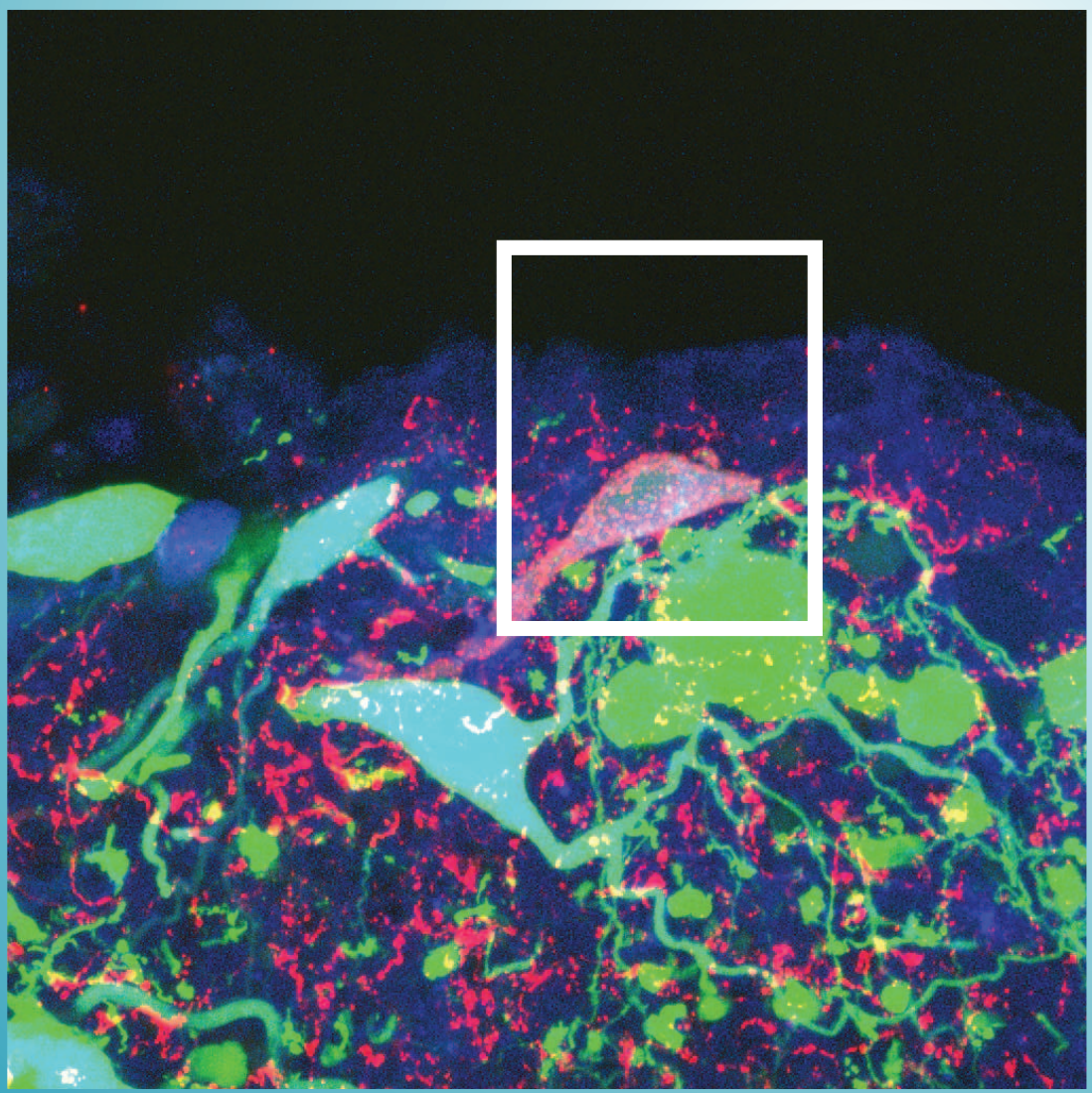
UNIVERSIDAD DE SANTIAGO DE COMPOSTELA
DEPARTAMENTO DE BIOLOGÍA CELULAR Y ECOLOGÍA
ÁREA DE BIOLOGÍA CELULAR

NEW INSIGHT INTO THE SEROTONERGIC SYSTEM OF SEA LAMPREY DURING DEVELOPMENT AND REGENERATION



DOCTORAL THESIS
MARÍA EUGENIA CORNIDE PETRONIO
SANTIAGO DE COMPOSTELA, 2013

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Universidad de Santiago de Compostela
DEPARTAMENTO DE BIOLOGÍA CELULAR Y ECOLOGÍA
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NEW INSIGHT INTO THE SEROTONERGIC SYSTEM OF SEA LAMPREY DURING DEVELOPMENT AND REGENERATION

MEMORIA

Que para optar al Grado de Doctor en Biología presenta

María Eugenia Cornide Petronio

Santiago de Compostela, 2013

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DE COMPOSTELA

MARÍA CELINA RODICIO RODICIO, CATEDRÁTICO DEL ÁREA DE BIOLOXÍA CELULAR DEL DEPARTAMENTO DE BIOLOXÍA CELULAR E ECOLOXÍA DE LA UNIVERSIDADE DE SANTIAGO DE COMPOSTELA,

Y

ANTÓN BARREIRO IGLESIAS, INVESTIGADOR DEL CENTRE FOR NEUROREGENERATION DE LA UNIVERSITY OF EDINBURGH,

CERTIFICAN:

Que la presente memoria titulada "New insight into the serotonergic system of sea lamprey during development and regeneration", (Nuevas perspectivas en el sistema serotonérgico de la lamprea de mar durante el desarrollo y regeneración) que para optar al Grado de **Doctor en Biología** presenta Dña. MARÍA EUGENIA CORNIDE PETRONIO, ha sido realizada bajo nuestra dirección, y considerando que constituye trabajo de TESIS DOCTORAL, autorizamos su presentación a la Comisión Académica correspondiente.

Y para que así conste, expedimos el presente certificado en Santiago de Compostela, a 31 de Mayo de 2013.

La Doctoranda

Los Directores de Tesis

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Gracias a todos los que me habéis apoyado a lo largo de estos años para realizar la tesis. No se habría hecho sin la ayuda de mis jefes Celina y Antón. Gracias por la oportunidad que me disteis y todo lo que me habéis enseñado. Gracias también a Ramón, por su experiencia y ayuda incalculables. A la familia, que siempre os habéis interesado por mi trabajo y sin lugar a dudas fuisteis un gran apoyo. Y a todos compañeros del departamento, en especial al lab 3, y por supuesto a mi querido lab 4. Gracias!

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GENERAL INTRODUCTION

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GENERAL INTRODUCTION

The sea lamprey

Lampreys belong to the most ancient group of extant vertebrates: cyclostomes or agnathans, which are jawless vertebrates (Hardisty and Potter, 1971). They hold a unique phylogenetic position between *Amphioxus* (protochordates) and gnathostomes (jawed vertebrates) (Fig. 1) (Hardisty, 1983; Kuratani et al., 2002; Takezaki et al., 2003). Living lampreys are represented by three families; two of them live in the southern hemisphere and one in the northern hemisphere (Hubbs and Potter 1971, Gill et al. 2003). Northern lampreys belong to the family Petromyzontidae, which contains 38 species (Lang et al. 2009). Three of those species live in the Iberian Peninsula: sea lamprey *Petromyzon marinus* L., European river lamprey *Lampetra fluviatilis* L. (only in Portugal), and European brook lamprey *L. planeri* Bloch. The first two species are anadromous and parasitic, and the other one is a freshwater resident and non-parasitic. *P. marinus* is found on both sides of the North Atlantic, on the east coast of North America and from the Barents Sea (Russia) to the Iberian Peninsula and Adriatic Sea (Italy) in Europe (Hardisty 1986). In Spain, *P. marinus* occurs in most rivers flowing into the Cantabrian Sea and the Atlantic Ocean, but also into the Mediterranean. In Galicia, they live in the rivers Eo, Masma, Ouro, Mera, Mandeo, Anllóns, Tambre, Ulla, Umia, Lérez and the last stretch of the river Miño (Mateus et al., 2012).

The life cycle of the sea lamprey is unique among vertebrates (Fig. 2). In the breeding season, adult lampreys build a nest in which they lay the fertilized eggs. The diameter of the eggs is around 1 mm, and every female can lay several hundreds of thousands. Fertilization is followed by an embryonic period that lasts for some 11 days after fertilization and then by a prolarval stage when the eggs hatch. The prolarval stage lasts between 22 and 25 days, at the end of which they become larvae. Larvae, known as

ammocoetes, burrow into the silt deposits of the river bed where they feed on microorganisms. At this stage, they are toothless and have subcutaneous, rudimentary eyes. The larval stage may last for up to seven years (Hardisty, 1951). After that, they undergo a metamorphosis that implies drastic changes in the morphology and the physiology of the animal, such as the acquisition of functional eyes, the development of the oral disc, the formation of the rough tongue, and changes in body coloration and osmoregulation mechanisms (Youson, 1980; Nieuwenhuys and Nicholson, 1998). Young adult lampreys migrate to the sea where they remain for 2 to 4 years feeding as parasites on any class of fish. At the end of the adult stage, lampreys stop feeding and return to the rivers where they spawn and die.

Lamprey as a model animal in Neurobiology

Lampreys hold a key phylogenetic position between *Amphioxus* and gnathostomes, which makes them unique species to study the evolution of morphological characters and the basic molecular mechanisms at the dawn of Craniates and throughout the evolution of this phylum (for a revision see Osório and Rétaux, 2008; see also Murakami and Watanabe, 2009). So, lampreys are a very important animal model for evolutionary and developmental ("evo-devo") studies. In the last years, the whole genome of sea lamprey has been sequenced and published in http://www.ensembl.org and evolutionary studies are being carried out from a genomic point of view. (see Smith et al., 2013).

Literature of comparative neuroanatomical studies of lampreys dates back to the middle 1800s. The gross anatomy of their nervous system and path of axonal fibers were extensively studied (e.g. Reissner, 1860; Johnston, 1905). Since these first studies, the cytoarchitecture of the lamprey nervous system has been well described in the literature of comparative neuroanatomy (see Nieuwenhuys and Nicholson, 1998).

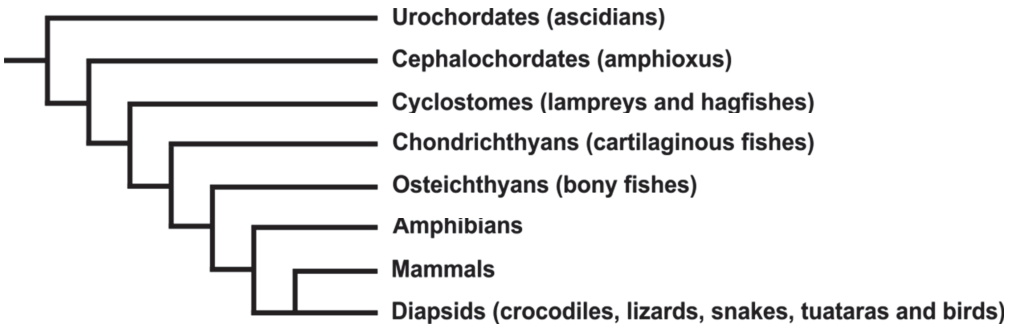


Figure 1. Vertebrate phylogeny.

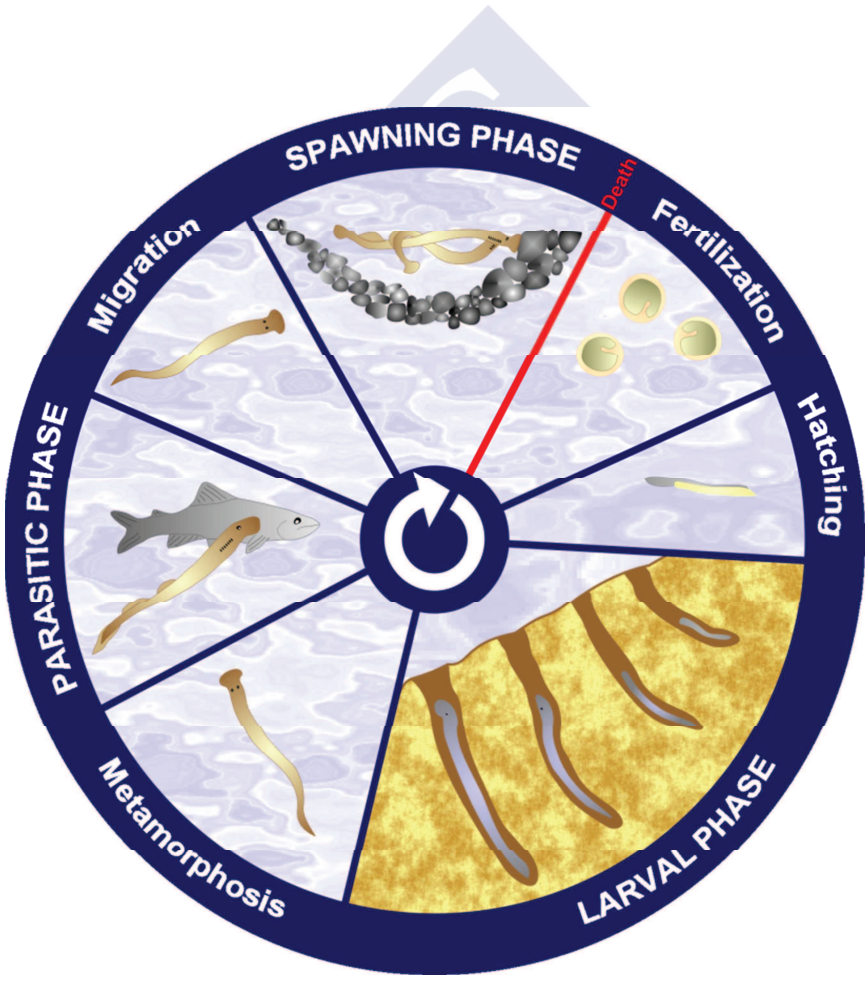


Figure 2. Life cycle of sea lamprey.

The lamprey nervous system has also been used to study the neuronal circuitry underlying locomotion in vertebrates (see Dubuc et al., 2008; see Grillner et al., 2008). The central nervous system (CNS) of the lamprey has all the main regions of the mammalian CNS but much fewer neurons; therefore, it is much easier to study the circuits for the coordination of locomotion than in other vertebrate species. Moreover, the isolated lamprey brainstem and spinal cord can be maintained alive in vitro for several days. Because of this, the motor pattern underlying locomotion in lampreys can be elicited through stimulation of the brainstem locomotor areas of the di- or rhombencephalon corresponding to the locomotor areas in mammals. In the isolated lamprey spinal cord, locomotion activity can be elicited by pharmacological activation and the fictive motor activity can be monitored by ventral root recordings. These preparations have been used extensively to investigate the organization of the locomotor pattern generating network in lampreys (for a revision see Grillner and Wallén, 2007).

Lampreys have also been established as a model organism for studies into spinal cord regeneration (Cohen et al., 1988). In contrast to mammals, lampreys can recover normal-appearing locomotion after a complete spinal cord transection, and the spinal axons regenerate selectively in their correct paths (Rovainen, 1976; Selzer, 1978; Wood and Cohen, 1979). An NIH convened panel proposed five criteria that should be met in order to demonstrate functional regeneration in the spinal cord (Guth et al., 1980). Lampreys are the only vertebrate that have fully met these criteria: the experimental lesion must cause disconnection of nerve processes, processes of CNS neurons must bridge the level of injury, the regenerated fibers must make junctional contacts, the regenerated fibers must generate postjunctional responses and changes in function must derive from regenerated connections (see Shifman et al., 2007). However, previous studies have shown that even in the presence of functional recovery only about 50% of all injured descending axons regenerate through the scar (Davis and McClellan, 1994; Jacobs et al., 1997), which probably indicates that plastic events are also involved in the recovery of function. This feature favours the use of lampreys as a vertebrate animal model to study the intrinsic molecular

mechanisms that favour or impede the regeneration of descending axons attempting to regenerate in the same permissive environment after spinal cord injury (Shifman and Selzer, 2000, 2007; Shifman et al., 2009; Jin et al., 2009). Lampreys have been also proposed as a model to study evolution from a regeneration point of view ("evo-rego") (Barreiro-Iglesias, 2012).

In last years, lampreys have also been used as an animal model to study the citotoxic effects of proteins related to Alzheimer's disease on neurons. The lamprey brain has large identifiable neurons that can be microinjected with plasmids encoding human proteins. For example, the tau protein, whose accumulation or aggregation is the intracellular hallmark of neurofibrillary degenerative diseases, such as Alzheimer's disease, has been studied in lampreys (Hall, 1999; Hall et al., 2000; Honson et al., 2009; Lee et al., 2012).

Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a biogenic monoamine that was identified as a neurotransmitter about 60 years ago. It is a hydrophilic substance that does not pass the lipophilic blood (brain barrier) readily. Thus, its discovery in the brain indicated that serotonin is synthesized in the brain, where it plays an important role in brain function. The synthesis and primary metabolic pathways of serotonin are shown in Figure 4. The initial step in the synthesis of serotonin is the facilitated transport of the amino acid l-tryptophan from the blood into the brain. The primary source of tryptophan is dietary protein. Serotonergic neurons contain the enzyme l-tryptophan-5-monooxygenase, more commonly termed tryptophan hydroxylase (Tph), which converts tryptophan to 5-hydroxytryptophan (5-HTP) (Fig. 4). Tph is known to be the rate-limiting enzyme for serotonin synthesis (see Hensler, 2006). It is synthesized in serotonergic cell bodies of the raphe nuclei and is found only in cells that synthesize serotonin; its distribution in the brain is similar

to that of serotonin itself. The enzyme requires both molecular oxygen and a reduced pteridine cofactor, such as L-erythro-tetrahydrobiopterin (BH4), for its activity. 5-HTP is rapidly decarboxylated by the enzyme aromatic amino acid decarboxylase (AADC, also acting in catecholamine synthesis) to produce serotonin (Fig. 4).

As with other biogenic amine transmitters, serotonin is stored primarily in vesicles and is released by an exocytose mechanism. Storage of serotonin in vesicles requires its active transport from the cytoplasm. Two synaptic vesicle transporters have been cloned with 12 transmembrane domains: the vesicular membrane transporter 1 and 2 ($VMAT_1$ and $VMAT_2$, respectively). Serotonergic synaptic vesicles are characterized to contain a specific protein that binds serotonin with high affinity in the presence of Fe^{2+}. This serotonin-binding protein is present in serotonergic cells derived ontogenetically from the neuroectoderm (Tamir et al., 1994).

The release of serotonin occurs by a calcium-dependent process of exocytosis. Serotonin binds to pre- and/or postsynaptic serotonin receptors to produce a chemical signalling; in the presynaptic cell the feedback signalling is mediated by autoreceptors. Once in the synaptic cleft, serotonin is recycled by reuptake via membrane transporters and restored in vesicles, or degraded by the mitochondrial monoamine oxidase enzyme (MAO). The serotonin transporter (SERT) is located on serotonergic neurons and regulates the concentration of serotonin in the synapse, thereby influencing synaptic transmission. On the other hand, MAO converts serotonin to 5-hydroxyindoleacetaldehyde, and this product is oxidized by an NAD+-dependent aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid (5-HIAA) (Fig. 4). The intermediate acetaldehyde can also be reduced by an NADH-dependent aldehyde reductase to form alcohol 5-hydroxytryptophol. Whether oxidation or reduction takes place depends on the ratio of NAD^+ to NADH in the tissue. In the brain, 5-HIAA is the primary metabolite of serotonin.

Serotonin is also the precursor of the hormone melatonin. In the pineal gland and retina serotonin is converted enzymatically to the hormone melatonin (5-methoxy-N-acetyltryptamine; Gern and Ralph, 1979) (Fig. 4).

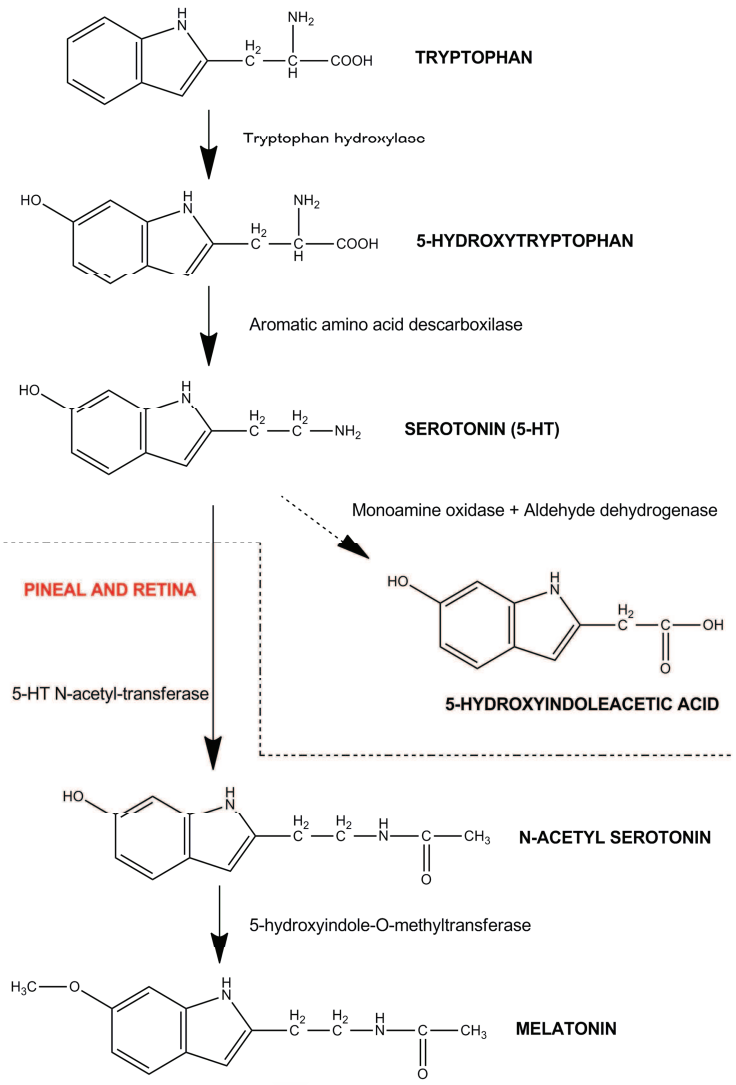


Figure 4. Serotonin synthesis and catabolism.

Serotonin receptors

The initial suggestion that there might be more than one type of receptor for serotonin came from experiments on the isolated guinea pig

ileum, which demonstrated that only a portion of the contractile response to serotonin could be blocked by high concentrations of morphine, whereas the remainder of the response could be blocked by low concentrations of dibenzyline (phenoxybenzamine) (Gaddum and Picarelli, 1957). In the 80s, the development of radioligand-binding assays furthered our understanding of subtypes of receptors for serotonin. Initially, a number of radioligands, such as (^{3}H)5-HT, (^{3}H)LSD and (^{3}H)spiperone, were used to label sites related to serotonin receptors (Leysen et al., 1979; Hamon et al., 1980; Peroutka et al., 1981; Leonhardt et al., 1989). These radioligands were originally proposed to label two classes of serotonin receptors in the brain. Binding sites that were labelled with high affinity by (^{3}H)5-HT were designated 5-HT_1 receptors, and binding sites labelled with high affinity by (^{3}H)spiperone were termed 5-HT_2 receptors. Subsequent studies with radioligans identify the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_2 binding sites. To date, at least 14 different serotonin receptor subtypes have been identified in mammals, which are grouped into seven families (5-HT_1–5-HT_7) (Kroeze and Roth 2006). Table 1 summarizes these different serotonin receptor subtypes in the mammalian CNS and their distribution. Serotonin receptors, except 5-HT_3, are members of the G protein receptor superfamily. This receptor family is characterized by the presence of seven transmembrane domains, an intracellular carboxy-terminus and an extracellular amino-terminus. It is the interaction of the receptor with the G protein that allows the receptor to modulate the activity of different effector systems, such as ion channels, phospholipase C and adenylyl cyclase. The 5-HT_1 receptor family contains receptors that are negatively coupled to adenylyl cyclase, whereas 5-HT_4, 5-HT_6 and 5-HT_7 receptors couple positively with adenylyl cyclase. The pharmacological profile of 5-HT_5 receptor family is very limited and different from the rest of the 5-HT receptors. Recently, a study by Nelson (2004) describes their affinities for different ligands, being similar to 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors. This suggested that they could be negatively coupled to adenylyl cyclase. 5-HT_2 receptors stimulate phosphoinositide-specific phospholipase C (PI-PLC). The transmembrane domains of G protein-coupled receptors are the most highly conserved regions of these proteins. The 5-HT_3 receptor differs from all other known subtypes of serotonin receptor

in that it is a member of the ligand-gated ion channel superfamily. Members of this receptor superfamily consist of five subunits, each of which possesses four transmembrane segments and a large, extracellular N-terminal region.

Cloning and physiological studies have been done also in invertebrates (for a review see Tierney, 2001) and in non-mammalian vertebrates (*Xenopus*: Lübbert et al., 1987; electric fish: Allee et al., 2008; zebrafish: Norton et al., 2008). Pharmacological and physiological studies have shown that lampreys have 5-HT_{1A}-like receptors and that they play a role in the modulation of the locomotion process (Wikström et al. 1995; Hill et al. 2003). However, despite the wide knowledge of the importance of serotonergic system of the lamprey CPG, the cloning and molecular characterization of the 5-HT receptors of lampreys has not been reported before nor its pattern of expression has been investigated.

The serotonergic system of vertebrates

The serotonergic system has been one of the first neurotransmitter systems of the CNS to be characterized histochemically (Parent, 1981; Parent and Northcutt, 1982; Parent, 1984). Serotonergic neurons are among the earliest cells to be born in the developing CNS and serotonin is one of the earliest neurotransmitters to appear in the nervous system. The anatomy of the serotonergic system has been thoroughly described in mammals (Frankfurt et al., 1981; Lidov and Molliver, 1982; Wallace and Lauder, 1983; Botchkina and Morin, 1993), chick (Wallace, 1985), reptils (Wolters et al., 1985; Rodrigues et al., 2008), anurans (van Mier et al., 1986; Zhao and Debski, 2005) and teleosts (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994). Non-mammalian vertebrates usually have serotonergic cells extensively distributed in the CNS (ray: Stuesse et al., 1990; sturgeon: Adrio et al., 1999; dogfish: Carrera et al., 2008). However, perikarya of these cells are largely restricted to the hindbrain in mammals (Rubenstein, 1998), although the serotonergic

RECEPTOR	DISTRIBUTION
5-HT_{1A}	Hippocampus, amygdala, septum, entorhinal cortex, hypothalamus, raphe nuclei, spinal cord
5-HT_{1B}	Substantia nigra, basal ganglia, superior colliculus, raphe nuclei, striatum, cerebellum, hippocampus, cerebral cortex, and nucleus accumbens
5-HT_{1D}	Not distinguishable from 5-HT_{1B}
5-HT_{1E}	Caudate and putamen, islands of Calleja, cortex, hippocampus, septum, hypothalamus
5-HT_{1F}	Cerebral cortex, striatum, hippocampus, olfactory bulb, claustrum, thalamus, amygdala, raphe nuclei
5-HT_{2A}	Claustrum, cerebral cortex, olfactory tubercle, striatum, nucleus accumbens
5-HT_{2B}	Hypothalamus, septum, amygdala, cerebellum
5-HT_{2C}	Choroid plexus, globus pallidus, cerebral cortex, hypothalamus, septum, substantia nigra, spinal cord
5-HT_3	Hippocampus, entorhinal cortex, amygdala, nucleus accumbens, solitary tract nerve, trigeminal nerve, motor nucleus of the dorsal vagal nerve, area postrema, spinal cord
5-HT_4	Hippocampus, striatum, olfactory tubercle, substantia nigra
5-HT_{5A}	Cortex, habenula, hippocampus, arcuate nucleus, raphe nuclei, parabigeminal nucleus, prepositus nucleus
5-HT_{5B}	Hippocampus, inferior olivary nucleus, raphe nuclei
5-HT_6	Striatum, olfactory tubercles, nucleus accumbens, cerebral cortex, hippocampus, hypothalamus, amygdala
5-HT_7	Cerebral cortex, septum, thalamus, hypothalamus, amygdala, superior colliculus

Table 1. Serotonin receptors present in the mammals CNS

system is involved in a large number of physiological functions resulting from the widespread serotonergic innervation of the brain.

Examples of the different functions of serotonin are the generation and modulation of sleep, pain, addiction, locomotion or sexual activity (Weiger, 1997). However, in addition to its role as neurotransmitter, serotonin is also involved in neuronal differentiation (Lauder and Krebs, 1978; Gromova et al., 1983; Haydon et al., 1984; Chubakov et al., 1986; Lauder, 1987; Goldberg and Kater, 1989; Goldberg et al., 1991; Meier et al., 1991; Lauder, 1993; Gaspar et al., 2003; Sodhi and Sanders-Busch, 2004). Growing evidence suggests that serotonin regulates the establishment of brain connectivity by modulating developmental cellular migration and cytoarchitecture (Daubert and Condron, 2010). Data obtained from multiple animal models also support the hypothesis that serotonin autoregulates branch morphology of serotonergic processes (Acklin and Nicholls, 1990; Couch et al., 2004). Moreover, a role of serotonin in the regulation of programmed cell death during brain development has been suggested (Persico et al., 2003).

Serotonergic cells with projections to the spinal cord have been reported in the rhombencephalon of different jawed vertebrates (zebrafish (McLean and Fetcho, 2004a, 2004b), amphibians (van Mier et al., 1986; Woolston et al., 1994), and rats (Aitken and Törk, 1988)) and they play a role in the modulation of locomotion at spinal levels (Jordan et al., 2008; Grillner et al., 2008). In mammals, paralysis caused by spinal cord injury results from a loss of supraspinal tracts that underlie voluntary initiation of locomotion and from a loss of their major source of neuromodulators, such as serotonin (Carlsson et al. 1963; Rekling et al., 2000; Jacobs et al. 2002; Jordan et al. 2008). In mammals, a loss of brainstem-derived serotonin after spinal cord injury acutely reduces motor neuron excitability and accordingly depresses all motor functions (Murray et al., 2010). Recent studies have also shown that, in rodents, autonomic dysreflexia after spinal cord injury is associated with the lack of serotonergic axonal reinnervation (Cormier et al., 2010). The critical importance of this neuromodulator is demonstrated by the repeated finding in animal models of locomotor activity being regained soon after spinal injury

with the exogenous application of drugs that activate the serotonin receptors (Viala and Buser 1971; Wiklund et al., 1978; Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996; McEwen et al. 1997; Landry and Guertin 2004; Madriaga et al. 2004) or even transplants of serotonin producing cells into the spinal cord (Giménez y Ribotta and Privat 1998; Ribotta et al. 2000).

The serotonergic system in lampreys

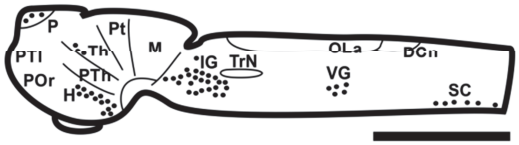
The general organization of the serotonergic central nervous system is well known in lampreys. The first studies of the serotonergic system in the brain of adult lampreys were performed by means of formaldehyde-induced fluorescence (Honma, 1969; Baumgarten, 1972) or immunocytochemistry (Steinbusch and Nieuwenhuys, 1979; Steinbusch et al., 1981; Pierre et al., 1992; Antri et al., 2006). The development of the serotonergic populations in the CNS of the sea lamprey from embryos to adults was studied by inmunohistochemistry (Abalo et al., 2007). A schematic drawing showing the organization of the serotonin-immunoreactive (-ir) system in the CNS of embryonic, larval and adult sea lamprey is shown in Figure 3. Serotonin-ir cell neurons are present in the sea lamprey's diencephalon, rhombencephalon and spinal cord. In addition, occasional serotonin-ir cells are also observed in the telencephalon. The different serotonin-ir brain populations observed in the adult sea lamprey are already observed at the end of the larval stage. Eight serotonin-ir neuronal populations are present in the CNS of the sea lamprey (Abalo et al., 2007): serotonin-ir cells of the pineal and parapineal organs, the populations of serotonin-ir cerebrospinal fluid-contacting (CSF-c) cells of the mammillary nucleus and tuberal nucleus of the hypothalamus, the population of the dorsal thalamus, pretectal population, cells of the isthmic population (dorsal, medial and ventral isthmic subgroups), vagal reticular group (caudal rhombencephalon) and serotonin-ir cells of the ventral margin of the spinal cord. Only the axons from caudal isthmic and vagal groups project to the rostral spinal cord (Barreiro-Iglesias et al., 2008).

In adult lampreys there are three sources of serotonin-ir in the spinal cord, namely, the ventral plexus, the dorsal root ganglia, and the brainstem (Harris-Warrick et al., 1985; Brodin et al., 1986; Zhang et al., 1996). Changes in the serotonergic innervation of the spinal cord have been evaluated after spinal cord injury in lampreys (Cohen et al., 2005). In lampreys, serotonin innervation is highly recovered in the segment immediately caudal to the lesion site with little recovery further away from the lesion, suggesting that some intraspinal reorganization occurs to achieve functional recovery.

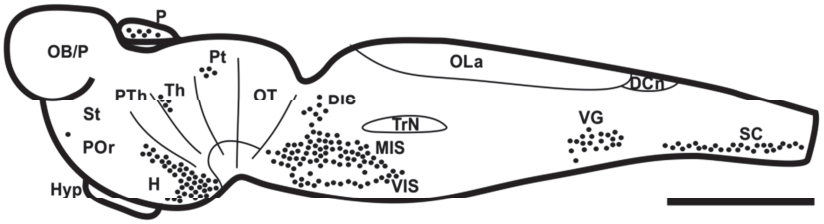
Colocalization of other classical neurotransmitters in serotonin-ir cells has been reported to occur with dopamine (DA; Schotland et al., 1995) and γ-aminobutyric acid (GABA; Barreiro-Iglesias et al., 2009b) neurotransmitters. Double-labelled cells for DA and serotonin were observed in spinal cord ventral midline neurons, below the central canal. Both neurotransmitters have presumably additive effects at the locomotor network level when released by these neurons, because both reduce after hyperpolarization through two distinctly different, but complementary, cellular mechanisms (Schotland et al., 1995). GABA inmunoreactivity was observed in about 87% of the serotonergic cells of the rostral tier of the dorsal thalamus (close to the zona limitans), and in about 61% in the isthmic ventral subgroup. In addition, occasional double-labelled cells for GABA and serotonin were observed in the hypothalamic tuberal nucleus and the pretectum (Barreiro-Iglesias et al., 2009b).

The peripheral distribution of serotonergic elements in the head of sea lampreys was also studied. Serotonergic cells were observed in the Vth, IXth, and Xth cranial nerve ganglia, the musculature, the taste buds, and in the gills; whereas serotonergic fibers were extensive in all tissues, especially in the musculature, except in the epidermis of the skin (Barreiro-Iglesias et al., 2009a).

PROLARVA



LARVA



ADULT

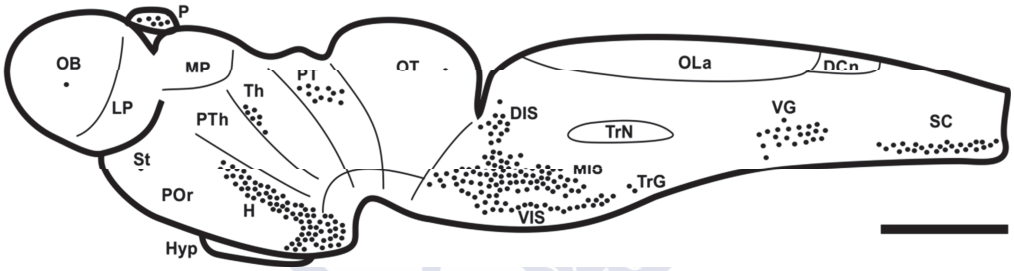


Figure 3. Serotonin distribution in lamprey CNS.

Brief introduction to the methods used in the thesis project

Animals

As mentioned above, the sea lamprey has a long and complex life cycle (Fig. 2, page 5), and embryonic, prolarval, larval, metamorphic and adult stages are distinguishable during its life cycle (see Hardisty and Potter, 1971). In the present investigation all these stages were used. The adult

lampreys used in experiments were bought from a local supplier, while postmetamorphic individuals and larvae were mainly obtained by digging the bed of the river Ulla (Galicia, Northwest Spain). The embryos and prolarvae were obtained in the laboratory by in vitro fertilization using sperm and ovules from mature spawning lampreys. Some larval and postmetamorphic sea lampreys were also obtained from streams feeding Lake Michigan.

All experiments were approved by the Ethics Committee of the University of Santiago de Compostela and conformed to European Community guidelines on animal care and experimentation to minimize any pain or discomfort.

Cloning

Total RNA from lamprey CNS was isolated using Trizol reagent (Invitrogen, La Jolla, CA, USA) and the first-strand cDNA synthesis reaction was catalysed using Superscript III Reverse Transcriptase with oligodT18 primers or random primers (Invitrogen). For polymerase chain reaction (PCR) cloning, degenerate oligonucleotide primers were designed based on alignments of mRNA sequences of different species deposited in the trace archives of the NCBI (National Center for Biotechnology Information, NIH, Bethesda, MD) and in the Ensembl database of the sea lamprey genome (http://www.ensembl.org/Petromyzon_marinus/Info/Index). The amplified fragments were cloned into pGEM-T vectors (Promega, Madison, WI) and sequenced. Sequence analysis and comparison was done using the Basic Local Alignment Search Tool (BLAST) on NCBI, on the first version of the assembled sea lamprey genome in the Ensembl database, with the CLC Sequence viewer 6 software (CLC bio A/S) and with the Vector NTI software (Invitrogen). For more details see Material and Methods sections of chapters 1 and 2.

Phylogenetic analysis

Protein sequences corresponding to different species were aligned with our cloned sequences using CLUSTALW. These alignments were used to construct different phylogenetic trees using the minimum evolution or the neighbor-joining method with Poisson corrected distances on amino acids and bootstrap analysis (1000 replications). MEGA 5 software was used to construct the phylogenetic trees (Tamura et al., 2011). For more details see Material and Methods sections of chapters 1 and 2.

In situ *hybridization*

Digoxigenin (DIG)-labelled riboprobes were synthesized using the amplified fragments of our cloned sequences as templates and following standard protocols using T7 polymerase (Roche Diagnostics, Germany). Whole-mount *in situ* hybridization of embryos and prolarvae was conducted using standard protocols, with the following adaptations. A proteinase K treatment (10 mg/ml) was applied for 30 min at room temperature following rehydration of embryos and prolarvae and an RNAse A treatment (100 mg/ml, 37ºC during 30 min) was added to the posthybridization washings. The sheep anti-DIG antibody conjugated to alkaline phosphatase (1:2000; Roche) was incubated overnight at 4ºC in blocking buffer solution. Staining was conducted in BM Purple (Roche) at 37ºC until the signal was clearly visible. *In situ* hybridization on cryostat sections was conducted as above with the following differences. The sections were not treated with proteinase K and the RNAse treatment step was conducted with a lower concentration (0.2 µg/mL). For more details see Material and Methods sections of chapters 1, 2 and 3.

mRNA quantification

The Feature J Hessian plug-in for Image J software was used according to an established protocol (Grider et al., 2006) to quantify in situ signal positive profiles. For statistical analysis, the program Prism (GraphPad Software, La Jolla, CA) was used. Variability of values is given as standard error of mean (SEM). Data from all groups of animals passed the Kormogolov-Smirnov normality test. So, data sets were statistically analysed using a one-way ANOVA with Bonferroni's multiple comparison test. For more details see Material and Methods section of chapter 3.

Spinal cord injury procedure

The spinal cord was exposed from the dorsal midline at the level of the fifth gill and a complete spinal cord transection was performed with a scalpel and the spinal cord cut ends were visualized under the microscope. The incision was closed with Histoacryl tissue glue (B. Braun Surgical, Tuttlingen, Germany). Each transected animal was examined 24 h after surgery to confirm that there was no movement caudal to the lesion site. Animals were allowed to recover in fresh water tanks at 19-20ºC. For more details see Material and Methods sections of chapters 3 and 4.

Characterization of serotonin-ir

Immunofluorescence experiments were conducted to characterize serotonin immunoreactive populations. Sections were incubated with a rabbit polyclonal anti-5-HT antibody (dilution 1:2500; Incstar, Still Water, MN) at 4ºC for 72 h. For indirect immunofluorescence detection of serotonin, Cy3-conjugated goat anti-rabbit antibody (dilution 1:200; Millipore, Temecula, CA) was used (incubation time: 1 h at room temperature). All antibodies were diluted in TBS containing 15% normal goat serum and 0.2% Triton X-100 as detergent. For more details see Material and Methods section of chapter 4.

Tract-tracing techniques

The spinal cord was exposed to apply crystals of the tracer Neurobiotin (NB; of 322.8 Da molecular weight Vector, Burlingame, CA) or tracer Fluoro-gold (FG; molecular weight, 532.6 Da: Fluorochrome LLC, Denver, CO USA) with the aid of a minute pin (00) 1 mm. The animals were maintained at 4ºC with appropriate aeration conditions in lamprey Ringer solution of the following composition: 137 mM NaCl, 2.9 mM KCl, 2.1 mM CaCl2, 2 mM HEPES, to allow transport of the tracer. For indirect detection of the tracer NB the sections were incubated at room temperature for 4 h with fluorescein isothiocyanate (FITC)-labelled avidin D (Vector Laboratories, Burlingame, CA) previously diluted to 1:1000 with TBS containing 0.3% Triton X-100. FG tracer was observed directly in confocal microscope with the ultraviolet laser. For more details see Material and Methods section of chapter 4.

Photographs

Whole-mounts and sections of *in situ* hybridization experiments were photographed with an Olympus photomicroscope (AX-70; Provis) equipped with a color digital camera (Olympus DP70, Tokyo, Japan) or using a Zeiss AxioCam CCD camera attached to a Zeiss Axioskop microscope with AxioVision software. Photomicrographs of immunofluorescence were taken with a spectral confocal laser microscope (Leica TCS-SP2). For more details see Material and Methods sections of each chapter.

Literature cited

Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R, Rodicio MC. (2007) Development of the serotonergic system in the

central nervous system of the sea lamprey. J Chem Neuroanat. 34: 29-46.

Acklin SE, Nicholls JG. (1990) Intrinsic and extrinsic factors influencing properties and growth patterns of identified leech neurons in culture. J Neurosci. 10: 1082-1090.

Adrio F, Anadón R, Rodríguez-Moldes I. (1999) Distribution of serotonin (5-HT)-immunoreactive structures in the central nervous system of two chondrostean species (*Acipenser baeri* and *Huso huso*). J Comp Neurol. 407: 333-348.

Aitken AR, Törk I. (1988) Early development of serotonin-containing neurons and pathways as seen in whole-mount preparations of the fetal rat brain. J Comp Neurol. 274: 32-47.

Allee SJ, Markham MR, Salazar VL, Stoddard PK. (2008) Opposing actions of 5HT1A and 5HT2-like serotonin receptors on modulations of the electric signal waveform in the electric fish *Brachyhypopomus pinnicaudatus*. Horm Behav. 53: 481-488.

Antri M, Cyr A, Auclair F, Dubuc R. (2006) Ontogeny of 5-HT neurons in the brainstem of the lamprey, *Petromyzon marinus*. J Comp Neurol. 495: 788-800.

Barreiro-Iglesias A. (2012) 'Evorego': studying regeneration to understand evolution, the case of the serotonergic system. Brain Behav Evol. 79: 1-3.

Barreiro-Iglesias A, Villar-Cerviño V, Anadón R, Rodicio MC. (2008) Descending brain-spinal cord projections in a primitive vertebrate, the lamprey: cerebrospinal fluid-contacting and dopaminergic neurons. J Comp Neurol. 511: 711-723.

Barreiro-Iglesias A, Aldegunde M, Anadón R, Rodicio MC. (2009a) Extensive presence of serotonergic cells and fibers in the peripheral nervous system of lampreys. J Comp Neurol. 512: 478-499.

Barreiro-Iglesias A, Cornide-Petronio ME, Anadón R, Rodicio MC. (2009b) Serotonin and GABA are colocalized in restricted groups of neurons in the larval sea lamprey brain: insights into the early evolution of neurotransmitter colocalization in vertebrates. J Anat. 215: 435-443.

Baumgarten HG. (1972) Biogenic monoamines in the cyclostome and lower vertebrate brain. Prog Histochem Cytochem. 4: 1-90.

Bolliet V, Ali MA. (1992) Immunohistochemical study of the development of serotoninergic neurons in the brain of the brook trout, *Salvelinus fontinalis*. Brain Behav Evol. 40: 234-249.

Bolliet V, Perreault S, Ali MA. (1994) Development of serotoninergic neurons in the brain of the mackerel, *Scomber scombrus*. An immunohistochemical study. J Fish Biol. 44: 241-253.

Botchkina GI, Morin LP. (1993) Development of the hamster serotoninergic system: cell groups and diencephalic projections. J Comp Neurol. 338: 405-431.

Brodin L, Buchanan JT, Hökfelt T, Grillner S, Verhofstad AA. (1986) A spinal projection of 5-hydroxytryptamine neurons in the lamprey brainstem; evidence from combined retrograde tracing and immunohistochemistry. Neurosci Lett. 67:53-57.

Carlsson A, Magnusson T, Rosengren E. (1963) 5-Hydroxytryptamine of the spinal cord normally and after transection. Experientia. 19: 359.

Carrera I, Molist P, Anadón R, Rodríguez-Moldes I. (2008) Development of the serotoninergic system in the central nervous system of a shark, the lesser spotted dogfish Scyliorhinus canicula. J Comp Neurol. 511: 804-831.

Chubakov AR, Gromova EA, Konovalov GV, Sarkisova EF, Chumasov EI. (1986) The effects of serotonin on the morpho-functional development of rat cerebral neocortex in tissue culture. Brain. 369: 285-297.

Cohen AH, Mackler SA, Selzer ME. (1988) Behavioral recovery following spinal transection: functional regeneration in the lamprey CNS. Trends Neurosci. 11: 227-231.

Cohen AH, Abdelnabi M, Guan L, Ottinger MA, Chakrabarti L.. (2005) Changes in distribution of serotonin induced by spinal injury in larval lampreys: evidence from immunohistochemistry and HPLC. J Neurotrauma. 22: 172-188.

Cormier CM, Mukhida K, Walker G, Marsh DR. (2010) Development of autonomic dysreflexia after spinal cord injury is associated with a lack of serotonergic axons in the intermediolateral cell column. J Neurotrauma. 27: 1805-1818.

Couch JA, Chen J, Rieff HI, Uri EM, Condron BG. (2004) robo2 and robo3 interact with eagle to regulate serotonergic neuron differentiation. Development. 131: 997-1006.

Cowley KC, Schmidt BJ. (1994) A comparison of motor patterns induced by *N*-methyl-D-aspartate, acetylcholine and serotonin in the in vitro neonatal rat spinal cord. Neurosci Lett. 171: 147-150.

