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Novel markers identify nervous system components of the holothurian nervous system

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Abstract

Echinoderms occupy a key position in the evolution of deuterostomes. As such, the study of their nervous system can shed important information on the evolution of the vertebrate nervous system. However, the study of the echinoderm nervous system has lagged behind when compared to that of other invertebrates due to the lack of tools available. In this study, we tested three commercially available antibodies as markers of neural components in holothurians. Immunohistological experiments with antibodies made against the mammalian transcription factors Pax6 and Nurr1, and against phosphorylated histone H3 showed that these markers identified cells and fibers within the nervous system of *Holothuria glaberrima*. Most of the fibers recognized by these antibodies were co-labeled with the well-known neural marker, RN1. Additional experiments showed that similar immunoreactivity was found in the nervous tissue of three other holothurian species (*Holothuria mexicana*, *Leptosynapta clarki* and *Sclerodactyla briareus*), thus extending our findings to the three orders of Holothuroidea. Furthermore, these markers identified different subdivisions of the holothurian nervous system. Our study presents three additional markers of the holothurian nervous system, expanding the available toolkit to study the anatomy, physiology, development and evolution of the echinoderm nervous system.

Keywords

Holothuria glaberrima (Echinodermata); holothurians; anatomical circuits; neuropeptides; comparative neuroscience

Introduction

Members of the phylum Echinodermata lie at a key position to understand the evolution of the vertebrate nervous system. Nonetheless, the information available on the nervous system of adult echinoderms, particularly information on cellular phenotypes, neurochemistry and

neural circuitry, is limited. One of the reasons for the small number of studies of echinoderm nervous systems lies in the fact that echinoderm nerve cells, when compared to those of other invertebrates, are small and protected by an endoskeleton of calcareous ossicles (Cobb 1978). An additional problem in studying the echinoderm nervous system is the difficulty in clearly identifying the neurons mainly due to a shortage of neuronal markers. Thus, neurobiologists have stayed away from using echinoderms in their studies. Nonetheless, in recent years, some aspects of echinoderm neurobiology have received particular attention. One of these is the larval embryonic system and its evolutionary relationship to those of other deuterostomes, including chordates (Bishop and Burke 2007; Byrne and Cisternas 2002; Chee and Byrne 1999; Dupont et al. 2009; Hirokawa et al. 2008; Katow et al. 2009; Murabe et al. 2008; Nakano et al. 2006; Yaguchi et al. 2006). Many of these larval studies were made possible by the development of an antibody that recognizes echinoderm synaptotagmin (Burke et al. 2006b); thus highlighting the importance of having markers for identifying nervous tissue components.

A handful of neuronal markers of the adult echinoderm nervous system have been described. Most of these are antibodies against neuropeptides or other neurotransmitter systems that recognize subpopulations of neurons or nerve fibers (Cottrell and Pentreath 1970; Diaz-Miranda et al. 1995; Diaz-Miranda et al. 1996; Elphick et al. 1995; Inoue et al. 2002; Newman and Thorndyke 1994; Newman et al. 1995). More recently, other markers have been obtained by our group that recognize neuronal cells and fibers of the sea cucumber *Holothuria glaberrima*. Among these are: RN1 a monoclonal antibody that recognizes a hitherto unknown epitope (Díaz-Balzac et al. 2007) and anti-calbindin antibodies that recognize the echinoderm ortholog of Calbindin-D32k (Díaz-Balzac et al. 2012).

In our quest of expanding the tools available for students of echinoderm neurobiology we have identified three new commercially available antibodies that recognize components of the holothurian nervous system. Two of them recognize transcription factors (Pax 6 and Nurr1), while the other was made against phosphorylated histone H3 (PH3). We present here the immunoreactivity of these markers in the radial nerve cords (RNCs), the peripheral nerves including those in the body wall, and in the large intestine of *H. glaberrima* and describe the neuronal and fiber populations that are identified. To demonstrate that the immunoreactivity is not a spurious artifact, we compared the immunoreactivity in *H. glaberrima* with that of representative species of three different holothurian orders: Apodida, Dendrochirotida, and Aspidochirotida. Our findings provide new tools for the identification of nervous system subdivisions and will help in the advancement of echinoderm cellular and molecular studies, as well as in understanding the evolution of the nervous system in this phylum.

