Regenerating the central nervous system: How easy for planarians!

ARTICLE *in* DEVELOPMENT GENES AND EVOLUTION · JANUARY 2008

Impact Factor: 2.44 · DOI: 10.1007/s00427-007-0188-6 · Source: PubMed

1 AUTHOR:

Francesc Cebrià
University of Barcelona
34 PUBLICATIONS **1,544** CITATIONS

SEE PROFILE

Dev Genes Evol (2007) 217:733–748
DOI 10.1007/s00427-007-0188-6

REVIEW

Regenerating the central nervous system: how easy for planarians!

Francesc Cebrià

Received: 8 August 2007 / Accepted: 3 October 2007 / Published online: 13 November 2007

Abstract The regenerative capabilities of freshwater planarians (Platyhelminthes) are very difficult to match. A fragment as tiny as 1/279th of the planarian body is able to regenerate a whole animal within very few days [Morgan. Arch Entwm 7:364–397 (1898)]. Although the planarian central nervous system (CNS) may appear quite morphologically simple, recent studies have shown it to be more complex at the molecular level, revealing a high degree of molecular compartmentalization in planarian cephalic ganglia. Planarian neural genes include homologues of well-known transcription factors and genes involved in human diseases, neurotransmission, axon guidance, signaling pathways, and RNA metabolism. The availability of hundreds of genes expressed in planarian neurons coupled with the ability to silence them through the use of RNA interference makes it possible to start unraveling the molecular mechanisms underlying CNS regeneration. In this review, I discuss current knowledge on the planarian nervous system and the genes involved in its regeneration, and I discuss some of the important questions that remain to be answered.

Keywords Planarian · Central nervous system · Neural genes · Regeneration · Axon guidance · Neurotransmission

Communicated by R.J. Sommer

F. Cebrià (✉)
Departament de Genètica, Facultat de Biologia and Institut de Biomedicina de la Universitat de Barcelona (IBUB),
Av. Diagonal 645, edifici annex, planta 1,
08028 Barcelona, Catalunya, Spain
e-mail: fcebrias@ub.edu

Introduction

Freshwater planarians display a biological feature that has fascinated scientists since it was first reported (Pallas 1766): They can regenerate whole animals from tiny pieces of their bodies. Although the ability to regenerate is a phenomenon found to a greater or lesser extent in many metazoans (Sánchez Alvarado 2000), very few species can compete with planarians. Planarians were defined as "… almost immortal under the edge of the knife" (Dalyell 1814), and in the late 1890s, authors such as Randolph (1897) and Morgan (1898) published several papers describing the amazing regenerative abilities of these animals. In the first half of the twentieth century, several groups carried out experiments to address issues such as how the regenerative blastema is formed, the distribution of neoblasts (planarian totipotent stem cells) in intact and regenerating animals, whether inductive or inhibitory mechanisms are involved in regeneration, or the role of the nervous system in the regenerative process (for a review of the classical literature, see Brondsted 1969). However, some of these basic questions still remain to be satisfactorily answered.

Research on planarian regeneration has been revitalized in the last 10 years by the application of modern molecular tools (Agata et al. 2003; Agata and Watanabe 1999; Baguñà 1998; Newmark and Sánchez Alvarado 2002; Reddien and Sanchez Alvarado 2004; Saló 2006; Sánchez Alvarado and Kang 2005). These new tools include whole-mount immunostaining and confocal microscopy (Cebrià et al. 1997; Reuter et al. 1995b; Reuter et al. 1996a), whole-mount in situ hybridization (Umesono et al. 1997), RNA interference (RNAi; Newmark et al. 2003; Reddien et al. 2005; Sánchez Alvarado and Newmark 1999), BrdU labeling of neoblasts (Newmark and Sánchez Alvarado 2000), expressed sequence tag (EST) collections (Ishizuka

et al. 2007; Sánchez Alvarado et al. 2002; Zayas et al. 2005), and a genome sequencing project (further information on the white paper proposal can be obtained from http://www.genome.gov/12512286). The use of these tools should allow us to improve our understanding of the regenerative process in planarians.

Unlike most invertebrate and vertebrate animals, planarians can regenerate a complete, functional central nervous system (CNS) in only a few days. Although, at first sight, planarians and their nervous system may appear simple, a closer look reveals greater complexity. This complexity is found at many levels: (1) At the cellular level, we find unipolar, bipolar, and multipolar neurons; (2) at the structural level, different types of vesicles and release sites have been described along with a wide range of neuroactive substances, including serotonin, dopamine, noradrenaline, and several neuropeptides (McVeigh et al. 2005; Reuter and Gustafsson 1995; Ribeiro et al. 2005); and finally, (3) at the gene level, the first high throughput analyses of the planarian CNS have shown its complexity in the number and degree of conservation of neural specific genes (Mineta et al. 2003), as well as in the molecular compartmentalization of the planarian CNS (Cebrià et al. 2002b; Nakazawa et al. 2003; Umesono et al. 1999). Despite this weight of accumulated knowledge and the newly available molecular markers, we are still some way from fully understanding how the planarian CNS is regenerated. After cutting a planarian into, say, ten pieces, it is remarkable to see how, in only a few days, each of these fragments regenerates a new head, where the new photoreceptors are easily distinguished and in which a new brain differentiates. However, although this process seems very easy for a tiny planarian fragment, it involves many events that have yet to be fully elucidated, such as how neoblasts become committed to a neural fate, how the new nervous system is rewired, how the regenerated nervous system integrates with the preexisting CNS, and how the nervous system itself influences planarian regeneration.

In this review, I will first discuss previous research characterizing the planarian nervous system at different levels and recent studies that have identified numerous planarian neural genes. I will then describe what we have learned so far about planarian neural regeneration by using these new markers and modern methods. Finally, I will discuss the available data on the influence that the nervous system may have on planarian regeneration.

Structure of the planarian nervous system

Freshwater planarians (Platyhelminthes) are among the simplest animals possessing a centralized nervous system. For a long time, Platyhelminthes were thought to occupy a basal position in metazoan phylogeny, and that led many scientists to focus on the "primitive" CNS of these organisms. In recent years, however, the phylogenetic position of Platyhelminthes has been reconsidered, and it is now widely accepted that they occupy a less basal position and are included within the Lophotrochozoan clade (Baguñà and Riutort 2004; Philippe et al. 2005; Ruiz-Trillo et al. 2002). However, the exact relationship of the Platyhelminthes with the other Lophotrochozoan phyla remains to be elucidated. Irrespective of the final phylogenetic position of Platyhelminthes, freshwater planarians display several features that make them interesting for scientists, the most popularly known being their remarkable regenerative capacity.

The planarian CNS consists mainly of an anterior brain or cephalic ganglia and a pair of ventral nerve cords (VNC) that run along the length of the animal (Fig. 1). Usually, the brain is bilaterally symmetrical, and its shape is frequently correlated with the shape of the head. The two halves of the brain are connected by one or more commissures, depending on the species (Agata et al. 1998; Cebrià et al. 2002c; Hyman 1951; Lentz 1968; Reuter et al. 1995b; Reuter et al. 1996a; Rieger et al. 1991). The brain is organized as a central neuropil surrounded by an outer layer of neuronal cell bodies (Morita and Best 1965; Morita and Best 1966; Oosaki and Ishii 1965), and it displays a typical spongy texture, as it is traversed by muscles and processes from secretory cells (Baguñà and Ballester 1978). A large number of different sensory nerves leave the brain and connect with the margins and sensory organs of the head (Hyman 1951; Rieger et al. 1991). Thus, MacRae (1967) described sensory nerve endings within the epithelium of the auricles of *Dugesia tigrina*. Because those cells looked similar to vertebrate olfactory cells, it was suggested that they could function as chemoreceptors (MacRae 1967). The planarian brain lies on top of the VNC that run below it (Agata et al. 1998; Cebrià et al. 2002c; Okamoto et al. 2005). In some cases, these cords continue anterior to the brain (Hyman 1951). Both at the molecular and morphological level, the brain and VNC can be considered as independent structures (Agata et al. 1998; Cebrià et al. 2002c; Okamoto et al. 2005). Although several pairs of nerve cords are observed (including dorsal and lateral cords) in lower Platyhelminthes and marine planarians, in most freshwater planarians, only the ventral cords are present (Hyman 1951). Based on comparisons of the nervous system in different groups of Platyhelminthes, it has been suggested that there is an evolutionary tendency toward a reduction of the number of nerve cords (Reisinger 1972). Along the length of the planarian VNCs, small clusters of neurons are grouped in more or less regularly spaced ganglia that, similar to the brain, have a spongy structure (Baguñà and Ballester 1978); from these ganglia,

transverse commissures extend connecting both VNCs and forming an orthogon (Fig. 1). Also, lateral nerves extend from the VNC and form bundles that branch near the edge of the animal and are continuous with a fine marginal plexus representing the submuscular nerve plexus (Lentz 1968).

In addition to the CNS, several peripheral nerve plexuses have been described in contact with the main cords in planarians (Koopowitz and Chien 1974; Koopowitz and Chien 1975; Reuter and Gustafsson 1995). In triclads, a subepidermal plexus is generally found (Hyman 1951). This plexus appears as a diffuse network of very thin processes (Baguñà and Ballester 1978); within it, the cells are sparse, bipolar, or multipolar, and consist of spindle-shaped ganglion and sensory cells (Lentz 1968). From this plexus, some nerve fibers penetrate the epithelium (Baguñà and Ballester 1978; Reuter and Gustafsson 1995). Also, some nerve bundles connect this subepidermal plexus with the submuscular one, situated below the muscle layers of the body wall (Baguñà and Ballester 1978; Lentz 1968). This submuscular plexus contains a fairly regular network of fibers with a small number of nerve cell bodies observed within it (Baguñà and Ballester 1978). Most cells in this plexus seem to be bipolar, although unipolar and multipolar cells can also be found (Lentz 1968). Other nerve plexuses are found around the gut diverticula, within the pharynx (one external and one internal), and around the testis and ovaries of mature sexual planarians (Baguñà and Ballester 1978; Reuter and Gustafsson 1995).