Daubert EA, Condron BG. (2010) Serotonin: a regulator of neuronal morphology and circuitry. Trends Neurosci. 33: 424-434.

Davis GR, McClellan AD. (1994) Extent and time course of restoration of descending brainstem projections in spinal cord-transected lamprey. J Comp Neurol 344: 65-82.

Dubuc R, Brocard F, Antri M, Fénelon K, Gariépy JF, Smetana R, Ménard A, Le Ray D, Viana Di Prisco G, Pearlstein E, Sirota MG, Derjean D, St-Pierre M, Zielinski B, Auclair F, Veilleux D. (2008) Initiation of locomotion in lampreys. Brain Res Rev. 57: 172-182.

Ekström P, Nyberg L, van Veen T. (1985) Ontogenetic development of serotoninergic neurons in the brain of a teleost, the three-spined

stickleback. An immunohistochemical analysis. Dev Brain Res. 17: 209-224.

Frankfurt M, Lauder JM, Azmitia EC. (1981) The immunocytochemical localization of serotonergic neurons in the rat hypothalamus. Neurosci Lett. 24: 227-232.

Gaddum JH, Picarelli ZP. (1957) Two kinds of tryptamine receptors. Br J Pharmacol. 12: 323-328.

Gaspar P, Cases O, Maroteaux L.. (2003) The developmental role of serotonin: news from mouse molecular genetics. Nature Rev Neurosci. 4: 1002-1012.

Gern WA, Ralph CL.. (1979) Melatonin synthesis by the retina. Science. 204: 183-184.

Gill HS, Renaud CB, Chapleau F, Mayden RL, Potter IC. (2003) Phylogeny of living parasitic lampreys (Petromyzontiformes) based on morphological data. Copeia. 2003: 687-703.

Giménez y Ribotta M, Privat A. (1998) Biological interventions for spinal cord injury. Curr Opin Neurol 11: 647-654.

Goldberg JI, Kater SB. (1989) Expression and function of the neurotransmitter serotonin during development of the *Helisoma* nervous system. Dev Biol. 131: 483-495.

Goldberg JI, Mills LR, Kater SB. (1991) Novel effects of serotonin on neurite outgrowth in neurons cultured from embryos of *Helisoma trivolvis*. J Neurobiol. 22: 182-194.

Grider MH, Chen Q, Shine HD. (2006) Semi-automated quantification of axonal densities in labeled CNS tissue. J Neurosci Methods. 155: 172-179.

Grillner S, Wallén P. (2007) Spinal motor functions in lamprey. In: Becker CG, Becker T (eds) Model organism in spinal cord regeneration. Wiley-VCH Verlag GmbH & Co, Weinheim, pp 129-145.

Grillner S, Wallén P, Saitoh K, Kozlov A, Robertson B. (2008) Neural bases of goaldirected locomotion in vertebrates-an overview. Brain Res Rev. 57: 2-12.

Gromova HA, Chubakov AR, Chumasov EI, Konovalov HV. (1983) Serotonin as a stimulator of hippocampal cell differentiation in tissue culture. Int J Dev Neurosci. 1: 339-349.

Guth L, Brewer CR, Collins WF, Goldberger ME, Perl ER. (1980) Criteria for evaluating spinal cord regeneration experiments. Exp Neurol. 69: 1-3.

Hall GF. (1999) PHF-Tau from Alzheimer brain is rapidly dephosphorylated and degraded when injected into neurons *in situ*. J Alzheimers Dis. 1: 379-386.

Hall GF, Chu B, Lee G, Yao J. (2000) Human tau filaments induce microtubule and synapse loss in an in vivo model of neurofibrillary degenerative disease. J Cell Sci. 113: 1373-1387.

Hamon M, Nelson DL, Herbet A, Glowinski J. (1980) Multiple receptors for serotonin in the rat brain. Adv Biochem Psychopharmacol. 21: 223-233.

Hardisty MW. (1951) Duration of the larval period in the brook lamprey (*Lampetra planeri*). Nature. 167: 38-39.

Hardisty MW. (1983) The significance of lampreys for biological research. Endeavour. 7: 110-115.

Hardisty MW. (1986) *Petromyzon marinus* (Linnaeus, 1758). In: Holcík J (ed) The freshwater fishes of Europe, vol. 1. Part I - Petromyzontiformes. Aula-Verlag, Wiesbaden, pp 94-116.

Hardisty MW, Potter IC. (1971) The behaviour, ecology and growth of larval lampreys. In Hardisty MW, Potter IC (eds) The biology of lampreys, vol. 1. Academic Press, London, pp 85-125.

Harris-Warrick RM, McPhee JC, Filler JA. (1985) Distribution of serotonergic neurons and processes in the lamprey spinal cord. Neuroscience. 14: 1127-1140.

Haydon PG, McCobb DP, Kater SB. (1984) Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. Science. 226: 561-564.

Hensler JG. (2006) Serotonin. In: Siegel GJ, Albers RW, Brady ST, Price DL (eds) Basic Neurochemistry. Elsevier Academic Press, San Diego, pp 227-249.

Hill RH, Svensson E, Dewael Y, Grillner S. (2003) 5-HT inhibits N-type but not L-type Ca(2+) channels via 5-HT1A receptors in lamprey spinal neurons. Eur J Neurosci. 18: 2919-2924.

Honma, S. (1969) Fluorescence microscopic observations on the brain of the lamprey, *Lampetra japonica*. Arch Histol Jpn. 31: 167-178.

Honson NS, Jensen JR, Abraha A, Hall GF, Kuret J. (2009) Small-molecule mediated neuroprotection in an in situ model of tauopathy. Neurotox Res. 15: 274-283.

Hubbs CL, Potter IC. (1971) Distribution, phylogeny and taxonomy. In: Hardisty MW, Potter IC (eds) The biology of lampreys, vol. 1. Academic Press, New York, pp 1-65.

Jacobs BL, Martin-Cora FJ, Fornal CA. (2002) Activity of medullary serotonergic neurons in freely moving animals. Brain Res Brain Res Rev. 40: 45-52.

Jacobs AJ, Swain GP, Snedeker JA, Pijak DS, Gladstone LJ, Selzer ME. (1997) Recovery of neurofilament expression selectively in regenerating reticulospinal neurons. J Neurosci. 17: 5206-5220.

Jin LQ, Zhang G, Jamison C, Takano H, Haydon PG, Selzer ME. (2009) Axon regeneration in the absence of growth cones: acceleration by cyclic AMP. J Comp Neurol. 515: 295-312.

Johnston JB. (1905) The cranial nerve components of *Petromyzon*. Morph Jb. 34: 149-203.

Jordan LM, Liu J, Hedlund PB, Akay T, Pearson KG. (2008) Descending command systems for the initiation of locomotion in mammals. Brain Res Rev. 57: 183-191.

Kiehn O, Kjaerulff O. (1996) Spatiotemporal characteristics of 5-HT and dopamineinduced rhythmic hindlimb activity in the in vitro neonatal rat. J Neurophysiol. 75: 1472-1482.

Kroeze WK, Roth BL.. (2006) Molecular biology and genomic organization of G protein-coupled serotonin receptors. In: Roth BL (ed) The receptors: the serotonin receptors: from molecular pharmacology to human therapeutics. Totowa, New Jersey, pp 1-38.

Kuratani S, Kuraku S, Murakami Y. (2002) Lamprey as an evo-devo model: lessons from comparative embryology and molecular phylogenetics. Genesis. 34: 175-183.

Landry ES, Guertin PA. (2004) Differential effects of 5-HT1 and 5-HT2 receptor agonists on hindlimb movements in paraplegic mice. Prog Neuropsychopharmacol Biol Psychiatry. 28: 1053-1060.

Lang NJ, Roe KJ, Renaud CB, Gill HS Potter IC, Freyhof J, Naseka AM, Cochran P, Espinosa Pérez H, Habit EM, Kuhajda BR, Neely DA, Reshetnikov YS, Salnikov VB, Stoumboudi MTh, Mayden RL. (2009) Novel relationships among lampreys (Petromyzontiformes) revealed by a taxonomically comprehensive molecular data set. In: Brown LR, Chase SD, Mesa MG, Beamish RJ, Moyle PB (eds) Biology, management, and conservation of lampreys in North America. Am Fish Soc Symp 72. AFS, Bethesda, MD, pp 41-56.

Lauder JM. (1987) Neurotransmitters as morphogenetic signals and trophic factors. In: Vernadakis A, Privat A, Lauder JM, Timiras PS, Giacobini E (eds) Model systems of development and aging of the nervous system. Martinus Nijhoff, Boston, pp 219-237.

Lauder JM. (1993) Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends Neurosci. 16: 233-240.

Lauder JM, Krebs H. (1978). Serotonin as a differentiation signal in early neurogenesis. Dev Neurosci. 1: 15-30.

Lee S, Kim W, Li Z, Hall GF. (2012) Accumulation of vesicle-associated human tau in distal dendrites drives degeneration and tau secretion in an in situ cellular tauopathy model. Int J Alzheimers Dis. doi: 10.1155/2012/172837.

Leonhardt S, Herrick-Davis K, Titeler M. (1989) Detection of a novel serotonin receptor subtype (5-HT1E) in human brain: interaction with a GTP-binding protein. J Neurochem. 53: 465-471.

Leysen JE, Gommeren W, Laduron PM. (1979) Distinction between dopaminergic and serotonergic components of neuroleptic binding sites in limbic brain areas. Biochem Pharmacol. 28: 447-448.

Lidov HGW, Molliver ME. (1982) Immunohistochemical study of the development of serotoninergic neurons in the rat CNS. Brain Res Bull. 9: 559-604.

Lübbert H, Hoffman BJ, Snutch TP, van Dyke T, Levine AJ, Hartig PR, Lester HA, Davidson N. (1987) cDNA cloning of a serotonin 5-HT1C receptor by electrophysiological assays of mRNA-injected *Xenopus* oocytes. Proc Natl Acad Sci USA. 84: 4332-4336.

Madriaga MA, McPhee LC, Chersa T, Christie KJ, Whelan PJ. (2004) Modulation of locomotor activity by multiple 5-HT and dopaminergic receptor subtypes in the neonatal mouse spinal cord. J Neurophysiol. 92: 1566-1576.

Mateus CS, Rodríguez-Muñoz R, Quintella BR, Alves MJ, Almeida PR. (2012) Lampreys of the Iberian Peninsula: distribution, population status and conservation. Endang Species Res. 16: 183-198.

McEwen ML, Van Hartesveldt C, Stehouwer DJ. (1997) L-DOPA and quipazine elicit air-stepping in neonatal rats with spinal cord transections. Behav Neurosci. 111: 825-833.

McLean DL, Fetcho JR. (2004a) Ontogeny and innervation patterns of dopaminergic, noradrenergic, and serotonergic neurons in larval zebrafish. J Comp Neurol. 480: 38-56.

McLean DL, Fetcho JR. (2004b) Relationship of tyrosine hydroxylase and serotonin immunoreactivity to sensorimotor circuitry in larval zebrafish. J Comp. Neurol. 480: 57-71.

Meier E, Hertz L, Schousboe A. (1991) Neurotransmitters as developmental signals. Neurochem Int. 19: 1-15.

Murakami Y, Watanabe A. (2009) Development of the central and peripheral nervous systems in the lamprey. Dev Growth Differ. 51: 197-205.

Murray K, Nakae A, Stephens MJ, Rank M, D´Amico J, Harvey P, Li X, Harris L, Ballou EW, Anelli R, Heckman CJ, Mashimo T, Vavrek R, Sanelli L, Gorassini MA, Bennett DJ, Fouad K. (2010) Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. Nat Med 16: 694-700.

Nelson DL.. (2004) 5-HT5 receptors. Curr Drug Targets CNS Neurol Disord. 3: 53-58.

Nieuwenhuys R, Nicholson C. (1998) Lampreys, Petromyzontoidea. In: Nieuwenhuys R, Donkelaar T, Nicholson C (eds) The central nervous system of vertebrates, vol. 1. Springer-Verlag, Berlin, pp 397-495.

Norton WH, Folchert A, Bally-Cuif L.. (2008) Comparative analysis of serotonin receptor (HTR1A/HTR1B families) and transporter (slc6a4a/b) gene expression in the zebrafish brain. J Comp Neurol. 511: 521-542.

Osório J, Rétaux S. (2008) The lamprey in evolutionary studies. Dev Genes Evol. 218: 221-235.

Parent A. (1981) The anatomy of serotonin-containing neurons across phylogeny. In: Jacobs BL, Gelperin A (eds) Serotonin neurotransmission and behaviour. MIT Press, Cambridge, pp 3-34.

Parent, A. (1984) Functional anatomy and evolution of monoaminergic systems. Amer. Zool. 24: 783-790.

Parent A, Northcutt RG. (1982) The monoamine-containing neurons in the brain of the garfish, *Lepisosteus osseus*. Brain Res Bull. 9: 189-204.

Peroutka SJ, Lebovitz RM, Snyder SH. (1981) Two distinct serotonin receptors with distinct physiological functions. Science. 212: 827-829.

Persico AM, Baldi A, Dell'Acqua ML, Moessner R, Murphy DL Lesch KP, Keller F. (2003) Reduced programmed cell death in brains of serotonin transporter knockout mice. Neuroreport. 14: 341-344.

Pierre J, Repèrant J, Ward R, Vesselkin NP, Rio JP, Miceli D, Kratskin I. (1992) The serotoninergic system of the brain of the lamprey, *Lampetra fluviatilis*: an evolutionary perspective. J Chem Neuroanat. 5: 195-219.

Rekling JC, Funk GD, Bayliss DA, Dong XW, Feldman JL. (2000) Synaptic control of motoneuronal excitability. Physiol Rev. 80: 767-852.

Reissner E. (1860) Beiträge zur Kenntnis vom Bau des Rükenmarks von *Petromyzon fluviatilis* L. Arch Anat Physiol. 77: 545-588.

Ribotta MG, Provencher J, Feraboli-Lohnherr D, Rossignol S, Privat A, Orsal D. (2000) Activation of locomotion in adult chronic spinal rats is achieved by transplantation of embryonic raphe cells reinnervating a precise lumbar level. J Neurosci. 20: 5144-5152.

Rodrigues SL, Maseko BC, Ihunwo AO, Fuxe K, Manger PR. (2008) Nuclear organization and morphology of serotonergic neurons in the brain of the Nile crocodile, *Crocodylus niloticus*. J Chem Neuroanat. 35: 133-145.

Rovainen CM. (1976) Regeneration of Müller and Mauthner axons after spinal transection in larval lampreys. J Comp Neurol. 168: 545-554.

Rubenstein JL.. (1998) Development of serotonergic neurons and their projections. Biol Psychiatry. 44: 145-150.

Schotland J, Shupliakov O, Wikström M, Brodin L, Srinivasan M, You ZB, Herrera-Marschitz M, Zhang W, Hökfelt T, Grillner S. (1995) Control of lamprey locomotor neurons by colocalized monoamine transmitters. Nature. 374: 266-268.

Selzer ME. (1978) Mechanisms of functional recovery and regeneration after spinal cord transection in larval sea lamprey. J Physiol. 277: 395-408.

Shifman MI, Selzer ME. (2000) Expression of the netrin receptor UNC-5 in lamprey brain: modulation by spinal cord transection. Neurorehabil Neural Repair. 14: 49-58.

Shifman MI, Selzer ME. (2007) Differential expression of class 3 and 4 semaphorins and netrin in the lamprey spinal cord during regeneration. J Comp Neurol. 501: 631-646.

Shifman MI, Jin L, Selzer ME. (2007) Regeneration in the lamprey spinal cord. In: Becker CG, Becker T (eds) Model organism in spinal cord regeneration. Wiley-VCH Verlag GmbH & Co, Weinheim, pp 229-262.

Shifman MI, Yumul RE, Laramore C, Selzer ME. (2009) Expression of the repulsive guidance molecule RGM and its receptor neogenin after spinal cord injury in sea lamprey. Exp Neurol. 217:242-251.

Smith JJ, Kuraku S, Holt C, Sauka-Spengler T, Jiang N, Campbell MS, Yandell MD, Manousaki T, Meyer A, Bloom OE, Morgan JR, Buxbaum JD, Sachidanandam R, Sims C, Garruss AS, Cook M, Krumlauf R, Wiedemann LM, Sower SA, Decatur WA, Hall JA, Amemiya CT, Saha NR, Buckley KM, Rast JP, Das S, Hirano M, McCurley N, Guo P, Rohner N, Tabin CJ, Piccinelli P, Elgar G, Ruffier M, Aken BL, Searle SM, Muffato M, Pignatelli M, Herrero J, Jones M, Brown CT, Chung-Davidson YW, Nanlohy KG, Libants SV, Yeh CY, McCauley DW, Langeland JA, Pancer Z, Fritzsch B, de Jong PJ, Zhu B, Fulton LL, Theising B, Flicek P, Bronner ME,

Warren WC, Clifton SW, Wilson RK, Li W. (2013) Sequencing of the sea lamprey (*Petromyzon marinus*) genome provides insights into vertebrate evolution. Nat Genet. 45: 415-421.

Sodhi MS, Sanders-Busch E. (2004) Serotonin and brain development. Int Rev Neurobiol. 59: 111-174.

Steinbusch HWM, Nieuwenhuys R. (1979) Serotoninergic neuron systems in the brain of the lamprey, *Lampetra fluviatilis*. Anat Rec. 193: 693-694.

Steinbusch HW, Verhofstad AA, Penke B, Varga J, Joosten HW. (1981) Immunohistochemical characterization of monoamine-containing neurons in the central nervous system by antibodies to serotonin and noradrenalin. A study in the rat and the lamprey (*Lampetra fluviatilis*). Acta Histochem. 24: 107-122.

Stuesse SL, Cruce WL, Northcutt RG. (1990) Distribution of tyrosine hydroxylase- and serotonin-immunoreactive cells in the central nervous system of the thornback guitarfish, *Platyrhinoidis triseriata*. J Chem Neuroanat. 3: 45-58.

Takezaki N, Figueroa F, Zaleska-Rutczynska Z, Klein J. (2003) Molecular phylogeny of early vertebrates: monophyly of the agnathans as revealed by sequences of 35 genes. Mol Biol Evol. 20: 287-292.

Tamir H, Liu K, Hsiung S, Adlersberg M, Gershon MD. (1994) Serotonin binding protein: Synthesis, secretion, and recycling. J Neurochem. 63: 97-107.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 28: 2731-2739.

Tierney AJ. (2001) Structure and function of invertebrate 5-HT receptors: a review. Comp Biochem Physiol A Mol Integr Physiol. 128: 791-804.

van Mier P, Joosten HW, van Rheden R, ten Donkelaar HJ. (1986) The development of serotonergic raphespinal projections in *Xenopus laevis*. Int J Dev Neurosci. 4: 465-475.

Viala D, Buser P. (1971) Methods of obtaining locomotor rhythms in the spinal rabbit by pharmacological treatments (DOPA, 5-HTP, D-amphetamine). Brain Res. 35: 151-165.

Wallace JA. (1985) An immunohistochemical study of the development of the central serotoninergic neurons in the chick embryo. J Comp Neurol. 236: 443-453.

Wallace JA, Lauder JM. (1983) Development of the serotoninergic system in the rat embryo: an immunocytochemical study. Brain Res Bull. 10: 459-479.

Weiger WA. (1997) Serotonergic modulation of behaviour: a phylogenetic overview. Biol Rev Camb Philos Soc. 72: 61-95.

Wiklund L, Björklund A, Nobin A. (1978) Regeneration of serotonin neurons in the rat brain after 5,6-dihydroxytryptamine-induced axotomy. Ann N Y Acad Sci. 305: 370-384.

Wikström M, Hill R, Hellgren J, Grillner S. (1995) The action of 5-HT on calcium-dependent potassium channels and on the spinal locomotor network in lamprey is mediated by 5-HT1A-like receptors. Brain Res. 678: 191-199.

Wolters JG, ten Donkelaar HJ, Steinbusch HW, Verhofstad AA. (1985) Distribution of serotonin in the brain stem and spinal cord of the lizard Varanus exanthematicus: an immunohistochemical study. Neuroscience. 14: 169-193.

Wood MR, Cohen MJ. (1979) Synaptic regeneration in identified neurons of the lamprey spinal cords. Science. 206: 344-347.

Woolston AM, Wedderburn JF, Sillar KT. (1994) Descending serotonergic spinal projections and modulation of locomotor rhythmicity in Rana temporaria embryos. Proc Biol Sci. 255: 73-79.

Youson JH. (1980) Morphology and physiology of lamprey metamorphosis. Can Fish Aquat Sci. 11: 1687-1710.

Zhang W, Pombal MA, el Manira A, Grillner S. (1996) Rostrocaudal distribution of 5-HT innervation in the lamprey spinal cord and differential effects of 5-HT on fictive locomotion. J Comp Neurol. 374: 278-290.

Zhao B, Debski EA. (2005) Serotonergic reticular formation cells in Rana pipiens: categorization, development, and tectal projections. J Comp Neurol. 487: 441-456.

Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HT_1–5-HT_7	Serotonin receptors 1-7
5-HTP	5-hydroxytryptophan
AADC	Aromatic amino acid decarboxylase
BH4	L-erythro-tetrahydrobiopterin
CNS	Central nervous system
CPG	Central pattern generator
CSF-c	Cerebrospinal fluid-contacting
FG	Fluoro-gold
GABA	γ-aminobutyric acid
-ir	-immunoreactive
MAO	Monoamine oxidase enzyme
NB	Neurobiotin
PCR	Polymerase chain reaction

Tph	Tryptophan hydroxylase
SCI	Spinal cord injury
SEM	Error of the mean
SERT	Serotonin transporter
VMAT	Vesicular membrane transporter

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JUSTIFICATION AND AIMS OF THE THESIS

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JUSTIFICATION AND AIMS OF THE THESIS

Justification and aims of the thesis

Our research group is involved in studies of nervous system evolution and spinal cord regeneration using the sea lamprey as a model. Several studies in mammals have demonstrated that a loss of brainstem-derived serotonin after spinal cord injury acutely reduces motor neuron excitability and accordingly depresses all motor functions (e.g. Cormier et al., 2010; Murray et al., 2010). Moreover, locomotor activity is regained after spinal injury with the exogenous application of drugs that activate the serotonin receptors (Viala and Buser 1971; Wiklund et al., 1978; Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996; McEwen et al. 1997; Landry and Guertin 2004; Madriaga et al. 2004) or with transplants of serotonin producing cells into the spinal cord (Giménez y Ribotta and Privat 1998; Ribotta et al. 2000). So, the regeneration of the serotonergic system and the activation of plastic events in this system are important for the recovery of function after spinal cord injury. In this work we attempted to increase our knowledge about the changes that occur in the serotonergic system of a regenerating vertebrate (i.e. a vertebrate species that recovers locomotion after spinal cord injury) like the sea lamprey after a complete spinal cord transection. This will provide the right tools and knowledge to study the molecular mechanisms and plastic events occurring in this system to lead to functional recovery after spinal cord injury.

In addition, since the sea lamprey is a very interesting model to understand nervous system evolution and for the study of the neuronal circuits underlying locomotion in vertebrates we also aimed to provide new insights on the study of the organization and development of the serotonergic system in this species by cloning and studying the expression of the rate-limiting enzyme for serotonin synthesis and the 5-HT_{1A}.

The specific aims of the thesis were:

- To clone the rate-limiting enzyme during serotonin synthesis, (Tph), and study its pattern of expression in the sea lamprey nervous system. Since lampreys hold a key phylogenetic position, this could provide new clues to understand the evolution of *Tph* genes in vertebrates. In addition, these results will offer a new tool for researchers studying the serotonergic system in both normal and pathological conditions using the sea lamprey as animal model.

- The cloning and the study of the pattern of expression of one of the major targets of the activity of serotonergic cells: 5-HT_{1A}. Since 5-HT_{1A} is central to serotonin response, a better knowledge of the 5-HT_{1A} in lampreys is an important step for the study of its role in the nervous system and its role in functional recovery after spinal cord injury. This is also important in an evolutionary and comparative context.

- To determine how spinal cord injury affects the expression of the 5-HT_{1A}. This will provide new knowledge to understand the plastic events that lead to the recovery of function in a regenerating vertebrate like the sea lamprey.

- To study if descending serotonergic axons of lampreys actually regenerate through the site of injury after a complete spinal cord transection. This will offer a model to allow studying the molecular mechanisms that control the successful regeneration of serotonergic axons in vertebrates.

The thesis has been organized in 4 chapters corresponding to the 4 main objectives proposed in the present investigation.

In the first chapter, we report the cloning of a *Tph* cDNA of the sea lamprey and the pattern of *Tph* mRNA expression in larval and postmetamorphic (young adult) sea lampreys using in situ hybridization. Phylogenetic analysis indicated that the lamprey *Tph* is an orthologue of *Tphs*

of other vertebrates. In the sea lamprey brain, the *Tph* transcript was expressed in perikarya of the pineal organ, the retina, the diencephalic and rhombencephalic nuclei reported previously with serotonin immunohistochemistry and in small cells of the spinal cord, with a pattern similar to that observed with anti-serotonin antibodies. This suggests that expression of this *Tph* gene is shared by all lamprey serotonergic brain populations.

In the second chapter, we cloned and characterized the 3D structure of the sea lamprey 5-HT_{1A}, and studied the expression of its transcript in the brain, spinal cord an retina by means of in situ hybridization. In phylogenetic analyses, the sea lamprey 5-HT_{1A} sequence clustered together with 5-HT_{1A} sequences of vertebrates and emerged as an outgroup to all gnathostome sequences. In situ hybridization analysis during prolarval, larval and adult stages showed a widespread expression of the lamprey 5-ht1a transcript and the comparison of its expression between the sea lamprey and other vertebrates reveals a conserved pattern in most brain regions. Expression of the 5-ht1a transcript was also observed in the larval lamprey retina, even years before the appearance of serotonergic amacrine cells. This is interesting finding is also discussed in this chapter.

In the third chapter, we report the development of a semiautomatic quantification method to analyse the changes in the expression of the sea lamprey 5th1a after spinal cord injury as revealed by in situ hybridization. A significant increase in the expression of the receptor was observed in the rostral and caudal spinal cord 1 week post lesion, while at later time points the expression returned to control levels.

In the fourth chapter, we used combined immunofluorescence and tract-tracing techniques to show that descending serotonergic neurons regenerate after complete spinal cord transection in the sea lamprey. We observed that the reinnervation of the caudal spinal cord by serotonergic axons corresponds to the sprouting from non-axotomized axons and also to regeneration of axotomized axons.

Literature cited

Cormier CM, Mukhida K, Walker G, Marsh DR. (2010) Development of autonomic dysreflexia after spinal cord injury is associated with a lack of serotonergic axons in the intermediolateral cell column. J Neurotrauma. 27: 1805-1818.

Cowley KC, Schmidt BJ. (1994) A comparison of motor patterns induced by *N*-methyl-D-aspartate, acetylcholine and serotonin in the in vitro neonatal rat spinal cord. Neurosci Lett. 171: 147-150.

Giménez y Ribotta M, Privat A. (1998) Biological interventions for spinal cord injury. Curr Opin Neurol 11: 647-654.

Kiehn O, Kjaerulff O. (1996) Spatiotemporal characteristics of 5-HT and dopamineinduced rhythmic hindlimb activity in the in vitro neonatal rat. J Neurophysiol. 75: 1472-1482.

Landry ES, Guertin PA. (2004) Differential effects of 5-HT1 and 5-HT2 receptor agonists on hindlimb movements in paraplegic mice. Prog Neuropsychopharmacol Biol Psychiatry. 28: 1053–1060.

Madriaga MA, McPhee LC, Chersa T, Christie KJ, Whelan PJ. (2004) Modulation of locomotor activity by multiple 5-HT and dopaminergic receptor subtypes in the neonatal mouse spinal cord. J Neurophysiol. 92: 1566-1576.

McEwen ML, Van Hartesveldt C, Stehouwer DJ. (1997) L-DOPA and quipazine elicit air-stepping in neonatal rats with spinal cord transections. Behav Neurosci. 111: 825-833.

Murray K, Nakae A, Stephens MJ, Rank M, D´Amico J, Harvey P, Li X, Harris L, Ballou EW, Anelli R, Heckman CJ, Mashimo T, Vavrek R, Sanelli L, Gorassini MA, Bennett DJ, Fouad K. (2010) Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. Nat Med 16: 694-700.

Ribotta MG, Provencher J, Feraboli-Lohnherr D, Rossignol S, Privat A, Orsal D. (2000) Activation of locomotion in adult chronic spinal rats is achieved by transplantation of embryonic raphe cells reinnervating a precise lumbar level. J Neurosci. 20: 5144-5152.

Viala D, Buser P. (1971) Methods of obtaining locomotor rhythms in the spinal rabbit by pharmacological treatments (DOPA, 5-HTP, D-amphetamine). Brain Res. 35: 151-165.

Wiklund L, Björklund A, Nobin A. (1978) Regeneration of serotonin neurons in the rat brain after 5,6-dihydroxytryptamine-induced axotomy. Ann N Y Acad Sci. 305: 370-384.

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CHAPTER 1
The sea lamprey tryptophan hydroxylase: a new insight into the evolution of the serotonergic system of vertebrates

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THE SEA LAMPREY TRYPTOPHAN HYDROXYLASE: A NEW INSIGHT INTO THE EVOLUTION OF THE SEROTONERGIC SYSTEM OF VERTEBRATES

Introduction

Serotonin or 5-hydroxytriptamine (5-HT) is an important neurotransmitter in the central nervous system (CNS) of vertebrates. Serotonin plays a variety of roles in the nervous system, where it participates in diverse functions like locomotion modulation, mood control and in regulating sleep, anxiety, drug abuse, food intake or sexual behaviour (see Veenstra-Vander Weele et al., 2000). Moreover, serotonin is found in the pineal organ as a precursor of melatonin and in different peripheral tissues. Neurons containing serotonin have been found in representative species from all major metazoan groups suggesting an early appearance during animal evolution (Lillesaar, 2011). Tryptophan hydroxylase (Tph) is known to be the rate-limiting enzyme for serotonin synthesis (see Hensler, 2006). Walther and coworkers (2003) reported the existence of two *Tph* genes (Tph1 and Tph2) in mammals. In humans, *Tph1* and *Tph2* genes are located on chromosomes 11 and 12, respectively (McKinney et al., 2005), and show different patterns of expression. In mammals, *Tph1* is expressed exclusively in the pineal organ and in peripheral tissues, whereas *Tph2* is expressed in brainstem raphe nuclei (Patel et al., 2004). In teleost fishes, like zebrafish, stickleback and medaka, there are three *Tph* genes (*Tph1a*, *Tph1b* and *Tph2*) (Lillesaar, 2011). In zebrafish, *Tph2* is expressed in serotonergic neurons of the raphe nuclei as in mammals (Teraoka, et al. 2004); whereas, *Tph1a* is expressed in the pineal organ, in amacrine cells of the retina, in restricted preoptic and posterior tubercular nuclei and in the caudal hypothalamus and *Tph1b* is transiently expressed in a restricted population in the preoptic nucleus (Bellipanni et al., 2002). The existence of an additional *Tph1* paralog in teleosts has been explained by the whole genome duplication

event that took place at the base of the teleost radiation more than 300 million years ago (Postlethwait et al., 1998; Taylor et al., 2003). However, it is not known when the initial duplication of the ancestral *Tph* gene occurred during animal evolution.

Lampreys belong to the oldest branch of extant vertebrates, the agnathans or jawless vertebrates. Their key phylogenetic position makes them invaluable animal models to understand molecular evolution. Interestingly, comparative studies on lampreys have provided important information about the evolution of the development and organization of both the peripheral (Barreiro-Iglesias et al., 2008b, 2009a, 2010, 2011) and central (Abalo et al., 2006, 2007, 2008; Barreiro-Iglesias et al., 2008a, 2009b, 2009c) serotonergic systems. In addition, lampreys have been also used as animal models to study the role that serotonin plays in the modulation of locomotion (Grillner et al., 2008) and the regeneration of serotonergic axons after spinal cord injury (Cohen et al., 2005; Cornide-Petronio et al., 2011).

In the present chapter, we aimed to identify and clone *Tph* cDNA/s of the sea lamprey and study the expression of *Tph* transcript/s in larval sea lampreys. This could provide new clues to understand the evolution of *Tph* genes in vertebrates and establish when the duplication/s of the ancestral *Tph* gen took place. In addition, present results offer a new tool for researchers studying the serotonergic system in both normal and pathological conditions using sea lamprey as an animal model.

Materials and methods

Animals

Wild-type larval (11–14 cm in length) and postmetamorphic sea lampreys (*Petromyzon marinus* L.) were obtained from streams feeding Lake Michigan and maintained in freshwater tanks at 16ºC with the appropriate

ventilation conditions until the day of use. Experiments were approved by the Institutional Animal Care and Use Committee at Temple University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and conformed to European Union (86/609/EEC) and Spanish (Royal Decree 223/1998) guidelines on animal care and experimentation. Animals were anesthetized by immersion in 0.1 % tricaine methanesulfonate (Sciencelab, Houston, TX, USA). For subsequent total RNA isolation, the brains and spinal cords were dissected and frozen in RNAlater® Soln., (Ambion, Austin, TX, USA). For *in situ* hybridization experiments, the heads of larval samples and brain/spinal cord of postmetamorphic samples were fixed in 4 % paraformaldehyde in phosphatebuffered saline (PBS) by immersion for 5 h at 4ºC, dehydrated in methanol and stored in 100 % methanol at -20ºC before use.

Cloning of tryptophan hydroxylase from sea lamprey CNS

Total RNA from lamprey CNS was isolated using Trizol reagent (Invitrogen, La Jolla, CA, USA). The first-strand cDNA synthesis reaction from total RNA was catalysed using Superscript III Reverse Transcriptase and oligodT18 primers. For polymerase chain reaction (PCR) cloning, oligonucleotide primers were designed based on alignments of rat *Tph1* and *Tph2* mRNA sequences (GenBank accession numbers: NM_001100634.2 and NM_173839.2, respectively) with lamprey genome sequences deposited in the trace archives of the NCBI (National Center for Biotechnology Information, NIH, Bethesda, MD) and in the Ensembl database of the sea lamprey genome (http://www.ensembl.org/Petromyzon_marinus/Info/Index). The amplified fragments were cloned into pGEM-T easy vectors (Promega, Madison, WI, USA) and sequenced. Sequence analysis and comparison was done using the Basic Local Alignment Search Tool (BLAST) on NCBI.

Phylogenetic analysis

Only a unique *Tph* sequence sharing homology with *Tph* sequences of other animals was cloned. The putative amino acid sequence of the cloned cDNA was aligned with *Tph* protein sequences of other animals (*D. melanogaster Tph*: NM_138236.1; *Danio rerio Tph1a, Tph1b, Tph2*: NM_178306.2, NM_001001843.2 and NM_214795.1, respectively; *Gallus gallus Tph1* and *Tph2*: U26428.1 and NM_001001301.1, respectively; *Rattus norvegicus Tph1* and *Tph2*: NM_001100634.2 and NM_173839.2, respectively) using Clustal X. A phylogenetic tree was constructed using the minimum evolution method with Poisson corrected distances on amino acids and bootstrap analysis (1,000 replications), using MEGA 5 software (Tamura et al., 2011). A reconciliation tree of the minimum evolution tree for the Tph sequences and the corresponding species tree (obtained from the NCBI database) was generated using Notung 2.6 software (Durand et al., 2006). Notung offers a united framework for incorporating duplication-loss parsimony into phylogenetic tasks. This parsimony principle asserts that gene duplication and gene loss are rare events. Notung's functions embody the assumption that, in the absence of information from other sources, the phylogenetic hypothesis that requires the fewest duplications and losses to explain the data is preferred.

Riboprobe synthesis

The cloned *Tph* cDNA was used as template for the generation of Digoxigenin (DIG)-labelled sense and antisense riboprobes for *in situ* hybridization. The *Tph* cRNA probe was transcribed from a sequence located in 5' end of the cloned cDNA (sense primer: AAGTCGAAGCGACGCAACTC; antisense primer containing the T7 overhang: ACTCACTATAGGGAGAAGAAATCTTCTTTGGAACCAGG). The sea lamprey *Tph* cDNA cloned in the pGEM-T Easy vector (Promega) was used as a template for probe generation by standard PCR protocols. The DIG incorporation into probes was controlled by dot blots. The length and integrity of the probes were

examined by gel electrophoresis. Sense RNA probes were generated as internal controls.

In situ *hybridization on cryostat sections*

Fixed heads of large larval sea lampreys or brains/spinal cords of postmetamorphic sea lampreys were rehydrated in PBS. These samples were cryoprotected with 30 % sucrose in PBS, embedded in Tissue Tek (Sakura, Torrance, CA, USA), frozen in liquid nitrogen-cooled isopentane, and cut serially on a cryostat in the transverse plane. *In situ* hybridization on sections was conducted using standard protocols, with the following adaptations. DIG-labelled probes were incubated with the samples at 70ºC overnight in a concentration of 1 µg/mL in hybridization mix. An RNAse treatment step was added to the posthybridization washings (0.2 µg/mL of RNAse A (Invitrogen) at 37ºC for 30 min). The sheep anti-DIG antibody (1:2000; Roche) was incubated overnight at 4ºC in blocking buffer solution. Staining was conducted in NBT/BCIP mix (Roche) at 37ºC until the signal was clearly visible. Then, sections were dehydrated and mounted. Expression of *Tph* transcript in regions reported previously to present serotonin-immunoreactive (-ir) cells and the absence of labelling when using the sense probe supported the specificity of labelling.

Photography

Images were captured digitally using a Zeiss AxioCam CCD camera attached to a Zeiss Axioskop microscope with AxioVision software. Images were imported into Adobe Photoshop CS4 (Adobe Systems, Inc., San Jose, CA) converted to black and white images and adjusted for brightness and contrast, and labels were added. The schematic drawing was done with Corel Draw 12 software.

Results

Several lamprey sequences sharing homology with *Tph* mRNAs of mammals were amplified by reverse transcriptase-PCR (RT-PCR). Among the clones obtained by RT-PCR, only one unique sequence was found. The sequence was submitted to GenBank (Bethesda, MD). GenBank accession number for the sea lamprey *Tph* sequence is JN870281. The deduced Tph protein sequence presents a conserved ACT domain and the conserved aromatic amino acid hydroxylase superfamily domain. Residues coordinating iron biding are also present in the deduced sequence of the protein. A conserved binding site for the cofactor BH4 (biopterin; (6R)-L-erythro-5,6,7,8 tetrahydrobiopterin) is also present in the protein sequence.

The sea lamprey putative Tph amino acid sequence was aligned with Tph family members of other taxa. The highest levels of identity were found with Tph1 protein sequences of gnathostome vertebrates (higher than 75 %). The alignment of Tph protein sequences was used to conduct phylogenetic analyses and construct a phylogenetic tree using the minimum evolution method in molecular evolutionary genetics analysis 5 (MEGA 5). The phylogenetic tree constructed is shown (Fig. 1A). Bootstrap resampling was used to assess the reliability of each node. The phylogenetic tree showed several significant features: (1) all vertebrate Tph amino acid sequences were clustered together, including the sea lamprey sequence, with the invertebrate fruit fly (*Drosophila melanogaster*) Tph as the sister group. (2) The analysis also confirmed the monophyly of each of the osteichthyan Tph1 and Tph2 classes, each containing at least one representative in tetrapods and teleosts, with strong statistical support (bootstraps of 84 and 100). (3) The sea lamprey Tph sequence was found to emerge as an outgroup of the branch leading to the Tph1 sequences of gnathostome vertebrates since it was more similar to Tph1 than to Tph2. Reconciliation of the species and sequence trees was done using the Notung software, the resulting tree had the same topology of the minimum evolution tree. This analysis suggested that the duplications of the ancestral *Tph* probably took place before the divergence of agnathans and jawed vertebrates (indicated with a D in Fig. 1A; but see discussion).

The anatomical organization of the serotonergic cells of the sea lamprey central and peripheral nervous systems have been previously studied using anti-5-HT antibodies (Abalo et al., 2006, 2007, 2008; Barreiro-Iglesias et al., 2008a, 2009b, 2009c). These immunohistochemical studies, both in larvae and adults, reported serotonergic perikarya in the pineal organ, retina, periventricular regions of the dorsal, ventral and caudal hypothalamus, thalamus, pretectum, three isthmic populations (dorsal, intermediate and ventral), caudal rhombencephalon and spinal cord, as well as numerous perikarya in the peripheral nervous system. No serotonergic cells were observed in the rhombencephalic raphe.

Present *in situ* hybridization experiments in larvae showed that in the sea lamprey, the *Tph* transcript was only expressed in cells of the CNS. In the brain and spinal cord, no appreciable differences were observed in the pattern of expression of the *Tph* transcript before and after metamorphosis, therefore, the results from both groups of animals are presented together. A schematic drawing showing the organization of *Tph*-expressing cells in the brain of the adult sea lamprey is shown in Fig. 1B. In both larvae and young adults, *Tph*-expressing perikarya were numerous in the pineal organ (Fig. 1C). In the forebrain, *Tph*-expressing perikarya were numerous in the dorsal hypothalamus (Fig. 1D), ventral hypothalamus, and mammillary region of the caudal hypothalamus (Fig. 1G), and more scarce in the thalamus (Fig. 1E) and the pretectum (Fig. 1F). Numerous *Tph*-expressing perikarya were observed in the isthmus (dorsal, intermediate and ventral populations; Fig. 1G). Large *Tph*-expressing neurons were observed in a rather compact group of the caudal rhombencephalon (Fig. 1H). The spinal cord contained scattered *Tph*-expressing neurons located ventral to the central canal (Fig. 1I). In all these regions, cell distribution and organization were similar to the reported distribution of serotonergic perikarya using anti-5-HT antibodies (see Abalo et al. 2007). Expression of the *Tph* transcript was not observed in any perikarya of the peripheral regions of larval sea lampreys (e.g., in the body wall muscles or the velar muscles; Fig. 2A, B) that have been shown to contain numerous 5-HT-ir

cells in previous studies (Barreiro-Iglesias et al. 2008b, 2009a, 2010, 2011). Peripheral tissues of adult lampreys were not investigated.

The retina of premetamorphic larvae was also investigated. The retina of premetamorphic larvae has two main regions: a central differentiated retina and a lateral, mostly neuroblastic, retina that has differentiated ganglion cells in the innermost layer. In the central retina, strong *Tph* transcript expression was observed in putative photoreceptors (Fig. 3A) and in putative amacrine cells. In the lateral retina, strong *Tph* transcript expression was observed in some putative amacrine cells (Fig. 3B), whereas weak expression was observed in putative neuroblasts (Fig. 3A).

Discussion

Here we reported for the first time, the cloning, phylogenetic analysis and expression of a *Tph* cDNA of an agnathan species, the sea lamprey. The cloned *Tph* sequence corresponds to the sequence annotated in Ensembl database of the sea lamprey genome as *Tph1* (ENSPMAG00000008400). Our phylogenetic analysis suggested that the cloned *Tph* sequence of the sea lamprey is an ortholog of *Tph1* sequences of gnathostome vertebrates and this indicates that the duplication of the ancestral *Tph* gene probably occurred before the separation of agnathans (jawless vertebrates) and gnathostomes (jawed vertebrates). However, the sea lamprey Tph sequence is outside of both the Tph1 and Tph2 gnathostome sequences in the tree, although closer to Tph1 than to Tph2 sequences. Moreover, if Tph2 sequences diverged faster from the ancestral Tph sequence than Tph1 sequences, any Tph phylogenetic tree based in similarity of sequences might be misleading regarding the evolutionary history of these genes. Accordingly, we cannot rule out the possibility that the duplication of the ancestral *Tph* gene may have occurred right after the divergence of lamprey and gnathostome lineages or at the time of the divergence. This has been suggested in other studies to explain the

whole-genome duplication events of vertebrates (Putnam et al., 2008). This last possibility is also suggested by available data of gene synteny, since in the Ensembl genome database the lamprey *Tph1* sequence is located close to three genes (the leucine-rich repeat-containing G protein-coupled receptor 5, transmembrane protein 19 and thyrotropin-releasing hormone degrading genes) that in amniotes are located close to the *Tph2* gene but not close to *Tph1*. In any case, recent research has detected that when studying several gene families many more gene duplications have occurred before the agnathan-gnathostome split than after (Kuraku et al., 2009), suggesting that whole-genome duplications occurred before the agnathan-gnathostome split.

Two partial sequences have been annotated as *Tph2* sequences in the Ensembl database of the sea lamprey genome (ENSPMAG00000006244 and ENSPMAG00000001558). To analyse these sequences with regards to what we are discussing here, we used BLAST comparisons. BLAST comparisons indicated that ENSPMAG00000006244 sequence corresponds with another member of the aromatic amino acid hydroxylase family (phenylalanine 4-hydroxylase), and therefore, it was not included in the present phylogenetic analysis. The ENSPMAG00000001558 sequence shows low similarity with *Tph* and tyrosine hydroxylase genes of vertebrates and invertebrates (less than 40 %), which suggests that it is not an ortholog of *Tph2*. In any case, further studies should investigate whether extant lamprey species have a second *Tph* gene.

Comparison of the expression patterns of *Tph* genes in the CNS of mammals (Patel et al. 2004), teleost fishes (Bellipanni et al., 2002; Teraoka et al., 2004) and lampreys (present results) reveals that *Tph2* is expressed in raphe nuclei of both mammals and teleosts, whereas, in the lamprey, brain rhombencephalic populations (isthmic and caudal populations) express the same *Tph* (*Tph1*) as in forebrain regions. Note that no serotonergic raphe cells have been described in lampreys, although isthmic and caudal serotonergic populations are considered as probable homologues of the raphe nuclei (Barreiro-Iglesias et al., 2008a). Since phylogenetic analysis of amino acid sequences indicated that the cloned lamprey *Tph* is probably an ortholog to *Tph1* of jawed vertebrates, it appears more parsimonious to argue that the

presence of *Tph1*-expressing cells in diencephalic nuclei, as observed in both zebrafish and sea lamprey, is a primitive condition in vertebrates. With regard to the rhombencephalic serotonergic populations, which express *Tph* (probable *Tph1*) in lampreys, the selection of *Tph2* to be expressed in raphe nuclei of the rhombencephalon may have occurred along the line leading to gnathostome vertebrates. Whether option for *Tph1* or *Tph2* expression in brain serotonergic neurons has a phylogenetic significance is not known.

In mammals, *Tph1* is primarily expressed in the pineal organ and in peripheral tissues (see above). In lampreys, *Tph* (*Tph1*) is also expressed in the pineal organ. However, unlike mammals, we have not found expression of this lamprey *Tph* in the peripheral nervous system. These results in the lamprey peripheral nervous system are in marked contrast with the abundance of serotonergic cells previously observed (Barreiro-Iglesias et al., 2008b; 2009a). Together, they predict the existence in lampreys of a second enzyme with the ability to hydroxylate tryptophan primarily expressed in the peripheral nervous system, again providing circumstantial support for the hypothesis that the duplication of the *Tph* gene occurred before the separation of lampreys and gnathostomes. The situation in lampreys appears to be similar to that of the invertebrate *D. melanogaster*, in which *Tph* (DTPH) expression has been reported only in CNS neurons and not in peripheral serotonergic neurons (Coleman and Neckameyer, 2005; Neckameyer et al., 2007; Bao et al., 2010). We have recently proposed that the extensive presence of serotonergic elements in the peripheral nervous system of lampreys suggests a close relationship with the organization of the system in invertebrate groups (Barreiro-Iglesias et al., 2009a). Future completion and assembly of the sea lamprey genome will help to investigate which enzyme is expressed in peripheral serotonergic neurons of lampreys and its phylogenetic relationship with the other known tryptophan hydroxylases.

