrite marker in MB neurons (Zhang *et al.*, 2007) and different aminergic neurons of the larval VNC (Vömel and Wegener, 2008). Similarly, ectopic expression of the GFP fusion protein Synaptotagmin1-GFP (Syt-GFP) has been shown to label specifically pre-synaptic sites of many neuronal types, including VNC aminergic neurons (Iyengar *et al.*, 2006; Vömel and Wegener, 2008; Zhang *et al.*,

2002; 2007). Thus, these markers can be ectopically expressed in the 5-HT neurons using *TRH-GAL4* in order to determine the synaptic nature of the larval optic neuropil 5-HT arborization.

As mentioned before, preliminary results indicate that Slit may play a role as trophic signalling molecule for the development and/or maintenance of the 5-HT arborization. It would be interesting to further examine this possibility by studying the morphogenesis of the serotonergic arbor for instance in viable *slit* mutants. In *Drosophila*, three Roundabout (Robo) receptors generally mediating responses to Slit have been cloned so far: Robo, Robo2 and Robo3 (reviewed in Dickson and Gilestro, 2006). Therefore, the development of the optic center 5-HT processes can be studied as well in larvae in which the expression of these receptors has been down-regulated specifically in the 5-HT neurons. Lastly, in order to investigate whether the larval photoreceptors might be the source of Slit-mediated signal, analysis of Slit expression in the larval photoreceptors should be performed.

My results indicate that a trophic signal deriving from the Rh6 photoreceptors is involved in the branching and/or its maintenance of the 5-HT arborization. Despite my demonstration that neither the Rh5 cells nor the LNv are required, additional cells might participate in this process. For instance, besides the neurons above mentioned, other cells such as GABAergic and glutamatergic neurons have been recently shown to project to the larval optic neuropil (Hamasaka *et al.*, 2005; 2007). Using similar approaches to those used in my studies, it would be interesting to analyze whether these cells are also

involved in the development of the 5-HT arbor or if the Rh6 cell subset represents indeed a unique trophic signalling source.

5.2.2. Additional studies regarding the modulation of the larval photobehaviour and the contribution of 5-HT neurons to this regulation

In order to determine how neuromodulation is integrated into neural circuits, it is necessary to identify the neurons that evoke neuromodulatory effects as well as the targets of such modulatory neurons. In this particular case, my observations suggest that the VNC 5-HT neurons are not required for the developmental regulation of the larval photobehaviour. Nevertheless, the identity and projection pattern of the serotonergic cells involved in this phenomenon remain elusive. For instance, behavioural analyses together with a modified version of MARCM incorporating TNT expression could be used to address these questions. In this way, mosaic larvae showing an increased response to light could be screened for the presence of GFP-labelled serotonergic clones. In addition, their neuronal projections could be mapped in respect to CNS landmarks such as identified major axonal tracts, neuropil compartments and/or secondary NB lineages previously used in other morphological studies (e.g. Iyengar *et al.*, 2006; Landgraf *et al.*, 2003b; Nassif *et al.*, 2003; Pereanu and Hartenstein, 2006; Vömel and Wegener, 2008; Younossi-Hartenstein, *et al.*, 2003; reviewed in Pereanu and Hartenstein, 2004). Furthermore, elucidation of the projection pattern of the potential 5-HT neurons involved in modulation of the larval photoresponse could also provide insights into the putative targets of these neurons.

My behavioural studies indicate that the larval photoresponse is developmentally regulated by 5-HT neurons and presumably to a less extent by CRZ neurons. Nevertheless, it appears that these two groups of cells do not represent the only modulatory input for this regulation. For instance, in addition to dopaminergic, serotonergic and corazonergic neurons, recent anatomical studies indicate that the CCAP/MIP-expressing neurons are *Ddc-GAL4* positive as well, at least in the case of the cells found in the VNC (Vömel and Wegener, 2008). As mentioned before, one should be careful when comparing relative contributions of different sets of neurons to a given output using *GAL4* constructs, as the differences observed between strains may be obscured by differences in the strength of the promoter used. Thus, the fact that *Ddc:TNT-G* and *TRH:TNT-G* larvae respond similarly does not exclude the possibility that CCAP/MIP cells could be contributing to the increased response to light observed in *Ddc:TNT-G* larvae. In order to investigate this, one could conduct behavioural analyses of larvae expressing TNT specifically in this group of neurons by means of the *CCAP-GAL4* driver. As well, it would be interesting to perform similar studies to examine whether other inhibitory inputs such as GABAergic or glutamatergic neurons could also form part of the neuronal network modulating larval photobehaviour during development.

It has been shown that CRZ and CCAP cells represent two groups of non-overlapping peptidergic cells (Park *et al.*, 2008; Santos *et al.*, 2007; Vömel and Wegener, 2008). In addition, it appears that the distribution pattern of these neurons is also different from that of VNC serotonergic neurons (Vömel and Wegener, 2008). However, whether the latter is also true in the brain hemispheres is yet unknown. To date, it is

widely accepted that the activity of neuronal circuits can be regulated by the action of different neuromodulators, which may or not be co-released by the same terminal, acting in conjunction to modulate neuronal network output (Nässel and Homberg, 2006). Moreover, co-expression of 'classical' neurotransmitters including BAs and neuropeptides by the same neuron/s has been shown to be quite common in both vertebrates and invertebrates (reviewed in Burnstock, 2004; Nässel, 2002). Thus, it would be of great interest to examine whether these two peptidergic neuronal groups also express 5-HT in *Drosophila* larva. This could be easily investigated by performing immunohistochemical studies. It is important to keep in mind that co-localization does not necessarily imply co-transmission. However, additional criteria such as post-synaptic co-localization of receptors and simultaneous release upon nerve stimulation may help to establish whether two substances are co-released from a given input terminal (reviewed in Burnstock, 2004).

5.3. Concluding remarks

The insect CNS including that of *Drosophila melanogaster* provides us with neuronal circuit models to study the contributions of extrinsic and intrinsic factors to normal circuit assembly and function. Furthermore, the neuronal networks controlling a given behaviour in insects such as *Drosophila* often comprise a small number of identified neurons, and in many cases only a few layers of neural processing lie in between sensory input and behavioural output (Birmingham and Tauck, 2003; Libersat, 2005). This, combined with *Drosophila* powerful genetics makes the fruit fly nervous

system particularly useful to study how changes in neuronal wiring and function can be associated not only with changes in CNS development but also with modifications in behaviour.

Serotonergic neurons are found in all animal phyla with a nervous system (reviewed in Hay-Scmidt, 2000; Weiger, 1997). In both vertebrates and invertebrates, serotonin has been shown to be an important signalling molecule regulating many behavioural responses including feeding, sexual behaviour and aggression (reviewed in Nichols and Nichols, 2008; Weiger, 1997). Using *Drosophila* larva as a model, I investigated the development of 5-HT neurons. In particular, my studies pinpoint the Rh5- and Rh6-expressing cells as well as the LNv as possible synaptic partners or targets of the 5-HT neurons. My results also revealed that the larval Rh6 photoreceptors are required for normal outgrowth and branching of the 5-HT processes found in the larval optic neuropil and that Rac signalling is also involved in this phenomenon. Regarding 5-HT neuronal function, I demonstrated that these neurons are important modulators of the larval response to light, a process that appears to be mediated by 5-HT1A_{Dro} receptors. Thus, my present findings have revealed signalling mechanisms underlying the development of 5-HT neurons in *Drosophila* larva, as well as provided new insights into the roles that these cells play in this organism. Furthermore, my research has paved the way for future studies aimed at further exploring extrinsic and intrinsic factors involved in the development of *Drosophila* larval 5-HT neurons, as well as the mechanisms underlying the regulation of larval photobehaviour.

Many questions regarding nervous system wiring and function including those that comprise serotonergic cells remain to be answered. Nevertheless, we can optimistically hope that future work on invertebrates like *Drosophila* will contribute with answers to some of these questions, providing new insights into fundamental mechanisms underlying the generation and function of invertebrate neural networks. Moreover, we can expect that, despite some unique structural and functional aspects of invertebrate nervous systems, many of the mechanisms underlying neuronal circuit elaboration and function uncovered in these model organisms in the future will also be valuable for mammalian systems.

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APPENDIX

APPENDIX A: Additional data regarding functional studies of *Drosophila* larval 5-HT neurons

Figure A1. Normal developmental timing of larvae expressing TNT in the Ddc neurons. In order to verify that larvae expressing active TNT in the Ddc neurons were wandering at the proper developmental time, emptying of their guts, characteristic of wandering stage, was measured by disappearance of blue-colored food from larval guts. A, B, photographs of representative early wandering *UAS-TNT-G/+;Ddc-GAL4/+* larva (A) and *UAS-TNT-VIF/+;Ddc-GAL4/+* larva (B). Early wandering 3rd instar *Ddc:TNT-G* larvae show only residues of blue food at the posterior end of their gut, comparatively similar to what is observed in *Ddc:TNT-VIF* larvae. This suggests that *Ddc:TNT-G* larvae reach the wandering stage at the expected developmental time.

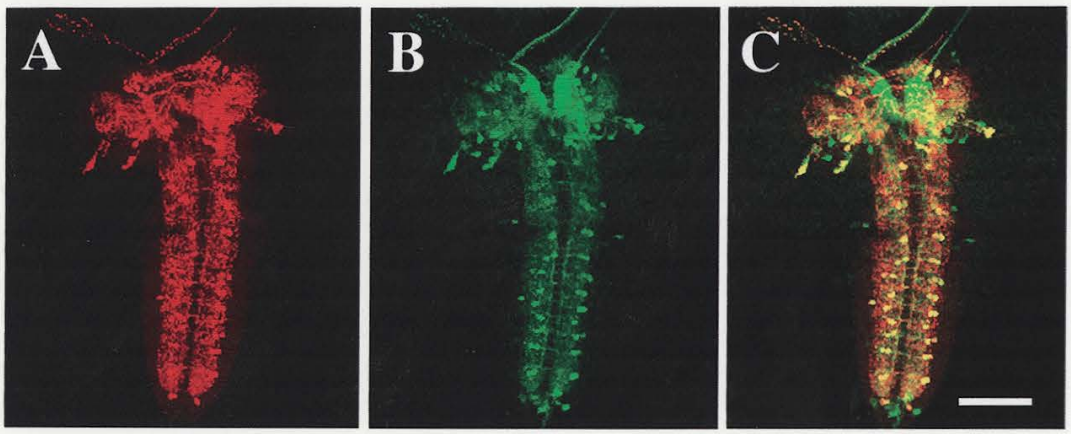
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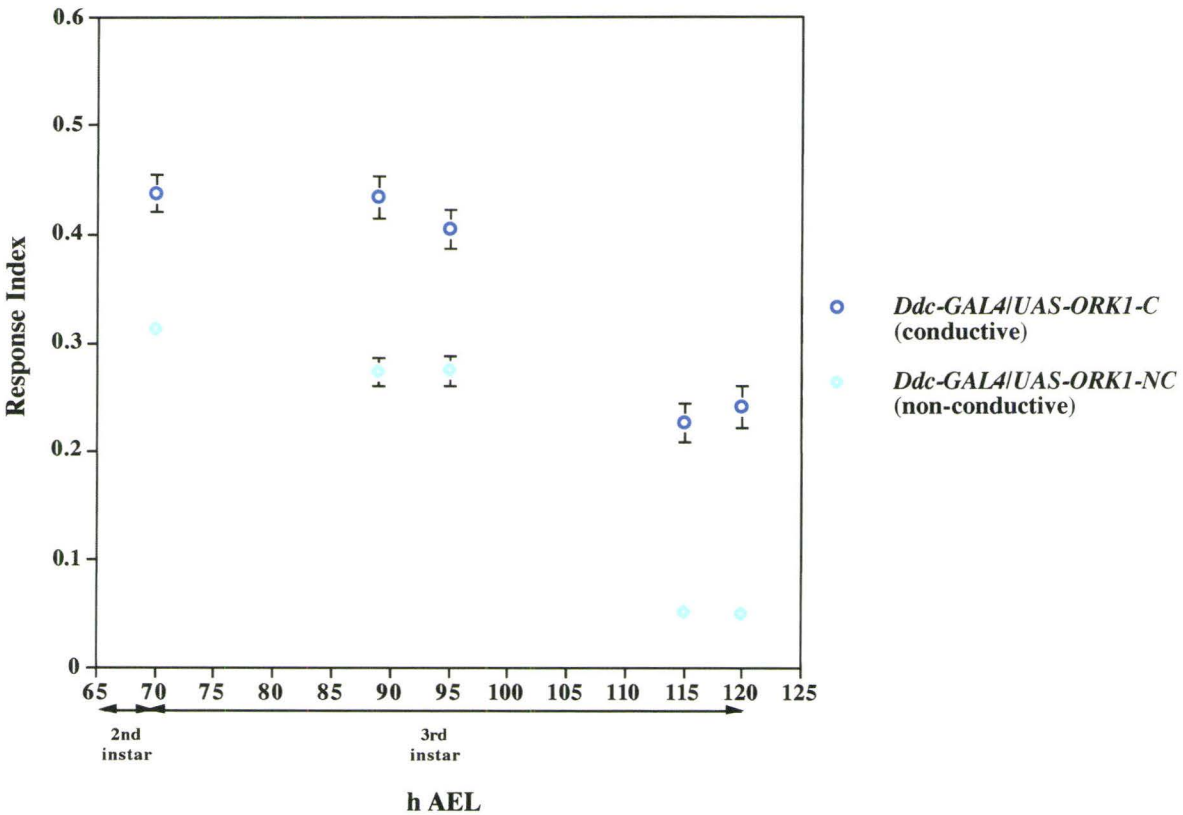
Figure A2. Expression of ORK1Δ-C in Ddc neurons increases the larval photoresponse. Response to light in the ON/OFF assay of *Ddc-GAL4/UAS-ORK1Δ-C* and *Ddc-GAL4/UAS-ORK1Δ-C* (control) larvae tested at different developmental stages. ORK1Δ-C represents a genetically modified constitutively open version of the wild type *Drosophila* open rectifier K^+ channel 1 (ORK1). On the contrary, ORK1Δ-NC is a non-conducting version of ORK1Δ-C (Nitabach *et al.*, 2002). RIs were obtained using the semi-automatic tracking system. Compared to what is observed in control larvae, targeted expression of the conductive form of ORK1Δ-C in the Ddc neurons increased the larval response to light from late 2nd to late 3rd instar stage. (late 2nd instar: *Ddc-GAL4/UAS-ORK1Δ-C*, N=18, RI=0.44; *Ddc-GAL4/UAS-ORK1Δ-NC*, N=15, RI=0.31; ANOVA: $F_{(1,31)} = 35.87$, $p < 0.001$; early foraging 3rd instar: *Ddc-GAL4/UAS-ORK1Δ-C*, N=16, RI=0.43; *Ddc-GAL4/UAS-ORK1Δ-NC*, N=15, RI=0.27; ANOVA: $F_{(1,29)} = 43.61$, $p < 0.001$; late foraging 3rd instar: *Ddc-GAL4/UAS-ORK1Δ-C*, N=15, RI=0.40; *Ddc-GAL4/UAS-ORK1Δ-NC*, N=17, RI=0.27; ANOVA: $F_{(1,30)} = 38.36$, $p < 0.001$; early wandering 3rd instar: *Ddc-GAL4/UAS-ORK1Δ-C*, N=17, RI=0.23; *Ddc-GAL4/UAS-ORK1Δ-NC*, N=17, RI=0.05; ANOVA: $F_{(1,32)} = 83.92$, $p < 0.001$; late wandering 3rd instar *Ddc-GAL4/UAS-ORK1Δ-C*, N=13, RI=0.24; *Ddc-GAL4/UAS-ORK1Δ-NC*, N=17, RI=0.05; ANOVA: $F_{(1,28)} = 110.52$, $p < 0.001$).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Ddc-GAL4/UAS-ORK1-NC (non-conductive)

Figure A3. Larvae expressing EKO in the Ddc neurons show increased response to light. Photoresponse in the ON/OFF assay of *UAS-EKO/+;Ddc-GAL4/+*, and the parental control *Ddc-GAL4/+* and *UAS-EKO/+*larvae tested at different developmental times. The electrically knockout (EKO) represents a genetically modified version of the wild type *Drosophila* shaker K^+ channel (White *et al.*, 2001). RIs were calculated by the semi-automatic tracking system and statistically analyzed using Tukey's pairwise comparisons. Compared to what is observed in parental control larvae, larvae in which expression of EKO was targeted to the *Ddc* neurons showed increased larval photobehaviour from late 2^{nd} to late 3^{rd} instar stage. (late 2^{nd} instar: *UAS-EKO/+;Ddc-GAL4/+*, N=22, RI=0.44; *Ddc-GAL4/+*, N=16, RI=0.37; *UAS-EKO/+*, N=20, RI=0.36; $p < 0.05$; early foraging 3^{rd} instar: *UAS-EKO/+;Ddc-GAL4/+*, N=17, RI=0.43; *Ddc-GAL4/+*, N=12, RI=0.36; *UAS-EKO/+*, N=13, RI=0.33; $p < 0.05$; late foraging 3^{rd} instar: *UAS-EKO/+;Ddc-GAL4/+*, N=18, RI=0.43; *Ddc-GAL4/+*, N=16, RI=0.32; *UAS-EKO/+*, N=18, RI=0.35, $p < 0.05$; early wandering 3^{rd} instar: *UAS-EKO/+;Ddc-GAL4/+*, N=20, RI=0.19; *Ddc-GAL4/+*, N=16, RI=0.06; *UAS-EKO/+*, N=20, RI=0.08, $p < 0.05$; late wandering 3^{rd} instar: *UAS-EKO/+;Ddc-GAL4/+*, N=20, RI=0.20; *Ddc-GAL4/+*, N=15, RI=0.06; *UAS-EKO/+*, N=19, RI=0.11, $p < 0.05$).

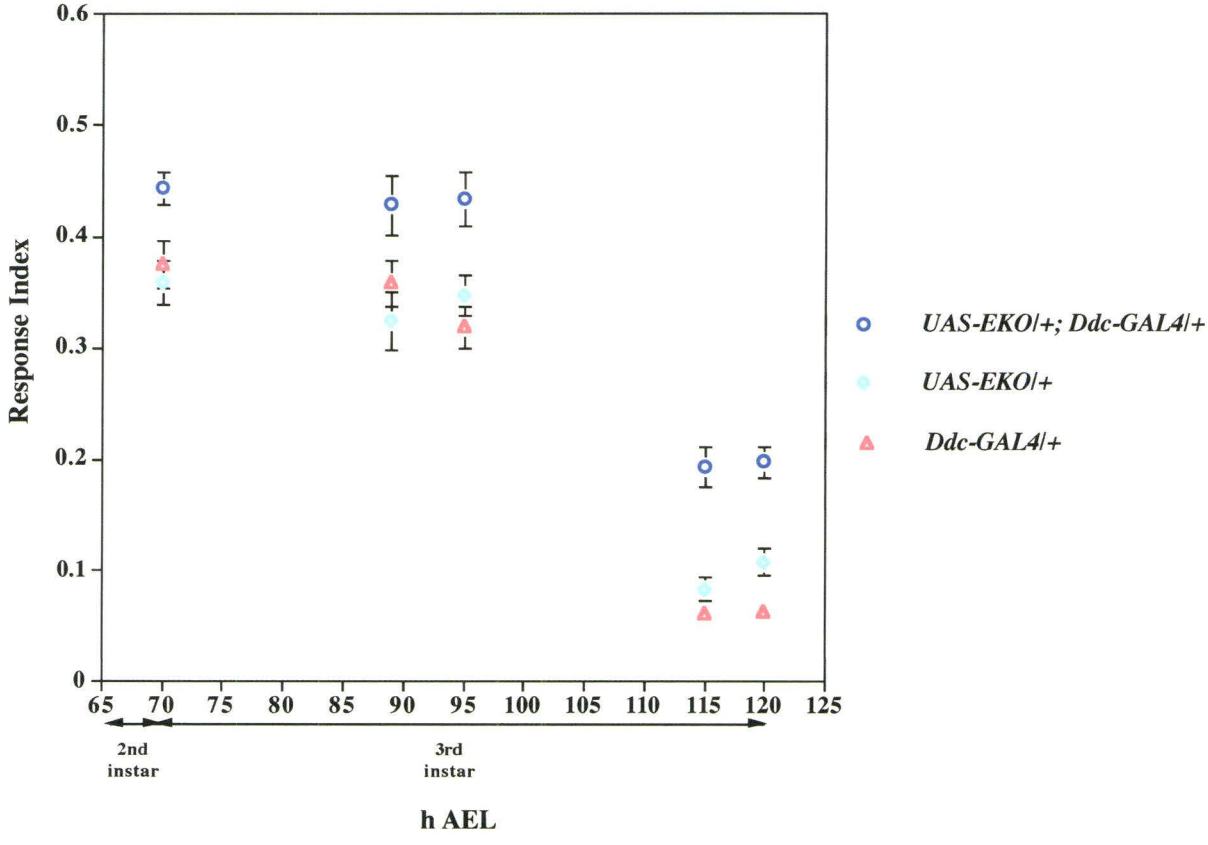
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Figure A4. Colocalization of neuronal TRH and 5-HT in the larval CNS. A-C, Low magnification single section confocal micrographs of a wandering 3rd instar *TRH-GAL4/UAS-mCD8-GFP* larval CNSs immunolabelled with anti-5-HT and detected by Texas Red-conjugated secondary (A, red). Tissue-specific expression of TRH was revealed by targeted expression of the *UAS-mCD8-GFP* construct (B, green). As seen in C (merge of A and B), TRH and 5-HT expressions are found colocalizing in most neurons and neurites. It appears that a small number of neurons only show expression of either 5-HT or TRH. One possible explanation is that, in those cells, these molecules are expressed at such low levels that they are undetectable by GFP labelling (in the case of TRH) or immunolabelling (5-HT). Scale bars: 40 μm.