Material and Methods

Animals

Adult specimens (10–15 cm in length) of the holothurian *Holothuria glaberrima* (Selenka, 1867) (Holothuroidea, Aspidochirotida), were collected from the rocky shores of the north coast of Puerto Rico and kept in seawater aquaria. Adult specimens (20–35 cm in length) of the holothurian *Holothuria mexicana* (Ludwig, 1875) (Holothuroidea, Aspidochirotida),

were collected from the seagrass beds of the north coast of Puerto Rico and kept in seawater aquaria. Adult specimens (10–15 cm in length) of the holothurian *Leptosynapta clarki* (Heding, 1928) (Holothuroidea, Apodida) and of the holothurian *Sclerodactyla briareus* (Aurivillius, 1891) (Holothuroidea, Dendrochirotida) were acquired from the Marine Biological Laboratory (Woods Hole, Massachusetts) and kept in seawater aquaria.

Tissue Sections

Specimens were anesthetized in 0.2% 1,1,1-trichloro-2-methyl-2-propanol (Sigma, St. Louis, MO) for 10 min and dissected by longitudinal section of the body wall. Samples were obtained from the ventro-lateral ambulacrum region and dorso-lateral body wall, which were divided into anterior, middle and posterior. In the case of *L. clarki* and *S. briareus*, the central body wall was dissected with the corresponding section of the digestive tract. All tissues were fixed in 4% paraformaldehyde at 4°C for approximately 1 h. Tissues were rinsed 3 times for 15 min with 0.1 M phosphate-buffered saline (PBS), and left in a 30% sucrose solution at 4°C for at least 24 h before proceeding to embed them in Tissue-Tek (Sakura Finetek, Torrance, CA). Cryostat tissue sections of 20 µm were cut and mounted on Poly-L-lysine-coated slides.

Immunohistochemistry

The indirect immunofluorescence method was followed (Díaz-Balzac et al. 2010a; Díaz-Balzac et al. 2007; García-Arrarás 1993). In brief, tissues were rinsed for 5 min in 0.1 M PBS, followed by a 1 h incubation in goat serum 1:50 (Invitrogen, Carlsbad, CA) and one rinse of 15 min in 1 % Triton X, and two other rinses in 0.1 M PBS. Subsequently, the primary antibodies (Table 1) were incubated overnight at room temperature. The primary antibodies used include the RN1 monoclonal antibody (Díaz-Balzac et al. 2007) raised against a homogenate of the radial nerve of *H. glaberrima* and used at a dilution of 1:100,000; the rabbit polyclonal αPH3 (Upstate Biotechnology 06-570 Lot. 21714 and DAM1416518) prepared against KLH-conjugated peptide ARK[pS]TGGKAPRKQLC, corresponding to amino acids 7–20 of human histone H3 and used at a 1:250 dilution; the rabbit polyclonal αnurr1 (Santa Cruz Biotechnology sc-990 Lot. K1903) prepared against a peptide mapping at the C-terminus of NURR1 of rat origin and used at a 1:500 dilution; the rabbit polyclonal αpax6 (Abcam ab5790 Lot. 464388) prepared against the synthetic peptide C-REEKLRNQRRQASNTPSHI, corresponding to amino acids 267–285 of mouse PAX6 and used in at a 1:100 dilution. Negative controls were performed in all experiments by incubating the tissue sections with rabbit serum during the incubation period of the primary antibody and following the normal immunostaining protocol.

The secondary FITC antibodies, goat anti-mouse (Biosource, Camarillo, CA, #AMI0408 Lot. 3501) and goat anti-rabbit (Biosource, Camarillo, CA, #ALI0408 Lot. 1502), were used at a 1:50 dilution for double-labeling indirect immunohistochemistry. Also, the Cy3 conjugated secondary antibodies, goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, #115-165-068 Lot. 47814) and goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, #111-165-144 Lot. 50694), were used at a 1:2,000 dilution for double-labeling indirect immunohistochemistry.

Cell nuclei were stained by either a 10 min rinse in 1 μ M Hoechst dye (Sigma, St. Louis, MO) or by adding 2 μ M DAPI (Sigma, St. Louis, MO) to the buffered glycerol solution used to mount the slides. In cases where double-labeling was performed, the two primary antibodies were applied simultaneously and later the two secondary antibodies were added together [57]. Tissues were examined and photomicrographs taken on a Leitz Laborlux fluorescent microscope with N2, I2/3 and D filters or on a Nikon Eclipse E600 fluorescent microscope with FITC, R/DII and DAPI filters. Cells were counted using the 100x objective. Images were recorded using the MetaVue software (version 6.0; MDS Analytical Technologies, Toronto, Canada) or the Spot Basic software (version 4.7; Diagnostic Instruments, Sterling Heights, MI), and Image J (version 1.37; NIH, Bethesda, MD). These were cropped, brightness and contrast adjusted, using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Results

Anti-pax6, anti-nurr1, and anti-PH3 identified subpopulations of neurons and fibers of *H. glaberrima*, however, the intensity and localization of the immunoreactivity varied with each antibody. In the following sections we will describe the presence and localization of cells and fiber in the RNCs, peripheral nerves, body wall and large intestine (Fig. 1A–B). The nervous system in these organs has been thoroughly described in previous studies by our group (Díaz-Balzac et al. 2010a; Díaz-Balzac et al. 2010b; Díaz-Balzac et al. 2007; Díaz-Miranda et al. 1995; Díaz-Miranda et al. 1996) and thus provides an excellent starting point for comparison of the immunoreactivity observed with the new markers.