Our results showed that the *Tph* transcript is present in the larval sea lamprey retina. *Tph* transcript expression has been also reported in cells of the retina of other vertebrate species, including photoreceptors and amacrine cells (*Xenopus*: Green et al., 1995; chicken: Chong et al., 1998, rat: Liang et al.,

2004). Tph is the first enzyme in the biosynthetic pathways of serotonin in amacrine cells and of melatonin in photoreceptor cells. Serotonin immunoreactive amacrine cells have been reported to be present in adult lamprey retina (Versaux-Botteri et al., 1991; Pierre et al., 1992; Villar-Cerviño et al., 2006; Abalo et al., 2008) and they are immunocytochemically detected from the onset of metamorphosis (Abalo et al., 2008), which coincides with the observation of *Tph* transcript expression found in this study. However, neither serotonin nor melatonin have been detected in photoreceptors of the retina of lampreys, in spite of the fact that serotonin is immunohistochemically detected in pineal photoreceptors (Tamotsu et al., 1990; 1997; Pombal et al., 1999) and melatonin is secreted by the pineal organ of lampreys (Bolliet et al., 1993; Samejima et al., 1997). The expression of the *Tph* transcript in retinal photorreceptors of lampreys strongly suggests that they synthesize melatonin and that this characteristic appeared early and has been conserved throughout the evolution of vertebrates. Melatonin has been proposed to have a local paracrine function related to photic adaptation in the retina (revised in Cahill and Besharse 1995). If it serves the same function in lampreys, it needs to be investigated. Although present results showed *Tph* expression in neuroblasts, serotonin has not been detected in these neuroblasts of the developing retina (Villar-Cerviño et al., 2006). The weak expression of *Tph* in these cells suggests that serotonin may be produced at low levels in neuroblasts, which could account for the lack of immunocytochemical detection. The presence of Tph in neuroblasts indicates that serotonin may be playing another role other than a neurotransmitter function. For example, it has been shown that serotonin acts as a regulator of axonal outgrowth in explants of retina (Lima et al., 1994; Schmeer et al., 2001), so serotonin may be also playing a developmental role in the larval retina.

In conclusion, our results indicate that the duplication of the ancestral *Tph* gene probably occurred before the separation of agnathans and gnathostomes and suggest that a second enzyme with the ability to hydroxylate tryptophan is expressed in the peripheral nervous system of

lampreys. This study also offers a new tool to further study the serotonergic system of lampreys under normal and pathological conditions.

Literature cited

Abalo XM, Villar-Cheda B, Barreiro-Iglesias A, Meléndez-Ferro M, Pérez-Costas E, Anadón R, Rodicio C. (2006) Development of the serotonergic system in the central nervous system of the sea lamprey. Int J Dev Neurosci. 24: 541.

Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R, Rodicio MC. (2007) Development of the serotonergic system in the central nervous system of the sea lamprey. J Chem Neuroanat. 34: 29-46.

Abalo XM, Villar-Cerviño V, Villar-Cheda B, Anadón R, Rodicio MC. (2008) Neurochemical differentiation of horizontal and amacrine cells during transformation of the sea lamprey retina. J Chem Neuroanat. 35: 225-232.

Bao X, Wang B, Zhang J, Yan T, Yang W, Jiao F, Liu J, Wang S. (2010) Localization of serotonin/tryptophan-hydroxylase-immunoreactive cells in the brain and suboesophageal ganglion of Drosophila melanogaster. Cell Tissue Res. 340: 51-59.

Barreiro-Iglesias A, Villar-Cerviño V, Anadón R, Rodicio MC. (2008a) Development and organization of the descending serotonergic brainstem-spinal projections in the sea lamprey. J Chem Neuroanat. 36: 77-84.

Barreiro-Iglesias A, Villar-Cerviño V, Villar-Cheda B, Anadón R, Rodicio MC. (2008b) Neurochemical characterization of sea lamprey taste buds and afferent gustatory fibers: presence of serotonin, calretinin, and CGRP

immunoreactivity in taste bud bi-ciliated cells of the earliest vertebrates. J Comp Neurol. 511: 438-453.

Barreiro-Iglesias A, Aldegunde M, Anadón R, Rodicio MC. (2009a) Extensive presence of serotonergic cells and fibers in the peripheral nervous system of lampreys. J Comp Neurol. 512: 478-499.

Barreiro-Iglesias A, Cornide-Petronio ME, Anadón R, Rodicio MC. (2009b) Serotonin and GABA are colocalized in restricted groups of neurons in the larval sea lamprey brain: insights into the early evolution of neurotransmitter colocalization in vertebrates. J Anat. 215: 435-443.

Barreiro-Iglesias A, Villar-Cerviño V, Anadón R, Rodicio MC. (2009c) A monoclonal antibody as a tool to study the subcommissural organ and Reissner's fibre of the sea lamprey: an immunofluorescence study before and after a spinal cord transection. Neurosci Lett. 464: 34-38.

Barreiro-Iglesias A, Anadón R, Rodicio MC. (2010) The gustatory system of lampreys. Brain Behav Evol. 75: 241-250.

Barreiro-Iglesias A, Romaus-Sanjurjo D, Senra-Martínez P, Anadón R, Rodicio MC. (2011) Doublecortin is expressed in trigeminal motoneurons that innervate the velar musculature of lampreys: considerations on the evolution and development of the trigeminal system. Evol Dev. 13: 149-158.

Bellipanni G, Rink E, Bally-Cuif L.. (2002) Cloning of two tryptophan hydroxylase genes expressed in the diencephalon of the developing zebrafish brain. Gene Expr Patterns. 2: 251-256.

Bolliet V, Ali MA, Anctil M, Zachmann A. (1993) Melatonin secretion in vitro from the pineal complex of the lamprey *Petromyzon marinus*. Gen Comp Endocrinol. 89:101-106.

Cahill GM, Besharse JC. (1995) Circadian rhythmicity in vertebrate retinas: Regulation by a photoreceptor oscillator. Progress in Retinal and Eye Research 14: 267-291.

Chong NW, Cassone VM, Bernard M, Klein DC, Iuvone PM. (1998) Circadian expression of tryptophan hydroxylase mRNA in the chicken retina. Brain Res Mol Brain Res. 61: 243-250.

Cohen AH, Abdelnabi M, Guan L, Ottinger MA, Chakrabarti L.. (2005) Changes in distribution of serotonin induced by spinal injury in larval lampreys: evidence from immunohistochemistry and HPLC. J Neurotrauma. 22: 172-188.

Coleman CM, Neckameyer WS. (2005) Serotonin synthesis by two distinct enzymes in Drosophila melanogaster. Arch Insect Biochem Physiol. 59: 12-31.

Cornide-Petronio ME, Ruiz MS, Barreiro-Iglesias A, Rodicio MC. (2011) Spontaneous regeneration of the serotonergic descending innervation in the sea lamprey after spinal cord injury. J Neurotrauma. 28: 2535-2540.

Durand D, Halldorsson BV, Vernot B. (2006) A hybrid micromacroevolutionary approach to gene tree reconstruction. J Comput Biol. 13: 320-335.

Green CB, Cahill GM, Besharse JC. (1995) Tryptophan hydroxylase is expressed by photoreceptors in *Xenopus laevis* retina. Vis Neurosci. 12: 663-670.

Grillner S, Wallén P, Saitoh K, Kozlov A, Robertson B. (2008) Neural bases of goal-directed locomotion in vertebrates-an overview. Brain Res Rev. 57: 2-12.

Hensler JG. (2006) Serotonin. In: Siegel GJ, Albers RW, Brady ST, Price DL (eds) Basic Neurochemistry. Elsevier Academic Press, San Diego, pp 227-249.

Kuraku S, Meyer A, Kuratani S. (2009) Timing of genome duplications relative to the origin of the vertebrates: did cyclostomes diverge before or after? Mol Biol Evol. 26: 47-59.

Liang J, Wessel JH 3rd, Iuvone PM, Tosini G, Fukuhara C. (2004) Diurnal rhythms of tryptophan hydroxylase 1 and 2 mRNA expression in the rat retina. Neuroreport. 15: 1497-1500.

Lillesaar C. (2011) The serotonergic system in fish. J Chem Neuroanat. 41: 294-308.

Lima L, Matus P, Urbina M. (1994) Serotonin inhibits outgrowth of goldfish retina and impairs the trophic effect of taurine. J Neurosci Res. 38: 444-450.

McKinney J, Knappskog PM, Haavik J. (2005) Different properties of the central and peripheral forms of human tryptophan hydroxylase. J Neurochem. 92: 311-320.

Neckameyer WS, Coleman CM, Eadie S, Goodwin SF. (2007) Compartmentalization of neuronal and peripheral serotonin synthesis in Drosophila melanogaster. Genes Brain Behav. 6: 756-769.

Patel PD, Pontrello C, Burke S. (2004) Robust and tissue-specific expression of TPH2 versus TPH1 in rat raphe and pineal gland. Biol Psychiatry. 55: 428-433.

Pierre J, Repérant J, Ward R, Vesselkin NP, Rio JP, Miceli D, Kratskin I. (1992) The serotoninergic system of the brain of the lamprey, *Lampetra fluviatilis*: an evolutionary perspective. J Chem Neuroanat. 5: 195-219.

Pombal MA, Yáñez J, Marín O, González A, Anadón R. (1999) Cholinergic and GABAergic neuronal elements in the pineal organ of lampreys, and tract-tracing observations of differential connections of pinealofugal neurons. Cell Tissue Res. 295: 215-223.

Postlethwait JH, Yan YL, Gates MA, Horne S, Amores A, Brownlie A, Donovan A, Egan ES, Force A, Gong Z, Goutel C, Fritz A, Kelsh R, Knapik E, Liao E, Paw B, Ransom D, Singer A, Thomson M, Abduljabbar TS, Yelick P, Beier D, Joly JS, Larhammar D, Rosa F, Westerfield M, Zon LI, Johnson SL,

Talbot WS. (1998) Vertebrate genome evolution and the zebrafish gene map. Nat Genet. 18: 345-349.

Putnam NH, Butts T, Ferrier DE, Furlong RF, Hellsten U, Kawashima T, Robinson-Rechavi M, Shoguchi E, Terry A, Yu JK, Benito-Gutiérrez EL, Dubchak I, Garcia-Fernàndez J, Gibson-Brown JJ, Grigoriev IV, Horton AC, de Jong PJ, Jurka J, Kapitonov VV, Kohara Y, Kuroki Y, Lindquist E, Lucas S, Osoegawa K, Pennacchio LA, Salamov AA, Satou Y, Sauka-Spengler T, Schmutz J, Shin-I T, Toyoda A, Bronner-Fraser M, Fujiyama A, Holland LZ, Holland PW, Satoh N, Rokhsar DS. (2008) The amphioxus genome and the evolution of the chordate karyotype. Nature. 453: 1064-1071.

Samejima M, Tamotsu S, Uchida K, Moriguchi Y, Morita Y. (1997) Melatonin excretion rhythms in the cultured pineal organ of the lamprey, Lampetra japonica. Biol Signals. 6: 241-246.

Schmeer C, Obregón F, Urbina M, Lima L.. (2001) Further characterization of 5-HT1A receptors in the goldfish retina: role of cyclic AMP in the regulation of the in vitro outgrowth of retinal explants. Neurochem Res. 26: 213-223.

Tamotsu S, Korf HW, Morita Y, Oksche A. (1990) Immunocytochemical localization of serotonin and photoreceptor-specific proteins (rod-opsin, S-antigen) in the pineal complex of the river lamprey, *Lampetra japonica*, with special reference to photoneuroendocrine cells. Cell Tissue Res. 262: 205-216.

Tamotsu S, Samejima M, Suzuki N, Morita Y. (1997) Three-dimensional reconstruction of serotonin-immunoreactive photoreceptors in the pineal organ of the river lamprey, *Lampetra japonica*. Biol Signals. 6: 184-190.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood,

evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 29: 2731-2739.

Taylor JS, Braasch I, Frickey T, Meyer A, Van de Peer Y. (2003) Genome duplication, a trait shared by 22000 species of rayfinned fish. Genome Res. 13: 382-390.

Teraoka H, Russell C, Regan J, Chandrasekhar A, Concha ML, Yokoyama R, Higashi K, Take-Uchi M, Dong W, Hiraga T, Holder N, Wilson SW. (2004) Hedgehog and Fgf signalling pathways regulate the development of tphR-expressing serotonergic raphe neurons in zebrafish embryos. J Neurobiol. 60: 275-288.

Veenstra-Vander Weele J, Anderson GM, Cook EH. (2000) Pharmacogenetics and the serotonin system: initial studies and future directions. Eur J Pharmacol. 410: 165-181.

Versaux-Botteri C, Dalil N, Kenigfest N, Repérant J, Vesselkin N, Nguyen-Legros J. (1991) Immunohistochemical localization of retinal serotonin cells in the lamprey (*Lampetra fluviatilis*). Vis Neurosci. 7: 171-177.

Villar-Cerviño V, Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Holstein GR, Martinelli GP, Rodicio MC, Anadón R. (2006) Presence of glutamate, glycine, and gamma-aminobutyric acid in the retina of the larval sea lamprey: comparative immunohistochemical study of classical neurotransmitters in larval and postmetamorphic retinas. J Comp Neurol. 499: 810-827.

Walther DJ, Peter JU, Bashammakh S, Hörtnagl H, Voits M, Fink H, Bader M. (2003) Synthesis of serotonin by a second tryptophan hydroxylase isoform. Science. 299: 76.

Abbreviations

CNS	Central nervous system
DIG	Digoxigenin
-ir	Immunoreactive
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase-PCR
Tph	Tryptophan hydroxylase gene
Tph	Tryptophan hydroxylase protein

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FIGURES

Figure 1 - A) The evolutionary history of Tph sequences was inferred using the minimum evolution method with Poisson corrected distances on amino acids and bootstrap analysis (1,000 replications). The percentages of replicate trees are shown next to the branches. *Capital D letters* in the tree indicate when the duplication of the ancestral Tphs took place as inferred by the Notung 2.6 software after reconciliation of the minimum evolution Tph sequences tree with the species tree obtained from the NCBI. B) Schematic drawing of a lateral view of the postmetamorphic sea lamprey brain showing the distribution of *Tph* expressing cells. C) Photomicrograph of the pineal organ (*PO*) of the larval sea lamprey showing the presence of numerous cells expressing the *Tph* transcript. D) Photomicrograph of a transverse section of the hypothalamus (*H*) of a postmetamorphic sea lamprey showing numerous cells expressing the *Tph* transcript in the dorsal hypothalamus (putative cerebrospinal fluid-contacting cells). E) Photomicrograph of a transverse section of the diencephalon of a postmetamorphic sea lamprey showing the presence of cells expressing the *Tph* transcript in the pretectal (*PT*) and thalamic (*Th*) regions. F) Photomicrograph of a transverse section of the hypothalamus of a postmetamorphic sea lamprey showing numerous cells expressing the *Tph* transcript in the mammillary (*Mm*) region. G) Photomicrograph of a transverse section of the rostral rhombencephalon of a postmetamorphic sea lamprey showing numerous cells expressing the *Tph* transcript in the isthmus. Note that populations extend from dorsal to ventral levels. H) Photomicrograph of a transverse section of the caudal rhombencephalon (*CRh*) of a postmetamorphic sea lamprey showing a group of large cells expressing the *Tph* transcript. I) Photomicrograph of a transverse section of the rostral spinal cord of a postmetamorphic sea lamprey showing a cell expressing the *Tph* transcript in the ventromedial region of the spinal cord (*SC*). The bars indicate the approximate level of the brain sections shown in photomicrographs C-I. *LP* lateral pallium, *MP* medial pallium, *OB* olfactory bulb, *ON* optic nerve. *Stars* indicate the ventricles. *Arrows* point to some of the *Tph* transcript expressing cells. *Scale bars*: 20 µm (I); 25 µm (C,,G, H); 50 µm (D, F), 75 µm (E).

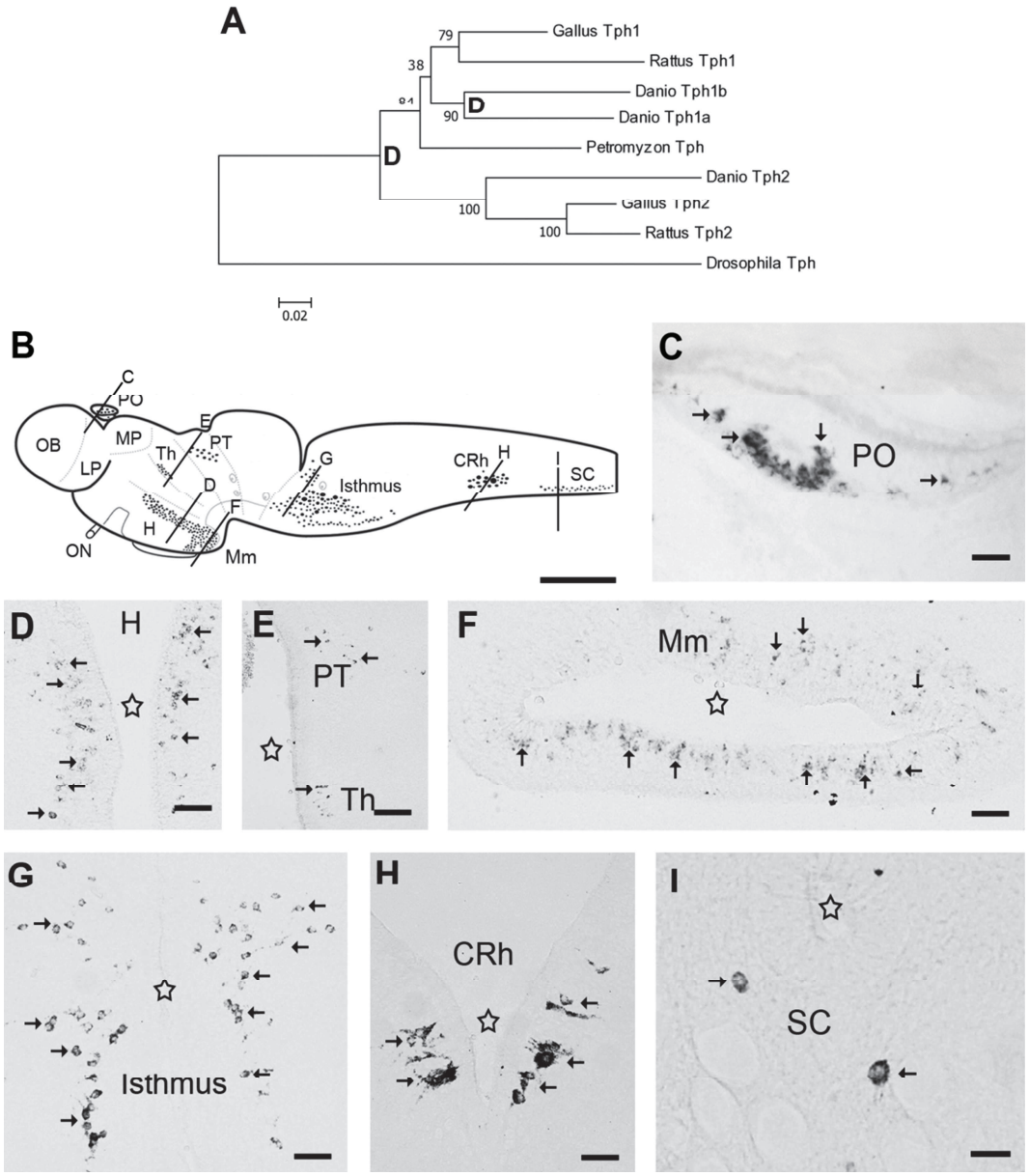
A
Gallus Tph1
Rattus Tph1
Danio Tph1b
Danio Tph1a
Petromyzon Tph
Danio Tph2
Gallus Tph2
Rattus Tph2
Drosophila Tph
79
38
90
100
100
D
D
0.02
B
C
PO
OB
MP
LP
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FIGURE 1

Figure 2 - A) Photomicrograph of a transverse section of the body wall of a larval sea lamprey showing the absence of cells expressing the *Tph* transcript in a region containing numerous serotonergic cells (see Barreiro-Iglesias et al. 2009a). Dark cells below the epidermis and among muscle fibers are melanin-containing cells (melanophores, indicated by *arrows*). B) Photomicrograph of a transverse section of the velum of a larval sea lamprey (which contains a large number of serotonergic cells; see Barreiro-Iglesias et al. 2009a) showing the absence of cells expressing the *Tph* transcript. *Arrowheads* indicate the epidermis. *Asterisks* indicate the muscles (body wall muscles in A and velar muscles in B). *Scale bars*: 25 µm.

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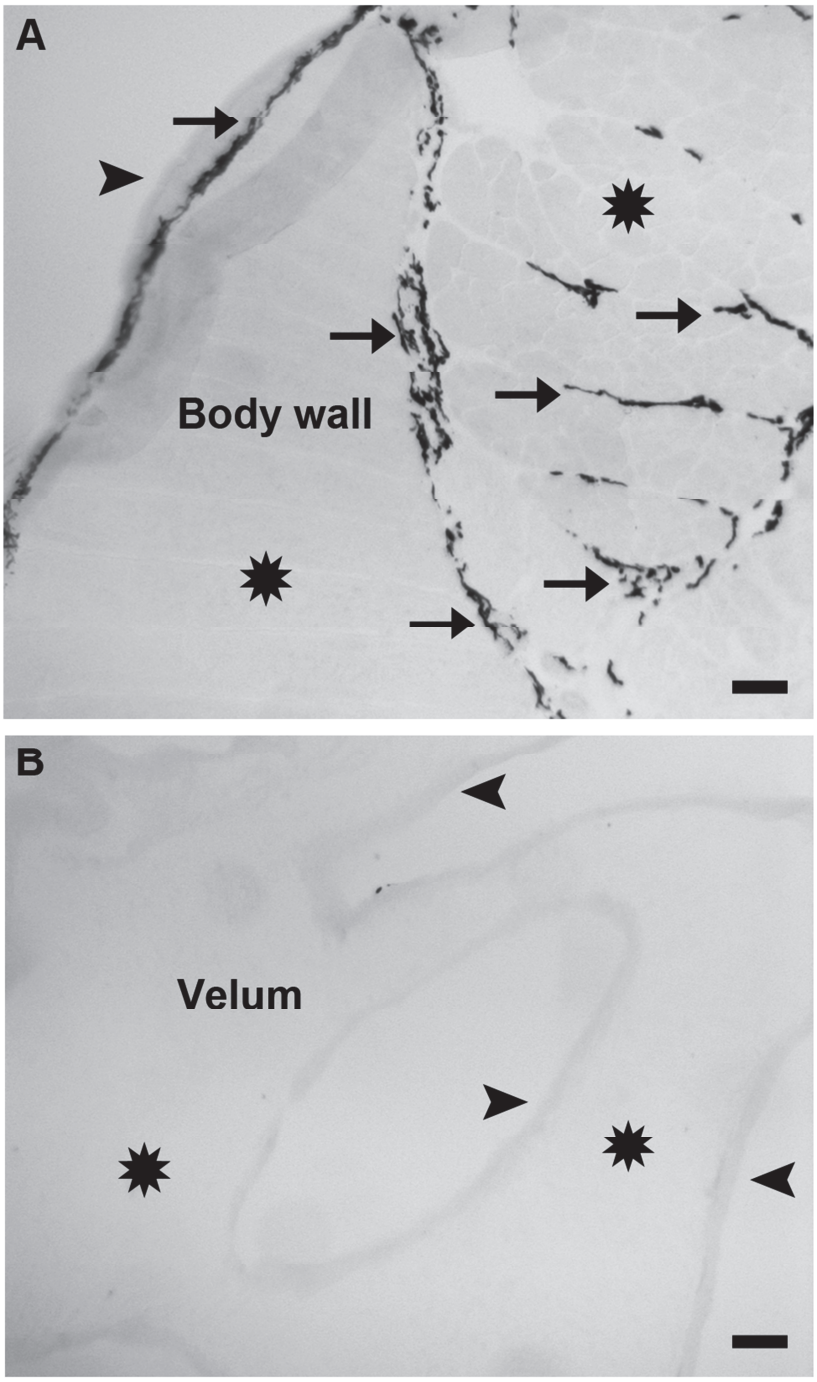

FIGURE 2

Figure 3 - A) Photomicrograph of a transverse section of the retina of a premetamorphic larval sea lamprey showing the presence of putative photoreceptors (*arrowhead*) and putative neuroblasts (*arrows*) expressing the *Tph* transcript. B) Photomicrograph of a transverse section of the retina of a premetamorphic larval sea lamprey showing the presence of the *Tph* transcript in putative amacrine cells. *Scale bars*: 25 µm (B), 60 µm (A).

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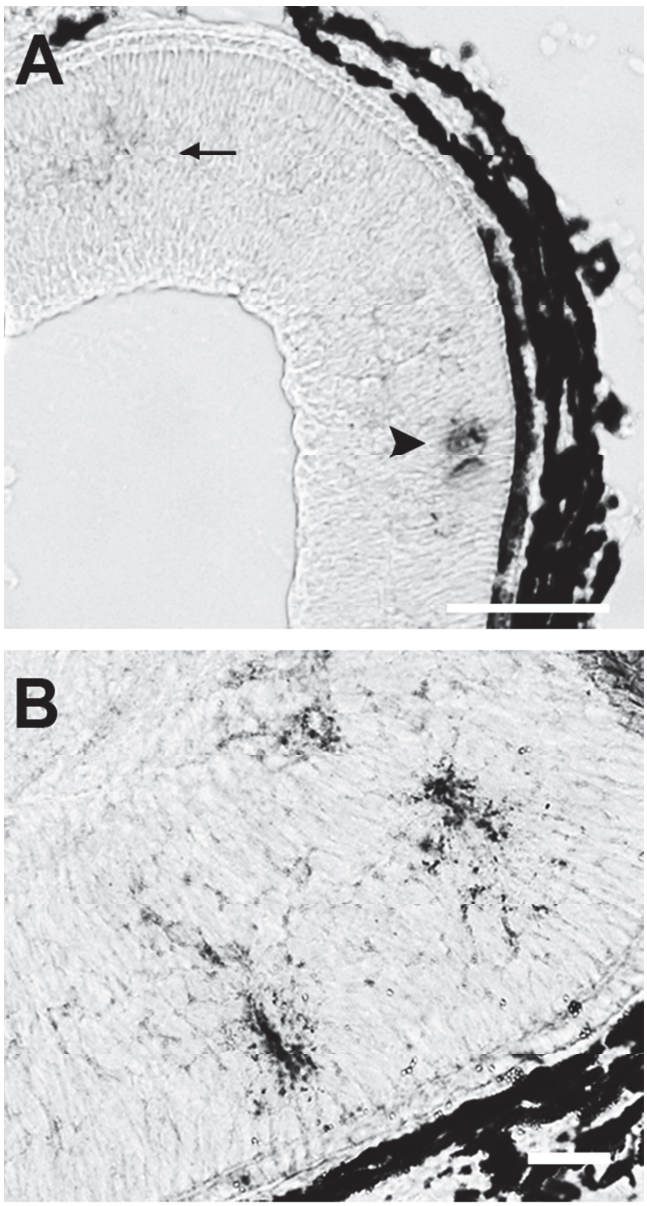

FIGURE 3

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CHAPTER 2
Serotonin 1A receptor (5-HT1A) of sea lamprey: cDNA cloning and expression in the central nervous system

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SEROTONIN 1A RECEPTOR (5-HT1A) OF SEA LAMPREY: cDNA CLONING AND EXPRESSION IN THE CENTRAL NERVOUS SYSTEM

Introduction

Serotonergic neurons are among the earliest cells to be born in the developing central nervous system. In mammals, perikarya of these cells are largely restricted to the hindbrain (Rubenstein, 1998), although the serotonergic system is involved in a large number of physiological functions resulting from its widespread innervation in the brain (Weiger, 1997). However, in addition to its role as a neurotransmitter, growing evidence suggests that serotonin also regulates the connectivity of the brain by modulating developmental cellular migration and cytoarchitecture (Daubert and Condron, 2010). Data obtained from multiple animal models also support the hypothesis that serotonin autoregulates branch morphology of serotonergic processes (Acklin and Nicholls, 1990; Couch et al., 2004).

To date, at least fourteen different serotonin receptor (5-HTR) subtypes have been identified in mammals, and have been grouped into seven families (5-HT$_1$–5-HT$_7$) (Kroeze and Roth, 2006). These serotonin receptors are G-protein-coupled receptors except the ligand-gated ion channel 5-HT$_3$ receptor. The serotonin 1A receptor (5-HT$_{1A}$) is a major determinant of the activity of serotonergic cells and of serotonin release because of its pre- and post-synaptic location (Chalmers and Watson, 1991). The 5-HT$_{1A}$ is estimated to have differentiated around 650 million years ago from the 5-HT$_1$ subfamily around the time during which vertebrates diverged from invertebrates (Peroutka and Howell, 1994). Most of the invertebrate 5-HTRs resemble either the mammalian 5-HT$_{1A}$ or the 5-HT$_7$ (Kroeze and Roth, 2006), suggesting that these two subclasses represent the ancestral archetypical 5-HTR.

The expression of the 5-HT_{1A} has been described in a few vertebrate species (e.g. Saudou et al., 1992; Thamm et al., 2010). The 5-ht1a transcript is widely expressed in the adult rat brain, particularly in the dorsal raphe nucleus, septum, hippocampus, entorhinal cortex, and interpeduncular nucleus (see Pompeiano et al., 1992). In early embryonic mice it is also expressed in the forebrain (Bonnin et al., 2006). The relationship between the expression of the 5-HT_{1A} receptor and anxiety, depression or aggressive behaviour has been thoroughly studied in rodents (reviewed in Popova, 2006). The distribution of the 5-ht1a mRNA has also been examined in the adult (Burnet et al., 1995) and developing (del Olmo et al., 1998) human brain. A study by Norton and coworkers (2008) has also reported widespread expression of the 5-ht1a in the brain of adult and larval zebrafish.

The sea lamprey, *Petromyzon marinus* L., belongs to an ancient lineage of vertebrates (Agnathans) that occupies a key phylogenetic position in the transition between *Amphioxus* (protochordates) and jawed vertebrates. Development of the sea lamprey is complex and begins with eggs laid in rivers (Hardisty and Potter, 1971). The embryonic period (12–13 days) is followed by a non-feeding prolarval phase (about 20–23 days) and a long larval stage (4–7 years) (Piavis, 1971). The larvae are blind filter feeders that, through complex metamorphosis, transform into young adults (postmetamorphic lampreys). The young adults descend to the sea and feed on teleost fish, and, after 1-2 years, mature sea lampreys migrate to rivers to breed and die (Hardisty and Potter, 1971).

The lamprey brain organization and basic circuitry follows the basic vertebrate plan. Lampreys are important model vertebrates for studies on neural circuitry, development and evolution (reviewed in Osório and Rétaux, 2008), and lamprey genes have been used as a key reference to understand molecular evolution in vertebrates (reviewed by Shifman et al., 2007). Lampreys are important models for the study of the vertebrate circuits controlling locomotion (reviewed in Grillner et al., 1995). The organization and development of central serotonin-immunoreactive (-ir) cells and fibers is well known (Antri et al., 2006; Abalo et al., 2007). A number of physiological studies

have found that serotonin modulates spinal cord and brain circuits (Christenson et al., 1989; El Manira et al., 1997; Antri et al., 2008). Pharmacological and physiological studies have shown that lampreys have 5-HT_{1A}-like receptors and that they play a role in the modulation of the locomotion process (Wikström et al., 1995; Hill et al., 2003). Serotonin modulates presynaptic inhibition via activation of G protein-coupled receptors (Blackmer et al., 2001). Despite the wide knowledge on the lamprey serotonergic system, the cloning and molecular characterization of the 5-HT receptors of lampreys has not been reported yet nor has its pattern of expression been investigated.

In the present study, we report the identification and cloning of the sea lamprey 5-ht1a cDNA and the central expression pattern of the 5-ht1a transcript during lamprey development from embryogenesis to adults. Our study allowed the characterization of the 5-HT_{1A} receptor, including its predicted three-dimensional structure and phylogenetic relationship. Moreover, we describe for the first time the central distribution of the 5-ht1a transcript in a lamprey species and compare its expression pattern with that of other species.

Material and methods

Animals

For *in situ* hybridization, sea lamprey, *Petromyzon marinus* L., prolarvae (n=30), early larvae (10 mm in length; n=6), medium-sized larvae (40-70 mm in length; n=3), large larvae (100–140 mm in length; n=5), and adults (n=3) were used. Twelve additional larvae were used for RNA extraction. Larvae and adults were collected from the River Ulla (Galicia, Northwest Spain) and maintained in fresh water with a bed of river sediment until use. The prolarvae and the early larvae were obtained from in vitro fertilized eggs reared in our laboratory. The stages of prolarvae (P) are indicated by age (i.e. P1 indicates 1 day post-hatching prolarvae, and so on; hatching of the broods occurred 11–

13 days after fertilization). To classify sea lamprey prolarvae, Piavis's staging system was adopted: hatchling (P0–P1), pigmentation (P2–P3), gill-cleft (P4–P7) and burrowing (P8–P23) stages (Piavis, 1971). All experiments were approved by the Bioethics Committee of the University of Santiago de Compostela and conformed to European Union (86/609/EEC) and Spanish (Royal Decree 223/1998) regulations for the care and handling of animals in research.

Cloning and sequencing of lamprey 5-ht1a

Larval lampreys were anesthetized by immersion in 0.05% benzocaine (Sigma, St. Louis, MO) and the brains were removed. Total RNA was isolated from lamprey brain using Trizol reagent (Invitrogen, La Jolla, CA). The first-strand cDNA synthesis reaction from total RNA was catalyzed by Superscript III reverse transcriptase (Invitrogen) using random primers (Invitrogen). For polymerase chain reaction (PCR) cloning, degenerate oligonucleotide primers were designed based on alignments of the *Danio rerio* (GenBank ID: NM_001123321), *Xenopus laevis* (GenBank ID: NM_001085830), *Mus musculus* (GenBank ID: MMU39391) and *Canis lupus familiaris* (GenBank ID: NM_001012397) 5-ht1a sequences with lamprey sequences deposited in the trace archives of the NCBI (National Centre for Biotechnology Information, NIH, Bethesda, MD). The amplified fragments were cloned into pGEM-T vectors (Promega, Madison, WI) and sequenced. Sequence analysis and comparison was done through Basic Local Alignment Search Tool (BLAST) on NCBI, on the first version of the assembled sea lamprey genome in the Ensembl database (http://www.ensembl.org), with the CLC Sequence viewer 6 software (CLC bio A/S) and with the Vector NTI software (Invitrogen).

Structural model in 3D of the 5-HT_{1A} was obtained with the SWISS-MODEL workspace in Vector NTI software. The SWISS-MODEL workspace is an integrated Web-based modelling expert system. For a given protein sequence, a library of experimental protein structures is searched to identify suitable templates. On the basis of sequence alignment between the target protein and the template structure, a three-dimensional model for the target protein is

generated. Model quality assessment tools are used to estimate the reliability of the resulting models (Bordoli et al., 2009).

Phylogenetic analysis

5-HT_{1A} protein published sequences (GenBank ID: *Apis mellifera*: NP_001164579.1; *Canis lupus familiaris*: NP_001012397.1; *Danio rerio*: NP_001116793.1; *Drosophila melanogaster* isoform A: NP_476802.1; *Drosophila melanogaster* isoform B: NP_725849.1; *Gallus gallus*: NP_001163999.1; *Homo sapiens*: NP_000515.2; *Mus musculus*: Q64264.1; *Oreochromis mossambicus*: AAP83427.1; *Rattus norvegicus*: NP_036717.1; *Vulpes vulpes*: Q6XXY0.1; *Xenopus laevis*; NP_001079299.1) were aligned with the 5-HT1A putative amino acid sequence of the sea lamprey. A sequence corresponding to an alternative copy of *Danio rerio* (NP_001139238.1), possibly generated by whole genome duplication, was also included in the alignment. All sequences were aligned with CLUSTALW, and a phylogenetic tree was constructed using the neighbor-joining method with Poisson corrected distances on amino acids and bootstrap analysis (1,000 replications), using MEGA 5 software (Tamura et al., 2011). An alternative phylogenetic tree was constructed with the minimum-evolution method with Poisson corrected distances on amino acids and bootstrap analysis (1,000 replications), using also MEGA version 5 software. Another phylogenetic tree was constructed using the neighbor-joining method with Poisson corrected distances on amino acids and bootstrap analysis (1,000 replications), using MEGA version 5 software, but including other 5-HTRs of the same species: 5-HT_{1B} (*Gallus gallus* (GenBank ID: ADC54223), *Mus musculus* (GenBank ID: NP_034612), *Rattus norvegicus* (GenBank ID: NP_071561), *Homo sapiens* (GenBank ID: NP_000854), *Canis lupus familiaris* (GenBank ID: AAQ89935), *Vulpes vulpes* (GenBank ID: AAP12468)), 5-HT_{1D} (*Homo sapiens* (GenBank ID: NP_000855), *Rattus norvegicus* (GenBank ID: NP_036984), *Canis lupus familiaris* (GenBank ID: NP_001003280)), 5-HT_{1E} (*Homo sapiens* (GenBank ID: NP_000856)), 5-HT1F (*Rattus norvegicus* (GenBank ID: NP_068629)), 5-HT_{2A}

(*Canis lupus familiaris* (GenBank ID: AAX39385), *Rattus norvegicus* (GenBank ID: NP_058950)), 5-HT_{2B} (*Xenopus laevis* (GenBank ID: NP_001082744), *Canis lupus familiaris* (GenBank ID: NP_001019804), *Homo sapiens* (GenBank ID: NP_000858), *Mus musculus* (GenBank ID: NP_032337), *Rattus norvegicus* (GenBank ID: NP_058946)), 5-HT_{2C} (*Homo sapiens* (GenBank ID: NP_000859), *Rattus norvegicus* (GenBank ID: NP_036897), *Canis lupus familiaris* (GenBank ID: NP_001006649)), 5-HT_4 (*Homo sapiens* (GenBank ID: CAA71462), *Mus musculus* (GenBank ID: NP_032339), *Rattus norvegicus* (GenBank ID: NP_036985)), 5-HT_{5A} (*Homo sapiens* (GenBank ID: NP_076917), *Mus musculus* (GenBank ID: NP_032340), *Rattus norvegicus* (GenBank ID: NP_037280)), 5-HT_{5B} (*Mus musculus* (GenBank ID: NP_034613), *Rattus norvegicus* (GenBank ID: NP_077371)), 5-HT_6 (*Homo sapiens* (GenBank ID: NP_000862), *Rattus norvegicus* (GenBank ID: NP_077341) *Gallus gallus* (GenBank ID: NP_001166911)) and 5-HT_7 (*Gallus gallus* (GenBank ID: NP_001165240), *Xenopus laevis* (GenBank ID: NP_001079253), *Homo sapiens* (GenBank ID: CAH69968), *Mus musculus* (GenBank ID: NP_032341), *Rattus norvegicus* (GenBank ID: NP_075227)).

In situ *hybridization*

A 596 bp fragment was obtained by PCR using 5´-GCGACATCTTCATCGCGCTGGA-3´ forward and 5´-TAAGCTTTAATACGACTCACTATAGGGAGAGCGGTCTCTGCCTCTTGCCGCC -3´ reverse primers. The reverse primer includes the sequence of the T7 promoter. The identity of the amplified fragments was confirmed by direct sequencing. Digoxigenin (DIG)-labelled riboprobes were synthesized using the amplified fragments as templates and following standard protocols using T7 polymerase (Roche Diagnostics, Germany).

Embryos and prolarvae were fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 12-36 h at 4ºC, dehydrated in methanol series and stored in 100% methanol at -20ºC until use. Whole-mount *in situ* hybridizations of embryos and P0-P3 prolarvae were conducted using standard protocols, with the following adaptations. A

proteinase K treatment (10 mg/ml) was performed for 30 min at room temperature following rehydration of embryos and prolarvae and an RNAse A treatment (100 mg/ml, 37ºC during 30 min) was added to the posthybridization washings. The sheep anti-Dig antibody conjugated to alkaline phosphatase (1:2000; Roche) was incubated overnight at 4ºC in blocking buffer solution. Staining was conducted in BM Purple (Roche) at 37ºC until the signal was clearly visible. After photography of the whole-mount hybridized prolarvae, they were cryoprotected with 30% sucrose in PBS embedded in Tissue Tek (Sakura, Torrance, CA), frozen in liquid nitrogen-cooled isopentane, and cut serially on a cryostat (20 µm thick) in transverse planes. Sections were mounted on subbed glass slides. Then, sections were rinsed in distilled water, dehydrated and then coverslipped with Mowiol® 4-88 mounting medium (Calbiochem, La Jolla, CA).

For *in situ* hybridization of larval and adult lampreys, they were fixed under deep anaesthesia with 0.05% benzocaine (Sigma) by immersion for 12 hours in 4% paraformaldehyde in PBS at 4ºC. The brains were removed, cryoprotected and sectioned as above. *In situ* hybridization of sections was conducted using standard protocols, with the following adaptations. The sections were incubated with DIG-labelled probes at 70ºC overnight in a concentration of 3.5 µg/mL in hybridization mix. An RNAse treatment step was added to the posthybridization washings (0.2 µg/mL of RNse A (Invitrogen) at 37ºC for 30 min). The sections were incubated with sheep anti-DIG antibody conjugated to alkaline phosphatase (1:2000; Roche) overnight at 4ºC in blocking buffer solution. Staining was conducted in BM Purple (Roche) at 37ºC until the signal was clearly visible. Then, sections were dehydrated and mounted in Mowiol® 4-88 (Calbiochem). As a control, hybridization was performed following the same procedure with a DIG-labelled sense probe (Cornide-Petronio et al., 2012), or omitting the hybridization step. No staining was observed in these controls.

Whole-mounts and sections were photographed with an Olympus photomicroscope (AX-70; Provis) equipped with a colour digital camera (Olympus DP70, Tokyo, Japan). Brightness and contrast of photomicrographs

were adjusted with Adobe Photoshop CS4 and plates were composed with Corel Draw X5 software.

Results

Characterization of the sea lamprey 5-HT$_{1A}$ receptor

A sea lamprey cDNA sequence sharing homology with the 5-ht1a sequences of others animals (zebrafish, frog, mouse and dog (for GenBank IDs see Material and methods)) was amplified by reverse transcriptase PCR (RT-PCR). The cloned 5-ht1a cDNA was a 1236 bp sequence that contains the start codon (ATG) and the stop codon (TGA) of the transcript (Fig. 1A). The cloned 5-ht1a sequence corresponds to the sequence annotated in Ensembl database of the sea lamprey genome as 5-ht1a (1 of 2) (ENSPMAG00000008256) located in the scaffold GL478629.

A putative 5-HT$_{1A}$ protein sequence with 412 amino acids (Fig.1B) was deduced using the Vector NTI software and its 3D structure was hypothesized by using the SWISS-MODEL workspace in Vector NTI software (Fig. 1C). The analysis of the predicted amino acid sequence revealed the presence of seven hydrophobic domains that belong to the superfamily of G-protein coupled receptors (Fig. 1D). A short N-terminal region and three extracellular loops were located in the extracellular part of the protein, whereas a short C-terminal end and three loops were located in the intracellular part (Fig. 1C and D). Conserved sequences were found in the second intracellular loop (DRY) and in the third intracellular loop, which forms a long hydrophilic domain (LARERKTVKTLG) (Fig. 1B).

Phylogenetic analysis

Percentages of similarity between the 5-ht1a cDNA of the sea lamprey and those of different vertebrate and invertebrate groups are shown in Table 1. The sea lamprey 5-ht1a sequence shows an average percentage of similarity of 63.5% with the 5-ht1a sequences of vertebrate species. The sea lamprey sequence shows the highest levels of identity with the 5-ht1a of *C. lupus familiaris* and *D. rerio* whereas it shows the lowest degrees of homology with those of *O. mossambicus* and *R. norvegicus*. The percentage of similarity of the sea lamprey 5-ht1a with those of the invertebrates *D. melanogaster* and *A. mellifera* is lower than 50% (Table 1). The deduced 5-HT$_{1A}$ protein sequence of the sea lamprey was aligned with 5-HT$_{1A}$ sequences of representative vertebrate and invertebrate species (Fig. 2). The aligned protein sequences were used to construct a phylogenetic tree using the neighbor-joining (Fig. 3) and the minimum-evolution methods. The topology of the trees was the same using both methods with minimum differences in the bootstrap values in the node connecting gnathostome sequences. All vertebrate 5-HT$_{1A}$ sequences were clustered together and the sea lamprey sequence was found to emerge as an outgroup to all gnathostome sequences (bootstrap, BP = 100). In order to confirm that the deduced protein sequence of the sea lamprey actually belongs to the 5-HT$_{1A}$ subgroup of serotonin receptors, an additional phylogenetic tree was constructed including other serotonin receptors by using the neighbor-joining method (Fig. 4). The deduced protein sequence of the sea lamprey was positioned in the same location as in the 5-HT$_{1A}$ phylogenetic trees. Interestingly, the invertebrate 5-HT$_{1A}$ sequences appeared as an independent group to all 5-HT$_1$ receptors of vertebrates. All vertebrate serotonin receptor sequences were clustered according to the kind of receptor subgroup they belong to.

	Petromyzon marinus
Canis lupus familiaris (NP_001012397.1)	66%
Danio rerio (NP_001116793.1)	66%
Mus musculus (Q64264.1)	64%
Xenopus laevis (NP_001079299.1)	64%
Gallus gallus (NP_001163999.1)	63%
Vulpes vulpes (Q6XXY0.1)	63%
Danio rerio 5-HT1A b (NP_001139238.1)	62%
Homo sapiens (NP_000515.2)	62%
Oreochromis mossambicus (AAP83427.1)	61%
Rattus norvegicus (NP_036717.1)	61%
Drosophila melanogaster a (NP_476802.1)	49%
Drosophila melanogaster b (NP_725849.1)	49%
Apis mellifera (NP_001164579.1)	39%

Table 1 - Percentage of homology between the deduced amino acid sequence of the sea lamprey 5-HT_{1A} and the sequences of 5-HT_{1A} of different vertebrate and invertebrate species.