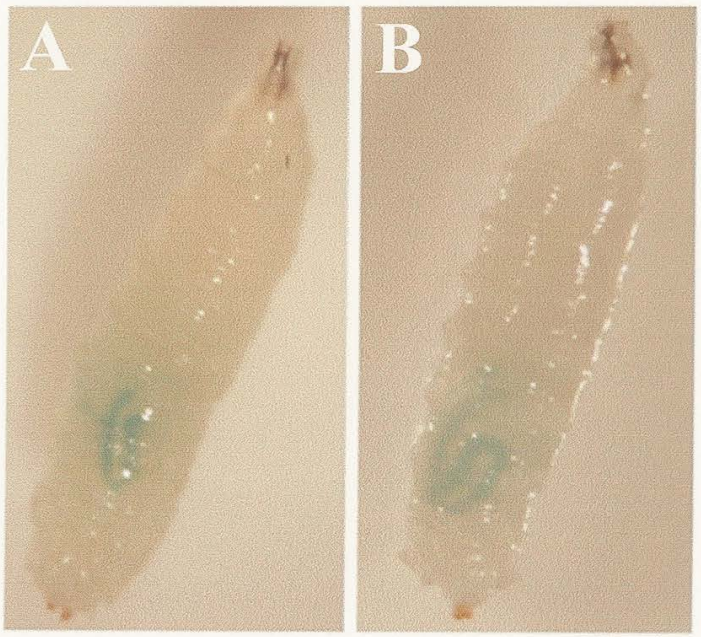
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Figure A5. Wandering 3rd instar *pBacTRH* mutant larvae display increased response to light during the ON/OFF assay. As measured by their RIs, wandering *pBacTRH* mutant larvae show photophobic response when compared with heterozygous (*pBacTRH/+*) parental control larvae in the same stage (early wandering stage: *pBacTRH*, N = 25, RI= 0.25; *pBacTRH/+*, N=24, RI= 0.11; ANOVA: $F_{(1,47)}$= 35.04, $p<0.001$; late wandering stage: *pBacTRH*, N = 25, RI= 0.23; *pBacTRH/+*, N=24, RI= 0.08; ANOVA: $F_{(1,47)}$= 51.72, $p<0.001$). *** $p<0.001$.

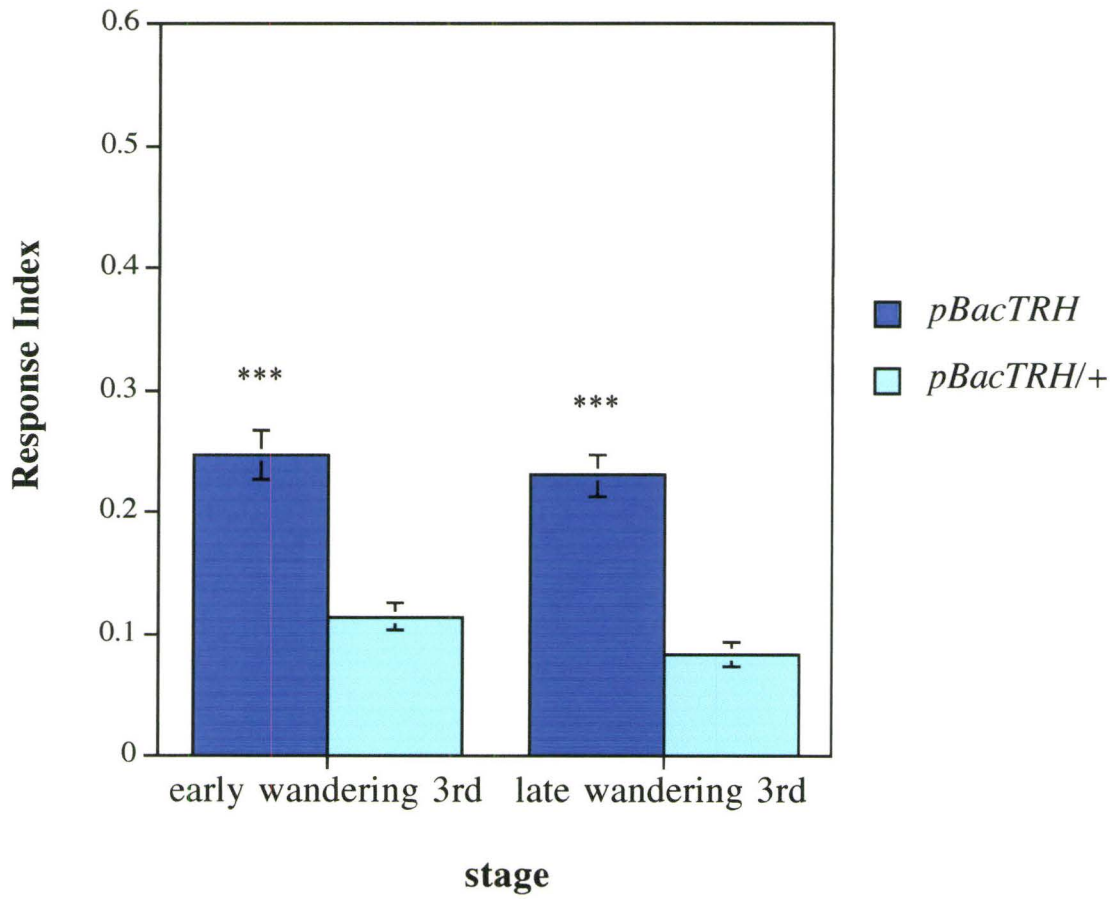
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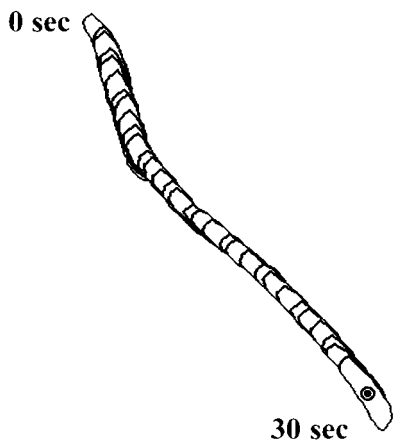
Figure A6. Silencing of Ddc neurons does not affect basic aspects of larval locomotion. Representative crawling patterns of foraging 3rd instar *Ddc:TNT* larvae in constant darkness. Since the response to light in the ON/OFF assay depends on the ability of the larva to move efficiently, larval locomotion was analyzed during 30 seconds in the absence of light. Perimeter stacks were generated using DIAS. Behavioural analysis using this software shows similar linear movement for both groups of larvae in constant dark conditions. Thus, locomotion of *UAS-TNT-G/+;Ddc-GAL4/+* larvae (A) appears normal when compared with that of *UAS-TNT-VIF/+;Ddc-GAL4/+* larvae (B, control).

A

UAS-TNT-G/+;Ddc-GAL4/+



B

UAS-TNT-VIF/+;Ddc-GAL4/+

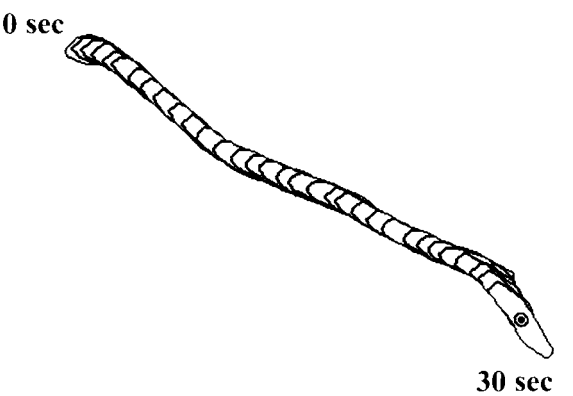


Figure A7. Expression of Slit in the Rh6 photoreceptors disrupts the proper development of the 5-HT arborization. A-C, Confocal micrographs of wandering 3rd instar larval brains immunolabelled with anti-5-HT detected by Texas Red-conjugated secondary (red). All larval photoreceptors were immunolabelled with 24B10 monoclonal antibody and detected by Alexa 488-conjugated secondary (A and B, green). Rh6 photoreceptors were labelled by targeted GPF expression (C, green). A, Wild type parental control *UAS-slit/+*. B, *GMR-GAL4/+;UAS-slit/+*. C, *UAS-mCD8-GFP/+;Rh6-GAL4/UAS-slit.* Targeted expression of Slit in either all or only the Rh6 photoreceptors causes a reduction in the development of the 5-HT processes. Scale bars: 10 μm.

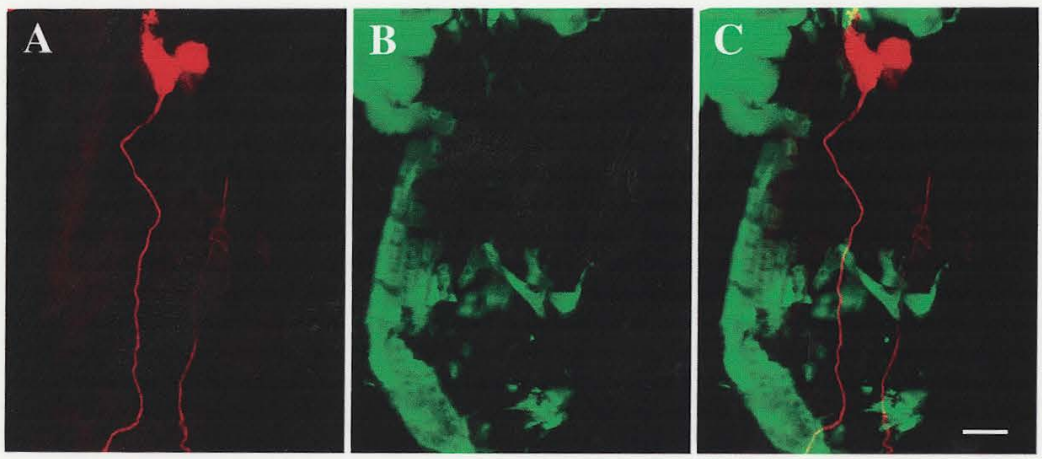

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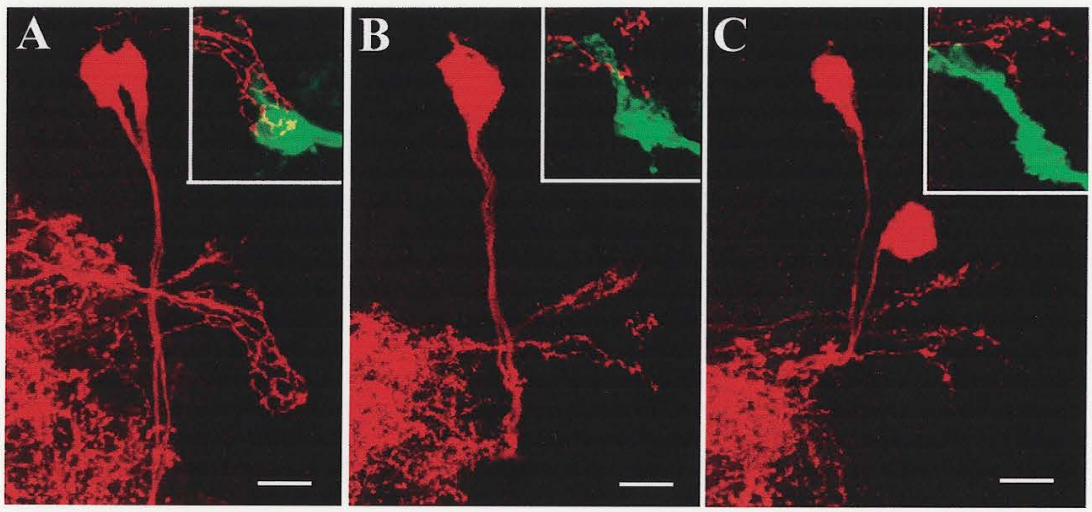
Figure A8. 5-HT1B$_{Dro}$ receptors are not expressed in *Drosophila* larval photoreceptors. (A-C) Confocal micrographs obtained at low magnification (20X) of a cuticle free-head portion of a wandering 3rd instar *5-HT1B$_{Dro}$-GAL4,UAS-mCD8-GFP* recombinant larva. A, Immunolabelling of larval photoreceptors with anti-24B10 and detected by Alexa 594-conjugated secondary (red). B, Expression pattern of 5-HT1B$_{Dro}$ receptors in the anterior portions of larval heads as revealed by targeted GFP expression (green). C, merge of A and B. As seen in C, no expression of 5-HT1B$_{Dro}$ receptors is found in the larval photoreceptors as observed by lack of signal colocalization. Scale bars: 30 μm.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C

Figure A9. Larval photoreceptors do not express 5-HT2$_{Dro}$ receptors. A-C, depict confocal micrographs of the anterior head portion of a 3rd instar wandering *pBac{GAL4D, EYFP}5-HT2^{PL0052}/UAS-tau-lacZ* larva. The enhancer trap line *pBac{GAL4D, EYFP}5-HT2^{PL00052}* contains a P-element inserted within the *5-HT2$_{Dro}$* gene, holding a variant of GAL4 (GAL4Δ) with both the amino-terminal DNA-binding domain and the carboxy-terminal activation domain present. This modified GAL4 protein is believed to be almost as good activator as the wild-type molecule but more stable. Together with a reporter line such as *UAS-tau-lacZ*, this enhancer trap reveals the expression of the neighbouring gene *5-HT2$_{Dro}$*. In addition, this construct contains the 3xP3 promoter sequence (an artificial promoter containing 3 Mmus/Pax6 homodimer binding sites) driving expression of EYFP (yellow GFP) in eye tissues. A, Expression pattern of 5-HT2$_{Dro}$ receptors in the anterior portions of the larval head as revealed by anti-lacZ antibody detected by Alexa 594-conjugated secondary (red). B, YFP expression in the larval photoreceptors (green). C, Merge of A and B. As observed in C, 5-HT2$_{Dro}$ receptors do not appear to be expressed in the larval photoreceptor cells. Scale bars: 10 μm.

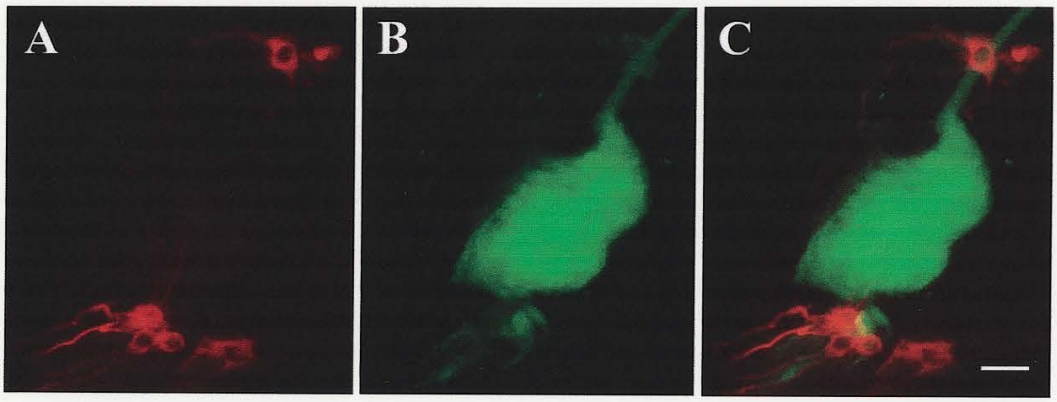

A
B
C

Figure A10. Photoresponse of 3rd instar foraging larvae over-expressing either 5-HT1A$_{Dro}$ or 5-HT7$_{Dro}$ receptors in the larval photoreceptors. Over-expression of either 5-HT1A$_{Dro}$ or 5-HT7$_{Dro}$ receptors in all larval photoreceptors was achieved by means of the *GMR-GAL4* driver. Tukey's pairwise comparisons revealed that ectopic expression of 5-HT1A$_{Dro}$ receptors in the photoreceptor cells caused only a slight reduction in the larval photoresponse (*GMR-GAL4/UAS-5HT1A$_{Dro}$*, N = 22, RI= 0.27; *GMR-GAL4/+*, N=31, RI= 0.38; *UAS-5HT1A$_{Dro}$ /+*, N = 30, RI = 0.36, ANOVA: $F_{(2,80)}$= 17.30, $p<0.05$). On the other hand, the response to light of larvae over-expressing 5-HT7$_{Dro}$ receptors was only significantly different to that of *GMR-GAL4/+* parental control larvae (*GMR-GAL4/UAS-5HT7$_{Dro}$*, N = 27, RI = 0.31; *GMR-GAL4/+*, N=31, RI= 0.38; *UAS-5HT7$_{Dro}$ /+*, N = 30, RI = 0.35, ANOVA: $F_{(2,85)}$= 10.35, $p<0.05$). * $p<0.05$.