Immunoreactivity in *H. glaberrima*

Radial nerve cord—The presence of immunoreactive neurons and fibers was initially determined in the radial nerve cord, the main component of the echinoderm nervous system. All three antibodies recognized fibers in both the ectoneural and hyponeural divisions of the radial nerve cord, although the density of fibers and the area of the neuropile that was identified differed with each antibody (Fig. 2). Anti-pax6 recognized extensively most of the neuropile showing a particularly dense fibers network in the apical mediolateral portions of the ectoneural component (Fig. 2A), as well as identifying individual cells in the ectoneural component of the radial nerve (Fig. 3A). Anti-PH3 identified the lateral and central regions of the ectoneural component, while in the hyponeural component the strongest immunoreactivity was found in the lateral regions (Fig. 2B). Anti-nurr1 showed specific immunoreactivity to the mediolateral area of the neuropile of the ectoneural component and in most of the hyponeural component (Fig. 2C). Most importantly, anti-nurr1 clearly identified cells in both components of the radial nerve cord and particularly in the hyponeural component (Fig. 3C).

Peripheral nerves—All antibodies also identified the peripheral nerves that originate both from the ectoneural and hyponeural components (Fig. 2D–F). The nerve that originates from the ectoneural compartment eventually gives rise to the podial nerve, a ganglionated nerve that borders (and innervates) the tube feet. Immunoreactivity to anti-pax6 and anti-PH3 (Fig. 3B) was observed in cells localized within the podial nerve.

Body wall connective tissue—The immunoreactivity of the fibers within the body wall connective tissue was particularly different for the three markers. Anti-pax6 identified a network of fibers that were associated with the connective tissue surrounding the tube feet (Fig. 2G). Interestingly, no other immunoreactivity with this antibody was detected in other areas of the body wall connective tissue. Anti-PH3 had little if any immunoreactivity within the connective tissue (Fig. 2H). Only some very rare discrete fibers were identified within the body wall dermis, which were clearly associated to the tube feet. Anti-nurr1 recognized many discrete fibers within the body wall dermis close to the circular body wall muscle (Fig. 2I). Few, if any anti-nurr1-immunoreactive fibers were observed in the most external dermis of the body wall or in the connective tissue near the tube feet.

Large intestine—This tissue also showed particular differences in the immunoreactivity of the three markers. Anti-pax6 identified fibers in the intestinal mesothelium and some fibers were observed to cross over into the intestinal connective tissue (Fig. 2J). In addition, a few, isolated neuroendocrine cells within the luminal epithelium were strongly immunoreactive to anti-pax6 (Fig. 3D). Some of these cells showed fibers that extended from the basal to the apical parts of the epithelium. Moreover, some cells appeared to have extensions that entered the connective tissue on the basal side. Anti-PH3 identified a small number of fibers in the intestinal mesothelium (Fig. 2K). These fibers were spatially restricted to the mesothelium and to the adjacent mesentery, and were never observed within the intestinal connective tissue. No neuroendocrine cell was observed to be immunoreactive to anti-PH3. Anti-nurr1 identified a network of fibers in the mesothelium and a few cells and fibers within the connective tissue layer (Fig. 2L). This antibody also identified a small subpopulation of neuroendocrine cells in the luminal epithelium (Fig. 3E).

In summary, the three markers: anti-pax6, anti-nurr1, and anti-PH3 showed immunoreactivity in well-described neural structures, such as the radial nerve cord, peripheral nerves, and intestinal plexi. The immunoreactivity was localized to fibers and cells that had a neural phenotype, suggesting that each marker recognized subpopulations of cells and fibers of the nervous system.