Early expression of the 5-ht1a in the sea lamprey

No expression of the 5-ht1a transcript was observed in the brain of hatchlings, in spite of the first serotonergic neuronal populations being present since late embryos (Villar-Cheda et al., 2006; Barreiro-Iglesias et al., 2008). *In situ* hybridization staining was observed for the first time in P1 prolarvae (Fig. 5A). The expression of the 5-ht1a transcript was observed in the presumptive nuclei of the supraoptic (Figs. 5A, 5C, D), posterior commissure (Fig. 5A, D) and postoptic commissure (Fig. 5A, E) tracts, and in the nucleus of the medial longitudinal fascicle (Fig. 5A, E). In these prolarvae, 5-ht1a expression was also

observed in the rhombencephalon (Figs. 5A, 5F-I) and in the rostral spinal cord (Figs. 5A, 5J). In the rhombencephalon, the staining was stronger in the isthmic (Fig. 5F), otic (Fig. 5H) and caudal (Fig. 5I) regions than in the trigeminal region (Fig. 5G). In peripheral regions, there was a weak expression in the first and second gills in some prolarvae (not shown). At P2 stage (Fig. 5B), a weak 5-ht1a expression was observed for the first time in the olfactory bulb/pallium primordium of the telencephalon. In these prolarvae, 5-ht1a expression increased in the diencephalic neuronal groups already observed in P1 prolarvae and was observed for the first time in the prethalamus, thalamus and caudal hypothalamus (Fig. 5B). In the mesencephalon, a weak 5-ht1a expression was observed in the tegmentum, whereas no signal was observed in the tectum. In the rhombencephalon, analysis of transverse sections showed 5-ht1a transcript in the same nuclei as in P1 prolarvae (not shown) and in more dorsal regions, mainly caudally. The peripheral expression of the transcript expanded to the posterior gills (Fig. 5B).

Expression of the 5-ht1a in the brain of larval sea lampreys

In the brain of larval lampreys, there was an almost ubiquitous expression of the 5-ht1a transcript in neuronal populations from the earlier stage studied (10 mm in body length). In sections of larval and adult brain, hybridization signal generally appeared as a granular material. Photomicrographs of representative transverse sections of the brain of large larvae are shown in Figure 6. In the olfactory bulbs (Fig. 6A), 5-ht1a expression was observed in most cells of the mitral and granular cell layers. In the lateral pallium (Fig. 6B), most cells in the periventricular layer showed numerous and small 5-ht1a positive dots (Fig. 7A), while the migrated cells showed larger and scarcer dots (Fig. 7B). The medial pallium (Fig. 6B) showed very weak expression (Fig. 7C), but the expression was more intense in the ventrally located subhippocampal lobe. In the subpallium (Fig. 6B), both the septum and striatum showed 5-ht1a expression, but the positive dots were larger in the striatal cell band. The ependymal layer of the telencephalon was mostly

devoid of 5-ht1a expression, but expression was present in cells of the septum (Fig. 7D), probably due to the presence of numerous cerebrospinal fluid-contacting (CSF-c) cells interspersed with ependymal cells (Meléndez-Ferro et al., 2002). The preoptic region presented numerous 5-ht1a positive dots in their neurons and some positive dots in cells in the ependymal layer (Fig. 6C).

In the diencephalon, both periventricular and migrated cells of the hypothalamus, prethalamus, thalamus, habenula and pretectum showed 5-ht1a expression (Fig. 6C-E), but in the migrated cells of the thalamus and prethalamus the expression was stronger than in those of the hypothalamus and pretectum, (Fig. 7E, F). The subcommissural organ did not show 5-ht1a expression (Figs. 6E, 7G). The transcript expression was considerably lower in all migrated diencephalic cells of smaller larvae, except in the habenula (Figs. 6C, D). In the pineal (Figs. 6B, 7H) and parapineal (Figs. 6B) organs, photoreceptors and ganglion cells showed 5-ht1a expression. The posterior tuberculum showed clear 5-ht1a expression (Fig. 6D). 5-ht1a expression was conspicuous in the nucleus of the medial longitudinal fascicle including the first and second Müller cells (Figs. 6F, 7I), although cells migrated laterally to it showed no or very scarce expression. Most cells in the hypothalamic ependymal layer showed 5-ht1a transcript. Some positive dots appeared to be located in dendritic clubs protruding into the third ventricle (Fig. 7J). Cells in the ependymal layer showing transcript expression were also present in other diencephalic regions.

In the mesencephalon, 5-ht1a expression was observed throughout the tegmentum (including the third Müller cell and the retinopetal neurons of the M5 nucleus of Schober), in the torus semicircularis (Fig. 6F), and in the optic tectum (Figs. 6F, 7K). The expression in the optic tectum in large larvae was different from that in larvae smaller than 100 mm in body length. In smaller larvae, the periventricular region with densely packed cells showed scarce 5-ht1a expression and the cells in the lateral regions showed no or very little expression. In the optic tectum of large larvae, the level of expression of 5-ht1a increased considerably, although it was lower than in other mesencephalic regions. The ependymal layer of the optic tectum lacked 5-ht1a expression.

In the rhombencephalon (Fig. 6G-K), there was strong 5-ht1a expression in periventricular populations of the alar plate (octavolateral nuclei (Fig. 7L), solitary tract nucleus (Fig. 7M), dorsal column nucleus and dorsal cells (Fig. 7N)) and the basal plate (reticular cells including the giant Müller and Mauthner cells (Fig. 7O) and visceromotor nuclei (Fig. 7P)). Expression of the 5-ht1a transcript was also observed in cells migrated to the lateral area, mainly in the alar plate (Fig. 7L).

Expression of 5-ht1a in the adult sea lamprey's brain

In the adult brain, the expression of 5-ht1a transcript was almost ubiquitous. With regard to larvae, the adult neurons are larger and the lateral areas of the brain increase considerably. The most important differences between the expression patterns in larvae and adults were noted in cells of the subcommissural organ, which in adults expressed 5-ht1a (Fig. 8A). In the mature optic tectum, the expression of the 5-ht1a transcript in tectal cells was weaker than in other brain regions (Fig. 8B). The adult medial pallium, which its differentiation is completed during the metamorphosis, showed clearly weaker 5-ht1a expression than other telencephalic regions (Fig. 8C).

Expression of the 5-ht1a transcript in the spinal cord

In the spinal cord of both larval and adult lampreys, most neuronal types (sensory cells, interneurons and motoneurons) appeared to express 5-ht1a (Fig. 8D-I). Two types of intraspinal sensory cells have been described in lampreys: dorsal cells and edge cells. Dorsal cells, located near the central canal (Fig. 8F), showed abundant 5-ht1a expression around the cell nucleus. Instead, edge cells (intraspinal proprioreceptor neurons) showed scant 5-ht1a reaction (Fig. 8H). A number of interneurons were located in the horizontally extended grey matter that corresponds to both the dorsal and ventral horns found in other vertebrates. 5-ht1a expression was observed in fine grains along

the region containing these neurons (Fig. 8D-E). With regard to motoneurons, they showed a number of 5-ht1a positive granules distributed through most of their large perikarya (Fig. 8G). In addition, 5-ht1a expression was observed in cells in the ependymal layer (Fig. 8I). In this layer, two types of small cells are present, CSF-c neurons and ependymal cells. However the location of 5-ht1a positive granules in one or in the two kinds of cells could not be assessed. Representative examples of 5-ht1a expression in spinal cells of larval and adult spinal cord are shown in Figures 8D-F and 8G-I, respectively.

Expression of the 5-ht1a transcript in the choroid plexus and brain vessels

Expression of the 5-ht1a transcript was also observed in the choroid plexus and brain vessels. The choroid plexus in lampreys is extensive, highly vascularised and covers dorsally the mesencephalic and rhombencephalic ventricles. In the choroid plexus, expression of 5-ht1a transcript was observed in the choroidal epithelial cells (Fig. 9A).

There are no blood vessels in the brain and spinal cord of larval lampreys nor in the spinal cord of adults. Expression of the 5-ht1a transcript was observed in endothelial cells of vessels in the adult sea lamprey brain (Fig. 9B).

Expression of the 5-ht1a in the retina

The retinas of late prolarvae, larvae and adults were investigated. In late prolarvae most of the retina is differentiated having only one type of photoreceptor, bipolar ganglion and pigment epithelium cells (Fig. 10A). Lateral to the differentiated part, there is a small lateral undifferentiated portion. The larval retina also presents these two regions (Fig. 10B, C), the differentiated central part (Fig. 10C) and the undifferentiated lateral part (Fig. 10B), which considerable increases with larval age. Most cells in the lateral retina are neuroblasts, although since the middle of the larval period, in the innermost part of the lateral retina there ganglion cells also differentiate. The

pigment epithelium cells of the differentiated retina showed melanin granules (Fig. 10B, C) that are not observed in the pigment epithelium of the lateral part (Fig. 10B). The entire adult retina is differentiated and presents two types of receptor cells, the long and short photoreceptors, as well as horizontal, bipolar, amacrine ganglion and pigment epithelium cells with melanin granules (Fig. 10D).

Expression of the 5-ht1a was observed in prolarval, larval and adult retinas. In the prolarval neural retina, all types of differentiated cells expressed the 5-ht1a transcript, whereas no expression was observed in the undifferentiated retina (Fig. 10A). The pattern of 5-ht1a expression was also different in the central and lateral retina of larvae. Photoreceptor, bipolar and ganglion cells in the central retina (Fig. 10C) and neuroblasts in the lateral retina (Fig. 10B) showed 5-ht1a expression. In the adult retina, photoreceptors showed the highest level of expression (Fig. 10D). The 5-ht1a transcript was mostly concentrated in the myoid of both short and long photorreceptors (Fig. 10E). Bipolar, putative amacrine and ganglion cells also showed high expression of 5-ht1a (Fig. 10D, F); whereas, the expression was very low in horizontal cells. Due to the great accumulation of melanin granules in the pigment epithelium of the central retina in larvae and adults, if its cells express or not the 5-ht1a could not be determined. However, it may be possible since the pigment epithelium cells in the lateral retina showed 5-ht1a expression (Fig. 10B).

Discussion

Relationships of the lamprey 5-ht1a gene and 5-HT$_{1A}$ sequence

The present study reports for the first time the isolation and characterization of the sea lamprey 5-ht1a cDNA and the distribution of the 5-ht1a transcript in the nervous system. The complete sequence of the 5-ht1a

cDNA from the start to stop codons was obtained and a 3D model of the translated 5-HT_{1A} protein sequence was hypothesized. The hypothesized 3D model shows that the protein belongs to the superfamily of G protein-coupled receptors in accordance with the structure of 5-HTR subtypes (Westkaemper and Roth, 2006). Moreover, the intracellular loops have characteristic sequences of G-protein coupled receptors. The second intracellular loop contained the DRY sequence that regulates ligand binding, G protein coupling and receptor stability (Arora et al., 1995; Alewijnse et al., 2000); and the third intracellular loop contains the G protein contact site and a PKC phosphorylation site of 5-HT_{1A}: LARERKTVKTLG (Lembo and Albert, 1995). The majority of G_i/G_o-coupled receptors have a threonine in this sequence preceded by a nonpolar/uncharged amino acid, but, in contrast with them, the 5-HT_{1A} has a valine preceded by a threonine (Malmberg and Strange, 2000). This molecular characteristic additionally supports that the sea lamprey cDNA obtained actually codes for a 5-HT_{1A}. In addition, the sequence of the G protein contact site of the G_i/G_o-coupled 5-HT1As seems to be highly conserved, already present before the split between agnates and gnathostomes. The first annotation of the sea lamprey genome in the Ensembl database located the sequence of LARERKTVKTLG in an intron. However, our data based on the 5-ht1a mRNA sequence indicate that this sequence is part of an exon, which is in agreement with the importance of the sequence in the G_i/G_o function. The present results also indicate that the lamprey gene consists of a single exon, without introns. 5-HT_1 receptor subclass genes have a single exon in humans (Stam et al., 1992), dog (van den Berg et al., 2005) and rat (Albert et al., 1990). This suggests that the 5-ht1a genomic organization is highly conserved in vertebrates. On the other hand, the amino end of the sea lamprey 5-HT_{1A} is very short in comparison with that of other animals, which supports the observation that the N-terminal segment of GPCRs is highly variable in length (Otaki and Firestein, 2001) and that in the serotonin receptors there is little conservation of the N-terminal sequence (Westkaemper and Roth, 2006). Moreover, several single-nucleotide polymorphisms (SNPs) in humans have been identified in the amino end of the 5-HT_{1A} without affecting the receptor function (Del Tredici et al., 2004).

In our analysis, we found a single 5-ht1a gene in the sea lamprey, as in mammals, birds and amphibians, but different to that reported in *D. rerio*. The presence of two genes in teleosts can be explained by the additional whole genome duplication that occurred in the actinopterygian lineage (Taylor et al., 2001). The 5-HT1A amino acidic sequence of the sea lamprey was located in the base of the phylogenetic tree of vertebrate 5-HT$_{1A}$, appearing as a sister member of the 5-HT$_{1A}$ receptor trunk of gnathostomes. In the phylogenetic tree of the 5-HTR sequences, each kind of receptor grouped in an independent cluster and the 5-HT$_{1A}$ branch maintained the structure of the previous phylogenetic tree. The location of the lamprey 5-HT$_{1A}$ sequence at the base of the vertebrate phylogenetic tree and its average of similarity (in agreement with the phylogenetic position of lamprey), suggest that the emergence of the 5-HT$_{1A}$ receptor occurred before the separation of agnathans and gnathostomes.

Comparative analysis of the 5-ht1a expression in the central nervous system

In the present study, the temporal sequence and spatial distribution of the 5-ht1a transcript during the development of the central nervous system is described for first time in a lamprey species. A high proportion of the larval and adult neurons express this 5-ht1a mRNA, mainly in perikarya. In many neurons, positive signal has a granular appearance, which is probably due to low expression of this mRNA in lamprey cells. Other *in situ* hybridization studies have found similar granular appearances of neurons expressing tyrosine hydroxylase (Barreiro-Iglesias et al., 2010), UNC5L2/3a (Barreiro-Iglesias et al., 2012) and Y1 receptor (Pérez-Fernández et al., 2012). The 5-ht1a-positive regions include those exhibiting serotonergic neurons (hypothalamus, pineal organ, isthmus, caudal rhombencephalon, spinal cord). This suggests that in different lamprey populations, 5-HT$_{1A}$ receptor might act as auto- and heteroreceptors, as proposed in zebrafish (Norton et al., 2008). In addition to the perikarya, our results showed positive hybridization dots in some dendrites coursing to the hypothalamic lateral area and to the ventricular surface (CSF-c neurons),

suggesting that local translation of the receptor mRNA occurs in these hypothalamic dendrites. Dendrites in animals phylogenetically distant as mammals (reviewed in Kindler and Kreienkamp, 2012) and *Aplysia* (Villareal et al., 2007), are able to locally translate proteins, including neurotransmitter receptors, but if the $5HT_{1A}$ is synthesized in dendrites has not been investigated yet. Two main functions have been proposed for CSF-c neurons, to sense the composition of the cerebrospinal fluid and/or to release substances into the ventricular system (reviewed in Vígh et al., 2004). Our results of 5-ht1a expression in CSF-c dendrites suggest that the $5\text{-}HT_{1A}$ receptor is involved in the detection of serotonin in the CSF. Alternatively, these CSF-c dendrites may receive inputs from other serotonergic neurons.

5-ht1a expression in glial cells

The ependymal layer showed 5-ht1a expression in regions lacking CSF-c neurons, i.e. in ependymal regions consisting only of tanycytes. Accordingly, our results showed that some lamprey glial cells express 5-ht1a, which is consistent with results in mammals. In the rat and musk shrew, 5-ht1a expression has been reported in astrocytes of the corpus callosum and brainstem white matter, in dentate gyrus radial glial cells and in ependymal cells of ventricular regions showing supraependymal serotonergic fibers (Dinopoulos and Dori, 1995; Hirunagi et al., 1995). $5\text{-}HT_{1A}$ immunoreactivity has been reported in adult brain tanycytes of *Macaca fascicularis* (Azmitia et al., 1996) and transient $5\text{-}HT_{1A}$ expression in glial cells of the developing rat hippocampus (Patel and Zhou, 2005). Rat ependymal cells respond to the released serotonin by increasing ciliary beat frequency (Nguyen et al., 2001) and mobilizing intracellular glycogen stores (Prothmann et al., 2001). In summary, expression of the $5\text{-}HT_{1A}$ receptor in glial cells appears to be shared by mammals and lampreys. Studies of 5-ht1a expression in other key groups (elasmobranches, teleosts, amphibians, reptiles and birds) are needed to know if this is a primitive character of vertebrates or if it is a phenomenon of convergent evolution.

Development of 5-ht1a/5-HT1A expressions in the brain

In the developing sea lamprey, the 5-ht1a transcript was detected for the first time after hatching and continued to be present throughout its development. We found no evidence of neuronal populations transiently expressing 5-ht1a in the sea lamprey's brain, which is in contrast with the transient expression of 5-ht1a mRNA reported in the septum, the medial and lateral preoptic area, the ventral lateral geniculate nucleus and the medial habenula of mice (Bonnin et al., 2006), and in the cerebellum of zebrafish (Norton et al., 2008), rat (Miquel et al., 1994) and human (del Olmo et al., 1998) (note that lampreys lack a true cerebellum; Pombal et al., 1996). The transient 5-ht1a expression has been interpreted as indicative of a developmental role for serotonin (Bonnin et al., 2006). Moreover, a role of the 5-HT_{1A} receptor in the refinement of the visual map of the frog has also been proposed (Butt et al., 2002). Whether this applies to the lamprey optic tectum is unclear, because expression of the 5-ht1a transcript appears long before the metamorphosis, when the tectum completes its differentiation (de Miguel and Anadón, 1987). Similarly, the 5-ht1a transcript is early expressed in the medial pallium of the larval sea lamprey, another highly immature region that completes its differentiation during the metamorphosis (Villar-Cheda et al., 2006).

The onset of 5-ht1a mRNA expression during development has been studied in the mouse prosencephalon, where the septum, striatum, preoptic area, hippocampus, dorsal thalamus and paraventricular hypothalamic nucleus express 5-ht1a mRNA at E14.5 (Bonnin et al., 2006). However, early expression of the 5-ht1a receptor is not observed in the lamprey telencephalon. Broad expression of 5-ht1a was found in the larval brain of both sea lamprey (present results) and zebrafish (taken the expression of 5-ht1aa and 5-ht1ab receptors together; Norton et al., 2008). Regions with conserved 5-ht1a expression between lampreys and zebrafish are the preoptic area, pretectum, thalamus, caudal hypothalamus, optic tectum and the rhombencephalon including the reticular formation. Main differences were the 5-ht1a expression in the sea lamprey telencephalon and rostral hypothalamus, but not in those of the zebrafish, and the comparatively stronger expression in

the optic tectum of larval zebrafish. To fully understand the significance of these differences between species, studies into other vertebrate groups are required.

5-ht1a expression in adult brains

In the adult brain, 5-ht1a expression is maintained in similar areas to those identified in larvae, both in lamprey (present results) and zebrafish (Norton et al., 2008). The patterns of 5-ht1a expression in adult brains were also reported in some mammals (Pompeiano et al., 1992). Regions with well-conserved expression of 5-ht1a among vertebrate species include the basal telencephalon, the hypothalamus and the rhombencephalic reticular formation. In contrast to adult sea lamprey and mammals, there is no expression of the 5-$HT1_{aa/b}$ receptor in the dorsal telencephalon (pallium) in adult zebrafish (Norton et al., 2008).

With regard to the 5-ht1a expression observed in the adult subcommissural organ (SCO), we previously showed that serotonergic fibers innervated it in larval lamprey (Barreiro-Iglesias et al., 2009). As far as we are aware, expression of the 5-ht1a receptor has not been reported in the SCO of other vertebrates, although 5-HT_{2A} receptor expression has been reported in the bovine SCO (Richter et al., 2004). Moreover, these authors have found that serotonin down-regulates the biosynthesis of spondin (a component of the Reissner fiber) by the SCO but apparently not its release. More studies are necessary to determine the role of serotonin receptors in the SCO and the relation with the Reissner fiber dynamic in lampreys and other vertebrates.

Functional significance of 5-ht1a distribution in the lamprey's brain

The analyses of the lamprey brain and spinal cord populations expressing the 5-ht1a mRNA and their comparison with the populations immunoreactive to serotonin (Antri et al., 2006; Abalo et al., 2007) indicates that

both serotonergic and non-serotonergic populations express the receptor, and that the receptor may be expressed in all serotonergic populations. The wide innervation of sea lamprey brain structures by serotonin-ir fibers (Antri et al., 2006; Abalo et al., 2007) would allow interaction of these fibers with numerous neurons. Moreover, ultrastructural immunocytochemical studies in the lamprey spinal cord have shown that serotonergic varicosities have no synaptic specializations, suggesting that released serotonin may act on neighbour and distant 5-HTRs (Christenson et al., 1990). Large reticulospinal neurons are among the brain populations expressing 5-ht1a. Numerous varicose serotonin-ir fibers are found close to their perikaryon and dendrites (Di Prisco et al., 1994), and activity of these cells elicited by sensory stimulation is subject of modulation by serotonin (Antri et al., 2008). Modulation of synapses of giant reticulospinal axons on spinal motoneurons by serotonin was also demonstrated (Buchanan and Grillner, 1991), which is consistent with the expression of this receptor. With regard the lamprey spinal cord, the interneurons and motoneurons involved in the spinal circuit for locomotion are modulated by local application of serotonin (Wallén et al., 1989). Moreover, pharmacological studies showed that actions of serotonin on the lamprey spinal locomotor network are mainly mediated by 5-HT$_{1A}$-like receptors (Wikström et al., 1995), which is consistent with the wide expression of 5-ht1a in sea lamprey spinal neurons. In the river lamprey, serotonin has been shown to be inhibitory for synapses of dorsal cell sensory axons on giant interneurons, and the application of an antagonist of 5-HT$_{1A}$ receptors does not counteracts this effect (El Manira et al., 1997). Sea lamprey dorsal cells express 5-ht1a mRNA. Therefore, further investigation is needed into whether these different results were due to differences in 5-ht1a expression between river and sea lampreys, or to the expression of additional serotonin receptors by dorsal cells.

In mammals, the 5-HT$_{1A}$ receptor is a major determinant of the activity of serotonergic cells and of serotonin release because of its location both pre- and post-synaptically (Chalmers and Watson, 1991). In the brainstem of mammals, where it is expressed on cells in the dorsal and medial raphe nuclei, the 5-HT$_{1A}$ receptor acts as a somatodendritic autoreceptor to inhibit cell firing

(Sprouse and Aghajanian, 1987). The postsynaptic 5-HT_{1A} (heteroreceptors) are expressed at high density in limbic areas and cortex in mammals (Nichols and Nichols, 2008) and as also mediate decreases of neuronal firing in the prefrontal cortex and the hippocampus (Sprouse and Aghajanian, 1988). Accordingly, the different pre- and post-synaptic functions of 5-HT_{1A} receptor appear to be an old characteristic of vertebrates.

5-ht1a expression in the choroid plexus and brain vessels

Here we report that the choroid plexus and vessels of the sea lamprey brain express the 5-ht1a transcript. As far as we are aware this is the first report showing expression of this serotonergic receptor in the choroid plexus. The ultrastructure of the choroid plexus of lampreys has been shown to be very similar to that of mammals (Bundgaard and van Deurs, 1982). However, in the mammalian plexus the main serotonin receptor appears to be the 5-HT_2 receptor (formerly named 5-HT_{1C}) (Pazos et al., 1984), which regulates the formation of the CSF (Kaufman et al., 1995). Cerebrovascular nerves that contain serotonin, which is a potent vasoconstrictor, innervate the major cerebral arteries of mammals (reviewed in Lincoln, 1995). Innervation of cerebral arteries by serotonin appears to be conserved in vertebrate evolution since it has been also reported in the lamprey *Entosphenus japonicus* (Iijima and Wasano, 1980). The vasoconstriction in cerebral arteries is mediated via the 5-HT_{1B} receptor (Nilsson et al., 1999); although the 5-HT_7 and the 5-HT_{1F} seem to be also implicated. If these serotonin receptors are expressed in the choroid plexus or brain arteries of lampreys needs to be investigated. On the other hand, the results in lampreys indicate that the 5-HT_{1A} could also be expressed in the plexus and cerebral arteries of mammals.

5-ht1a expression in the retina

Our results showed that the 5-ht1a transcript is expressed in the neural retina and in the pigment epithelium of the sea lamprey. Expression of the 5-ht1a transcript has been also reported in *Xenopus* (Marracci et al., 1997), rabbit and human (Sharif and Senchyna, 2006) retinas. Pharmacological research also indicates the presence of the 5-HT$_{1A}$ in the retina of goldfish (Lima et al., 1994; Urbina et al., 1996). New research has to be done in representatives of all vertebrate groups before an evolutionary picture can emerge. The expression of 5-ht1a transcript in pigment epithelium cells is a characteristic shared by lampreys (present results) and humans (Nash and Osborne, 1997), but new research is needed in other species to get a complete picture trough the vertebrate linage to understand the evolution of the retinal serotonergic system.

The patterns of 5-ht1a expression in adult and larval retina of the sea lamprey revealed striking differences that could be related to the developing state and suggests that it could be also playing a developmental role apart from its role in auto- and hetero- neurotransmitter reception. Three types of cells (amacrine, photoreceptors and neuroblasts) that expressed the 5-ht1a transcript also expressed *Tph* mRNA indicating that they synthesise serotonin (Chapter 1, present thesis). So, 5-HT$_{1A}$ may act as autoreceptor in these cells. The expression of the 5-ht1a transcript in neuroblasts suggests that possible developmental effects of serotonin in the lamprey retina could be mediated by 5-HT$_{1A}$ receptors. In explants of the goldfish retina 5-HT$_{1A}$ receptor agonists inhibited the outgrowth of neurites (Lima et al., 1994; Urbina et al., 1996), but if the same occurs *in vivo* in the lamprey retina needs to be investigated. As in other vertebrates, serotonin in photoreceptors of lampreys could have a metabolic function as an intermediary for melatonin synthesis, but the presence of 5-HT$_{1A}$ receptor could indicate that they could also detect serotonin, independent of its origin. In the retina of rats, this appears to play important roles in photoreceptor survival due to the neuroprotective effect of the 5HT$_{1A}$ receptor against oxidative damage (Collier et al., 2011). Retinal degeneration due to both aging and oxidative stress induces photoreceptor

and retinal epithelium degeneration (Marc et al., 2008; Collier et al., 2011; Thampi et al., 2012). So, a protective role of the 5-HT_{1A} in the retinal epithelium cells of lampreys, at least during development, is also suggested, indicating that it would be a primitive character of the vertebrate retina.

Other types of cells that expressed the 5-ht1a transcript are bipolar and ganglion cells. Serotonin amacrine cells are located in the inner part of the inner nuclear layer and, occasionally, close to the inner limiting membrane and their processes ramify on both sides of the inner plexiform layer (Villar-Cerviño et al., 2006). These processes can establish synaptic contacts to bipolar and ganglion cells, therefore, in these cells the 5-HT_{1A} probably acts as heteroreceptor. In addition, horizontal cells appear to have a low level of expression of the 5-ht1a transcript. So, they could be detecting diffuse serotonin by volume transmission.

In conclusion, the present study reports for the first time the isolation and the distribution of the sea lamprey 5-ht1a cDNA, with a G_i/G_o-coupling region highly conserved in vertebrates. Most of the larval and adult serotonergic and other populations express this receptor mRNA, suggesting that the different pre- and post-synaptic functions of 5-HT_{1A} are an old characteristic of vertebrates. In addition, the expression pattern of the 5-ht1a in the developing retina strongly suggest that serotonin is involved in the development of the retina in lampreys.

Literature cited

Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R, Rodicio MC. (2007) Development of the serotonergic system in the central nervous system of the sea lamprey. J Chem Neuroanat. 34: 29-46.

Acklin SE, Nicholls JG. (1990) Intrinsic and extrinsic factors influencing properties and growth patterns of identified leech neurons in culture. J Neurosci. 10: 1082-1090.

Albert PR, Zhou QY, Van Tol HH, Bunzow JR, Civelli O. (1990) Cloning, functional expression, and mRNA tissue distribution of the rat 5-hydroxytryptamine1A receptor gene. J Biol Chem. 265: 5825-5832.

Alewijnse AE, Timmerman H, Jacobs EH, Smit MJ, Roovers E, Cotecchia S, Leurs R. (2000) The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H(2) receptor. Mol Pharmacol. 57: 890-898.

Antri M, Cyr A, Auclair F, Dubuc R. (2006) Ontogeny of 5-HT neurons in the brainstem of the lamprey, *Petromyzon marinus*. J Comp Neurol. 495: 788-800.

Antri M, Auclair F, Albrecht J, Djeudjang N, Dubuc R. (2008) Serorotoninergic modulation of sensory transmission to brainstem reticulospinal cells. Eur J Neurosci. 28: 655-667.

Arora KK, Sakai A, Catt KJ. (1995) Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor. J Biol Chem. 270: 22820-22826.

Azmitia EC, Gannon PJ, Kheck NM, Whitaker-Azmitia PM. (1996) Cellular localization of the 5-HT1A receptor in primate brain neurons and glial cells. Neuropsychopharmacology. 14: 35-46.

Barreiro-Iglesias A, Villar-Cheda B, Abalo XM, Anadón R, Rodicio MC. (2008) The early scaffold of axon tracts in the brain of a primitive vertebrate, the sea lamprey. Brain Res Bull. 75: 42-52.

Barreiro-Iglesias A, Villar-Cerviño V, Anadón R, Rodicio MC. (2009) A monoclonal antibody as a tool to study the subcommissural organ and

Reissner's fibre of the sea lamprey: an immunofluorescence study before and after a spinal cord transection. Neurosci Lett. 464: 34-38.

Barreiro-Iglesias A, Laramore C, Shifman MI, Anadón R, Selzer ME, Rodicio MC. (2010) The sea lamprey tyrosine hydroxylase: cDNA cloning and in situ hybridization study in the brain. Neuroscience. 168: 659-669.

Barreiro-Iglesias A, Laramore C, Shifman MI. (2012) The sea lamprey UNC5 receptors: cDNA cloning, phylogenetic analysis and expression in reticulospinal neurons at larval and adult stages of development. J Comp Neurol. 520:4141-4156.

Blackmer T, Larsen EC, Takahashi M, Martin TF, Alford S, Hamm HE. (2001) G protein betagamma subunit-mediated presynaptic inhibition: regulation of exocytotic fusion downstream of Ca2+ entry. Science. 292: 293-297.

Bonnin A, Peng W, Hewlett W, Levitt P. (2006) Expression mapping of 5-HT1 serotonin receptor subtypes during fetal and early postnatal mouse forebrain development. Neuroscience. 141: 781-794.

Bordoli L, Kiefer F, Arnold K, Benkert P, Battey J, Schwede T. (2009) Protein structure homology modeling using SWISS-MODEL workspace. Nat Protoc. 4: 1-13.

Buchanan JT, Grillner S. (1991) 5-Hydroxytryptamine depresses reticulospinal excitatory postsynaptic potentials in motoneurons of the lamprey. Neurosci Lett. 122: 71-44.

Bundgaard M, van Deurs B. (1982) Brain barrier systems in the lamprey. II. Ultrastructure and permeability of the choroid plexus. Brain Res. 240:65-75.

Burnet PW, Eastwood SL, Lacey K, Harrison PJ. (1995) The distribution of 5-HT1A and 5-HT2A receptor mRNA in human brain. Brain Res. 676: 157-168.

Butt CM, Zhao B, Duncan MJ, Debski EA. (2002) Sculpting the visual map: the distribution and function of serotonin-1A and serotonin-1B receptors in the optic tectum of the frog. Brain Res. 931: 21-31.

Chalmers DT, Watson SJ. (1991) Comparative anatomical distribution of 5-HT1A receptor mRNA and 5-HT1A binding in rat brain - a combined in situ hybridisation/in vitro receptor autoradiographic study. Brain Res. 561: 51-60.

Christenson J, Franck J, Grillner S. (1989) Increase in endogenous 5-hydroxytryptamine levels modulates the central network underlying locomotion in the lamprey spinal cord. Neurosci Lett. 100: 188-192.

Christenson J, Cullheim S, Grillner S, Hökfelt T. (1990) 5-hydroxytryptamine immunoreactive varicosities in the lamprey spinal cord have no synaptic specializations - an ultrastructural study. Brain Res. 512: 201-209.

Collier RJ, Patel Y, Martin EA, Dembinska O, Hellberg M, Krueger DS, Kapin MA, Romano C. (2011) Agonists at the serotonin receptor (5-HT(1A)) protect the retina from severe photo-oxidative stress. Invest Ophthalmol Vis Sci. 52: 2118-2126.

Cornide-Petronio ME, Anadón R, Rodicio MC, Barreiro-Iglesias A. (2012) The sea lamprey tryptophan hydroxylase: new insight into the evolution of the serotonergic system of vertebrates. Brain Struct Funct. 218: 587-593.

Couch JA, Chen J, Rieff HI, Uri EM, Condron BG. (2004) robo2 and robo3 interact with eagle to regulate serotonergic neuron differentiation. Development. 131: 997-1006.

Daubert EA, Condron BG. (2010) Serotonin: a regulator of neuronal morphology and circuitry. Trends Neurosci. 33: 424-434.

de Miguel E, Anadón R. (1987) The development of retina and the optic tectum of *Petromyzon marinus*, L. A light microscopic study. J Hirnforsch. 28: 445-456.

del Olmo E, López-Giménez JF, Vilaró MT, Mengod G, Palacios JM, Pazos A. (1998) Early localization of mRNA coding for 5-HT1A receptors in human brain during development. Brain Res Mol Brain Res. 60: 123-126.

Del Tredici AL, Schiffer HH, Burstein ES, Lameh J, Mohell N, Hacksell U, Brann MR, Weiner DM. (2004) Pharmacology of polymorphic variants of the human 5-HT1A receptor. Biochem Pharmacol. 67: 479-490.

Di Prisco GV, Dubuc R, Grillner S. (1994) 5-HT innervation of reticulospinal neurons and other brainstem structures in lamprey. J Comp Neurol. 342: 23-34.

Dinopoulos A, Dori I. (1995) The development of the serotonergic fiber network of the lateral ventricles of the rat brain: a light and electron microscopic immunocytochemical analysis. Exp Neurol. 133: 73-84.

El Manira A, Zhang W, Svensson E, Bussières N. (1997) 5-HT inhibits calcium current and synaptic transmission from sensory neurons in lamprey. J Neurosci. 17: 1786-1794.

Grillner S, Deliagina T, Ekeberg O, el Manira A, Hill RH, Lansner A, Orlovsky GN, Wallén P. (1995) Neural networks that co-ordinate locomotion and body orientation in lamprey. Trends Neurosci. 18: 270-279.

Hardisty MW, Potter IC. (1971) The general biology of adult lampreys. In: Hardisty MW, Potter IC (eds) The biology of lampreys, vol. 1. Academic Press, London, pp 127-206.

Hill RH, Svensson E, Dewael Y, Grillner S. (2003) 5-HT inhibits N-type but not L-type Ca(2+) channels via 5-HT1A receptors in lamprey spinal neurons. Eur J Neurosci. 18: 2919-2924.

Hirunagi K, Ishikawa A, Namikawa T, Uryu K. (1995) Immunocytochemical identification of serotonergic supraependymal nerve fibers in the third ventricle of the house musk shrew, *Suncus murinus*. Ann Anat. 177: 297-304.

Iijima T, Wasano T. (1980) A histochemical and ultrastructural study of serotonin-containing nerves in cerebral blood vessels of the lamprey. Anat Rec. 198:671-80.

Kaufman MJ, Hartig PR, Hoffman BJ. (1995) Serotonin 5-HT2C receptor stimulates cyclic GMP formation in choroid plexus. J Neurochem. 64:199-205.

Kindler S, Kreienkamp HJ. (2012) Dendritic mRNA targeting and translation. Adv Exp Med Biol. 970: 285-305.

Kroeze WK, Roth BL. (2006) Molecular biology and genomic organization of G protein-coupled serotonin receptors. In: Roth BL (ed) The receptors: The serotonin receptors: From molecular pharmacology to human therapeutics. Totowa, New Jersey, pp 1-38.

Lembo PM, Albert PR. (1995) Multiple phosphorylation sites are required for pathway-selective uncoupling of the 5-hydroxytryptamine1A receptor by protein kinase C. Mol Pharmacol. 48: 1024-1029.

Lima L, Matus P, Urbina M. (1994) Serotonin inhibits outgrowth of goldfish retina and impairs the trophic effect of taurine. J Neurosci Res. 38: 444-450.

Lincoln J. (1995) Innervation of cerebral arteries by nerves containing 5-hydroxytryptamine and noradrenaline. Pharmacol Ther. 68:473-501.

Malmberg A, Strange PG. (2000) Site-directed mutations in the third intracellular loop of the serotonin 5-HT(1A) receptor alter G protein coupling from G(i) to G(s) in a ligand-dependent manner. J Neurochem. 75: 1283-1293.

Marc RE, Jones BW, Watt CB, Vazquez-Chona F, Vaughan DK, Organisciak DT. (2008) Extreme retinal remodeling triggered by light damage: implications for age related macular degeneration. Mol Vis. 14: 782-806.

Marracci S, Cini D, Nardi I. (1997) Cloning and developmental expression of 5-HT1A receptor gene in *Xenopus laevis*. Brain Res Mol Brain Res. 47: 67-77.

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B, Abalo XM, Rodríguez-Muñoz R, Rodicio MC, Anadón R. (2002) Ontogeny of gamma-aminobutyric acid-immunoreactive neuronal populations in the forebrain and midbrain of the sea lamprey. J Comp Neurol. 446: 360-376.

Miquel MC, Kia HK, Boni C, Doucet E, Daval G, Matthiessen L, Hamon M, Vergé D. (1994) Postnatal development and localization of 5-HT1A receptor mRNA in rat forebrain and cerebellum. Brain Res Dev Brain Res. 80: 149-157.

Nash MS, Osborne NN. (1997) Pharmacologic evidence for 5-HT1A receptors associated with human retinal pigment epithelial cells in culture. Invest. Ophthalmol. Vis. Sci. 38: 510-519.

Nguyen T, Chin WC, O'Brien JA, Verdugo P, Berger AJ. (2001) Intracellular pathways regulating ciliary beating of rat brain ependymal cells. J Physiol. 531: 131-140.

Nichols DE, Nichols CD. (2008) Serotonin receptors. Chem Rev. 108: 1614-1641.

Nilsson T, Longmore J, Shaw D, Olesen IJ, Edvinsson L.. (1999) Contractile 5-HT1B receptors in human cerebral arteries: pharmacological characterization and localization with immunocytochemistry. Br J Pharmacol. 128:1133-1140.

Norton WH, Folchert A, Bally-Cuif L. (2008) Comparative analysis of serotonin receptor (HTR1A/HTR1B families) and transporter (slc6a4a/b) gene expression in the zebrafish brain. J Comp Neurol. 511: 521-542.

Osório J, Rétaux S. (2008) The lamprey in evolutionary studies. Dev Genes Evol. 218: 221-235.

Otaki JM, Firestein S. (2001) Length analyses of mammalian G-protein-coupled receptors. J Theor Biol. 211: 77-100.

Patel TD, Zhou FC. (2005) Ontogeny of 5-HT1A receptor expression in the developing hippocampus. Brain Res Dev Brain Res. 157: 42-57.

Pazos A, Hoyer D, Palacios JM. (1984) The binding of serotonergic ligands to the porcine choroid plexus: characterisation of a new type of serotonin recognition site. Eur J Pharmacol. 106: 539-546.

Pérez-Fernández J, Megías M, Pombal MA. (2012) Distribution of a Y1 receptor mRNA in the brain of two lamprey species, the sea lamprey (*Petromyzon marinus*) and the river lamprey (*Lampetra fluviatilis*). J Comp Neurol. 521: 426-47.

Peroutka SJ, Howell TA. (1994) The molecular evolution of G protein-coupled receptors: focus on 5-hydroxytryptamine receptors. Neuropharmacology. 33: 319-324.

Piavis GW. (1971) Embryology. In: Hardisty MW, Potter IC (eds) The Biology of Lampreys, vol. 1. Academic Press, London, pp 361-400.

Pombal MA, Rodicio MC, Anadón R. (1996) Secondary vestibulo-oculomotor projections in larval sea lamprey: anterior octavomotor nucleus. J Comp Neurol. 372: 568-580.

Pompeiano M, Palacios JM, Mengod G. (1992) Distribution and cellular localization of mRNA coding for 5-HT1A receptor in the rat brain: correlation with receptor binding. J Neurosci. 12: 440-453.

Popova NK. (2006) From genes to aggressive behavior: the role of serotonergic system. Bioessays. 28: 495-503.

Prothmann C, Wellard J, Berger J, Hamprecht B, Verleysdonk. (2001) Primary cultures as a model for studying ependymal functions: glycogen metabolism in ependymal cells. Brain Res. 920: 74-83.

Richter HG, Tomé MM, Yulis CR, Vío KJ, Jiménez AJ, Pérez-Fígares JM, Rodríguez EM. (2004) Transcription of SCO-spondin in the subcommissural organ: evidence for down-regulation mediated by serotonin. Brain Res Mol Brain Res. 129: 151-162.

Rubenstein JL. (1998) Development of serotonergic neurons and their projections. Biol Psychiatry. 44: 145-150.

Saudou F, Boschert U, Amlaiky N, Plassat JL, Hen R. (1992) A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns. EMBO J. 11: 7-17.

Shifman MI, Jin L-Q, Selzer ME. (2007) Regeneration in the lamprey spinal cord. In: Becker CG, Becker T (eds) Model organisms in spinal cord regeneration. Wiley-VCH Verlag, Weinheim, pp 229-262.

Sprouse JS, Aghajanian GK. (1987) Electrophysiological responses of serotoninergic dorsal raphe neurons to 5-HT1A and 5-HT1B agonists. Synapse. 1: 3-9.

Sprouse JS, Aghajanian GK. (1988) Responses of hippocampal pyramidal cells to putative serotonin 5-HT1A and 5-HT1B agonists: a comparative study with dorsal raphe neurons. Neuropharmacology. 27: 707-715.

Stam NJ, Van Huizen F, Van Alebeek C, Brands J, Dijkema R, Tonnaer JA, Olijve W. (1992) Genomic organization, coding sequence and functional expression of human 5-HT2 and 5-HT1A receptor genes. Eur J Pharmacol. 227: 153-162.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 28: 2731-2739.

Taylor JS, Van de Peer Y, Braasch I, Meyer A. (2001) Comparative genomics provides evidence for an ancient genome duplication event in fish. Philos Trans R Soc Lond B Biol Sci. 356: 1661-1679.

Thamm M, Balfanz S, Scheiner R, Baumann A, Blenau W. (2010) Characterization of the 5-HT1A receptor of the honeybee (*Apis*

mellifera) and involvement of serotonin in phototactic behavior. Cell Mol Life Sci. 67: 2467-2479.

Thampi P, Rao HV, Mitter SK, Cai J, Mao H, Li H, Seo S, Qi X, Lewin AS, Romano C, Boulton ME. (2012) The 5HT1a receptor agonist 8-Oh DPAT induces protection from lipofuscin accumulation and oxidative stress in the retinal pigment epithelium. PLoS One. 7: e34468.

Urbina M, Schmeer C, Lima L. (1996) 5HT1A receptor agonist differentially increases cyclic AMP concentration in intact and lesioned goldfish retina. In vitro inhibition of outgrowth by forskolin. Neurochem Int. 29: 453-460.

van den Berg L, Kwant L, Hestand MS, van Oost BA, Leegwater PA. (2005) Structure and variation of three canine genes involved in serotonin binding and transport: the serotonin receptor 1A gene (htr1A), serotonin receptor 2A gene (htr2A), and serotonin transporter gene (slc6A4). J Hered. 96: 786-796.

Vígh B, Manzano e Silva MJ, Frank CL, Vincze C, Czirok SJ, Szabó A, Lukáts A, Szél A. (2004) The system of cerebrospinal fluid-contacting neurons. Its supposed role in the nonsynaptic signal transmission of the brain. Histol Histopathol. 19: 607-628.

Villar-Cheda B, Pérez-Costas E, Meléndez-Ferro M, Abalo XM, Rodríguez-Muñoz R, Anadón R, Rodicio MC. (2006) Cell proliferation in the forebrain and midbrain of the sea lamprey. J Comp Neurol. 494: 986-1006.

Villar-Cerviño V, Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Holstein GR, Martinelli GP, Rodicio MC, Anadón R. (2006) Presence of glutamate, glycine, and gamma-aminobutyric acid in the retina of the larval sea lamprey: comparative immunohistochemical study of classical neurotransmitters in larval and postmetamorphic retinas. J Comp Neurol. 499: 810-827.

Villareal G, Li Q, Cai D, Glanzman DL.. (2007) The role of rapid, local, postsynaptic protein synthesis in learning-related synaptic facilitation in *Aplysia*. Curr Biol. 17: 2073-2080.

Wallén P, Buchanan JT, Grillner S, Hill RH, Christenson J, Hökfelt T. (1989) Effects of 5-hydroxytryptamine on the afterhyperpolarization, spike frequency regulation, and oscillatory membrane properties in lamprey spinal cord neurons. J Neurophysiol. 61: 759-768.

Weiger WA. (1997) Serotonergic modulation of behaviour: a phylogenetic overview. Biol Rev Camb Philos Soc. 72: 61-95.

Westkaemper RB, Roth BL.. (2006) Structure and function reveal insights in the pharmacology of 5-HT receptor subtypes. In: Roth BL (ed) The receptors: The serotonin receptors: from molecular pharmacology to human therapeutics. Totowa, New Jersey, pp 39-58.

Wikström M, Hill R, Hellgren J, Grillner S. (1995) The action of 5-HT on calcium-dependent potassium channels and on the spinal locomotor network in lamprey is mediated by 5-HT1A-like receptors. Brain Res. 678: 191-199.

Abbreviations

5-HT	Serotonin (5-hydroxytryptamine)
5-HTR	Serotonin receptor
5-ht1a	Serotonin 1A receptor gene
5-HT_{1A}	Serotonin 1A receptor protein
CSF-c	cerebrospinal fluid-contacting
Dig	Digoxigenin
-ir	Immunoreactive
P0-P23	0 to 23 days post-hatching prolarvae
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase-PCR

FIGURES

Figure 1 - 5-ht1a cDNA of the sea lamprey and its deduced protein and 3D model. A) Sequence of the 5- ht1a cDNA with the start codon (ATG) and the stop codon (TGA) highlighted in grey. B) Sequence of the deduced 5-HT_{1A} protein with sequence corresponding to the G_i/G_o-coupled 5-HT_{1A} receptor highlighted in grey. C) The deduced 3D model of the 5-HT_{1A} protein colored according to the hydrophobicity grade of the amino acids: blue as the minor hydrophobicity grade and red as the major grade. D) Analysis of the predicted 5-HT_{1A} protein in BLAST belonging this in the rhodopsin family (indicated as 7tm).

