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

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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.

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

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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-