Planarian homologs of *netrin* and *netrin receptor* are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture

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Summary

Conserved axon guidance mechanisms are essential for proper wiring of the nervous system during embryogenesis; however, the functions of these cues in adults and during regeneration remain poorly understood. Because freshwater planarians can regenerate a functional central nervous system (CNS) from almost any portion of their body, they are useful models in which to study the roles of guidance cues during neural regeneration. Here, we characterize two netrin homologs and one netrin receptor family member from *Schmidtea mediterranea*. RNAi analyses indicate that *Smed-netR* (*netrin receptor*) and *Smed-netrin2* are required for proper CNS regeneration and that *Smed-netR* may mediate the response to *Smed-netrin2*. Remarkably, *Smed-netR* and *Smed-netrin2* are also required in intact planarians to maintain the proper patterning of the CNS. These results suggest a crucial role for guidance cues, not only in CNS regeneration but also in maintenance of neural architecture.

Key words: Neural regeneration, Netrin receptor, Axon guidance, Planarian, *Schmidtea mediterranea*

Introduction

The precise wiring of the nervous system is essential for its proper function. During development, axons navigate in response to a variety of cues – some attractive, some repulsive – which they must interpret properly to reach their targets. The functions of several of these cues (e.g. netrins, slits, NCAMs, semaphorins, ephrins) in the wiring of the developing nervous system have been studied extensively (Araújo and Tear, 2003; Guan and Rao, 2003). During regeneration of the nervous system, damaged axons need to re-grow and project toward their targets, thereby re-establishing proper functional connections. Thus, one might expect that many of the same mechanisms that guide axons and neurons during embryogenesis also function during cell renewal and regeneration. However, due to the limited regenerative abilities of vertebrates and common invertebrate models, the roles of axonal guidance cues in central nervous system (CNS) regeneration remain to be explored.

Freshwater planarians have been classic models for studying regeneration (Brøndsted, 1969). Recent work has applied modern techniques to unravel the cellular and molecular basis of the planarian's regenerative abilities (Newmark and Sánchez Alvarado, 2002; Saló and Baguñà, 2002; Agata et al., 2003). Planarian plasticity is also shown by the ability of these animals to grow or de-grow depending on culture conditions; these processes are dependent on the balance between cell proliferation and death (Romero and Baguñà, 1991). Both regeneration and cell turnover in intact animals depend on a population of stem cells known as neoblasts (Baguñà et al., 1989; Newmark and Sánchez Alvarado, 2000), which can differentiate into all cell types in the flatworm, including neurons.

The planarian CNS consists of two cephalic ganglia (the brain) and two ventral nerve cords (VNCs) that run the length of the body (Agata et al., 1998; Cebrià et al., 2002a). After amputation, planarians can regenerate a complete CNS de novo within 1 week (Reuter et al., 1996; Cebrià et al., 2002a). Recent studies have shown both the complexity of this CNS at the molecular level, as well as a high degree of evolutionary conservation between planarian and vertebrate neural genes (Umesono et al., 1997; Umesono et al., 1999; Cebrià et al., 2002a; Cebrià et al., 2002b; Cebrià et al., 2002c; Pineda and Saló, 2002; Mineta et al., 2003; Nakazawa et al., 2003). Therefore, planarians are an attractive model in which to study regeneration and renewal of the CNS.

We report the characterization of two planarian netrins and one member of the Deleted in Colorectal Cancer (DCC) family of netrin receptors. Netrins are secreted molecules that act as chemoattractants or chemorepellents for guiding axons during development (Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995; Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996). There are two families of single-pass transmembrane receptors for netrin: DCC and UNC5. The DCC receptors mediate the attractive effects of netrins (Chan et al., 1996; Keino-Masu et al., 1996), whereas the UNC5-type mediate repulsion, either alone or by

association with DCC (Hong et al., 1999; Keleman and Dickson, 2001). The DCC family belongs to the immunoglobulin (Ig) superfamily and includes DCC and neogenin in vertebrates (Fearon et al., 1990; Vielmetter et al., 1994; Keino-Masu et al., 1996), *frazzled* in *Drosophila melanogaster* (Kolodziej et al., 1996) and UNC-40 in *Caenorhabditis elegans* (Chan et al., 1996). Recent work has suggested possible roles for netrin and its receptors in the adult nervous system as well as during the regeneration of some neural cells (Madison et al., 2000; Petrausch et al., 2000; Ellezam et al., 2001; Manitt et al., 2001; Astic et al., 2002; Manitt and Kennedy, 2002); however, no functional evidence has been presented. Outside the CNS, netrin and its receptors function in the proper morphogenesis of several tissues and organs (Hinck, 2004).

Here we show that a *netrin receptor* gene from *Schmidtea mediterranea* (*Smed-netR*) is required for the proper patterning of the cephalic ganglia and the outgrowth of the VNCs during planarian regeneration. Our results suggest that in intact and regenerating animals, *Smed-netR* and *Smed-netrin2* might function to establish and maintain the relationship between the brain and the VNCs. *Smed-netR* may mediate the response to *Smed-netrin2* in the CNS, and both genes also play roles in targeting the photoreceptor axons to the brain visual center. Finally, the morphological defects observed after *Smed-netR* and *Smed-netrin2* RNAi translate into some behavioral abnormalities. These results show that planarians are a suitable model in which to characterize the function of axon guidance cues in the regeneration and maintenance of the nervous system.

Materials and methods

Organisms

A clonal line (Sánchez Alvarado et al., 2002) of the diploid, asexual strain of *Schmidtea mediterranea* (Benazzi et al., 1972) was used. Animals were maintained at 18°C in 1× Montjuïch salts (1.6 mmol/l NaCl, 1.0 mmol/l $CaCl_2$, 1.0 mmol/l $MgSO_4$, 0.1 mmol/l $MgCl_2$, 0.1 mmol/l KCl and 1.2 mmol/l $NaHCO_3$ prepared in Milli-Q water). Planarians 4-6 mm in length were starved for at least 1 week before use and kept at 21°C.

Isolation of *Smed-netR*, *Smed-netrin1* and *netrin2* and phylogenetic analyses

Expressed sequence tag (EST) Clone H.108.3a (Sánchez Alvarado et al., 2002) encodes a predicted protein similar to the DCC family of netrin receptors. In order to identify additional 5′ sequence, a series of RACE reactions was performed. To identify planarian *netrin* homologs, we took advantage of the ongoing *S. mediterranea* Genome Project. Netrin proteins from different organisms were used in tblastn searches of *S. mediterranea* genomic sequences (NCBI Trace Archives). Genomic clones encoding predicted proteins similar to netrins were identified and assembled using Sequencher 4.2.2 (Gene Codes Corp.). Specific primers were designed to amplify both putative netrin genes. RACE was then used to obtain additional cDNA sequences.

For phylogenetic analyses, maximum likelihood trees were made with Phyml (http://atgc.lirmm.fr/phyml/) with WAG model of substitution and with a gamma distribution; node support was obtained by Tree-Puzzle5.2 (1000 quartet-puzzling replicates) (Schmidt et al., 2002).

Whole-mount immunostaining and Hoechst labeling

Immunostaining was carried out essentially as described in Sánchez Alvarado and Newmark (Sánchez Alvarado and Newmark, 1999). We used the following monoclonal antibodies: VC-1, specific for photosensitive cells (kindly provided by Kiyokazu Agata, used at 1:10,000), anti-tubulin Ab-4 (NeoMarkers, used at 1:200) to visualize the axon bundles of the VNCs and the transverse commissures, and anti-phospho-tyrosine P-Tyr-100 (Cell Signaling Technology, used at 1:500) to visualize the brain and the ganglia of the VNCs. Highly cross-absorbed Alexa Fluor 488 goat anti-mouse IgG secondary antibodies (Molecular Probes) were used at 1:400. For double immunostaining with anti-phospho-tyrosine and VC-1, after staining with anti-phospho-tyrosine, planarians were fixed in 4% formaldehyde for 1 hour at room temperature (RT), washed 3×10 minutes in PBS, blocked and incubated with VC-1 labeled using the Zenon One Mouse IgG_1 Labeling Kit (Molecular Probes) for 4 hours at RT. They were then washed 3×10 minutes in PBS and re-fixed in 4% formaldehyde. Samples were labeled with 0.5 μg/ml Hoechst for nuclear staining, mounted in Vectashield (Vector Laboratories), and observed through a Nikon TE 2000-S inverted microscope and a CARV spinning disk confocal (AttoBioscience). Images were collected using a CoolSnap HQ camera (Photometrics) and Metamorph software v6.1.