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A

5´_1_atggaagctcacaacatcacaggcagcgcgccggccctggcagtgagtgcggcgcaggggaacacagcgagccct
cgtccgtagcctacaccgtgatctcgtcgctttgcttgaccgccttaatcctgtgcgcattttcggcaactcgtgcgtgatcgccgc
tatcgcgctggagcggactctgcagaacgtggccaactactgatcggctctctggccgtcaccgacctgttggtgtccatcctgg
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aaggatttcagaacgccttcaagaagatcattaaatgtaagttctgcaggcagtga_1236_3´

B

MEAHNITGSAPALAVSAAQGNSSEPSSVAYTVISSLCLTALILCAIFGNSCVIAAIALERTLQNV
ANYLIGSLAVTDLLVSILVLPMAALYQVLDRWTLGQVVCDIFIALDVLCCTSSILHLCVIALDR
YWAITNPIDYVNKRTPRRAVIMISLTWLVGFSISIPPMLGWRTPEDRADPNACKISQDHGYTI
YSTFGAFYIPLILMLVLYSRIFRAARFRIRKNVKKATEKKSFDAPFAISNRAEMPSAAAEAETA
APPSPAPCNGDSKQRSHRDLALPEAVGARSPSSLAHSSARPHLSPAMMPTQQQQHGNGTGAD
ACRRKTALARERKTVKTLGIIMGTFIFCWLPFFIVALISPFCGERCHLPPGVFAVINWLGYSN
SLLNPIIYAYFNKDFQNAFKKIIKCKFCRQ*

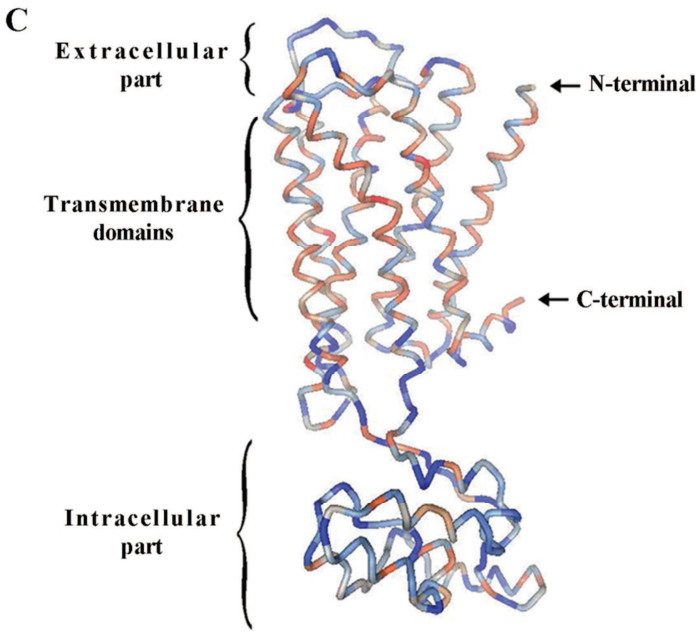

FIGURE 1

Figure 2 - Alignment of the sea lamprey 5-HT_{1A} deduced amino acid sequence with the putative amino acid sequences of 5-HT_{1A} of different vertebrates (see Experimental Procedures for GenBank accession numbers). At the bottom of the alignment, the consensus sequence and graphic indicating the percentage of amino acid conservation are shown.

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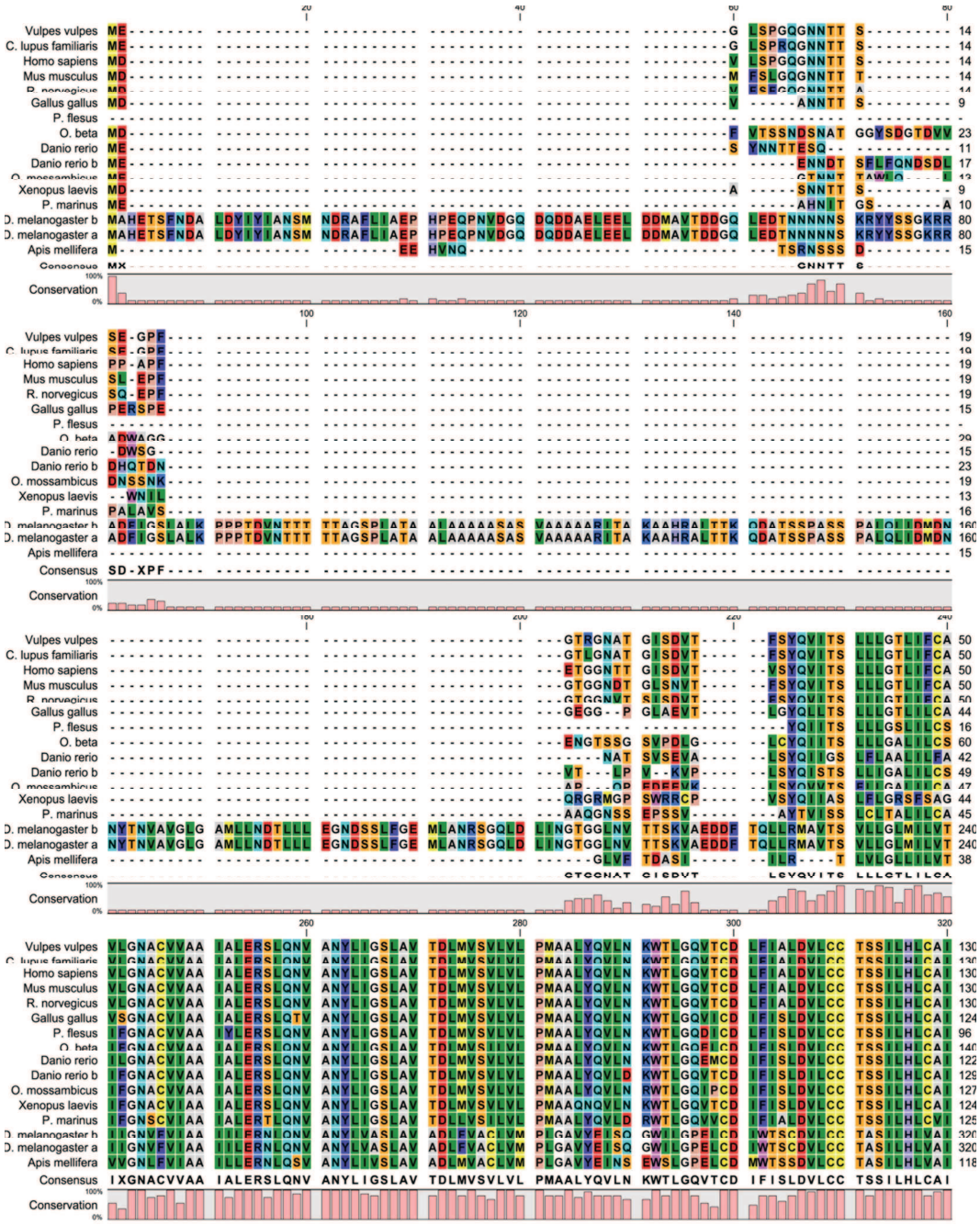
Vulpes vulpes
C. lupus familiaris
Homo sapiens
Mus musculus
R. norvegicus
Gallus gallus
P. flesus
O. beta
Danio rerio
Danio rerio b
O. mossambicus
Xenopus laevis
P. marinus
D. melanogaster b
D. melanogaster a
Apis mellifera
Consensus
Conservation
100%
0%

FIGURE 2

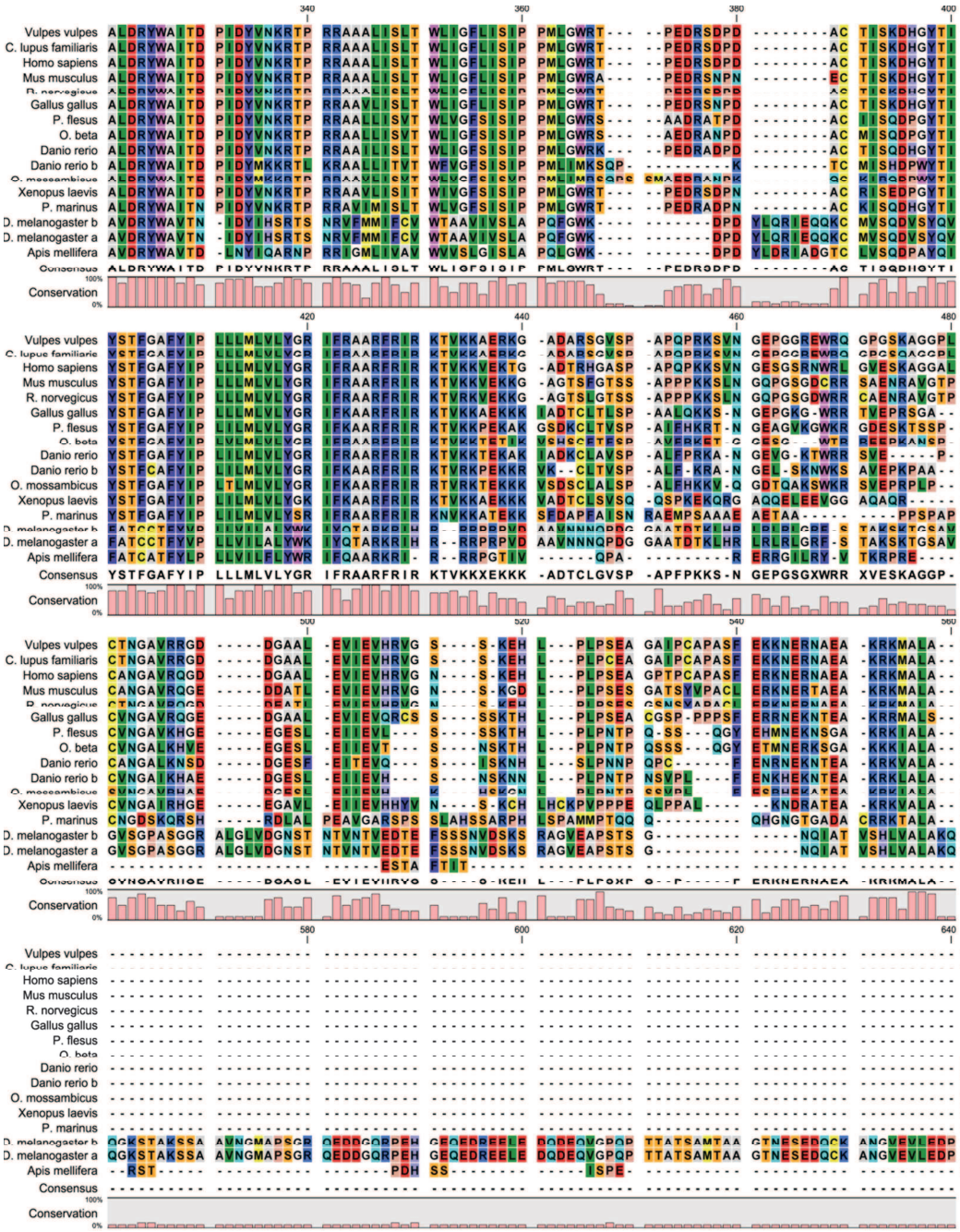

FIGURE 2 (*Continuation*)

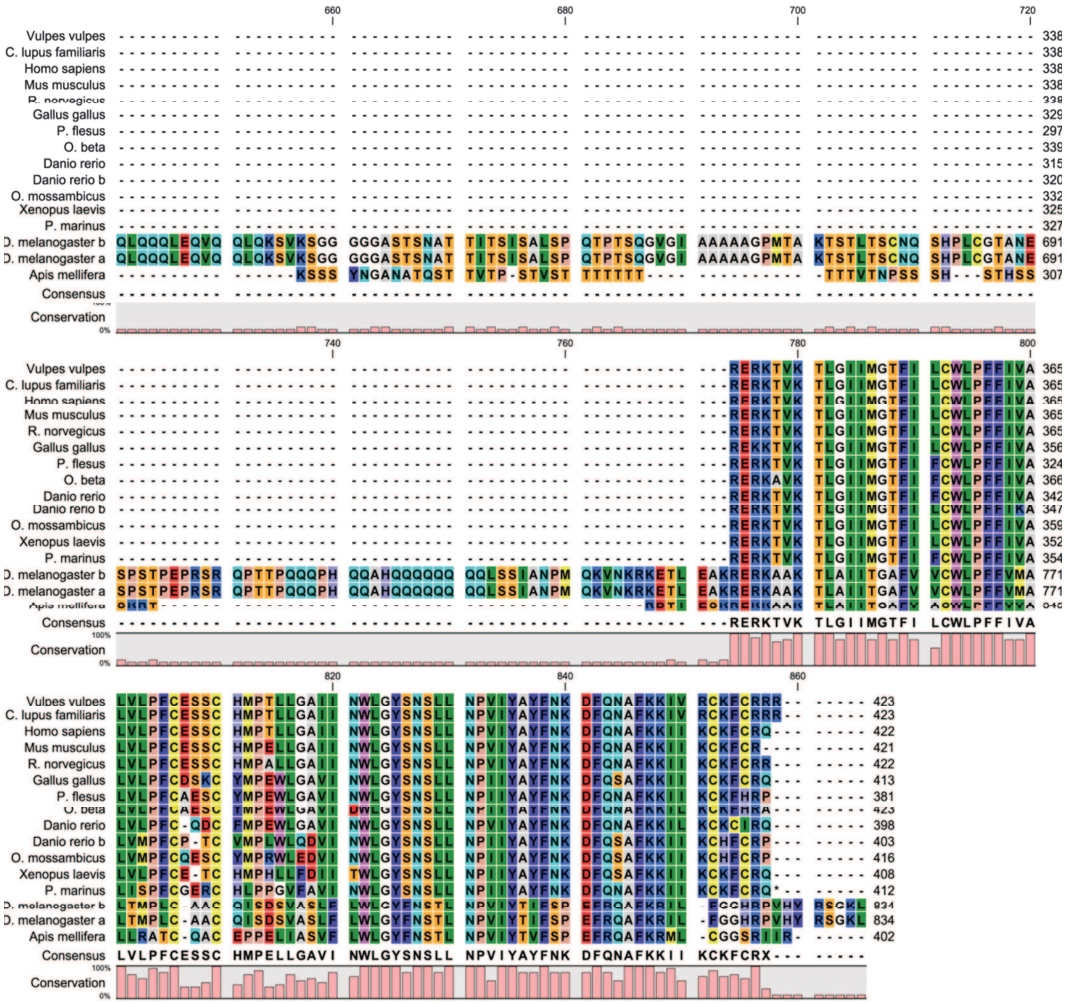

FIGURE 2 (*Continuation*)

Figure 3 - Phylogenetic tree of 5-HT_{1A} of different species according the neighbor-joining method. The bootstraps are indicated. Scale indicates 0.05 amino acid substitutions per locus.

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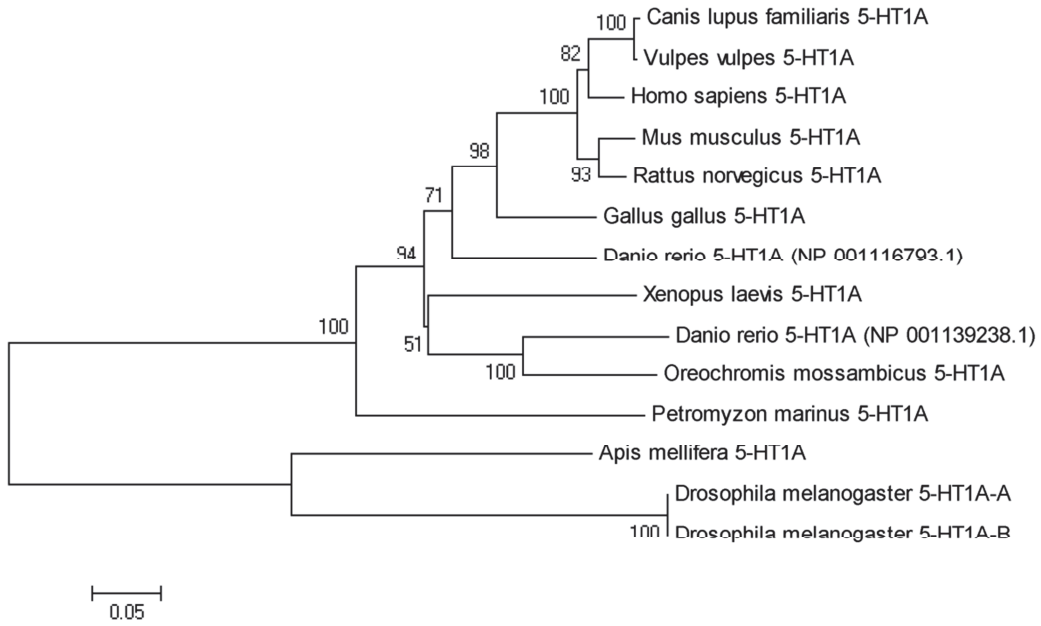
100
Canis lupus familiaris 5-HT1A
82
Vulpes vulpes 5-HT1A
100
Homo sapiens 5-HT1A
Mus musculus 5-HT1A
98
93
Rattus norvegicus 5-HT1A
71
Gallus gallus 5-HT1A
94
Danio rerio 5-HT1A (NP 001116793.1)
Xenopus laevis 5-HT1A
100
51
Danio rerio 5-HT1A (NP 001139238.1)
100
Oreochromis mossambicus 5-HT1A
Petromyzon marinus 5-HT1A
Apis mellifera 5-HT1A
Drosophila melanogaster 5-HT1A-A
100
Drosophila melanogaster 5-HT1A-B
0.05

FIGURE 3

Figure 4 - Phylogenetic tree of the different 5-HT receptors of different species according the neighbor-joining method. The bootstraps are indicated. Scale indicates 0.1 amino acid substitutions per locus.

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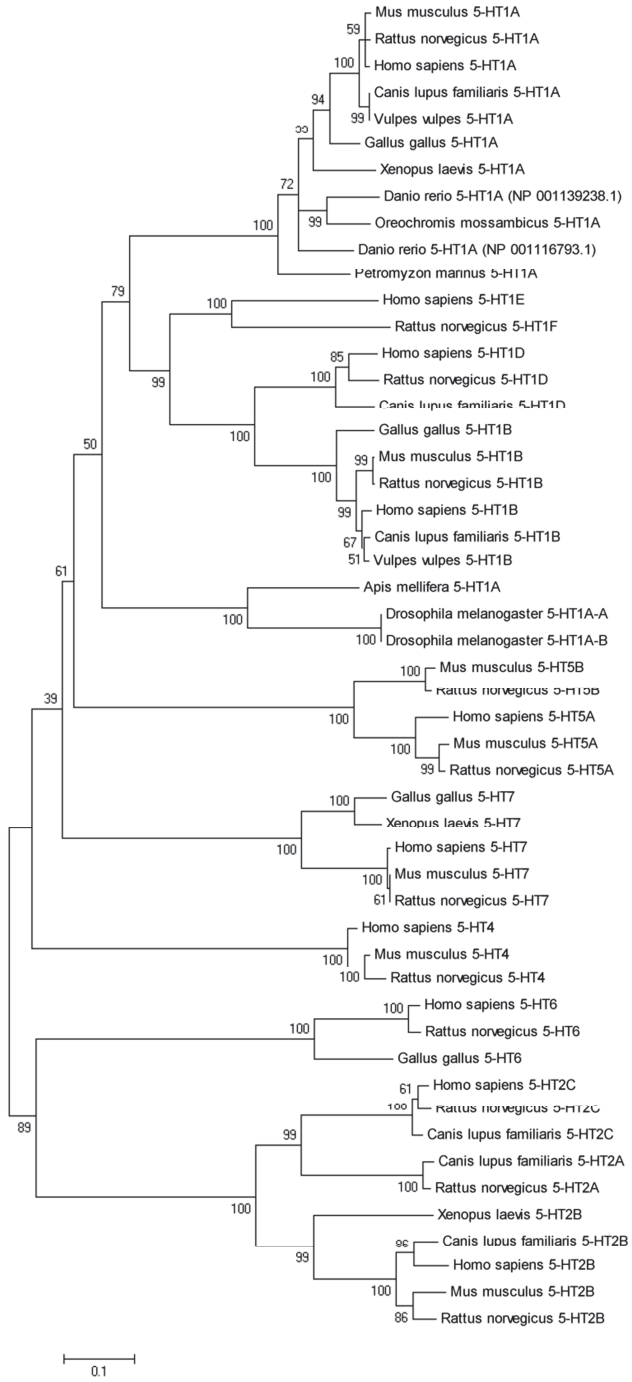
Mus musculus 5-HT1A
Rattus norvegicus 5-HT1A
Homo sapiens 5-HT1A
Canis lupus familiaris 5-HT1A
Vulpes vulpes 5-HT1A
Gallus gallus 5-HT1A
Xenopus laevis 5-HT1A
Danio rerio 5-HT1A (NP 001139238.1)
Oreochromis mossambicus 5-HT1A
Danio rerio 5-HT1A (NP 001116793.1)
Petromyzon marinus 5-HT1A
Homo sapiens 5-HT1E
Rattus norvegicus 5-HT1F
Homo sapiens 5-HT1D
Rattus norvegicus 5-HT1D
Gallus gallus 5-HT1B
Mus musculus 5-HT1B
Rattus norvegicus 5-HT1B
Homo sapiens 5-HT1B
Canis lupus familiaris 5-HT1B
Vulpes vulpes 5-HT1B
Apis mellifera 5-HT1A
Drosophila melanogaster 5-HT1A-A
Drosophila melanogaster 5-HT1A-B
Mus musculus 5-HT5B
Homo sapiens 5-HT5A
Mus musculus 5-HT5A
Rattus norvegicus 5-HT5A
Gallus gallus 5-HT7
Homo sapiens 5-HT7
Mus musculus 5-HT7
Rattus norvegicus 5-HT7
Homo sapiens 5-HT4
Mus musculus 5-HT4
Rattus norvegicus 5-HT4
Homo sapiens 5-HT6
Rattus norvegicus 5-HT6
Gallus gallus 5-HT6
Homo sapiens 5-HT2C
Canis lupus familiaris 5-HT2C
Canis lupus familiaris 5-HT2A
Rattus norvegicus 5-HT2A
Xenopus laevis 5-HT2B
Canis lupus familiaris 5-HT2B
Homo sapiens 5-HT2B
Mus musculus 5-HT2B
Rattus norvegicus 5-HT2B
0.1

FIGURE 4

Figure 5 - Wholemount *in situ* hybridization (A-B) and sections of the same heads (C-J) in P1 and P2 stages of sea lamprey. A) Photomicrograph of a wholemount P1 stage showing the expression of the 5-ht1a transcript in the nuclei of the supraoptic tract, of the tract of the posterior commissure, of the tract of the postoptic commissure, of the medial longitudinal fascicle, isthmic nucleus, trigeminal region, otic region, caudal rhombencephalon and rostral spinal cord. B) Photomicrograph of a wholemount P2 stage showing the expression of 5-ht1a in the olfactory bulbs/pallium primordium, nuclei of the supraoptic tract, of the tract of the postoptic commissure, of the medial longitudinal fascicle, hypothalamus, prethalamus, thalamus, pretectum, tegmentum mesencephalic, isthmic nucleus, caudal rhombencephalon, and rostral spinal cord. Thin arrow corresponds with the boundary between the thalamus and pretectum. Thick arrow corresponds with the optic tectum. C-J) Photomicrographs of transversal sections of the wholemount-stained P1 stage confirmed the expression of 5- ht1a in the corresponding zones (*arrowed*) and showing a low expression in the trigeminal region G). *cH,* caudal hypothalamus; *cRho,* caudal rhombencephalon; *I-VI,* first-to-sixth gill pouches; *Is,* isthmus; *N,* notochord; *nMLF,* nucleus of the medial longitudinal fascicle; *nSOT,* nucleus of the supraoptic tract; *nTPC,* nucleus of the tract of the posterior commissure; *nTPOC,* nucleus of the tract of the postoptic commissure; *OB/PP,* olfactory bulb/lateral pallium primordium; *Or,* otic region; *ov*, otic vesicle; *PT,* pretectum; *PTh,* prethalamus; *SC,* spinal cord; *Th,* thalamus; *tM,* mesencephalic tegmentum; *Tr,* trigeminal region. *Scale bars*: 250 µm (A-B), 20 µm (C-J).

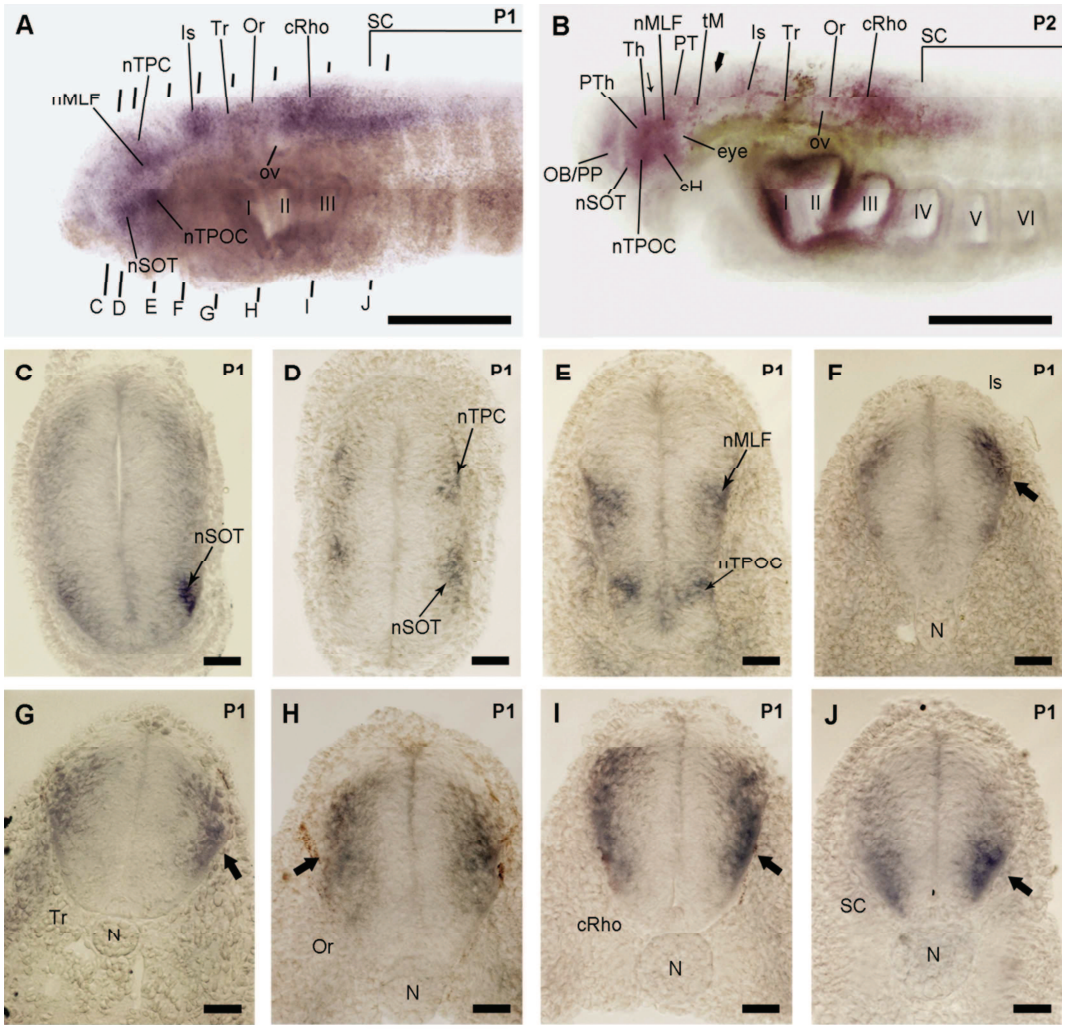
A
P1
Is
Tr
Or
cRho
SC
nTPC
nMLF
ov
I
II
III
nTPOC
nSOT
C D E F G H I J
B
P2
nMLF
tM
Th
PT
Is
Tr
Or
cRho
SC
PTh
eye
ov
OB/PP
nSOT
cH
nTPOC
I
II
III
IV
V
VI
C
P1
nSOT
D
P1
nTPC
nSOT
E
P1
nMLF
nTPOC
F
P1
Is
N
G
P1
Tr
N
H
P1
Or
N
I
P1
cRho
N
J
P1
SC
N

FIGURE 5

Figure 6 - Schematic drawing of transverse sections of the brain of a large sea lamprey larva (100 mm in length) representing the expression of the 5-ht1a transcript. Anatomical regions are indicated on the right and the expression density of the 5-ht1a transcript is represented by dots on the left. The level of the sections is indicated in the upper right figure. The correspondence with photomicrographs in other figures is indicated by squared areas. *Asterisks* indicate the ventricles. *dC*, dorsal cell; *DC*, dorsal column; *DCn*, dorsal column nucleus; *Dm*, dorsomedial pallial neuropil; *DN*, dorsal nucleus; *dV*, nucleus of the trigeminal descending root; *fr*, fasciculus retroflexus; *Gl*, glomerular layer; *Gr*, granular layer; *H*, hypothalamus; *Ha*, habenula; *Is*, isthmus; *IsRF*, isthmic reticular formation; *IX/Xm*, glossopharyngeal/vagal motor nuclei; *LC*, lateral column; *LP*, lateral pallium; *M1-M2*, first and second Müller cells; *MB*, Müller bulbar cells; *Ml*, mitral cell layer; *MLF*, medial longitudinal fascicle; *mN*, motoneurons; *MN*, medial nucleus; *MP*, medial pallium; *Mr*, mammillary region; *MRF*, medial rhombencephalic reticular formation; *Mth*, Mauthner cell; *nMLF*, nucleus of the medial longitudinal fascicle; *OB/P*, olfactory bulb/lateral pallium; *OLa*, octavolateral area; *OT*, optic tectum; *P*, pineal organ; *POr*, preoptic region; *Pp*, parapineal organ; *pr*, preoptic recess; *PRF*, posterior rhombencephalic reticular formation; *PT*, pretectum; *PTh*, prethalamus; *PTu*, posterior tuberculum; *Rho*, rhombencephalon; *SC*, spinal cord; *SCO*, subcommissural organ; *SL*, subhippocampal lobe; *SO*, spino-occipital motor column; *Sol*, nucleus of the solitary tract; *Sp*, septum; *St*, striatum; *Th*, thalamus; *TS*, torus semicircularis; *VC*, ventral column; *VIIm*, facial motor nucleus; *VN*, ventral nucleus. *Scale bar*: 100 µm.

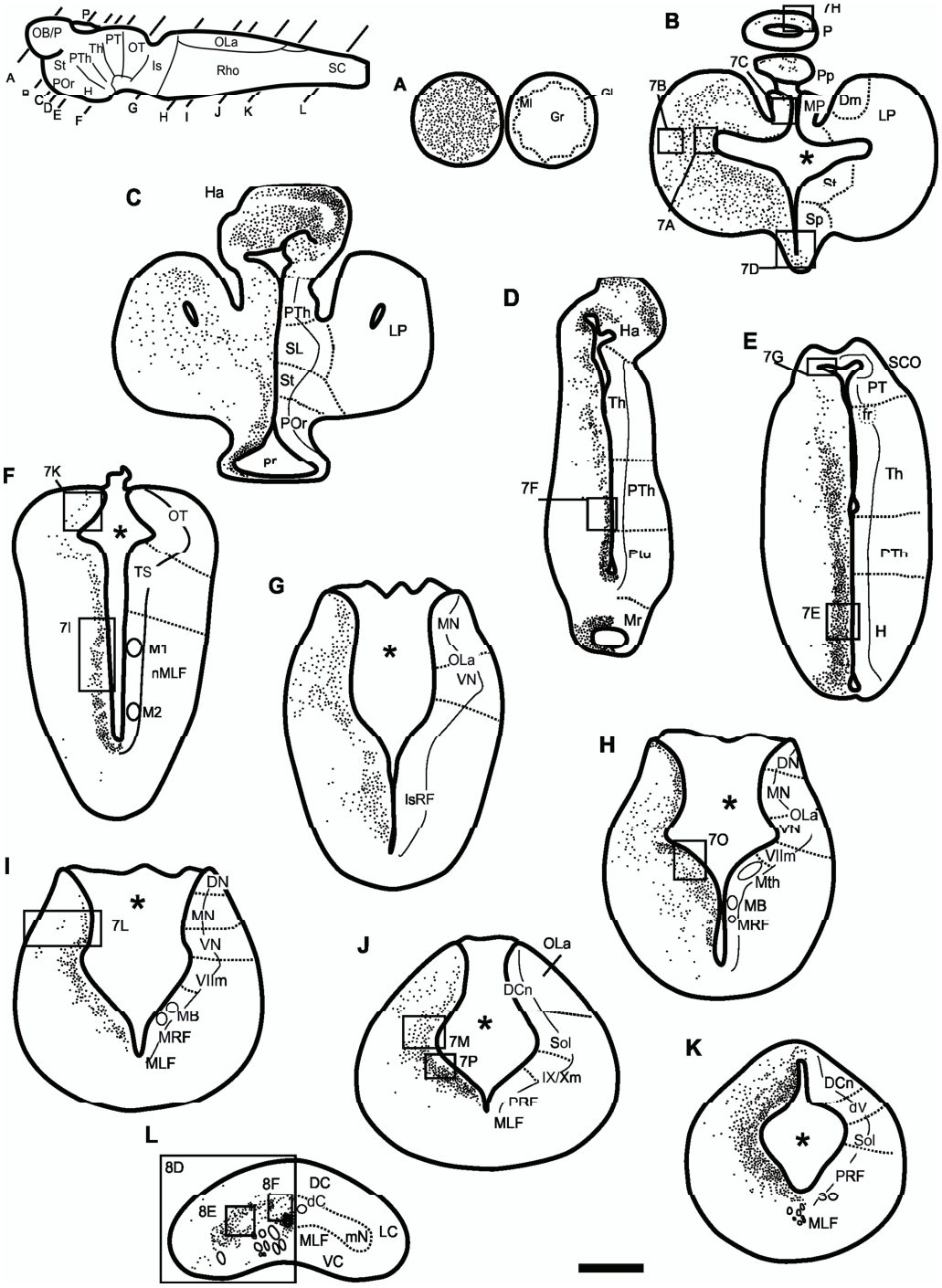
OB/P
P
PT
OT
OLa
PTh
Th
St
Is
Rho
SC
POr
H
A
B
C
D
E
F
G
H
I
J
K
L
Mi
Gr
7H
7C
7B
Pp
MP
Dm
LP
St
Sp
7A
7D
Ha
PTh
SL
LP
St
POr
Pr
Th
7F
Mr
7G
SCO
PT
Ir
7E
7K
TS
7I
M1
nMLF
M2
MN
OLa
VN
IsRF
DN
7O
Vllm
Mth
MB
MRF
7L
MLF
DCn
7M
7P
Sol
IX/Xm
PRF
dV
8D
8F
DC
8E
dC
mN
LC
VC

FIGURE 6

Figure 7 - Photomicrographs of transverse sections through the brain of a large sea lamprey larva (100 mm in length) showing distribution of the 5-ht1a transcript. A) Section through the periventricular cell layer of the lateral pallium showing cells with 5-ht1a transcript. B) Section of the lateral pallium showing perikarya of migrated cells with positive dots corresponding with the 5-ht1a transcript. C) Section of the medial pallium showing very weak 5-ht1a expression in occasional cells of the periventricular cell layer. D) Photomicrograph of the septum showing positivity to 5-ht1a transcript in most cells, including cells of the ependymal layer. E) Section of the hypothalamus with 5-ht1a positive cells. F) Section of the prethalamus showing the expression in periventricular cells. G) Section of the subcommissural organ showing the lack of 5-ht1a expression. H) Section of the pineal organ with 5-ht1a expression in photoreceptors (*thin arrow*) and ganglion cells (*thick arrow*). I) Section of the nucleus of the medial longitudinal fascicle showing the expression of the 5-ht1a transcript in the giant Müller cells M1. Expression is also observed in small cells. J) Section of the mammillary region showing 5-ht1a positivity in dendritic clubs protruding into the posterior hypothalamic recess (*arrows*). K) Section of the optic tectum with scant 5-ht1a transcript expression. L) Section of the octavolateral area showing expression of the 5-ht1a transcript in both periventricular and migrated cells. M) Section of the solitary tract nucleus showing 5-ht1a transcript expression. N) Section showing dorsal cells (primary sensory neurons) expressing 5-ht1a transcript. O) Section of the lateral reticular formation showing 5-ht1a expression in the giant Mauthner cell. P) Section of the vagal visceromotor nucleus showing 5-ht1a expression in motoneurons. *Asterisks* indicate the ventricle. *Numbers* in figures indicate correspondence with levels in Figure 6. *dC*, dorsal cell; *DCn*, dorsal column nucleus; *Gl*, glomerular layer; *H*, hypothalamus; *LP*, lateral pallium; *M1*, first Müller cell; *Ml*, mitral cell layer; *MP*, medial pallium; *Mth*, Mauthner cell; *Mr*, mammillary region; *nMLF*, nucleus of the medial longitudinal fascicle; *OLa*, octavolateral area; *OT*, optic tectum; *P*, pineal organ; *POr*, preoptic region; *PTh*, prethalamus; *SCO*, subcommissural organ; *Sol*, nucleus of the solitary tract; *Sp*, septum; *St*, striatum; *IX/Xm*, glossopharyngeal/vagal motor nuclei. *Scale bars*: 50 µm (D, I, L, M, O, P), 20 µm (A-C, E-H, J, K, N).

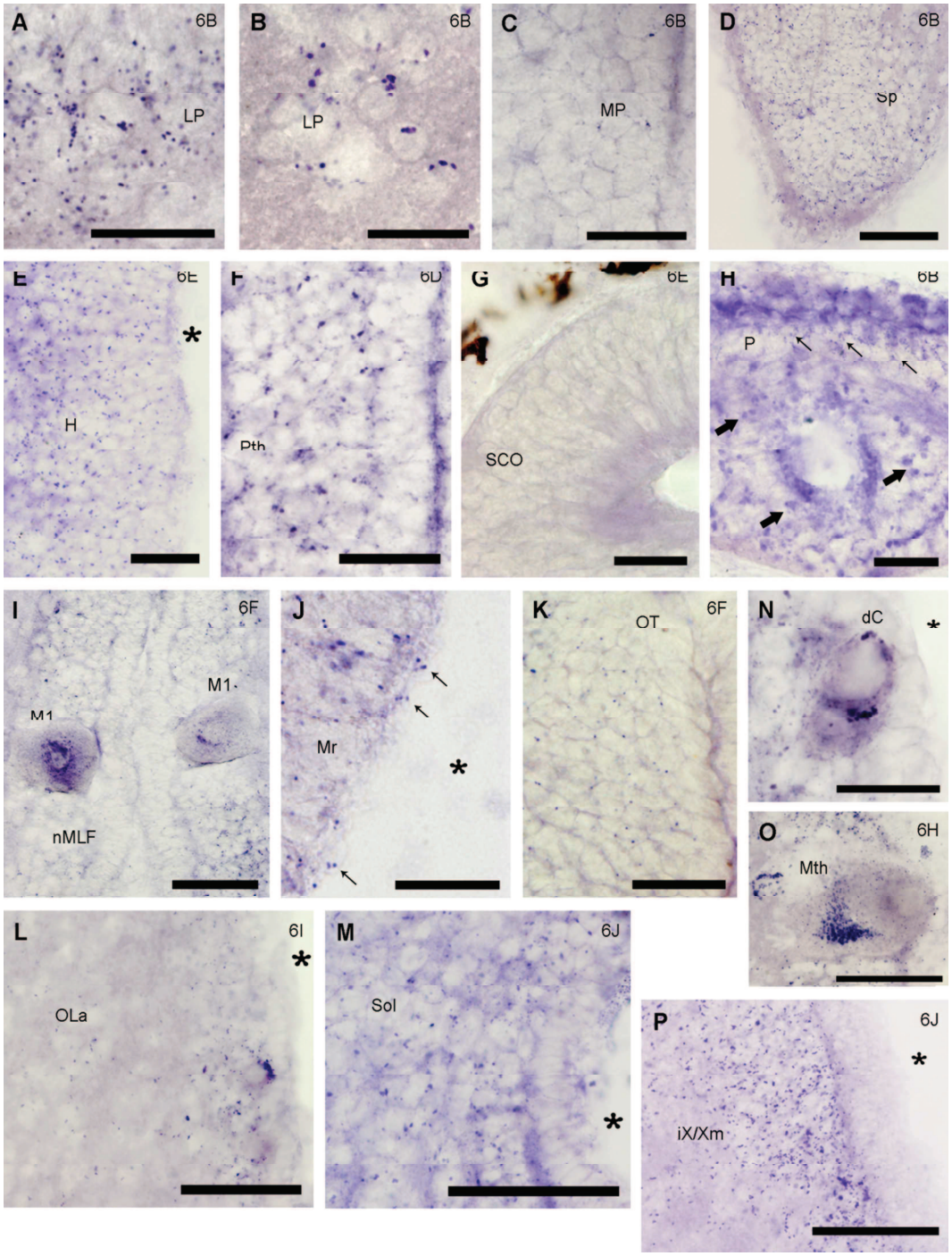
A
6B
LP
B
6B
LP
C
6B
MP
D
6B
Sp
E
6E
*
H
F
6D
Pth
G
6E
SCO
H
6B
P
I
6F
M1
M1
nMLF
J
Mr
*
K
OT
6F
N
dC
*
O
6H
Mth
L
6I
*
OLa
M
6J
Sol
*
P
6J
*
iX/Xm

FIGURE 7

Figure 8 - Photomicrographs of sections of the brain (A-C) and spinal cord (D-I) showing the expression of the 5-ht1a transcript in larval (D-F) and adult (A-C, G-I) sea lamprey. A) Section of the subcommissural organ showing 5-ht1a expression. B) Section of the optic tectum with scarce 5-ht1a expression in periventricular cell layers. C) Section of the medial pallium showing weak 5-ht1a expression in neurons of the periventricular cell layer. D) Section of the larval spinal cord showing the expression of 5-ht1a transcript in the gray matter. E) Detail of the lateral horn showing 5-ht1a transcript expression in a large neuron (*arrow*) and in small cells. F) Detail of the dorsal cell (primary sensory neuron) expressing 5-ht1a transcript. G) Section of the adult spinal cord showing 5-ht1a expression in a motoneuron. H) Section of the lateral spinal cord showing 5-ht1a expression in an edge cell of an adult. I) Section of the central canal of an adult lamprey showing cells in the ependymal layer with 5-ht1a expression. *Asterisks* correspond with the ventricle. *Start* corresponds with an axon of the medial longitudinal fascicle. *Numbers* in figures indicate correspondence with levels in Figure 6. *CSF-c*, cerebrospinal fluid-contacting; *dC*, dorsal cell; *DC*, dorsal column; *eC*, edge cell; *IC*, lateral cell; *LC*, lateral column; *mN*, motoneurons; *MP*, medial pallium; *OT*, optic tectum; *SCO*, subcommissural organ; *VC*, ventral column. *Scale bars*: 100 µm (D), 50 µm (A-C, E), 20 µm (B, F-I).

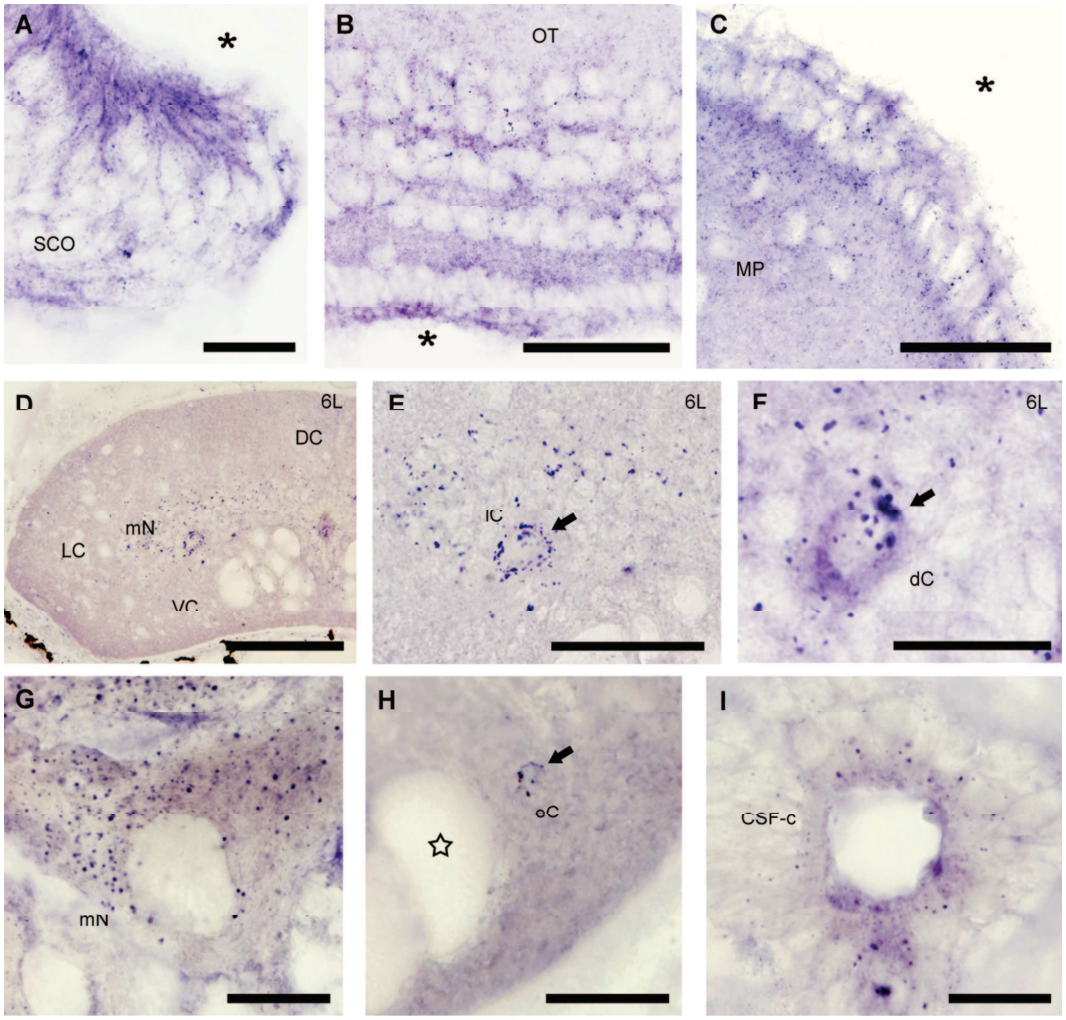

FIGURE 8

Figure 9 - Photomicrographs of sections of the choroid plexus (A) and brain vessels (B) showing the expression of the 5-ht1a transcript in sea lamprey. A) Section of the choroid plexus showing 5-ht1a expression. B) Longitudinal section of the brain vessels with 5-ht1a expression. *Asterisk* corresponds with the ventricle. *Scale bars*: 100 µm (A), 50 µm (B).