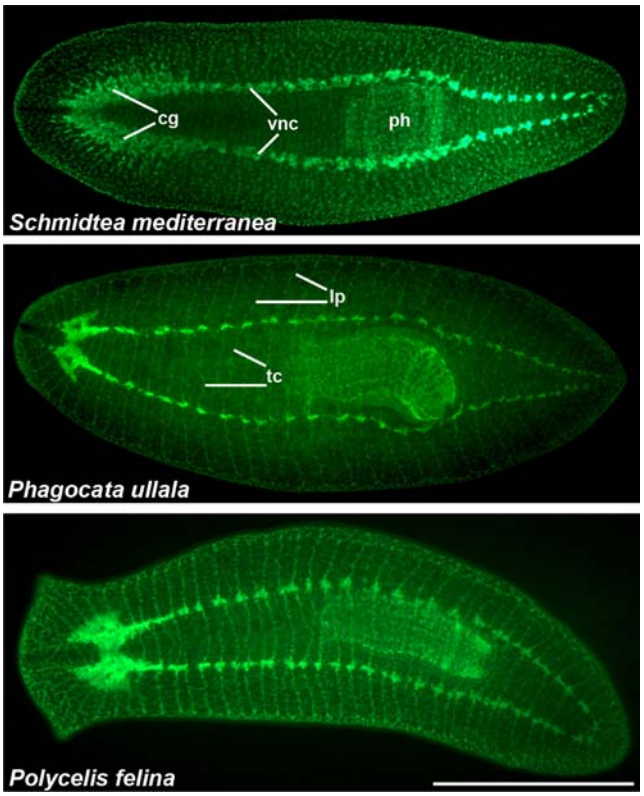

Fig. 1 Planarian CNS. Whole-mount immunostaining with an anti-synapsin (3C11) antibody in three different species of freshwater planarians. Note the differences in the CNS between different planarian species. *Phagocata ullala* and *Polycelis felina* possess smaller and more compact brains. Also, their VNCs display a more defined orthogonal pattern than *Schmidtea mediterranea*. Anterior is shown to the *left*. *cg* Cephalic ganglia, *vnc* ventral nerve cord, *ph* pharynx, *tc* transverse commissure, *lp* lateral process. Scale bar, 1mm for *S. mediterranea*, 0.8 mm for *P. ullala* and 0.5 mm for *P. felina*

Types of planarian neurons

Together with unipolar neurons, similar to those found in invertebrates, freshwater planarians possess bipolar and multipolar neurons in their nervous system (Bullock and Horridge 1965; Lentz 1968; Rieger et al. 1991). Up to seven types of neurons besides glia-like cells were classically identified by cytological methods based on their size, number of processes, and properties of the nuclei (reviewed in Bullock and Horridge 1965). Golgi impregnation and methylene blue staining revealed the complexity of the branching pattern in the processes of different planarian neural cells (Bullock and Horridge 1965). In planarians, bipolar neurons are typically found in transverse commissures between the VNCs, where they can function as interneurons (Rieger et al. 1991). Also, sensory neurons are usually bipolar (Reuter and Gustafsson 1995). At the fine structural level, glia-like cells have been described between brain neurons and along the VNCs (Morita and Best 1966). These accessory or supporting cells lack vesicles and have very little cytoplasm with very few organelles (Golubev 1988; Reuter and Gustafsson 1995). Although glia-like cell bodies are difficult to distinguish (Rieger et al. 1991), sheathing of axons and commissural fibers has been described in the neuropil of planarians (Trawicki et al. 1988). However, the presence of glial cells in the planarian nervous system has yet to be convincingly shown by the use of specific molecular markers. Two novel genes named *1020HH* and *eye53*, with no similarity to sequences in current databases, are expressed in discrete cells in the brain and along or surrounding the VNCs; these genes could be associated with some kind of supportive role for neural cells (Cebrià et al. 2002b, c). *1020HH* and *eye53* are also expressed surrounding the axons of the photosensitive cells, and as they code for putative secreted molecules, it has been proposed that they could be neurotrophic factors required in the synapses between the photosensitive cells and their targets (Inoue et al. 2004). Recently, a planarian homologue of *slit* (an axon guidance molecule) has been shown to be expressed along the midline of *Schmidtea mediterranea* (Cebrià et al. 2007). In both vertebrates and invertebrates, *slit* is expressed in glial cells located along the midline, where it functions as a

repulsive cue (reviewed in Dickson and Gilestro 2006). As planarian *slit* shows a conserved repulsive role at the midline (Cebrià et al. 2007), the possibility is raised that *slit*-positive cells could be some kind of planarian glial cells. Further characterization of these cells and detailed expression and functional analyses of planarian homologues of genes such as *gcm* (glial cells missing), glial maturation factor B, and glial factor-1, identified from an EST database (Zayas et al. 2005) should help to reveal the distribution of glia-like cells in the planarian nervous system.

Planarian neurons are characterized by a high content of secretory vesicles (Oosaki and Ishii 1965; Reuter and Gustafsson 1989). The different types of vesicles have been used to distinguish different types of neurons in the planarian brain. Thus, Oosaki and Ishii (1965) described three types of vesicles in the brain of *D. gonocephala*: small clear vesicles, dense vesicles, and neurosecretory vesicles, which apparently were not seen intermingled in the same nerve cells. This large number of vesicles indicates a secretory nature, and in fact, the term neurosecretory has long been used to describe the planarian nervous system (reviewed in Reuter and Gustafsson 1995; Rieger et al. 1991). Neurosecretory monopolar cells are numerous on the posteroventral surface of the brain of *Polycelis nigra* (Lender and Klein 1961). Neurons possessing dense vesicles were found to be associated with the photoreceptors of *D. dorotocephala*; their number changes with the circadian cycle, and they may secrete melatonin (Morita et al. 1987; Morita et al. 1988).

Neuroactive molecules

The presence of various neuroactive substances has been observed in all groups of Platyhelminthes using a variety of approaches (reviewed in McVeigh et al. 2005; Reuter and Gustafsson 1995; Ribeiro et al. 2005; Rieger et al. 1991). As planarians lack a circulatory system, it is thought that neurosecretory substances are released close to the target cells. Immunoreactivity to serotonin (5-hydroxytryptamine [5-HT]) has been reported in a few species of freshwater planarians, including *Girardia tigrina* (Reuter et al. 1995b), *Dendrocoelum lacteum*, and *Polycelis tenui* (Reuter et al. 1996a), as well as in the terrestrial planarian *Bipalium kewense* (Fernandes et al. 2003). In most cases, serotonin-positive cells are found in the CNS, in both the brain and the VNCs, as well as in the peripheral nerve plexuses, such as the pharyngeal, submuscular, and subepithelial plexuses. Also, immunostaining against gamma-aminobutyric acid (GABA) has been shown in the CNS and the subepidermal plexus of *G. tigrina* (Eriksson and Panula 1994). Two families of neuropeptides, the FMRFamide-like peptides and the neuropeptide Fs, have been shown to be present in planarians (McVeigh et al. 2005). An antiserum against neuropeptide F from *Moniezia expansa* (Maule et al. 1992) labels the CNS and peripheral plexuses (submuscular and pharyngeal) of freshwater (Reuter et al. 1995b) and marine (Reuter et al. 1995a) planarians. In addition, immunohistochemistry with antibodies against other invertebrate neuropeptides labels the nervous system of different groups of Platyhelminthes (reviewed in Reuter and Gustafsson 1995), for instance, enkephalin-positive neurons in planarians (Venturini et al. 1983).

In the various groups of Platyhelminthes (including planarians) analyzed, aminergic and peptidergic substances do not seem to colocalize, leading to an apparent compartmentalization of the different neuronal populations (Maule et al. 1990; Reuter et al. 1996a; Reuter and Palmberg 1989; Wikgren and Reuter 1985). Further analyses with additional markers against different neuroactive molecules should allow a fine map of the planarian nervous system to be developed and the different neuronal populations characterized.

Gene expression in the planarian CNS

Umesono et al. (1997) reported the first whole-mount in situ hybridization of a planarian gene (an *orthopedia* homologue) expressed in the CNS. Later, the same authors showed how planarian homologues of the Otx family defined different compartments in the brain of *D. japonica* (Umesono et al. 1999). Thus, *Djotp* is expressed in the lateral branches of the brain, *DjotxB* is expressed in the spongy central region of the brain, and *DjotxA* is expressed in more medial regions. In an attempt to identify a large number of neural genes in planarians, the laboratory of Kiyokazu Agata constructed a complementary DNA (cDNA) library from the planarian head. From this library, 3101 non-redundant ESTs were reported; blast homology searches revealed that 116 of those clones showed significant similarity to genes related to the nervous system (Mineta et al. 2003). More than 95% of those planarian neural genes were found in *Drosophila*, *Caenorhabditis elegans*, and humans, indicating a high degree of conservation. Among them, 42 clones were associated with neurotransmission (choline transporter, acetylcholine receptor, and neuropeptide Y receptor), 33 clones were associated with axon guidance (N-CAM and netrin), 21 clones were categorized under brain morphogenesis/neural differentiation (frizzled, noggin, and the BMP receptor), and 21 clones showed homology to genes involved in sensory systems (arrestin, rhodopsin, and mechanosensory protein 2; Mineta et al. 2003). Interestingly, two of those clones showed homology to vertebrate genes such as sortilin1 and noggin that are not found in *Drosophila* or *C. elegans*

(Mineta et al. 2003). A second approach that the laboratory of Kiyokazu Agata took to isolate neural-specific genes was to construct a microarray to compare head and trunk cDNAs on the basis that the cephalic ganglia occupy most of the head. The microarray contained 1640 non-redundant ESTs (Nakazawa et al. 2003). Whole-mount in situ hybridizations showed that, among the 30 genes with highest head/trunk expression ratio, 23 of them were expressed in the cephalic ganglia (Nakazawa et al. 2003). Those neural genes included planarian homologues of N-CAM, FGF receptor-like, acetylcholine receptor, and synaptotagmin; interestingly, 8 out of those 23 genes showed no sequence similarity to known genes (Nakazawa et al. 2003).

Whole-mount in situ hybridizations with some of the genes identified through the strategies described above, together with data from other laboratories, clearly show that, although the planarian brain may appear quite simple at the morphological level, at a molecular level, it is rather more compartmentalized. Although most genes that are expressed in the cephalic ganglia are also expressed in the VNCs, several examples of genes specifically expressed in the brain have been reported. Those examples include *nou-darake* (Cebrià et al. 2002a), *brain factor-1* (Koinuma et al. 2003), and *DSCAM* (Fusaoka et al. 2006). In contrast, to my knowledge, no gene expression specific to the VNCs has yet been described. The three molecular domains into which the planarian brain was first divided by the expression of homologues of the Otx family (Umesono et al. 1999) can be further subdivided (Cebrià et al. 2002b). Thus, some genes are specifically expressed in the lateral branches of the brain, which project to the head margins; those genes include homologues of *otp* (Umesono et al. 1997), *noggin* (Ogawa et al. 2002a), the *Six/so* family (Pineda and Salo 2002), a glutamate receptor (clone 1008HH), and a GTP-binding protein Gi1 alpha subunit (Cebrià et al. 2002b). Even within the brain branches, some genes such as a *GTP-binding protein Gi1* alpha subunit and *synaptotagmin VII* are mainly expressed in the distal half of those lateral branches (Cebrià et al. 2002b). Other genes are expressed in the central spongy region of the brain but not in the lateral branches; among those genes, we find *Pax6* (Pineda et al. 2002), *wnt-5* (Marsal et al. 2003), a glutamate receptor (clone 1406), and novel genes *1020HH* and *Eye53* (Cebrià et al. 2002b; Inoue et al. 2004). Recently, it has been reported that planarian homologues of *Wnt* and *frizzled* genes show complementary expression patterns within the brain, as *DjWntA* is expressed in the posterior half of the brain, whereas *DjfzA* is detected in the anterior half (Kobayashi et al. 2007). Table 1 summarizes some of the neural genes characterized in freshwater planarians.

