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Morphological and Functional Recovery of the Planarian Photosensing System during Head Regeneration

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ABSTRACT—When exposed to light, planarians display a distinctive light avoidance behavior known as negative phototaxis. Such behavior is temporarily suppressed when animals are decapitated, and it is restored once the animals regenerate their heads. Head regeneration and the simple but reproducible phototactic response of planarians provides an opportunity to study the association between neuronal differentiation and the establishment of behavior in a simple, experimentally tractable metazoan. We have devised a phototaxis assay system to analyze light response recovery during head regeneration and determined that light evasion is markedly re-established 5 days after amputation. Immunohistological and *in situ* hybridization studies indicate that the photoreceptors and optic nerve connections to the brain begin by the fourth day of cephalic regeneration. To experimentally manipulate the light response recovery, we performed gene knockdown analysis using RNA interference (RNAi) on two genes (*1020HH* and *eye53*) previously reported to be expressed at 5 days after amputation and in the dorso-medial region of the brain (where the optic nerves project). Although RNAi failed to produce morphological defects in either the brain or the visual neurons, the recovery of the phototactic response normally observed in 5-day regenerates was significantly suppressed. The data suggest that *1020HH* and *eye53* may be involved in the functional recovery and maintenance of the visual system, and that the phototaxis assay presented here can be used to reliably quantify the negative phototactic behavior of planarians.

Key words: planarian, visual system, neurotrophic factor, RNAi, neuronal maturation

INTRODUCTION

The brain acts as an information-processing center in which a variety of external signals are reinterpreted into defined cellular functions that often result in distinct animal behaviors. Although much has been learned about some of the specific neuronal activities underpinning the transformation of a neuronal stimulus into a behavioral response (*i.e.*, long-term potentiation) (Robbins, 1998; Bailey, 1999), much remains to be understood about the developmental, molecular and cellular processes that determine the onset and maintenance of stimulus perception and its association to the corresponding physiological response.

The relative simplicity of the planarian brain, combined with its molecular accessibility, and complex behavioral traits provides us with an opportunity to study in detail the ontogeny of stimulus/response events. The central nervous system (CNS) of planarians is composed of a bi-lobed brain in the anterior region of the animal and two longitudinal ventral nerve cords along the body (Rieger *et al.*, 1991; Reuter and Gustaffson 1995; Baguñà, 1998; Agata *et al.*, 1998).

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Recent studies have revealed that the planarian brain is divided into several functional and structural domains as defined by the discrete expression of three *otd/Otx*-related homeobox genes and other neural-specific genes (Umesono *et al.*, 1997, 1999; Cebrià *et al.*, 2002a, 2002b; Mineta *et al.*, 2003; Nakazawa *et al.*, 2003). These results suggest that the relatively simple planarian brain is nevertheless well organized and underscored by a surprisingly complex set of regulatory genetic events.

One of the stereotypical behaviors displayed by planarians is negative phototaxis. The organ system responsible for detecting light in these animals (the so-called "visual system") has been the subject of many studies (Taliaferro, 1920; MacRae, 1964; Carpenter *et al.*, 1974; Kuchiiwa *et al.*, 1991; Asano *et al.*, 1998). Light sensing organs ("eyes") located on the dorsal side of the body are composed of two cell types: pigment cells and visual neurons (photoreceptor cells). The pigment cells are arranged into a semilunar eyecup, while the visual bipolar neurons are located outside of the eyecup. Dendrites from the visual neurons distribute inside of the pigment eyecup to form a rhabdomeric structure containing opsins (Asano *et al.*, 1998; Orii *et al.*, 1998; Azuma *et al.*, 1999; Saló *et al.*, 2002). The axons of the visual neurons project caudally onto the dorso-medial region of the brain and some of the fibers form an optic chiasma, which integrates photosensory inputs from the left and right sides of the animal (Agata *et al.*, 1998; Sakai *et al.*, 2000).

Because of the extraordinary regenerative capacity of planarians (Agata and Watanabe, 1999; Sánchez Alvarado, 2000, 2003; Newmark and Sánchez Alvarado, 2002; Saló and Baguñà, 2002; Agata *et al.*, 2003), the visual system can be regenerated by adults after amputation. When an animal is decapitated at a region posterior to the auricles, the trunk piece can regenerate a brain, including the visual neurons. In fact, the function of the brain is completely restored 1 week after amputation. Recently, extensive morphological analyses of the process of planarian brain regeneration have been carried out using a variety of gene expression markers (Cebrià *et al.*, 2002a). These studies revealed that the set of brain-expressed genes investigated thus far can be catalogued into three categories according to their expression timing. However, the molecular mechanisms of the restitution of the planarian visual system during head regeneration are still unknown. In order to better understand the regeneration of the visual system and to lay a foundation for future behavioral studies, we have morphologically and functionally analyzed the process of photosensory recovery during head regeneration using molecular markers, RNAi and a phototaxis assay system described in the present study.