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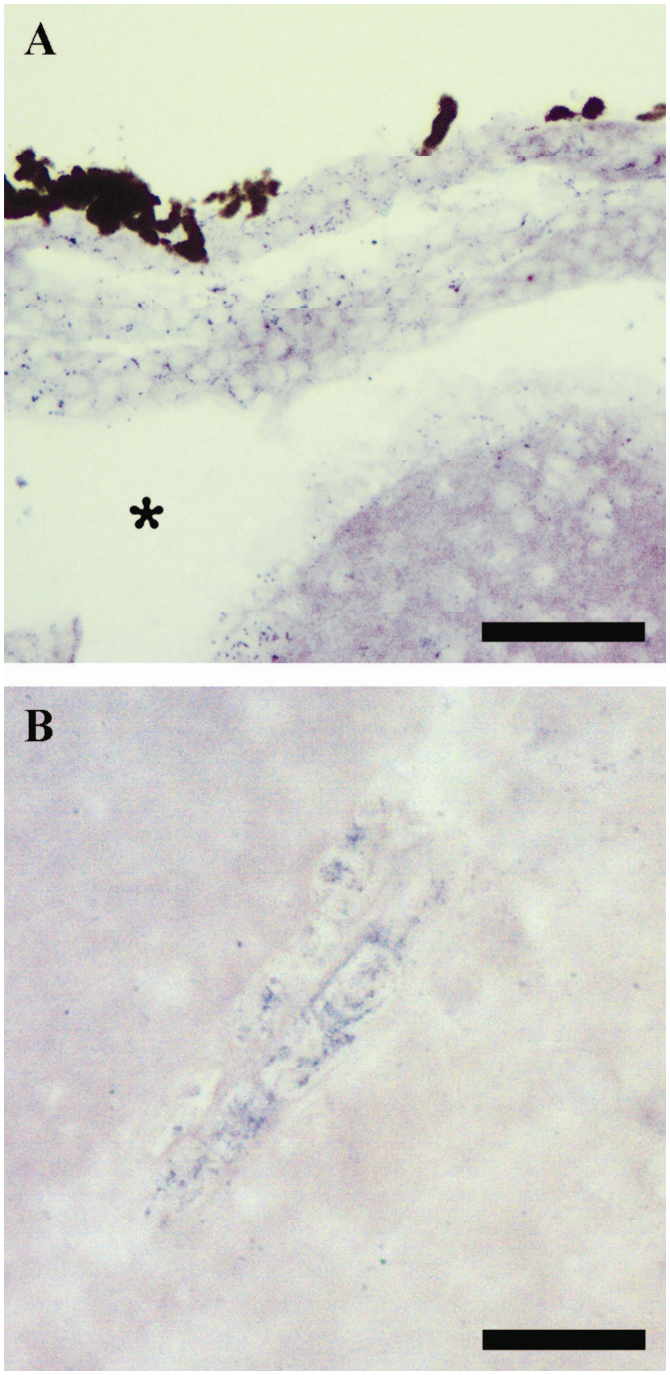

FIGURE 9

Figure 10 – 5-ht1a expression in the retina of the sea lamprey. A) Photomicrograph of a transverse section of the retina in a prolarval P27 showing the presence of 5-ht1a transcript. B) Photomicrograph of a transverse section of the lateral retina in larval (75 mm) showing the presence of the 5-ht1a transcript mainly in the inner neuroblastic layer, but also in the outer neuroblastic layer and pigmentary epithelium. C) Photomicrograph of a transverse section of the central retina in larval (75 mm) showing the presence of the 5-ht1a transcript mainly in the inner and outer nuclear layers. D) Photomicrograph of a transverse section of the retina in adults showing the presence of the 5-ht1a transcript in the inner nuclear layer and photoreceptor layer. E) Detail of photoreceptor layer showing 5-ht1a expression mostly concentrated in the myoid of both short (*headarrow*) and long (*arrows*) photorreceptors. F) Detail of the inner nuclear layer showing 5ht1a expression in the ganglion and amacrine cells. Some ganglion cells out of the inner nuclear layer also show the presence of the5ht1a transcript (*arrow*). HCL, horizontal cell sublayer; *INbL*, inner neuroblastic layer; *INL*, inner nuclear layer; *IPL*, inner plexiform/optic fibre layer; *ON*, optic nerve; *ONbL*, outer neuroblastic layer; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *P*, photoreceptor; *PE*, pigment epithelium. *Scale bars*: 25 µm (E, F), 50 µm (A-D).

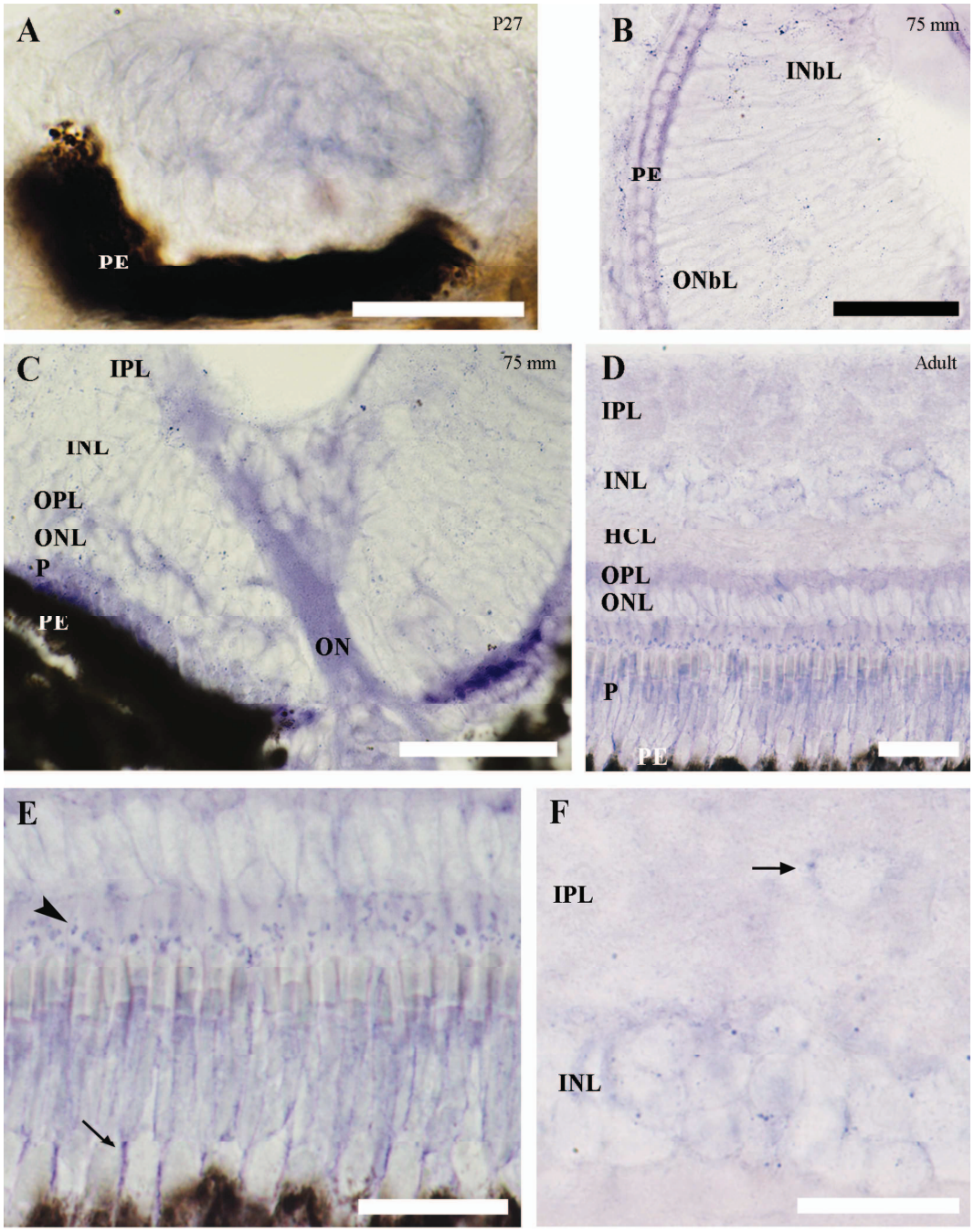
A
P27
PE
B
75 mm
INbL
PE
ONbL
C
IPL
75 mm
INL
OPL
ONL
P
PE
ON
D
Adult
IPL
INL
HCL
OPL
ONL
P
PE
E
F
IPL
INL

FIGURE 10

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CHAPTER 3
Traumatic injury induces changes in the expression of the serotonin 1A receptor in the spinal cord of lampreys

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TRAUMATIC INJURY INDUCES CHANGES IN THE EXPRESSION OF THE SEROTONIN 1A RECEPTOR IN THE SPINAL CORD OF LAMPREYS

Introduction

In mammals, including humans, spinal cord injury (SCI) leads to permanent disability and an irreversible loss of function because damaged axons do not regenerate across the trauma zone to reconnect to their appropriate targets. In contrast to mammals, lampreys recover locomotion several weeks after a complete spinal cord transection and severed descending axons from the brain are able to regenerate across the trauma zone and reinnervate the spinal cord below the site of injury (reviewed in Rodicio and Barreiro-Iglesias, 2012).

In last decades, lampreys have also become the vertebrate model in which the neuronal circuitry underlying the locomotion process has been studied in more detail (Buchanan, 1999; Grillner et al., 2008). Studies on lampreys have shown that the spinal cord has an intrinsic neuronal circuitry that controls locomotion by generating a coordinated rhythmic output; this circuitry is known as the central pattern generator (CPG; see Grillner et al., 2008). In most vertebrates, the CPG is at rest and needs to be activated and modulated by descending systems from the brain. These command centres are located in the brainstem and in the diencephalic and mesopontine locomotor regions and they control and activate the spinal CPG (Ronan, 1989; Swain et al., 1993; Grillner et al., 2008). Serotonin (5-HT), which is mainly derived from the descending projections of the raphe nuclei of the brainstem, plays a critical role in the locomotor CPG by modulating rhythm and coordination (Pearlstein et al., 2011). The presence of descending serotonergic innervation originated in the brainstem is highly conserved in all vertebrates, from lampreys to mammals (Barreiro-Iglesias et al., 2008). In addition to descending

projections, the spinal cord of lampreys also contains intrinsic spinal serotonergic cells, which are located ventrally to the central canal (Abalo et al., 2007; Barreiro-Iglesias et al., 2009). In lampreys, the spinal serotonergic system is turned on during locomotion (Wang et al., 2011) and serotonin is endogenously released during fictive locomotion (Biró et al., 2006). The serotonergic system in the lamprey spinal locomotor network contributes significantly to rhythm generation and the generation of fictive locomotion (Zhang and Grillner 2000). This is mediated, at least in part, by the serotonin 1A receptor (5-HT_{1A}) (Zhang and Grillner 2000). In lampreys, serotonin slows fictive swimming by reducing the slow postspike after hyperpolarization in spinal neurons (including interneurons and motoneurons) (Hill et al., 2003; Biró et al., 2006), which is caused by a reduction in Ca(2+) currents through the activation of 5-HT_{1A} (Wikström et al., 1995; Hill et al., 2003).

After SCI in mammals, the loss of serotonin coming from the brainstem reduces the excitability of motor neurons (Murray et al., 2010). This leads to a compensatory overexpression of autoactive 5-HT receptors of the family 2 (5-HT_{2A}: Fuller et al. 2005; Ung et al., 2008; Navarrett et al., 2012; 5-HT_{2C}: Husch et al., 2012; Ren et al., 2013) that contribute to motor neuronal excitability and compensate for the lack of agonist, but cause muscle spasms and spasticity at the same time and therefore, the impairment of motor behaviours (e.g. locomotion, ejaculation or micturition) (D´Amico et al., 2013; Perrier, et al., 2013). Nowadays, less attention has been paid to the study of the changes in expression after SCI of other serotonin receptors like the 5-HT_{1A} despite its key role in spinal CPG (see above). The 5-HT_{1A} is expressed in the spinal cord of humans (Perrin et al., 2011) and is specifically expressed in motor neurons of mammals (see Perrier et al., 2013). It has been previously shown that 5-HT_{1A} expression increases in lumbar levels after a thoracic spinal cord transection in rats (Otoshi et al., 2009) and cats (Giroux et al., 1999). Moreover, expression of 5-HT_{1A} has been shown to increase in the rat spinal cord after a nearly complete loss of descending serotonergic fibers by chemical denervation with 5,7-dihydroxytryptamine as well (Laporte et al., 1995). Overexpression of the 5-HT_{1A} after SCI has been related to the lack of agonist and seems to be

modulated by sensory inputs (Otoshi et al., 2009). However, the functional consequences or the physiological role of the overexpression of 5-HT_{1A} after SCI are unclear at the moment (Otoshi et al., 2009).

A study by Cohen and coworkers (2005) reported that, in contrast to mammals, the serotonergic innervation of the spinal cord of lampreys is partially recovered below the site of injury after a complete spinal cord transection. In addition, a recent study by our group has also shown that severed descending serotonergic axons spontaneously regenerate through the site of spinal cord transection in lampreys (Cornide-Petronio et al., 2011). So, lampreys appear to be a very interesting model on which to study the mechanisms that lead to the regeneration of the serotonergic system after SCI and to understand the plastic events occurring in this system leading to successful recovery. In Chapter 2, we have reported the cloning of the sea lamprey 5ht1a cDNA and the pattern of expression of the 5ht1a transcripts in the brain and spinal cord (Cornide-Petronio et al., 2012). Here, to further characterise the lamprey model to study the regeneration of the serotonergic system after SCI we investigated the changes in the expression of 5ht1a transcripts after a complete spinal transection and compared our results to those previously reported in mammalian models. Our study shows that in lampreys SCI acutely induces an overexpression of the 5-HT_{1A} both above and below the site of injury and that a few weeks after the injury the expression returns to levels similar to those observed in the control situation.

Material and Methods

Animals

Mature larval sea lampreys (100-140 mm in body length; 5-7 years old), *Petromyzon marinus* L., were caught in the river Ulla (Galicia, Northwest Spain) and maintained in fresh water with a bed of river sediment and appropriate

ventilation and temperature conditions until the day of use. Before experiments, all animals were deeply anesthetized with 0.1% tricaine methanesulfonate (Sigma, St. Louis, MO) in fresh water. The experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and conformed to European Union and Spanish regulations for the care and handling of animals in research.

Surgical Procedures

Larval sea lampreys (n=20) were randomly assigned to any of the following 4 experimental groups: control animals without injury (n= 5), 1 week post-lesion (n= 5), 3 weeks post-lesion (n= 5), and 7 weeks post-lesion (n= 5). To perform a complete spinal cord transection, the spinal cord was exposed from the dorsal midline at the level of the fifth gill, a complete SC transection was performed with a scalpel (# 11) and the spinal cord cut ends were visualized under the stereomicroscope. The spinal cord was gently realigned using a pair of fine forceps and the animals were then kept on ice for 1 hour to allow the wound to air dry. During this time, the animals are kept on a paper towel soaked with ringer solution not in direct contact with the ice; the low temperature keeps the animals alive while allowing the wound to air dry. After this hour the animals were returned to fresh water tanks and each transected animal was examined 24 hours after surgery to confirm that there was no movement caudal to the lesion site. The spinal cord transection was tentatively considered complete if on stimulation of the head, an animal could move only its head and body rostral to the lesion site. Animals were allowed to recover in fresh water tanks at 19-20ºC, since larval lampreys are known to be able to recover functionality at this temperature (Cohen et al., 1999).

In situ *hybridization*

Larval lampreys were fixed by immersion for 12 hours in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) at 4ºC. The portions of the spinal cord rostral and caudal to site of injury were cut out and cryoprotected with 30% sucrose in PBS overnight. Transverse sections (thickness of 18 µm) of the first 250 µm of the rostral and caudal spinal cord portions from the site of transection were obtained with a cryostat. *In situ* hybridization of spinal cord sections with a specific riboprobe for the 5-HT_{1A} was conducted as previously described (Chapter 2, Cornide-Petronio et al., 2012). *In situ* hybridization experiments were performed in parallel with animals from the 4 different experimental groups and the colorimetric reaction was stopped simultaneously for all the sections from the different groups of animals. A single batch of the riboprobe was used for all the animals from all groups.

Quantification of the number of 5-HT_{1A} profiles

We used an Olympus photomicroscope (AX-70; Provis) with a 20x Apochromate 0.75 lens and equipped with a colour digital camera (Olympus DP70, Tokyo, Japan) for acquiring images of a half of the spinal cord transverse sections. Images were always taken with the same microscope and software settings for all sections. As previously described, the 5-HT_{1A} *in situ* hybridization colour reaction appears as a dotted labelling (Chapter 2; Cornide-Petronio et al., 2012).

For the quantification of the 5-HT_{1A} positive profiles we adapted a semiautomatic quantification method that was previously used to quantify 5-HT and TH immunofluorescent positive profiles in cross sections of the adult zebrafish SC (Kuscha et al., 2012). Positive 5-ht1a profiles were quantified for each half spinal cord section using the dorsoventral midline of the central canal as a landmark and were only quantified in the grey matter (where most spinal cord neurons are located). To decrease background and increase edge and shape detection of the small positive profiles, the threshold was adjusted

for each image at the initial border of the histogram and the Feature J Hessian plug-in for Image J was used (smallest eigenvalue Hessian tensor; smoothing scale to 1.0) according to an established protocol (Grider et al., 2006). Upon conversion to binary images, positive profiles of the grey matter were quantified by using the ``analyse particle'' function of Image J with particle size from 1 to infinity and circularity from 0.00 to 1.00.

Numbers of positive profiles were counted for 12 sections (18 µm-thick) of the 250-µm rostral and caudal to the lesion site. Then the average number of positive profiles per half section was calculated for the rostral and caudal stumps of the spinal cord for each animal. In control animals, 12 18-µm-thick sections of a 250-µm segment of half spinal cord at the level of the fifth gill were analyzed.

Statistical analysis

For statistical analysis, the program Prism (GraphPad Software, La Jolla, CA) was used. Variability of values is given as statistical error of mean (SEM). Data from all groups of animals passed the Kormogolov-Smirnov normality test. Data sets were statistically analysed using a one-way ANOVA with Bonferroni's multiple comparison test.

Figure preparation

Brightness and contrast of photomicrographs were minimally adjusted and figure plates prepared and lettering added using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA). Schematic drawings were done using Corel Suit X5.

Results

Selection of the experimental time points

Axon regeneration in the spinal cord of lampreys has two main phases related to the behaviour of descending axons: retraction and growth. During the first two weeks after a complete spinal cord transection, the cut descending axons retract up to 2 mm (Roederer et al., 1983; Yin and Selzer, 1983; Zhang et al., 2005). Thereafter, many axons regenerate in the rostral stump for two weeks, reach the lesion site by the fourth week, and finally enter the caudal stump between five to seven weeks later (Yin and Selzer, 1983). The distances of regenerated axons have generally been limited to a few millimetres beyond the transection (Shifman et al., 2007). It should be noted that this is an intermittent process in which periods of axonal retraction and growth are intermingled, although retraction is more prominent initially and axon growth predominates afterwards (Jin et al., 2009). Due to this, we decided to study the expression of 5-HT_{1A} in the spinal cord at 3 different time points after the complete spinal cord transection: 1 week post-lesion (wpl) (when descending axons from the brain would be mainly retracting from the site of injury and degenerating caudally); 3 wpl (when the descending axons would be mainly re-growing and re-innervating the rostral stump) and 7 wpl (when at least some of descending axons would be already re-innervating the caudal the stump).

Expression of the 5-ht1a in the spinal cord

As described in Chapter 2, in control animals the expression of the receptor was observed mainly in the grey matter of the spinal cord and it appeared as a dotted labelling (Fig. 1A) which allowed us to quantify the number of positive profiles per half section using an adaptation of a previously described semiautomatic quantification method. The widespread distribution of the signal indicated that most neuronal types of the spinal cord would express 5-ht1a in both unlesioned and lesioned larval lampreys. Dorsal cells,

located near the central canal (Fig. 1B), showed abundant 5-ht1a expression around the cell nucleus. The horizontally extended grey matter corresponds to both the dorsal and ventral horns found in other vertebrates and contents interneurons and motoneurons. 5-ht1a expression was observed as fine grains along the region containing these neurons (Fig. 1B). 5-ht1a expression was also observed in the region of cerebrospinal fluid-contacting (CSF-c) cells and ependymal cells of the ependymal layer of all larval groups (Fig. 1B).

Quantitative changes in 5-ht1a expression after a complete spinal cord transection

First, we quantified the number of 5-ht1a positive profiles in hemisections of the spinal cord, both rostral and caudal to the lesion site in control and 1, 3 and 7 wpl larvae. Quantification of the average number of 5ht1a positive profiles per hemisection in the rostral stump of the spinal cord at 1 wpl indicated a significant increase of 221% as compared to control values (unlesioned: 179.5 ± 11.01; 1wpl: 575.1 ± 40.74; ANOVA, $p<0.0001$) (Fig. 2A, B and C). At 3 and 7 wpl, the average number of 5ht1a positive profiles per hemisection in the rostral spinal cord were similar to that of unlesioned animals and they were significantly inferior to the level of expression at 1 wpl (3 wpl: 156.2 ± 46.19; 7 wpl: 257.3 ± 63.29; ANOVA, $p<0.0001$) (Fig. 2A-E).

Quantification of the average number of 5ht1a profiles per hemisection in the caudal stump of the spinal cord at 1 wpl indicated a significant increase of 139% as compared to control values (unlesioned: 179.5 ± 11.01; 1 wpl: 428.6 ± 46.69; ANOVA, $p=0.0084$) (Fig. 2F-H). At 3wpl, the average number of 5ht1a positive profiles per hemisection was not significantly different from the values in 1 wpl or unlesioned animals (3 wpl: 298.6 ± 74.55) (Fig. 2B, F-H). At 7 wpl, the average number of 5ht1a profiles per section in the caudal spinal cord was significantly inferior to 1 wpl values (a 104% reduction) (7 wpl: 209.4 ± 34.16; ANOVA, $p=0.0084$) (Fig. 2F, G and H) and was not significantly different from the values in unlesioned animals (Fig. 2B, F and G).

A more detailed analysis was done by quantifying by zones, distinguishing between the periventricular layer (ependymocites and CSF-c cells) and the horizontally extended grey matter (interneurons and motoneurons). Quantification of the average number of 5-ht1a profiles in the periventricular layer of rostral stump of the spinal cord at 1 wpl indicated a significant increase of 162% as compared to control values (unlesioned: 67.19 ± 10.53; 1 wpl: 176.0 ± 13.72; ANOVA, p= 0.0002) (Fig. 3A-C). At 3 wpl, the average number of 5ht1a profiles was significantly inferior to 1 wpl values (a 69% reduction) (3 wpl: 54.50 ± 17.65; ANOVA, p=0.0002) and was not significantly different from the values in unlesioned animals (Fig. 3A). At 7 wpl, the average number of 5ht1a profiles was significantly inferior to 1 wpl values (a 46% reduction) (7 wpl: 94.67 ± 18.99; ANOVA, p=0.0002) and was not significantly different from the values in unlesioned animals (Fig. 3A, B and D). The average number of 5ht1a positive profiles at 1 wpl, 3 wpl and 7 wpl in the ventricular layer of caudal stump were not significantly different from the values in unlesioned animals (unlesioned: 67.19 ± 10.53; 1 wpl: 90.81 ± 16.93; 3 wpl: 52.20 ± 14.55; 7 wpl: 79.05 ± 10.19; ANOVA, p=0.2450) (Fig. 3B, E-G).

Quantification of the average number of 5ht1a profiles in interneurons and motoneurons of the rostral stump of the spinal cord at 1 wpl indicated a significant increase of 148% as compared to control values (unlesioned: 140.8 ± 5.902; 1 wpl: 349.3 ± 34.81; ANOVA, p= 0.0002) (Fig. 4A-C). At 3 wpl, the average number of 5ht1a profiles was significantly reduced respect to 1 wpl values (a 67% reduction) (3 wpl: 114.9± 30.93; ANOVA, p=0.0002) and was not significantly different from the values in unlesioned animals (Fig. 4A). At 7 wpl, the average number of 5ht1a profiles was significantly inferior to 1 wpl values (a 52% reduction) (7 wpl: 166.4 ± 42.83; ANOVA, p=0.0002) and was not significantly different from the values in unlesioned animals (Fig. 4A, B and D). Quantification of the average number of 5ht1a profiles in interneurons and motoneurons of caudal stump of the spinal cord at 1 wpl indicated a significant increase of 122% as compared to control values (unlesioned: 140.8 ± 5.902; 1 wpl: 312.3 ± 45.08; ANOVA, p= 0.0047) (Fig. 4E-G). At 3wpl, the average number of 5ht1a positive profiles per section was not significantly different from

the values in 1 wpl or unlesioned animals (3 wpl: 185.0± 35.64) (Fig. 4E). At 7 wpl, the average number of 5ht1a profiles was significantly inferior to 1 wpl values (a 54% reduction) (7 wpl: 141.2 ± 30.21; ANOVA, p= 0.0047) and was not significantly different from the values in unlesioned animals (Fig. 4B, E-G).

Discussion

Here, we report for the first time changes in the expression of the 5-HT_{1A} after a complete spinal cord transection in a regenerating vertebrate (i.e. a vertebrate species that spontaneously recovers locomotion and regenerates its spinal cord after SCI). The main findings of the present investigation are: (1) there is a marked general increase in the expression of the 5-HT_{1A} acutely (1 wpl) after a complete spinal cord transection both rostrally and caudally to the site of injury; (2) the expression of the 5-HT_{1A} returns to levels similar to those found in unlesioned animals a few weeks (7 wpl) after the injury both rostrally and caudally to the site of transection and (3) when looking at different spinal cord regions the cells in the periventricular layer respond in a different way rostrally (acute overexpression of the 5-ht1a) and caudally (no changes in the expression of the receptor).

Studies in mammals (Otoshi et al., 2009; Giroux et al., 1999) have shown that SCI induces an overexpression of the 5-HT_{1A} in lumbar levels after a thoracic SCI. This has been shown by immunofluorescence experiments after a spinal cord transection in rats (Otoshi et al., 2009) and by ligand binding autoradiography experiments after a spinal cord transection in cats (Giroux et al., 1999). So, present results in lampreys and previous results in mammals (Giroux et al., 1999; Otoshi et al., 2009) indicate that overexpression of the 5-HT_{1A} below the site of spinal transection is a common feature of regenerating and non-regenerating vertebrates and that this overexpression occurs both at the transcript (present study) and protein (Giroux et al., 1999; Otoshi et al., 2009) levels. In addition, both in cats (Giroux et al., 1999) and lampreys (present

study) the level of expression of the 5-HT1A in the caudal spinal cord returns to control levels in the chronic situation after SCI (8 to 21 weeks in cats (Giroux et al., 1999) and 7 weeks in lampreys (see above)).

In lampreys, we also observed a marked overexpression of the 5-HT_{1A} in the rostral spinal cord 1 week after the spinal cord transection (see above). Giroux and co-workers (1999) also investigated the expression of the 5-HT_{1A} at cervical levels after the thoracic transection in cats and reported the absence of any changes in expression at this rostral level (Giroux et al., 1999). This might reveal a significant difference between the situation in non-regenerating and regenerating vertebrates. It should be noted that this difference between cats and lampreys might also be due to differences in the methods used to study the expression of the 5-HT_{1A}, differences in the chosen time points after transection (2 or 4 weeks in cats (Giroux et al., 1999) and 1 week in lampreys (present study)) or differences in the distance from the site of injury. In any case, since this difference between cats and lampreys was not observed for the caudal spinal cord our results suggest that an overexpression of the 5-HT_{1A} in rostral levels after SCI could be part of the plastic events that lead to the recovery of function in a regenerating vertebrate like the sea lamprey as compared to non-regenerating mammals.

When looking at the changes in receptor expression by zones (periventricular region and horizontally extended grey matter) our results showed that these spinal cord regions basically followed the same pattern of changes detected in the spinal cord as a whole with exception of the caudal periventricular layer. In this spinal cord region, no significant differences were observed 1 wpl as compared to the significant overexpression detected in the rostral periventricular layer. This suggests that different molecular signals act on the periventricular cells of the rostral and caudal spinal cord after a complete spinal cord transection and thus affecting their response to the injury.

The cause/s for these changes in the expression of the 5-HT_{1A} after SCI are not completely clear at the moment. However, present and previous results indicate that the lack of serotonin due to the degeneration of

serotonergic fibers after the injury could induce a compensatory overexpression of the 5-HT_{1A}. This is supported by the study by Laporte and coworkers (1995) in which the authors observed an increase in the expression of the 5-HT_{1A} in the SC of rats after a nearly complete loss of serotonergic fibers due to a treatment with the toxin 5,7-dihydroxytryptamine. Along this line, the fact that in lampreys (present results) the return to control levels was faster and more pronounced rostrally than caudally could be related to the slower and incomplete serotonergic reinnervation of the caudal spinal cord after the injury (Cohen et al., 2005; Cornide-Petronio et al., 2011). However, the fact that in chronically injured cats the expression also returns to control levels could indicate that the lack of agonist only has an acute effect on the expression of the receptor. In rats, the overexpression of the 5-HT_{1A} after a spinal cord transection is completely neutralized when the spinal cord is deafferented (i.e. there is a lack of sensory input) (Otoshi et al., 2009). So, present and previous data suggest that the upregulation in 5-HT_{1A} that occurs after a spinal cord transection in vertebrates might depend both on the lack of agonist and also on the presence of sensory input.

If the upregulation of 5-HT_{1A} expression after SCI is beneficial for the recovery of function is not known at the moment. However, the fact that it occurs in a regenerating vertebrate like the sea lamprey suggests that it could be one of the plastic events leading to a recovery of function after injury in this species. In mammals, treatment with the 5-HT_{1A} specific agonist 8-OH-DPAT acutely elicits some locomotor-like movements in completely spinal cord transected mice (Lapointe and Guertin, 2008) and improves long-term locomotor recovery in rats with a thoracic complete transection (Antri et al., 2003), increases bladder capacity and suppresses bladder reflex activity in cats with spinal cord injury (Gu et al., 2004; Tai et al., 2006), enhances respiratory activity in rats with a cervical or thoracic spinal cord injury (Teng et al., 2003; Choi et al., 2005; Zimmer and Goshgarian, 2006) and reduces allodynia and hyperalgesia in rats with spinal cord injury (Colpaert et al., 2004; Hains et al., 2003; Wu et al., 2003). So, based on the observations in lampreys and previous data indicating that activation of the receptor is beneficial after

SCI, a higher availability of 5-HT_{1A} could be beneficial for behavioural recovery to compensate for a reduced availability of the agonist.

Literatured cited

Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R, Rodicio MC. (2007) Development of the serotonergic system in the central nervous system of the sea lamprey. J Chem Neuroanat. 34: 29-46.

Antri M, Mouffle C, Orsal D, Barthe JY. (2003) 5-HT1A receptors are involved in short- and long-term processes responsible for 5-HT-induced locomotor function recovery in chronic spinal rat. Eur J Neurosci. 18: 1963-1972.

Barreiro-Iglesias A, Cornide-Petronio ME, Anadón R, Rodicio MC. (2009) Serotonin and GABA are colocalized in restricted groups of neurons in the larval sea lamprey brain: insights into the early evolution of neurotransmitter colocalization in vertebrates. J Anat. 215: 435-443.

Barreiro-Iglesias A, Villar-Cerviño V, Anadón R, Rodicio MC. (2008) Development and organization of the descending serotonergic brainstem-spinal projections in the sea lamprey. J Chem Neuroanat. 36: 77-84.

Biró Z, Hill RH, Grillner S. (2006) 5-HT Modulation of identified segmental premotor interneurons in the lamprey spinal cord. J Neurophysiol. 96: 931-935.

Buchanan JT. (1999) The roles of spinal interneurons and motoneurons in the lamprey locomotor network. Prog Brain Res. 123: 311-321.

Choi H, Liao WL, Newton KM, Onario RC, King AM, Desilets FC, Woodard EJ, Eichler ME, Frontera WR, Sabharwal S, Teng YD. (2005) Respiratory

abnormalities resulting from midcervical spinal cord injury and their reversal by serotonin 1A agonists in conscious rats. J Neurosci. 25: 4550-4559.

Cohen AH, Abdelnabi M, Guan L, Ottinger MA, Chakrabarti L.. (2005) Changes in distribution of serotonin induced by spinal injury in larval lampreys: evidence from immunohistochemistry and HPLC. J Neurotrauma. 22: 172-188.

Cohen AH, Kiemel T, Pate V, Blinder J, Guan L.. (1999) Temperature can alter the function outcome of spinal cord regeneration in larval lampreys. Neuroscience. 90: 957-965.

Colpaert FC, Wu WP, Hao JX, Royer I, Sautel F, Wiesenfeld-Hallin Z, Xu XJ. (2004) High-efficacy 5-HT1A receptor activation causes a curative-like action on allodynia in rats with spinal cord injury. Eur J Pharmacol. 497: 29-33.

Cornide-Petronio ME, Anadón R, Barreiro-Iglesias A, Rodicio MC. (2012) Serotonin 1A receptor (5-HT1A) of the sea lamprey: cDNA cloning and expression in the central nervous system. Brain Struct Funct. DOI: 10.1007/s00429-012-0461-y.

Cornide-Petronio ME, Ruiz MS, Barreiro-Iglesias A, Rodicio MC. (2011) Spontaneous regeneration of the serotonergic descending innervation in the sea lamprey after spinal cord injury. J Neurotrauma. 28: 2535-2540.

D'Amico JM, Murray KC, Li Y, Chan KM, Finlay MG, Bennett DJ, Gorassini MA. (2013) Constitutively active 5-HT2/α1 receptors facilitate muscle spasms after human spinal cord injury. J Neurophysiol. 109: 1473-1484.

Fuller DD, Baker-Herman TL, Golder FJ, Doperalski NJ, Watters JJ, Mitchell GS. (2005) Cervical spinal cord injury upregulates ventral spinal 5-HT2A receptors. J Neurotrauma. 22: 203-213.

Giroux N, Rossignol S, Reader TA. (1999) Autoradiographic study of alpha1- and alpha2-noradrenergic and serotonin1A receptors in the spinal cord of normal and chronically transected cats. J Comp Neurol. 406: 402-414.

Grider MH, Chen Q, Shine HD. (2006) Semi-automated quantification of axonal densities in labeled CNS tissue. J Neurosci Methods. 155: 172-179.

Grillner S, Wallen P, Saitoh K, Kozlov A, Robertson B. (2008) Neural bases of goal-directed locomotion in vertebrates–an overview. Brain Res Rev. 57: 2-12.

Gu B, Olejar KJ, Reiter JP, Thor KB, Dolber PC. (2004) Inhibition of bladder activity by 5-hydroxytryptamine1 serotonin receptor agonists in cats with chronic spinal cord injury. J Pharmacol Exp Ther. 310: 1266-1272.

Hains BC, Willis WD, Hulsebosch CE. (2003) Serotonin receptors 5-HT1A and 5-HT3 reduce hyperexcitability of dorsal horn neurons after chronic spinal cord hemisection injury in rat. Exp Brain Res. 149: 174-186.

Hill RH, Svensson E, Dewael Y, Grillner S. (2003) 5-HT inhibits N-type but not L-type Ca(2+) channels via 5-HT1A receptors in lamprey spinal neurons. Eur J Neurosci. 18: 2919-2924.

Husch A, Van Patten GN, Hong DN, Scaperotti MM, Cramer N, Harris-Warrick RM. (2012) Spinal cord injury induces serotonin supersensitivity without increasing intrinsic excitability of mouse V2a interneurons. J Neurosci. 32: 13145-13154.

Jin LQ, Zhang G, Jamison C Jr, Takano H, Haydon PG, Selzer ME. (2009) Axon regeneration in the absence of growth cones: acceleration by cyclic AMP. J Comp Neurol. 515: 295-312.

Kuscha V, Barreiro-Iglesias A, Becker CG, Becker T. (2012) Plasticity of tyrosine hydroxylase and serotonergic systems in the regenerating spinal cord of adult zebrafish. J Comp Neurol. 2012 520: 933-951.

Lapointe NP, Guertin PA. (2008) Synergistic effects of D1/5 and 5-HT1A/7 receptor agonists on locomotor movement induction in complete spinal cord-transected mice. J Neurophysiol. 100: 160-168.

Laporte AM, Fattaccini CM, Lombard MC, Chauveau J, Hamon M. (1995) Effects of dorsal rhizotomy and selective lesion of serotonergic and noradrenergic systems on 5-HT1A, 5-HT1B, and 5-HT3 receptors in the rat spinal cord. J Neural Transm Gen Sect.100: 207-223.

Murray KC, Nakae A, Stephens MJ, Rank M, D'Amico J, Harvey PJ, Li X, Harris RL, Ballou EW, Anelli R, Heckman CJ, Mashimo T, Vavrek R, Sanelli L, Gorassini MA, Bennett DJ, Fouad K. (2010) Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. Nat Med. 16: 694-700.

Navarrett S, Collier L, Cardozo C, Dracheva S. (2012) Alterations of serotonin 2C and 2A receptors in response to T10 spinal cord transection in rats. Neurosci Lett. 506: 74-78.

Otoshi CK, Walwyn WM, Tillakaratne NJ, Zhong H, Roy RR, Edgerton VR. (2009) Distribution and localization of 5-HT(1A) receptors in the rat lumbar spinal cord after transection and deafferentation. J Neurotrauma. 26: 575-584.

Pearlstein E, Bras H, Deneris ES, Vinay L. (2011) Contribution of 5-HT to locomotion - the paradox of Pet-1(-/-) mice. Eur J Neurosci. 33: 1812-1822.

Perrier JF, Rasmussen HB, Christensen RK, Petersen AV. (2013) Modulation of the intrinsic properties of motoneurons by serotonin. Curr Pharm Des. Jan 21.

Perrin FE, Gerber YN, Teigell M, Lonjon N, Boniface G, Bauchet L, Rodriguez JJ, Hugnot JP, Privat AM. (2011) Anatomical study of serotonergic innervation and 5-HT(1A) receptor in the human spinal cord. Cell Death Dis. 2: e218.

Ren LQ, Wienecke J, Chen M, Møller M, Hultborn H, Zhang M. (2013) The time course of serotonin 2C receptor expression after spinal transection of rats: an immunohistochemical study. Neuroscience. 236: 31-46.

Rodicio MC, Barreiro-Iglesias A. (2012) Lampreys as an animal model in regeneration studies after spinal cord injury. Rev Neurol. 55: 157-166.

Roederer E, Goldberg NH, Cohen MJ. (1983) Modification of retrograde degeneration in transected spinal axons of the lamprey by applied DC current. J Neurosci. 3: 153-160.

Ronan M. (1989) Origins of the descending spinal projections in petromyzontid and myxinoid agnathans. J Comp Neurol. 281: 54-68.

Shifman MI, Jin L-Q, Selzer ME. (2007) Regeneration in the lamprey spinal cord. In: Becker CG, Becker T (eds) Model organisms in spinal cord regeneration. Wiley-VCH Verlag, Weinheim, pp 229-262.

Swain GP, Snedeker JA, Ayers J, Selzer ME. (1993) Cytoarchitecture of spinal-projecting neurons in the brain of the larval sea lamprey. J Comp Neurol. 336: 194-210.

Tai C, Miscik CL, Ungerer TD, Roppolo JR, de Groat WC. (2006) Suppression of bladder reflex activity in chronic spinal cord injured cats by activation of serotonin 5-HT1A receptors. Exp Neurol. 199: 427-437.

Teng YD, Bingaman M, Taveira-DaSilva AM, Pace PP, Gillis RA, Wrathall JR. (2003) Serotonin 1A receptor agonists reverse respiratory abnormalities in spinal cord-injured rats. J Neurosci. 23: 4182-4189.

Ung RV, Landry ES, Rouleau P, Lapointe NP, Rouillard C, Guertin PA. (2008) Role of spinal 5-HT2 receptor subtypes in quipazine-induced hindlimb movements after a low-thoracic spinal cord transection. Eur J Neurosci. 28: 2231-2242.

Wang D, Grillner S, Wallén P. (2011) 5-HT and dopamine modulates CaV1.3 calcium channels involved in postinhibitory rebound in the spinal network for locomotion in lamprey. J Neurophysiol. 105: 1212-1224.

Wikström M, Hill R, Hellgren J, Grillner S. (1995) The action of 5-HT on calcium-dependent potassium channels and on the spinal locomotor network in lamprey is mediated by 5-HT1A-like receptors. Brain Res. 678: 191-199.

Wu WP, Hao JX, Xu XJ, Wiesenfeld-Hallin Z, Koek W, Colpaert FC. (2003) The very-high-efficacy 5-HT1A receptor agonist, F 13640, preempts the development of allodynia-like behaviors in rats with spinal cord injury. Eur Pharmacol. 478: 131-137.

Yin HS, Selzer ME. (1983) Axonal regeneration in lamprey spinal cord. J Neurosci. 3: 1135-1144.

Zhang W, Grillner S. (2000) The spinal 5-HT system contributes to the generation of fictive locomotion in lamprey. Brain Res. 879: 188-192.

Zhang G, Jin LQ, Sul JY, Haydon PG, Selzer ME. (2005) Live imaging of regenerating lamprey spinal axons. Neurorehabil Neural Repair. 19: 46-57.

Zimmer MB, Goshgarian HG. (2006) Spinal activation of serotonin 1A receptors enhances latent respiratory activity after spinal cord injury. J Spinal Cord Med. 29: 147-155.

Abbreviations

5-HT	Serotonin or 5-hydroxytriptamine
5-ht1a	Serotonin 1A receptor gene
5-HT_{1A}	Serotonin 1A receptor protein

CPG	Central pattern generator
CSF-c	Cerebrospinal fluid-contacting
SC	Spinal cord
SCI	Spinal cord injury
Wpl	Week post-lesion

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FIGURES

Figure 1 - A) Photomicrograph of a transverse section of the spinal cord of a large larval sea lamprey (body length of 100 mm) showing the distribution the 5-ht1a transcript. B) Schematic drawing of a transverse section of the spinal cord of a larval sea lamprey (100 mm in length) representing the expression of the 5-ht1a transcript. *dC,* dorsal cell; *iN,* interneurons; *mN,* motoneurons; *PL,* periventricular layer. *Scale bars*: 50 µm.

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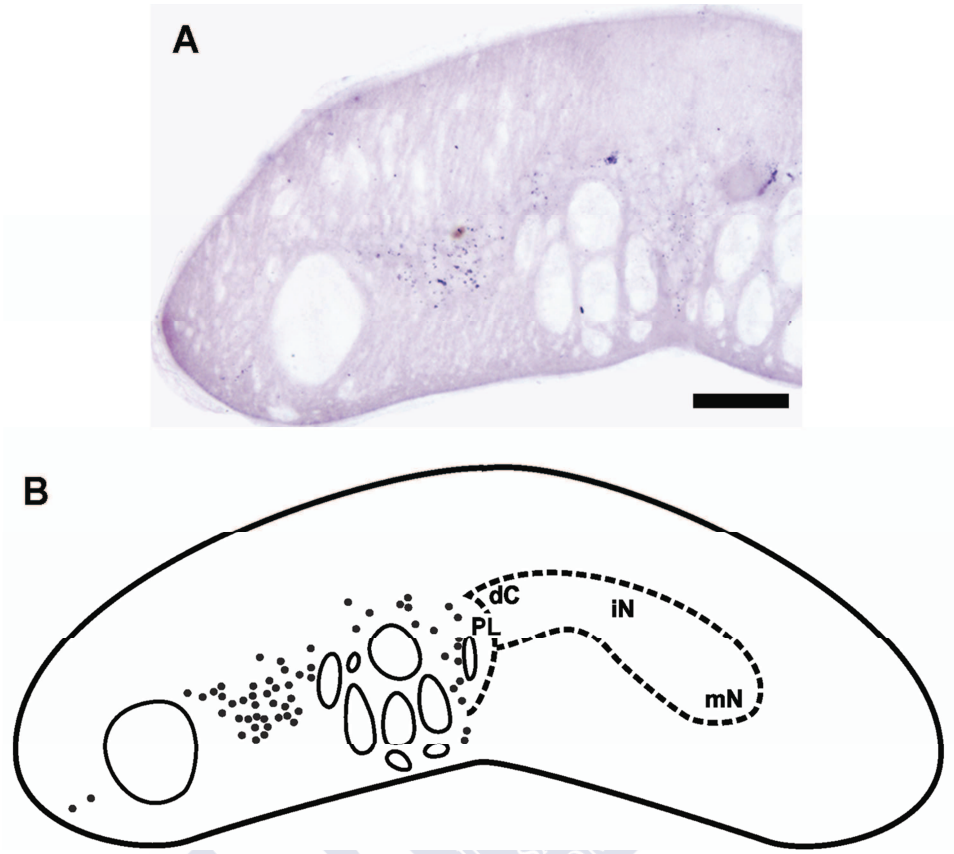

FIGURE 1

Figure 2 - Quantitative changes in the expression of the 5-HT_{1A} in the rostral (A-E) and caudal (F-J) spinal cord after a complete spinal cord transection. A) Graphic showing significant changes (*asterisks*) in the number of 5-HT_{1A} positive profiles in the rostral spinal cord: control: 179.5 ± 11.01; 1wpl: 575.1 ± 40.74; 3 wpl: 156.2 ± 46.19; 7 wpl: 257.3 ± 63.29 ANOVA, $p<0.0001$. B-E). Photomicrographs of transverse sections of the rostral spinal cord showing the distribution of the 5-ht1a transcripts in control, 1 wpl, 3 wpl and 7 wpl animals are shown in panels B-E. F) Graphic showing significant changes (*asterisks*) in the number of 5-HT_{1A} positive profiles in the caudal spinal cord: control: 179.5 ± 11.01; 1 wpl: 428.6 ± 46.69; 3 wpl: 298.6 ± 74.55; 7 wpl: 209.4 ± 34.16; ANOVA, $p=0.0084$. Photomicrographs of transverse sections of the caudal spinal cord showing the distribution of the 5-ht1a transcripts in control, 1 wpl, 3 wpl and 7 wpl animals are shown in panels G-I. *Scale bars*: 50 µm.