Whole-mount in-situ hybridization

After fixing and bleaching the planarians as previously described (Umesono et al., 1997), samples were loaded into an Insitu Pro hybridization robot (Intavis) and processed as described in Sánchez Alvarado et al. (Sánchez Alvarado et al., 2002). Samples were observed through a Leica MZ125 stereomicroscope or a Nikon Eclipse TE200 inverted microscope. Images were collected using a MicroFire digital camera (Optronics).

RNAi analyses

Double-stranded RNAs (dsRNAs) of *Smed-netR*, *netrin1*, *netrin2* and *semcap-1* were synthesized by in-vitro transcription (MegaScript, Ambion) and injected into planarians as described (Sánchez Alvarado and Newmark, 1999). Injected planarians were amputated prepharyngeally, allowed to regenerate, and then processed for immunostaining. For long-term experiments, intact planarians of the same size and physiological stage were used; injected planarians were starved for the entirety of the experiment. The samples were re-injected 2 or 3 weeks after the first round of injections. In all the RNAi experiments, control animals were injected with water. To analyze the efficacy of *Smed-netR* RNAi, two non-overlapping fragments of *Smed-netR* cDNA were used to synthesize dsRNA and as an in-situ hybridization probe (see Fig. S1A in the supplementary material). Similarly, two non-overlapping fragments of *Smed-netrin2* cDNA were used for dsRNA synthesis and in-situ hybridization (see Fig. S1B in the supplementary material).

Phototaxis assay

A circle of white light (NCL 150, Volpi USA) 4.4 cm in diameter was directed from above to the center of a Petri dish (8.5 cm in diameter); planarians were placed at the center of the circle and the time they needed to move toward the dark regions of the dish was measured. The assay was performed in a dark room.

Results

Smed-netR is expressed in the CNS of intact and regenerating planarians

A 4326 bp cDNA sequence with significant similarity to the DCC family of netrin receptors was obtained from *S. mediterranea* (see Materials and methods; Fig. S2 in the supplementary material). This partial *Smed-netR* included four putative Ig domains, five putative fibronectin type III repeats, a putative transmembrane domain, and a cytoplasmic portion with a region highly similar to the P3 domain (Fig. 1A; Fig.

S2 in the supplementary material). The P3 domain mediates the multimerization of these receptors and is necessary for chemoattraction (Stein et al., 2001). However, the intracellular P1 and P2 domains were not conserved in *Smed-netR*.

In intact planarians, *Smed-netR* was highly expressed throughout the CNS (Fig. 1B,C). *Smed-netR* was also expressed in the nerve ganglia of the pharynx, in cells that are likely to be the neurons of the submuscular plexus, and in the photosensitive cells. During anterior regeneration, (body pieces regenerating a new head), *Smed-netR* was first expressed in the blastema at day 1 (Fig. 1D). By day 2 *Smed-netR* was detected within two clusters of cells that correspond to the new brain primordia (Fig. 1E). As regeneration proceeded, these clusters increased in size and differentiated into the new brain, in which *Smed-netR* continued to be expressed (Fig. 1F-I).

During posterior regeneration (head pieces regenerating new pharynx and tail), *Smed-netR* was expressed within both the regeneration blastema and the new pharynx. The first signal appeared around day 3 of regeneration (Fig. 1J). At day 5, *Smed-netR* was highly expressed in the regenerated VNCs and throughout the newly forming pharynx (Fig. 1K). As regeneration proceeded, the expression of *Smed-netR* within the pharynx became restricted to the nerve ganglia (Fig. 1L).

Smed-netR is necessary for proper patterning of the CNS and peripheral nervous system during regeneration

RNA-interference (RNAi) (Fire et al., 1998) is a powerful technique for studying gene function in planarians: it results in gene-specific knockdowns throughout the animal, including the CNS (Sánchez Alvarado and Newmark, 1999; Pineda et al., 2000; Cebrià et al., 2002c; Newmark et al., 2003; Reddien et al., 2005). Following *Smed-netR* RNAi, the expression of this gene was inhibited in both the newly regenerated tissues and throughout the uninjured region (see Fig. S1A in the supplementary material). All the planarians injected with *Smed-netR* dsRNA showed consistent defects in the patterning of the regenerated nervous system (88 dsRNA-injected samples versus 73 control-injected samples that regenerated normal nervous systems).

Planarians normally regenerate cephalic ganglia indistinguishable from those observed in intact animals, with two lobes connected by a thin, anterior commissure (Fig. 2A). Nuclear staining showed the cellular organization of the new ganglia, with most of the neuronal cell bodies in the periphery surrounding a central neuropil (Oosaki and Ishii, 1965; Morita and Best, 1965; Morita and Best, 1966) (Fig. 2B). Lateral neuronal projections that extend from the cephalic ganglia toward the head periphery were evident (Fig. 2C).

After *Smed-netR* RNAi, however, the pattern of the newly regenerated cephalic ganglia was dramatically disrupted. The two ganglia appeared shorter and wider compared with controls, and the anterior commissure that connected them was thickened significantly [Fig. 2D; 91.3±3.4 μm in dsRNA-injected samples (n=16) versus 41.6±1.2 μm in controls (n=30); mean±s.e.m.; P<0.001, t-test]. Although the morphology of these ganglia was abnormal, the cell bodies were mainly localized to the periphery (Fig. 2E). The lateral

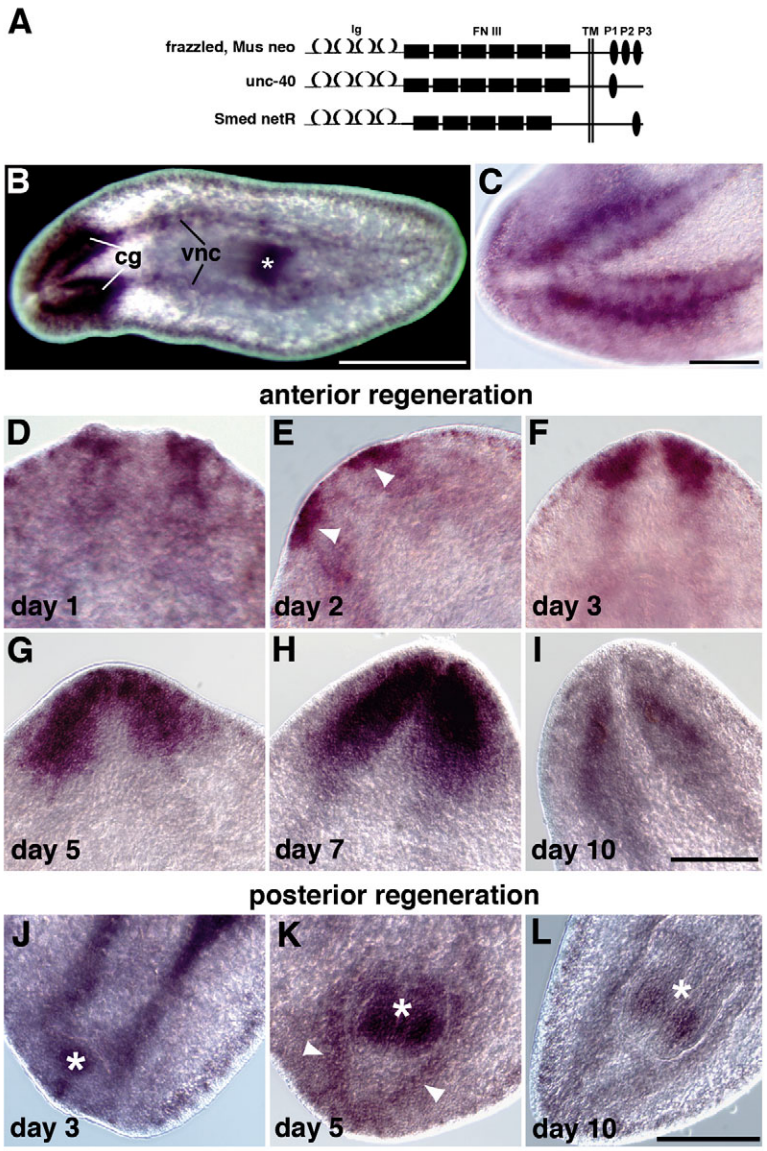

Fig. 1. Domain structure and expression patterns of *Smed-netR* in the intact and regenerating nervous system visualized by whole-mount in-situ hybridization. (A) Domain structure of Smed-netR compared to other members of the DCC family: frazzled (*Drosophila*), neogenin (mouse) and unc-40 (*C. elegans*). Smed-netR contains five predicted fibronectin type III repeats (NCBI CDD) (Marchler-Bauer and Bryant, 2004). (B,C) In intact planarians, *Smed-netR* is expressed in the cephalic ganglia, in the VNCs, in the nerve ganglia of the pharynx (asterisk) and in a few cells around the brain region. (D-I) *Smed-netR* expression during anterior regeneration. Arrowheads in E point to the brain primordia. (J-L) *Smed-netR* expression during posterior regeneration. Arrowheads in K point to the regenerated VNCs. Asterisks indicate the new pharynx. (B) Dark field image; (C-L) Nomarski differential interference contrast microscopy. All images are ventral views. (B-C) Anterior to the left. (D-L) Anterior to the top. Scale bars: 400 μm in B; 100 μm in C; 500 μm in D-I; 400 μm in J-L. cg, cephalic ganglia.