Neural specificity

To further confirm the neural specificity of these markers, we performed double-labeling experiments with the neural marker RN1. This is a monoclonal antibody that has been shown to identify many nervous structures in *H. glaberrima* (Díaz-Balzac et al. 2007). The immunoreactivity by this antibody is particularly conspicuous in fibers and in particular those associated with the connective tissues. Overall, structures that were recognized with our antibodies were also identified with RN1. In many areas the high density of fiber immunoreactivity precluded the possibility of determining whether a particular fiber co-expressed both markers. Thus, we focused most of our attention to the body wall dermis and the circular muscle. These are the areas where individual fibers could best be observed. These experiments showed that most, if not all, of the fibers immunoreactive to anti-pax6 and anti-nurr1 were co-labeled by RN1 (Fig. 4A–C and G–I). In contrast, most of the anti-PH3 immunopositive fibers were not co-labeled with RN1. These PH3-immunoreactive fibers were usually found associated with larger nerves that were strongly RN1

immunoreactive, but in those cases where individual anti-PH3 immunoreactive fibers could be clearly distinguished, these did not show RN1 immunoreactivity (Fig. 4D–F).

Immunoreactivity in other holothurian species

To determine that the immunoreactivity by these antibodies was not a particular cross-reactivity limited to *H. glaberrima* we tested the antibodies in representatives of three different Holothuroidea orders. These were *Holothuria mexicana* (Aspidochirotida), *Leptosynapta clarki* (Apodida) and *Sclerodactyla briareus* (Dendrochirotida). The immunoreactivity was analyzed in some of the structures previously described in *H. glaberrima*.

Radial nerve cord—All three markers identified either fibers or neural-like cell bodies in both ectoneural and hyponeural components of the RNC. Nonetheless, each antibody identified cell and/or fiber populations that were specific both to the species and to the antibody itself. Anti-pax6 immunoreactivity showed the most similarity across species, immunoreactivity was observed within the lateral regions of the ectoneural and hyponeural components of the three species. In *H. mexicana*, immunoreactivity was also observed in fibers located in the central region of the ectoneural component (Fig. 5A). *L. clarki*, also showed central region immunoreactivity although in this species the central immunopositive fibers were less in number when compared to the lateral regions (Fig. 5B). In *S. briareus*, a small number of immunoreactive fibers were identified in the apical-lateral regions of the ectoneural component, while a larger number of immunoreactive fibers were identified in the lateral regions of the hyponeural component (Fig. 5C). However, in contrast to the other species, a few unipolar cell bodies were visible in the apical-lateral region of *S. briareus* (not shown).

Anti-PH3 also identified fibers in the lateral regions of the ectoneural component in *H. mexicana* and all through the hyponeural component (Fig. 5D). Anti-PH3 immunoreactivity in *L. clarki* was similar in localization to that of anti-pax6, but the number of immunopositive fibers appeared to be smaller (Fig. 5E). In *S. briareus*, the most prominent immunoreactivity of anti-PH3 in the RNC was that of a large number of cell bodies with neuronal-like morphologies resembling unipolar cells throughout both the ectoneural and hyponeural (Fig. 5F). Finally, anti-nurr1 identified fibers in the lateral regions of both components of the RNC in *H. mexicana* and *L. clarki* (Fig. 5G–H). In *H. mexicana*, the immunopositive fibers in the ectoneural component were mainly restricted to the basal regions where the peripheral nerves originate. In contrast to the two other species, in *S. briareus*, anti-nurr1 immunoreactivity was observed throughout the whole RNC, without a clear separation into regions (Fig. 5I).

Peripheral nerves and body wall connective tissue—In general terms, *H. mexicana* showed similar immunoreactivity patterns for the three antibodies when compared to *H. glaberrima* (Fig. 6A–C). The three antibodies identified the peripheral nerves that originated from both ectoneural and hyponeural components. Also, similar to *H. glaberrima*, anti-pax6 recognized a large number of fibers in the connective tissue that surrounds the tube feet (Fig. 6D), but showed little or no immunoreactivity in other areas of the connective tissue. There

was little or no immunoreactivity of fibers in the connective tissue with anti-PH3 or anti-nurrl.

A similar, but not identical, distribution of the immunoreactivity was observed with the three markers in representatives of the other two orders of holothurians. Immunoreactivity to anti-pax6 or anti-PH3 within the connective tissue differed in *L. clarki* and *S. briareus*. No immunoreactivity was observed with either marker in *L. clarki*, while in *S. briareus*, a population of fibers and cells with bipolar morphologies was identified with anti-pax6 (Fig. 6E). Also, anti-PH3 immunopositive cell bodies with unipolar or multipolar morphologies were observed in the body wall connective tissue of *S. briareus* (Fig. 6F). Immunoreactivity to anti-nurrl also differed in these two species. In *L. clarki*, anti-nurrl identified the peripheral nerves localized under the circular body wall muscle (Fig. 6G). Being an apodid, instead of tube feet, *L. clarki* has dorsal papillae, which are directly innervated by the anti-nurrl immunoreactive peripheral nerves and these fibers end in bipolar cells with small fibers extending to the papillae's cuticle. In *S. briareus*, anti-nurrl immunopositive fibers were also observed in peripheral nerves localized under the circular body wall muscle and in the podial nerve (Fig. 6H). However, no immunoreactivity was observed in the body wall connective tissue of *L. clarki* with anti-nurrl, while in *S. briareus*, anti-nurrl identified a few fibers that were dispersed throughout the connective tissue (Fig. 6I).