In summary, a large number of neural-specific genes defining different molecular domains in the planarian CNS (Fig. 2) are now available and can be used to unravel the process of neural regeneration in these animals.

The planarian CNS as a model to study neural regeneration

In contrast to most animals, freshwater planarians are a model in that we can study the regeneration of the CNS from a population of stem cells found in intact adult animals. Analysis of nervous system regeneration in planarians (Fig. 3) can provide us not only with valuable information to understand how stem cells are regulated, how they are determined in the neural lineage, and how they differentiate into new neural cells that integrate into the pre-existing CNS, but also information that may be of critical importance in enhancing the poor regenerative capacity of higher animals, including humans. This potential is important to be able to treat spinal cord injuries and neurodegenerative diseases such as Alzheimer's or Parkinson's disease. In vertebrates, for instance, one of the reasons that neural regeneration is blocked after axonal damage is the formation of a glial scar together with the presence of inhibitory molecules (Busch and Silver 2007; Galtrey and Fawcett 2007; Niclou et al. 2006). However, what happens in planarians? Do the glial-like cells described in some previous studies have a role on regeneration? Are inhibitory molecules upregulated after injury? Unfortunately, very little is known about the molecular and cellular events that take place just immediately after amputation of the planarian CNS.

Early stages of CNS regeneration

Based on immunostaining with an antiserum against 5-HT, Reuter et al. (1996b) suggested that the regeneration of the nervous system in the planarian *G. tigrina* is the result of two processes: (1) an initial outgrowth of the original main longitudinal nerve cords that consequently send processes transversely—these processes indicate the position of the developing anterior commissure, and (2) new nerve cells in front of the commissure differentiate from neoblasts and fasciculate with the fibers from the old VNCs, giving rise to the new brain. However, 5-HT is not the most appropriate marker for analysis of very early stages of regeneration. Whole-mount in situ hybridizations for a collection of neural-specific genes combined with the results of immunohistochemistry with an anti-synaptotagmin antibody in *D. japonica* showed that two small clusters of cells defining the new brain primordia appear within the blastema at day 1 of regeneration before any outgrowth from the truncated VNCs was observed with the available markers (Cebrià et al. 2002c). Those findings suggest that the brain

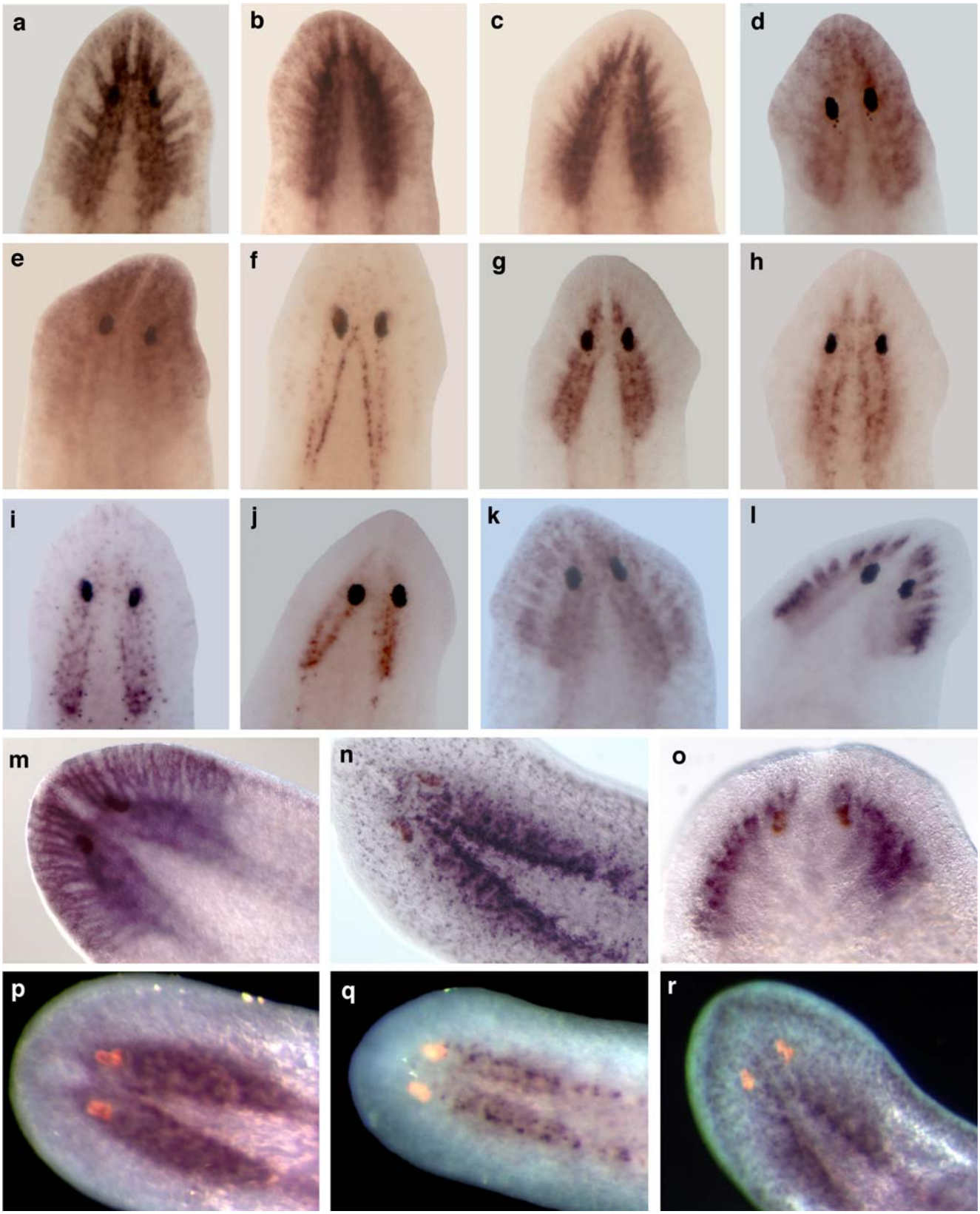
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r

Fig. 2 Molecular domains in the planarian brain. Whole-mount in situ hybridizations showing expression in the cephalic ganglia of two common species used in planarian regeneration research: *Dugesia japonica* (**a**–**l**) and *Schmidtea mediterranea* (M–R). **a** Clone 944HH, N-CAM; **b** clone 2814HH, synapsin; **c** clone 4307HH, nicotinic acetylcholine alpha receptor; **d** clone 721HH, FGF receptor-like; **e** 1993HH, unknown; **f** clone 639E, unknown; **g** clone 953HH, very low-density lipoprotein binding protein; **h** clone 793E, unknown; **i** clone 53E, unknown; **j** clone 1020HH, unknown; **k** clone 3491HH, synaptotagmin VII; **l** clone 1791HH, G protein alpha subunit; **m** clone H.10.2f, unknown; **n** clone H.117.5e, anosmin-1; **o** glutamate receptor; **p** Smed_roboA; **q** Smed_netrin2; **r** Smed_netrin1. Clones **a**–**l** were described in Nakazawa et al. (2003) and Mineta et al. (2003). Clones **m**–**n** were reported in Sánchez Alvarado et al. (2002). Clone **o** has not been described previously (F. Cebrià and P. Newmark, unpublished data). Clone **p** was reported in Cebrià and Newmark (2007). Clones **q** and **r** were described in Cebrià and Newmark (2005)

primordia differentiate independently of the presence of processes from the preexisting nerve cords.

Morphological and molecular data suggest that the planarian brain and VNCs can be seen as distinct structures (Agata et al. 1998; Cebrià et al. 2002c). The challenge is, therefore, to understand how differentiation of the new cephalic ganglia within the blastema is related to regeneration of the truncated nerve cords from the stump region. This will clearly require the use of new markers for those early stages and double labeling using available brain-specific genes and antibodies against neural processes from the VNC to clarify how the brain and nerve cords regenerate with respect to each other. It is interesting to notice that, in some cases, it is possible to regenerate more or less well-organized cephalic ganglia on top of completely disorganized VNCs. This is what happens after RNAi for netrin and a netrin receptor homologue (Cebrià and Newmark 2005). In those experiments, the silencing of either of the two genes resulted in the regeneration of morphologically abnormal cephalic ganglia that still retained their basic organization as the neuronal cell bodies were located at the periphery of a central neuropil. In contrast, the VNCs underneath this brain completely lost their organization and appeared as a disorganized meshwork of axonal processes and cell bodies (Cebrià and Newmark 2005). Thus, although it is obvious that the brain and the VNCs are intimately related in intact animals, several data suggest that

Table 1 Some genes expressed within the planarian CNS

Gene	Homology	Expression pattern	Species	Reference
Djotp	Orthopedia	Brain lateral branches	Dj	Umesono et al. 1997
PC2	Pro-hormone convertase	General	Dj	Agata et al. 1998
DjotxA	Orthodenticle	Cephalic ganglia (medial region)	Dj	Umesono et al. 1999
DjotxB	Orthodenticle	Cephalic ganglia	Dj	Umesono et al. 1999
GtPax6a	Pax6	General	Gt	Pineda et al. 2002
Djnlg	Noggin-like	Brain lateral branches	Dj	Ogawa et al. 2002a
nou-darake	Fibroblast growth factor receptor-like	Cephalic ganglia	Dj	Cebrià et al. 2002a
DjFGFR1 and 2	Fibroblast growth factor receptor	Cephalic ganglia	Dj	Ogawa et al. 2002b
1008HH	Glutamate receptor	Brain lateral branches	Dj	Cebrià et al. 2002b
4307HH	Nicotinic acetylcholine receptor	General	Dj	Cebrià et al. 2002b
Gtsix3	Six/so	Brain lateral branches	Gt	Pineda and Saló 2002
Gtwnt-5	Wnt	General	Gt	Marsal et al. 2003
DjXnp	SNF2-like	Cephalic ganglia	Dj	Rossi et al. 2003
DjFoxG	Brain factor-1	Cephalic ganglia	Dj	Koinuma et al. 2003
Djeya	Eyes absent	Cephalic ganglia	Dj	Mannini et al. 2004
DjPHM	Peptidylglycine α.hydroxilating monooxigenase	General	Dj	Asada et al. 2005
Inx3 and 4	Innexins	General	Dj	Nogi et al. 2005
Smed-netR	Netrin receptor	General	Sm	Cebrià and Newmark 2005
DjCAM	N-CAM	General	Dj	Fusaoka et al. 2006
DjDSCAM	DSCAM	Cephalic ganglia	Dj	Fusaoka et al. 2006
Smed-roboA	Roundabout	General	Sm	Cebrià and Newmark 2007
DjCHC	Clathrin heavy chain	General	Dj	Inoue et al. 2007
DjwntA	Wnt	Posterior half of the brain	Dj	Kobayashi et al. 2007
DjfzA	frizzled	Anterior half of the brain	Dj	Kobayashi et al. 2007
DjTPH	Tryptophan hydroxylase	Serotonergic neurons	Dj	Nishimura et al. 2007b
DjTH	Tyrosine hydroxylase	Dopaminergic neurons	Dj	Nishimura et al. 2007a