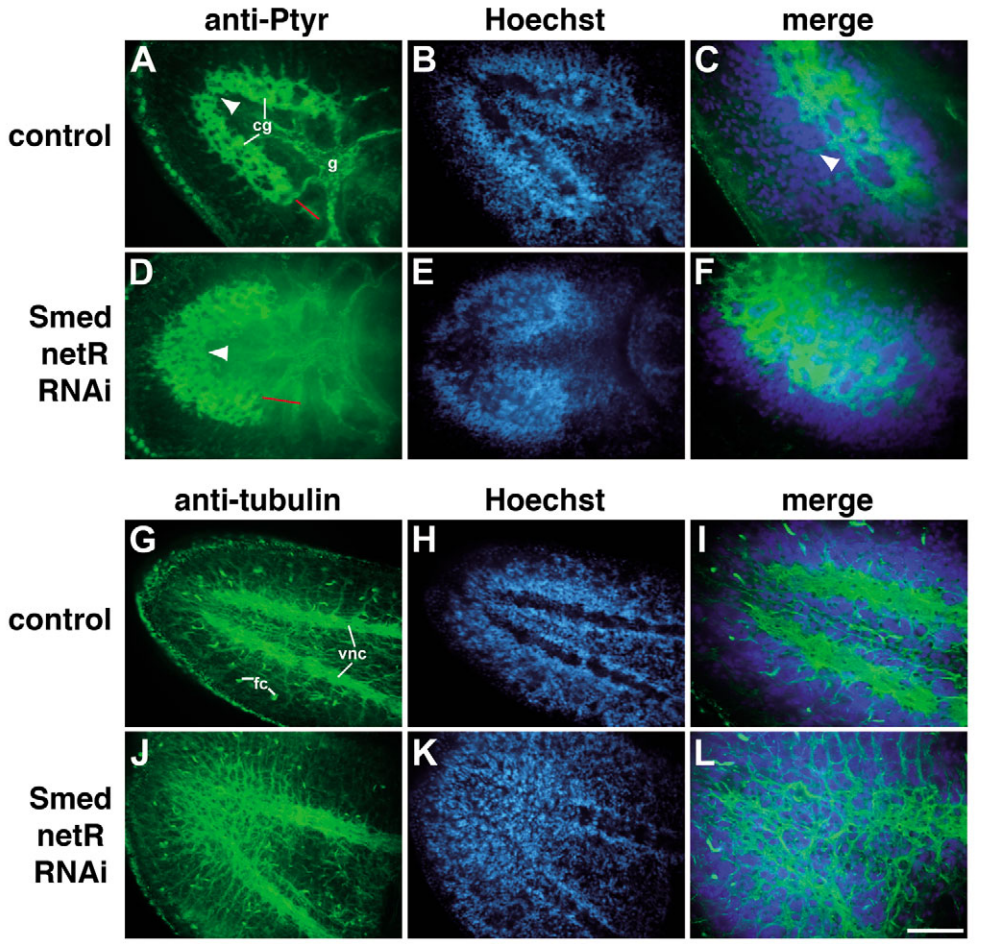

Fig. 2. Defects in anterior CNS regeneration after *Smed-netR* RNAi. (A-F) Eleven-day regenerated cephalic ganglia in control (A-C) and dsRNA-injected (D-F) animals. In A and D, arrowheads point to the brain's anterior commissure and the red lines mark the positions of the VNCs. Note the lateral expansion of the cephalic ganglia in D. (B,E) Nuclear labeling of the same planes as A and D, respectively, showing the peripheral localization of the neuronal cell bodies. (C,F) Higher magnification of merged images of A and B, and D and E, respectively. Arrowhead in C points to a lateral branch projecting from the cephalic ganglia; the branches are reduced or absent in *Smed-netR* dsRNA-injected animals (F). (G-L) Eighteen-day regenerated VNCs in control (G-I) and dsRNA-injected (J-L) animals. By contrast to the parallel nerve cords observed in G and H, the *Smed-netR* dsRNA-injected animals regenerate a disorganized neural meshwork (J,K). (I,L) Higher magnification of merged images of G and H, and J and K, respectively. (A,B) Confocal projections through 10.4 μm. (D,E) Confocal projections through 12 μm. (C,F) Merge of two single confocal planes. (G-L) Single confocal planes. Anterior to the left. Scale bar: in L, 100 μm for A-B, D-E, G-H, J-K; 50 μm for C,F,I,L. cg, cephalic ganglia; g, gut; fc, flame cells.

projections were thinner and shorter, or even absent, compared with controls (Fig. 2F). Neural cell bodies appeared to occupy the regions that normally contain lateral projections, giving rise to a continuous layer of cells (compare Fig. 2C,F).

The planarian cephalic ganglia lie dorsal to the anterior portion of the VNCs that run below them (Agata et al., 1998; Cebrià et al., 2002a). During regeneration, not only do new cephalic ganglia differentiate, but the truncated VNCs must also grow and re-establish their connections with the new brain. The VNCs regenerate as two parallel cords connected by transverse commissures (Fig. 2G). Similar to the cephalic ganglia, the cell bodies of the VNC neurons are located in the periphery of the axon bundles (Fig. 2H,I). After *Smed-netR* RNAi, the VNCs regenerated in a disorganized meshwork of axonal projections (Fig. 2J). The cell bodies of these neurons did not organize properly in two parallel cords, but rather appeared uniformly distributed in the region below the new cephalic ganglia (Fig. 2K,L).

During regeneration, the remaining, uninjured tissues are remodeled to restore proper body proportions. We analyzed the effects of *Smed-netR* RNAi on the nervous system in the uninjured, posterior region of planarians that were regenerating a new head. The submuscular plexus consists of a network of thin nerve fibers (Fig. 3A). After *Smed-netR* RNAi, the fibers of the submuscular plexus appeared wider and more disorganized than controls (Fig. 3B). Ectopic nerve fibers were observed between the two VNCs (Fig. 3D) and the bundles of nerve fibers that constitute the VNCs did not appear as tightly compacted (compare Fig. 3C,D).

During posterior regeneration, planarians normally regenerate VNCs that grow into the new tail and connect to each other by a few, thin processes (Fig. 3E). By contrast, after *Smed-netR* RNAi, the VNCs regenerated abnormally: ectopic