Intestine—In this tissue, observations regarding each antibody were most different when compared across species. Anti-pax6 immunoreactivity in *H. mexicana* was heavily present in fibers of the mesothelium, with some fibers traversing the connective tissue (Fig. 7A). In *L. clarki* the fiber immunoreactivity was restricted to the connective tissue, but here a few unipolar and bipolar cells were identified (Fig. 7B). These were usually present in the connective tissue proximal to the mesothelium. No immunoreactivity to anti-pax6 was observed in *S. briareus* intestine.

Anti-PH3 immunoreactivity was restricted to the mesothelium of *H. mexicana* (Fig. 7C). In *L. clarki*, anti-PH3 identified a population of fibers in the connective tissue, with solitary fibers extending through the border between the connective tissue and the luminal epithelium (Fig. 7D). Anti-PH3 recognized many neural-like cell bodies in *S. briareus* with unipolar and multipolar morphologies in all layers of the intestine (Fig. 7E–G). Finally, anti-nurrl recognized a small subpopulation of fibers in *H. mexicana*'s mesothelium (Fig. 7H). No immunoreactivity to this antibody was observed in *L. clarki*, and, in the case of *S. briareus*, fibers and cells were observed in the mesothelium with sparse fibers localized also in the connective tissue (Fig. 7I).

Discussion

Neural specificity of novel antibodies

In this study we describe the immunoreactivity of three different antibodies in four different holothurian species representing the three orders of Holothuroidea. Our results show that the immunoreactivity of the three markers is specific to nervous system components. However, since the interpretation of our results depends on the fact that the markers are indeed

recognizing nervous structures, it is important to further discuss the nature of the epitopes and what is known about their relationship with the nervous system in other species.

Nurr1 is a nuclear orphan receptor/transcription factor found in the vertebrate nervous system that has been associated with the differentiation of catecholaminergic neurons (Zetterstrom et al. 1997). This protein is highly conserved across taxa (Burke et al., 2006a), thus it is not surprising that cross-reactivity to this molecule is found in holothurians. In fact, a Nurr1-like protein (XP_786266) was also predicted, in another echinoderm, *S. purpuratus*, as part of the sea urchin genome sequencing project (Burke et al. 2006a). Pax6, also a transcription factor, is an important regulator of vertebrate eye development and was initially identified in mice (Hill et al. 1991). Since then its presence has been documented in a wide range of species ranging from *Drosophila* to vertebrates. In echinoderms, Pax6 was isolated from the embryo of the echinoid *Paracentrotus lividus* (Pascoe, 1874) (Echinoidea, Echinoidea) (Czerny and Busslinger 1995). It is expressed during the gastrula stage and in the podia of adult sea urchins (Czerny and Busslinger 1995). In the adult tube feet, Pax6 is expressed exclusively in photoreceptors cells, suggesting that its main function, as a key transcription factor required for the differentiation of photosensory cells, is conserved in echinoderms (Agca et al. 2011; Lesser et al. 2011; Ullrich-Luter et al. 2011). However, some of our results suggest that the epitopes being recognized by these two markers in *H. glaberrima* might differ from those of which they were designed against. The principal finding suggesting that these antibodies are not recognizing PAX6 and NURR1 homologs in the holothurians is the lack of immunoreactivity in the cell's nuclei, where it would be expected, since both molecules are transcription factors and as such their main function is exerted in the nucleus.

Notwithstanding which molecule they might be recognizing, it is clear that in holothurians immunoreactivity to these two markers was found to be restricted to fibers in nervous structures and to cells with a neuronal morphology. Close inspection of individual fibers within the circular and longitudinal muscles showed that the fibers identified with these 2 antibodies were similar to those previously described to be immunopositive to anti-GFSKLYFamide, RN1, anti-TH, or anti-calbindin. Moreover, double-labeling experiments using RN1, showed that most, if not all, of the fibers immunoreactive to anti-pax6 and anti-nurr1 were co-labeled by RN1, a bona fide neuronal marker (Díaz-Balzac et al. 2007). Therefore, the available evidence favors our contention that these antibodies are indeed recognizing nervous elements in *H. glaberrima*.