The term "general" is used for those genes that are broadly expressed in both the brain (cephalic ganglia) and the VNCs, independent of the number or subpopulations of neurons that they label.
Dj *Dugesia japonica*, Gt *Girardia tigrina*, Sm *Schmidtea mediterranea*

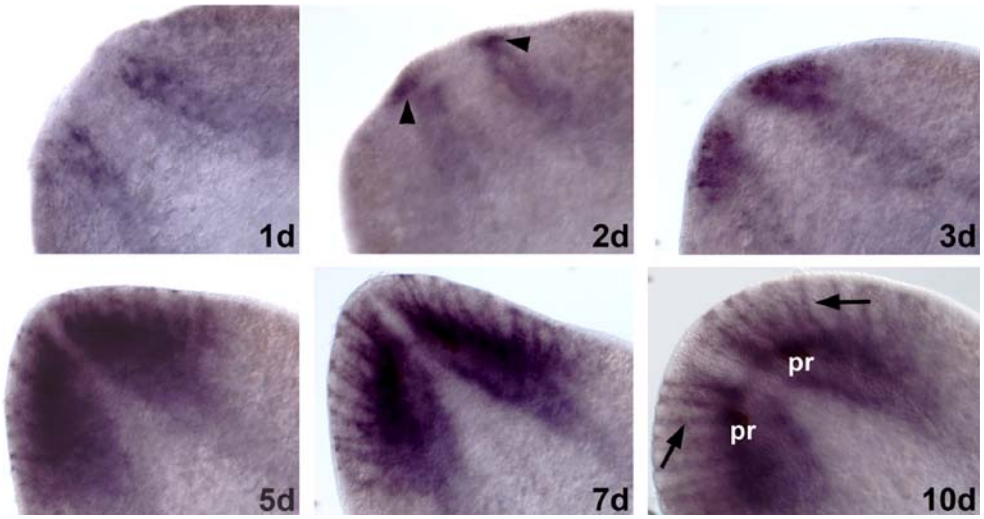

Fig. 3 Central nervous system regeneration in *Schmidtea mediterranea*. Whole-mount in situ hybridization with EST clone H.10.2f (Sánchez Alvarado et al. 2002). With this marker, the brain primordia are first detected within the blastema at day 2 of regeneration (*arrowheads*). By day 10, a mature brain with well-differentiated lateral branches (*arrows*) is observed. Also, by this time, the new photoreceptors (*pr*) are completely differentiated

different specific factors might be responsible for the initial determination of the brain primordia and growth of the amputated nerve cords.

In recent years, a working model (Fig. 4) has been proposed for the regeneration of the planarian CNS (Cebrià et al. 2002c; Inoue et al. 2007; Kobayashi et al. 2007), where there would be an initial stage (24–36 h of regeneration; stage 1 in Fig. 4) in which the brain primordia form within the blastema and are then patterned to define different molecular domains. Next, an intermediate stage would be characterized by the growth of the primordia, regeneration of the VNCs in the blastema and formation of neural circuits (2–3 days of regeneration; stage 2). Finally, at later stages, from 4–5 days (stage 3 in Fig. 4), the regenerated CNS would recover most of its functionality.

To date, no factor has been clearly shown to be responsible for the initial formation of the brain primordia. However, as more genes are being isolated from different EST projects, as well as from "in silico" searches of the genome, candidate genes that may play a role in this early stage of CNS regeneration can be tested. Candidate genes for the patterning of the brain primordia between 1 and 2 days of regeneration have already been postulated in *D. japonica*. Otx-related genes have been found to define distinct mediolateral domains in the planarian brain (Umesono et al. 1997; Umesono et al. 1999). Unfortunately, however, no functional characterization of these genes has been reported, so further analysis will be required to confirm their proposed role in brain patterning. A recent study showed that a homologue of the Wnt family is required for the proper anteroposterior patterning of the planarian brain (Kobayashi et al. 2007). The authors showed that *DjwntA* and its putative frizzled receptor (*DjfzA*) display complementary expression patterns in the brain; *DjwntA* is expressed in the posterior half of the brain, whereas *DjfzA* is restricted in the anterior half (Kobayashi et al. 2007). RNAi knockdowns of *DjwntA* resulted in posterior expansion of the brain, differentiation of ectopic eyes, and longer visual axons projecting to the expanded brain (Kobayashi et al. 2007), findings which clearly implicate the Wnt signaling pathway in brain patterning. Also, a different member of the Wnt family isolated in *G. tigrina*, *Gtwnt-5*, is expressed in a subpopulation of neurons in the CNS, and it has been suggested that it could have a role in mediolateral patterning of the CNS (Marsal et al. 2003), although support from functional analyses needs to be provided.

Genes required in intermediate and late stages of CNS regeneration

The intermediate stages of CNS regeneration would be characterized by the growth of the cephalic ganglia and the formation of proper neural networks. A clathrin heavy chain (CHC) homologue has recently been shown to be required at this stage (Inoue et al. 2007). After RNAi for *DjCHC*, the brain primordia seem to form normally; however, after that, the regenerated brain shows many morphological defects. In vitro cell cultures of neurons from *DjCHC*-knockdown animals showed that those neurons extended significantly fewer and shorter neurites as compared to the controls (Inoue et al. 2007), suggesting that this gene is important for axonal growth. Other neural genes that play an important role at this stage of regeneration are homologues of members of the immunoglobulin superfamily of neural cell adhesion molecules. Thus, *DjCAM* and *DjDSCAM* are expressed in the mature CNS and appear in the brain primordia within the regenerative blastema 2 days after amputation, whereas RNAi analyses suggest that they have a role in axonal development (Fusaoka et al. 2006). *DjCAM* knockdown results in impaired axon fasciculation in the lateral brain

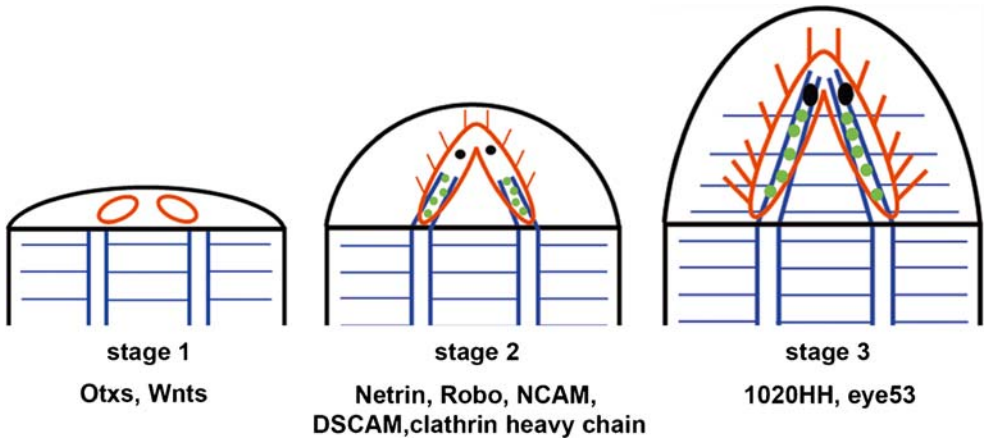

Fig. 4 Hypothetical model of planarian central nervous system (CNS) regeneration. Some of the genes postulated to have a function at each stage are indicated. At stage 1 (24–36 h of regeneration), the brain primordia (*red circles*) are determined within the blastema. Probably at this stage, the brain primordia are already be divided into different molecular domains along their anteroposterior and mediolateral axes (Kobayashi et al. 2007; Marsal et al. 2003; Umesono et al. 1999). Stage 2 (2–3 days of regeneration) would be characterized by the outgrowth of the truncated ventral nerve cords (*in blue*) into the blastema where they would reestablish proper connections with the growing cephalic ganglia (*in red*). The rewiring of the CNS and formation of new neural circuits would be mediated by factors such as homologues of axon guidance cues and cell-adhesion molecules (*green circles*; Cebrià and Newmark 2005; Cebrià and Newmark 2007; Fusaoka et al. 2006). A different factor recently shown to play a role at this stage is a planarian homologue of a clathrin heavy chain gene (Inoue et al. 2007). Finally, at stage 3 (from 4–5 days of regeneration), the newly regenerated CNS would recover all its functions (Inoue et al. 2004)

branches that project toward the head periphery (Fusaoka et al. 2006). A much more severe phenotype is obtained after *DjDSCAM* knockdown: The gross morphology of the regenerated brain is aberrant with a disorganized neuropil and a reduced number of abnormal lateral branches, along with disorganization of the axonal tracts in those branches (Fusaoka et al. 2006). Those results clearly suggest that *DjDSCAM* is required for proper regeneration of the neural network in the cephalic region.

To identify genes that might play a role in late stages of CNS regeneration, the laboratory of Kiyokazu Agata analyzed the function of two novel genes named *eye53* and *1020HH* (Inoue et al. 2004). Those two genes had been shown to be first expressed in the regenerating cephalic ganglia at day 5 after amputation (Cebrià et al. 2002c), suggesting that they could have a role in the final stage of neural regeneration at the time when the CNS would recover most of its functionality. The planarian visual system usually comprises a pair of photoreceptors in the anterior region; these photoreceptors contain pigmented and photosensitive cells, which have axonal processes that project in a very stereotypical pattern (Agata et al. 1998; Okamoto et al. 2005). During regeneration, the normal pattern of visual axon projections is reestablished after 5 days (Inoue et al. 2004). As *eye 53* and *1020HH* are expressed in the photosensitive cells and cells surrounding the visual axons, Inoue et al. (2004) established a behavioral assay to analyze the function of these genes in the photosensory system. Planarians typically display negative phototaxis, moving away from light. After RNAi for *eye53* or *1020HH*, the animals regenerated a morphologically normal brain and visual system but did not show a normal photophobic response, suggesting that these two late-expressed genes are required for the functional recovery of the planarian visual system (Inoue et al. 2004).