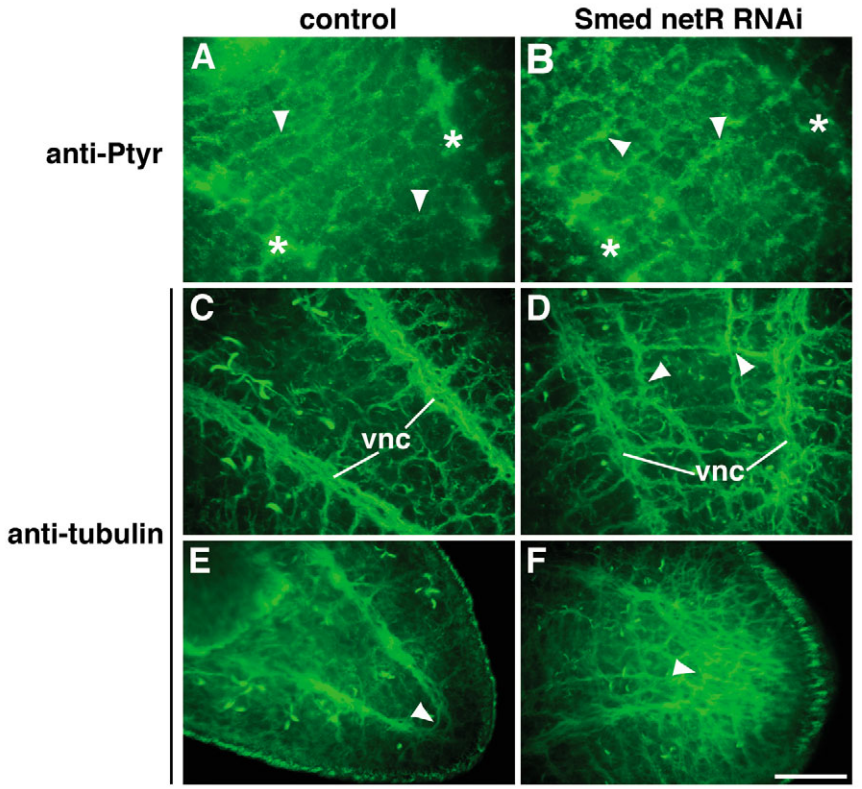

Fig. 3. Effects of *Smed-netR* RNAi upon uninjured regions of the animal and posterior regeneration. (A-B) Submuscular nerve plexus in the uninjured post-pharyngeal region of control (A) and dsRNA-injected (B) regenerating animals. The arrowheads point to some nerve fibers of the plexus. Asterisks mark the position of the VNCs deeper within the animal. (C-D) VNCs in the post-pharyngeal region of control (C) and dsRNA-injected (D) animals undergoing head regeneration. Ectopic nerve fibers appear between the VNCs of dsRNA-injected animals (arrowheads in D). (E-F) Newly formed VNCs in the regenerated tail 15 days after amputation; control (E) and dsRNA-injected (F) animals. Arrowheads in E and F point to the posterior end of the VNCs. (A) Confocal projection through 2 μm. (B) Confocal projection through 2.5 μm. (C,D) Single confocal planes. (E) Confocal projection through 2 μm. (F) Confocal projection through 2.5 μm. (A-C,E-F) Anterior to the left. (D) Anterior to the top. Scale bar: in F, 50 μm for A-D; 100 μm for E,F.

nerve fibers were found between them and a dense, disorganized neural meshwork was observed in the most posterior end (Fig. 3F). This phenotype is strikingly similar to that observed in the anteriorly regenerating nerve cords (compare Fig. 2J and Fig. 3F). These results indicate that during regeneration *Smed-netR* is required for proper patterning of the new brain and normal growth and patterning of the VNCs.

Smed-netR is required for proper targeting of the photoreceptor axons

Planarian photoreceptors consist of two pigmented eye-cups and clusters of photosensitive cells. The photosensitive cells are bipolar neurons: dendritic processes enter the eye-cup to form rhabdomeres and axonal projections grow posteriorly. Some axons project ipsilaterally to the visual center of the cephalic ganglion; others project contralaterally, forming an optic chiasm (Okamoto et al., 2005).

Following head amputation, planarians regenerate a normal visual system. An optic chiasm forms along the posterior extent of the commissure that connects both ganglia, and the visual axons project posteriorly to target the visual center in the brain (Fig. 4A). After *Smed-netR* RNAi, all animals showed aberrant axonal projections (Fig. 4A) (Newmark et al., 2003). These abnormal phenotypes could be classified into three groups: (1) those lacking posterior axonal projections toward the brain visual center, with an anterior ectopic projection along the midline (Fig. 4A; n=17/51); (2) those lacking posterior projections to the visual center with no ectopic anterior projections (Fig. 4A; n=21/51); and (3) those with more severe disruptions (Fig. 4A; n=13/51). In spite of the abnormal pattern of the cephalic ganglia and the defects in axonal targeting to the brain visual center, the projection of the visual axons along the posterior domain of the brain commissure proceeded normally in most cases (Fig. 4A). The photoreceptors are normally positioned very close to the anterior end of the brain commissure; after *Smed-netR* RNAi, however, the brain commissure was wider and extended anteriorly relative to the photoreceptors (Fig. 4A).

Photophobic response is altered after *Smed-netR* RNAi

We sought to determine whether the mispatterning of the regenerated CNS and the mistargeting of the visual axons observed after *Smed-netR* RNAi led to behavioral defects. *Smed-netR* dsRNA-treated planarians moved normally, responded to mechanical stimuli, were able to evaginate their pharynges in response to food and could eat (data not shown). However, the response to light was significantly different between *Smed-netR* dsRNA-treated animals and controls.

Planarians normally display negative phototaxis: they respond to light by moving away from the source (Taliaferro, 1920; Inoue et al., 2004). *Smed-netR* RNAi knockdown animals showed a statistically significant difference in the time required to move away from light [48.8±3.7 seconds in controls (n=34) versus 84.8±8.7 seconds after *Smed-netR* RNAi (n=37); mean±s.e.m.; P<0.005, t-test; Fig. 4B]. To ensure that the observed differences resulted from knocking down *Smed-netR*, as an additional control we tested planarians treated with dsRNA corresponding to *Semcap*-1, a gene that regulates the distribution of the transmembrane semaphorin M-SemF (Wang et al., 1999). *Smed-semcap-1* is expressed throughout the CNS and in the pharynx (see Fig. S3 in the supplementary material). *Smed-semcap-1* RNAi-treated planarians regenerated cephalic

ganglia connected by a significantly thinner anterior commissure [26.2±1.4 μm in dsRNA-injected samples (n=17) versus 41.6±1.2 μm in controls (n=30); mean±s.e.m.; P<0.001, t-test; Fig. 4B and Fig. S3 in the supplementary material]. Also, the visual axons projected much more posteriorly than controls (Fig. 4B); the ratio between the lengths of the visual axons and the cephalic ganglia from the optic chiasm was 0.49±0.01 in control animals and increased to 0.76±0.01 after *Smed-semcap-1* RNAi. These phenotypes are roughly opposite those observed after *Smed-netR* RNAi. No differences in the time required to move away from light were observed between controls and *Smed-semcap-1* dsRNA-injected animals [49.6±4.5 seconds in dsRNA-injected samples (n=18) versus 48.8±3.7 seconds in controls (n=34); mean±s.e.m.; Fig. 4B].

The slowed photophobic response of *Smed-netR* dsRNA-injected animals did not seem to be caused by slower movements. Controls and *Smed-semcap-1* dsRNA-injected animals moved away from the light following a rather direct course. By contrast, after *Smed-netR* RNAi the planarians turned more often, following much more irregular paths. These results are similar to those described for planarians with both eyes removed (Taliaferro, 1920), or after RNAi knockdowns for genes expressed either within or surrounding the photoreceptor axons (Inoue et al., 2004).

Smed-netR is necessary for maintaining neuronal pattern in intact planarians

To analyze the function of *Smed-netR* in intact adult planarians, we carried out long-term RNAi experiments. After 6 weeks of treatment, axonal projection length from the optic chiasm to the most posterior extent was significantly reduced in *Smed-netR* dsRNA-injected planarians [35.6±6.2 μm in dsRNA-injected samples (n=14) versus 63.4±4.9 μm in controls (n=22); mean±s.e.m.; P<0.005, t-test] (Fig. 5A,B). This reduction ranged from no posterior growth away from the chiasm to less drastic (but significant) shortening of visual axons. By contrast to photoreceptor regeneration, reduction in visual axon length was the only abnormal phenotype observed in the photoreceptors following *Smed-netR* RNAi in intact planarians.

Strikingly, *Smed-netR* RNAi resulted in dramatic patterning defects in the CNS of intact, non-regenerating animals. Four weeks after the first round of injections, the CNS of control animals (n=8/8) retained its normal pattern, with the brain positioned on top of the VNCs (Fig. 5C-F). Nuclear staining shows the cell bodies located normally around the periphery of both the brain and the VNCs (Fig. 5D,F). By contrast, in *Smed-netR* dsRNA-injected planarians (n=8/8), the ventral region of the brain appeared to have expanded laterally with respect to the VNCs (Fig. 5G,I). This expansion is also evident after nuclear staining with Hoechst; the cell bodies from the ventral portion of the brain were clearly separated from the cell bodies of the VNCs (compare brackets in Fig. 5F and 5J). Also, ectopic nerve fibers appeared between the VNCs in the cephalic region, giving rise to a disorganized meshwork similar to that observed during regeneration (data not shown). In the submuscular nerve plexus, the fibers appeared wider and slightly disorganized when compared with controls (Fig. 5K,M). Outside the cephalic region, ectopic nerve fibers appeared between the VNCs, which also looked loosely organized relative to controls (compare Fig. 5L and 5N). All these defects are similar to those observed during regeneration following *Smed-netR* RNAi. The defects observed in the CNS of intact planarians after *Smed-netR* RNAi were first apparent after 2 weeks of dsRNA injection (11/17 dsRNA-injected planarians showed defects in the CNS compared with 15/15