Anti-PH3 immunoreactivity represents a very difficult case. This is a commercial antibody known to identify dividing cells in a large number of species (Friocourt et al. 2008; Karashima et al. 2000). However, this is apparently not the case in *H. glaberrima*, where the immunoreactivity was very specific to cells and fibers of neuronal morphology and localization. This was documented by the neuronal-like morphology of their soma, their localization within well-known nervous system structures and their long fibers that extended from/to the RNCs showing nervous-like varicosities. These morphological features strongly suggest that they represent nervous system components. Similarly, immunoreactivity of anti-PH3 in other holothurian species also appears to be mainly restricted to nervous system elements. The fact that the fibers identified by anti-PH3 were not co-labeled by RN1 is not

entirely surprising in view of recent unpublished evidence from our laboratory showing that although RN1 identifies a large component of the nervous system of *H. glaberrima*, this antibody is not pan-neural and therefore does not recognize all nervous system cells or fibers.

The fact that the PH3 antibody is recognizing nervous-related structures in holothurians is puzzling, particularly in view of the fact that no immunoreactivity is observed in the nuclei, where histones are localized and where previous studies have documented its immunoreactivity in dividing cells (Friocourt et al. 2008; Karashima et al. 2000). Thus, we propose that the antigen against which this polyclonal antibody was raised, shares some similarity to a neural-specific antigen(s) found in holothurians. Other such cases can be found in the scientific literature, exemplified by a monoclonal antibody to horseradish peroxidase that recognizes the *Drosophila* nervous system (Sun and Salvaterra 1995).

Subdivisions of the holothurian nervous system

At the morphological and anatomical level the echinoderm nervous system has been traditionally divided into the ectoneural and hyponeural components of the radial nerve cord and the enteric nervous system (Garcia-Ararras et al. 1999; Hyman 1955). None of the three novel markers used in this study showed any specificity for one of these divisions, as all of the markers identified fibers or cells within the three subdivisions. Such a broad range of immunoreactivity is not surprising in view that other markers of the holothurian nervous system have shown similar ranges. These include the immunoreactivity against the neuropeptides galanin and GFSKLYFamide (Diaz-Miranda et al. 1995; Diaz-Miranda et al. 1996), the calcium-binding protein calbindin (Diaz-Balzac et al. 2012), and immunoreactivity to the antibody RN1 that recognizes a yet undetermined neural-specific epitope (Diaz-Balzac et al. 2007).

Comparisons of the new and the well-studied markers in *H. glaberrima*, which has been the holothurian that has been studied in most detail in terms of its nervous system, do allow us to make some key observations. First, none of the immunoreactivity patterns of the new markers is identical to those of the previously used markers. This suggests that the group of cells and fibers that is identified is either a subdivision of those previously described using other markers or a different component altogether. For example, both anti-pax6 and anti-nurr1 appear to identify subdivisions of the nervous system recognized by RN1, while anti-PH3 identifies a new (RN1-negative) subdivision. In the case of anti-pax6, although there are similarities to the immunoreactivity of anti-GFSKLYFamide with that of anti-pax6, there are also some particular differences that demonstrate that they are not recognizing the same neural subpopulations. This is best observed by their immunoreactivity within the radial nerve cord, particularly the large group of anti-pax6 immunoreactive fibers that was observed in the two apical central regions that are not observed with anti-GFSKLYFamide. Similarly, anti-nurr1 is the first marker that appears to preferentially identify cells of the hyponeural component over those of the ectoneural component, contrary to anti-galanin, which preferentially identifies cells and fibers of the ectoneural component (Diaz-Miranda et al. 1996). This is particularly surprising in view of the fact that in vertebrates, Nurr1 function has been associated with the catecholaminergic neurotransmitter system

(Zetterstrom et al. 1997), but previous studies from our group have shown that the holothurian catecholaminergic system is present within the ectoneural component of the radial nerve cord (Díaz-Balzac et al. 2010b). Finally, most of the cells immunoreactive to anti-PH3 and anti-pax6 were found outside of the radial nerve cord, suggesting that they correspond to a peripheral nervous system component and that most of the fibers identified by these antibodies within the radial nerve cord itself might correspond to afferent fibers originating from the somas localized in peripheral regions, such as the podial nerve or enteric nervous system.