The role of axon guidance cues during planarian CNS regeneration

When seeing the complexity of the neural networks in the CNS of both vertebrates and invertebrates, one cannot avoid wondering how axons find their proper targets, in many cases, after navigating a considerable distance through complex extracellular environments. It was Ramón y Cajal who already suggested in his neurotropic theory that some unknown forces guided the axons to their targets (reviewed in Ramón y Cajal 1928). Today, it is known that the proper wiring of the nervous system depends on several families of well-conserved axon guidance cues such as netrins, slit, ephrins, and semaphorins, which regulate not only axonal growth but also other aspects such as neural cell migration (for reviews, see Araújo and Tear 2003, Chilton 2006, and Guan and Rao 2003). Some of these factors act as repulsive cues for the growing axons, whereas others serve as attractants, and still, others may function as either repulsive or attractive cues depending on the context.

During regeneration of the CNS, damaged axons need to regrow and project toward their original targets, reestablish-

ing proper functional connections. However, in most animals, axonal projections in the CNS do not usually regenerate after injury because of intrinsic and environmental factors (for reviews, see Goldberg 2003; Selzer 2003).

Planarians provide a good model in which to analyze the function of these guidance cues during the regeneration and maintenance of the CNS. Recent studies have addressed the function of planarian homologs of *netrin* (Cebrià and Newmark 2005), *robo* (Cebrià and Newmark 2007), and *slit* (Cebrià et al. 2007) during neural regeneration. In all cases, those genes have been shown to be essential for the proper regeneration of the CNS. However, for some of the abnormal phenotypes obtained, it is not yet clear if they are caused by direct axon-guidance defects or abnormal fasciculation or cell migration. For example, planarian netrins are expressed not at the midline but bilaterally along the VNCs or along the medial region of the cephalic ganglia (Cebrià and Newmark 2005). After silencing either *Smed_netrin2* or *Smed_netR*, commissural axons still cross the midline. In fact, the anterior commissure connecting the two cephalic ganglia appears much wider, although that could be the result of either more axonal projections crossing the midline in that region or defasciculation of a normal number of projections (Cebrià and Newmark 2005). On the other hand, silencing of *Smed_roboA* results in a thinner anterior commissure, and in some cases, a complete loss of the commissure (Cebrià and Newmark 2007). Thus, planarian *netrin/netR* and *robo* could be seen as having opposing effects; however, it is noticeable that the effects seen in planarians seem to be the opposite of what has been observed in other systems, where netrins attract commissural axons toward the midline (*netrin* and *netrin receptor* mutants show fewer or no axonal projections crossing the midline) and robo mediates the repulsive role of midline slit (in *slit* and *robo* mutants commissural axons get trapped at the midline). *Smed-slit* seems to display a more conserved function in planarians as a midline repellent. This gene is expressed at the midline, and its silencing results in collapse of the whole regenerated CNS at the midline (Cebrià et al. 2007), a situation that resembles what happens to commissural axons in *Drosophila* and vertebrate *slit* mutants.

Unexpectedly, silencing of these cues in planarians also causes defects that go beyond axon guidance. Thus, after silencing *Smed_slit*, ectopic neural tissue and eyes differentiate around the midline, suggesting that this gene could somehow regulate the behavior of neural precursors at the planarian midline (Cebrià et al. 2007); however, further data will be necessary to clarify the origin of those ectopic neural cells. Finally, differentiation of ectopic pharynges and dorsal outgrowths with a cephalic identity is observed after *Smed_roboA* RNAi (Cebrià and Newmark 2007). Those defects seem to correlate with the failure of the newly differentiated cephalic ganglia to reestablish proper connections with the regenerating VNCs, suggesting that, in the absence of a proper brain/VNC connection, a neurally derived signal would change some positional information and trigger the differentiation of those ectopic structures (Cebrià and Newmark 2007).

Proper targeting of visual axons depends on multiple guidance cues

The axonal projections of planarian photosensitive cells display a highly stereotypical pattern in which some axons project ipsilaterally and others project contralaterally to form an optic chiasm (Fig. 5; Okamoto et al. 2005). Given this highly stereotypical pattern of visual projections and the fact that, in higher invertebrates and vertebrates, different cues are essential for guiding the retinal axons, planarians are an attractive model in which to study the function of these axon guidance cues during the regeneration of the visual system.

Similar to the situation described in vertebrates (reviewed in Inatani 2005), several studies have shown that different cues seem to control different stages of the development and guidance of planarian visual axons (Fig. 5). For instance, *Smed_netrin2* and *Smed_netR* are required for the final targeting of the visual axons to the visual center of the brain (Fig. 5; Cebrià and Newmark 2005). Netrins are expressed in the visual center and would act as attractants for the axons of the photosensitive cells that express a netrin receptor (Cebrià and Newmark 2005). Moreover, *netrin2*/*netR* also seem to regulate the projections that form the chiasm, as RNAi knockdown of those genes leads to the generation of axons that project ectopically in an anterior direction along the midline (Fig. 5; Cebrià and Newmark 2005). Interestingly, the length of the projections of the photosensitive axons toward the visual center seems to be regulated not only by netrins but also by other cues such as a homologue of a semaphorin cytoplasmic domain-associated protein (*semcap-1*). After RNAi silencing of this gene, visual axons project abnormally to a more posterior region of the brain (Fig. 5; Cebrià and Newmark 2005). A *robo* homologue also seems to be required for the regeneration of proper visual pattern. After silencing of *Smed_roboA*, ectopic projections forming loops were observed (Fig. 5; Cebrià and Newmark 2007). The shape and localization of those loops showed some variability, making it more difficult to determine the exact function of *Smed_roboA*. One possibility is that *Smed_roboA* could have a role in preventing or correcting pathfinding errors, as has been described for a zebrafish *robo* homologue (Hutson and Chien 2002). In the absence of *Smed_roboA* function, ectopic projections that, under

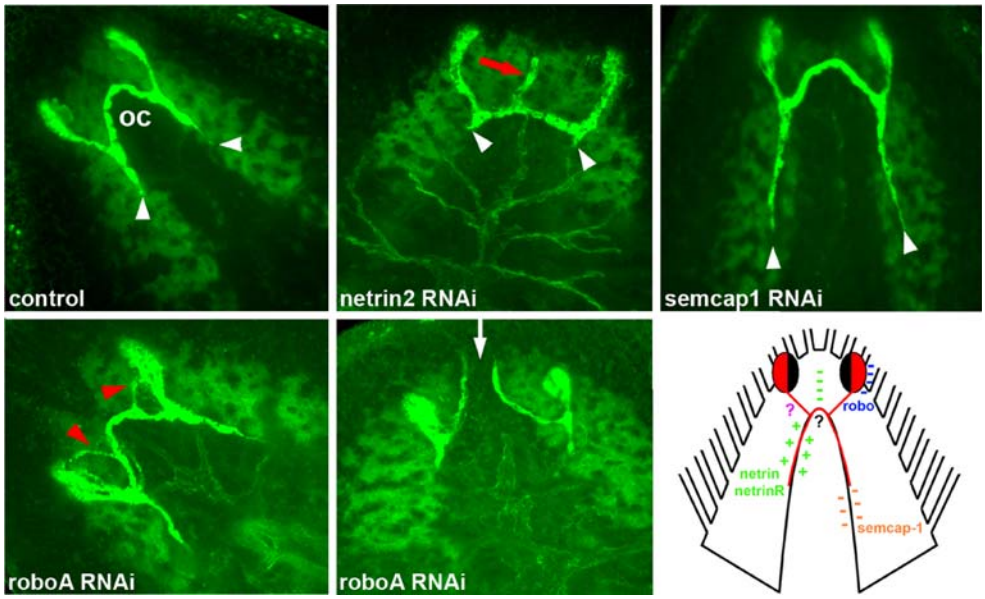

Fig. 5 Cues involved in visual axon guidance during regeneration. Double whole-mount immunostaining with an anti-phosphotyrosine antibody (*light green*) to label the cephalic ganglia and the monoclonal antibody VC-1 (*bright green*; Sakai et al. 2000) to reveal the stereotypical pattern of visual axon projections. Control planarians regenerate a normal pattern, and the visual axons project correctly to the visual center of the brain (*white arrowheads*). After *Smed_netrin2* or *Smed_netR* RNAi, the visual axons do not grow from the optic chiasm (*oc*) to the visual center (*white arrowheads*). In some cases, ectopic visual axons project anteriorly along the midline (*red arrow*; Cebrià and Newmark 2005). After *Smed_semcap1* RNAi, the visual axons project to more posterior regions of the brain compared to the controls (*white arrowheads*; Cebrià and Newmark 2005). After *Smed_roboA* RNAi, ectopic visual projections form loops (*red arrowheads*). In the absence of the anterior brain commissures, the visual axons project anteriorly without crossing the midline (*white arrow*). The *diagram* shows a proposed model in which different molecules act as positive (attractant) or negative (repulsive) cues for the growth and guidance of visual axons. *Question marks* refer to putative cues that remain to be identified

normal conditions, would be corrected could give rise to the observed ectopic loops.

Therefore, at least four different genes (*netrin2*, *netrin receptor*, *semcap1*, and *roboA*) have been shown to play a role in the regeneration of planarian visual axons (Cebrià and Newmark 2005; Cebrià and Newmark 2007). However, additional unknown cues may also be involved in visual axon patterning, as suggested in the model shown in Fig. 5. For example, what are the cues that guide visual axons from the eye-cup toward the point at which some of them project ipsilaterally to the visual center of the brain and others cross the midline? Also, when the visual axons project contralaterally to form the chiasm, they always do it along the posterior end of the anterior brain commissure, which connects the two cephalic ganglia (Fig. 5). After *Smed_roboA* RNAi, the anterior brain commissure is absent in some animals; in all those cases, the visual axons do not cross the midline and instead keep projecting anteriorly (Fig. 5; Cebrià and Newmark 2007). This observation suggests that some factor(s) that guide the visual axons across the midline may be present in the anterior commissure. Interestingly, a similar abnormal anterior projection of visual axons is observed after RNAi for a BMP-1 homologue (Reddien et al. 2005), although, in that case, it was not shown whether the anterior commissure was present or not. Future experiments should try to isolate additional cues involved in those different aspects of visual axon development. It would be interesting to characterize the role of planarian homologues of ephrins in this process, as these factors have been shown to be important for guidance of vertebrate retinal axons (reviewed in Inatani 2005).