MATERIALS AND METHODS

Animals

A clonal strain (GI) of the planarian *Dugesia japonica* derived from the Iruma river (Gifu, Japan), established by Dr. Kenji Watanabe at the Himeji Institute of Technology, and maintained in autoclaved tap water at 22–24°C, was used in this study. In all experiments, planarians 8–10 mm in length that had been starved for 2 weeks were used. For cephalic regeneration studies, animals were cut at a portion posterior to the auricle on ice.

Phototaxis assay

A schematic representation of the phototaxis assay system is shown in Fig. 2A. A planarian was put into a 60 × 30 × 10 mm container filled with 10 ml of autoclaved tap water at 22–24°C. The container was painted black except for one clear side. The container was exposed to 500 lux of white light from a horizontal position on the clear side of the container. Planarian behavior was recorded using an overhead digital video camera (Sony, Tokyo) for 90 seconds. Using a computer and SMART v2.0 behavior analysis software (Panlab, Spain), we analyzed the time animals spent in a target quadrant located in the dark end of the container opposite to the clear side (Fig. 2B) during the 90-sec test. Data were analyzed by one-way analysis of variance (ANOVA) and the statistical significance of differences between test results was determined by Student's t test; p values greater than 0.05 were taken as not significant (NS).

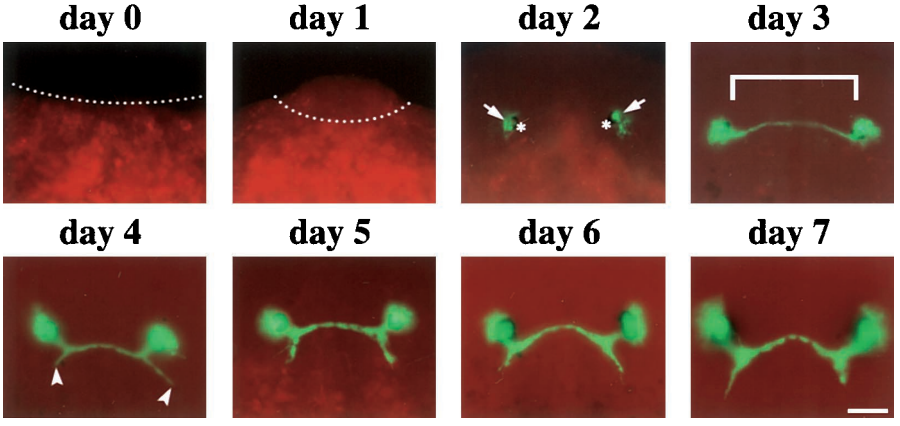

Fig. 1. Regeneration of the planarian visual system can be visualized by whole-mount immunostaining using an anti-arrestin antibody. Regeneration of both pigment cells (asterisks) and photoreceptor cells (arrows) was detected within 2 days after amputation. Connection between the right and left visual neurons (white bracket) was formed on day 3. Projections of visual neurons to the brain (arrowheads) was established around day 4. Dashed lines indicate the borderline between the newly formed blastema and the old stump region. Bar, 50 μm.

Whole-mount immunostaining with VC-1 monoclonal antibody

Planarians were treated with 2% hydrochloric acid in 5/8 Holtfreter solution for 5 min and then fixed in Carnoy's (ethanol, chloroform and glacial acetic acid in a proportion of 6:3:1) for 2 hr at 4°C. After fixation, they were bleached in 6% hydrogen peroxide (H_2O_2) in methanol for 14 hr under light. The animals were rehydrated in a series of decreasing concentrations of methanol in TPBS (PBS containing 0.1% Triton X-100) for 30 min at each step and blocked in 10% goat serum in TPBS for 2 hr at 4°C. The planarians were then incubated with an ascites fluid containing monoclonal antibody VC-1 against visual neurons (Sakai *et al*., 2000) diluted 1/5000 in 10% goat serum in TPBS for 12 hr at 4°C with shaking. The samples were washed with 10% goat serum in TPBS for 5 hr (with several changes of medium) and VC-1 was detected with Alexa Fluor 488-conjugated goat anti-mouse IgG(H+L) (Molecular Probes, Eugene OR, USA) diluted 1/400 in 10% goat serum in TPBS for 12 hr in the dark. After the samples were washed for several hours with several changes of TPBS, fluorescence was detected with a BX62 microscope (Olympus, Tokyo).

Whole-mount *in situ* hybridization

Animals were treated with 2% HCl for 5min at 4°C and then fixed in Carnoy's (ethanol, chloroform and glacial acetic acid in a proportion of 6:3:1) for 2 hr at 4°C. Hybridization was carried out using 20 ng/ml of digoxygenin (DIG)-labelled riboprobes (Roche Diagnostics, Basel, Switzerland), as previously described (Umesono *et al*., 1997; Agata *et al*., 1998).

