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Organization of the nervous system in the model planarian *Schmidtea mediterranea*: An immunocytochemical study

Francesc 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

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Abstract

Freshwater planarians are an emerging model in which to study regeneration at the molecular level. These animals can regenerate a complete central nervous system (CNS) in only a few days. In recent years, hundreds of genes expressed in the nervous system have been identified in two popular planarian species used by several laboratories: *Dugesia japonica* and *Schmidtea mediterranea*. Functional analyses of some of those neural genes have allowed the process of CNS regeneration to begin to be elucidated in those animals. However, additional work is required to characterize the different neuronal populations. Thus, the identification or generation of antibodies that act as markers for specific neuronal cell types would be extremely useful not only in obtaining a more detailed characterization of the planarian nervous system but also for the analysis of phenotypes obtained by RNA interference. Here, I have used five different antibodies to describe different neuronal populations in the freshwater planarian *S. mediterranea.* This study represents a first step in characterizing the organization of the nervous system of this species at the cellular level.

Keywords: Planarian; *Schmidtea mediterranea*; Nervous system; Regeneration; Serotonin; Neuropeptide F; GYRFamide; Allatostatin

1. Introduction

Freshwater planarians (Phylum Platyhelminthes) are a classical model in which to study regeneration (Newmark and Sánchez Alvarado, 2002; Agata et al., 2003; Reddien and Sánchez Alvarado, 2004; Saló, 2006). Their regenerative capacity is such that if, for example, you decapitate a planarian the resulting headless fragment will regenerate an anterior region including the cephalic ganglia in only a few days (Reuter et al., 1996b; Cebrià et al., 2002c). Unravelling how planarians can regenerate a complete, functional central nervous system (CNS) is an attractive field of study not only for a better understanding of the molecular basis of regeneration but also because it could provide insights into ways in which we might enhance the very limited neural regenerative capabilities shown by most animals, including humans (Cebrià, 2007).

The planarian CNS consists mainly of a brain or cephalic ganglia and a pair of longitudinal ventral nerve cords that run along the length of the animal (Agata et al., 1998; Cebrià et al., 2002c). In recent years, hundreds of nervous system-specific genes have been identified in the two most common species of freshwater planarians used by several laboratories, *Dugesia japonica* and *Schmidtea mediterranea* (Mineta et al., 2003; Nakazawa et al., 2003; Zayas et al., 2005). In situ hybridizations for many of those genes have defined different molecular domains within the planarian CNS (Umesono et al., 1999; Cebrià et al., 2002b) and have helped to start dissecting the process of CNS regeneration (Cebrià et al., 2002c). Also, functional analyses based on RNA-interference (RNAi) have provided data on the important roles played by several of those genes in the process of neural regeneration (Cebrià et al., 2002a; Cebrià and Newmark, 2005; Fusaoka et al., 2006; Cebrià and Newmark, 2007; Inoue et al., 2007; Kobayashi et al., 2007; Takano et al., 2007). However, the number of antibodies that can be used to characterize in greater detail the different neuronal populations in those planarian species is much more limited. Thus, additional markers would be useful to identify the different types of nerve cells within the planarian nervous system. Recently, antibodies against tryptophan hydroxylase and tyrosine hydroxylase have been generated and used as

* Tel.: +34 934034795; fax: +34 934034420.
E-mail address: fcebrias@ub.edu.

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markers of serotonergic and dopaminergic neurons, respectively, in *D. japonica* (Nishimura et al., 2007a,b). Also, few antibodies from different sources have been reported to label the central nervous system of *S. mediterranea* (Robb and Sanchez Alvarado, 2002). Here, five different antibodies against synapsin, serotonin, allatostatin, GYRFamide and neuropeptide F have been used to characterize the nervous system of *S. mediterranea*. As expected, the antibodies used label distinct neuronal populations and generate basic information about the organization of the nervous system in this model species.

2. Materials and methods

2.1. Organisms

Freshwater planarians from the species *S. mediterranea* (Benazzi et al., 1972) were maintained in the laboratory at 20 °C in a 1:1 (v/v) mixture of tap water and distilled water treated with AquaSafe (TetraAqua). Animals were starved for at least 1 week prior to experiments.