The influence of the nervous system on planarian regeneration

The extent to which planarian regeneration is under the influence of the nervous system is an important issue that still needs to be clarified. When considering this question, it should be kept in mind that such an influence could be found in multiple processes, such as cell proliferation, differentiation, and/or migration, and regulation of more general morphogenetic events. Over the years various experimental observations have suggested a role for the nervous system in planarian regeneration. However, the exact nature of that neural influence is currently unknown.

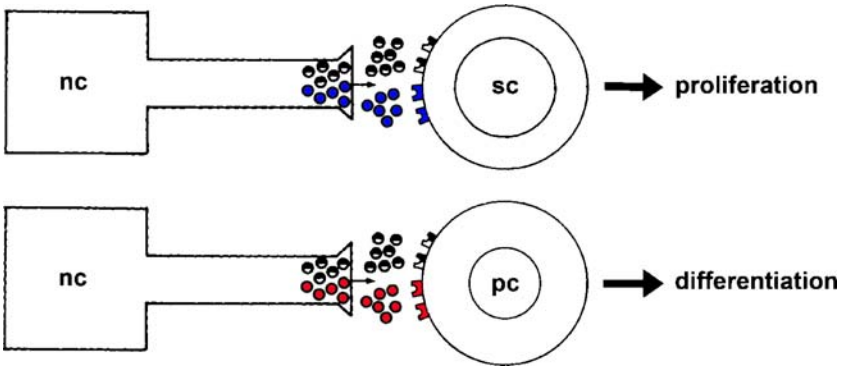

Fig. 6 Possible actions of neurotransmitters on regeneration. Different molecules from nerve cells (*nc*) could signal through receptors in planarian stem cells (*sc*) to control their proliferation either activating or inhibiting it. On the other hand, the same (*black* and *white* circles) or different neural factors (*red circles*) could act on already committed precursor cells (*pc*) regulating their differentiation (adapted from Baguñà et al. 1989)

Classical evidences of a neural influence on regeneration

Several experiments indicated that overall regeneration was inhibited by removal of the nervous system in some species (Child 1904a,b; Morgan 1905). However, in other species, lateral pieces without any VNC could regenerate a head (Morgan 1898; Morgan 1900). Bondi (Bondi 1959) described that, during head regeneration, a planarian with a very shortened lateral nerve cord on one side formed symmetrical blastemas, but the eyes corresponding to the longer nerve cord appeared 3 days before those corresponding to the shorter nerve cord. Later, Lender and Gripon (1962) found that the retardation of eye formation because of the shorter nerve cord was only 24 h, but they nevertheless observed a delay. Sperry et al. (1973) showed that lateral pieces with no VNC give rise to "head-hump" regenerates, which have an abnormally large head with no differentiation of post-cephalic structures. They suggested a head-inhibiting influence of the nerve cords during regeneration. Kishida and Kurabuchi (1978) analyzed regenerating fragments deprived of nerve cords and observed that regeneration was delayed by about 2 days compared to normal regenerating pieces. Wolff and Lender (1950a, b) suggested an inductive role of the brain for eye differentiation, with the eye-inducing factor being of a neuro-humoral nature (Lender 1955). Using a different kind of approach, Stéphan-Dubois and Lender (1956) concluded that wound neurohormones would trigger the migration of neoblasts at the beginning of regeneration and suggested that the nervous system was involved in this early event in the regenerative process. Lender and Klein (1961) and Lender (1964) observed secretory cells in the brain and found that their number increased during subsequent regeneration. Similarly, Sauzin-Monnot (1972) described neurosecretory cells all along the nerve cords and observed an accumulation of neurosecretory granules 6 h after amputation.

The application of modern techniques and the availability of numerous molecular markers allow us to revisit these classical observations and try to characterize the nature of the neural factors involved in regeneration. Remarkably, although a neural influence has also been postulated in other regenerating systems such as the amphibian limb and tail (Dinsmore and Mescher 1998; Singer 1952), no definitive molecule has been reported in those cases. Thus, the benefits of understanding the role of the nervous system during planarian regeneration are clear.

Nou-darake restricts brain differentiation to the head

In 2002, a new gene related to the FGF receptor family was reported called *nou-darake* ("brains everywhere" in Japanese; Cebrià et al. 2002a). This gene is expressed in the entire cephalic region (brain and surrounding tissues), and RNAi led to an unexpected posterior expansion of brain tissues along the length of the body of treated animals. Those results seemed to suggest that *nou-darake* could be involved in an inhibitory pathway that would restrict the differentiation of brain tissues to the anterior region. That the planarian brain could have a role in inhibiting a new brain in more posterior regions was also postulated on the basis of classical observations (reviewed in Brondsted 1969); however, our observations with *nou-darake* provided the first molecular support for that possibility. Unfortunately, the function of other FGF receptors or FGF ligands has not been characterized in these animals. One feature of the *nou-darake* knockdown phenotype is that the ectopic brain tissue does not differentiate throughout the body but rather on top of the preexisting VNCs. This situation is somewhat reminiscent of what happens in some species of Platyhelminthes that reproduce asexually by paratomy, that is, where the differentiation of the new organs starts before fissioning of the animal. In *Microstomum lineare*, it has

been observed that the first cells of the new brain appear in close contact with the existing nerve cords, suggesting an important role of those cords in the differentiation of the new cephalic ganglia (Reuter and Gustafsson 1996; Reuter and Palmberg 1989). In both cases, it could be supposed that some neural factor derived from the VNCs would affect neoblasts (or neural precursors) around them to regulate their proliferation and/or differentiation.

Morphogenesis defects are associated to abnormal nervous system regeneration

More recently, abnormal regeneration of the CNS has been correlated with morphogenesis defects. After *Smed_roboA* RNAi, the newly differentiated cephalic ganglia appear disconnected from the truncated VNCs, which themselves do not appear to regenerate normally; in those cases, differentiation of ectopic pharynges and dorsal outgrowths are observed (Cebrià and Newmark 2007). Based on those observations, it has been proposed that, in the absence of a properly connected CNS (cephalic ganglia and VNC), putative neurally derived signals could be present in the surrounding tissues, altering the behavior of the neoblasts and inducing the morphogenesis defects observed (Cebrià and Newmark 2007). The use of the functional genomics techniques that are now available may help to identify those putative neural signals.

Neurotransmitters and planarian regeneration

Finally, an alternative approach that can be used to unravel the influence of the nervous system on planarian regeneration is to systematically characterize the role of neurotransmitters and growth factors in this process. In other systems, such molecules have been also implicated in regulating the fate of stem cells or neural precursors. In planarians, neurons positive for serotonin, FMRFamide, EGF receptor, met-enkephalin (opioid neuropeptide), and neuropeptide F have been detected by immunohistochemistry (Reuter et al. 1995a, b; Venturini et al. 1983). A peptide named GYRFfamide was the first neuropeptide isolated in planarians (Johnston et al. 1995), and a neuropeptide receptor from *Girardia tigrina* has been reported (Omar et al. 2007). Recently, dopaminergic and serotonergic neurons have been described after in situ hybridization for tyrosine hydroxylase and tryptophan hydroxylase, respectively (Nishimura et al. 2007a, b). Also, by using agonists or antagonists of some of these neurotransmitters, it has been proposed that serotonin and dopamine increase the rates of planarian regeneration (Franquinet 1979; Franquinet and Le Moigne 1979), that substance P and substance K could have a stimulatory effect on neoblast proliferation (Baguñà et al. 1989; Bayascas et al. 1997), that somatostatin might inhibit proliferation (Bautz and Schilt 1986), and that exposure of regenerating pieces to neuropeptide F or FMRFamide may stimulate pharyngeal regeneration (Sheiman et al. 2004). However, most of these observations are based on indirect pharmacological approaches. By combining current tools such as ESTs and genomic information, RNAi-based functional analyses, and cell culturing of neural cells (Asami et al. 2002), it should be possible to first characterize these factors in the planarian genome and then analyze their role during regeneration (Fig. 6).

Conclusions and future prospects

There is no doubt that, in the last 10 years, planarians have regained their popularity as a model in which to study regeneration. As modern techniques have been applied to them, we have begun to characterize, for example, some of the molecular factors that control stem cell proliferation, differentiation, and/or maintenance. Numerous genes expressed in the planarian CNS have now been reported, and analysis of their expression patterns has revealed a high degree of compartmentalization. Also, RNAi experiments have begun to provide data on the role of several genes in brain patterning and in the proper wiring of the regenerating CNS. In summary, we now have the tools to unravel how neural regeneration is accomplished in these animals. Some of the important questions that should be answered in the near future include how many different neuronal populations are found in the planarian CNS, how neoblasts become committed to the neural lineage, and what is the role of the nervous system during regeneration.

Acknowledgment I would like to thank Kiyokazu Agata, Chiyoko Kobayashi, Yoshihiko Umesono, Phillip A. Newmark, Tingxia Guo, Ricardo M. Zayas, and the laboratory of Emili Saló for helpful discussions on the planarian CNS; Hidefumi Orii and Emili Saló for their helpful comments; and Miquel Vila Farré for providing the specimens of *Polycelis felina* and *Phagocata ullala* used for the immunostaining shown in Fig. 1. I also thank Dr. Iain Patten for the advice on the English style in a version of the manuscript. The antibody 3C11 (anti-synapsin) used in Fig. 1 and developed by Dr. E. Buchner was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, USA. F.C. is supported by the Beatriu de Pinós (Generalitat de Catalunya) and Ramón y Cajal (Ministery of Education and Culture, Spain) programs.