***In situ* hybridization and immunostain of sections**

Planarians were fixed in relaxant solution (1% NHO_3, 2.25% formalin, 50 μM $MgSO_4$). Fixed worms were embedded in paraffin and serially sectioned at 8 μm. Hybridization on histological section was basically performed as described by Kobayashi *et al*. (1998). Detection of DIG-labeled probes was performed by using TSA Alexa Fluoro 488 detection kit (Molecular Probe, Eugene OR, USA), as described in the manufacturer's protocol. After *in situ* hybridization, immunostaining was performed using a monoclonal antibody against the visual neurons. Signals were detected with Alexa Fluoro 594 goat anti-mouse IgG(H+L) (Molecular probes, Eugene, OR,

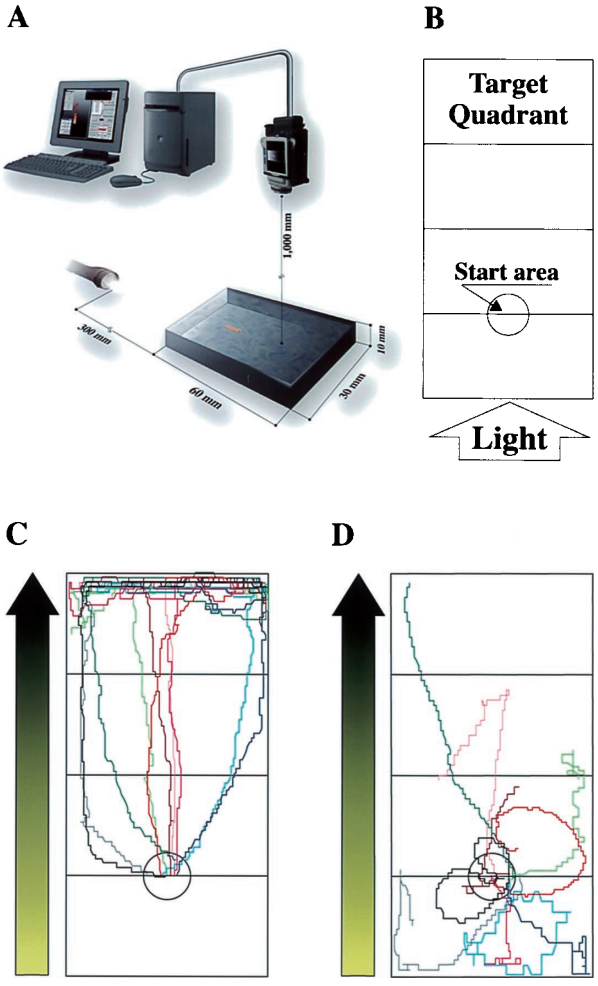

Fig. 2. A schematic drawing of the phototaxis assay system (A) and the container compartment (B). Distribution of 90-second trajectories representing the movements of intact planarians (C) and headless planarians (D). Each colored line indicates the trajectory of an individual planarians.

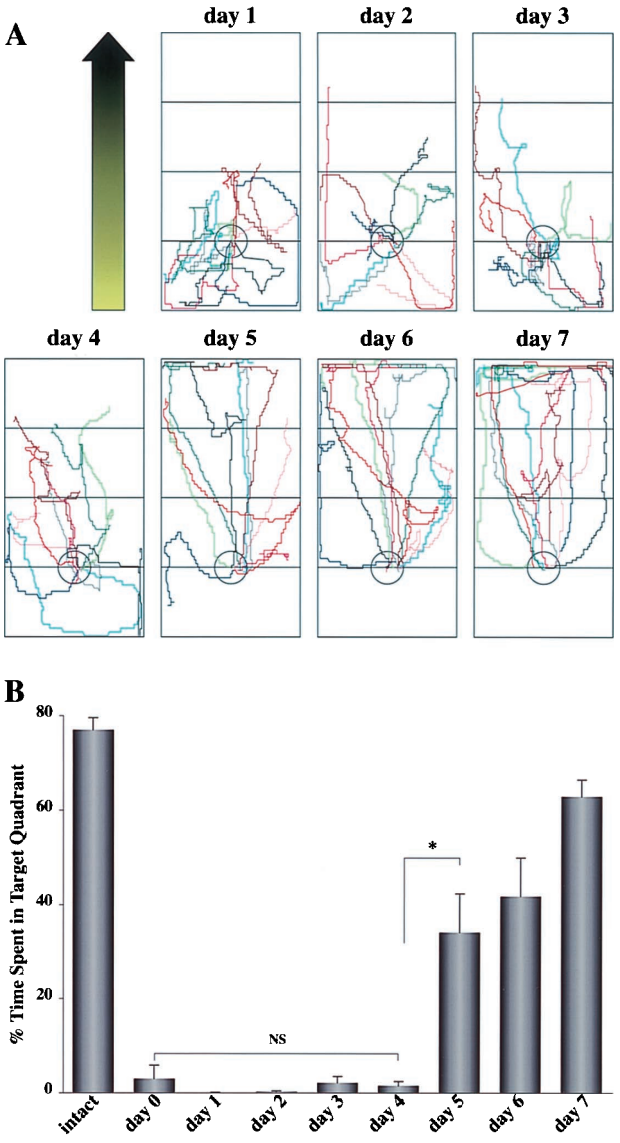

Fig. 3. (A) Distribution of traced trajectories of planarian movements during head regeneration. Each colored line indicates an individual planarian trajectory. (B) The time spent in the target quadrants is represented as mean±SEM from 10 independent animals. *, $p<0.005$; NS, not significantly different; one-way ANOVA followed by Tukey's multiple comparison test.