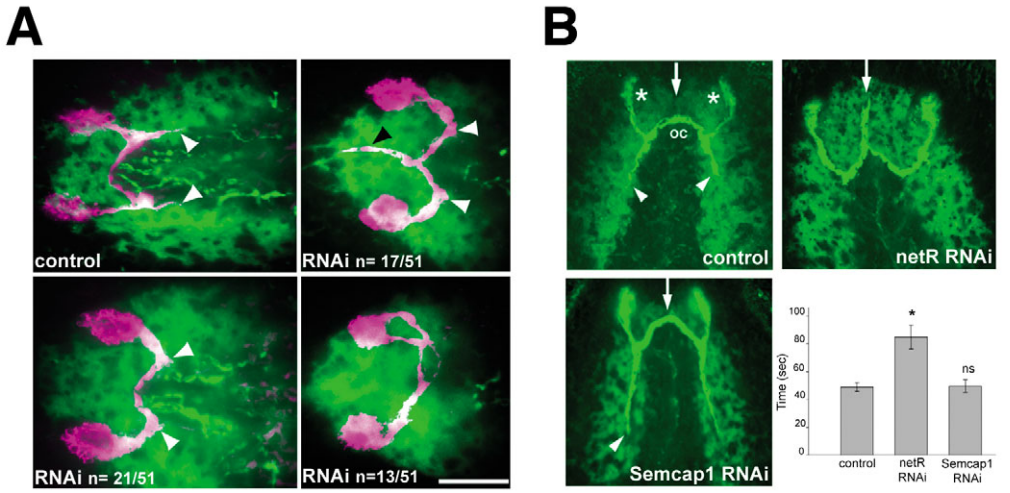

Fig. 4. Defects in visual axon targeting in regenerating planarians and in phototactic behavior after *Smed-netR* RNAi. (A) Confocal projections showing the visual axons (VC-1 staining, magenta) relative to the cephalic ganglia (anti-phospho-tyrosine staining, green). In control animals, the visual axons target the brain visual center (arrowheads, n=45/50; 5/50 show minor defects in the visual pattern different from those described below). In *Smed-netR* dsRNA-injected animals the visual axons do not project posteriorly to the brain visual center (white arrowheads). A black arrowhead in the upper right panel labels an ectopic anterior projection along the midline. All the samples are 2-week regenerants. Anterior to the left. (B) Phototaxis assays. Twenty-five days after *Smed-netR* RNAi, the negative response to light is significantly slower compared with control and *Smed-semcap1* RNAi animals (bottom right panel; *P<0.005; ns, non significant). After *Smed-semcap1* RNAi, the anterior commissure is thinner compared with controls and *Smed-netR* dsRNA-treated animals (white arrows). White arrowheads point to the posterior end of the visual axons, which project more posteriorly after *Smed-semcap1* RNAi. Asterisks indicate the position of the eye-cups. Anterior to the top. Scale bar for A: 100 μm. oc, optic chiasm.

normal controls), and could still be observed after 6 weeks (9/9 dsRNA-injected planarians showed defects in the CNS compared to 10/10 normal controls). Thus, in addition to being required for proper CNS regeneration, *Smed-netR* is also required to maintain the architecture of the CNS and visual system in intact planarians.

Two planarian netrin homologs have distinct expression patterns in the CNS

To isolate potential ligands for *Smed-netR,* we identified two planarian netrin homologs from whole genome shotgun sequences (see Fig. S4 in the supplementary material). *Smed-netrin1* encodes a predicted protein highly similar to *Djnet1*, a netrin homolog from the planarian *Dugesia japonica* (Cebrià et al., 2002a). Like *Djnet1*, *Smed-netrin1* was expressed in two rows of cells in the region where the brain and the VNCs overlap (Fig. 6A,B); it was also detected throughout the body in cells close to the lateral edges. *Smed-netrin1* also showed a pattern of expression similar to *Djnet1* during regeneration (data not shown). Given their sequence similarity and their similar expression patterns, *Smed-netrin1* and *Djnet1* are probable orthologs. *Smed-netrin2* showed a distinct expression pattern: small cell clusters were observed in the brain region posterior to the photoreceptors and along the VNCs (Fig. 6C,D). Cross-sections at the level of the cephalic ganglia showed that *Smed-netrin2*-positive cells were found mainly between the cephalic ganglia and the VNCs (data not shown). During anterior regeneration, *Smed-netrin2*-expressing cells were first observed within the blastema 2-3 days after amputation (Fig. 6E-G). At day 3 the *Smed-netrin2*-expressing cells within the blastema formed a discontinuous arc that connected the VNCs (Fig. 6F). As regeneration proceeded, the