References

Agata K, Watanabe K (1999) Molecular and cellular aspects of planarian regeneration. Semin Cell Dev Biol 10:77–33

Agata K, Soejima Y, Kato K, Kobayashi C, Umesono Y, Watanabe K (1998) Structure of the planarian central nervous system (CNS) revealed by neuronal cell markers. Zoolog Sci 15:433–440

Agata K, Tanaka T, Kobayashi C, Kato K, Saitoh Y (2003) Intercalary regeneration in planarians. Dev Dyn 226:308–316

Araújo SJ, Tear G (2003) Axon guidance mechanisms and molecules: lessons from invertebrates. Nat Rev Neurosci 4:10–92

Asada A, Orii H, Watanabe K, Tsubaki M (2005) Planarian peptidylglycine-hydroxylating monooxygenase, a neuropeptide processing enzyme, colocalizes with cytochrome b561 along the central nervous system. FEBS J 272:942–955

Asami M, Nakatsuka T, Hayashi T, Kou K, Kagawa H, Agata K (2002) Cultivation and characterization of planarian neuronal cells isolated by fluorescence activated cell sorting (FACS). Zoolog Sci 19:1257–1265

Baguñà J (1998) Planarians. In: Geraudie J, Ferretti J (eds) Cellular and molecular basis of regeneration: from invertebrates to humans. Wiley, Chichester, pp 135–165

Baguñà J, Ballester R (1978) The nervous system in planarians: peripheral and gastrodermal plexuses, pharynx innervation, and the relationship between central nervous system structure and the acoelomate organization. J Morph 155:237–252

Baguñà J, Riutort M (2004) The dawn of bilaterian animals: the case of acoelomorph flatworms. Bioessays 26:1046–1057

Baguñà J, Salo E, Romero R (1989) Effects of activators and antagonists of the neuropeptides substance P and substance K on cell proliferation in planarians. Int J Dev Biol 33:261–266

Bautz A, Schilt J (1986) Somatostatin-like peptide and regeneration capacities in planarians. Gen Comp Endocrinol 64:267–272

Bondi C (1959) Osservazioni sui rapporti tra rigenerazione degli occhi e sistema nervoso in *Dugesia lugubris*. Arch Zool Ital 44:141–150

Brondsted HV (1969) Planarian regeneration. Pergamon, New York

Bullock TH, Horridge GA (1965) Platyhelminthes. Structure and function in the nervous systems of invertebrates. Freeman, San Francisco, CA

Busch SA, Silver J (2007) The role of extracellular matrix in CNS regeneration. Curr Opin Neurobiol 17:120–127

Cebrià F, Newmark PA (2005) Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. Development 132:3691–3703

Cebrià F, Newmark PA (2007) Morphogenesis defects are associated with abnormal nervous system regeneration following roboA RNAi in planarians. Development 134:833–837

Cebrià F, Vispo M, Newmark PA, Bueno D, Romero R (1997) Myocyte differentiation and body wall muscle regeneration in the planarian Girardia tigrina. Dev Genes Evol 207:306–316

Cebrià F, Kobayashi C, Umesono Y, Nakazawa M, Mineta K, Ikeo K, Gojobori T, Itoh M, Taira M, Sanchez Alvarado A, Agata K (2002a) FGFR-related gene nou-darake restricts brain tissues to the head region of planarians. Nature 419:620–624

Cebrià F, Kudome T, Nakazawa M, Mineta K, Ikeo K, Gojobori T, Agata K (2002b) The expression of neural-specific genes reveals the structural and molecular complexity of the planarian central nervous system. Mech Dev 116:199–204

Cebrià F, Nakazawa M, Mineta K, Ikeo K, Gojobori T, Agata K (2002c) Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. Dev Growth Differ 44:135–146

Cebrià F, Guo T, Jopek J, Newmark PA (2007) Regeneration and maintenance of the planarian midline is regulated by a slit orthologue. Dev Biol 307:394–406

Child CM (1904a) Studies on regulation. V. The relation between the central nervous system and regeneration in Leptoplana: posterior regeneration. J Exp Zool 1:463–512

Child CM (1904b) Studies on regulation. VI. The relation between the central nervous system and regulation in Leptoplana: anterior and lateral regeneration. J Exp Zool 1:513–558

Chilton JK (2006) Molecular mechanisms of axon guidance. Dev Biol 292:13–24

Dalyell JG (1814) Observations on some interesting phenomena in animal physiology, exhibited by several species of planariae. Edinburgh House, London, UK

Dickson BJ, Gilestro GF (2006) Regulation of commissural axon pathfinding by slit and its Robo receptors. Annu Rev Cell Dev Biol 22:651–675

Dinsmore CE, Mescher AL (1998) The role of the nervous system in regeneration. In: Ferretti P, Géraudie J (eds) Cellular and molecular basis of regeneration: from invertebrates to humans. Wiley, Chichester, pp 79–108

Eriksson KS, Panula P (1994) Gamma-aminobutyric acid in the nervous system of a planarian. J Comp Neurol 345:528–536

Fernandes MC, Alvares EP, Gama P, Silveira M (2003) Serotonin in the nervous system of the head region of the land planarian *Bipalium kewense*. Tissue Cell 35:479–486

Franquinet R (1979) The role of serotonin and catecholamines in the regeneration of the Planaria *Polycelis tenvis*. J Embryol Exp Morphol 51:85–95

Franquinet R, Le Moigne A (1979) Relation entre les variations des taux de sérotonine et d' AMP cyclique au cors de la régénération d' une planaire. Biol Cell 34:71–76

Fusaoka E, Inoue T, Mineta K, Agata K, Takeuchi K (2006) Structure and function of primitive immunoglobulin superfamily neural cell adhesion molecules: a lesson from studies on planarian. Genes Cells 11:541–555

Galtrey CM, Fawcett JW (2007) The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system. Brain Res Rev 54:1–18

Goldberg JL (2003) How does an axon grow? Genes Dev 17:941–958

Golubev AI (1988) Glia and neuroglia relationships in the central nervous system of the Turbellaria (Electron microscopic data). Fortschr Zool 36:31–37

Guan KL, Rao Y (2003) Signalling mechanisms mediating neuronal responses to guidance cues. Nat Rev Neurosci 4:941–956

Hutson LD, Chien CB (2002) Pathfinding and error correction by retinal axons: the role of astray/robo2. Neuron 33:205–217

Hyman LH (1951) The invertebrates: Platyhelminthes and Rhynchocoela. McGraw-Hill, New York

Inatani M (2005) Molecular mechanisms of optic axon guidance. Naturwissenschaften 92:549–561

Inoue T, Kumamoto H, Okamoto K, Umesono Y, Sakai M, Sanchez Alvarado A, Agata K (2004) Morphological and functional recovery of the planarian photosensing system during head regeneration. Zoolog Sci 21:275–283

Inoue T, Hayashi T, Takechi K, Agata K (2007) Clathrin-mediated endocytic signals are required for the regeneration of, as well as homeostasis in, the planarian CNS. Development 134:1679–1689

Ishizuka H, Maezawa T, Kawauchi J, Nodono H, Hirao Y, Nishimura O, Nakagawa H, Sekii K, Tasaka K, Tarui H, Agata K, Hoshi M, Kobayashi K, Sakakibara Y, Matsumoto M (2007) The *Dugesia ryukyuensis* database as a molecular resource for studying switching of the reproductive system. Zoolog Sci 24:31–37

Johnston RN, Shaw C, Halton DW, Verhaert P, Baguñà J (1995) GYIRFamide: a novel FMRFamide-related peptide (FaRP) from the triclad turbellarian, *Dugesia tigrina*. Biochem Biophys Res Commun 209:689–697

Kishida Y, Kurabuchi S (1978) The role of the nervous system in the planarian regeneration. I. Regeneration of body fragments deprived of ventral nerve cords. Annot Zool Jpn 51:90–99

Kobayashi C, Saito Y, Ogawa K, Agata K (2007) Wnt signaling is required for antero-posterior patterning of the planarian brain. Dev Biol 306:714–724

Koinuma S, Umesono Y, Watanabe K, Agata K (2003) The expression of planarian brain factor homologs, DjFoxG and DjFoxD. Gene Expr Patterns 3:21–27

Koopowitz H, Chien P (1974) Ultrastructure of the nerve plexus in flatworms. I. Peripheral organization. Cell Tissue Res 155: 337–351

Koopowitz H, Chien P (1975) Ultrastructure of nerve plexus in flatworms. II. Sites of synaptic interactions. Cell Tissue Res 157:207–216

Lender T (1955) Some properties of the organisine of eye regeneration in the planaria *Polycelis nigra*. C R Hebd Seances Acad Sci 240:1726–1728

Lender T (1964) Mise en évidence et role de la neurosécrétion chez les planaires d'eau douce (Turbellariés, Triclades). Ann d'Endocrin 25:61–65

Lender T, Gripon P (1962) La régénération des yeux et du cerveaux de *Dugesia lugubris* en présence de deux troncs nerveaux inégaux. Bull Soc Zool Fr 87:387–395

Lender T, Klein N (1961) Mise en évidence de cellules sécrétices dans le cerveau de la Planaire POlycelis nigra. Variation de leur nombre au cours de la régénération postérieure. CR Acad Sci 253:331–333

Lentz TL (1968) Primitive nervous systems. Yale University Press, New Haven, CT

MacRae EK (1967) The fine structure of sensory receptor processes in the auricular epithelium of the planarian *Dugesia tigrina*. Z Zellforsch 82:479–494

Mannini L, Rossi L, Deri P, Gremigni V, Salvetti A, Saló E, Batistoni R (2004) *Djeyes absent* (*Djeya*) controls prototypic planarian eye regeneration by cooperating with the transcription factor *Djsix-1*. Dev Biol 269:346–359

Marsal M, Pineda D, Salo E (2003) Gtwnt-5 a member of the wnt family expressed in a subpopulation of the nervous system of the planarian *Girardia tigrina*. Gene Expr Patterns 3:489–495

Maule AG, Halton DW, Johnston CF, Shaw C, Fairweather I (1990) The serotoninergic, cholinergic and peptidergic components of the nervous system in the monogenean parasite, *Diclidophora merlangi*: a cytochemical study. Parasitology 100:255–273

Maule AG, Shaw C, Halton DW, Brennan GP, Johnston CF, Moore S (1992) Neuropeptide F (*Moniezia expansa*): localization and characterization using specific antisera. Parasitology 105:505–512

McVeigh P, Kimber MJ, Novozhilova E, Day TA (2005) Neuropeptide signalling systems in flatworms. Parasitology 131:S41–S55

Mineta K, Nakazawa M, Cebrià F, Ikeo K, Agata K, Gojobori T (2003) Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. Proc Natl Acad Sci U S A 100:7666–7671

Morgan TH (1898) Experimental studies of the regeneration of *Planaria maculata*. Arch Entwicklungsmech Org 7:364–397

Morgan TH (1900) Regeneration in planarians. Arch Entwicklungsmech Org 10:58–119

Morgan TH (1905) "Polarity" considered as a phenomenon of gradation of materials. J Exp Zool 2:495–506

Morita M, Best JB (1965) Electron microscopic studies on Planaria. II. Fine structure of the neurosecretory system in the planarian *Dugesia dorotocephala*. J Ultrastruct Res 13:396–408

Morita M, Best JB (1966) Electron microscopic studies of Planaria. 3. Some observations on the fine structure of planarian nervous tissue. J Exp Zool 161:391–411

Morita M, Hall F, Best JB, Gern W (1987) Photoperiodic modulation of cephalic melatonin in planarians. J Exp Zool 241:383–388