USA). Cell nuclei were labeled with Hoechst No. 33342 (Sigma, St Louis, MO, USA).

Synthesis of double-stranded RNA

Double-strand RNA (dsRNA) was basically synthesized as previously described by Sánchez Alvarado and Newmark (1999). pBluscript SK+ containing the appropriate cDNA inserts were linearized for *in vitro* transcription. *1020HH* containing 424 bp including a putative full-length open reading frame was digested with *Bam* HI and *Kpn* I to synthesize antisense (T7) or sense (T3) RNAs, respectively; *eye53* containing 436 bp including a putative full-length open reading frame was digested with *Pst* I and *Xho* I to synthesize antisense (T7) or sense (T3) RNAs, respectively. The RNAs ware denatured for 20 min at 65°C, and annealed for 40 min at 37°C. After ethanol precipitation, dsRNA was resuspended in H_2O Electrophoretic mobilities of dsRNA and single-strand RNA were assessed in 2.0% agarose gel.

Microinjections and amputations

Intact planarians were injected with dsRNA three times (32 nl/injection) for seven consecutive days using a Drummond Scientific Nanoject injector (Broomall, PA, USA). Control animals were injected with green fluorescence protein (GFP) dsRNA a gene that is not found in planarians. Four hr after the third injection, planarians were amputated immediately posterior to the auricles and the resulting headless pieces were used for the phototaxis assay.

RESULTS

Process of regeneration of visual neurons

To investigate the regeneration of visual neurons during head regeneration, we performed whole-mount immunostaining with a monoclonal anti-arrestin antibody (VC-1) that is specific to photoreceptor neurons (Sakai *et al.*, 2000). Fig. 1 shows the progressive development of these neurons during the process of regeneration. In days 0 and 1 regenerants, VC-1-positive cells could not be detected. However, two days after amputation, a pair of small clusters of pigment cells and visual neurons could be detected on each side of the cephalic blastema, although no axonal projections were observed at this stage. On the third day after amputation, VC-1 positive axonal projections are seen crossing the midline of the animal in a lateral orientation. Projections from the VC-1-positive cells towards the cephalic ganglia become apparent at around 4 days after amputation, and by morphological appearance, the regeneration of the photoreceptor system appears to be complete at this stage as well. After 5 days of regeneration, the newly established visual neurons and axonal projections were observed to gradually continue their growth.

Phototactic behavior in intact and headless planarians

In order to understand how the recovery of negative phototactic behavior occurs, we developed a phototaxis assay system to measure and quantify this behavior in intact and headless planarians. Essentially, the assay consists in tracing the movements of planarians in response to light. The setup to carry out these measurements is shown in Fig. 2A, B. When we traced the movements of intact animals, all of them (n=10) moved away from the light source and reached the dark side (target quadrant) within 20.8±2.5 seconds on average. The animals then spent most of their time in the target quadrant (value±SEM, n= number that reached the target quadrant, 76.9±2.5%, n=10; Figs. 2C and 4). In contrast, when we traced the movement of headless animals, we found that they could not recognize the direction of the light and showed random movements (Fig. 2D). Most of them (9 of 10) did not reach the dark side within the assay time period (90 seconds). In contrast to the movement of intact animals, which moved away from the light, the head-

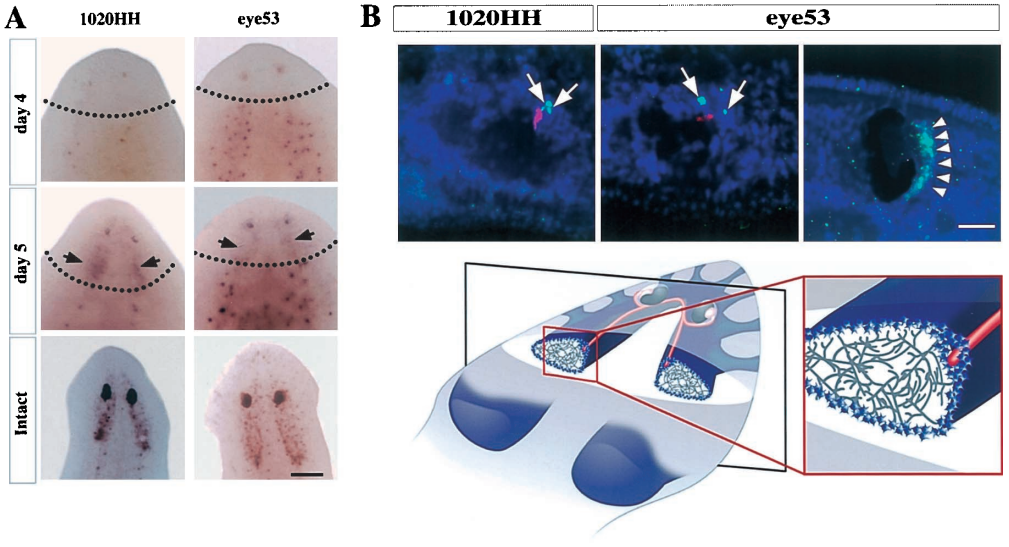

Fig. 4. (A) Expression patterns of *1020HH* and *eye53* during head regeneration. *In situ* hybridization signal is undetectable in day 4 regenerants, but detectable in day 5 regenerants. Bar, 200 μm. (B) Section *in situ* hybridization of *1020HH* and *eye53*. Detection of the target genes' mRNA is in green (arrows), while photoreceptor neurons are in red by using VC-1 antibody. Positive cells were detected in the cells surrounding the visual axons (schematic drawing in bottom panel). *eye53* is also expressed in mature photoreceptor cells (arrowhead). Bar, 50 μm.