Comparative neuroanatomy of holothurians

We explored the three marker's immunoreactivity in *H. mexicana*, *L. clarki* and *S. briareus* in order to show that their immunoreactivity in *H. glaberrima* was not spurious and that these antibodies were indeed recognizing nervous elements by showing that they also did so in other holothurian species. The fact that all three species show the immunoreactivity to be restricted to nervous elements supports our argument. It is particularly important to emphasize that the three species are from three different orders, including the most basal order, Apodida. Thus, our results suggest that the markers will serve to identify specific nervous system components in all holothurian species and that they might serve to identify neural structures in other echinoderm groups as well. As expected, because of their phylogenetic proximity, *H. mexicana* has the most similar immunoreactivity to *H. glaberrima* for the three markers. Nonetheless, there are evident differences in the immunoreactivity among the representative species of the three different orders. The most intriguing finding was that of anti-PH3 immunopositive cells in the radial nerve of *S. briareus*; since most of the immunoreactive cells are elongated cells within the neuropile that have not been described previously.

Comparisons of the antibody immunoreactivity of the enteric nervous system showed several differences among the three different species. The most striking being those observed in *L. clarki* where little, if any immunoreactivity, was observed in the mesothelium while anti-pax6 recognized isolated neurons with long fiber extensions in the submucosa. It is possible that these are properties of the enteric nervous system of apodids. However, some of the differences observed in the enteric nervous system of the three species could also be due to variations associated with the level of the intestinal tissue that was studied and not necessarily species differences. Such differences have been well documented in *H. glaberrima* where, for example an intestinal catecholaminergic plexus is observed in the esophagus and descending small intestine but is not found in other intestinal regions (Díaz-Balzac et al. 2010b). In this respect, it is important to highlight that the posterior (large) intestine of *H. mexicana* was studied, but for the other two species the sections were obtained at mid-body level, thus it is not clear if the intestinal tissue at that level corresponds (anatomically or functionally) to a more small intestine (nutrient absorptive function) or large intestine (feces compaction function) type.

A recent report characterizing the distribution of FMRFamide- and histamine-like immunoreactivities in the nervous system of *L. clarki* (Hoekstra et al. 2012) provides for a comparison of the results obtained in this species with our three novel markers. In general

terms, the immunoreactivity of all the antibodies is found within the same structures, in small subset of cells and fibers. Anti-histamine was reported to identify a large number of fibers within the RNC, while anti-nurr1 was limited to the lateral sides of the ectoneural and hyponeural components. Upon close inspection of their immunoreactivity, they seem to share a similar distribution. This is particularly evident in the identification of bipolar cells in the papillae and of fibers in the longitudinal and circular muscles. Thus, although anti-histamine appears to recognize a larger group of neural structures, it is possible that both anti-histamine and anti-nurr1 are identifying the same sub-set of cells and/or fibers. However, whether cells or fibers co-express some of these marker cannot be determined with the available antibodies since all of them are made in the same species (rabbit) ruling out the possibility of doing double-labeling studies.

In summary, our results provide evidence that antibodies raised against two transcription factor orthologs (Pax6 and Nurr1) identify subpopulations of cells and fibers of the holothurian nervous system. A third marker, anti-PH3, with a still unknown epitope, identified a novel subdivision of the nervous system, which is not recognized by RN1. Putting aside the nature of the epitopes recognized by the antibody markers, our results highlight several components or subdivisions of the holothurian nervous system, and moreover, provide markers to identify them in several species. Therefore, the discovery of these novel markers serves to expand the toolkit available for studies of the holothurian nervous system, and possibly of other echinoderm as well. Future experiments should be aimed at determining if these components correspond to specific anatomical and functional circuits. More importantly, the nature of the epitope being recognized by these markers should also be investigated, although it might prove to be difficult due to the polyclonal nature of the antibodies.

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Fig. 1.

The holothurian nervous system. Transverse section through the body wall (A), depicting the longitudinal muscle, circular muscle, radial nerve cord, peripheral nerve, dermis, and epidermis. Transverse section through the large intestine (B) depicting its three main layers; the serosa, which is composed of the coelomic epithelium, longitudinal muscle, and circular muscle; the submucosa, which is composed of the connective tissue; and mucosa, which is composed of the luminal epithelium. CE, coelomic epithelium; CM, circular muscle; CT, connective tissue; DE, dermis; EP, epidermis; LE, luminal epithelium; LM, longitudinal muscle; PN, peripheral nerve; RNC, radial nerve cord.

Fig. 2.

Anti-pax6, anti-PH3 and anti-nurr1 immunoreactivity was observed in various nervous system components of *H. glaberrima*. Cross sections of radial nerve cord (A–C), peripheral nerves (D–F), body wall connective tissue (G–I) and large intestine (J–L) were labeled by anti-pax6 (A, D, G, J), anti-PH3 (B, E, H, K) and anti-nurr1 (C, F, I, L). Scale bar = 40 μ m A–C, F, and J–L; and 20 μ m D–E, and G–I.

Fig. 3.