Morita M, Hall FL, Best JB (1988) An optic neurosecretory cell in the planarian. Fortschr Zool 36:207–210

Nakazawa M, Cebrià F, Mineta K, Ikeo K, Agata K, Gojobori T (2003) Search for the evolutionary origin of a brain: planarian brain characterized by microarray. Mol Biol Evol 20:784–791

Newmark PA, Sánchez Alvarado A (2000) Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. Dev Biol 220:142–153

Newmark PA, Sánchez Alvarado A (2002) Not your father's planarian: a classic model enters the era of functional genomics. Nat Rev Genet 3:210–219

Newmark PA, Reddien PW, Cebrià F, Sánchez Alvarado A (2003) Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. Proc Natl Acad Sci U S A 100 (Suppl 1):11861–11865

Niclou SP, Ehlert EM, Verhaagen J (2006) Chemorepellent axon guidance molecules in spinal cord injury. J Neurotrauma 23:409–421

Nishimura K, Kitamura Y, Inoue T, Umesono Y, Sano S, Yoshimoto K, Inden M, Takata K, Taniguchi T, Shimohama S, Agata K (2007a) Reconstruction of dopaminergic neural network and locomotion function in planarian regenerates. Dev Neurobiol 67:1059–1078

Nishimura K, Kitamura Y, Inoue T, Umesono Y, Yoshimoto K, Takeuchi K, Taniguchi T, Agata K (2007b) Identification and distribution of tryptophan hydroxylase (TPH)-positive neurons in the planarian *Dugesia japonica*. Neurosci Res 59:101–106

Nogi T, Levin M (2005) Characterization of innexin gene expression and functional roles of gap-junctional communication in planarian regeneration. Dev Biol 287:314–335

Ogawa K, Ishihara S, Saito Y, Mineta K, Nakazawa M, Ikeo K, Gojobori T, Watanabe K, Agata K (2002a) Induction of a *noggin*-like gene by ectopic DV interaction during planarian regeneration. Dev Biol 250:59–70

Ogawa K, Kobayashi C, Hayashi T, Orii H, Watanabe K, Agata K (2002b) Planarian fibroblast growth factor receptor homologs expressed in stem cells and cephalic ganglions. Develop Growth Differ 44:191–204

Okamoto K, Takeuchi K, Agata K (2005) Neural projections in planarian brain revealed by fluorescent dye tracing. Zoolog Sci 22:535–546

Omar HH, Humphries JE, Larsen MJ, Kubiak TM, Geary TG, Maule AG, Kimber MJ, Day TA (2007) Identification of a platyhelminth neuropeptide receptor. Int J Parasitol 37:725–733

Oosaki T, Ishii S (1965) Observations on the ultrastructure of nerve cells in the brain of the planarian, *Dugesia gonocephala*. Z Zellforsch 66:782–793

Pallas PS (1766) Miscellanea zoologica, quibus novae imprimis atque obscurae animalium species Hagae Comitum, apud Pterum van Cleef, Holland

Philippe H, Lartillot N, Brinkmann H (2005) Multigene analyses of bilaterian animals corroborate the monophyly of Ecdysozoa, Lophotrochozoa, and Protostomia. Mol Biol Evol 22:1246–1253

Pineda D, Salo E (2002) Planarian Gtsix3, a member of the Six/so gene family, is expressed in brain branches but not in eye cells. Gene Expr Patterns 2:169–173

Pineda D, Rossi L, Batistoni R, Salvetti A, Marsal M, Gremigni V, Falleni A, Gonzalez-Linares J, Deri P, Saló E (2002) The genetic network of prototypic planarian eye regeneration is Pax6 independent. Development 129:1423–1434

Ramón y Cajal S (1928) Degeneration and regeneration of the nervous system. Oxford University Press, London

Randolph H (1897) Observations and experiments on regeneration in planarians. Arch Entwicklungsmech Org 5:352–372

Reddien PW, Sanchez Alvarado A (2004) Fundamentals of planarian regeneration. Annu Rev Cell Dev Biol 20:725–757

Reddien PW, Bermange AL, Murfitt KJ, Jennings JR, Sanchez Alvarado A (2005) Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. Dev Cell 8:635–649

Reisinger E (1972) Die Evolution des Orthogons der Spiralier und daB Archicoelomatenproblem. Z Zool Syst Evolutionforsch 10:1–43

Reuter M, Gustafsson M (1989) "Neuroendocrine cells" in flatworms—progenitors to metazoan neurons? Arch Histol Cytol 52(Suppl): 253–263

Reuter M, Gustafsson MK (1995) The flatworm nervous system: pattern and phylogeny. Exs 72:25–59

Reuter M, Gustafsson M (1996) Neuronal signal substances in asexual multiplication and development in flatworms. Cell Mol Neurobiol 16:591–616

Reuter M, Palmberg I (1989) Development and differentiation of neuronal subsets in asexually reproducing *Microstomum lineare*. Immunocytochemistry of 5-HT, RF-amide and SCPB. Histochemistry 91:123–131

Reuter M, Gustafsson MK, Sahlgren C, Halton DW, Maule AG, Shaw C (1995a) The nervous system of Tricladida. I. Neuroanatomy of *Procerodes littoralis* (Maricola, Procerodidae): an immunocytochemical study. Invert Neurosci 1:113–122

Reuter M, Gustafsson MK, Sheiman IM, Terenina N, Halton DW, Maule AG, Shaw C (1995b) The nervous system of Tricladida. II. Neuroanatomy of *Dugesia tigrina* (Paludicola, Dugesiidae): an immunocytochemical study. Invert Neurosci 1:133–143

Reuter M, Gustafsson MKS, Mäntylä K, Grimmelikhuijzen CJP (1996a) The nervous system of Tricladida. III. Neuroanatomy of *Dendrocoelum lacteum* and *Polycelis tenuis* (Plathelminthes, Paludicola): an immunocytochemical study. Zoomorphology 116:111–122

Reuter M, Sheiman IM, Gustafsson MKS, Halton DW, Maule AG, Shaw C (1996b) Development of the nervous system in *Dugesia tigrina* during regeneration after fission and decapitation. Invertebr Reprod Dev 29:199–211

Ribeiro P, El-Shehabi F, Patocka N (2005) Classical transmitters and their receptors in flatworms. Parasitology 131:S19–S40

Rieger RM, Tyler S, Smith JPS III, Rieger GE (1991) Platyhelminthes: turbellaria. In: Harrison FW, Bogitsh BJ (eds) Microscopic anatomy of invertebrates, vol. 3. Wiley–Liss, New York, pp 7–140

Rossi L, Deri P, Andreoli I, Gremigni V, Salvetti A, Batistoni R (2003) Expression of DjXnp, a novel member of the SNF2-like ATP-dependent chromatin remodelling genes, in intact and regenerating planarians. Int J Dev Biol 47:293–298

Ruiz-Trillo I, Paps J, Loukota M, Ribera C, Jondelius U, Baguna J, Riutort M (2002) A phylogenetic analysis of myosin heavy chain type II sequences corroborates that Acoela and Nemertodermatida are basal bilaterians. Proc Natl Acad Sci U S A 99:11246–11251

Sakai F, Agata K, Orii H, Watanabe K (2000) Organization and regeneration ability of spontaneous supernumerary eyes in planarians—eye regeneration field and pathway selection by optic nerves. Zoolog Sci 17:375–381

Saló E (2006) The power of regeneration and the stem-cell kingdom: freshwater planarians (Platyhelminthes). Bioessays 28:546–559

Sánchez Alvarado A (2000) Regeneration in the metazoans: why does it happen? BioEssays 22:578–590

Sánchez Alvarado A, Kang H (2005) Multicellularity, stem cells, and the neoblasts of the planarian *Schmidtea mediterranea*. Exp Cell Res 306:299–308

Sánchez Alvarado A, Newmark PA (1999) Double-stranded RNA specifically disrupts gene expression during planarian regeneration. Proc Natl Acad Sci U S A 96:5049–5054

Sánchez Alvarado A, Newmark PA, Robb SM, Juste R (2002) The *Schmidtea mediterranea* database as a molecular resource for studying Platyhelminthes, stem cells and regeneration. Development 129:5659–5665

Sauzin-Monnot MJ (1972) Étude ultrastructurale du tissue nerveux et des produits de secretion nerveuse, au cours des premières heures de regeneration de la planaire Polycelis nigra (Turbellarié-Triclade) au niveau de la blessure. Ann Embryol Morphogen 5:257–265

Selzer ME (2003) Promotion of axonal regeneration in the injured CNS. Lancet Neurol 2:157–166

Sheiman IM, Kreshchenko ND, Sedel, nikov ZV, Groznyi AV (2004) Morphogenesis in planarians *Dugesia tigrina*. Ontogenez 35:285–290

Singer M (1952) The influence of the nerve in regeneration of the amphibian extremity. Q Rev Biol 27:169–200

Sperry PJ, Ansevin KD, Tittel FK (1973) The inductive role of the nerve cord in regeneration of isolated postpharyngeal body sections of *Dugesia dorotocephala*. J Exp Zool 186:159–174

Stéphan-Dubois F, Lender Th (1956) Corrélation humorales dans le régénération des planaires paludicoles. Ann Sci Nat Zool 11. ser

Trawicki W, Czubaj A, Moraczewski J (1988) The brain ultrastructure of *Dendrocoelum lacteum* (O.F. Muller). Fortschr Zool 36:195–200

Umesono Y, Watanabe K, Agata K (1997) A planarian orthopedia homolog is specifically expressed in the branch region of both the mature and regenerating brain. Dev Growth Differ 39:723–727

Umesono Y, Watanabe K, Agata K (1999) Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes. Dev Genes Evol 209:31–39

Venturini G, Carolei A, Palladini G, Margotta V, Lauro MG (1983) Radioimmunological and immunocytochemical demonstration of Met-enkephalin in planaria. Comp Biochem Physiol C 74: 23–25

Wikgren MC, Reuter M (1985) Neuropeptides in a microturbellarian whole-mount immunocytochemistry. Peptides 6(Suppl 3): 471–475

Wolff E, Lender T (1950a) Sur le déterminisme de la régénération des yeux chez une planaire d'eau douce *Polycelis nigra*. CR Séance Soc Biol 144:1213

Wolff E, Lender T (1950b) Sur le role organisateur du cerveau dans la régénération des yeux chez une planaire d'eau douce. CR Acad Sci 230:2238–2239

Zayas RM, Hernandez A, Habermann B, Wang Y, Stary JM, Newmark PA (2005) The planarian Schmidtea mediterranea as a model for epigenetic germ cell specification: analysis of ESTs from the hermaphroditic strain. Proc Natl Acad Sci U S A 102: 18491–18496