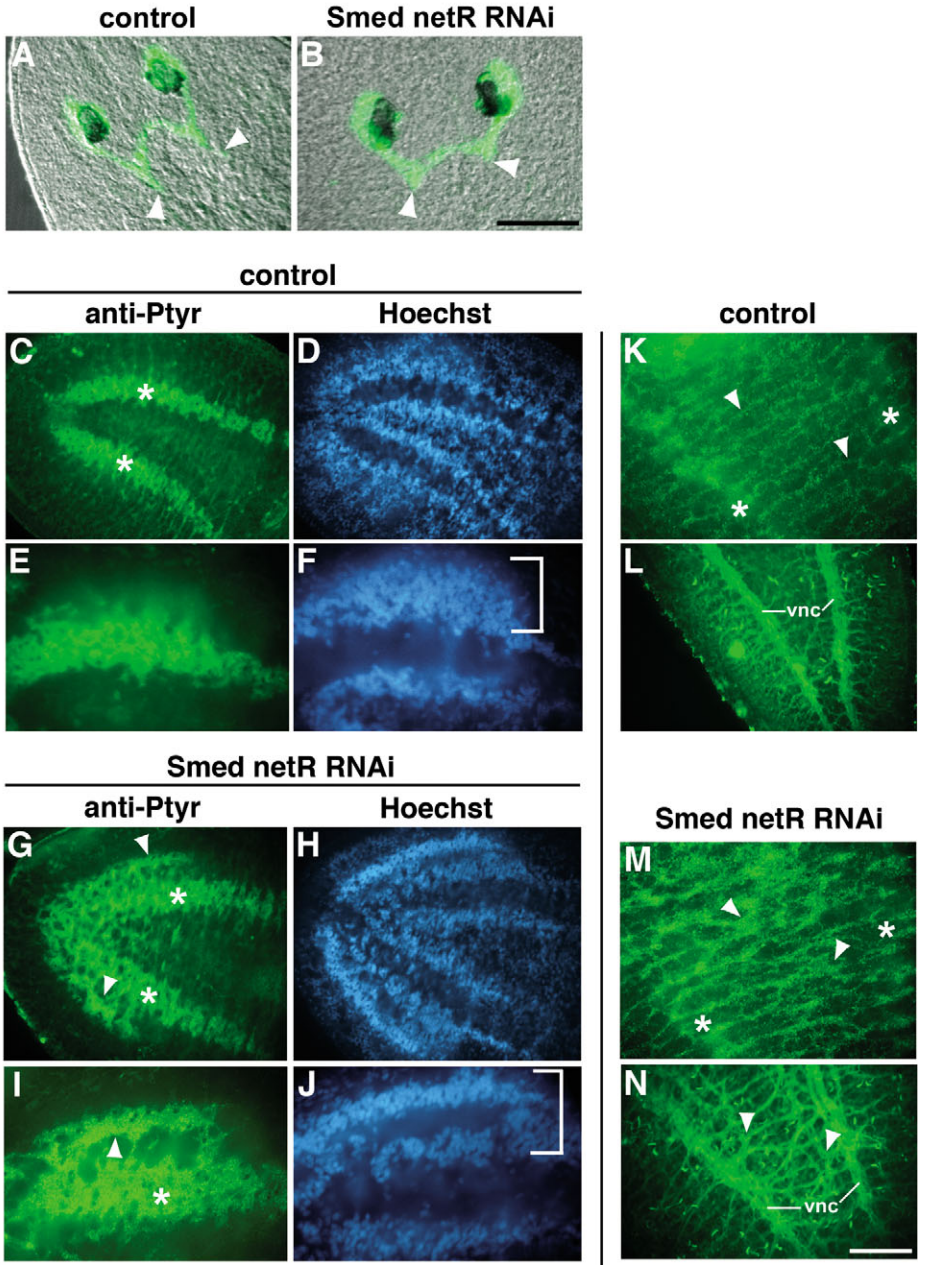

Fig. 5. Defects in visual axon projections and nervous system architecture in intact planarians after long-term *Smed-netR* RNAi treatment. (A-B) Merged confocal projections (VC-1 staining in green) and Nomarski (DIC) images (in gray), showing the visual axons of control (A) and dsRNA-injected (B) planarians. Arrowheads point to the posteriorly projecting visual axons. (C-J) Cephalic ganglia visualized by anti-phospho-tyrosine and Hoechst staining: (C-F) control animals; (G-J) *Smed-netR* dsRNA-injected animals. Single confocal sections were taken at the plane in which the ventral region of the brain overlaps with the dorsal portion of the VNCs (asterisks in C). (E,F) Higher magnification of C and D, respectively. The bracket in F indicates the juxtaposition of ventrolateral brain cells and the dorsolateral VNC cells. (G-J) The ventrolateral region of the brain (arrowheads in G and I) appears shifted laterally with respect to the VNCs (asterisks in G and I). (I,J) Higher magnifications of G and H, respectively. The bracket in J indicates the separation between ventrolateral brain cells (at the top of bracket) and the VNC cells (at the bottom of the bracket), yielding two discontinuous rows of nuclei. (K,M) Anti-phospho-tyrosine staining to visualize the submuscular nerve plexus from controls (K) and *Smed-netR* dsRNA-injected planarians (M). In K and M, arrowheads point to the plexus and the asterisks mark the position of the VNCs out of the focal plane. (L,N) Anti-tubulin staining to visualize the posterior VNCs of controls (L) and *Smed-netR* dsRNA-injected planarians (N). In N, arrowheads point to ectopic processes. All the samples shown were analyzed 4 weeks after RNAi treatment; C-J, L and N show single confocal planes; K and M show confocal projections through 2.5 μm. (A-B,K-N) Anterior to the top. (C-J) Anterior to the left. Scale bar: in B, 100 μm for A,B; in N, 100 μm for C,D,G,H,L,N and 50 μm for E,F,I,J,K,M.

Table 1. Summary of the phenotypes observed in the regenerated visual system after *Smed netrin1* and *netrin2* RNAi

	Normal	No posterior projections	Short posterior projections	Ectopic anterior projections	Others
Control	16/16	0/16	0/16	0/16	0/16
Smed netrin 1 RNAi	20/20	0/20	0/20	0/20	0/20
Smed netrin 2 RNAi	0/20	4/20	12/20	3*/20	1/20
Smed netrin 1+2 RNAi	0/20	10/20	7/20	1*/20	2/20

*Also show short posterior projections.

original pattern of two parallel rows of *Smed-netrin2*-positive cells was restored, posterior to the photoreceptors (Fig. 6G).

Smed-netrin2 RNAi phenotypes resemble those of *Smed-netR*

To examine the function(s) of these netrins in planarians, we performed a series of RNAi experiments. Following *Smed-netrin1* RNAi, planarians regenerated normally and no defects were observed, either in the cephalic ganglia or the VNCs (*n*=26/26; Fig. 7A,B). By contrast, after *Smed-netrin2* RNAi, the regenerated CNS showed phenotypes similar to those described for *Smed-netR* (*n*=28/29). Thus, the new cephalic ganglia were wider, the anterior commissure was significantly thickened, and the overall pattern was highly disorganized (compare Fig. 7C and Fig. 2D). Also, the VNCs regenerated in a disorganized meshwork of axonal projections (compare Fig. 7D and Fig. 2J). In the uninjured posterior region of an anterior-regenerating animal, ectopic projections appeared between the two VNCs, which also looked slightly disorganized (compare Fig. 7E and Fig. 3D). Finally, during posterior regeneration the VNCs grew into the blastema as a disorganized axonal meshwork (compare Fig. 7F and Fig. 3F). Defects in the architecture of the nervous system were also observed in intact, non-regenerating planarians (*n*=7/7). Two weeks after *Smed-netrin2*-dsRNA injections, ectopic projections appeared between the VNCs below the cephalic ganglia (Fig. 7G) and in the post-pharyngeal region (Fig. 7H).

With respect to the targeting of the photoreceptor axons, after *Smed-netrin1* RNAi the visual system regenerated normally (Fig. 8A). However, after *Smed-netrin2* RNAi the visual axons showed phenotypes similar to those observed after *Smed-netR* RNAi (Fig. 8B,C). In some cases there were no posterior projections from the chiasm to the brain visual center (Fig. 8B) or there were ectopic anterior projections along the midline (Fig. 8C). By contrast to *Smed-netR* RNAi, after *Smed-netrin2* RNAi short posterior projections from the chiasm were seen in the majority of cases (Fig. 8C, Table 1). *Smed-netrin1*; *netrin2* double RNAi knockdowns resulted in phenotypes similar to those observed after *Smed-netrin2* RNAi alone (Fig. 8D,E). However, the proportion of animals with no posterior projections of the visual axons increased in the double knockdowns (Table 1), suggesting that *Smed-netrin1* and *netrin2* may play synergistic roles in the proper targeting of these axons. No additional or more severe phenotypes were observed after *Smed-netrin2*; *Smed-netR* double RNAi knockdowns (Fig. 8F), suggesting that both genes may function in the same pathway.

In order to determine whether the phenotypes observed in the CNS after *Smed-netrin2* RNAi also led to behavioral defects, we tested the light responsiveness of these animals. As shown in Fig. 8G, no differences were observed after *Smed-netrin1* RNAi [38.7±3.3 seconds in controls (*n*=10) versus 38.3±5.6 seconds after *Smed-netrin1* RNAi (*n*=10); mean±s.e.m.]. However, after either *Smed-netrin2* or *Smed-netrin1; netrin2* RNAi, the regenerated animals showed a significant delay in photophobic response (80.8±23.6 seconds after *Smed-netrin2* RNAi (*n*=9) and 72.5±12.02 seconds after *Smed-netrin1*; *netrin2* RNAi (*n*=12); mean±s.e.m.; $P<0.05$; *t*-test).

Finally, we carried out long-term RNAi experiments to determine whether *Smed-netrin1* and *netrin2* play roles in maintaining photoreceptor axonal projections. After 4.5 weeks of RNAi for *Smed-netrin1* and *netrin2*, the length of the photoreceptor projections from the chiasm to the brain visual center was shortened (Fig. 8H). The ratio between visual

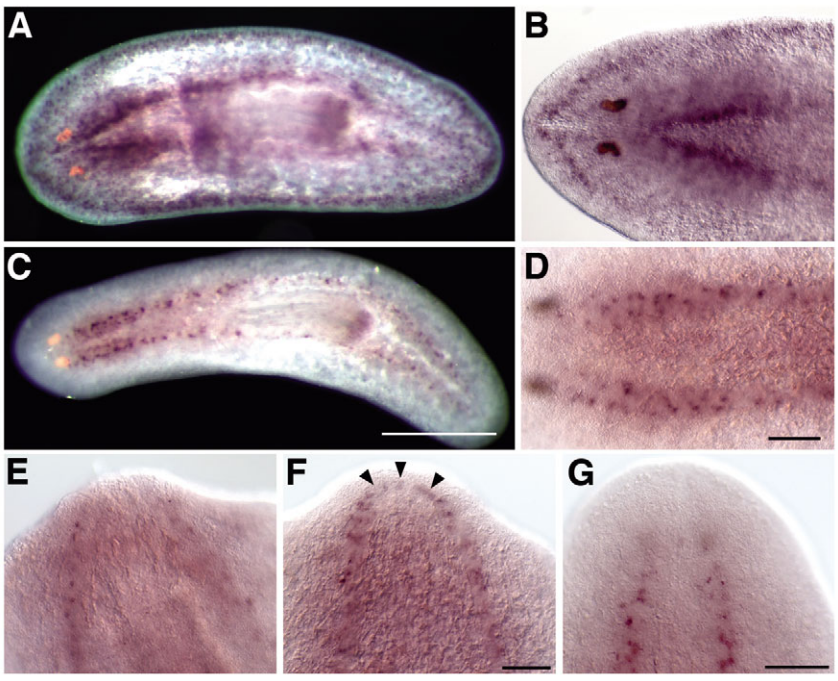

Fig. 6. Expression patterns of *Smed-netrin1* and *netrin2* in intact and regenerating CNS visualized by whole-mount in-situ hybridization. (A-D) Expression of *Smed-netrin1* (A,B) and *netrin2* (C,D) in intact animals. Expression of *Smed-netrin2* during anterior regeneration: (E) 2 days; (F) 3 days; (G) 10 days. Arrowheads in F point to positive cells within the blastema. (A-D) Anterior to the left. (E-G) Anterior to the top. Scale bars: 500 μm in A,C; 100 μm in B,D; 100 μm in E-G.