less animals meandered independently from the direction of the incident light (Fig. 2D). As such, the aimless movement of the decapitated animals resulted in the covering of much larger distances to reach the target quadrant (35.7±0.8 mm) (Fig. 3B).Once there, the animals spent almost 0% of their time in the target quadrant (2.8±2.8%, Fig. 3B day 0). The data indicates that this assay can be used to carefully quantify planarian phototactic behavior.

Functional recovery of negative phototaxis during head regeneration

Next, we subjected animals at different stages of cephalic regeneration to the phototaxis assay described above in order to quantify their respective behaviors. Fig. 3 shows the traced movement detected and provides a quantification of the recovery of negative phototaxis during head regeneration. Fig. 3A shows the traces obtained for each day of regeneration. On day 2, although visual neurons have formed (Fig. 1), the animals were unable to recognize the direction of the light and moved in a random way similar to that observed for Day 0 decapitated animals (Fig. 2D). Recovery of negative phototaxis, however, could be detected in a few of the 3-day regenerants, a stage at which the photoreceptor neurons have already began to display the growth of axons (Fig. 1). At 4 days of regeneration, most of the animals displayed a negative phototactic response (Fig. 3A, day 4). Nevertheless, the negative phototactic response was still weak in 4-day regenerates as most of these animals did not reach the target quadrant within the 90-second duration of the assay (Fig. 3A, day 3). Strong, functional recovery of the light evasion behavior, however, was observed at 5 days after amputation, even though morphological recovery was completed on day 4 (Fig. 1). In order to better analyze the data, functional recovery was quantified and plotted graphically (Fig. 3B). The average time spent by the animals (n=10) in the target quadrant (the darkest quadrant in the assay chamber) during the 90-sec test interval was measured to determine the ability of animals to recognize the light and move to the dark end. These analyses clearly indicated that although the response was very weak at day 4 (value±SEM, n= number that reached the target quadrant, 1.4±0.9%, n=3), the negatively phototactic response to light was precipitously up-regulated 5 days after amputation (34.0±8.2%, n=8).

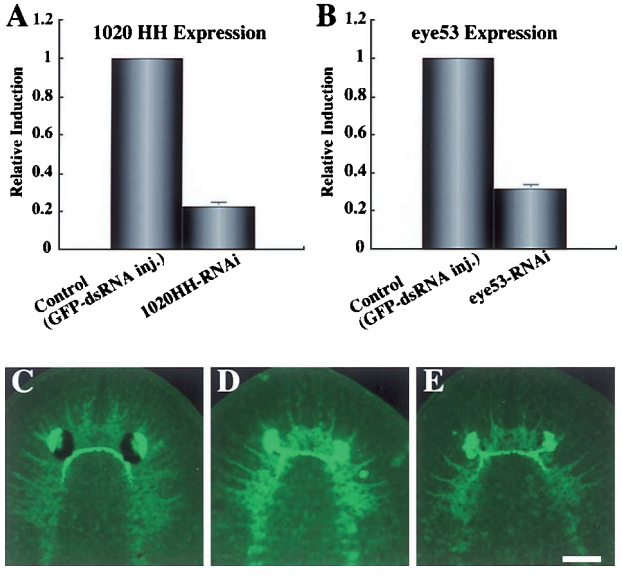

Fig. 5. mRNA levels of *1020HH* (A) and *eye53* (B) after RNAi tratment as measured by real-time PCR (mean±SEM from 3 analyses). The expression of both genes is significantly reduced by RNAi. Immunostaining of the control [GFP-dsRNA injected (C)], *1020HH*-RNAi (D) and *eye53*-RNAi (E) animals using anti-DjSYT (synaptotagmin) and VC-1 antibodies. There is no observation on morphological defects in either the brain or the visual neurons in RNAi animals. Bar, 100 μm.