2.2. Immunostaining

Depending on the primary antibody used, animals were fixed in two different ways. For anti-neuropeptide F and anti-SYNORF1 (3C11) antibodies, animals were killed by immersion in 2% HCl for 5 min on ice and then fixed in Carnoy's solution for 2 h at 4 °C. For anti-5HT, anti-allatostatin (5F10) and anti-GYRFamide antibodies, animals were killed by immersion in 37% formaldehyde for 1 min on ice and then fixed in 4% paraformaldehyde for 2 h at 4 °C. The samples were incubated in Proteinase K (20 μg/ml in PBS containing 0.3% Triton X-100) for 8 min at 37 °C, followed by post-fixation in 4% paraformaldehyde for 2 h at 4 °C. In both cases, after fixation, all of the animals were processed as described previously (Cebrià and Newmark, 2005). The following primary antibodies were used: anti-SYNORF1, a mouse monoclonal antibody specific for synapsin (3C11, Developmental Studies Hybridoma Bank) used at a dilution of 1:50; anti-allatostatin (5F10, Developmental Studies Hybridoma Bank) used at a dilution of 1:2; anti-5HT (Sigma, S5545) used at a dilution of 1:1000; anti-GYRFamide (Johnston et al., 1996) used at a dilution of 1:1000; anti-neuropeptide F (Maule et al., 1992) used at a dilution of 1:1000; and TMUS13 (Cebrià et al., 1997), which is specific for muscle fibers, used at a dilution of 1:10. The following secondary antibodies were used: goat anti-mouse conjugated to Alexa 488 (Molecular Probes), goat anti-rabbit conjugated to Alexa 568 (Molecular Probes) and goat anti-guinea pig conjugated to Alexa 488 (Molecular Probes). Samples were mounted in SlowFade Gold antifade reagent (Invitrogen). Confocal laser scanning microscopy was performed with a Leica TCS 4D (Leica Lasertechnik, Heidelberg) adapted to an inverted microscope (Leitz DMIRB).

2.3. In situ hybridization and Western blot

Whole-mount in situ hybridization was performed as previously described (Umesono et al., 1997), modified with the triethanolamine treatment (Nogi and Levin, 2005). EST clone PL04024B1B02 from a Smed database (Zayas et al., 2005) was used to synthesize the antisense riboprobe. For fluorescence

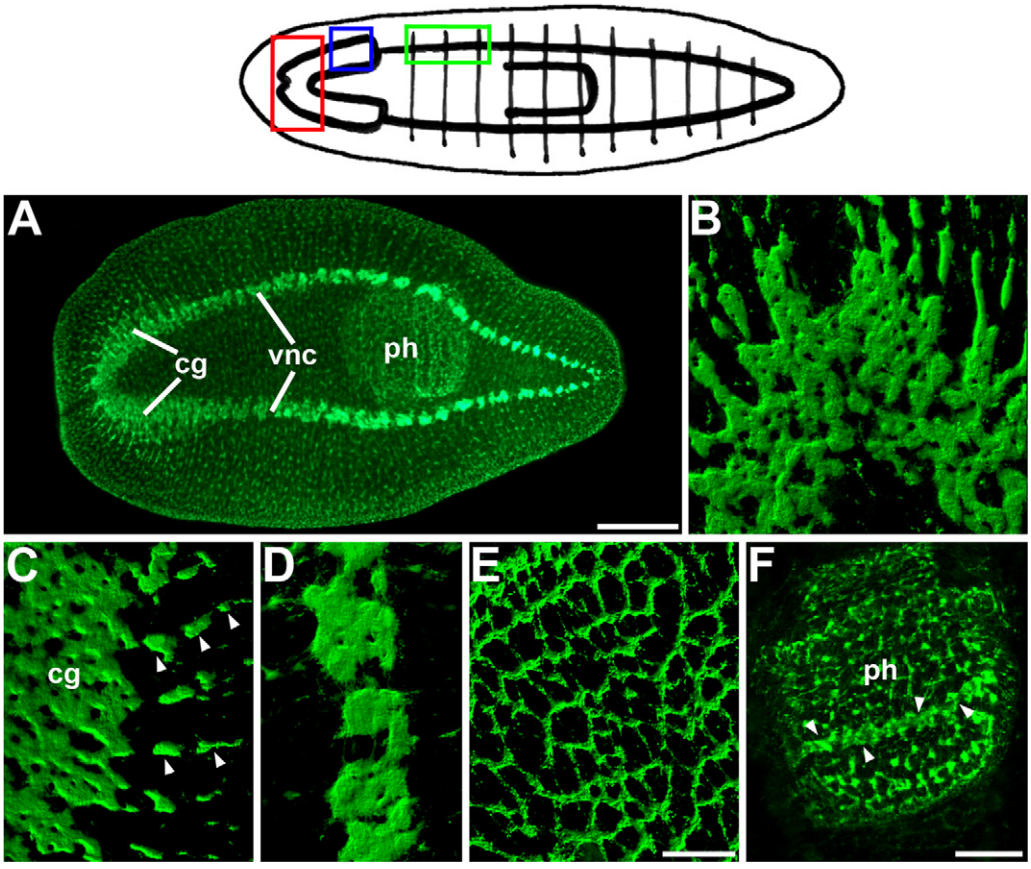

Fig. 1. Anti-SYNORF1 immunostaining of the *Schmidtea mediterranea* nervous system. Schematic representation of a planarian and its CNS indicating the regions depicted in (B) red box, (C) blue box and (D) green box. (A) Whole-mount immunostaining on an intact planarian. (B) Anterior commissure connecting the two cephalic ganglia. (C) Cephalic ganglion with lateral branches (arrowheads) projecting towards the periphery of the head. (D) View of the ganglia-like knots of the ventral nerve cords. (E) Submuscular plexus in the head region. (F) Pharyngeal plexus. Arrowheads point to a strongly labelled nerve ring close to the distal end of the pharynx. In (A) anterior to the left; in (B–F) anterior to the top. cg, cephalic ganglia; vnc, ventral nerve cords; ph, pharynx. Scale bars: 0.5 mm (A); 50 μm (B–E); 100 μm (F).