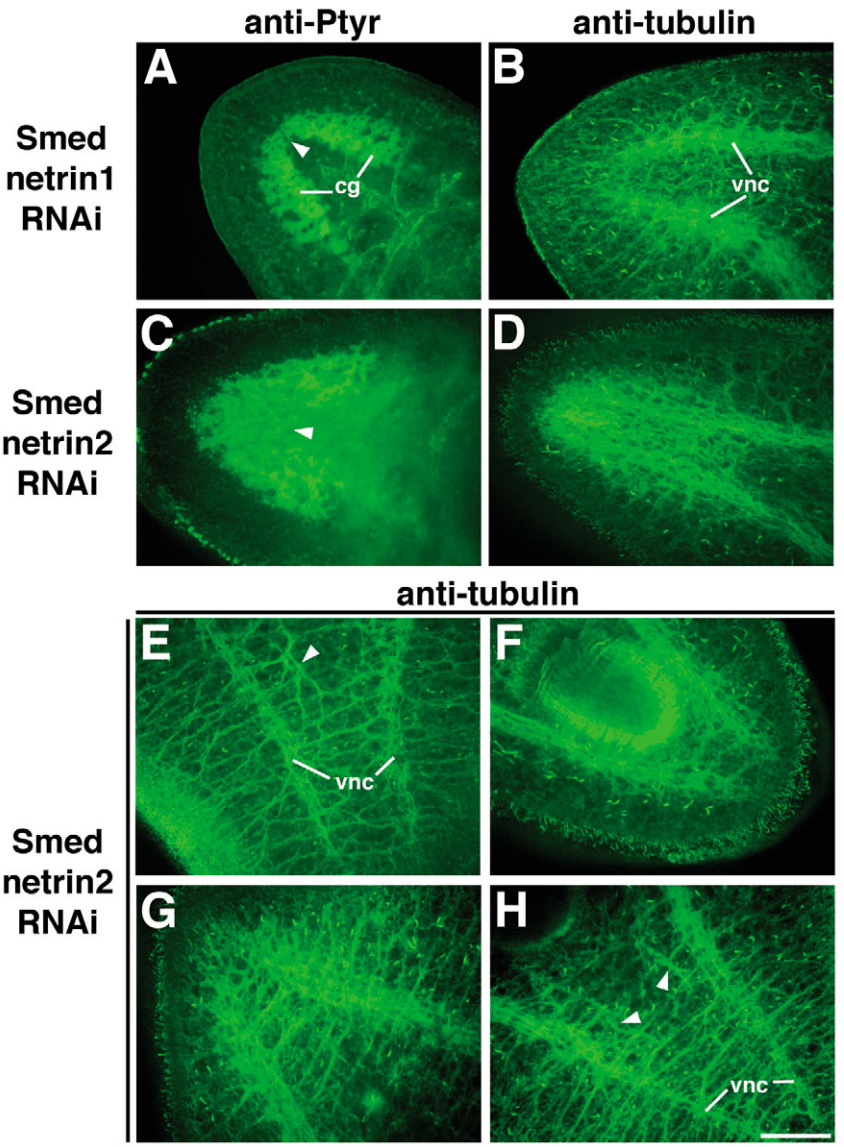

Fig. 7. Effects of *Smed-netrin1* and *netrin2* RNAi in the regeneration and maintenance of the planarian CNS. (A-B) Normal cephalic ganglia (A) and VNCs (B) regenerate after *Smed-netrin1* RNAi. Arrowhead points to the commissure connecting the cephalic ganglia. (C-D) After *Smed-netrin2* RNAi the anterior commissure is thickened (arrowhead in C) and the ganglia are wider than normal. The VNCs regenerate in a disorganized meshwork of projections (D). (E) Posterior uninjured region of an anterior regenerating animal showing ectopic processes (arrowhead) between the VNCs after *Smed-netrin2* RNAi. (F) Newly regenerated tail region showing abnormal regeneration of the VNCs after *Smed-netrin2* RNAi. All regenerants were fixed 2 weeks after amputation. (G-H) Disorganized neural pattern with ectopic axonal processes in the cephalic (G) and post-pharyngeal (H) regions of intact planarians 2 weeks after *Smed-netrin2* RNAi. B,D,E,G and H are single confocal planes; A shows confocal projections through 5.6 μm; C,F show confocal projections through 4.8 μm. (A-D,F-H) Anterior to the left. (E) Anterior to the top. Scale bar: 100 μm. cg, cephalic ganglia.

axon length and the length of cephalic ganglia from the optic chiasm was 0.55±0.03 in controls (n=10) and was significantly reduced after RNAi knockdowns of *Smed-netrin 2* (0.35±0.01; n=13; P<0.005; t-test), *Smed-netrin 1* (0.45±0.02; n=9; P<0.05; t-test) and double *Smed-netrin1*; *netrin2* (0.32±0.03; n=9; P<0.005; t-test). *Smed-netrin2* knockdowns resulted in greater reductions in visual axon length than *Smed-netrin1* knockdowns (P<0.05, t-test). Together, the above results indicate that, like *Smed-netR*, *Smed-netrin2* is required for the normal regeneration and maintenance of the planarian nervous system.

Discussion

Proper patterning of the planarian CNS during regeneration depends on *Smed-netR*

The data presented above indicate that *Smed-netR* is required for the proper regeneration of the planarian nervous system. The planarian cephalic ganglia lie dorsal to the VNCs; both structures are closely associated, yet they can be considered independent of one another (Agata et al., 1998, Cebrià et al., 2002a). During the first day of regeneration, the brain primordia appear within the blastema, before any outgrowth from the amputated VNCs can be observed (Cebrià et al., 2002a). As regeneration proceeds, both the brain and the VNCs must grow and re-establish their close association. After *Smed-netR* RNAi, this association was somehow lost and the patterns of the new brain and VNCs were disrupted: the VNCs did not grow as two parallel cords and instead formed a disorganized neural meshwork below the brain; the new cephalic ganglia were shorter and wider and extended more laterally to the VNCs. These results suggest that *Smed-netR* plays an important role in establishing the proper relationship between the cephalic ganglia and the VNCs during regeneration.

Smed-netR is also required for the patterning of the nervous system outside the cephalic region. During posterior regeneration following *Smed-netR* RNAi knockdowns, the VNCs did not grow normally and showed disorganization similar to that observed in anteriorly regenerating VNCs. Moreover, the uninjured VNCs were slightly disorganized – the axonal bundles appeared to be less tightly associated – and ectopic nerve fibers appeared between them. Similarly, the fibers that constitute the peripheral submuscular nerve plexus appeared wider and slightly disorganized. At present, however, we cannot determine whether the ectopic axonal projections derived from defasciculation, improper outgrowth of the VNCs, and/or from ectopic neurons.

During development, DCC family members play key roles in axonal growth and guidance, as well as in neural migration (Chan et al., 1996; Keino-Masu et al., 1996; Fazeli et al., 1997; Deiner et al., 1997; Gong et al., 1999; Murase and Horwitz,

2002). However, the function of *DCC* genes following CNS injury and regeneration remains unclear. Some studies have shown that in mammals *DCC* is downregulated in axotomized retinal ganglion cell (RGC) neurons (Petrausch et al., 2000; Ellezam et al., 2001) and that *DCC* is not upregulated in regenerating olfactory axons (Astic et al., 2002). By contrast, our results indicate that in planarians *Smed-netR* is upregulated within the blastema, is expressed in the newly differentiating brain as well as the VNCs, and is essential for proper regeneration of the CNS.

Smed-netR is required for targeting the photoreceptor axons to the brain visual center

Despite the stereotypical pattern of axonal projections displayed by planarian visual cells, little is known about the cues that guide these projections. The results presented here clearly indicate that silencing *Smed-netR* resulted in a failure to target properly the brain visual center during regeneration (Fig. 4) (Newmark et al., 2003). However, other aspects of the outgrowth of the photoreceptor axons were not affected; thus, the photoreceptor cells associated normally with the pigmented eye-cups and sent axonal projections posteriorly to the region where the chiasm is formed. Also, in about two-thirds of the samples, the axons projected along the posterior domain of the cephalic commissure, giving rise to a partially formed chiasm (Fig. 4A). These results suggest that different stages of the development and guidance of planarian photoreceptor axons are governed by different guidance cues; the final step in this process – targeting the appropriate brain region – seems to require *Smed-netR*. A similar situation is found in vertebrates in which different guidance cues such as Netrin and DCC (Deiner et al., 1997), Slit (Plump et al., 2002) and Semaphorins (Oster et al., 2003) play roles in the projection of the retinal axons from the retina to their targets. *Smed-netR* function in targeting the visual axons suggests that axon guidance mechanisms involved in patterning the visual system have been evolutionarily conserved.