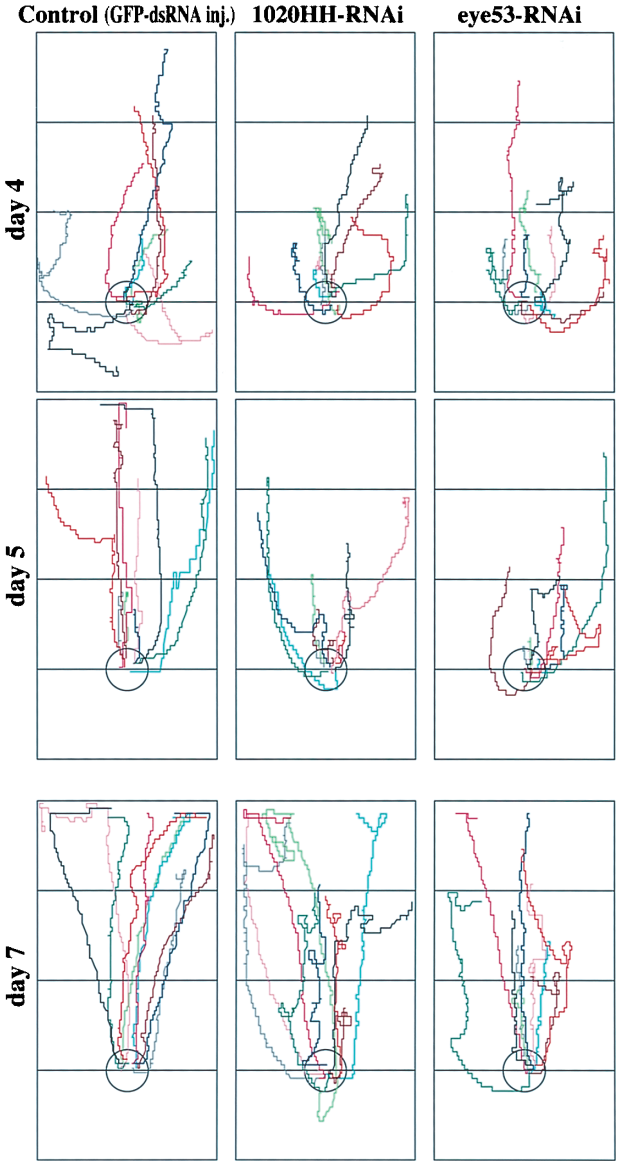

Fig. 6. Traced movement trajectories of control and RNAi-treated (*1020HH* and *eye53*) planarians at later stages of cephalic regeneration. Each animal is represented by a different color.

1020HH and *eye53* are discreetly expressed in the photoreceptor system on the fifth day of regeneration

In previous studies, we have classified neural specific genes into three classes according to their expression timing during the process of brain regeneration (Cebrià *et al.*, 2002a). Two of them, *1020HH* and *eye53*, started to be expressed in day 5 regenerants and were thus categorized as late-expressing genes (Fig. 4A) (Cebrià *et al.*, 2002a). Interestingly, when the expression patterns of *1020HH* and *eye53* are analyzed at the cellular level by carrying out *in situ* hybridizations on thin paraffin sections, we noticed that the positive cells detected surrounded the axons of the photoreceptor neurons projecting into the brain (Fig. 4B). Even though both of these genes are expressed in the few cells surrounding the visual axons, they do not appear to be co-expressed (data not shown). In addition to expression in the cells of the brain, *eye53* was also detected in the photoreceptor neurons once they had fully regenerated, *i.e.*, after their axons had properly targeted the brain. Expression timing and position of these genes suggests that they might be involved in the functional recovery of the phototactic behavior in planarians during head regeneration.

Abrogation of *1020HH* and *eye53* transcripts by RNAi affects phototactic behavior in planarians

To investigate whether *1020HH* and *eye53* are required for the functional recovery of phototactic behavior in planarians, we generated gene knockdown planarians by using RNAi and analyzed their phototactic behavior. Although reduction of *1020HH* and *eye53* mRNAs after RNAi was confirmed by real-time PCR (Fig. 5A, B), RNAi animals did not display any overt morphological abnormalities (Fig. 5C–E). However, they did present defects in their ability to functionally recover their phototactic behavior 5 days after decapitation (Fig. 6). The RNAi-treated animals when compared to controls show a statistically significant slower recovery of their phototactic behavior (Fig. 7A). In order to make sure that this difference did not arise from an effect of the RNAi on locomotor activity, we compared the velocity of movements in mm/sec between control and treated animals. The results shown in Fig. 7B clearly indicate that the rate of displacement of experimental and control animals was not

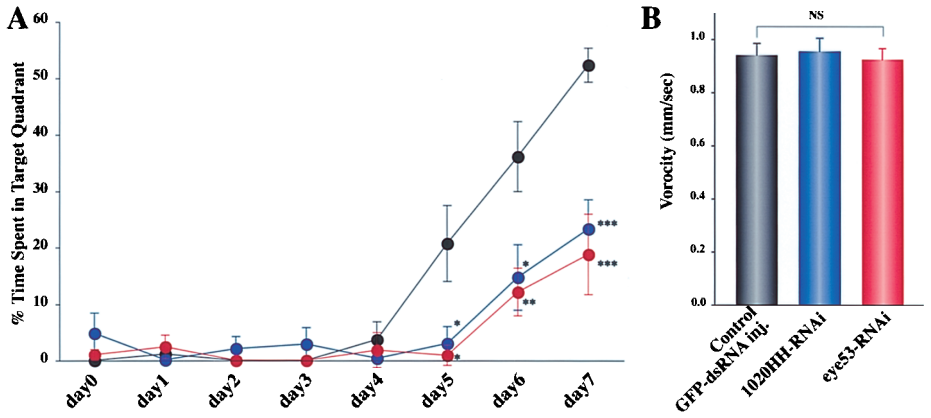

Fig. 7. Quantification of traced movement trajectories of control and RNAi-treated animals. (A) The time spent in the target quadrant represented as mean±SEM from 10 independent animals. (B) The Maximum speed during the assay of the animals. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; NS, not significantly different; one-way ANOVA followed by Tukey's multiple comparison test.