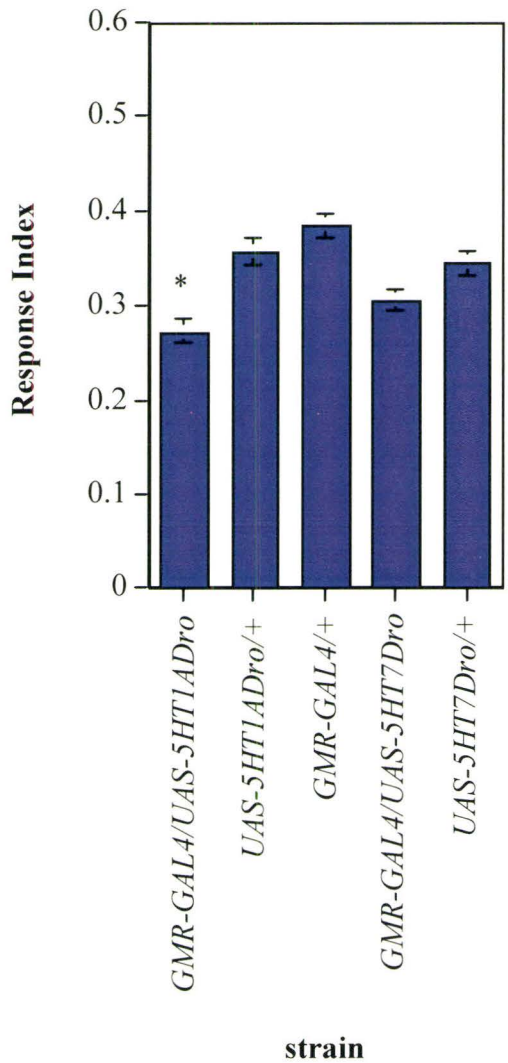
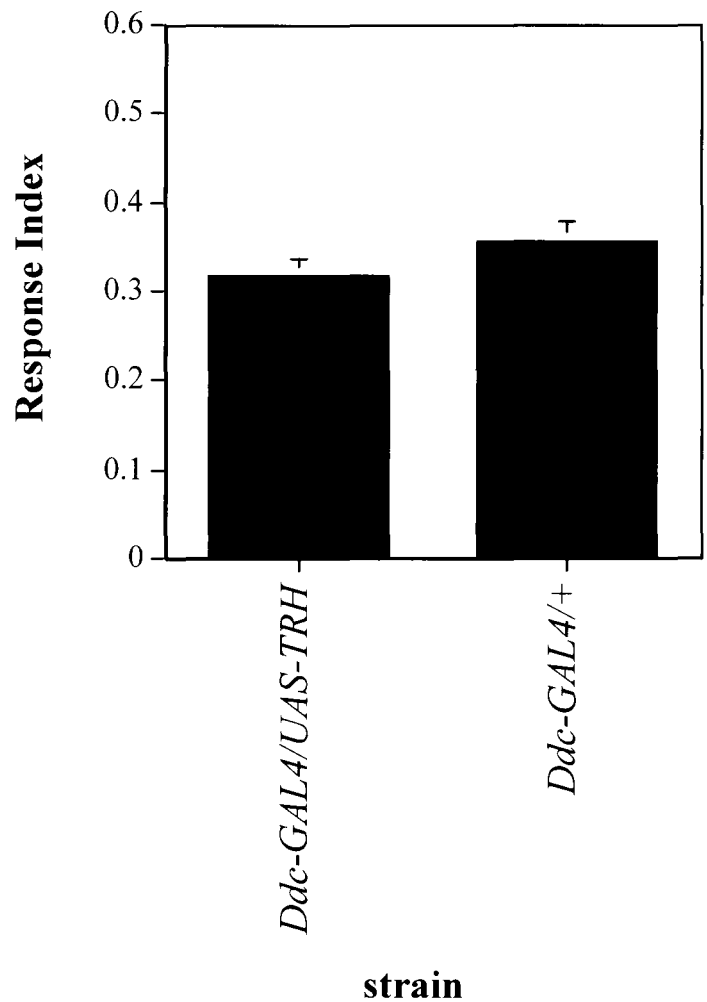

Response Index
0.6
0.5
0.4
0.3
0.2
0.1
0
*
GMR-GAL4/UAS-5HT1ADro
UAS-5HT1ADro/+
GMR-GAL4/+
GMR-GAL4/UAS-5HT7Dro
UAS-5HT7Dro/+
strain

Figure A11. Normal response to light in the ON/OFF assay of larvae with up-regulated levels of TRH in Ddc neurons. Up-regulation of 5-HT biosynthesis and presumably therefore of its release was induced by over-expressing TRH in *Ddc-GAL4*-expressing cells. Surprisingly, increased levels of serotonin specifically in Ddc neurons including serotonergic neurons did not cause a decrease in the response to light of 3rd instar early foraging larvae as expected (*Ddc-GAL4/UAS-TRH*, N = 20, RI= 0.32; *Ddc-GAL4/+*, N = 12, RI = 0.36; ANOVA: $F_{(1,30)} = 1.91$, $p = 0.18$).


Response Index
0.6
0.5
0.4
0.3
0.2
0.1
0
Ddc-GAL4/UAS-TRH
Ddc-GAL4/+
strain

APPENDIX B: Manuscript

Rodriguez Moncalvo VG, and Campos AR. (2005). Genetic dissection of trophic interactions in the larval optic neuropil of *Drosophila melanogaster*. Dev Biol, 286(2):549-58.

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Genetic dissection of trophic interactions in the larval optic neuropil of *Drosophila melanogaster*

Verónica G. Rodriguez Moncalvo, Ana Regina Campos *

Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON, Canada L8S 4K1

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Abstract

The larval visual system of *Drosophila melanogaster* consists of two bilateral clusters of 12 photoreceptors, which express Rhodopsin 5 and 6 (Rh5 and Rh6) in a non-overlapping manner. These neurons send their axons in a fascicle, the larval optic nerve (LON), which terminates in the larval optic neuropil. The LON is required for the development of a serotonergic arborization originating in the central brain and for the development of the dendritic tree of the circadian pacemakers, the small ventral lateral neurons (LNv) [Malpel, S., Klarsfeld, A., Rouyer, F., 2002. Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during *Drosophila* brain development. Development 129, 1443–1453; Mukhopadhyay, M., Campos, A.R., 1995. The larval optic nerve is required for the development of an identified serotonergic arborization in *Drosophila melanogaster*. Dev. Biol., 169, 629–643]. Here, we show that both Rh5- and Rh6-expressing fibers overlap equally with the 5-HT arborization and that it, in turn, also contacts the dendritic tree of the LNv. The experiments described here aimed at determining whether Rh5- or Rh6-expressing fibers, as well as the LNv, influence the development of this serotonergic arborization. We conclude that Rh6-expressing fibers play a unique role in providing a signal required for the outgrowth and branching of the serotonergic arborization. Moreover, the innervation of the larval optic neuropil by the 5-HT arborization depends on intact Rac function. A possible role for these serotonergic processes in modulating the larval circadian rhythmicity and photoreceptor function is discussed.
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Keywords: Rhodopsin; Photobehavior; *Drosophila*; Serotonin; Larva; Mutant; Photoreceptors; Pacemaker

Introduction

Assembly of neural circuits requires the orchestration of multiple intrinsic and extrinsic signals (for reviews, see Grueber and Jan, 2004; Jan and Jan, 2003; McAllister, 2000). For instance, the role of neurotrophins in dendrite development and maintenance is well established (Huang and Reichardt, 2001; McAllister, 2001; McAllister et al., 1995). Recently, receptor–ligand systems initially identified on the basis of their function in axon guidance have been added to the growing list of extrinsic cues that modulate dendrite development (Furrer et al., 2003; Polleux et al., 2000; Whitford et al., 2002). Furthermore, the role of afferent neurotransmission in dendritic development is also well established and conserved in many organisms (Li et al., 2002; Rajan and Cline, 1998; Rajan et al., 1999; Sin et al., 2002; Wong and Ghosh, 2002).

Although the underlying mechanisms by which extrinsic factors exert their effects on dendritic and axonal growth remain largely unknown, accumulating evidence in different model systems has revealed links between some of these factors and the activation of Rho GTPases (Fan et al., 2003; Hu et al., 2001; Li et al., 2002; Sin et al., 2002; Yamashita et al., 1999). Moreover, these studies support the notion that members of the Rho family of GTPases function as key integrators of extrinsic and intrinsic cues that regulate the underlying dendritic and axonal cytoskeleton.

This report examines the interaction among three groups of neural processes in the *Drosophila melanogaster* larval optic neuropil. The central nervous system of *Drosophila* like in many other insects follows a typical organization in which the somata of neurons and glia form an outer layer surrounding the inner neuropil where axons and dendrites are segregated (Campos et al., 1995; Nassif et al., 2003). By the end of embryogenesis, the larval optic nerve (LON) terminates within the optic lobe anlagen in the larval optic neuropil area. The larval visual system

* Corresponding author. Fax: +1 905 522 6066.
E-mail address: camposa@mcmaster.ca (A.R. Campos).

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of *Drosophila* consists of a pair of bilateral visual organs also known as Bolwig's organs, situated just anterior to the cephalopharyngeal skeleton (Green et al., 1993; Steller et al., 1987). Each of these visual organs is made up of 12 photoreceptor cells that differentiate during stage 13 of embryogenesis (Campos et al., 1995; Green et al., 1993) and are divided in 2 subsets: ~4 cells expressing the blue-absorbing opsin Rh5 (λ_{max} = 437 nm) and ~8 cells expressing the green-absorbing opsin Rh6 (λ_{max} = 508 nm) (Malpel et al., 2002).

It has been previously shown that the LON overlaps with a serotonergic arborization that originates from cell bodies located in the central brain (Mukhopadhyay and Campos, 1995). More recently, it has been reported that the LON terminus overlaps with the dendritic arborization of a subset of circadian pacemaker neurons, the small ventral lateral neurons (LNv) (Malpel et al., 2002). Interestingly and similar to what was previously shown for the serotonergic arborization, the development of the LNv dendritic arbor is dependent on contact with the LON (Malpel et al., 2002; Mukhopadhyay and Campos, 1995).

Here, we show that both Rh5- and Rh6-expressing fibers overlap with the 5-HT arborization, which in turn, also contacts the dendritic tree of the LNv. The results of cell ablation experiments indicate that the presence of Rh6-expressing fibers is necessary for the development of the serotonergic arborization. Moreover, suppression of synaptic activity by targeted expression of tetanus toxin light chain (TNT) in Rh6-expressing fibers prevents the full development of this 5-HT arborization, suggesting that this is at least in part an activity-dependent process. Finally, our results implicate Rac signaling in the development of the serotonergic arborization.

Materials and methods

Fly stocks

All *D. melanogaster* stocks were raised at 25°C in standard medium containing inactivated yeast, sucrose, agar, 10% and tegosept in ethanol to prevent mold growth. Cell ablation was achieved by targeted expression of the cell death genes *head involution defective* (*hid*) or *reaper* (*rpr*) using the *Drosophila* stocks *yw; P[UAS-hid]/P[UAS-hid]*) (Grether et al., 1995) and *+/+;P[UAS-rpr]/P[UAS-rpr]* (White et al., 1996) respectively. Neuronal silencing was achieved by targeted expression of tetanus toxin light chain (*TNT*) or non-inactivating Shaker K^+ channel protein (electrically knock out or EKO) (White et al., 2001). Three forms of *TNT* were used: the active forms *w; P[UAS-TNT-E]* and *w; P[UAS-TNT-G]* and the inactive control *w; P[UAS-TNT-VIF]* (Sweeney et al., 1995). The strain *GMR-hid* was used to ablate all photoreceptors (Grether et al., 1995). *GMR-Gal4* (Bloomington Stock Center, Indiana University, IN, #1104), *Rh6-Gal4* and *Rh5-Gal4* transgenic strains allowed expression of the target genes in all larval photoreceptors, Rh6- or Rh5-expressing larval photoreceptors cells respectively. In the case of the *Rh6-Gal4* and *Rh5-Gal4* strains, the co-expression of *P[UAS-mCD8:GFP]* construct (Bloomington Stock Center, Indiana University, IN, #5137) allowed the visualization of Rh5 and Rh6 termini. In addition, a *Ddc-Gal4* line (HL836, third chromosome) kindly provided by Jay Hirsch (University of Virginia, VA) and the *P [UAS-mCD8: GFP]* construct (Bloomington Stock Center, Indiana University, IN, #5130) were used to recombine both transgenes in the same chromosome and to target the expression of the green fluorescent protein (GFP) in the serotonergic cells. Similarly, *yw; P [PDF-Gal4]* and the *P [UAS-mCD8: GFP]* construct (Bloomington Stock Center, Indiana University, IN, #5137) were recombined to express GFP specifically in the LNv. Standard wild type stock *Oregon-R* (*OR*) was used. For *Rac* mutant analysis, the *Rac2*-specific null mutant homozygous viable $Rac2^{\Delta}$ *ry* stock, the *yw;* $Rac1^{J11}$ *FRT2A/TM6B* stock (which contains a null allele copy of $Rac1^{J11}$) and the *yw;* $Rac1^{J11}$ $Rac2^{\Delta}$ *FRT2A/TM6B* stock were utilized (Bloomington Stock Center, Indiana University, IN, #6675, 6674 and 6677 respectively).

Histology, immunohistochemistry and imaging

Late wandering third instar larval brains were dissected, fixed and incubated with the appropriate primary antibody according to a previously published protocol (Mukhopadhyay and Campos, 1995). In order to visualize photoreceptor axons, the mouse monoclonal antibody anti-CHAOPTIN (24B10, 1:100), which recognizes CHAOPTIN, a glycoprotein expressed specifically on the photoreceptor cell plasma membrane, was used (Van Vactor et al., 1988; Zipursky et al., 1984). 5-HT neurons were labeled using rabbit anti-serotonin (1:200) (Protos Biotech Corp., NY). Accordingly, the secondary antibodies used were Alexa 488-conjugated goat anti-mouse IgG (1:200) (Molecular Probes Inc., Eugene, OR) and Texas Red-conjugated goat anti-rabbit IgG (1:200) (Jackson InmunoResearch Laboratories, Inc., West Grove, PA). The specimens were viewed in a Nikon Eclipse ∈800 microscope. Confocal images were obtained with a Bio-Rad Radiance MRC 600 Krypton/Argon laser confocal microscope using the LaserSharp software. Each image consisted of *z*-stack of 2 to 25 sections approximately at 1 μm intervals, and they were adjusted for brightness and contrast using Adobe Photoshop 5.0 for Macintosh. In the case of cell ablation and neuronal silencing, all specimens were first analyzed blind.

Behavioral assays

Third instar foraging larvae at 84–90 h after egg laying (AEL) were harvested following the protocol described previously (Busto et al., 1999). Photobehavioral assays were carried out using a semi-automated assay system previously used in our laboratory (Busto et al., 1999; Hassan et al., 2005). Briefly, individual larvae were placed on a test arena of non-nutritive agar and were exposed to alternative 10-s pulses of light and dark for a total of at least 60 s. The tracking program controlled the periodicity of the light stimulus while allowing a stylus/tablet-based tracking of larval locomotion. The light stimulus was controlled by a serial device MacIO microcontroller (MacBrick, Netherlands) and by a relay to obtain a 10-s periodicity of the light pulse. At the end of each assay, the macro automatically calculated a response index, RI = [(total distance traveled in the dark period − total distance traveled in the light period) / total distance traveled in both the periods]. Since the response to light in this assay depends on the ability of the larva to move efficiently, larval locomotion in constant darkness was measured as a control.

Statistical analysis

Minitab 10.5 Xtra for Macintosh was used in the statistical analysis of samples. Statistical tests employed in the analysis of data included one-way analysis of variances (ANOVAs), Tukey–Kramer post-hoc multiple comparison tests and normality test on the residuals of ANOVA using Rootogram test.

Results

Both the Rh5- and the Rh6-expressing fibers overlap with the larval optic neuropil 5-HT arborization

The LON is formed by two groups of axons distinguished by the non-overlapping expression of Rh5 and Rh6 (Malpel et al., 2002). In late third instar larvae, the LON is found intimately associated with a 5-HT arborization in the larval optic center (Figs. 1A–C and Mukhopadhyay and Campos, 1995). However, whether both sets of photoreceptor cells are involved in this contact was not known. In order to address this question, brains dissected from wandering third instar larvae in which the Rh5-

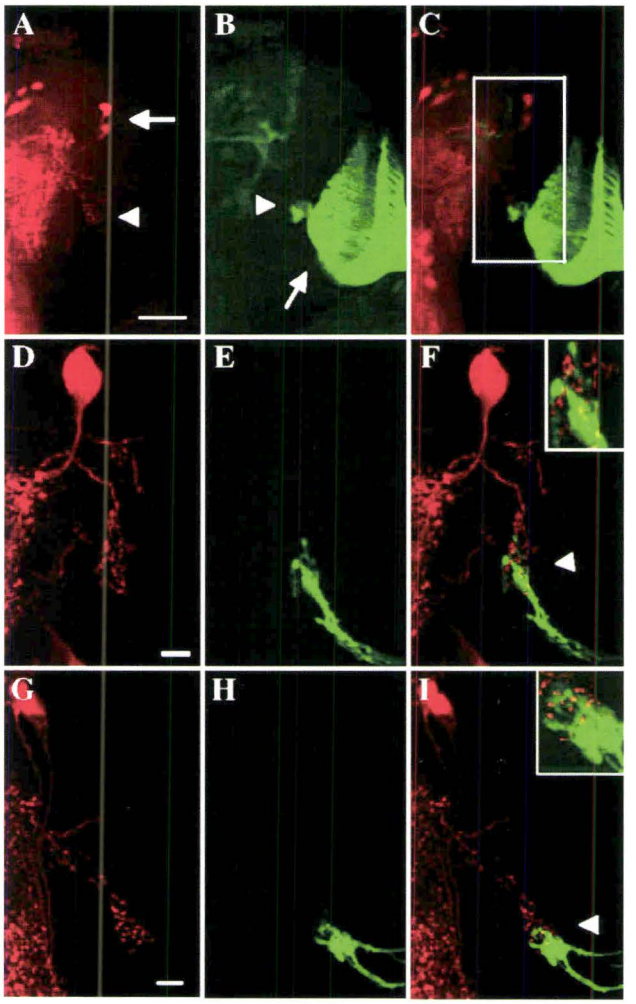

Fig. 1. The termini of Rh5 and Rh6 expressing photoreceptors overlap with a 5-HT arborization in the larval optic neuropil. (A–C) Low magnification confocal micrographs of a wild type wandering third instar larval hemisphere immunolabeled with anti-5-HT detected by Texas Red-conjugated secondary (red in panel A and in all subsequent panels and figures) and 24B10 monoclonal antibody detected by Alexa 488-conjugated secondary (B, green), showing the relationship between the LP1 serotonergic cell bodies (arrow in panel A), the serotonergic arborization in the larval optic center (arrowhead in panel A), the LON (arrowhead in panel B) and the developing adult retinal projection (arrow in panel B). (C) Merge of panels A and B. The box represents the area studied in all panels and subsequent figures. (D–I) High magnification confocal micrographs of GFP expression in Rh5 or Rh6 specific photoreceptors by targeted expression of the *UAS-CD8-GFP* construct using either the *Rh5-Gal4* or *Rh6-Gal4* drivers (D–F, *UAS-CD8-GFP; Rh5-Gal4*; G–I, *UAS-CD8-GFP; Rh6-Gal4*). (D) The LP1 cell bodies lie near the 5-HT arborization in the larval optic neuropil. (E) The termini of the Rh5-expressing photoreceptor axons in the same region. (F) Merge of panels D and E showing the overlapping between the 5-HT arborization and the Rh5 photoreceptors termini (arrowhead). The inset shows a higher magnification of this region. (G–I) Same as in panels D–F but showing the overlap of the Rh6 photoreceptor termini with the 5-HT arborization in the larval optic neuropil (arrowhead in panel I). Scale bar in panel A represents 40 μm and is valid for panels B and C. Scale bar in panels D and G represents 10 μm and is valid for panels E, F, H and I.

specific photoreceptors neurons express CD8-GFP under the regulation of the *Rh5* promoter (*yw; UAS-CD8-GFP; Rh5-GAL4*, $N = 18$) were labeled with 5-HT antibody (Figs. 1D–F). Similarly, aged specimens dissected from strains in which CD8-GFP was specifically expressed in Rh6 neurons were labeled with 5-HT (*w; UAS-CD8-GFP; Rh6-GAL4*, $N = 18$) (Figs. 1G–I). At the level of resolution afforded by the confocal microscope, while variable from specimen to specimen, the termini of either subset of photoreceptor axons did not display any distinguishing characteristics. Likewise, the degree of overlap between both subset of photoreceptor axons and the 5-HT arborization in the larval optic neuropil was apparently the same (Figs. 1F and I).

The 5-HT arborization is intimately associated with the dendritic tree of the LNv in the larval optic neuropil

It has been previously reported that the larval LNv contact both the Rh5- and the Rh6-expressing fibers and that this interaction with the LON provides trophic support for the development of the LNv dendritic tree (Malpel et al., 2002). We asked whether the LNv would also contact the serotonergic arborization in the larval optic center. To that end, larval brains in which the LNv were labeled by virtue of the targeted expression of CD8-GFP (*yw; PDF-GAL4, UAS-CD8-GFP*, $N = 11$) were treated with 5-HT antibody (Fig. 2). As shown in Fig. 2C, the 5-HT arborization was found in intimate association with the dendritic tree of the larval LNv (arrowhead).