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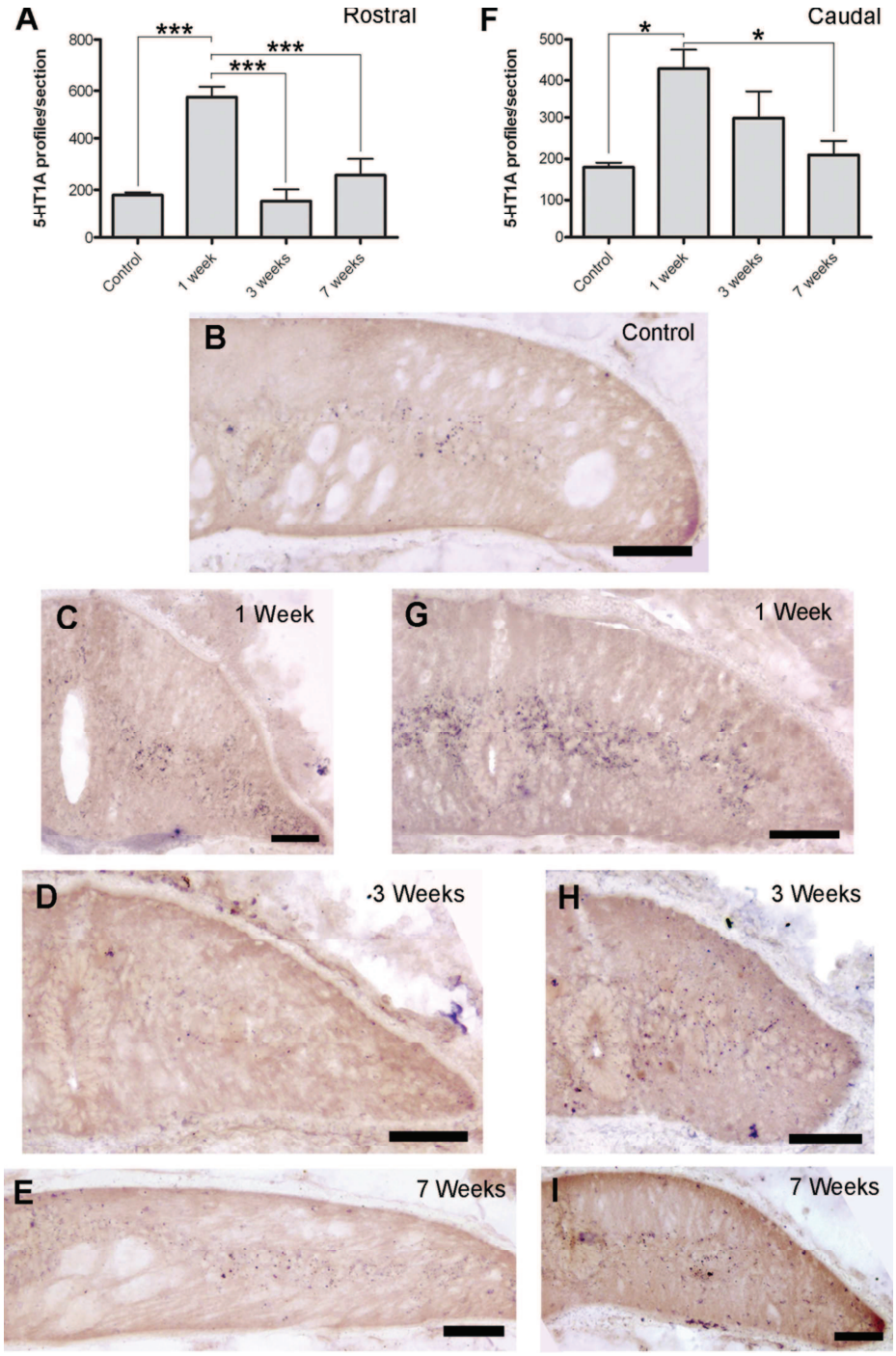
A
Rostral
5-HT1A profiles/section
800
600
400
200
0
***
***
***
Control
1 week
3 weeks
7 weeks
F
Caudal
5-HT1A profiles/section
500
400
300
200
100
0
*
*
Control
1 week
3 weeks
7 weeks
B
Control
C
1 Week
G
1 Week
D
3 Weeks
H
3 Weeks
E
7 Weeks
I
7 Weeks

FIGURE 2

Figure 3 - Quantitative changes in the expression of the 5-HT_{1A} in the rostral (A-D) and caudal (E-G) periventricular layer of the spinal cord after a complete spinal cord transection. A) Graphic showing significant changes (*asterisks*) in the number of 5-HT_{1A} positive profiles in the rostral spinal cord: control: 67.19 ± 10.53; 1 wpl: 176.0 ± 13.72; 3 wpl: 54.50 ± 17.65; 7 wpl: 94.67 ± 18.99; ANOVA, p= 0.0002. B-D). Photomicrographs of transverse sections of the rostral spinal cord showing the distribution of the 5-ht1a transcripts in control, 1 wpl, 3 wpl and 7 wpl animals are shown in panels B-D. E) Graphic showing no significant changes in the number of 5-HT_{1A} positive profiles in the caudal spinal cord: control: 67.19 ± 10.53; 1 wpl: 90.81 ± 16.93; 3 wpl: 52.20 ± 14.55; 7 wpl: 79.05 ± 10.19; ANOVA, p=0.2450. F-G) Photomicrographs of transverse sections of the caudal spinal cord showing the distribution of the 5-ht1a transcripts in control, 1 wpl, 3 wpl and 7 wpl animals are shown in panels F-G. *Scale bars*: 50 µm

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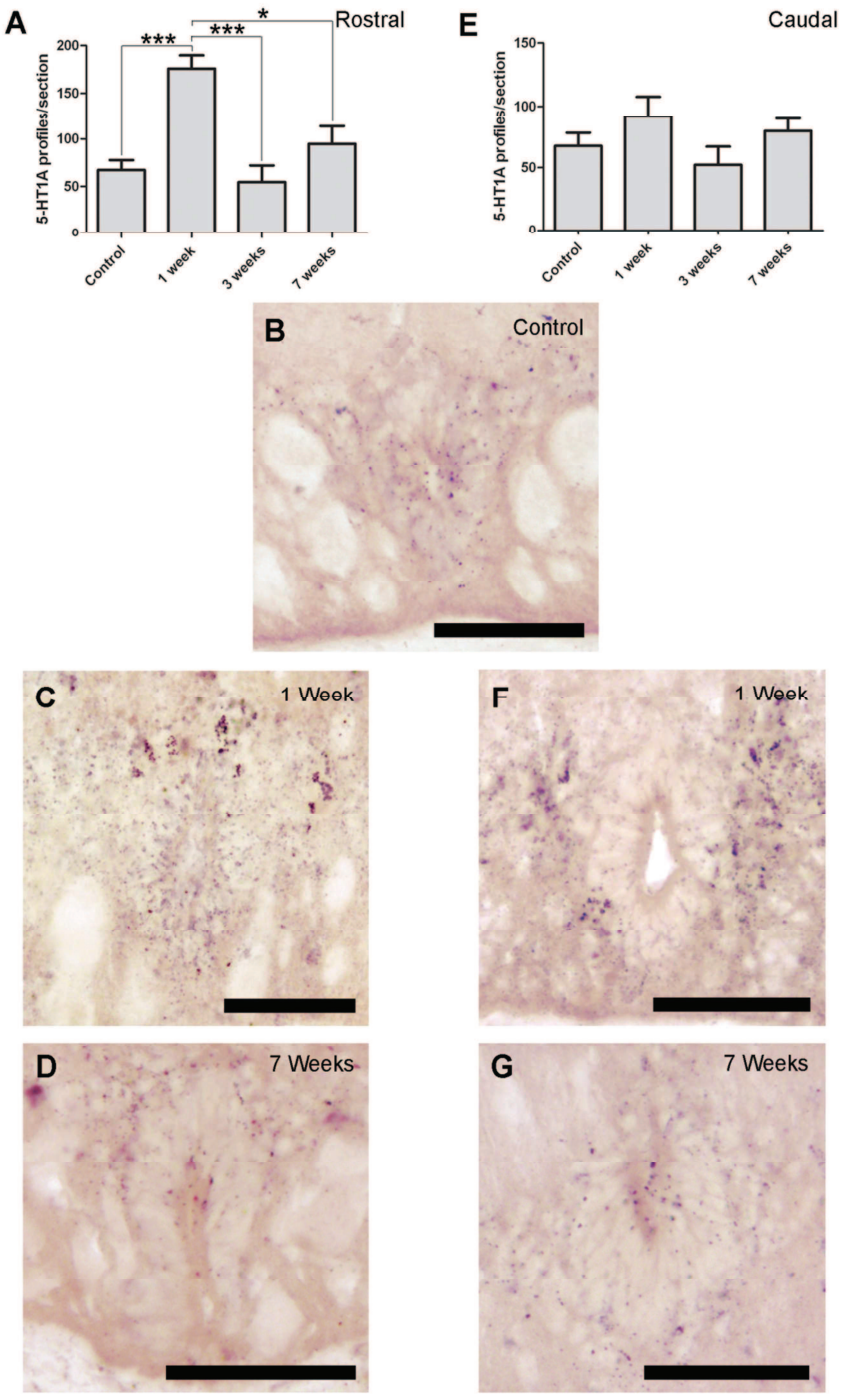
A
Rostral
5-HT1A profiles/section
200
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C
1 Week
F
1 Week
D
7 Weeks
G
7 Weeks

Figure 3

Figure 4 - Quantitative changes in the expression of the 5-HT_{1A} in the rostral (A-D) and caudal (E-G) interneurons and motoneurons of the spinal cord after a complete spinal cord transection. A) Graphic showing significant changes (*asterisks*) in the number of 5-HT_{1A} positive profiles in the rostral spinal cord: control: 140.8 ± 5.902; 1 wpl: 349.3 ± 34.81; 3 wpl: 114.9± 30.93; 7 wpl: 166.4 ± 42.83; ANOVA, p= 0.0002. B-D). Photomicrographs of transverse sections of the rostral spinal cord showing the distribution of the 5-ht1a transcripts in control, 1 wpl, 3 wpl and 7 wpl animals are shown in panels B-D. E) Graphic showing significant changes (*asterisks*) in the number of 5-HT_{1A} positive profiles in the caudal spinal cord: control: 140.8 ± 5.902; 1 wpl: 312.3 ± 45.08; 3 wpl: 185.0± 35.64; 7 wpl: 141.2 ± 30.21; ANOVA, p= 0.0047. F-G) Photomicrographs of transverse sections of the caudal spinal cord showing the distribution of the 5-ht1a transcripts in control, 1 wpl, 3 wpl and 7 wpl animals are shown in panels F-G. *Scale bars*: 50 µm.

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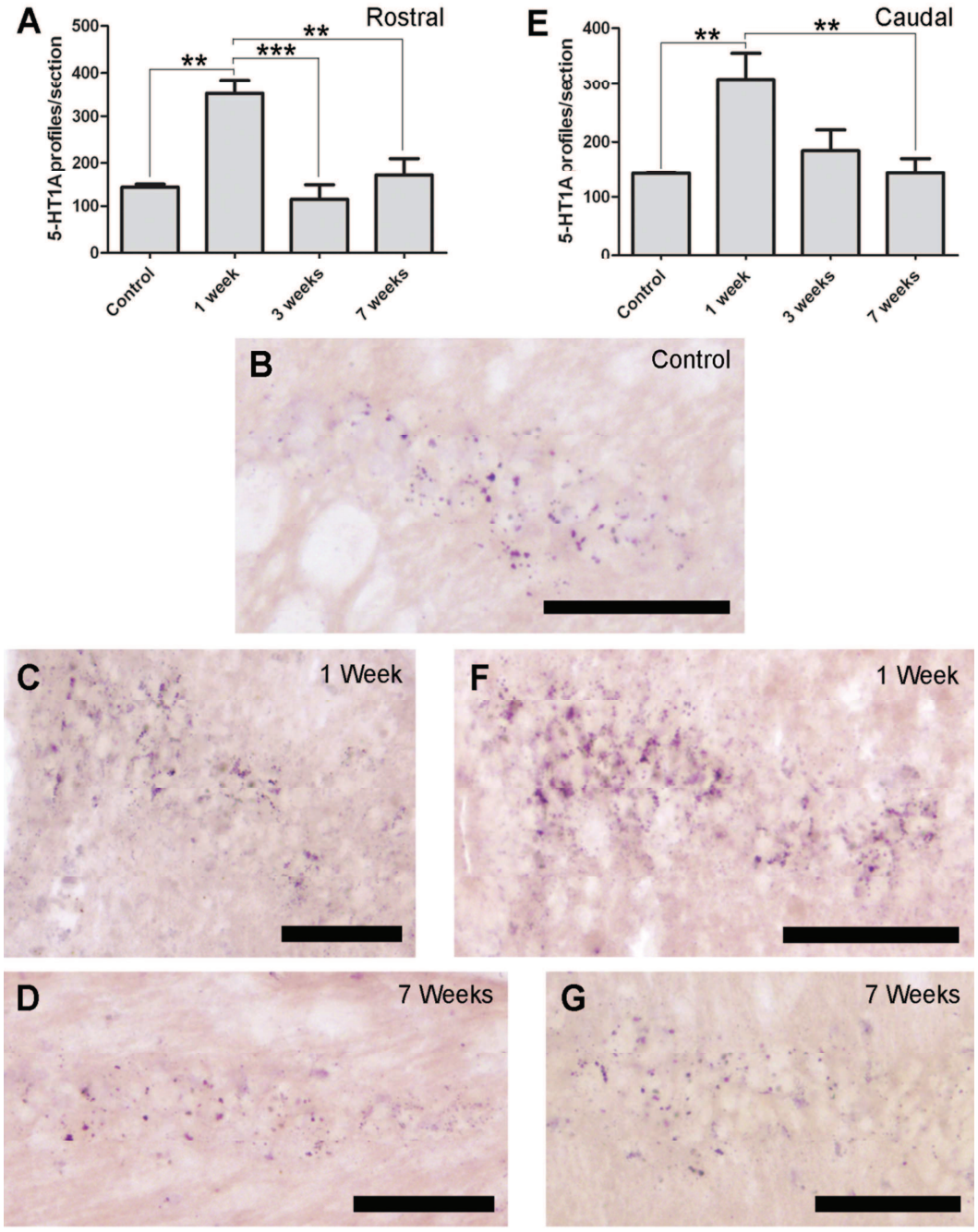
A
Rostral
5-HT1A profiles/section
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C
1 Week
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1 Week
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FIGURE 4

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CHAPTER 4
Spontaneous regeneration of the serotonergic descending innervation in the sea lamprey after spinal cord injury

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SPONTANEOUS REGENERATION OF THE SEROTONERGIC DESCENDING INNERVATION IN THE SEA LAMPREY AFTER SPINAL CORD INJURY

Introduction

After spinal cord injury (SCI), axons in the mammalian central nervous system do not spontaneously regenerate across the trauma zone and are thus unable to reconnect to their appropriate targets, which lead to permanent loss of function. Studies in animal models in which spinal cord (SC) is able to regenerate spontaneously may provide important clues for establishing new therapies for patients with SCI (Barreiro-Iglesias, 2010). In contrast to mammals, lampreys can recover normal-appearing locomotion after a complete SC transection and the spinal axons regenerate selectively in their correct paths (Rovainen, 1976; Selzer, 1978; Wood and Cohen, 1979). In lampreys, axonal regeneration is directionally specific (Mackler et al., 1986; Yin et al., 1984) and leads to selective synapse formation, with normal types of target neurons distal to the lesion site (Mackler and Selzer, 1987). Moreover, spinal axons of lampreys grow preferentially through a hemisection scar rather than around it (Lurie and Selzer, 1991a, 1991b, 1991c), suggesting that reactive glial cells support axon regeneration. However, previous studies have shown that only about 50% of all injured descending axons regenerate through the scar (Davis and McClellan, 1994; Jacobs et al., 1997). These features favour the use of lampreys as a vertebrate animal model to study the intrinsic molecular mechanisms whereby neurons favour or impede the regeneration of descending axons in the same permissive environment after SCI (e.g. Shifman and Selzer, 2000, 2007; Shifman et al., 2009; Jin et al., 2009).

The presence of serotonergic cells with projections to the SC has been reported in the rhombencephalon (raphe nuclei) of different jawed

vertebrates, such as zebrafish (McLean and Fetcho, 2004a, b), amphibians (van Mier et al., 1986; Woolston et al., 1994) and rats (Aitken and Törk, 1988). Descending serotonergic projections to the SC play a role in the modulation of locomotion at spinal levels in both mammals (see Jordan et al., 2008) and lampreys (see Grillner et al., 2008). Moreover, the development and anatomical organization of the serotonergic SC-projecting neurons in the brainstem is highly conserved between lampreys and mammals (Barreiro-Iglesias et al., 2008). In mammals, paralysis caused by SCI results from a loss of supraspinal tracts that underlie voluntary initiation of locomotion (e.g. corticospinal or reticulospinal glutamatergic tracts (Rekling et al., 2000; Murray et al., 2010)). Furthermore, the paralysis that occurs following SCI also occurs because of loss of descending brainstem tracts that provide spinal neurons with their major source of neuromodulators, such as serotonin (or 5-hydroxytriptamine; 5-HT) (Murray et al., 2010). In mammals, a loss of brainstem-derived serotonin after SCI acutely reduces motoneuron excitability and accordingly depresses all motor functions (Murray et al., 2010). Recent studies have also shown that, in rodents, autonomic dysreflexia after SCI is associated with the lack of serotonergic axonal reinnervation (Cormier et al., 2010). Regeneration of the serotonergic descending projections will therefore probably benefit therapies designed to facilitate recovery of function after SCI.

Spontaneous regeneration of the serotonergic descending projections after SCI has not been experimentally demonstrated in any vertebrate species. Immunofluorescence experiments carried out at spinal levels in the sea lamprey have shown that serotonergic processes reinnervate the SC below the lesion site after a complete SC transection (Cohen et al., 2005). However, regeneration of the severed serotonergic projections from brainstem neurons was not conclusively demonstrated in the latter study. Thus, recovery of serotonergic innervation below the site of injury may be due to axonal sprouting from intrinsic spinal neurons.

In the present study, we combined immunofluorescence and tract-tracing methods to show that descending serotonergic neurons of the caudal rhombencephalon regenerate their axons across the lesion site after complete

SC transection in the sea lamprey. This study offers a new and interesting model for investigating the intrinsic molecular mechanisms that favour regeneration of the descending serotonergic axons in vertebrates, which may provide valuable information for developing new therapies for patients with SCI.

Materials and Methods

Mature larval sea lampreys (100-140 mm in body length) were caught in the river Ulla (Galicia, northwest Spain) and maintained in fresh water with a bed of river sediment and appropriate ventilation and temperature conditions until use. Before experiments, all animals were deeply anesthetized with 0.05% benzocaine (Sigma, St. Louis, MO) in fresh water. The experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and conformed to the European Union (86/609/EEC) and Spanish (Royal Decree 223/1998) regulations for the care and handling of animals in research.

In experimental animals (n= 5), the SC was exposed from the dorsal midline at the level of the fifth gill, complete SC transection was performed with a scalpel and the SC cut ends were visualized under the microscope. The incision was closed with Histoacryl tissue glue (B. Braun Surgical, Tuttlingen, Germany). Each transected animal was examined 24 h after surgery to confirm that there was no movement caudal to the lesion site. Transection was tentatively considered complete if on stimulation of the head, an animal could move only its head and body rostral to the lesion site. Animals were allowed to recover in fresh water tanks at 19-20^{o}C, since lampreys are known to be able to recover full functionality at this temperature (Cohen et al., 1999). After a recovery period of five months, the SC was again exposed and crystals of the tracer Neurobiotin (NB; of 322.8 Da molecular weight Vector, Burlingame, CA) were applied with the aid of a minute pin (00) 1 mm caudal to the injury site,

(at the level of the sixth gill) (Figs. 1A-C). The incision was again closed with Histoacryl and the animals were maintained at 4ºC with appropriate aeration conditions in lamprey Ringer solution of the following composition: 137 mM NaCl, 2.9 mM KCl, 2.1 mM CaCl2, 2 mM HEPES, for 5 days to allow transport of the tracer.

After the incubation period, larvae were deeply anesthetized, and the heads (from the snout to the seventh gill) were fixed by immersion in 4% paraformaldehyde in 0.4 M Tris buffer pH 7.4 (TBS) for 6 h. The samples were rinsed in TBS and then cryoprotected with 30% sucrose in TBS overnight, embedded in Tissue Tek (Sakura, Torrance, CA), frozen in nitrogen-cooled isopentane, and cut serially on a cryostat (20 µm thick) in transverse (from the snout to the third gill) and horizontal (from the third gill to the seventh gill) planes. Sections were incubated with a rabbit polyclonal anti-5-HT antibody (dilution 1:2500; Incstar, Still Water, MN) at 4ºC for 72 h. For indirect immunofluorescence detection of 5-HT, Cy3-conjugated goat anti-rabbit antibody (dilution 1:200; Millipore, Temecula, CA) was used (incubation time: 1 h at room temperature). All antibodies were diluted in TBS containing 15% normal goat serum and 0.2% Triton X-100 as detergent. For indirect detection of the tracer the sections were incubated at room temperature for 4 h with fluorescein isothiocyanate (FITC)-labelled avidin D (Vector Laboratories, Burlingame, CA) previously diluted to 1:1000 with TBS containing 0.3% Triton X-100. Sections were rinsed in distilled water and then coverslipped with mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA).

Photomicrographs were taken with a spectral confocal laser microscope (Leica TCS-SP2), and adjusted for brightness and contrast with Adobe Photoshop 7 or CS2 software. Cell number and sizes were counted and measured in confocal stacks with the aid of Leica confocal software (LITE). To avoid counting cells twice, series of photographs were aligned with LITE, and the positions of double labelled cells in pairs of consecutive sections were compared. This unbiased approach made it unnecessary to introduce any correction factor for possible overestimation of cell numbers. The total number

of serotonergic descending cells was counted in each animal and the minor axes of these cells were measured. Statistical comparisons were performed by a *Student's t*-test, with IBM SPSS 18.0 software. Data are reported as means ± standard deviations.

Control larvae (n=3) were processed as above for NB labelling at the level of the sixth gill and serotonin immunofluorescence, but without prior transection of the SC at the level of the fifth gill. In order to confirm that the neurons labelled with NB in injured animals actually correspond to neurons that reinnervated levels below the lesion site, some larvae (n=4) were processed in the same way as the experimental larvae, but the tracer NB was applied one week post-injury, when severed axons are suffering retraction but regrowth has not started (see Shifman et al., 2007). In order to determine whether the serotonergic neurons labelled with NB after the SC transection actually correspond to neurons with regenerated axons and not to non-injured neurons that regrowth their axons from above, some larvae (n=3) were processed in the same way as the experimental group, but the tracer Fluoro-gold (FG; molecular weight, 532.6 Da: Fluorochrome LLC, Denver, CO USA) was applied to the SC at the level of the fifth gill at the time of SC transection.

Results

Serotonin-immunoreactive (-ir) fibers of the SC have two origins in the sea lamprey: intrinsic fibers arising from serotonin-ir cells of the ventral margin of the SC (Abalo et al., 2007; Barreiro-Iglesias et al., 2009) and extrinsic serotonin-ir fibers of supraspinal origin (Barreiro-Iglesias et al., 2008). We have previously reported the development and organization of the descending serotonergic projections to the SC of the sea lamprey by means of combined tract-tracing and immunofluorescence techniques (Barreiro-Iglesias et al., 2008). In mature larval sea lampreys, two brainstem nuclei bear serotonin-ir cells that project to the rostral SC (at the level of the second/third gill, which is

close to the brainstem-SC transition), the isthmic and vagal serotonergic nuclei (Fig. 1D; Barreiro-Iglesias et al., 2008). Here, we performed control experiments in which the tracer NB was applied at the level of the sixth gill of non-injured animals. In these animals, serotonergic descending cells were only observed in the caudal portion of the vagal nucleus of the caudal rhombencephalon (Fig. 1D). The number of double labelled cells in the vagal nucleus was 18.7 ± 4.2 (n=3), and the size of these descending serotonin-ir neurons was 11.3 ± 2.6 μm in the minor axis (Fig. 2A-D). The experiments in control larvae revealed that the serotonergic descending cells of the isthmic nucleus probably only project to the rostral SC (at the level of the second/third gill), whereas some serotonergic descending cells of the vagal nucleus project to more caudal SC levels (at the level of the sixth gill).

In experimental larvae, complete SC transection was done at the level of the fifth gill to ensure that all serotonergic descending neurons projecting to the SC at the level of the sixth gill were axotomized. Five months post-injury and after application of the tracer NB at the level of the sixth gill (1 mm caudal to the injury site), double labelled serotonin-ir cells were also observed in the vagal nucleus (Fig. 2E-H) and not in the isthmic nucleus. As in normal animals, serotonin-ir descending neurons of the vagal nucleus with regenerated axons were observed in the caudal portion of the nucleus. The number of serotonin-ir neurons labelled by the tracer NB five months after complete SC transection in the vagal nucleus was 7 ± 1.4 (n=5), and the size of these neurons was 8.7 ± 1.8 μm in the minor axis. Statistical analysis revealed a significant reduction in the size of neurons between control and experimental larvae ($P < 0.05$). When the tracer NB was applied at the level of the sixth gill to larvae one week post-injury (n=4), no double labelled cells were observed in the 5-HT-ir the vagal nucleus or in any other brain region. In larvae in which the tracer FG was applied at the level of the fifth gill at the time of SC transection and NB was applied at the level of the sixth gill five months later, between 1 and 3 serotonin-ir neurons labelled by both FG and NB were observed five months after the injury in the vagal nucleus (n=3; Fig. 2I). In addition, serotonin-ir

cells only labelled by NB (6 ± 4.3) or only labelled with FG (9.5 ± 3.5) were also observed (data not shown).

Discussion

Previous studies in invertebrates have shown the spontaneous regeneration of axotomized serotonergic neurons (Murphy et al., 1985; Chiasson et al., 1994; Koert et al., 2001), but this phenomenon has not previously been described in any vertebrate species. This is therefore the first experimental study reporting the spontaneous regeneration of at least part of the serotonergic descending projection after complete SC transection in a vertebrate species. A previous study in the sea lamprey reported the recovery of serotonin expression below the site of injury after complete SC transection in the mid body region (Cohen et al., 2005). However, in the previous study regeneration of the serotonergic descending projection was not experimentally assessed. Since the lamprey SC possesses serotonergic neurons (Harris-Warrick et al., 1985; Abalo et al., 2007; Barreiro-Iglesias et al., 2009), it was not known if reinnervation of the SC below the site of injury was due to actual regeneration of the severed descending axons or to compensatory reinnervation from intraspinal serotonergic cells. Here we experimentally demonstrated that, after complete SC transection, at least some of the serotonergic descending neurons of the caudal rhombencephalon regenerate to levels caudal to the lesion site.

The number of serotonergic descending cells in the vagal nucleus revealed by application of FG at the level of the fifth gill was approximately half the number revealed by application of NB at the sixth gill level. This strongly suggests that FG was metabolized or not transported in about 50% of the neurons projecting to the level of the fifth gill. With this in mind, comparison of the results obtained in control and experimental animals revealed some interesting findings: 1) a population of serotonergic cells of about 40% of the

original population of descending serotonergic neurons in the vagal nucleus reinnervated levels caudal to the lesion site after complete SC transection; 2) the size of the descending serotonergic cells after SCI was significantly smaller than the descending serotonergic cells in control larvae, and 3) at least 20% of the descending serotonergic neurons regenerate their axons after axotomy (this may be as much as 40% if FG is metabolized or not well transported). In the sea lamprey, the regenerative abilities differ greatly between identified reticulospinal neurons and neuronal groups (Jacobs et al., 1997). In previous studies by other groups, neurons regenerating 20% of the times or less have been considered as "bad regenerating" reticulospinal neurons (e.g. Shifman and Selzer, 2000; Shifman et al., 2009). In the present study, we report the regenerative ability of a neuronal population defined by its neurochemistry and not only but its anatomical location. In addition, the present results indicate that sprouting of non-axotomized neurons from above may help to compensate the lost descending serotonergic innervation. This is consistent with recent data in mammals, which show that serotonergic neurons have a better sprouting ability than other neurons of the central nervous system (Hawthorne et al., 2011). In any case, the serotonergic descending projection is not completely restored after SC transection in mature larval sea lampreys. This indicates that partial recovery of the serotonergic descending innervation below the SC lesion site may be sufficient to allow functional recovery of locomotion.

It is not known why complete restoration of the serotonergic descending projection does not occur. A recent study in the sea lamprey has shown that the axons of some identifiable reticulospinal neurons do not regenerate after complete SC transection, because they undergo a process of delayed cell death and eventually disappear (Shifman et al., 2008). Thus, the absence of regeneration of the entire serotonergic descending projection after axotomy may also be due to activation of cell death or atrophy. Shrinkage of the cell body of the serotonergic neurons with regenerated axons (present results) may support the idea that because of the SCI event, axotomy affects the viability of the serotonergic neurons and that only those neurons

that "escape" from the cell death process would regenerate their axons. Another possible explanation for the smaller size of the regenerated cells is that the serotonergic cells with small cell somas are better regenerators than the serotonergic cells with larger somas. In the present study, only 25% of the descending serotonergic cells had small somas (around 8.3 μm) in control animals, whereas about 40% of the descending serotonergic cells regenerated the axons, and almost all of them had small somas, after complete SC transection. The difference in the percentage of cells with small somas in all descending cells of the vagal group between control and experimental groups supports the idea that the somas of serotonergic neurons shrink after axotomy.

The molecular processes guiding the growth of the regenerating serotonergic axons are not known. In invertebrates, it has been shown that serotonin released from the regenerating axon may autoregulate this process by fine-tuning axon guidance and branching by inducing local collapse responses in the extending neurite (Koert et al., 2001). The sea lamprey may serve as a vertebrate model in which to study the possible role of this neurotransmitter in the autoregulation of axonal re-growth of the severed axon.

The serotonergic descending projection to the SC modulates locomotor activity and appears to promote an optimal function of the central pattern generator networks (CPGs) in vertebrates, from lampreys (Grillner et al., 2008) to mammals (Jordan et al., 2008). The regeneration of this system after SCI probably contributes to restoring the locomotor CPGs. Previous studies in rats have shown that, after complete SC transection, transplantation of embryonic raphe cells enhances recovery of motor functions and that this enhancement is serotonin-related (Majczyński et al., 2005). In addition, other studies have reported that combinations of Schwann cell bridge, olfactory-ensheathing glia transplatation and chondroitinase ABC treatment promote the regeneration of descending serotonergic fibers and recovery of locomotion after SCI in rats (Fouad et al., 2005; Vavrek et al., 2007). However, it should be noted that all forms of therapy that involve cell transplantation require labour-intensive in

vitro propagation and manipulation, followed by transplantation and establishment of the cells at appropriate sites in injured patients (Barreiro-Iglesias, 2010). Finding a non-invasive way of activating the regeneration of the severed serotonergic descending processes in vivo would be a good way of avoiding cell transplantation. Future studies in the sea lamprey model should identify cellular mechanisms underlying the spontaneous regeneration of serotonergic descending axons after SCI, allowing potential application of these mechanisms to enhance spontaneous sprouting or to induce regeneration of severed serotonergic axons in mammals. This direction of research will be important for generating therapies for humans with SCI.

Literature cited

Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R, Rodicio MC. (2007) Development of the serotonergic system in the central nervous system of the sea lamprey. J Chem Neuroanat. 34: 29-46.

Aitken AR, Törk I. (1988) Early development of serotonin-containing neurons and pathways as seen in wholemount preparations of the fetal rat brain. J Comp Neurol. 274: 32-47.

Barreiro-Iglesias A, Villar-Cerviño V, Anadón R, Rodicio MC. (2008) Development and organization of the descending serotonergic brainstem spinal projections in the sea lamprey. J Chem Neuroanat. 36: 77-84.

Barreiro-Iglesias A, Cornide-Petronio ME, Anadón R, Rodicio MC. (2009) Serotonin and GABA are colocalized in restricted groups of neurons in the larval sea lamprey brain: insights into the early evolution of neurotransmitter colocalization in vertebrates. J Anat. 215: 435-443.

Barreiro-Iglesias A. (2010) Targeting ependymal stem cells in vivo as a non-invasive therapy for spinal cord injury. Dis Model Mech. 3: 667-668.

Chiasson BJ, Baker MW, Croll RP. (1994) Morphological changes and functional recovery following axotomy of a serotonergic cerebrobuccal neurone in the land snail *Achatina fulica*. J Exp Biol. 192: 147-167.

Cohen AH, Kiemel T, Pate V, Blinder J, Guan L.. (1999) Temperature can alter the function outcome of spinal cord regeneration in larval lampreys. Neuroscience. 90: 957-965.

Cohen AH, Abdelnabi M, Guan L, Ottinger MA, Chakrabarti L.. (2005) Changes in distribution of serotonin induced by spinal injury in larval lampreys: evidence from immunohistochemistry and HPLC. J Neurotrauma. 22: 172-188.

Cormier CM, Mukhida K, Walker G, Marsh DR. (2010) Development of autonomic dysreflexia after spinal cord injury is associated with a lack of serotonergic axons in the intermediolateral cell column. J Neurotrauma. 27: 1805-1818.

Davis GR, McClellan AD. (1994) Extent and time course of restoration of descending brainstem projections in spinal cord-transected lamprey. J Comp Neurol. 344: 65-82.

Fouad K, Schnell L, Bunge MB, Schwab ME, Liebscher T, Pearse DD. (2005) Combining Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord. J Neurosci. 25: 1169-1178.

Grillner S, Wallén P, Saitoh K, Kozlov A, Robertson B. (2008) Neural bases of goal-directed locomotion in vertebrates-an overview. Brain Res Rev. 57: 2-12.

Harris-Warrick RM, McPhee JC, Filler JA. (1985) Distribution of serotonergic neurons and processes in the lamprey spinal cord. Neuroscience. 14: 1127-1140.

Hawthorne AL, Hu H, Kundu B, Steinmetz MP, Wylie CJ, Deneris ES, Silver J. (2011) The unusual response of serotonergic neurons after CNS injury: lack of axonal dieback and enhanced sprouting within the inhibitory environment of the glial scar. J Neurosci. 31: 5605-5616.

Jacobs AJ, Swain GP, Snedeker JA, Pijak DS, Gladstone LJ, Selzer ME. (1997) Recovery of neurofilament expression selectively in regenerating reticulospinal neurons. J Neurosci. 17: 5206-5220.

Jin LQ, Zhang G, Jamison C, Takano H, Haydon PG, Selzer ME. (2009) Axon regeneration in the absence of growth cones: acceleration by cyclic AMP. J Comp Neurol. 515: 295-312.

Jordan LM, Liu J, Hedlund PB, Akay T, Pearson KG. (2008) Descending command systems for the initiation of locomotion in mammals. Brain Res Rev. 57: 183-191.

Koert CE, Spencer GE, van Minnen J, Li KW, Geraerts WP, Syed NI, Smit AB, van Kesteren RE. (2001) Functional implications of neurotransmitter expression during axonal regeneration: serotonin, but not peptides, auto-regulate axon growth of an identified central neuron. J Neurosci. 21: 5597-5606.

Lurie DI, Selzer ME. (1991a) The need for cellular elements during axonal regeneration in the sea lamprey spinal cord. Exp Neurol. 112: 64-71.

Lurie DI, Selzer ME. (1991b) Axonal regeneration in the adult lamprey spinal cord. J Comp Neurol. 306: 409-416.

Lurie DI, Selzer ME. (1991c) Preferential regeneration of spinal axons through the scar in hemisected lamprey spinal cord. J Comp Neurol. 313: 669-679.

Mackler SA, Yin HS, Selzer ME. (1986) Determinants of directional specificity in the regeneration of lamprey spinal axons. J Neurosci. 6: 1814-1821.

Mackler SA, Selzer ME. (1987) Specificity of synaptic regeneration in the spinal cord of the larval sea lamprey. J Physiol. 388: 183-198.

Majczyński H, Maleszak K, Cabaj A, Sławińska U. (2005) Serotonin-related enhancement of recovery of hind limb motor functions in spinal rats after grafting of embryonic raphe nuclei. J Neurotrauma. 22: 590-604.

McLean DL, Fetcho JR. (2004a) Ontogeny and innervation patterns of dopaminergic, noradrenergic, and serotonergic neurons in larval zebrafish. J Comp Neurol. 480: 38-56.

McLean DL, Fetcho JR. (2004b) Relationship of tyrosine hydroxylase and serotonin immunoreactivity to sensorimotor circuitry in larval zebrafish. J Comp Neurol. 480: 57-71.

Murphy AD, Barker DL, Loring JF, Kater SB. (1985) Sprouting and functional regeneration of an identified serotonergic neuron following axotomy. J Neurobiol. 16: 137-151.

Murray KC, Nakae A, Stephens MJ, Rank M, D'Amico J, Harvey PJ, Li X, Harris RL, Ballou EW, Anelli R, Heckman CJ, Mashimo T, Vavrek R, Sanelli L, Gorassini MA, Bennett DJ, Fouad K. (2010) Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT2C receptors. Nat Med. 16: 694-700.

Rekling JC, Funk GD, Bayliss DA, Dong XW, Feldman JL. (2000) Synaptic control of motoneuronal excitability. Physiol Rev. 80: 767-852.

Rovainen CM. (1976) Regeneration of Müller and Mauthner axons after spinal transection in larval lampreys. J Comp Neurol. 168: 545-554.

Selzer ME. (1978). Mechanisms of functional recovery and regeneration after spinal cord transection in larval sea lamprey. J Physiol. 277: 395-408.

Shifman MI, Selzer ME. (2000) Expression of the netrin receptor UNC-5 in lamprey brain: modulation by spinal cord transection. Neurorehabil Neural Repair. 14: 49-58.

Shifman MI, Selzer ME. (2007) Differential expression of class 3 and 4 semaphorins and netrin in the lamprey spinal cord during regeneration. J Comp Neurol. 501: 631-646.

Shifman MI, Jin L-Q, Selzer ME. (2007) Regeneration in the lamprey spinal cord. In: Becker CG, Becker T (eds) Model organisms in spinal cord regeneration. Wiley-VCH Verlag, Weinheim, pp 229-262.

Shifman MI, Zhang G, Selzer ME. (2008) Delayed death of identified reticulospinal neurons after spinal cord injury in lampreys. J Comp Neurol. 510: 269-282.

Shifman MI, Yumul RE, Laramore C, Selzer ME. (2009) Expression of the repulsive guidance molecule RGM and its receptor neogenin after spinal cord injury in sea lamprey. Exp Neurol. 217: 242-251.

van Mier P, Joosten HW, van Rheden R, ten Donkelaar HJ. (1986) The development of serotonergic raphespinal projections in *Xenopus laevis*. Int J Dev Neurosci. 4: 465-475.

Vavrek R, Pearse DD, Fouad K. (2007) Neuronal populations capable of regeneration following a combined treatment in rats with spinal cord transection. J Neurotrauma. 24: 1667-1673.

Wood MR, Cohen MJ. (1979) Synaptic regeneration in identified neurons of the lamprey spinal cords. Science. 206: 344-347.

Woolston AM, Wedderburn JF, Sillar KT. (1994) Descending serotonergic spinal projections and modulation of locomotor rhythmicity in *Rana temporaria* embryos. Proc Biol Sci. 255: 73-79.

Yin HS, Mackler SA, Selzer ME. (1984) Directional specificity in the regeneration of lamprey spinal axons. Science. 224: 894-896.

Abbreviations

5-HT	Serotonin (5-hydroxytryptamine)
SC	Spinal cord
SCI	Spinal cord injury

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FIGURES

Figure 1 - A-C) Photomicrographs of horizontal sections of the rostral spinal cord at the level of the fourth-fifth gill (A), the lesion site at the level of the fifth gill (B) and the site of NB application at the level of the sixth gill (C) of a five months post-injury larval sea lamprey. D) Schematic drawing of a lateral view of the mature larval sea lamprey brain showing the location of rhombencephalic serotonergic cells (grey), of serotonergic cells with descending processes at the level of the second/third gill (red), of serotonergic cells with descending processes at the level of the sixth gill (blue) and of serotonergic cells with regenerated axons at the level of the sixth gill (green). *Scale bars*: 150 μm (A, B and C).

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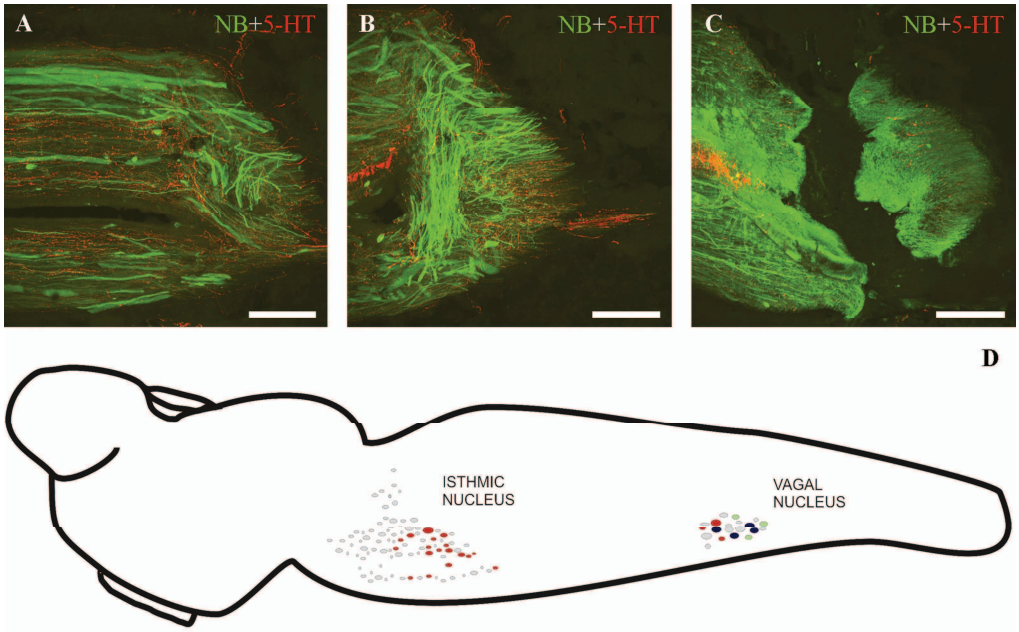

FIGURE 1

Figure 2 - Photomicrographs of transverse sections of the serotonergic reticulospinal group of the vagal region of mature larval sea lampreys as revealed by combined retrograde tract-tracing using NB (green channel), FG (blue channel) and 5-HT immunofluorescence (red channel). A-D) Serotonergic descending cells of the vagal nucleus of control larval sea lampreys. E-H) Photomicrographs showing the presence of 5-HT-ir neurons with regenerated axons in the vagal nucleus (white arrows) (E and G) and details of these neurons (F and H). I) Triple labeled serotonergic cell in the vagal nucleus showing the existence of true axonal regeneration of descending serotonergic neurons. Asterisks indicate the ventricle. *Scale bars*: 12.5 μm (B, D, F, H and I), 37.5 μm (A, C, E and G).

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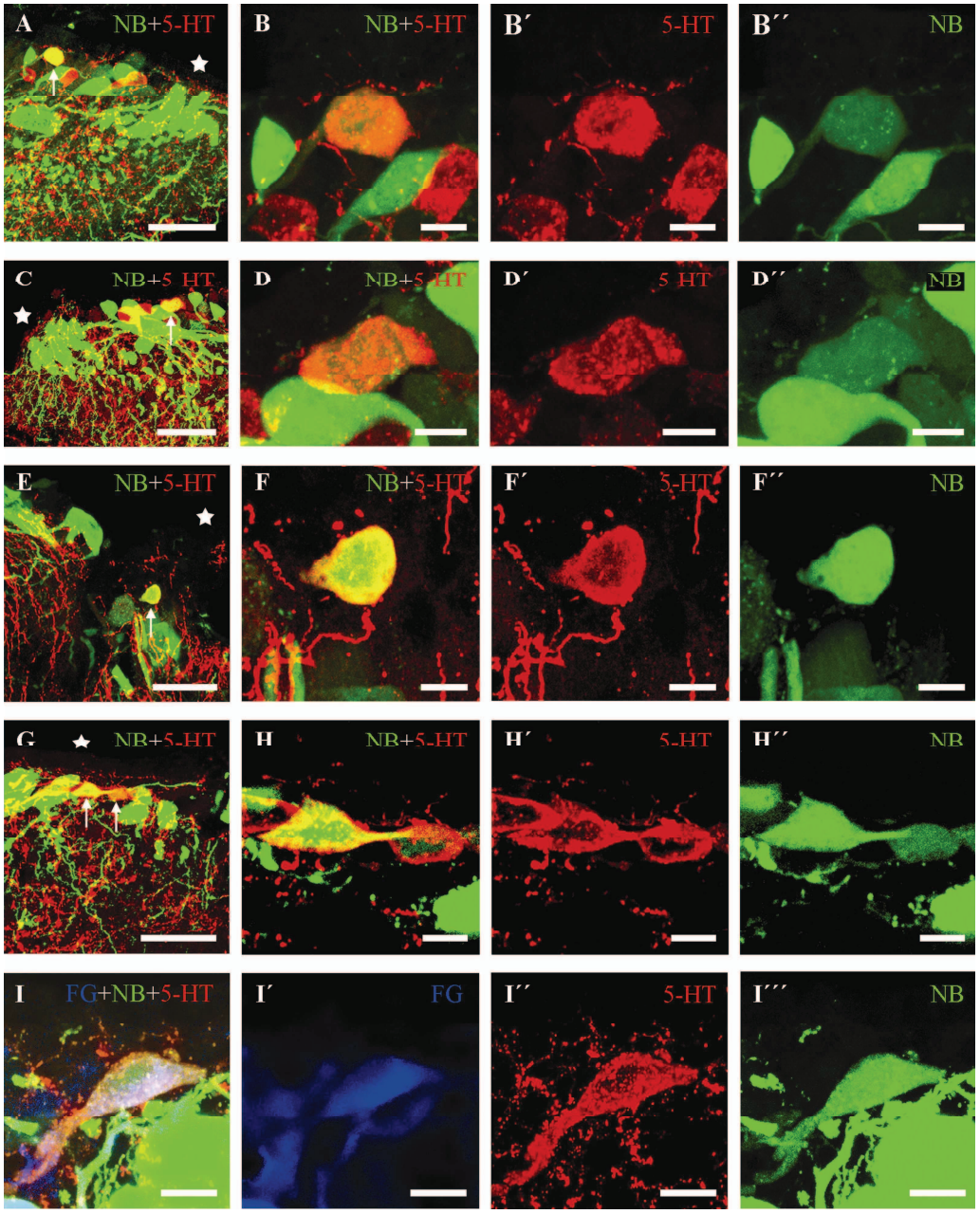
A
NB+5-HT
B
NB+5-HT
B′
5-HT
B′′
NB
C
NB+5 HT
D
NB+5-HT
D′
5 HT
D′′
NB
E
NB+5-HT
F
NB+5-HT
F′
5-HT
F′′
NB
G
NB+5-HT
H
NB+5-HT
H′
5-HT
H′′
NB
I
FG+NB+5-HT
I′
FG
I′′
5-HT
I′′′
NB

FIGURE 2

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GENERAL DISCUSSION

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GENERAL DISCUSSION

General discussion

Sea lampreys are interesting animal models for comparative neuroanatomical research and for the study of the neuronal circuitry underlying locomotion in vertebrates. Due to lampreys hold a phylogenetic position between *Amphioxus* and gnathostomes, they are key species to study the evolution of morphological characters and the basic molecular mechanisms at the dawn of Craniates and throughout the evolution of this phylum. Moreover, lampreys have been also established as a model organism for studies of spinal cord regeneration because, in contrast to mammals, they are capable of recovering apparently normal locomotion after a complete spinal cord transection.

The main aim of this thesis project has been the analysis of some aspects of the serotonergic system during development and spinal cord regeneration in the sea lamprey. This has been done by means of combined serotonin immunofluorescence and tract-tracing techniques to demonstrate the serotonergic neurons that project to the spinal cord and *in situ* hybridization for the detection of the rate-limiting enzyme for serotonin synthesis and a serotonin receptor, after their cloning.