Fig. 8. Nucleotide sequence and deduced amino acid of *1020HH* and *eye53*. Signal peptides are indicated in red boxes. The complete cDNA sequence of *1020HH* and *eye53* has been deposited in DDBJ/EMBL/GenBank databases with the accession number AB126830 and AB126831, respectively.

affected by the knockdown of either *1020HH* or *eye53*.

The late regeneration expressing genes *1020HH* and *eye53* encode secreted proteins

In order to gain a better understanding of the molecular activities of *1020HH* and *eye53*, we isolated and sequenced their full-length cDNAs and the deduced amino acid composition of their products. The complete cDNA sequence of *1020HH* and *eye53* have been submitted to DDBJ/EMBL/GenBank databases under accession number AB126830 and AB126831, respectively. *1020HH* encodes a protein of 82 amino acid residues with a signal peptide in its amino terminal region (Fig. 8). The product of *eye53* consists of 85 amino acid residues and also has a signal peptide in its amino terminus. These analyses suggest that the products of both of these genes may be secreted from the cells surrounding the visual axons and thus may function to enhance the functional recovery of the visual connections.

DISCUSSION

Three steps in planarian eye regeneration

Although the genes required for eye formation have been extensively studied in planarians (Pineda *et al*., 2000, 2002; Saló *et al*., 2002), little attention has been paid to the process of regeneration of the visual system. Here, we have analyzed the process of regeneration of the planarian visual system by using a monoclonal antibody (VC-1) specific to the photoreceptor neurons (Sakai *et al*., 2000), and a novel, quantitative phototaxis assay system. Immunostaining analysis using the VC-1 monoclonal antibody revealed both the appearance and morphological differentiation of the neurons fated to become part of the photoreceptors. This process can be divided into at least three steps (Fig. 1). The first step involves the formation of two bilaterally symmetric visual-cell clusters in the dorsal side of the anterior blastema at 2 days of regeneration. The second step is characterized by the projection of contralateral axons running parallel to the anterior-most part of the cephalic ganglia. This begins sometime after the second day of regeneration (Fig. 1, day 2). At the end of this stage, no caudally directed axonal projections to the brain can be detected. The third step is defined by the appearance of posteriorly directed axonal projection onto the brain (Figs. 1 and 9).

This complex choreography of axonal ontogeny suggests that a number of regulatory events must be occurring during the late stages of regeneration to produce the stereotypical anatomy of the developed photoreceptor neurons. Cebrià *et al*. (2002a) have shown that a planarian netrin homologue (*Djnetrin*) begins to be weakly expressed 3 days after amputation when the optic nerves have just started to project onto the brain. Moreover, *Djnetrin* is expressed in the dorso-medial region of the brain where the optic nerves project. Netrins are diffusible proteins that may act as both attractive and repulsive cues depending on the receptors expressed in the axonal growth cones (Chisholm and Tesier-Lavigne 1999; Kennedy, 2000). These observations suggest that *Djnetrin* may be involved in the proper projection of visual neurons to the brain. Future investigation involving the elimination of netrin by RNAi should help define its putative role in regulating photoreceptor axonal projections. Still, the expression pattern of *Djnetrin* is insufficient to explain the chiasma formed by the left and right visual axons (Fig. 1, day 3), because no expression of *Djnetrin* has been detected by day 3 of head regeneration. Therefore, it is very likely that other as yet undetermined mechanisms might be working in the formation of the optic chiasma at the early stages of photoreceptor regeneration.

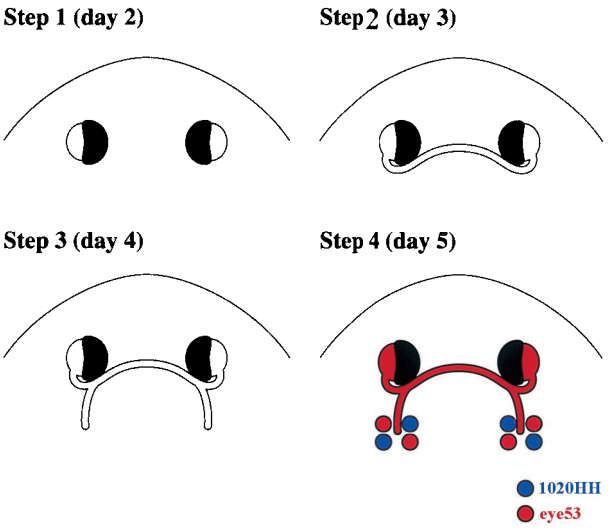

Fig. 9. Schematic drawing of the regeneration of the planarian visual system. The first step involves the formation of two, laterally restricted visual cell-clusters in the dorsal side of the anterior blastema by 2 days of regeneration. The second step is characterized by the appearance of contralateral axonal projections directed towards the midline and perpendicular to the antero-posterior axis of the animal. At this stage, no projections are detected directed towards the brain. The third step is distinguished by the appearance of posteriorly directed axonal projections from the photoreceptor neurons onto the brain. The fourth step is functional recovery. *1020HH* and *eye53* are candidates for genes involved in the final maturation of functional synapses between the photoreceptor neurons and the planarian brain.