The development of the serotonergic arborization does not depend upon the presence of the Rh5-expressing fibers or the larval LNv

In order to determine whether the Rh5-expressing fibers influence the development of the 5-HT arborization, we investigated the impact of absence of Rh5 fibers on the integrity of the serotonergic arborization as seen by anti 5-HT

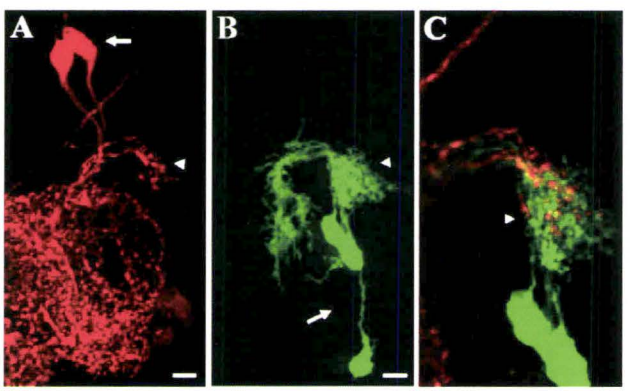

Fig. 2. The dendritic arborization of the LNv overlaps with the serotonergic innervation of the larval optic neuropil. Confocal micrographs of brains dissected from wandering third instar larvae (*PDF-Gal4, UAS-CD8-GFP*) in which GFP (green) expression is targeted to the main circadian pacemakers, the LNv, and labeled with anti 5-HT antibody (red) and detected as mentioned before. (A) LP1 serotonergic cells (arrow) and the larval optic neuropil 5-HT arborization (arrowhead). (B) LNv (arrow) and their dendritic tree (arrowhead) in the larval optic center. (C) Higher magnification merge of panels A and B, showing the overlap between the LNv dendrites and the 5-HT arborization. Scale bars: 10 μm.

immunolabeling. For this purpose, the Rh5-expressing photoreceptors were ablated by targeted expression of the cell death gene *hid* under the regulation of the *Rh5-GAL4* driver. In this and all subsequent experiments, cell ablation was assessed by the concomitant expression of the CD8-GFP reporter or by photoreceptor-specific protein CHAOPTIN staining (Zipursky et al., 1984). As shown in Fig. 3B, no obvious defect in the 5-HT arborization (arrowhead) is observed when the Rh5-expressing photoreceptors are absent (*yw; UAS-CD8-GFP/UAS-hid; Rh5-GAL4/+*, $N = 31$), suggesting that this subset of photoreceptor cells is not required for the proper innervation of the larval optic neuropil by the serotonergic arborization. A similar result was observed when the LNv were ablated due to targeted expression of *rpr* death gene by *PDF-GAL4*. In these specimens, the serotonergic arborization is indistinguishable from that of wild type samples, suggesting that it develops normally in the absence of the LNv (Fig. 3D, arrowhead) (*UAS-CD8-GFP, PDF-GAL4/UAS-rpr*, $N = 14$).

Rh6-expressing fibers are required for the development of the serotonergic arborization

In order to examine whether the Rh6-expressing fibers are required for normal development of the serotonergic arborization, the Rh6 cells and their axons were ablated by targeted expression of *hid* driven by the *Rh6-GAL4* element. No 5-HT labeling was detected in the larval optic neuropil area when the Rh6 fibers were absent (Fig. 4 compare A to C) (*UAS-CD8-GFP/UAS-hid; Rh6-GAL4/+*, $N = 26$). These results are indistinguishable from those obtained by the complete absence of the LON in *glass* mutants as previously reported by Mukhopadhyay and Campos (1995) or due to the expression of *hid* under the *glass multimer reporter (GMR)* promoter (Fig. 4B, $N = 16$). Thus, these observations suggest that, among the larval photoreceptors, the presence of the Rh6-expressing fibers is specifically required for the development of the serotonergic arborization.

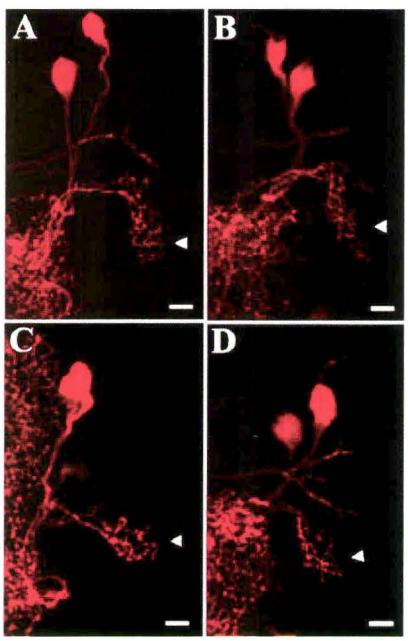

Fig. 3. Ablation of the Rh5-specific photoreceptors or LNv does not affect the development of the 5-HT arborization. The Rh5 photoreceptors or the LNv were ablated by targeted expression of the dead genes *hid* or *rpr* respectively, and the dissected brains were labeled with 5-HT antibody as described before. In all specimens, ablation was nearly complete as determined by the absence of expression of GFP in the targeted cells. (A) Parental strain (*UAS-CD8-GFP/+*; *Rh5-Gal4/+*) showing the stereotypical location of the 5-HT arborization (arrowhead). (B) *UAS-CD8-GFP/UAS-hid*; *Rh5-Gal4/+*. Ablation of Rh5 photoreceptors has no apparent effect in the development of the 5-HT arborization (arrowhead). (C) Parental strain (*PDF-Gal4, UAS-CD8-GFP/+*) showing the stereotypical location of the 5-HT arborization (arrowhead). (D) *PDF-Gal4, UAS-CD8-GFP/UAS-rpr*. Similar to what is observed for the Rh5 photoreceptors, the LNv are not required for the development of the 5-HT arborization (arrowhead). Scale bars: 10 μm.

Simultaneous ablation of Rh5 photoreceptors and LNv does not disrupt the development of the 5-HT arborization

The results presented above do not address whether Rh6 projection is sufficient for the development of the 5-HT arborization in the larval optic neuropil. In order to assess this, one would need to eliminate all other neurons known to project to the larval optic neuropil except the Rh6 photoreceptor cells. While the identity of all neurons that project to the larval optic center is not known, two other types of neurons, namely the Rh5 photoreceptors and the LNv, have been shown not to be required individually for the presence of 5-HT labeling in the larval optic center. Given that there are only 3–4 Rh5 axons and a similar number of LNv axons, it is possible that reduction of a putative trophic support provided by either Rh5 or LNv alone is not sufficient to impact the development of the 5-HT arborization. In order to address this question and to determine whether the requirement of Rh6 projection for the development of the 5-HT arborization in the larval optic neuropil is a feature unique of these neurons, we ablated Rh5 photoreceptors and LNv simultaneously by the targeted expression of *rpr* as described above. No obvious defect in the 5-HT arborization is observed when both the LNv and the Rh5-expressing fibers are absent (arrowhead in Fig. 5B) (*PDF-GAL4, UAS-CD8-GFP/UAS-rpr; Rh5-Gal4/+*, $N = 16$). Thus, these results further confirm that the larval LNv and Rh5-expressing fibers are not required for the normal development of the 5-HT arborization.

Absence of 5-HT arborization in LON ablated larvae is due to reduced branching and not lack of 5-HT expression

Disruption in the development of the serotonergic arborization as a consequence of afferent ablation is inferred by the absence of 5-HT immunolabeling in the larval optic neuropil. As such, these results do not distinguish between reduction in 5-HT synthesis and/or transport or impaired branching of these neurons. In order to distinguish between these alternatives, we sought to visualize these neurons and their projections by targeting the expression of GFP using a Gal4 driver regulated by the *Dopa decarboxylase* (*Ddc*) gene regulatory region (Li et al., 2000). In these larvae, serotonergic as well as dopaminergic neurons and their projections can be visualized

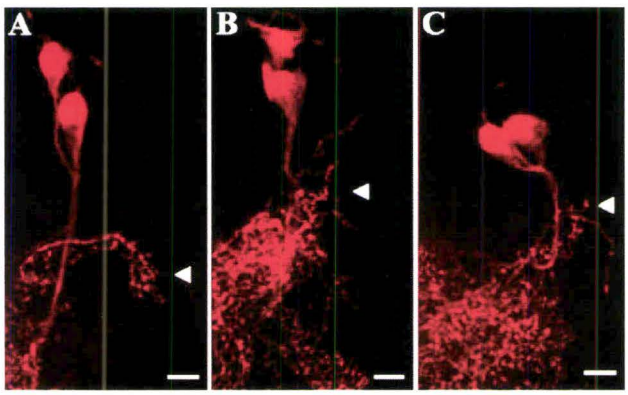

Fig. 4. The Rh6 photoreceptors are required for the proper development of the 5-HT arborization. (A) Wild type parental control (*UAS-CD8-GFP/+; Rh6-Gal4/+*) showing the stereotypical 5-HT innervation of the larval optic neuropil (arrowhead). (B) *GMR-hid*. Ablation of the larval eye by the targeted expression of the cell death gene *hid* in all photoreceptors causes a significant reduction in the 5-HT arborization (arrowhead). (C) *UAS-CD8-GFP/UAS-hid; Rh6-Gal4/+*. Ablation of all Rh6 photoreceptors by targeted expression of *hid* in these cells causes a similar reduction in the serotonergic arborization (arrowhead). Scale bars: 10 μm.

by virtue of GFP expression driven by the *Ddc* gene promoter. The GFP-expressing projection that corresponds to the serotonergic arborization in the larval optic neuropil can be identified due to its stereotypic position relative to other landmarks and its intimate association with the terminus of the larval optic nerve (Figs. 6A–C). Ablation of larval photoreceptors by expression of the cell death gene *hid* in these larvae appears to impair the branching of the 5-HT arborization as seen by the absence of GFP as well as 5-HT labeling (Figs. 6D–F). These results demonstrate that the LON provides a putative trophic signal required for the branching of this arborization rather than for the expression and localization of 5-HT.

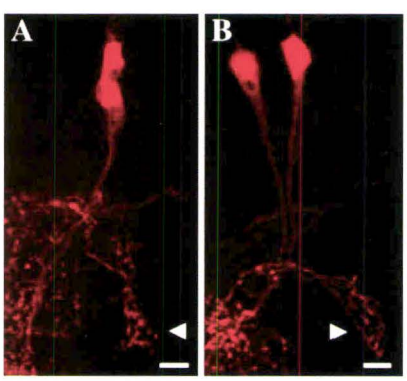

Fig. 5. Normal development of the larval optic neuropil 5-HT arborization in the absence of both the LNv and the Rh5-expressing fibers. Both A and B panels depict confocal micrographs of third instar larval brains labeled with anti 5-HT antibody and detected as before (red). (A) Brain dissected from parental strain (*PDF-Gal4, UAS-CD8-GFP/+; Rh5-Gal4/+*) showing the stereotypical location of the 5-HT arborization (arrowhead). (B) *PDF-Gal4, UAS-CD8-GFP/UAS-rpr; Rh5-Gal4/+*. The development of the 5-HT arborization is normal (arrowhead) in the absence of both the LNv and the Rh5 photoreceptors. Scale bars: 10 μm.

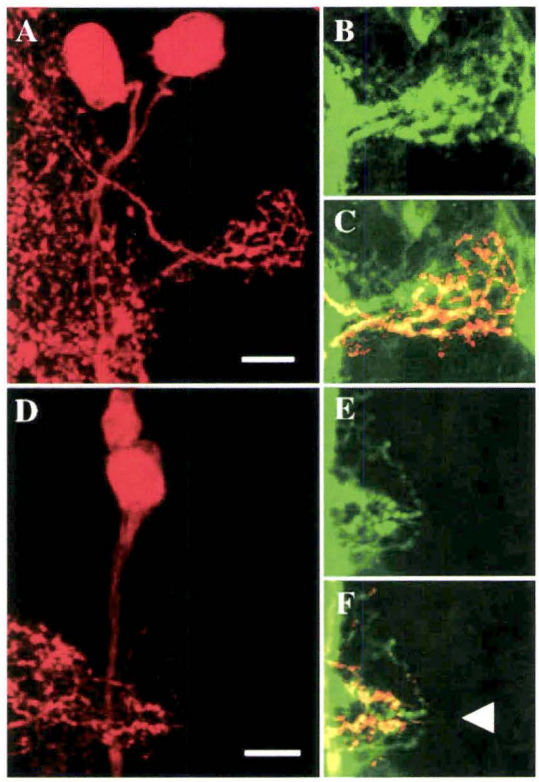

Fig. 6. Absence of 5-HT arborization in LON ablated larvae is due to reduced branching and not lack of 5-HT expression. In order to visualize the development of the serotonergic arborization independently from the expression of 5-HT, a *Ddc-Gal4* driver was used to target GFP expression to these neurons. Panels A–C depict the wild type parental control specimen (*Ddc-Gal4, UAS-CD8-GFP*) showing the expected 5-HT arborization in the larval optic neuropil (A) and Ddc-regulated GFP expression in the same structure (B and C). Ablation of all photoreceptors in these flies by introducing the *GMR-hid* construct (*GMR-hid; Ddc-Gal4, UAS-CD8-GFP*) dramatically reduces this arborization as seen by the lack of *Ddc*-driven GFP expression (D) as well as 5-HT staining (E and F) (arrowhead). Scale bars: 10 μm.

Suppression of synaptic activity in the Rh6-expressing fibers disrupts the branching of the 5-HT arborization

In an attempt to investigate whether synaptic activity of Rh6-expressing fibers may influence the development of the 5-HT arborization, a weak tetanus-toxin light chain allele (TNT-E) or a strong tetanus-toxin light chain allele (TNT-G) was expressed under the control of *Rh6-GAL4* driver. The TNT gene product cleaves synaptobrevin, thereby inhibiting synaptic vesicle docking (Sweeney et al., 1995). In CNS specimens dissected from larvae in which Rh6 photoreceptors expressed TNT-E, a blind analysis revealed that 37.9% of the lobes displayed a notable alteration in the branching of the 5-HT arborization (N = 56, data not shown). A more penetrant phenotype was observed when these cells expressed the stronger TNT-G allele. In these specimens, 79.3% of the lobes displayed a similar reduction of the 5-HT branching as seen when the less active form of TNT was expressed (Fig. 7D, N = 30). Comparable results were obtained when TNT-E or TNT-G were expressed under the control of general photoreceptor driver *GMR-GAL4*. In these specimens, 39.7% of the *GMR-Gal4xUAS-TNT-E* (N = 26; data not shown) and 72.7% of the

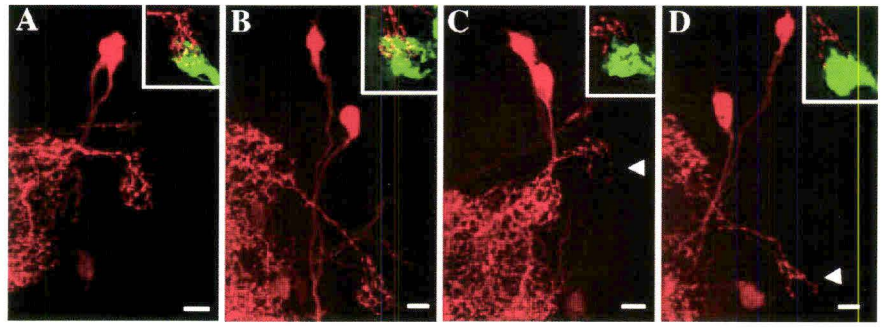

Fig. 7. Suppression of synaptic activity in the Rh6-expressing fibers disrupts the branching of the 5-HT arborization. In order to determine whether the induction of the 5-HT arborization by the larval Rh6 photoreceptor axons is an activity-dependent process, tetanus-toxin light chain (TNT) that suppresses synaptic activity was expressed in all larval photoreceptors or in the Rh6 photoreceptors only. The morphology of the Rh6 photoreceptor termini or the LON terminus was largely normal as evaluated by the concomitant expression of GFP or 24B10 monoclonal antibody staining detected by Alexa 488-conjugated secondary (green) respectively (insets). (A) Wild type parental control *GMR-Gal4/+*. (B) Control in which an inactive form of TNT is expressed in all photoreceptors (*GMR-Gal4/UAS-TNT-VIF*). (C) Expression of TNT-G in all photoreceptors (*GMR-Gal4/UAS-TNT-G*) reduces the extent of 5-HT arborization (arrowhead). (D) A similar phenotype is seen (arrowhead) when expression of TNT-G is restricted to Rh6 photoreceptors only (*UAS-CD8-GFP/UAS-TNT-G; Rh6-Gal4/+*). Scale bars: 10 μm.

GMR-Gal4xUAS-TNT-G lobes (Fig. 7C; $N = 22$) showed such 5-HT branching disruption. In all these cases, the larval photoreceptors developed normally as determined by the pattern of photoreceptor-specific protein CHAOPTIN staining (Zipursky et al., 1984) (Fig. 7, insets).

In order to dissect the role of electrical versus synaptic activity in the development of the serotonergic arborization, we expressed a genetically modified Shaker K^+ channel (the EKO channel) under the control of the *Rh6-GAL4* element. This K^+ channel attenuates electrical activity by being activated at potentials close to E_k and by remaining open (White et al., 2001). Confocal micrographs of third instar larval brains labeled with 5-HT antibody did not reveal any impact on the development of the 5-HT arborization due to the expression of the EKO channels in the Rh6 cells ($N = 27$; data not shown). A similar result was observed when the EKO channel was expressed in all larval photoreceptor cells through the use of the *GMR-Gal4* driver ($N = 14$; data not shown). It is possible that partial suppression of excitability was achieved by expression of these modified channels in these cells. However, behavioral assays carried out with third instar *GMR-GAL4/UAS-EKO* larvae revealed a significant reduction in the response to light compared to controls and as revealed by their mean RIs (*GMR-Gal4/UAS-EKO*, $N = 14$, RI = 0.11; *UAS-EKO/+*, $N = 13$, RI = 0.40; OR, $N = 16$, RI = 0.34; ANOVA: $F_{(2,40)} = 41.51$, $P < 0.001$). Taken together, these results suggest that the innervation of the larval optic neuropil by the 5-HT arborization does not depend on evoked synaptic activity of the Rh6-expressing fibers. Moreover, these results support the notion that spontaneous synaptic activity is sufficient to induce the branching of this serotonergic arborization.

Rac signaling is required for the branching of the 5-HT arborization

While the identity of the LON-derived signal is not known yet, results obtained in other model systems point to Rac GTPases as possible integrators in the activity-dependent development of the serotonergic arborization. Therefore, we investigated the integrity of this projection in larvae with reduced *Rac* function. To this end, we took advantage of a mutant chromosome carrying null mutations in two *Rac* genes (*Rac1* and *Rac2*) present in the *Drosophila* genome (Hakeda-Suzuki et al., 2002; Ng et al., 2002). Heterozygotes carrying at least one wild type copy of either one of the *Rac* genes survive until after the third instar larval stage. Moreover, organisms homozygous for just the *Rac2* null allele are viable. The level of reduction in Rac signaling afforded by these heteroallelic combinations did not cause any major developmental defect in the third instar larval brain as seen by the normal overall 5-HT staining (data not shown). Similarly, the larval photoreceptors developed appropriately as determined by the pattern of photoreceptor-specific protein CHAOPTIN staining (Zipursky et al., 1984) (Fig. 8, insets).

Therefore, we reasoned that the residual *Rac* function provided by one wild-type copy of *Rac1* or *Rac2* was sufficient

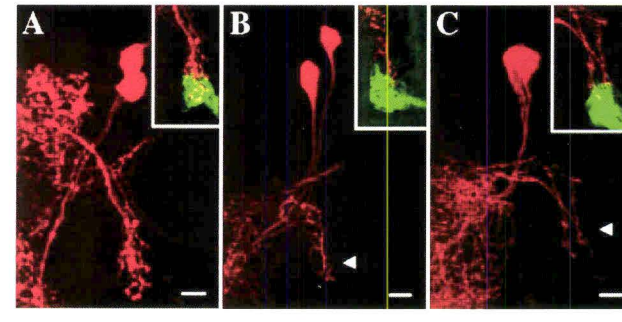

Fig. 8. Induction of 5-HT arborization by the larval optic nerve depends on Rac signaling. Brains from larvae in which Rac function was reduced by mutations in either one of the two Rac genes were dissected and processed for 5-HT labeling as before. The morphology of the larval optic nerve was normal as visualized using the photoreceptor specific 24B10 monoclonal antibody (green). The following panels depict representative confocal micrographs. (A) Wild type control *OR*. (B) *Rac1 Rac2/Rac2* mutant brain. (C) *Rac1 Rac2/Rac1* mutant brain. As seen in panels B and C (arrowheads), both mutants show a reduction in the branching of the serotonergic arborization. The insets in panels B and C show a higher magnification of the 5-HT arborization as its reduced overlap with the LON. Scale bars: 10 μm.

for most of the basic developmental processes required for the assembly of the larval circuitry that takes place earlier during embryogenesis. However, it was possible that inductive processes, such as that which takes place in the larval optic neuropil, may require Rac signaling which is above the level of that provided by the heteroallelic combinations as described before.

The integrity of the 5-HT arborization and the degree of overlap with the LON were analyzed in blind experiments. Fig. 8 depicts representative confocal micrographs of these specimens. Development of the 5-HT arborization was markedly reduced in 83% of CNSs dissected from larvae carrying only one functional copy of either *Rac1* ($N = 42$) or *Rac2* ($N = 22$) (Figs. 8B, C). While the degree of reduction of the 5-HT arborization was similar in all mutant combinations analyzed, the penetrance of this phenotype varied considerably. Marked disruption in the 5-HT arborization was seen in 37% of the *Rac2/Rac2* CNSs (data not shown). Interestingly, the reduction of the 5-HT arborization caused by reduced Rac function is similar to that found in larvae in which synaptic transmission was suppressed by the targeted expression of *TNT* (Fig. 7) and less than that observed when the entire LON was ablated (Fig. 4).