Anti-pax6, anti-PH3 and anti-nurr1 immunoreactivity was observed within cells of the nervous system of *H. glaberrima*. Arrowheads indicate neuron-like cells labeled by anti-pax6 in the ectoneural component of the radial nerve cord (A), by anti-PH3 in the podial nerve (B) and by anti-nurr1 in the hyponeural component of the radial nerve cord (C). Neuroendocrine-like cells in the large intestine mucosa are immunopositive to anti-pax6 (D) and by anti-nurr1 (E). Scale bar = 20 μ m A–E.

Fig. 4.

Double labeling of neuronal fibers with RN1 and the novel markers (anti-pax6, anti-PH3 or anti-nurr1) in the body wall or muscle tissue of *H. glaberrima*. Cross sections of body wall showing double labeling for anti-pax6 (A), RN1 (B) and the overlay (C). Body wall cross sections showing double labeling of fibers in the circular muscle with anti-PH3 (D), RN1 (E) and the overlay (F). Double labeling of anti-nurr1 (G), RN1 (H) and overlay (J) in transverse sections of the body wall. Most, if not all, of the fibers labeled with anti-pax6 and anti-nurr1 (arrows) are co-labeled by both markers, contrary to fibers labeled by anti-PH3 (see arrows in D) which are not the co-labeled by RN1 (arrowheads in E). Scale bar = 20 μ m A–E.

Fig. 5.

Anti-pax6, anti-PH3 and anti-nurr1 immunoreactivity in nervous system components of *H. mexicana*, *L. clarki* and *S. briareus*. Anti-pax6 (A–C), anti-PH3 (D–F) and anti-nurr1 (G–I) immunoreactivity in the radial nerve cords of *H. mexicana* (A, D, G), *L. clarki* (B, E, H) and *S. briareus* (C, F, I) show the particular immunoreactivity patterns of each marker in each species. Scale bar = 40 μ m A, D–E, and G; and 20 μ m B–C, F, and H–I.

Fig. 6.

Anti-pax6, anti-PH3 and anti-nurr1 immunoreactivity in nervous system components of *H. mexicana*, *L. clarki* and *S. briareus*. Cross sections of the body wall show the immunoreactivity in peripheral nerves (A–C) and fibers (arrows) within the body wall connective tissue of *H. mexicana* (D). Cells (arrowheads) immunopositive to anti-pax6 (E) and anti-PH3 (F) are shown in the body wall connective tissue of *S. briareus*. Anti-nurr1 immunoreactivity was observed in fibers localized in the peripheral nerves of both *L. clarki* (G) and *S. briareus* (H) as well as in the body wall connective tissue of *S. briareus* (J). Scale bar = 40 μ m A, D, and H–I; and 20 μ m B–C, E–F, and G.

Fig. 7.

Anti-pax6, anti-PH3 and anti-nurr1 immunoreactivity was observed in the enteric nervous system components of *H. mexicana*, *L. clarki* and *S. briareus*. Cross sections of intestinal tissue show the immunoreactivity of anti-pax6 (A–B), anti-PH3 (C–H) and anti-nurr1 (I–J) in the enteric nervous system of the three species. Anti-pax6 immunoreactivity was observed in the mesothelium plexus of *H. mexicana* (A) and neuron-like cells (arrowhead) in the submucosa of *L. clarki* (B). Anti-PH3 immunoreactivity was observed in fiber localized in the mesothelium of *H. mexicana* (C), *L. clarki* (D), and cells *S. briareus*. Anti-PH3 immunopositive cells (arrowheads) were identified in the luminal plexus (E) submucosa (F), and mesothelium (G) of *S. briareus*. Anti-nurr1 immunoreactivity was observed in fibers of the mesothelium of *H. mexicana* (H) and in fibers and cells (arrowheads) of the mesothelium of *L. clarki* (I). Scale bar = 40 μ m A–C, and H; 20 μ m in D and I; and 8 μ m E–G.

Table 1

Antibodies used in this study

Antigen	Raised in	Immunogen	Source	Dilution used
Unknown (RN1)	mouse (monoclonal)	Homogenate of the radial nerve of <i>H. glaberrima</i>	Dr. García-Arrarás lab (Díaz-Balzac et al. 2007)	1:100,000
Histone H3	rabbit (polyclonal)	Amino acids 7-20 of human histone H3	Millipore (06-570)	1:250
Nurr1	rabbit (polyclonal)	Peptide mapping at the C-terminus of Nurr1 of rat	Santa Cruz Biotechnology (sc-990)	1:500
Pax6	rabbit (polyclonal)	synthetic peptide C-REEKLRNQRRQASNTPSHI	Abcam (ab5790)	1:100

For more detailed information, see Materials and Methods.