In mammals, adult retinal axons fail to regenerate spontaneously after injury of the optic nerve; however, in an appropriate environment, injured adult retinal axons can re-grow and establish new synapses (Vidal-Sanz et al., 1987). Although *DCC* is expressed in adult rat retina and RGCs, its expression is downregulated after optic nerve lesion (Petrausch et al., 2000; Ellezam et al., 2001), suggesting that it may not contribute to RGC axon regeneration induced by a peripheral nerve graft (Ellezam et al., 2001). By contrast, goldfish retina continues to grow throughout life by adding new RGCs. In adults, *DCC* is expressed in young growing RGC axons, and during regeneration it is upregulated in all the RGCs, correlating with the regenerative capabilities of injured fish retinal axons (Petrausch et al., 2000). Similarly, *Smed-netR* is expressed in the photoreceptors of intact adult planarians and is necessary for the proper targeting of photoreceptor axons to the brain visual center during regeneration. These resemblances suggest that the capacity of fish and planarians to regenerate their visual axons may depend upon the upregulation of axon guidance cues after injury or amputation.

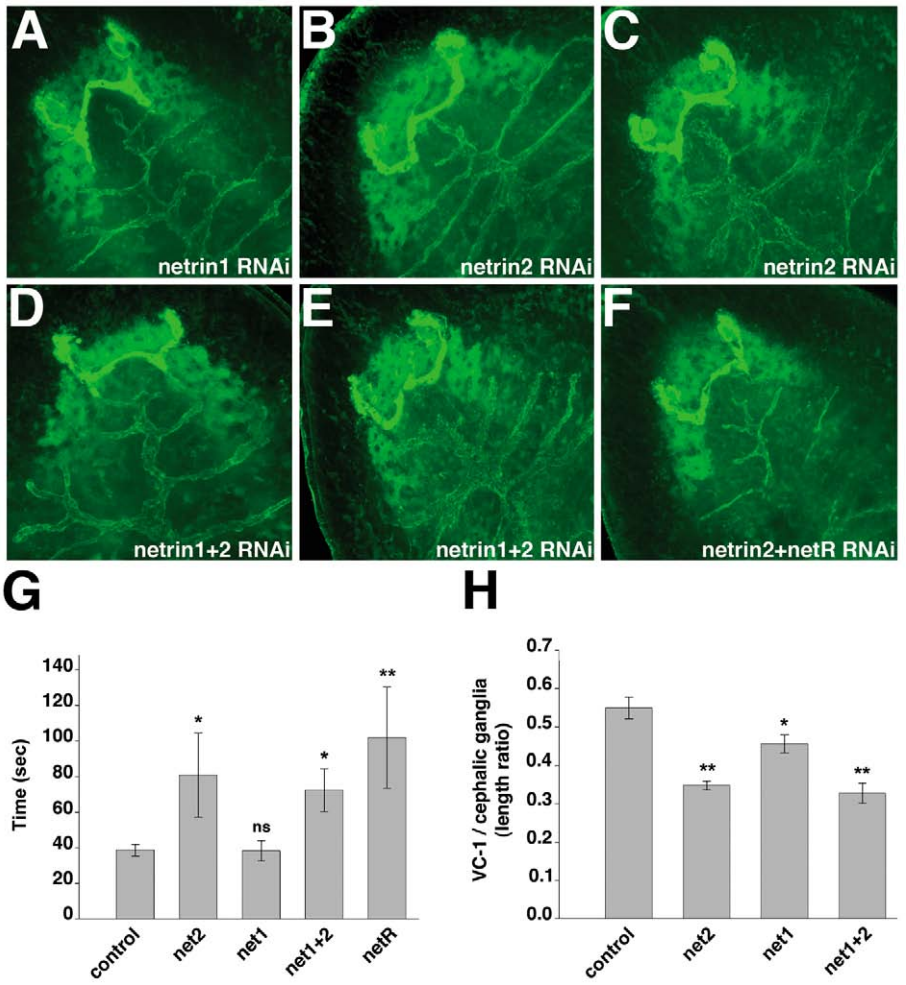

Fig. 8. Defects in visual axon targeting in regenerating and intact planarians and in phototactic behavior after RNAi for *Smed-netrins.* (A-F) Confocal projections showing the visual axons (VC-1 staining, bright green) relative to the cephalic ganglia (anti-phospho tyrosine staining, pale green) on 14-day regenerants. After *Smed-netrin2* (B,C) and *Smed-netrin1* + *netrin2* (D,E) RNAi no posterior projections (B,E) or shorter (C,D) than in controls are observed. Anterior to the upper left corner. (G) Phototaxis assay. After *Smed-netrin2*, *Smed-netrin1* + *netrin2* and *Smed-netR* RNAi the negative response to light is significantly slower compared with controls and after *Smed-netrin1* RNAi. Eighteen days of regeneration. **P*<0.05; ***P*<0.005; ns, non significant. (H). The ratio between the length of the posterior axonal projections of the photosensitive cells and the cephalic ganglia is reduced in intact planarians 4.5 weeks after *Smed-netrin1*, *Smed-netrin2* and *Smed-netrin1* + *2* RNAi treatment. **P*<0.05; ***P*<0.005.

Smed-netR function in intact planarians

Planarians grow and de-grow continuously, depending on culture temperature and food availability. Growth and de-growth are the result of alterations in the balance between cell proliferation and death (Baguñà, 1976; Romero and Baguñà, 1991). Planarians are somehow able to monitor the relative sizes of their structures and the number of cells in order to maintain body proportions, in spite of the continuous remodeling that they are undergoing (Oviedo et al., 2003).

Our results show that in intact planarians *Smed-netR* is necessary to maintain the proper architecture of the nervous system. Within a few weeks of *Smed-netR* RNAi, the cephalic ganglia expanded laterally relative to the VNCs, as if the ganglia and the VNCs were no longer properly associated. Also, ectopic nerve fibers appeared between the VNCs, which appeared to be defasciculated. These phenotypes are remarkably similar to those observed during regeneration following *Smed-netR* RNAi. Moreover, a significant shortening of the projections of the visual axons was observed in dsRNA-injected intact planarians. By contrast, control animals starved for a few weeks retained normal patterning of the brain, VNCs and visual system. The phenotypes observed in intact planarians after *Smed-netR* RNAi might be explained either by disorganization of the pre-existing structures and/or by guidance defects of newly generated cells. Further experiments are required to distinguish between these two possibilities, but the fact that structural defects were detected throughout the entire brain as early as 2 weeks after dsRNA injections suggests that *Smed-netR* plays an important role in maintaining the organization of the mature nervous system.

Expression of *netrin* and *netrin receptors* has been detected in the CNS of adult vertebrates (Manitt and Kennedy, 2002); however, their functions remain to be elucidated. Some studies suggest that Netrin-1 may function as a short-range cue mediating cell-cell interactions in the adult CNS (Manitt et al., 2001). Outside the CNS there is growing evidence that axon guidance cues also play roles in the morphogenesis of a variety of tissues and organs (Hinck, 2004). Thus, during development of the mammary gland, Netrin-1 seems to act through Neogenin as a short-range attractant mediating proper cell adhesion, rather than guidance (Srinivasan et al., 2003). Similarly, *Smed-netR* could mediate cell-cell interactions in the planarian CNS in order to establish and maintain proper connectivity between the cephalic ganglia and the VNCs, as well as to maintain bundling of the VNCs. The requirement of *Smed-netR* function for patterning the nervous system in both regenerating and intact planarians indicates that the same mechanisms may operate during regeneration of new tissues and the remodeling of pre-existing structures during growth and de-growth.

Smed-netrins are required for regeneration and maintenance of the CNS

Several studies have suggested that DCC functions as a receptor for Netrins to mediate a neuronal chemoattractant response (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Serafini et al., 1996). Here, we reported the isolation of two netrin homologs from *S. mediterranea*. *Smed-netrin2* RNAi led to defects in the CNS that were very similar to those observed after *Smed-netR* RNAi, indicating that *Smed-netR* could act as a receptor to mediate the response to *Smed-netrin2*. Although RNAi for *Smed-netrin1* did not result in CNS defects during regeneration, the observation that the double *Smed-netrin1*; *Smed-netrin2* RNAi knockdowns resulted in more severe visual axon phenotypes than *Smed-netrin2* alone (Table 1) suggests that both *Smed-netrins* may act synergistically. RNAi experiments in intact non-regenerating planarians support a role for *Smed-netrin1* in the maintenance of the visual axon pattern.

Our data clearly indicate that *Smed-netrin 2* is required not only to regenerate a proper CNS but also to maintain neural architecture in intact animals. To our knowledge this represents the first evidence of netrin function in the adult CNS. In contrast to other animals, planarian netrins are not expressed along the midline; rather they are expressed bilaterally along each of the two VNCs. This expression pattern, together with the results of RNAi knockdowns, suggests that *Smed-netrin2* probably functions to establish and maintain the structure of the CNS, rather than to guide commissural axons across the midline. The soon-to-be completed genome sequence of *S. mediterranea* will aid the identification and functional characterization of other axon guidance cues in planarians, and help us to understand how these remarkably plastic animals are able to regenerate and maintain their CNS.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/16/3691/DC1

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