The overall integrity of the 5-HT system and the visual system morphology suggest that reduction of Rac function caused by these heteroallelic combinations does not have a pleiotropic effect on the nervous system development that would explain the disruption of the 5-HT arborization. Thus, although the present results do not provide a direct link between the LON-derived signal and Rac activation, they strongly suggest that a Rac-dependent signaling pathway is involved in the transduction of the signal provided by the LON for the development of this arborization.

Discussion

The dendritic arbor of the LNv and the termini of Rh5 and Rh6 photoreceptors overlap equally with the serotonergic arborization in the larval optic neuropil (Figs. 1 and 2). Ablation of two of these groups of neurons, the 3–4 Rh5 photoreceptors and the 4 LNv, did not disrupt the development of the 5-HT arborization (Figs. 3 and 5). However, ablation of Rh6-expressing fibers that originate from circa 8 retinular neurons had a drastic effect on the development of this 5-HT arborization (Fig. 4C). These observations are consistent with those found in *glass* (gl) mutants (Mukhopadhyay and Campos, 1995) and in *GMR-hid* larval brains (Fig. 4B) in which the LON is absent, suggesting that the Rh6 photoreceptors are uniquely required for the development of this serotonergic arborization.

The role of afferent activity in the development of postsynaptic partners is well documented in various systems (Miller and Kaplan, 2003; Sin et al., 2002; Van Aelst and Cline, 2004; Wong and Ghosh, 2002). Consistent with these observations is the finding that disruption of synaptic activity of the Rh6 fibers by targeted expression of tetanus-toxin light chain ("weak", TNT-E or "strong", TNT-G) caused reduction in the branching of the 5-HT arborization in the majority of specimens (Fig. 7D). Targeted expression of TNT completely eliminates evoked synaptic transmission and decreases spontaneous synaptic vesicle release by about 50% (Deitcher et al., 1998; Sweeney et al., 1995). Attenuation of electrical activity due to the expression, in Rh6 cells or in all photoreceptors, of the modified K^+ channel EKO did not have any effect on the development of the 5-HT arborization. It is possible that only partial suppression of excitability was achieved in these larvae, similar to what was observed for adult photoreceptors (White et al., 2001). However, behavioral assays carried out with third instar *GMR-GAL4/UAS-EKO* larvae revealed a significant reduction of the response to light compared to wild type larvae, suggesting that evoked potentials had been significantly suppressed. These observations are consistent with the finding that maintenance of larvae in the dark throughout development does not alter the morphology of the 5-HT projection in the larval optic neuropil (M. Mukhopadhyay and A.R. Campos, personal communication). Similarly, larvae carrying mutations in the *norpA* gene encoding the light-activated phospholipase C required for phototransduction did not reveal any disruption in the development of the 5-HT arborization (data not shown).

Taken together, these results suggest that proper branching of the 5-HT arborization in the larval optic neuropil does not depend on evoked synaptic activity of the Rh6-expressing fibers but may rely on spontaneous neurotransmitter release. Alternatively, expression of TNT disrupts this process independently of its effects on synaptic physiology. A requirement for synaptobrevin function for the proper expression of the neural cell adhesion molecule FasII has been reported in *Drosophila* (Baines et al., 2002; Hiesinger et al., 1999). Consistent with these observations is the demonstration that manipulation in the level of FasII expression mimics some aspects of the phenotypic consequences of synaptic suppression due to expression of TNT (Baines et al., 2002).

Thus, the partial disruption of the 5-HT arborization by targeted expression of TNT on the LON afferents, by comparison to the complete disruption observed when Rh6 photoreceptors are ablated, may be explained by either partial suppression of spontaneous neurotransmitter release or by a synaptic vesicle release-independent effect such as modification of FasII expression. Alternatively, Rh6 fibers may provide an activity-independent trophic support that potentiates and/or maintains activity-dependent processes, similar to what is found in other systems. For example, in the developing cortex Sema3A, neurotrophins and Slit interact to specify the basic morphology of cortical neurons. As development proceeds, the control of further growth and branching is shifted to activity-dependent mechanisms that rely on global and local increases of intracellular calcium (reviewed by Wong and Ghosh, 2002).

Our observations suggest a role for *Rac1* and/or *Rac2* signaling in the transduction of the signal provided by the LON (Fig. 8). These results are consistent with previous studies suggesting a requirement for the Rho family of small GTPases, notably, RhoA, Rac and Cdc42 in neuronal morphogenesis. For instance, Hakeda-Suzuki et al. (2002) and Ng et al. (2002) have

shown that *Drosophila* MB neurons mutant for *Rac1* and *Rac2* present defects in axon growth, guidance and branching. Interestingly, Rac has also been shown to be important for dendritic branching stability and morphogenesis of dendritic spines (reviewed in Govek et al., 2005; Luo, 2002; Van Aelst and Cline, 2004). For example, Lee et al. (2003) have shown that *Rac1* mutant *Drosophila* DA neurons developed fewer dendritic branches than wild type neurons in the third instar larval stage. Our observations are unique in *Drosophila* in that they suggest a role for Rac signaling in activity-dependent neuronal morphogenesis. Alternatively, Rac signaling may be required for the synaptobrevin-dependent developmental process discussed above and reported previously (Baines et al., 2002; Hiesinger et al., 1999).

Drosophila adults that have been reared in complete darkness since embryogenesis still display rhythmic behavior albeit not in synchrony with other individuals in the population (e.g. Sehgal et al., 1992). These observations support the notion that a circadian clock is assembled during embryogenesis and is functional as the larva hatches. Synchronization of the circadian clock or its entrainment can be achieved by light treatment as early as the end of the first instar, indicating that, in addition, photic input pathways are in place by the end of embryogenesis which are capable of resetting the pacemaker neurons (Malpel et al., 2004; Sehgal et al., 1992). Recent reports demonstrate a role for the larval visual system as a photic input pathway in entrainment during larval development (Malpel et al., 2004). Whether all or a subset of the 12 larval photoreceptors found in each of the two larval eyes are equally involved in this process has not yet been addressed. Similarly, it is not known whether the larval visual system functions as an entrainment input pathway equally throughout larval development. Relevant to these questions are our previous observations that indicate that Rh6-expressing photoreceptors are not involved in the basal response to light measured in our behavioral assay (Hassan et al., 2005). The results reported here demonstrate that these same photoreceptors (Rh6) are uniquely required for the induction of a 5-HT arborization that innervates the larval optic neuropil and that, it in turn, overlaps with the dendritic arborization of the main larval pacemaker neurons, the LNv. Thus, it is possible that the larval photoreceptor function, as a circadian input pathway, is modulated by their interaction with the 5-HT arborization that takes place during the third instar larval stage.

Several reports demonstrate the presence of circadian rhythms in the visual system of insects. Of note are the extensive analysis of Meinertzhagen and colleagues that established the existence in *Musca domestica* and *D. melanogaster* adults of circadian oscillation in the number of synapses between the outer photoreceptors (R1–R6) and the first order lamina interneurons L1 and L2 and in the diameter of L1 and L2 axons (Pyza and Meinertzhagen, 1993, 1995, 1999). These neuroanatomical changes are believed to be regulated by the neuromodulators 5-HT and pigment dispersing factor (PDF) present in two sets of neurons that innervate the optic lobe neuropiles of adult flies (Chen et al., 1999; Meinertzhagen and Pyza, 1996; Pyza and Meinertzhagen, 1996). This conclusion is supported by the observation that, in the *Caliphora* compound eyes, the circadian rhythmicity of the light-evoked response measured in electroretinograms (ERG) is affected by injection of 5-HT and PDF (Chen et al., 1999). A recent report detailing circadian oscillation in the larval response to light (Mazzoni et al., 2005) supports the hypothesis that the 5-HT arborization described here may be modulating larval visual system function.

Alternatively, the fact that 5-HT processes innervating the larval optic neuropil are found overlapping with the dendritic arborization of the LNv may suggest a direct modulation of the *Drosophila* larval pacemaker neurons. It has been shown that serotonin, in addition to its function in behavior, also plays a role in modulating circadian locomotor activity and heart rate in insects. For instance, it has reported that injection of the specific neurotoxin 5,7-DHT, which causes selective degeneration of serotonergic neurons, modified the level of locomotor activity and period of circadian rhythmicity in the blowfly (Cymborowski, 2003). Furthermore, it has been demonstrated that serotonin increases heart rate in *Drosophila* (Johnson et al., 1997, 2002), supporting the idea of a direct modulatory effect of this neurotransmitter on pacemaker cells.

Previous developmental analysis of the 5-HT arborization has shown that the contact between the LON and the serotonin process in the larval optic center occurs during late second–early third instar larval stage (Mukhopadhyay and Campos, 1995). After that, the 5-HT processes undergo further branching. These observations suggest that this serotonergic arborization may have a role in the function of the larval visual system during the third instar larval stage. Interestingly, it is towards the end of this stage that the larva becomes progressively less photophobic, attaining photo neutrality just before pupariation (Sawin-McCormack et al., 1995). Moreover, it has been shown that 5-HT is able to modulate the voltage dependency of K^+ channels in *Drosophila* adult photoreceptors (Hevers and Hardie, 1995; Kauranen and Weckstrom, 2004). Hence, it is possible that the innervation of the larval optic center by this 5-HT arborization plays a role in the modulation of the photobehavior that occurs during the foraging–wandering transition (Sawin-McCormack et al., 1995).

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APPENDIX C: Manuscript

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Photic Input Pathways That Mediate the *Drosophila* Larval Response to Light and Circadian Rhythmicity Are Developmentally Related but Functionally Distinct

JANA HASSAN, BALAJI IYENGAR, NADIA SCANTLEBURY, VERONICA RODRIGUEZ MONCALVO, AND ANA REGINA CAMPOS*
Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

ABSTRACT

The *Drosophila melanogaster* larval photosensory organ that mediates the response to light consists of bilaterally symmetrical clusters of 12 photoreceptors. These are distinguished on the basis of expression of the rhodopsins Rh5 and Rh6. The Rh6-expressing cells correspond to the Hofbauer-Buchner (H-B) eyelet found later in the posterior margin of the adult compound eye and recently shown to function as an input pathway in the entrainment of circadian rhythmicity in adult *Drosophila*. In addition, the axons of the larval photoreceptors are found in intimate association with a subset of the main circadian pacemaker neurons located in the developing accessory medulla, the small ventral lateral neurons (LNv). The observed spatial overlap between components of the circadian circuitry, input pathway, and pacemaker neurons—and the larval visual organ—suggest a functional relationship between these two photosensory input pathways. In this study we determined the requirement of specific rhodopsin-expressing photoreceptors including the presumptive H-B eyelet and pacemaker neurons in the larval locomotory response to visual stimuli. Our results demonstrate that two of the most important components of the neuronal circuitry underlying circadian rhythmicity in *Drosophila*, namely, the extraretinal H-B cluster and the circadian pacemakers, while in intimate association with the larval visual system are not required for the larval motor response to light. J. Comp. Neurol. 481:266–275, 2005. © 2004 Wiley-Liss, Inc.

Indexing terms: rhodopsin; behavior; *Drosophila*

A comparison between the organization of the *Drosophila melanogaster* adult compound eye and the simpler larval visual system suggest that the latter may represent a specific subset of the adult retina. In the adult compound eye each of the 750 ommatidia contain eight photoreceptors: six outer photoreceptors (R1–R6) and two inner photoreceptors (R7 and R8) (reviewed in Ready, 1993). The outer photoreceptors express the blue-green-sensitive rhodopsin 1 (Rh1) as well as a UV-sensitive photostable pigment that transfers energy to Rh1 (Kirschfeld and Franceschini, 1977a,b; reviewed by Hardie, 1985), while the inner photoreceptors express rhodopsin 3 (Rh3), rhodopsin 4 (Rh4), or rhodopsin 5 (Rh5). The pattern of rhodopsin expression in the two inner photoreceptors subdivides the ommatidia into two major classes: pale (P) and yellow (Y). The P subtype comprises 30% of the ommatidia and express Rh3 in R7 cells and Rh5 in R8 cells. The remaining 70%, comprising the Y subtype, express Rh4 in R7 and Rh6 in R8. Rh3 and Rh4 are UV-sensitive, while Rh5 (λ_{max} = 437 nm) and Rh6 (λ_{max} = 508 nm) are blue and green, respectively (Chou et al., 1996, 1999; Papatsenko et al., 1997; Salcedo et al., 1999). Therefore, the inner pho-

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Dr. Balaji Iyengar's current address is Department of Physiology, University of Toronto, Ontario M5S 1A8, Canada.

*Correspondence to: Ana Regina Campos, Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON L8S 4K1, Canada. E-mail: camposa@mcmaster.ca

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toreceptors detect a broader range of wavelengths and have been proposed to house the *Drosophila* color discrimination system (Pichaud et al., 1999), while retinal cells R1 through R6 represent a high sensitivity system required for motion detection (Heisenberg and Buchner, 1977; reviewed by Hardie, 1985). A third, less prevalent class of ommatidia containing UV-sensitive Rh3 in both R7 and R8 inner photoreceptors is located in the dorsal rim area of the compound eye and has been proposed to detect skylight polarization (Hardie, 1985; Wolf et al., 1980).

The visual system that mediates the *Drosophila* larva's response to light, the so-called Bolwig's organ, consists of bilaterally symmetrical clusters of 12 photoreceptors that send their axonal projections to the optic lobe primordium in the brain hemispheres (Steller et al., 1987; Busto et al., 1999; Campos et al., 1995; Hassan et al., 2000). This structure mediates all response to light detected in the laboratory as seen by the lack of response of larvae mutant for the *glass (gl)* gene (Busto et al., 1999; Hassan et al., 2000). Rhodopsins Rh5 and Rh6 are expressed in nonoverlapping sets of larval retinular cells, suggesting that these photoreceptors are most similar to the adult R8 receptors (Malpel et al., 2002).

The larval response to light has been assayed in a variety of paradigms (Gordesky-Gold et al., 1995; Lilly and Carlson, 1990; Busto et al., 1999; Hassan et al., 2000). In the ON/OFF assay used in this report to study larval motor response to light, an individual larva is subjected to intermittent pulses of light and dark (10 seconds) and its behavior is recorded. The path recording of the larval movement is used to measure various locomotory parameters during the light and dark pulses, such as path length, change of direction at the transition between the light and dark pulses, and head-swinging behavior (Busto et al., 1999). Analysis of these behaviors in wildtype strains demonstrated that light modulates locomotion as seen by the reduction in the distance traveled and marked increase in head-swinging behavior that occurs during the light pulse. The response to light detected in the ON/OFF assay has been shown to require the larval visual system and the same phototransduction cascade required for adult visual system function (Busto et al., 1999). Interestingly, the response to light detected in a previously used population assay and in the ON/OFF assay is abolished upon the transition from the foraging to the wandering period of the third larval instar when the larva leaves the food substrate to search for a site to undergo metamorphosis (Sawin-McCormack et al., 1995; Iyengar and Campos, unpubl. results). This behavioral transition correlates with the appearance of a serotonergic arborization overlapping the larval optic nerve terminus in the optic neuropil (Mukhopadhyay and Campos, 1995). At the level of resolution afforded by confocal microscopy, no changes in the terminus of the photoreceptor axons were detected during the transition from foraging to wandering (Moncalvo and Campos, unpubl. results).

In addition to a visual system involved in the perception and analysis of the spatial world, animals also rely on a specialized form of visual function for the entrainment of circadian rhythms. In *Drosophila* adults, an extraretinal cluster of photoreceptor cells was described lying in the posterior margin of the compound eye (Hofbauer and Buchner, 1989). This structure, named the Hofbauer-Buchner (H-B) eyelet, has recently been examined in more detail, regarding its pattern of projection, developmental origin, and role in circadian rhythmicity (Helfrich-Forster et al., 2001, 2002; Yasuyama and Meinertzhagen, 1999). In the adult stage these photoreceptors project to the main circadian pacemaker neurons, the ventral lateral neurons (LNv), and express Rh6 exclusively (Helfrich-Forster et al., 2002). Interestingly, these photoreceptors have been shown to be already present in the larva and represent the Rh6-expressing photoreceptor of the Bolwig's organ or larval visual system (Helfrich-Forster et al., 2002).

Taken together, these observations suggest a developmental and spatial overlap between components of the circadian circuitry and the larval visual organ. They also suggest functional redundancy between these two photosensory pathways. Here, we address the question of whether there is a functional overlap between the pathways involved in light detection and circadian rhythmicity by determining the requirement of specific Rhodopsin-expressing photoreceptors, which includes the presumptive H-B eyelet, and the circadian pacemakers, in the modulation of locomotion by light in the *Drosophila* larva.

MATERIALS AND METHODS

Fly stocks

All *D. melanogaster* stocks were raised at 25°C in standard medium containing inactivated yeast, sucrose, agar, 10% tegosept in ethanol and acid mix (phosphoric acid and propionic acid) supplemented with vitamin A. We used the *y w; P[PDF-Gal4]/CyO* (kindly provided by Paul Taghert), *w*; P[UAS-mCD8:GFP]* (*Drosophila* Stock Center, Bloomington, IN), strains to recombine both transgenes onto the same second chromosome, thus resulting in *y w; P[PDF-Gal4], P[UAS-mCD8:GFP]; +/+*. The *Rh6-Gal4* and *Rh5-Gal4* transgenic strains (kindly provided by Claude Desplan) allowed expression of the cell death gene *head involution defective (hid)*, the reporter *β-Galactosidase (lacZ)*, and of the tetanus neurotoxin light chain (*TNT*) specifically in Rh6 and Rh5-expressing larval photoreceptors cells, respectively (Pichaud and Desplan, 2001; Malpel et al., 2002) and were thus used to ablate or silence Rh5 or Rh6 cells independently. The converse experiment, rescue of Rh5 or Rh6 cells in a *gl* mutant background, is not possible as *gl* transcription factor is required for the development of all photoreceptor cells. Two forms of *TNT* were used, the active (*TNTE* and *TNTG*) and inactive control (*IMPTNTQ4A*) (Sweeney et al., 1995). Standard wildtype stocks Canton-S (CS) and Oregon-R (OR) were used. In addition, the following *Drosophila* stocks were used: *+/+;P[UAS-rpr]/P[UAS-rpr]* (White et al., 1996) *y, w; P[UAS-hid]/P[UAS-hid]* (Grether et al., 1995), *w; P[UAS-TNT]* (Sweeney et al., 1995); *+/+, P[UAS-τ:lacZ]/P[UAS-τ:lacZ]* (*Drosophila* Stock Center).

Behavioral Assays

Third instar foraging larvae at ~84–90 hours after egg laying (AEL) were harvested following the protocol described previously (Busto et al., 1999). Photobehavioral assays were carried out using a semi-automated assay system previously reported (Busto et al., 1999; Leventis et al., 2001). Briefly, individual larvae were placed on a test arena of non-nutritive agar and were exposed to alternative pulses of light and dark (10 seconds each) for a total of at least 1 minute. The tracking program controlled the periodicity of the light stimulus while allowing a stylus/tablet-based tracking of larval locomotion. The light stim-

ulus was gated by controlling a serial device MacIO microcontroller (MacBrick, Netherlands) and a relay to obtain a 10-second periodicity of the light pulse. At the end of each assay, the macro automatically calculated a response index, RI = [(total distance traveled in the dark period–total distance traveled in the light period)/total distance traveled in both the periods]. The head-swinging behavior was measured from video recordings as previously described (Busto et al., 1999). Because the response to light in this assay depends on the ability of the larva to move efficiently, larval locomotion in the absence of light transitions, i.e., constant dark, was measured for all genotypes as a control. In all genotypes locomotion in the absence of light was not significantly different from control wildtype and parental stocks (Hassan, M.Sc. thesis, 2000, data not shown).

Statistical analysis

Minitab 10.5 Xtra for Macintosh and Microsoft Excel software were used in the statistical analysis of samples. Statistical tests employed in the analysis of data included normality tests, one-way analysis of variances (ANOVAs), and Tukey-Kramer post-hoc multiple comparison tests. In the statistical analysis of Response Indices, all strains were compared to wildtype or parental control strains, thus involving between strain analysis. Head-swinging behavior was analyzed for each strain and comparison of each individual strain in light versus dark conditions was carried out. A normality test was conducted on the residuals of ANOVA using Rootogram test (Minitab 10.5 for Macintosh). For datasets that were not normal, a Mann-Whitney U-Test was applied to determine significance. Unpaired Student's *t*-test was employed for comparisons of mutant genotypes with their respective controls to analyze the role of lateral neurons in the larval visual-motor behavior in the ON/OFF assay.