Functional recovery of the visual system does not coincide with the completion of its regeneration

By taking advantage of the regeneration biology of planarians and the devised phototactic assay, we analyzed the process of functional recovery of the planarian visual system during head regeneration. Day 3 regenerants were found to have already established an optic chiasma, yet were unable to negatively respond to incident light. These results indicate that axonal connection between the photoreceptor neurons and the brain are required to elicit negative phototaxis in planarians. Interestingly, even though the regeneration of the optic nerves appears to be morphologically complete by the fourth day of regeneration (Fig. 1), these animals display a very weak phototactic response (Fig. 3). Robust, functional

recovery of negative phototaxis was only observed by day 5 of regeneration (Fig. 3). Taken together, the data suggests that an additional process taking place four to 5 days after decapitation may be required for the functional regeneration of the visual system. These results are consistent with previously reported data (Asano *et al*., 1998). However, the authors of this work speculated that the appearance of rhodopsin-like proteins in the regenerating eyes corresponded to the recovery of negative phototactic behavior. This is unlikely since both of the arrestin mRNA and protein (antigen of VC-1) can be detected in 2-day regenerants (Fig. 1). Furthermore, recent studies in our laboratory clearly indicate that rhodopsin mRNA can be strongly detected 2 days after amputation (data not shown), suggesting that other components may be involved in the functional recovery of day 5 regenerants.

1020HH and *eye53* are novel genes implicated in the functional recovery of phototactic behavior

In previous work, we reported on the identification of a number of CNS-specific genes whose expression became detectable only on the fifth day of cephalic regeneration (Cebrià *et al*., 2002a). RNAi of these genes, however, failed to produce detectable morphological phenotypes, precluding further functional characterization (Fig. 5C–E). We reasoned that the function of these genes may have more to do with the localized regulation of brain activity rather than with brain regeneration per se. Thus, if brain function was being affected by the RNAi treatment, we surmised that by subjecting the animals to behavioral tests we may uncover specific phenotypes. Phenotypes for both *1020HH* and *eye53* were thus detected, allowing for the establishment of a functional correlation between their timing of expression and the recovery of phototaxis in the regenerating worms.

Expression of *1020HH* and *eye53* were detected in a dorso-medial region of the brain where the planarian homeobox gene *DjotxA* is also expressed (Umesono *et al*., 1999) (Fig. 4A). Umesono *et al*. (1999) suggested that this region might be important for photo-recognition in the brain. Interestingly, after careful examination, we noticed that the positive cells are found surrounding the visual axons (Fig. 4B). Thus, the timing and location of the *1020HH* and *eye53* expression clearly imply that these genes may help regulate the visual axons and perhaps help maintain their connection to the cephalic ganglia. Analyses of animals in which *1020HH* and *eye53* were targeted for silencing by RNAi demonstrated that these genes were necessary for the functional recovery of negative phototaxis in regenerating animals (Figs 6 and 7A). In addition, silencing of *1020HH* and *eye53* did not affect locomotion (Fig. 7B), indicating that the RNAi-induced behavioral defect is specific and that these genes may play a crucial role in the functional restitution of the planarian visual system after day 5.

While we could not find sequence similarity of the open reading frames of the *1020HH* and *eye53* to any other known proteins, sequencing analysis revealed that the products of both genes may be secreted (Fig. 8). It has been reported that neurotrophic factors may be essential for the fine-tuning or activation of synaptic plasticity of the visual systems (Caleo and Maffei, 2002; Berardi *et al*., 2003). Based on our results, we speculate that the secretion of *1020HH* and *eye53* may be acting as secreted neurotrophic factors capable of promoting the formation and maintenance of functional synapses between the visual neurons and their target neurons (Fig. 9). This hypothesis will have to be tested at both the ultrastructural and electrophysiological level in order to determine if *1020HH* and *eye53* can in fact affect synaptogenesis. Interestingly, when *1020HH* and *eye53* are silenced together, no additive effect is detected (data not shown), suggesting that these two genes may participate in the same functional restitution pathway. Taken together, these data illustrate the usefulness of the phototaxis assay in helping to detect functional defects not associated with gross morphological phenotypes.

Finally, we have shown that the process of phototactic restitution includes not only the regeneration of the organ, but also its functional recovery, and that these two processes are separated from each other by at least 24 hr. Because many of the genes identified as being specific to later stages of brain regeneration failed to produce overt phenotypes by RNAi (Cebrià *et al*., 2002a), our data suggest that a battery of assays should be developed to test if the silencing of this group of genes by RNAi can cause changes in behavior in these organisms, as shown here for *1020HH* and *eye53*. The tracing assay presented here for evaluating planarian behavior should be amenable to modifications tailored to test other neural functions such as chemotaxis, mechanosensation, learning and memory. Combined with RNAi (Sánchez Alvarado and Newmark, 1999), this assay system should prove rather useful in the functional characterization of neural genes during the regeneration and maintenance of the planarian central and peripheral nervous systems.

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