Histology

Early foraging and late wandering third instar larval brains were dissected, fixed, and incubated with primary 24B10 monoclonal antibody that recognizes Chaoptin, a photoreceptor-specific cell adhesion molecule (Van Vactor et al., 1988; Zipursky et al., 1984) according to a previously published protocol (Mukhopadhyay and Campos, 1995). Alternatively, the monoclonal antibody 22C10 that recognizes the microtubule binding protein Futsch was used (Fujita et al., 1982; Hummel et al., 2000; Zipursky et al., 1984). It labels all sensory neurons and a subset of central nervous system neurons. The secondary antibody used was either HRP-conjugated goat antimouse antibody or Cy3 or Texas Red conjugated goat antimouse antibody (Jackson ImmunoResearch, West Grove, PA). Visualization of the LNv was achieved using the recombinant strain PDF-Gal4, UAS-mCD8:GFP that expressed green fluorescent protein specifically in these neurons (Renn et al., 1999). All specimens were viewed using Zeiss Axioskop compound microscope or Bio-Rad MRC 600 Krypton/Argon laser confocal microscopy. Images were exported as Tiff files. All photomicrographs were produced using Adobe PhotoShop 5.0.

RESULTS

Subset of the larval photoreceptors is required for the larval response to light

Previous experiments using mutations in the *gl* (gl^{60j}) gene and ablation of all larval photoreceptors using the cell death gene *hid* regulated by the *gl* gene promoter demonstrated that all retinular cells were required for the responses to light detected in the ON/OFF assay. Additionally, it has been reported that foraging larvae are repelled by near UV, blue, and green wavelengths of the visual spectrum (Warrick et al., 1999). The presence of Rh5 and Rh6 rhodopsins in the larval visual system supports these earlier results, suggesting that larval vision may be maximally sensitive to blue and green wavelengths, respectively (Malpel et al., 2002).

We asked whether photoreceptors expressing the blue wavelength-sensitive Rh5 rhodopsin or the green wavelength-sensitive Rh6 are equally important for mediating the *Drosophila* larval response to light. To that end, we ablated either Rh5 or Rh6-positive neurons using the Gal4/UAS binary control system to target the expression of the cell death gene *hid* (Grether et al., 1995). Parallel experiments in which *hid* and a reporter construct (*UAS-mCD8:GFP*) were expressed under the control of the same driver elements (*Rh5-Gal4* or *Rh6 Gal4*) demonstrated that expression of *hid* in larval photoreceptors caused complete ablation in all samples examined (*Rh5-Gal4; UAS-hid* n = 31 and *Rh6-Gal4; UAS-hid* n = 26).

Ablation of Rh5-specific photoreceptors by the targeted expression of the cell death gene *hid* caused a severe reduction in response to light as measured in the ON/OFF assay (Fig. 1). In contrast, the *Rh6-Gal4*-mediated expression of *UAS-hid* did not affect larval response to light (Fig. 1). Similar results were obtained when tetanus neurotoxin light chain (*TNT*) was selectively expressed in Rh5 or Rh6 photoreceptors. The *TNTE* (active form) gene product abrogates synaptic transmission by cleaving Synaptobrevin, which is an essential component of synaptic vesicle exocytosis (Sweeney et al., 1995). We observed a similar loss in response only when the larvae expressed *TNTE* in Rh5-specific photoreceptors but not in Rh6 receptors (Fig. 2). In all genotypes locomotion in the absence of light was not significantly different from control wildtype and parental stocks, indicating that low response in this assay is not due to reduced locomotion (Hassan, M.Sc. thesis, 2000, data not shown).

In the adult visual system outer photoreceptors differ from inner photoreceptors with respect to the type of response to light they mediate. The outer R1-R6 photoreceptors are required for motion control in the adult (Heisenberg and Buchner, 1977), while the inner photoreceptors are thought to play a role in phototaxis (Gerresheim, 1988) and house the putative color discrimination system (Pichaud et al., 1999). We speculated that the Rh5 and Rh6 photoreceptors present in the larval visual system might be similarly distinguished. Therefore, we investigated the role of these photoreceptors in another kind of motor response to light that can be measured in our assays, the increase in head swinging in the presence of light.

We found that head-swinging behavior in response to light stimulation is severely reduced in larvae lacking Rh5 photoreceptors (Fig. 3). Silencing the Rh5 photoreceptors through expression of *TNTE* similarly abrogates head swinging (Fig. 4). Ablation or inactivation of Rh6-expressing photoreceptors, on the other hand, does not affect larval head swinging in response to light stimulation (Figs. 3, 4).

We concluded that the larval response to light as measured in these two behavioral paradigms is solely medi-

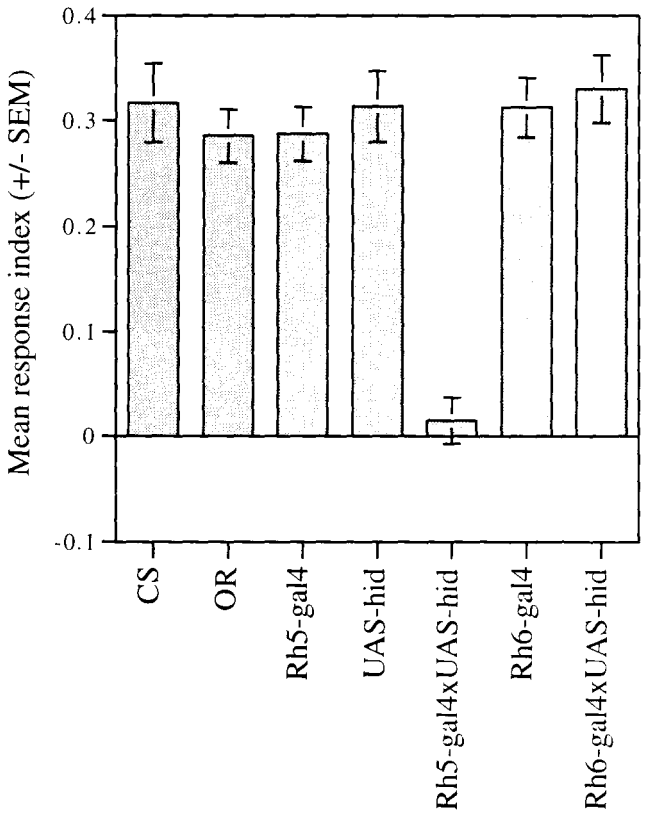

Fig. 1. Targeted expression of the cell death gene *hid* in Rh5-specific photoreceptors but not Rh6 disrupts the larval response to light. The ON/OFF assay measures the relative distance traveled by a larva in the presence and in the absence of light. A response index (RI) was derived for each larva and a mean RI was then determined for each genotype. The mean RIs for the wildtype strains (*OR*, n = 20; *CS*, n = 20), parental strains (*Rh5-Gal4*, n = 30; *UAS-hid* n = 17), and the strains in which *hid* is targeted to Rh5-expressing cells (*Rh5-Gal4xUAS-hid*, n = 25) are significantly different (ANOVA: $F_{(4,107)} = 22.00$, $P < 0.001$). In strains in which cell death gene *hid* is targeted to Rh6-expressing cells (*Rh6-Gal4xUAS-hid*, n = 15, ANOVA: $F_{(4,91)} = 0.25$, $P = 0.910$), the response to light is not affected as compared to wildtype (*OR*, n = 20; *CS*, n = 20) or parental strains (*Rh6-Gal4*, n = 24; *UAS-hid*, n = 17). We conclude that integrity of the Rh5-expressing photoreceptors is required and sufficient to mediate the response to light in the ON/OFF Assay.

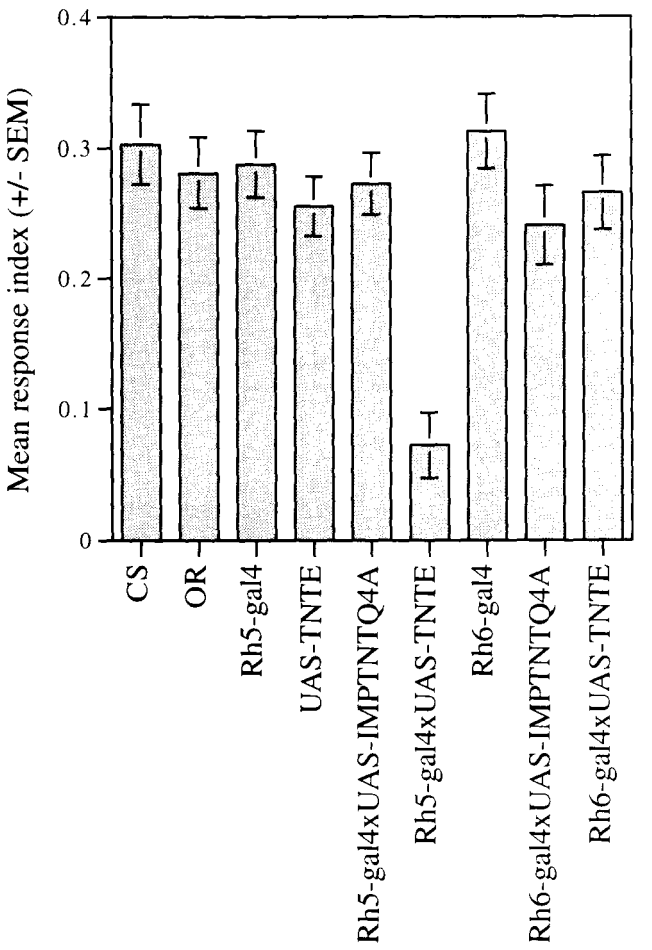

Fig. 2. Targeted expression of *TNT* in Rh5-specific photoreceptors but not Rh6 disrupts the larval response to light. The mean RIs for the wildtype strains (*OR*, n = 20; *CS*, n = 20), parental strains (*Rh5-Gal4*, n = 30; *UAS-TNTE*, n = 20), control strain in which an inactive form of *TNT* is expressed in Rh5 cells (*Rh5-Gal4xUAS-IMPTNTQ4A*, n = 20) are significantly different from that obtained for the larvae in which the active form of *TNT* has been targeted to Rh5-expressing cells (*Rh5-Gal4xUAS-TNTE*, n = 20, ANOVA: $F_{(5,124)} = 10.26$, $P < 0.001$). In the strain in which the active form of *TNT* is targeted to Rh6-expressing cells (*Rh6-Gal4xUAS-TNTE*, n = 20) the response to light is not significantly different from that of wildtype strains (*OR*, n = 20; *CS*, n = 20), parental strains (*Rh6-Gal4*, n = 24; *UAS-TNTE*, n = 20), or the control strain (*Rh6-Gal4xUAS-IMPTNTQ4A*, n = 20, ANOVA $F_{(5,118)} = 1.01$, $P = 0.417$). The observations demonstrate that synaptic transmission in Rh5 photoreceptors is required and sufficient for the larval response to light measured in this assay.

ated by the Rh5 photoreceptors. Rh5-expressing photoreceptors represent a small subset of the photoreceptor neurons (3–4 cells) present in the larval eye (Malpel et al., 2002). These observations demonstrate that the Rh6 neurons comprising the majority of the photoreceptors in the larval visual system are dispensable for larval response to light measured in the ON/OFF assay.

Ablation or functional impairment of Rh5 or Rh6 photoreceptors does not prevent the proper projection of the remaining photoreceptor axons

It is conceivable that Rh5-expressing photoreceptors in the larva may play a role in the development of Rh6-expressing neurons. Thus, in this scenario the ablation of Rh5 cells may result in a nonautonomous behavioral phenotype due to the additional disruption in the development of the Rh6 neurons. In order to address this question we investigated the morphology of the Rh6-expressing photoreceptor projection in larvae whose Rh5-expressing cells were ablated.

As expected in specimens lacking Rh6 neurons, the number of photoreceptor axons were noticeably reduced (Fig. 5, compare panels A to B and D to E). This is not the case when Rh5 neurons were ablated, given that those represent only ~4 out of 12 photoreceptor neurons (Fig. 5, compare panel A to C). Most importantly, the projection pattern of the remaining axons in either case was indistinguishable from wildtype. We concluded that the behav-

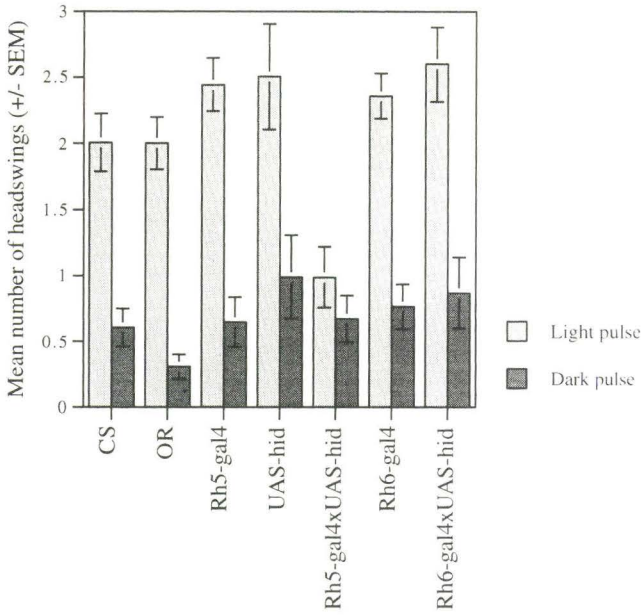

Fig. 3. Modulation of head-swinging behavior is disrupted by targeted expression of the cell death gene *hid* to Rh5 but not Rh6-expressing cells. The number of head swings (HS) were counted in the light and in the dark for each larva and mean HS in both conditions was determined for each genotype. Wildtype larvae (*OR*, n = 18; *CS*, n = 18) exhibit a significant increase in HS during light pulses relative to that during dark pulses (ANOVA: *OR* $F_{(1,34)} = 61.08$, $P < 0.001$; Mann-Whitney U: *CS* $P < 0.001$). Increased HS in light vs. dark pulses is also observed for parental strains (*Rh5-Gal4*, n = 26; *UASg-hid*, n = 17) (ANOVA: *Rh5-Gal4* $F_{(1,50)} = 42.26$, $P < 0.001$; Mann-Whitney U: *UASg-hid*, $P = 0.008$). When *hid* is targeted to Rh5-expressing cells (*Rh5-Gal4xUASg-hid*, n = 20) the larval response to light, as measured by head-swinging behavior, is abolished (Mann-Whitney U: *Rh5-Gal4xUASg-hid*, $P = 0.355$). A significant difference in HS in light and dark pulses is also observed for parental strain (*Rh6-Gal4*, n = 23) (ANOVA *Rh6-Gal4* $F_{(1,44)} = 43.12$, $P < 0.001$; Mann-Whitney U: *UASg-hid*, $P = 0.008$). However, in larvae in which *hid* is targeted to Rh6-expressing cells (*Rh6-Gal4xUASg-hid*, n = 8) the response to light as measured by increased HS in light, is normal (ANOVA: Rh6-*Gal4xUASg-hid*, $F_{(1,14)} = 19.66$, $P = 0.001$).

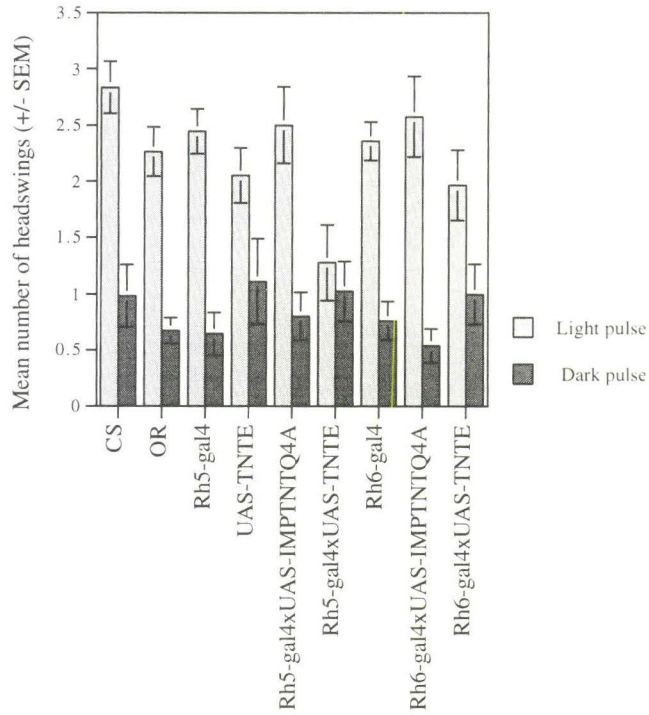

Fig. 4. Targeted expression of *TNT* to Rh5 but not Rh6-expressing cells disrupts modulation of head-swinging behavior. Wildtype larvae (*OR*, n = 17; *CS*, n = 20) exhibit an increase in HS during light pulses relative to that during dark pulses (ANOVA: *OR* $F_{(1,32)} = 40.40$, $P < 0.001$; Mann-Whitney U: *CS*, $P < 0.001$). Significant differences in HS in light and dark pulses are also observed for parental (*Rh5-Gal4*, n = 26; *UAS-TNTE*, n = 19 ANOVA: *Rh5-Gal4* $F_{(1,50)} = 42.26$, $P < 0.001$; Mann-Whitney U: *UAS-TNTE*, $P < 0.001$) and control strains (*Rh5-Gal4xUAS-IMPTNTQ4A*, n = 17 ANOVA: *Rh5-Gal4xUAS-IMPTNTQ4A* $F_{(1,32)} = 17.87$, $P < 0.001$. Increased HS in the dark vs. the light is abolished when the active form of *TNT* is targeted to *Rh5*-expressing cells. (*Rh5-Gal4xUAS-TNTE*, n = 20) (Mann-Whitney U: *Rh5-Gal4xUAS-TNTE*, $P = 0.821$). A significant difference in HS in light and dark pulses is also observed for the parental strain (*Rh6-Gal4*, n = 23) (ANOVA: *Rh6-Gal4* $F_{(1,44)} = 43.12$, $P < 0.001$) the control strain (*Rh6-Gal4xUAS-IMPTNTQ4A*, n = 15) (ANOVA: *Rh6-Gal4xUAS-IMPTNTQ4A* $F_{(1,16)} = 9.99$, $P = 0.006$) and the strain in which the active form of *TNT* is targeted to Rh6-expressing cells (*Rh6-Gal4xUAS-TNTE* n = 17). There is no significant reduction in the larval response to light, as measured by head-swinging behavior in the strain in which the active form of *TNT* is targeted to Rh6-expressing cells (Mann-Whitney U: Rh6-*Gal4xUAS-TNTE* $F_{(1,14)} = 5.08$, $P = 0.041$).

ioral consequence of ablating Rh5 neurons is not due to a nonautonomous effect on the development of Rh6 cells.

Circadian pacemaker neurons contacted by the larval optic nerve do not play a role in the larval response to light

The larval photoreceptor axons appear to contact the pacemaker neurons, LNv, in the brain (Fig. 6B) (Helfrich-Forster, 1997; Helfrich-Forster et al., 2002; Kaneko et al., 1997; Malpel et al., 2002). These neurons are essential for the maintenance and entrainment of circadian rhythmicity (Blanchardon et al., 2001; Renn et al., 1999). There are two sets of LNv in the adult brain, the small Lnv, already present in the larva and the object of the present investigation, and the large LNv, found only in adults. In the third instar larva 4 of the 5 small LNv are identified by the expression of pigment-dispersing factor (PDF) (Helfrich-Forster, 1997; Park and Hall, 1998; Renn et al., 1999). In strains that lack the larval visual system due to the targeted expression of Hid in all photoreceptors (*GMR-Hid*) the dendritic arborization of the small LNv is severely reduced (Malpel et al., 2002). These observations suggest that in the larva these pacemaker neurons may mediate light-triggered motor behaviors in addition to their role as a photic input pathway for circadian rhythmicity. In order to test this hypothesis we asked whether integrity of the LNv is required for larval response to light as determined by the ON/OFF assay.

We used strains carrying a *PDF* promoter fused to Gal4 to target the expression of the cell death gene *rpr* for ablation and *TNT* for silencing of pacemaker neurons (Renn et al., 1999). The inclusion of the *UAS-mCD8:GFP* element into the *PDF-Gal4* containing chromosome allowed the morphological examination of the targeted neurons. There are eight PDF-expressing neurons per larval brain, four in each hemisphere. The cell bodies are located

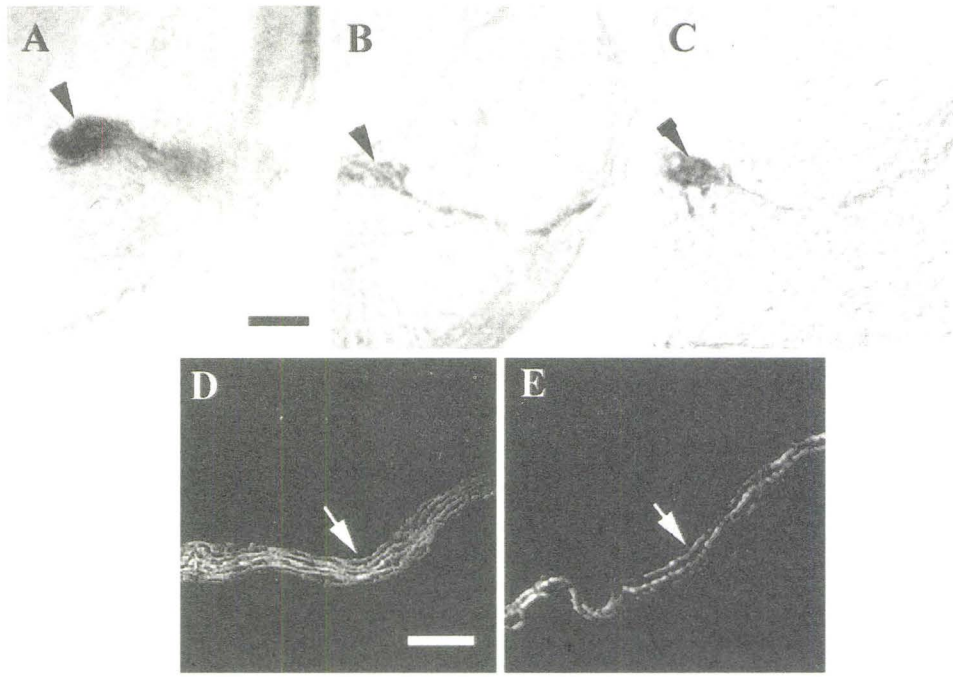

Fig. 5. Ablation of either Rh5 or Rh6-expressing cells does not affect the projection pattern of the remaining photoreceptor neurons. **A–C:** Light photomicrographs of larval brains dissected from third instar foraging larvae and immunolabeled with the photoreceptor-specific 24B10 monoclonal antibody and viewed under Normarski microscopy. **D,E:** High-magnification confocal micrograph of the larval optic nerve immunolabeled with the monoclonal antibody 22C10. Ablation of Rh-specific photoreceptors was achieved by the targeted expression of cell death gene *hid*. Wildtype (A); *Rh6-Gal4; UASg-hid* (B) (n = 25); *Rh5-Gal4; UAS-hid* (C) (n = 20); wildtype larval optic nerve (D); *Rh6-Gal4; UAS-hid* larval optic nerve (E) (n = 10). Arrowheads point to the terminus of the larval optic nerve (A,B). Arrows indicate individual axons (E,F). A–C were shot at the same magnification. D,E were shot at the same magnification. Scale bars = 20 μm in A; 10 μm in D.

medially in the brain hemispheres and send their projections posteriorly and dorsally. The dendritic arborization of the lateral neurons contact the terminus of the larval optic nerve (Fig. 6A,B) (Malpel et al., 2002). Larvae in which the pacemaker neurons expressed *rpr* were tested in the ON/OFF behavioral assay prior to morphological analysis. After the completion of the assay each larva was examined for the integrity of the LNv by virtue of the concomitant expression of the *UAS-mCD8:GFP* element and grouped as described below.

Expression of *UAS-TNTE* transgene did not cause any drastic change in the morphology of these neurons (Fig. 6C), whereas expression of *rpr* resulted in varying degrees of ablation of the normal complement of eight PDF neurons per larval brain (Fig. 6D–F). Larvae were grouped according to the severity of the disruption of LNv morphology as judged by the number of remaining neurons. In group 1 (G1) dissected CNSs showed 4–6 remaining neurons with reduced dendritic arborizations. CNSs from group 2 (G2) had 2–3 cells per brain and no visible dendritic arborization. Group 3 (G3) samples showed an almost complete ablation of these neurons; no cellular profiles could be identified at that level of resolution. A representative example of each one of these categories is shown in Figure 6D–F, respectively.

The comparison of the response indices between these three groups showed that variable degree of ablation of the LNv does not significantly correlate with any change in the larval response to light (Fig. 7). In particular, larvae completely lacking these neurons (G3) responded in a manner that is indistinguishable from the wildtype larvae in the ON/OFF assay (Fig. 7). Consistent with these findings, we found that the targeted expression of *TNTE* to these neurons did not affect the larval response to light.

In order to confirm these observations, another Gal4 driver, *timeless (tim)*-Gal4 was used to impair the function of these LNv pacemaker neurons (Blau and Young, 1999). The *tim* gene is expressed in the PDF containing LNv as well as other two groups of pacemaker neurons, the larval dorsal neurons 1 and larval dorsal neurons 2 ($DN1^L$ and $DN2^L$, respectively). The *tim-Gal4* driver was employed to drive the expression of *TNTG* or *rpr*. Ablation of PDF neurons by *rpr* under the control of the *tim-Gal4* was considerable but not uniform (n = 15). In 73.3% of the specimens there were only 1–3 out of the 8 PDF neurons present per CNS. In the remaining 26.7% there were 4–5 cells left. Consistent with previous results, no significant change in the response to light was detected by ablation or inactivation of PDF neurons using the *tim-Gal4* driver (Fig. 7). Thus, we conclude that the LNv pacemaker neurons are not required for larval motor response to light as measured in the ON/OFF assay.

DISCUSSION

The repertoire of visually guided behaviors of the *D. melanogaster* adult includes courtship, walking, flight, and landing. By contrast, the *Drosophila* larva possessing a simpler nervous system is faced with a less challenging environment and displays a more restricted repertoire of light-modulated behaviors. During the foraging stage, from the first instar to the beginning of the third instar, the *Drosophila* larva has as its primary concern to remain immersed in the food substrate. Consistent with this preference, the larva is repelled by light until the onset of the wandering stage, when it initiates a search for an adequate site to undergo metamorphosis. At this point in larval development it ceases to respond to light in laboratory assays (Sawin-McCormack et al., 1995).

One of the most interesting behaviors modulated by light input is biological rhythmicity (reviewed by Hall, 2003; Harmer et al., 2001). Entrainment of biological rhythms requires the precise alignment of biological time with solar time. Different input pathways have evolved

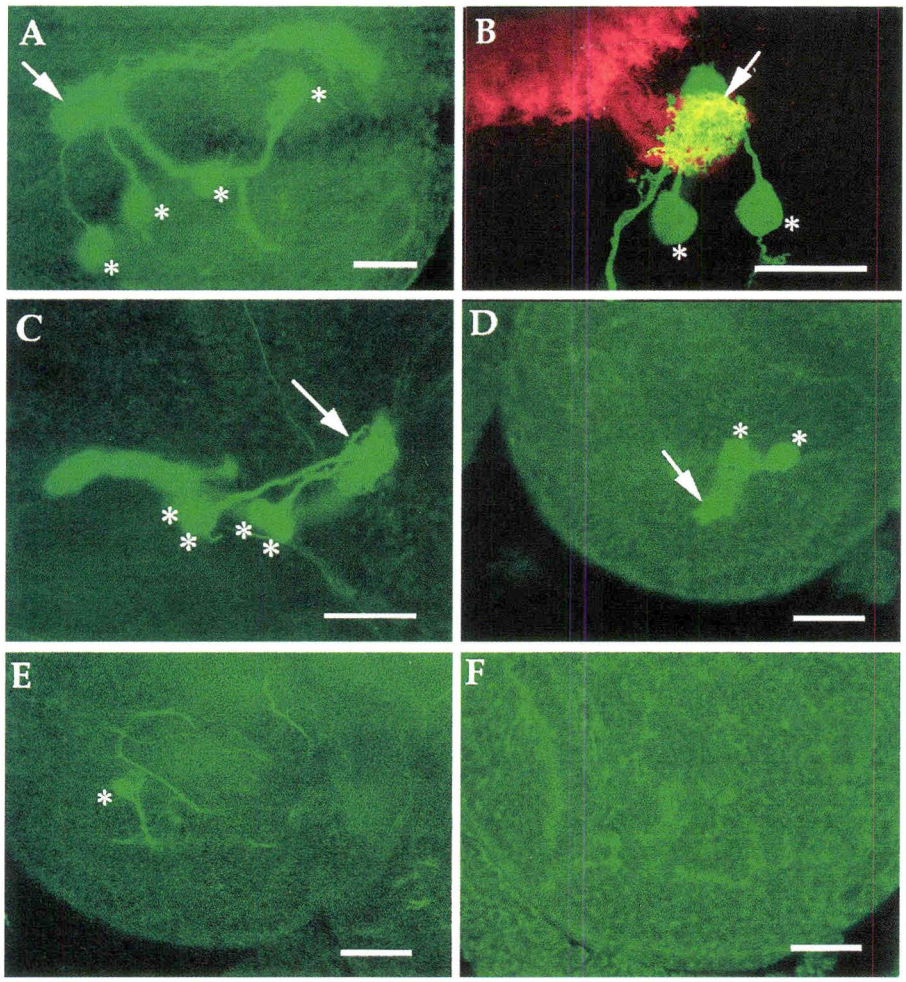

Fig. 6. Ablation and inactivation of LNv. **A:** Lateral view of the left brain hemisphere in the *y, w; PDF-Gal4, UAS-mCD8:GFP* larva. The axons of the four neurons fasciculate and send their projections dorsally. In all panels the arrow indicates the dendritic arborization where the larval photoreceptor axons make contact and the asterisk indicates the cell body of these neurons. **B:** Double-labeling of *y, w; PDF-Gal4, UAS-mCD8:GFP* with the 24B10 antibody that labels the larval visual system. **C:** Morphology of lateral neurons in the *PDF-Gal4, UAS-mCD8:GFP/UAS-TNTE* heterozygous larva. The dendritic arborization (arrow) and the cell bodies (asterisk) are apparently not affected by the expression of the tetanus toxin gene product. **D–F:** Variation in the degree of ablation of lateral neurons in *PDF-Gal4, UAS-mCD8:GFP/UAS-rpr* genotype larvae; a single brain hemisphere is shown in each case. D: Representative brain hemisphere of group 1, 2 GFP-labeled cell bodies are clearly seen; the dendritic arborization was visible in this group, although reduced in its extent. E: Representative sample that belongs to group 2; each CNS in this group contained 2–3 GFP labeled neurons, dendritic arborizations of lateral neurons in this group was absent. F: Representative sample of group 3, in which no cells can be detected. Scale bars = 25 μm.

that use twilight transition (dawn and dusk) as the primary cue to adjust circadian phase (reviewed by Zordan et al., 2001). These in turn modulate the activity of endogenous oscillators, the so-called circadian pacemakers or clocks (reviewed by Allada et al., 2001). Non-mammalian vertebrates employ a variety of extraretinal photoreceptors that originate in the embryonic forebrain such as the intracranial pineal body, or deep brain photoreceptors as input pathways (Foster and Soni, 1998; Shand and Foster, 1999). Mammals, on the other hand, are viewed as an exception, as they use their visual organs both for image detection as well as for entrainment of biological rhythms (reviewed by King and Takahashi, 2000).

Circadian-regulated biological rhythms have been the focus of intense investigations in *Drosophila*. The neuronal circuitry that describes the relationship between the photic input pathway, central pacemaker neurons such as the lateral neurons and output pathways, have been partially elucidated. Similarly, the identity, biochemical relationship, and function of many of the intrinsic components of the molecular clock are well established (reviewed by Hall, 2003). In contrast, only recently have the identity and function of the circadian photoreceptors and photopigments been unraveled (Emery et al., 2000a,b; Helfrich-Forster et al., 2001, 2002). Mutations in the gene encoding the flavoprotein CRY cannot phase shift, suggesting that this molecule represents the circadian photoreceptor molecule (Emery et al., 1998; Stanewsky et al., 1998). In these mutants overexpression of CRY in the LN-v is sufficient to rescue their circadian defects, demonstrating that these neurons, besides their function as central pacemakers, play the additional role of deep brain extra-ocular photoreceptors (Emery et al., 2000b).

The so-called H-B extraretinal organ, located in the posterior margin of the adult retina, has been recently reexamined regarding its developmental origin, axonal projection, and role as a circadian input pathway (Helfrich-Forster et al., 2001, 2002; Malpel et al., 2002; Yasuyama and Meinertzhagen, 1999). These distinct photoreceptors derive from Bolwig's organ, the larval photosensory organ, whose terminus in turn is found in the larva, in close association with the LNvs pacemakers. The current view is that the H-B eyelet together with the compound eyes and the LNv, the latter using CRY as a photopigment, constitute the *Drosophila* photic input pathway required for circadian entrainment, (Helfrich-Forster et al., 2001, 2002; reviewed by Hall, 2003).

The close association between components of the circadian circuitry and the visual system suggests that in the *Drosophila* larva light detection and regulation of the

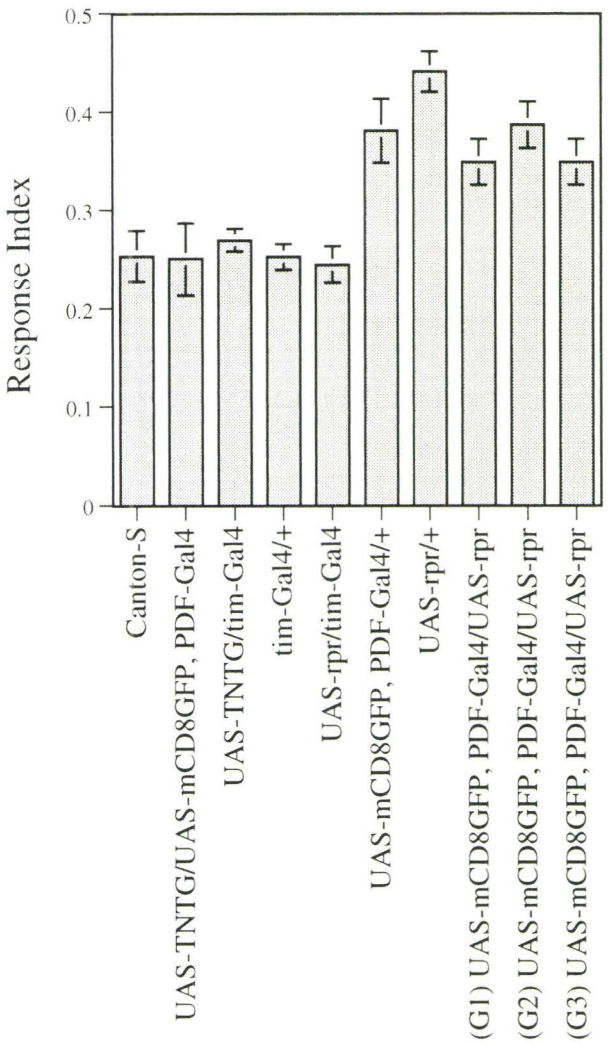

Fig. 7. Circadian pacemaker neurons are not required for larval response to light. Larvae in which the expression of the cell death gene *reaper* was targeted to the small LNv under the control of the PDF-Gal4 element were grouped with respect to the degree of ablation (G1, G2, and G3). In addition, silencing of these neurons was achieved by targeted expression of TNTE under the same Gal 4 driver. Ablation or silencing of these pacemaker neurons did not significantly disrupt the larval response to light as determined by the response index in the ON/OFF assay. (*Canton-S*, n = 10; *PDF-Gal4, UAS-mCD8: GFP/ UAS-TNTE*, n = 15; *PDF-Gal4, UAS-mCD8: GFP/UAS-rpr*, G1 n = 14, G2 n = 20, G3 n = 11, *PDF-Gal4, UAS-mCD8: GFP/+* n = 13, *UAS-rpr/+* n = 17.) Two sample Student's *t*-tests: (Canton-S vs. *PDF-Gal4, UAS-mCD8: GFP/UAS-TNTE*, T = −0.07, $P = 0.94$, DF = 22); (*PDF-Gal4, UAS-mCD8: GFP/+* vs. *UAS-rpr/+*, T = 1.57, $P = 0.13$, DF = 21); (*PDF-Gal4, UAS-mCD8: GFP/+* vs. G1, T = −0.79, $P = 0.44$, DF = 22); (*PDF-Gal4, UAS-mCD8: GFP/+* vs. G2, T = 0.16, $P = 0.88$, DF = 23); (*PDF-Gal4, UAS-mCD8: GFP/+* vs. G3, T = −0.79, $P = 0.44$, DF = 20). Similarly, ablation or silencing of these and other pacemaker neurons using the *tim-Gal4* driver did not significantly disrupt the larval response to light (*tim-Gal4/+*, n = 25; *tim-Gal4/UAS-rpr*, n = 25; *tim-Gal4/UAS-TNTG*, n = 25). Two sample *t*-tests: *tim-Gal4/UAS-TNTG* vs. *tim/+*, T = 0.98, $P = 0.33$, DF = 47; *tim-Gal4/UAS-rpr* vs. *tim/+*, T = −0.35 $P = 0.73$ DF = 43).

biological time are served by the same neuronal circuitry. In this article, we elucidated the role of some of the neuronal components underlying circadian rhythmicity in the larval response to light in the ON/OFF assay. To that end, targeted expression of cell death genes as well as *TNT*-induced neuronal silencing of a small number of specific neurons was employed.

Surprisingly, our results indicate that only a small number of photoreceptors, characterized by the expression of Rh5, are required for the larval photobehavior defined here by two distinct motor outputs modulated by light, namely, head swinging and distance traveled. The Rh6 photoreceptors comprising ~8 out of 12 photoreceptors present in the larval eye are not required for the larval response to light. Interestingly, these photoreceptors have been recently shown to be retained through metamorphosis and form all or part of the H-B extraretinal eyelet found in the posterior margin of the adult compound eye (Helfrich-Forster et al., 2002; Malpel et al., 2002; Yasuyama and Meinertzhagen, 1999). Action spectra as well as cell ablation experiments support the notion that these photoreceptors, in particular those expressing Rh6, function as one of the photic input pathways for circadian entrainment in the adult *Drosophila* (Helfrich-Forster et al., 2001, 2002). Our results suggest that the Rh6 photoreceptors, while in intimate association with the larval eye, may play an exclusive role as a circadian input pathway photoreceptor. Alternatively, they may function in a response to light not detected in our assay.

Recently, it was reported that the development and/or maintenance of the dendritic arborization of the LNvs require contact with the larval optic nerve, suggesting that the larval optic nerve provides trophic support to these neurons (Malpel et al., 2002). These results also indicate that the LNvs may function as one of the larval optic nerve first-order interneurons and may thus be functionally relevant for the larval response to light.

In order to address these questions we ablated or inhibited the activity of LNvs. Surprisingly, this had no effect in the larval response to light as detected in our assays. In conclusion, our results demonstrate that two of the most important components of the neuronal circuitry underlying circadian rhythmicity in *Drosophila,* namely, the extraretinal H-B cluster and the LNvs, while developmentally related and in intimate association with the larval visual system, are functionally independent from this structure.

Previous experiments in our laboratory failed to detect circadian regulation in the larval response to light in the ON/OFF assay (Busto, M.Sc. thesis, 1998, and data not shown). These experiments were restricted to investigating the response to light as determined by the reduction in the distance traveled during the light pulse versus the dark pulse. The assays conducted for the current report therefore did not take into consideration circadian time and aimed at investigating the role of these neurons in the basal response to light at different points during the circadian cycle. Therefore, it is possible that the two other behaviors triggered by light such as increase in head-swinging behavior or change in the direction of larval path display circadian rhythmicity which may thus be regulated by the central pacemakers.

The vast majority of the studies investigating the molecular genetic mechanisms underlying entrainment of circadian rhythmicity in *Drosophila* employ the adult form. It is known, however, that entrainment of biological rhythms can occur from the first larval instar (Sehgal et al., 1992). The presence in the larva of receptors and

pacemakers that will later be incorporated into the adult nervous system suggest that these cells perform the same role earlier in development. Furthermore, they suggest that oscillation of the molecular clock is maintained in these cells through metamorphosis. Our studies demonstrate that both LNv and Rh6 photoreceptors are not required for the larval response to light. The crucial role played by the Rh5 neurons in the larval response to light further support the notion of unique pathways serving entrainment of circadian rhythms.

ACKNOWLEDGMENTS

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APPENDIX D: Manuscript

Verónica G. Rodriguez Moncalvo and Ana R. Campos. (2008). Mosaic Analysis with a Repressible Cell Marker. Binder, M.D., Hirokawa, N., Windhorst, U. (Eds). Encyclopedia of Neuroscience. Springer, Berlin Heidelberg, New York, 2401-05.

de-represses Smoothened activity. The mechanism of Patched/Smoothened interaction is not yet known.

Intracellular components of both the Hedgehog and the Wnt signaling cascade are multiprotein complexes containing a ►scaffold protein and kinases. These complexes phosphorylate and stabilize β-catenin downstream of Wnts, and control levels of Gli repressor and Gli activators downstream of Shh.

Wnt signaling includes three different pathways, the canonical and the non-canonical pathway with the latter being subdivided into planar cell polarity (PCP) and Ca^{2+} pathways. The canonical pathway involves the stabilization of β-catenin. In the absence of Wnt, β-catenin is recruited to a multi-component complex consisting of the scaffold protein Axin, the tumor suppressor APC and two kinase families, CK1 and GSK. Subsequent ►ubiquitination results in degradation of β-catenin. If Wnt binds to the surface receptor Frizzled and co-receptors, such as Lrp5/6, phosphorylation of β-catenin is suppressed. Thus, β-catenin accumulates and can be transported to the nucleus, where it activates transcription by binding to LEF/TCF transcription factors.

In general signaling downstream of morphogens has only been studied in detail during early stages of development, including neural induction, differentiation and patterning. Signaling involved in later stages of development, such as axon guidance or synaptogenesis is not well understood. In axon guidance along the longitudinal axis of the spinal cord, the receptor for Shh is Hedgehog-interacting protein and no longer Patched [9,10]. Wnts appear to use Frizzled receptors for both axon guidance and earlier functions, but the intracellular signaling pathways have not been identified and do not seem to be identical to any of the well-known pathways (see above). In contrast to the classical roles of morphogens in tissue patterning, their role in axon guidance does most likely not involve changes in gene transcription but is restricted to more rapid changes in signaling affecting directly the cytoskeleton of growth cones.

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Mosaic Analysis with a Repressible Cell Marker

Veronica G. Rodriguez Moncalvo, Ana R. Campos
Department of Biology, McMaster University, Hamilton, ON, Canada

M

Synonyms

MARCM

Definition

Mosaic Analysis with a Repressible Cell Marker or MARCM is a set of genetic tools developed in the fruitfly *Drosophila melanogaster* for the positive labeling of individual cells or groups of cells derived from the same lineage. In addition, labeled cells may be generated that are homozygous mutant for genes of interest or that express constructs that modulate gene expression and/or function. This system was created by Liqun Luo and Tzumin Lee [1] and has been used extensively in this model organism for the analysis of neuronal differentiation, cell lineage and other biological processes outside of the nervous system.

Characteristics

Overview

The nervous system is arguably one of the most complex tissues in the animal kingdom. Even in the simplest of the model organisms, its assembly requires not only the generation of a large number of diverse cell types but also the complex wiring of these cells. Therefore, the study of nervous system development and function is aided by the identification and genetic manipulation of a small number of neurons. Genetic tools unique to the *Drosophila* model system have

greatly facilitated the analysis of the molecular mechanisms underlying neuronal pattern formation by providing investigators with exquisite spatial and temporal control of gene expression and function. In this context, mobile DNA elements (►P-elements), carrying heterologous transcription factors (e.g. *GAL4*) under the regulation of *Drosophila*-specific promoters have been used extensively for the targeted expression of reporter molecules, genetically modified alleles or more recently constructs capable of mediating gene silencing via RNA interference (►RNAi).

The ability to induce, isolate and characterize the phenotypic consequence of single gene mutations has been fundamental to our current understanding of nervous system development and function in *Drosophila* and other model organisms. Relevant genes are for the most part expressed in a temporal and spatial complex pattern, which is reflected in the pleiotropic phenotype displayed by mutant organisms. Moreover, homozygous mutant animals may not survive to adulthood making it difficult to study the consequence of lack of gene function beyond a certain stage. In order to overcome these limitations, developmental biologists have relied on genetic ►mosaic organisms in which homozygous mutant clones are generated in an otherwise wild type and heterozygous background. This approach has been used extensively in *Drosophila melanogaster* as well as in mice and *C. elegans*, to investigate the stage-specific cell autonomous requirement of gene function. Recent improvements to this system include the ability to generate small homozygous clones at specific times during development and to unambiguously identify individual homozygous mutant clones such that the cellular phenotype can be studied appropriately.

Mechanism

Traditionally, genetic mosaics in *Drosophila* have been generated through chromosomal loss (e.g. ring X chromosome) or X-ray induced mitotic recombination. More recently, sequence-specific recombination systems (FLP/FRT or Cre/LoxP) have been introduced allowing efficient gene-specific mitotic recombination. While the generation of genetically distinct somatic clones is technically straightforward, a reliable way to unambiguously label specific cell types within mutant clones has been missing. In the past, external markers have been used to infer the genotype of internal tissue. An improvement on this approach was the introduction of reporter constructs whose loss would mark the presence of homozygous clones in an otherwise heterozygous-labeled organism. The shortcoming of this method was that mutant clones still remained unlabelled and thus not available to detailed morphological analysis.

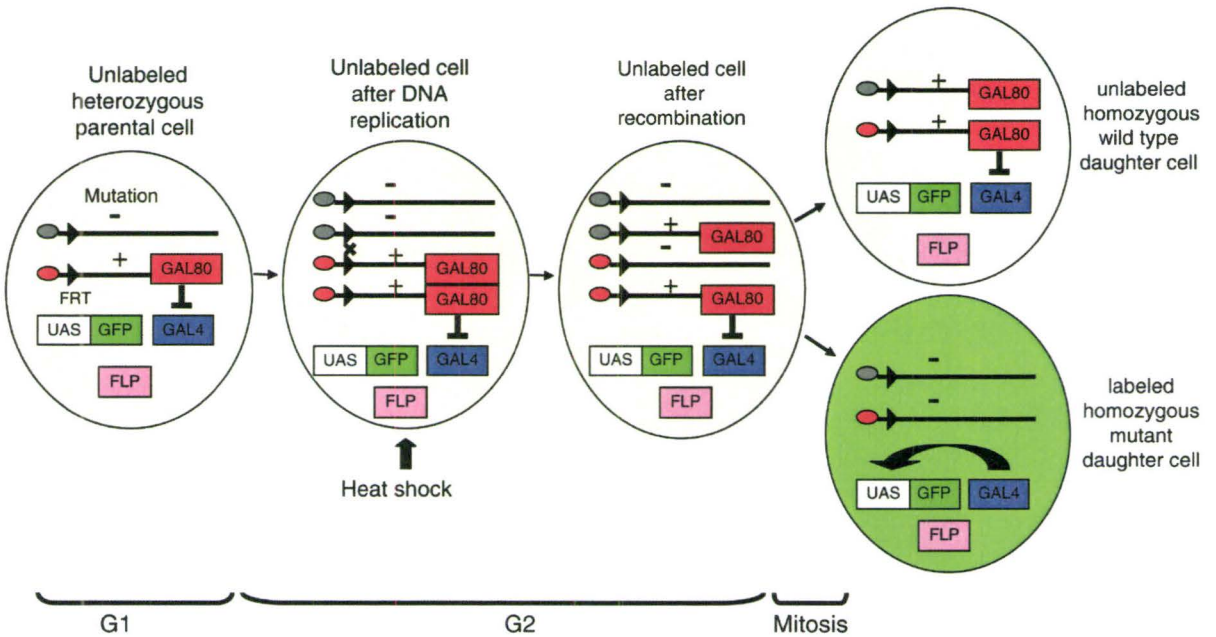
MARCM is a major advance because it combines the ability to positively label small numbers of cells with the FLP-FRT recombination system previously used to generate genetic mosaics (Fig. 1). This was made possible by the introduction of a repressible cell marker. In MARCM, the expression of reporter genes such as ►GFP, driven by a tissue-specific *GAL4* construct, is repressed due to the ubiquitous expression of a *GAL4* inhibitor, *GAL80*. The *GAL80* gene is inserted in the same chromosomal arm as the wild type allele of a gene of interest *X*. The homologous chromosome carries a mutant allele of the gene *X* and no copy of *GAL80*. Mitotic recombination generates two daughter cells that differ from the parental cell regarding the genotype of the *FRT*-bearing chromosome in that they are homozygous for one or the other homologous chromosome. Therefore, the mitotic recombination event not only yields homozygous mutant cells but also relieves in these cells the repression of the *GAL4* construct through the loss of *GAL80* ►transgene. The outcome is the generation of flies carrying single or multiple cells derived from a single progenitor that are homozygous mutant for a gene of interest. Specific cell types within the mutant clone will be positively labeled by the expression of a reporter construct that facilitates their morphological analysis. The size of the labeled clone depends upon the timing of FLP expression (refer to [2] for a detailed protocol). Moreover, as illustrated below, MARCM can be used to label clones of specific neurons without manipulating gene function. In this context, MARCM has been employed to investigate the developmental architecture – pattern of projection and clonal relationship – of specific neurons (see Fig. 2 for examples of MARCM generated clones).

The caveat is that, while labeling is found only in cells homozygous for the mutant chromosome (not carrying the *GAL80* repressor), not all mutant cells are labeled. Labeling of mutant cells is restricted to the cell types in which *GAL4* driver is expressed. Modifications to this method introduced recently address this issue but have not yet been used as extensively as MARCM [3].

Components

All constructs described below are found within P-element vectors and were inserted into the *Drosophila* genome via ►P-element mediated transformation.

1. *GAL4* is a yeast transcription factor that binds to specific DNA elements known as upstream activating sequence (*UAS*) and activates RNA transcription of reporter genes. It is often referred to as a "driver element." In *Drosophila*, expression of GAL4 under the control of tissue-specific regulatory sequences has been employed to activate the expression of reporter genes such as *GFP* or *β-Galactosidase* in specific cell types. Alternatively, one can increase the expression of a target gene (up-regulation), by introducing a full-length cDNA downstream from the *UAS* or silence a gene



Mosaic Analysis with a Repressible Cell Marker. Figure 1 Schematic representation of the MARCM system. MARCM requires two *FRT* sites (™) situated at the same location and one copy of the *GAL80* gene downstream to one of the *FRT* sites. The genes encoding Flipase (FLP) recombinase, the tissue-specific *GAL4* driver, and the *UAS-GFP* may be located anywhere in the genome. Additionally, the *FRT*-bearing, non-*GAL80* chromosome may carry a mutation (−) distal to the *FRT* site. A brief heat shock induces *FLP* expression. At the *FRT* sites, FLP recombines the wild type (+) *GAL80*-containing chromosome with its homologous mutant (−) chromosome. The resulting wild type (+/+) daughter cell will carry two copies of *GAL80*, which suppresses GAL4-dependent expression of the *UAS-GFP* (unlabeled cell). In the other daughter cell, which may be homozygous mutant for a gene of interest (−/−), the absence of GAL80 allows for GAL4-mediated expression of the GFP (labeled cell). (Adapted from [2]).

(knock-down), by introducing a construct capable of mediating RNA interference (RNAi).

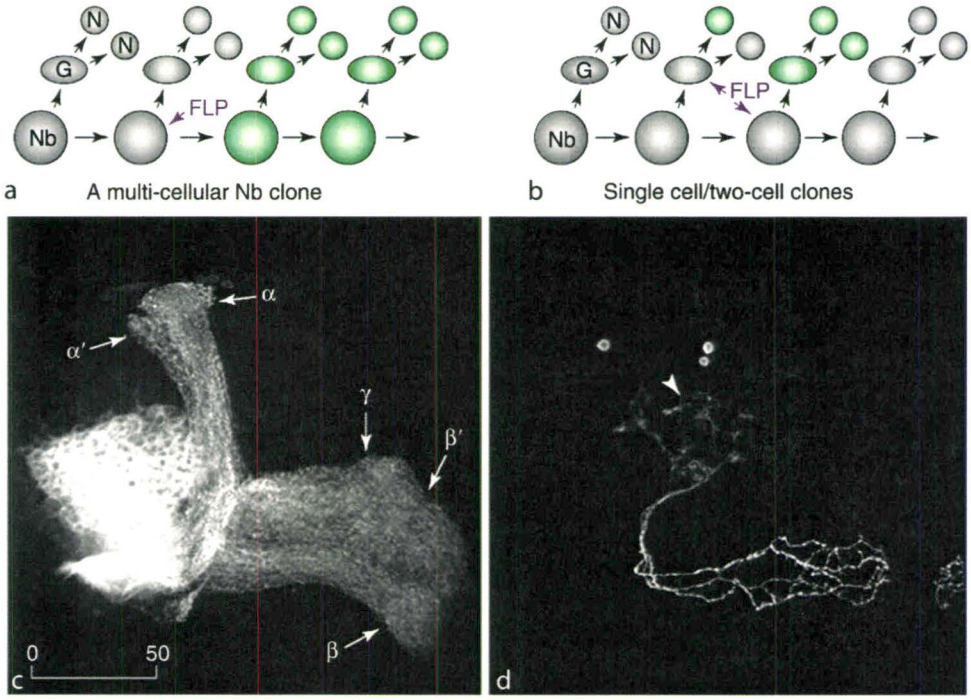
2. *UAS-mCD8-GFP* This construct encodes a *GAL4* responsive reporter gene. In this case, the reporter is the green fluorescent protein (GFP), which has been fused to the transmembrane mouse lymphocyte marker CD8. This allows for targeting of GFP to the cell surface.
3. Flipase (FLP) recombinase. FLP recombinase is a yeast enzyme that catalyzes mitotic recombination at FRT sites. *Drosophila* strains have been created carrying the FLP gene under the regulation of a ubiquitous promoter, such as the *tubulin* gene promoter (*tubP*), or a conditional promoter such as that of the *Heat Shock Protein 70* gene (*HSP70*).
4. *FRT* sites. *FRT* sites are DNA sequences recognized by FLP recombinase. High frequency mitotic recombination is catalyzed by FLP at these sites. In flies heterozygous for a recessive allele of a gene of interest (+/−) in which FRT sequences are also present in the same chromosome, mitotic recombination at these sites yields homozygous mutant clones (−/−) as well as homozygous wild type twin clones (+/+). The latter are indistinguishable from the wild type heterozygous background (+/−).
5. *tubP-GAL80*- GAL80 is a yeast protein that represses GAL4 function as a transcription factor. In MARCM, the *GAL80* gene is under the control of the *tubulin* promoter (*tubP*) thereby providing ubiquitous repression of GAL4 function. Mitotic recombination at *FRT* sites catalyzed by the *FLP* gene product eliminates the *GAL80* gene from one of the daughter cells thereby relieving GAL4 from GAL80 repression while at the same time inducing a loss of heterozygosity event in the same chromosome.

Uses of MARCM

Since it was first published, MARCM has been used extensively. It has become an essential component of the ever-expanding *Drosophila* genetics toolbox. Below we describe briefly a few examples in which the use of MARCM system played an essential role in the genetic analysis of nervous system development.

Neuronal Morphogenesis

MARCM has been effective in the investigation of axonal and dendritic branching patterns as well as neuronal wiring and circuitry formation. For instance, Grueber and colleagues employed MARCM to study dendrite



Mosaic Analysis with a Repressible Cell Marker. Figure 2 MARCM clones in *Drosophila* mushroom bodies (MB). A neuroblast (Nb) generates a series of ganglion mother cells (GMC, G in Fig.). Each GMC generates two post-mitotic neurons (N). (a) A GAL80-negative Nb (GAL80–) gives rise to a multi-cellular clone. (b) If a GMC loses GAL80, a two-neuron labeled clone is generated. If mitotic recombination occurs in a dividing GMC, only one of the two post-mitotic neurons will be labeled. (c) and (d) Confocal images of MARCM clones of MB neurons. (c) A MB Nb clone produced by an early mitotic recombination event consists of hundreds of neurons at the adult stage visualized by mCD8-GFP expression. There are five axon bundles in the adult MB: γ, β′ and β projecting towards the midline and α′ and α projecting dorsally. (d) Single cell labeling shows that each cell body extends a single process from which dendrites (*arrowhead*) branch out. (Modified with permission from Lee T, Luo L (2001) Trends Neurosci 24(5):251–254).

branching morphology and the establishment of dendritic territories of specific neurons of the *Drosophila* third instar larva peripheral nervous system [4]. They focused their studies on the dendritic arborization neurons (da), which spread their dendrites in a two-dimensional coverage of the larval epidermis. By examining single cell clones generated using MARCM and labeled by the expression of a pan-neuronal driver (*elav-GAL4*), da neurons were grouped into four morphological classes (I-IV) according to differences in dendrite branching complexity. Most importantly, these authors reported that neurons of the same class show dendritic exclusion or heteroneuronal tiling whereas those in different classes show extensive overlap of their dendritic fields. These pioneer studies set the stage for further investigations addressing the molecular genetic mechanisms underlying dendritic branching and tiling briefly discussed below.

Gene Function Requirement

The MARCM system has been successfully employed to assess the role of candidate genes in different biological processes. Of particular note are recent findings that further elucidate the role of *Down's syndrome cell adhesion molecule* (*Dscam*) in dendrite self-avoidance or isoneuronal tiling. The *Drosophila Dscam* gene shows a remarkable degree of alternative splicing with the potential to generate more than 38,000 different isoforms and has been implicated in axonal and dendritic patterning. Using MARCM, three different groups addressed the cell autonomous requirement for *DsCam* gene in the patterning of the larval epidermis da sensory neurons [5,6,7]. These workers showed that *Dscam* mediates isoform-specific homophilic interactions required for self-avoidance within a single sensory neuron arbor. Interestingly, heteroneuronal tiling such as that of class II and IV sensory neurons is not affected by *Dscam* mutations suggesting the existence of an additional pathway. Thus, the current view of the molecular underpinnings of dendrite morphogenesis in *Drosophila* has been made possible by the high level of resolution afforded by single cell labeling and genetic manipulation unique to the MARCM system.

Mosaic Genetic Screens

Mutant screens constitute a powerful tool in the identification of genes essential for diverse biological processes. A forward genetic approach can be combined with the MARCM system, thereby bypassing pleiotropic

effect of mutations (i.e. early lethality) and increasing the sensitivity of the phenotype analysis. This strategy is well illustrated in the report of Reuter et al. [8]. These investigators carried out a genetic screen aimed at identifying genes that play a role in the morphogenesis of the larval MB neurons. To that end, homozygous mutant clones generated by MARCM were examined by virtue of expression of MB-specific *GAL4* drivers, which in turn activated the transcription of target reporter constructs (*UAS- mCD8-GFP*). In order to increase the frequency of MB clones, FLP expression was heat- induced in newly hatched larvae. At that time, the only dividing neuroblasts are those giving rise to the MB neurons. Nearly 20% of the genome was sampled by this approach. Larvae bearing mutant clones showing abnormal distribution of GFP, large cells, defective axonal transport and abnormal axon and dendrite morphogenesis were isolated. Further characterization of these mutations led to identification of new genes that play a role in neuronal morphogenesis as well as discovery of new functions of previously identified genes.

Cell Lineage Analysis

The ability to induce mitotic recombination at different times during development makes MARCM particularly well suited for cell lineage analysis. Several investigators have taken advantage of these properties to investigate clonal relationships in the olfactory glomeruli and the mushroom body (MB), the area of the insect brain involved in olfaction-mediated learning and memory. As one of the earliest contributions of MARCM system, Lee et al. [9] showed that, in the *Drosophila* CNS, a single identified neuroblast sequentially gives rise to at least three distinct types of neurons. More interestingly, their projection into different MB lobes depends upon their birth order [9]. Similar strategy when applied to clonal relationship of projecting neurons of the *Drosophila* olfactory system demonstrated that their dendritic arborizations in the antennal lobe and thus odour representation, depends upon their birth order (reviewed in [10]).

Acknowledgements

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MOSAIC Model

Definition

Modular selection and identification control (MOSAIC) model, proposed for solving a large-scale sensorimotor problem using multiple pairs of a forward (estimation) model and an inverse (control) model. An important ingredient in the model is how well a forward model predicts movement outcome or reward, defined as a responsibility signal. Those responsibility signals in turn determine which controllers will be used for a particular movement and which internal models will be updated accordingly. The MOSAIC model attempts to decompose a large-scale sensorimotor problem automatically by making each module specialized for a particular situation or task.

►Theories on Motor Learning

Mossy Fibers

Definition

Most afferents from the brainstem or spinal cord to the cerebellum terminate in the granule cell layer of the