We reported for the first time, the cloning, phylogenetic analysis and expression of a tryptophan hydroxilase enzyme (Tph) and serotonin 1A receptor (5-HT_{1A}) cDNAs of the sea lamprey. Our phylogenetic analysis suggested that both cloned sequences of the sea lamprey are orthologs of *Tph1* and 5-HT_{1A} sequences of gnathostome vertebrates, and that the duplication of the ancestral *Tph* gen and the emergence of the 5-HT_{1A} receptor occurred before the separation of agnathans (jawless vertebrates)

and gnathostomes (jawed vertebrates). These results confirm one more time that the sea lampreys are key species for evo-devo studies.

In contrast to other vertebrates in which the *Tph2* is expressed in raphe nuclei, a single *Tph1* transcript is shared by diencephalic and rhombencephalic serotonergic populations in the sea lamprey (Patel et al. 2004; Bellipanni et al. 2002; Teraoka et al. 2004). Conversely, in spite of the abundance of serotonergic cells present in the peripheral nervous system of lampreys, a trait shared with invertebrates (Barreiro-Iglesias et al. 2008; 2009), the *Tph1* transcript, which is expressed in the peripheral nervous system of gnathostomes, is not expressed in it. Only one *Tph* expression has been reported in central nervous system neurons of *D. melanogaster* neurons (Coleman and Neckameyer 2005; Neckameyer et al. 2007; Bao et al. 2010). All together these data predict the existence of a second *Tph* gen in lampreys and suggests a close relationship of the peripheral serotonergic system of lampreys to that of invertebrate groups. In addition, the *Tph* transcript expression in the photoreceptors may indicate that in lampreys and in other vertebrates (Gern and Ralph, 1979; Iuvone et al., 1997; Redburn and Mitchell, 1989) the photoreceptors synthetize melatonin.

Serotonin is involved in different physiological functions, as the generation and modulation of sleep, pain, addiction or locomotion. Apart from its role as a neurotransmitter, serotonin is also involved in other functions as neuronal differentiation or in the regulation of programmed cell death during brain development. The serotonin expression in the retina of lampreys (Villar-Cerviño et al., 2006; Abalo et al., 2008) has not showed any clue about it activity as developmental factor, however, the observation of expression of the 5-ht1a transcript in neuroblasts of the larval retina years before the appearance of serotonergic amacrine cells supports the hypothesis of a role of this receptor in the development of the retina.

The cloning of *Tph and* 5-HT_{1A} genes in lampreys offers new tools for researchers studying the development, organization and evolution of the

serotonergic system in both normal and pathological conditions using the sea lamprey as animal model.

As serotonin modulates the central pattern generator (CPG) in the spinal cord during locomotion (Christenson et al., 1989; Schwartz et al., 2005), the study of the 5-HT_{1A} receptor could help in further physiological studies. In addition, lampreys have been established as a model organism for studies of spinal cord regeneration (reviewed in Rodicio and Barreiro-Iglesias, 2012), therefore the study of the changes in this system after a lesion is also important. We reported that 5-HT_{1A} expression increases acutely 1 week post-lesion after a complete spinal cord transection both rostrally and caudally to the site of injury, and returns to levels similar to those found in unlesioned animals 3-7 weeks post-lesion. The overexpression of the 5-HT_{1A} could be an acute compensatory response to the lack of serotonin due to the degeneration of serotonergic fibers after spinal cord injury and help to achieve recovery of function and/or an response to lesions in the neurons.

The serotonin that modulated CPG is originated from descending serotonergic projections (Wallén et al.,1989), so we also decided to study the regeneration of these projections in lampreys. We reported that at least 20% of the serotonergic descending neurons of the caudal rhombencephalon regenerate to levels caudal to the lesion site. Moreover, we also proposed that the sprouting of non-axotomized neurons from above the site of spinal cord transection may help to compensate for the absence of a complete regeneration of severed descending serotonergic fibers. Interestingly, the size of the descending serotonergic cells after spinal cord injury was significantly smaller than in control larvae, suggesting that axotomy affects the viability of the serotonergic neurons and that only those neurons that "escape" from the cell death process would regenerate their axons. Our study offers a new and interesting model for investigating the intrinsic molecular mechanisms that favour the true regeneration of descending serotonergic fibers, which may provide valuable information for developing new therapies for patients with spinal cord injury.

In conclusion, the present thesis project could establish the basis to future research in the serotonergic system using the sea lamprey as an animal model, since we have done the phylogenetic analysis and studied the expression of a *Tph* and the serotonin 1A receptor of the sea lamprey. Moreover, the present thesis project could also contribute to future research in the molecular mechanisms that favour spinal cord regeneration after an injury in lampreys, and specifically the regeneration of the serotonergic system.

Literature cited

Abalo XM, Villar-Cerviño V, Villar-Cheda B, Anadón R, Rodicio MC. (2008) Neurochemical differentiation of horizontal and amacrine cells during transformation of the sea lamprey retina. J Chem Neuroanat. 35: 225-232.

Bao X, Wang B, Zhang J, Yan T, Yang W, Jiao F, Liu J, Wang S. (2010) Localization of serotonin/tryptophan-hydroxylase-immunoreactive cells in the brain and suboesophageal ganglion of Drosophila melanogaster. Cell Tissue Res. 340: 51-59.

Barreiro-Iglesias A, Villar-Cerviño V, Villar-Cheda B, Anadón R, Rodicio MC. (2008) Neurochemical characterization of sea lamprey taste buds and afferent gustatory fibers: presence of serotonin, calretinin, and CGRP immunoreactivity in taste bud bi-ciliated cells of the earliest vertebrates. J Comp Neurol. 511: 438-453.

Barreiro-Iglesias A, Aldegunde M, Anadón R, Rodicio MC. (2009) Extensive presence of serotonergic cells and fibers in the peripheral nervous system of lampreys. J Comp Neurol. 512: 478-499.

Bellipanni G, Rink E, Bally-Cuif L. (2002) Cloning of two tryptophan hydroxylase genes expressed in the diencephalon of the developing zebrafish brain. Gene Expr Patterns. 2: 251-256.

Christenson J, Franck J, Grillner S. (1989) Increase in endogenous 5-hydroxytryptamine levels modulates the central network underlying locomotion in the lamprey spinal cord. Neurosci Lett. 100: 188-192.

Coleman CM, Neckameyer WS. (2005) Serotonin synthesis by two distinct enzymes in Drosophila melanogaster. Arch Insect Biochem Physiol. 59: 12-31.

Gern WA, Ralph CL.. (1979) Melatonin synthesis by the retina. Science. 204: 183-184.

Iuvone PM, Bernard M, Alonso-Gomez A, Greve P, Cassone VM, Klein DC. (1997) Cellular and molecular regulation of serotonin N-acetyltransferase activity in chicken retinal photoreceptors. Biol Signals. 6: 217-224.

Neckameyer WS, Coleman CM, Eadie S, Goodwin SF. (2007) Compartmentalization of neuronal and peripheral serotonin synthesis in Drosophila melanogaster. Genes Brain Behav. 6: 756-769.

Patel PD, Pontrello C, Burke S. (2004) Robust and tissue-specific expression of TPH2 versus TPH1 in rat raphe and pineal gland. Biol Psychiatry. 55: 428-433.

Redburn DA, Mitchell CK. (1989) Darkness stimulates rapid synthesis and release of melatonin in rat retina. Vis Neurosci. 3: 391-403.

Rodicio MC, Barreiro-Iglesias A. (2012) Lampreys as an animal model in regeneration studies after spinal cord injury. Rev Neurol. 55: 157-166.

Schwartz EJ, Gerachshenko T, Alford S. (2005) 5-HT prolongs ventral root bursting via presynaptic inhibition of synaptic activity during fictive locomotion in lamprey. J Neurophysiol. 93: 980-988.

Teraoka H, Russell C, Regan J, Chandrasekhar A, Concha ML, Yokoyama R, Higashi K, Take-Uchi M, Dong W, Hiraga T, Holder N, Wilson SW. (2004) Hedgehog and Fgf signalling pathways regulate the development of tphR-expressing serotonergic raphe neurons in zebrafish embryos. J Neurobiol. 60: 275-288.

Villar-Cerviño V, Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Holstein GR, Martinelli GP, Rodicio MC, Anadón R. (2006) Presence of glutamate, glycine, and gamma-aminobutyric acid in the retina of the larval sea lamprey: comparative immunohistochemical study of classical neurotransmitters in larval and postmetamorphic retinas. J Comp Neurol. 499: 810-827.

Wallén P, Christenson J, Brodin L, Hill R, Lansner A, Grillner S. (1989) Mechanisms underlying the serotonergic modulation of the spinal circuitry for locomotion in lamprey. Prog Brain Res. 80: 321-327.

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SUMMARY AND CONCLUSIONS

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SUMMARY AND CONCLUSIONS

Summary

Sea lampreys belong to the most ancient group of extant vertebrates: cyclostomes or agnathans, therefore they hold a unique phylogenetic position between *Amphioxus* (protochordates) and gnathostomes (jawed vertebrates). Due to this fact, they are a key species to study the evolution of morphological characters and the basic molecular mechanisms at the dawn of Craniates and throughout the evolution of this phylum. In addition, in the last years, the whole-genome of sea lamprey has been sequenced and published in http://www.ensembl.org, so new evolutionary studies have been possible at a molecular level. Lamprey is also as an interesting animal for comparative neuroanatomical research and for the study of neuronal circuitry underlying locomotion in vertebrates. The central nervous system (CNS) of the lamprey has all the main regions of the mammalian CNS, but much fewer neurons so the excitability of the circuits for the coordination of locomotion movements is easier to study than in other species. Moreover, the isolated lamprey brainstem and spinal cord can be maintained alive in vitro for several days. Due to this, the motor pattern underlying locomotion in lampreys can be elicited through the stimulation of the brainstem locomotor areas of the di- or rhombencephalon corresponding to the locomotor areas in mammals. Finally, the lamprey has also been established as a model organism for studies of spinal cord regeneration and other nervous system diseases. In contrast to mammals, lampreys can recover normal-appearing locomotion after complete spinal cord transection, and the spinal axons regenerate selectively in their correct paths. Moreover, lamprey is the only vertebrate that has fully met the NIH criteria to demonstrate functional regeneration in the spinal cord:

1. The experimental lesion must cause disconnection of nerve processes.
2. Processes of CNS neurons must bridge the level of injury.

3. The regenerated fibers must make junctional contacts.
4. The regenerated fibers must generate postjunctional responses.
5. Changes in function must derive from regenerated connections.

The serotonergic system is thoroughly described in vertebrates. Serotonergic cells are extensively distributed in the CNS of vertebrates except in mammals, where these cells are largely restricted to the hindbrain. Serotonergic neurons are among the earliest cells to be born in the developing CNS, so serotonin is one of the earliest neurotransmitters to appear in the nervous system. Examples of the different functions of serotonin are the generation and modulation of sleep, pain, addiction, locomotion or sexual activity. In addition to its role as a neurotransmitter, serotonin is also involved in neuronal development, in the modulation of the development of cellular migration and cytoarchitecture, in the autoregulation of branch morphology of serotonergic processes, or in the regulation of programmed cell death during brain development. In mammals, a loss of brainstem-derived serotonin after spinal cord injury acutely reduces motor neuron excitability and accordingly depresses all motor functions. The critical importance of serotonin is demonstrated by the repeated finding in animal models that locomotor activity can be regained soon after spinal injury with the exogenous application of drugs that activate serotonin receptors or even transplants of serotonin producing cells into the spinal cord.

The development and organization of the serotonergic system have been also studied in the central nervous system and in peripheral tissues of the sea lamprey by means of immunohistochemical methods. Moreover, previous studies show colocalization of serotonin with GABA and dopamine. We aimed to increase our knowledge of the serotonergic system of lampreys and on the present thesis project we focus in the study of 1) the rate-limiting enzyme for serotonin synthesis (Tph), 2) the serotonin 1A receptor, 3) the expression changes of this receptor during spinal cord regeneration and 4) the regeneration of descending serotonergic projections after spinal cord injury. The thesis has been organized in 4 chapters corresponding to the 4 main objectives proposed. In the first chapter of the thesis, the cloning of a *Tph*

cDNA and its pattern of expression in larval and young adult sea lampreys are reported. In the second chapter, the serotonin 1A receptor, one of the major targets of the activity of serotonergic cells, is studied. In the third chapter, the study of the serotonin 1A receptor during spinal cord regeneration was conducted. Finally, the spontaneous regeneration of descending serotonergic projections after spinal cord injury is reported in chapter four.

CHAPTER 1: The sea lamprey tryptophan hydroxylase: new insight into the evolution of the serotonergic system of vertebrates

Recent research has shown that at least two tryptophan hydroxylase (*Tph*) genes are present in gnathostome vertebrates, but it is not known when the duplication of the ancestral *Tph* gene took place during evolution. By their position as an out-group of gnathostomes, lampreys (agnathans) are key models to understand molecular evolution in vertebrates. In this chapter, the cloning of a *Tph* cDNA and its pattern of expression in larval and young adult sea lampreys are reported. The deduced Tph protein sequence presents a conserved ACT domain and the conserved aromatic amino acid hydroxylase superfamily domain. Residues coordinating iron biding and a conserved binding site for the cofactor BH4 are also present in the deduced sequence of the protein. Phylogenetic analysis indicated that the lamprey *Tph* is an orthologue of *Tphs* of other vertebrates and suggested that the duplication of the ancestral *Tph* gene occurred before the separation of agnathans and gnathostomes, although alternative hypothesis are also discussed in the present study. In the sea lamprey brain, the *Tph* gene is expressed in the pineal organ, the retina, and the diencephalic and rhombencephalic nuclei of the sea lamprey reported previously with serotonin immunohistochemistry and in small cells of the spinal cord, with a pattern similar to that observed with anti-serotonin antibodies. This result differs with the situation in other vertebrates in which different *Tph* genes are expressed in different populations: *Tph1* is primarily expressed in the pineal organ and in peripheral tissues while *Tph2* is expressed in raphe nuclei. Our results suggest that the selection of *Tph2* to be

expressed in raphe neurons may have occurred along the line leading to gnathostomes. No *Tph* expression was observed in peripheral serotonergic cells, which, unlike in other vertebrates, are widely distributed in lampreys, suggesting that a second *Tph* gen could exist in lampreys.

Tph transcript expression was also observed in retina of premetamorphic larvae, with different pattern of expression between the central and the lateral retinas. In the central retina, *Tph* transcript expression was observed in putative photoreceptors and in putative amacrine cells and in the lateral retina, it was observed in putative amacrine cells and in putative neuroblasts. Since Tph is the first enzyme in the biosynthetic pathways of serotonin in amacrine cells and of melatonin in photoreceptor cells and no serotonin was previously reported to be detected in retinal photoreceptors of lampreys, our results indicate that, as that of other vertebrate species, they synthesize melatonin and that this character has appeared early and has been conserved in vertebrate evolution. In addition, the expression of the Tph transcript in neuroblast suggests that in lampreys serotonin plays a role in the development of the retina.

CHAPTER 2: Serotonin 1A receptor (5-HT_{1A}) of the sea lamprey: cDNA cloning and expression in the central nervous system

Serotonergic cells are among the earliest neurons to be born in the developing central nervous system and serotonin is known to regulate the development of the nervous system. One of the major targets of the activity of serotonergic cells is the serotonin 1A receptor (5-HT_{1A}), an ancestral archetypical serotonin receptor. In this study, we cloned and characterized the 3D structure of the sea lamprey serotonin 1A receptor, and we also studied the expression of its transcript in the central nervous system during prolarval, larval and adult stages by means of *in situ* hybridization. The cloned sequence indicates that second intracellular loop of the receptor contains the DRY sequence that regulates ligand binding, G protein coupling and receptor stability, whereas the third intracellular loop contains the G protein contact site

and a PKC phosphorylation site of 5-HT_{1A}: LARERKTVKTLG. This sequence seems to be highly conserved, and as the Tph sequence, already present before the split between agnates and gnathostomes. In phylogenetic analyses, the sea lamprey serotonin 1A receptor sequence emerged as an outgroup to all gnathostome sequences. There is a widespread expression of the receptor during all developmental states, from post hatching day 1 (P1) to adult without evidences of neuronal populations transiently expressing the receptor. In P1 prolarvae 5-ht1a mRNA expression was observed in diencephalic nuclei, the rhombencephalon and rostral spinal cord. At P2 prolarval stage the 5-ht1a expression extended to other brain areas including telencephalic regions. 5-ht1a expression in larvae was observed throughout almost all the main brain regions with the strongest expression in the olfactory bulbs, lateral pallium, striatum, preoptic region, habenula, prethalamus, thalamus, pretectum, hypothalamus, rhombencephalic reticular area, dorsal column nucleus and rostral spinal cord. In adults, the 5-ht1a transcript was also observed in cells of the subcommissural organ. Comparison with other vertebrates reveals a conserved pattern in most of the brain regions, likely reflecting the ancestral vertebrate condition.

Our results also have showed 5-ht1a expression in the brain vessels, choroid plexus and in the retina. In the retina 5-ht1a expression was observed all the differentiated cells in the central retina of prolarvae and larvae (photorreceptors, bipolar and ganglion cells), in neuroblast of the lateral retina of larvae and in all neuronal cells of adult retinas, with strong expression in amacrine, bipolar and ganglion cells and weak expression in horizontal cells. These results indicate that $5HT_{1A}$ may be an autoreceptor in cells synthetizing serotonin, a postsynaptic heteroreceptor in bipolar and ganglion cells, act by volumen transmission on horizontal cells and have a developmental role during retinal development.

CHAPTER 3: Traumatic injury induces changes in the expression of the serotonin 1A receptor in the spinal cord

In contrast to mammals, lampreys recover locomotion several weeks after a complete spinal cord transection and severed descending axons from the brain are able to regenerate across the trauma zone and reinnervate the spinal cord below the site of injury. After spinal cord injury in mammals, the loss of serotonin coming from the brainstem reduces the excitability of motor neurons and this leads to a compensatory overexpression of serotonin receptors. In this study, we adapted a semiautomatic quantification method to quantify the changes in the expression of the 5-HT_{1A} in the spinal cord after a complete spinal cord transection as seen by *in situ* hybridization. We performed a complete spinal cord transection at the level of the fifth gill, and animals were allowed to recover for 1, 3 or 7 weeks post-lesion. Our results showed that there is a marked increase in the expression of the 5-HT_{1A} acutely (1 wpl) after a complete spinal cord transection both rostrally and caudally to the site of injury. Then, the expression of the 5-HT_{1A} returns to levels similar to those found in unlesioned animals a few weeks (3-7 wpl) after the injury both rostrally and caudally to the site of transection. Interestingly, overexpression of the 5-HT_{1A} in rostral levels after spinal cord injury has not been reported in mammals, suggesting that this rostral overexpression could be part of the plastic events that lead to the recovery of function in a regenerating vertebrate like the sea lamprey as compared to non-regenerating mammals. It suggested that that the lack of serotonin due to the degeneration of serotonergic fibers after the injury could induce a compensatory overexpression of the 5-$HT1_{1A}$. The overexpression of the 5-HT_{1A} in rostral levels after spinal cord injury in sea lamprey has not been observed in mammals, suggesting that the overexpression could be part of the plastic events that lead to the recovery of function in a regenerating vertebrate like the sea lamprey. The analysis of changes in receptor expression by zones (periventricular region and horizontally extended grey matter) has shown that they, except the caudal periventricular layer, basically followed the same pattern of changes detected in the spinal cord as a whole. In the caudal

periventricular layer no significant differences were observed between control and experimental animals at any time post lesion. This suggests that different molecular signals act on the periventricular cells of the rostral and caudal spinal cord after a complete spinal cord transection and thus affecting their response to the injury.

Present and previous results indicate that the lack of serotonin due to the degeneration of serotonergic fibers after the injury could induce a compensatory overexpression of the 5-HT_{1A}. Based on our observations in lampreys and previous data in this and other vertebrates indicating that the activation of the receptor is beneficial after spinal cord injury, a higher availability of 5-HT_{1A} could be beneficial for behavioural recovery to compensate for a reduced availability of the agonist.

CHAPTER 4: Spontaneous regeneration of the serotonergic descending innervation in the sea lamprey after spinal cord injury

In contrast to mammals, lampreys are capable of recovering apparently normal locomotion after complete spinal cord transection, and the spinal axons regenerate selectively in their correct paths. Descending serotonergic projections to the spinal cord play a role in the modulation of locomotion at spinal levels in both mammals and lampreys. In this study, we used combined immunofluorescence and tract-tracing techniques to investigate if the serotonergic descending axons regenerate across the lesion site after complete spinal cord transection. The spinal cord of mature larval sea lampreys was transected at the level of the fifth gill, then after a recovery period of 5 months, the spinal cord was exposed again, 1mm caudal to the injury site, and the tracer neurobiotin was applied. Double-labelled cells were observed in the caudal portion of the serotonin-immunoreactive vagal nucleus indicating that the serotonergic descending neurons of the caudal rhombencephalon (vagal nucleus) regenerate their axons across the lesion site. The size of the descending serotonergic cells after spinal cord injury was significantly smaller in regenerated than in control larvae, suggesting that

axotomy affects the viability of the serotonergic neurons and that only those neurons that "escape" from the cell death process would regenerate their axons. In order to investigate whether the reinnervation was due to sprouting from axons above the injury site or to regeneration of axotomized axons, the experiments were performed again, but the tracer fluoro-gold was applied at the time of transection. Triple-labelled cells were observed in the vagal nucleus, indicating that at least part of the reinnervation corresponds to true regeneration. Since the 40% of the original population of descending serotonergic neurons in the vagal nucleus reinnervated levels caudal to the lesion site after complete spinal cord transection but only the 20% of the neurons corresponds to true regeneration, our results have also indicated that sprouting of non-axotomized neurons from above may help to compensate the lost descending serotonergic innervation. This study provides a new and interesting model for investigating the intrinsic molecular mechanisms involved in regeneration of the serotonergic descending axons in vertebrates. Use of this model may provide valuable information for proposing new therapies for patients with spinal cord injury.

Some chapters of this Doctoral Thesis have been already published in the form of research articles with the following references:

- Chapter 1: Brain Struct Funct (2013) 218: 587-593.
- Chapter 2 will be published as 3 different articles. The expression of the serotonin 1A receptor in the brain and spinal cord has been already published (Brain Struct Funct. 2012, DOI 10.1007/s00429-012-0461-y). The results of the expression of the receptor together with that of the expression of *Tph* in the retina will be submitted for publication to Experimental Eye Research, and the results of the expression in the choroid plexus and brain vessels will be submitted to Neuroscience Letters.
- Chapter 3 will submitted for publication to Journal of Neurotrauma.
- Chapter 4: J Neurotrauma (2011) 28: 2535-2540.

For this thesis project we used animals from all lamprey developmental stages: embryonic, prolarval, larval, metamorphic and adult. Cloning and sequencing methods were used for characterizing the *Tph* and the serotonin 1A receptor. The first-strand cDNA synthesis reaction was catalysed using Superscript III Reverse Transcriptase with oligodT18 primers or random primers. For PCR cloning, degenerate oligonucleotide primers were designed based on alignments of mRNA sequences of different species deposited in the trace archives of the NCBI and in the Ensembl database of the sea lamprey genome. The alignments of our cloning sequences with protein sequences corresponding to different species were used to construct different phylogenetic trees by the minimum evolution method or the neighbor-joining method.

In addition, *in situ* hybridization techniques were used to study the expression of these transcripts in the central nervous system. Digoxigenin (DIG)-labelled riboprobes were synthesized using the amplified fragments of our cloning sequences as templates and following standard protocols using T7 polymerase. *In situ* hybridization was conducted using standard protocols, with the following adaptations. A proteinase K treatment was performed following rehydration of samples and an RNAse A treatment was added to the post-hybridization washes. The sheep anti-DIG antibody conjugated to alkaline phosphatase was incubated overnight at 4ºC in blocking buffer solution. Staining was conducted in BM Purple at 37ºC until the signal was clearly visible. To quantify our mRNA profiles during spinal cord regeneration, the Feature J Hessian plug-in for Image J software was used. For statistical analysis, the program Prism was used with the Kormogolov-Smirnov normality test.

Tract-tracing methods (using neurobiotin or fluoro-gold as retrograde tracers) in combination with serotonin immunofluorescence were used to determine the ability of the descending serotonergic axons to regenerate after a spinal cord injury. The spinal cord was exposed from the dorsal midline at the level of the fifth gill, a complete spinal cord transection was performed with a scalpel and the spinal cord cut ends were visualized under the microscope. Each transected animal was examined 24 h after surgery to confirm that there

was no movement caudal to the lesion site and then they were allowed to recover in fresh water tanks at 19-20ºC. The spinal cord was exposed after the recovery period and crystals of the tracer neurobiotin were applied with the aid of a minute pin (00). The animals were maintained at 4ºC with appropriate aeration conditions in lamprey Ringer solution to allow transport of the tracer. For indirect detection of the tracer, sections were incubated at room temperature for 4 h with fluorescein isothiocyanate (FITC)-labelled avidin. The tracer fluoro-gold (FG) was applied at the time of the lesion with the aid of a minute pin (00). The FG tracer was observed directly in confocal microscope with the ultraviolet laser. Serotonin immunofluorescence was performed using a rabbit polyclonal anti-5-HT antibody at 4ºC for 72 h. For indirect immunofluorescence detection of serotonin, Cy3-conjugated goat anti-rabbit antibody was used.

All experiments were approved by the Ethics Committee of the University of Santiago de Compostela and conformed to European Community guidelines on animal care and experimentation to minimize pain and discomfort.

Conclusions

The analysis of the results obtained in the present Thesis led us to the following conclusions:

1. Phylogenetic analysis of the *Tph* sequences suggests that the *Tph* of sea lamprey is an ortholog of *Tph1* sequences of gnathostome vertebrates.
2. The expression of the *Tph* transcript in the diencephalic nuclei of sea lampreys suggests that it is a primitive condition of vertebrates, whereas its expression in rhombencephalic populations is a specific condition of extant lampreys that could be either a primitive or recently evolved character.
3. Lack of expression of the *Tph* transcript in peripheral tissues suggests the existence in lampreys of a second enzyme with the ability to hydroxylate tryptophan primarily expressed in the peripheral nervous system.
4. Expression of *Tph* transcript in the retina suggest that it could synthetized serotonin in amacrine cells and melatonin in photoreceptor cells, whereas its presence in neuroblasts suggests that serotonin may be playing another role other than a neurotransmitter function in the development of the retina.
5. Phylogenetic analysis of 5-HT_{1A} sequences and the high conserved G_i/G_o-coupling region of 5-HT_{1A} suggest that the emergence of the 5-HT_{1A} receptor occurred before the separation of agnathans and gnathostomes.
6. The widespread expression of 5-ht1a mRNA in lamprey brain regions containing serotonergic neurons and also in those lacking these neurons indicates that it may act as a heteroceptor as well as an autoreceptor, indicating that the different pre- and post-synaptic functions of the 5-HT_{1A} are an old character of vertebrates.

7. The expression of the 5-ht1a transcript starts after hatching in P1 and continues along the development without evidences of neuronal populations transiently expressing the receptor.
8. The expression of the 5-ht1a transcript in the somas, in basal dendrites and in glial cells appears to be a characteristic shared by mammals and lampreys indicating that it may be a primitive character of vertebrates.
9. The expression of the 5-ht1a transcript in the retina during the different developmental stages of the sea lamprey suggests that, in addition to be an auto- and heroreceptor, it could play a role in retinal development.
10. 5-HT_{1A} expression increases acutely 1 week post-lesion after a complete spinal cord transection both rostrally and caudally to the site of injury and then returns to levels similar to those found in unlesioned animals 3 to 7 weeks post-lesion, similar to what is been observed in mammals.
11. Over-expression of the 5-HT_{1A} could be an acute compensatory response to the lack of serotonin due to the degeneration of serotonergic fibers after the spinal cord injury.
12. At least 20% of the serotonergic descending neurons of the caudal rhombencephalon regenerate to levels caudal to the lesion site whereas the sprouting of non-axotomized neurons from above the spinal cord transection may help to compensate for absence of complete regeneration.
13. The size of the descending serotonergic cells after spinal cord injury was significantly smaller than in control larvae, suggesting that axotomy affects the viability of the serotonergic neurons and that only those neurons that "escape" from the cell death process would regenerate their axons.

RESUMEN Y CONCLUSIONES

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RESUMEN Y CONCLUSIONES

Resumen

La lamprea de mar pertenece al grupo más antiguo de vertebrados actuales: ciclóstomos o agnatos, de modo que ocupan una posición filogenética única entre *Amphioxus* (protocordados) y gnatóstomos (vertebrados con mandíbula). Por ello, la lamprea es una especie clave en estudios de evolución de los caracteres morfológicos y de los mecanismos moleculares básicos en la base de los Craniados y a través de la evolución de este filo. Además, en los últimos años, el genoma de la lamprea ha sido secuenciado y publicado en http://www.ensembl.org, de modo que se han realizado estudios evolutivos bajo un punto de vista genómico. La lampea es también un modelo animal interesante para estudios de neuroanatomía comparada y para estudios de los circuitos neuronales subyacentes a la locomoción en vertebrados. El sistema nervioso central (SNC) de la lamprea presenta las principales regiones del SNC de mamíferos, pero con muchas menos neuronas. De este modo, la excitabilidad de los circuitos para la coordinación de los movimientos durante la locomoción es más fácil de estudiar que en otras especies. Además, el cerebro y médula espinal aislados se pueden mantener vivos in vitro durante varios días. Debido a ello, el patrón motor subyacente de la locomoción en lamprea puede obtenerse a través de la estimulación de áreas locomotoras del di- o rombencéfalo correspondientes a áreas locomotoras de mamíferos. Por último, la lamprea también se ha establecido como animal modelo en estudios de regeneración de la médula espinal y para otras enfermedades del sistema nervioso. A diferencia de los mamíferos, la lamprea puede recuperar una locomoción aparentemente normal tras una sección completa de la médula espinal, ya que los axones descendentes regeneran selectivamente en las rutas

correctas. Además, la lamprea es el único vertebrado que cumple todos los criterios del NIH para demostrar regeneración funcional de la médula espinal:

1. La lesión experimental debe causar la desconexión de los procesos nerviosos.
2. Los procesos de las neuronas del SNC deben atravesar la lesión tras la regeneración.
3. Las fibras regeneradas deben establecer nuevos contactos sinápticos.
4. Las fibras regeneradas deben generar respuestas sinápticas.
5. Los cambios funcionales tienen que deberse a la formación de conexiones regeneradas.

El sistema serotonérgico está ampliamente descrito en vertebrados. Las células serotonérgicas están ampliamente distribuidas en el SNC de vertebrados excepto en mamíferos, donde estas células están restringidas al rombencéfalo a pesar de la amplia inervación serotonérgica del cerebro. Las neuronas serotonérgicas son una de las primeras en aparecer en el desarrollo del SNC, de modo que la serotonina es unos los primeros neurotransmisores que aparecen en el sistema nervioso. Ejemplos de las funciones de la serotonina son la generación y modulación del sueño, dolor, adicción, locomoción o actividad sexual. Sin embargo, además de su función como neurotransmisor, la serotonina está involucrada en la diferenciación neuronal, en la modulación del desarrollo de la migración celular y citoarquitectura, en la autorregulación de la morfología de las ramificaciones serotonérgicas o en la regulación de la muerte celular programada durante el desarrollo del cerebro. En mamíferos, una pérdida de la serotonina procedente de las células del rombencéfalo tras una sección de la médula espinal reduce intensamente la excitabilidad de las motoneuronas y por tanto reduce las funciones motoras. La importancia crucial de la serotonina se demuestra por los hallazgos en diferentes modelos animales en los que la actividad locomotora se recupera pronto tras una sección de la médula espinal por la aplicación exógena de fármacos que activan los receptores de serotonina o por transplantes de células que producen serotonina en la médula espinal.

El desarrollo y organización del sistema serotonérgico ha sido ampliamente estudiado en el sistema nervioso central y tejidos periféricos de la lamprea. Además, trabajos previos han demostrado la colocalización de la serotonina con otros neurotransmisores como GABA y dopamina. Nos propusimos incrementar nuestros conocimientos del sistema serotonérgico de la lamprea y en el presente proyecto de tesis nos centramos en el estudio de 1) la enzima limitante en la síntesis de serotonina (Tph), 2) del receptor 1A de serotonina, 3) los cambios en expresión del este receptor durante la regeneración de la médula espinal, y 4) la regeneración espontánea de la inervación serotonérgica descendente. La tesis se ha organizado en 4 capítulos correspondientes a los 4 objetivos principales propuestos. En el primer capítulo de la tesis, se describe el clonaje del ADNc de la *Tph* y su expresión en larvas y jóvenes adultos de lamprea de mar. En el segundo capítulo, se ha estudiado el receptor 1A de serotonina, una de las principales dianas de la actividad de las células serotonérgicas. En el tercer capítulo, se estudió el receptor 1A de serotonina durante la regeneración de la médula espinal. Por último, estudiamos la regeneración espontánea de la inervación descendente serotonérgica tras una lesión de médula espinal.

CAPÍTULO 1: Triptófano hidroxilasa en lamprea de mar: nuevas perspectivas en la evolución del sistema serotonérgico de vertebrados

Investigaciones recientes han mostrado que al menos dos genes de triptófano hidroxilasa (*Tph*) están presentes en los vertebrados gnatóstomos, pero no se conoce cuándo tuvo lugar la duplicación del gen ancestral de la *Tph* durante la evolución. Por su posición filogenética como un grupo separado de los gnatóstomos, las lampreas (agnatos) son un modelo clave para entender la evolución molecular en vertebrados. En este trabajo, clonamos el ADNc de la *Tph* de lamprea de mar y se estudió el patrón de expresión de su ARNm en larvas y adultos jóvenes. La secuencia proteica deducida de la Tph presenta un dominio conservado ACT y el dominio de amino ácido aromático de la superfamilia hidroxilasa. Residuos de unión a

hierro están también presentes en la secuencia deducida de la proteína. Un sitio conservado de unión al cofactor BH4 también se encuentra en la secuencia. Los análisis filogenéticos indican que el gen *Tph* de lamprea es un ortólogo de las *Tphs* de otros vertebrados, sugiriendo que la duplicación del gen ancestral *Tph* ocurrió antes de la separación entre agnatos y gnatóstomos, aunque hipótesis alternativas también se discuten en este estudio. En el cerebro de la lamprea de mar, el transcrito *Tph* se expresa en las células del órgano pineal, de la retina, de los núcleos diencefálicos y rombencefálicos y en pequeñas células de la médula espinal, con un patrón similar a lo observado con anticuerpos contra serotonina. Esto difiere con la situación de otros vertebrados en los que diferentes genes *Tph* se expresan en las distintas poblaciones: *Tph1* se expresa principalmente en el órgano pineal y en los tejidos periféricos, mientras que *Tph2* se expresa en el núcleo del rafe. Nuestros resultados sugieren que la selección de expresión de *Tph2* en las neuronas del rafe pudo haber ocurrido a lo largo de la línea de la línea evolutiva que llevó a los gnatóstomos. No se observó expresión de *Tph* en las células serotonérgicas del sistema nervioso periférico, que, a diferencia de otros vertebrados, están ampliamente distribuidas en lamprea, sugiriendo que un segundo gen *Tph* podría existir en lamprea.

También se observó expresión de *Tph* en la retina de larvas premetamórficas, con diferente patrón de expresión entre las retinas central y lateral. En la retina central, se observó expresión *Tph* en supuestos fotorreceptores y en supuestas células amacrinas, y en la retina lateral se observó en supuestas células amacrinas y supuestos neuroblastos. Ya que la Tph es la primera enzima en la biosíntesis de serotonina en las células amacrinas y de melatonina en los fotorreceptores, y que no se ha descrito previamente detección de sertonina en los fotorreceptores de la reina de lamprea, nuestros resultados indicant que, como en otros vertebrados, sintetizan melatonina y que es éste en un carácter que ha aparecido temprano y que se ha conservado en la evolución de los vertebrados. Además, la expresión de Tph en neuroblastos sugiere que en la lamprea la serotonina tiene una función en el desarrollo de la retina.

CAPÍTULO 2: El receptor de serotonina 1A (5-HT_{1A}) de la lamprea de mar: clonación de ADNc y expresión en el sistema nervioso central

Las células de serotonina son de las primeras en aparecer durante el desarrollo del sistema nervioso central y se conoce que la serotonina regula el desarrollo del sistema nervioso. Una de las mayores dianas de la actividad de las células serotonérgicas en el receptor de serotonina 1A (5-HT_{1A}), un arquetipo ancestral de receptores de serotonina. En este estudio, clonamos y caracterizamos la estructura en 3D del 5-HT_{1A} de lamprea, y estudiamos la expresión de su transcrito en el sistema nervioso central mediante hibridación *in situ*. La secuencia clonada indica que el receptor tiene un segundo bucle intracelular que con la secuencia DRY, que regula la unión de ligando, el acoplamiento de proteína G y la estabilidad del receptor, mientras que el tercer bucle intracelular presentó el sitio de contacto de proteínas G y el sitio de fosforilación PKC del 5-HT_{1A}: LARERKTVKTLG. Esta secuencia parece estar altamente conservada, y al igual que la secuencia Tph, presente incluso antes de la separación entre agnatos y gnatóstomos. En el análisis filogenético, la secuencia 5-HT_{1A} de la lamprea se agrupa junto con las secuencias 5-HT_{1A} de vertebrados, y aparece como un grupo separado de todas las secuencias de gnatóstomos. Hay una amplia expresión del receptor durante todos los estadios de desarrollo, desde el día 1 tras la eclosión (P1) a adulto, sin evidencias de poblaciones neuronales transitorias que expresaran el receptor. En prolarvas P1 la expresión del ARNm 5-ht1a se observó en núcleos diencefálicos, el rombencéfalo y la médula espinal rostral. En prolarvas P2 la expresión de 5-ht1a se extendió a otras áreas del cerebro incluidas regiones telencefálicas. La expresión de 5-ht1a en larvas se observó a lo largo de las regiones principales del cerebro, siendo más fuerte en los bulbos olfatorios, palio lateral, estriado, región preóptica, habénula, pretálamo, tálamo, pretecho, hipotálamo, área reticular rombencefálica, núcleo de la columna dorsal y médula espinal rostral. En adultos, también se observa expresión de 5-ht1a en células del órgano subcomisural. La comparación de expresión de 5-ht1a entra la lamprea y otros vertebrados muestra un patrón conservado en

la mayoría de regiones del cerebro, lo que refleja una condición ancestral entre vertebrados.

Nuestros resultados también mostraron expresión de 5-ht1a en los vasos del cerebro, en los plexos coroideos, y en la retina. La expresión de 5-ht1a en la retina se observó en todas las células diferenciadas de la retina central de prolarvas y larvas (fotorreceptores, células bipolares y células ganglionares), en neuroblastos de la retina lateral de larvas, y en todas las células neuronales de la retina de adultos, con una fuerte expresión en células amacrinas, bipolares y ganglionares y escasa expresión en células horizontales. Estos resultados indican que $5HT_{1A}$ podría ser un autorreceptor en células que sintetizan serotonina, un heterorreceptor postsináptico en células bipolares y ganglionares, que actuaría por transmisión por volumen en células horizontales, y que tendría una función de desarrollo durante el desarrollo de la retina.

CAPÍTULO 3: Lesiones traumáticas inducen cambios en la expresión del receptor 1A de serotonina en la médula espinal

A diferencia de mamíferos, la lamprea recupera la locomoción varias semanas después de una lesión completa de la médula espinal y una alta proporción de los axones descendentes procedentes del cerebro son capaces de cruzar y reinervar la zona caudal a la lesión. Tras un daño de médula espinal en mamíferos, la pérdida de serotonina procedente de los axones descendentes reduce la excitabilidad de las motoneuronas, lo que lleva a una respuesta compensatoria de sobre-expresión de receptores serotonérgicos. En este estudio, utilizamos un método semiautomatico para cuantificar la expresión de $5\text{-}HT_{1A}$ en la médula espinal de lamprea tras una lesión medular mediante hibridación *in situ*. La médula fue completamente seccionada a nivel de la quinta branquia y los animales se recuperaron entre 1, 3 ó 7 semanas tras la lesión. Nuestros resultados muestran un marcado incremento de la expresión de $5\text{-}HT_{1A}$ 1 semana después de la sección completa de la médula espinal, tanto rostral como caudalmente al lugar de

la lesión. Más adelante, la expresión de 5-HT_{1A} regresa a niveles similares a los de animales sin lesión (3-7 semanas), tanto rostral como caudalmente al lugar de la lesión. Resulta interesante que la sobre-expresión de 5-HT_{1A} en niveles rostrales tras un daño medular no se ha descrito en mamíferos, sugiriendo que la sobre-expresión rostral puede formar parte de los eventos plásticos que llevan a la recuperación de la función locomotora en vertebrados regeneradores como la lamprea en comparación con animales no regeneradores como mamíferos. Esto sugiere que la pérdida de serotonina como consecuencia de la degeneración de las fibras serotonérgicas tras una lesión de médula espinal induce una sobre-expresión de 5-HT_{1A}. Esta sobre-expresión a niveles rostrales de la lesión no se ha descrito en mamíferos, sugiriendo que la sobre-expresión de este receptor podría ser parte en los eventos plásticos que conllevan una recuperación de la función locomotora en vertebrados regeneradores como la lamprea. El análisis de los cambios en la expresión del receptor por zonas (region periventricular y la region gris extendida horizontalmente) ha mostrado que, excepto la capa ventricular caudal, básicamente siguen el mismo patrón de cambios detectados en la médula espinal entera. En la capa periventricular caudal no se observaron diferencias estadísticamente significativas entre los controles y los animales lesionados a ningún tiempo de recuperación. Esto sugiere que diferentes señales moleculares actuarían en las células periventriculares de la zona rostral y de la zona caudal de la médula espinal tras una sección completa de la médula y por consiguiente afectarían a su respuesta ante la lesión.

Éstos y previos resultados indican que la pérdida de serotonina como consecuencia de la degeneración de las fibras serotonérgicas tras una lesión podría inducir una sobre-expresión compensatoria de 5-HT_{1A}. Basándonos en observaciones en lamprea y en datos previos en los que se indica que la activación del receptor es beneficiosa tras daño de la médula espinal, una disponibilidad mayor del 5-HT_{1A} podría ser beneficiosa para la recuperación para compensar la escasa disponibilidad de su agonista.

CAPÍTULO 4: Regeneración espontánea de la inervación serotonérgica descendente tras una lesión medular en la lamprea de mar

A diferencia de mamíferos, las lampreas son capaces de recuperar una locomoción aparentemente normal después de una lesión completa de la médula, ya que los axones descendentes regeneran selectivamente en las rutas correctas. La inervación descendente serotonérgicas a la médula está implicada en la modulación de la locomoción a nivel medular tanto en mamíferos como en lampreas. En este estudio, se utilizaron técnicas combinadas de inmunofluorescencia y trazado neuronal para mostrar que en lampreas la se regenera la inervación descendente serotonérgica desde el rombencéfalo caudal después de una sección completa de la médula. La médula de larvas maduras fue seccionada a nivel de la quinta branquia, y tras cinco meses, la médula fue nuevamente seccionada 1 mm caudal al lugar de la primera lesión y se aplicó el trazador neurobiotina. Se observó doble marcaje en la porción caudal del núcleo vagal serotonérgico indicando que las neuronas serotonérgicas descendentes del rombencéfalo caudal (núcleo vagal) regeneran sus axones a través del lugar de la lesión. El tamaño de las células serotonérgicas descendentes tras un daño completo de la médula espinal fue significativamente más pequeño que en las células serotonérgicas descendentes en larvas controles, sugiriendo que la axotonía afecta a la viabilidad de las neuronas serotonérgicas y que sólo aquellas neuronas capaces de "escapar" de los procesos de muerte celular regenerarían sus axones. Para investigar si la reinervación de niveles caudales se debe a la extensión y ramificación de axones que no lesionados o a la regeneración de axones que fueron seccionados, se llevaron a cabo nuevos experimentos utilizando la metodología anterior pero aplicando el trazador fluoro-gold en el momento de la primera sección medular. Se observó triple marcaje en la parte caudal del núcleo vagal, indicando que al menos una parte de la reinervación se corresponde con verdadera regeneración. Ya que sólo el 40% de la población original de neuronas serotonérgicas en el núcleo vagal reinervó niveles caudales al lugar de la lesión tras una sección completa de la médula espinal pero sólo el 20% corresponde a verdadera regeneración, nuestros resultados también indicarían que el crecimiento de los

axones de neuronas no axotomizadas situados justo antes del lugar de la lesión ayudarían a compensar la pérdida de la inervación serotonérgica descendente. Este estudio proporciona un nuevo e interesante modelo para investigar los mecanismos moleculares intrínsecos involucrados en la regeneración de los axones descendentes serotonérgicos en vertebrados. El uso de este modelo proporcionará valiosa información para proponer nuevas terapias con pacientes con lesiones medulares.

Algunos capítulos de esta Tesis Doctoral han sido publicados en forma de artículos de investigación con las siguientes referencias:

- Capítulo 1: Brain Struct Funct (2013) 218: 587-593.
- El Capítulo 2 será publicado en tres artículos diferentes. La expresión del receptor 1A de serotonina en el cerebro y la médula espinal ya ha sido publicada (Brain Struct Funct. 2012, DOI 10.1007/s00429-012-0461-y). Los resultados de la expresión del receptor junto con la expresión de Tph en la retina se enviará para su publicación en la revista Experimental Eye Research, y los resultados de la expresión en los plexos coroideos y los vasos sanguíneos en el cerebro se enviará para su publicación en la revista Neuroscience Letters.
- El Capítulo 3 se enviará para su publicación en la revista Journal of Neurotrauma.
- Capítulo 4: J Neurotrauma (2011) 28: 2535-2540.

Para este proyecto de tesis hemos utilizado todos los estadíos de desarrollo de la lamprea: embriones, prolarvas, larvas, metamórficos y adultos. Se utilizaron técnicas de clonaje y secuenciación para la caracterización de la enzima *Tph* y el receptor 1A de serotonina. La primera hebra de ADNc en la reacción de síntesis fue catalizada usando la Superscript III Reverso Transcriptasa con cebadores oligodT18 o cebadores aleatorios. Para clonación por PCR, se diseñaron cebadores de oligonucleótidos degenerados

mediante alineamientos de secuencias de ARNm de distintas especies depositadas en archivos del NCBI y en la base de datos del genoma de la lamprea Ensembl. Los alineamientos de nuestras secuencias clonadas y secuencias proteicas correspondientes a otras especies se utilizaron para construir diferentes árboles filogenéticos mediante los métodos de mínima evolución y neighbor-joining.

Además, técnicas de hibridación *in situ* se usaron para estudiar la expresión de estos genes en el sistema nervioso central. Ribosondas unidas a Digoxigenina (DIG) se sintetizaron utilizando cebadores sintetizados a partir de nuestras secuencias clonadas y siguiendo protocolos estándares con la polimerasa T7. Hibridaciones *in situ* se realizaron usando protocolos estándar con las siguientes modificaciones. Se realizó un tratamiento con proteinasa K seguido de un rehidratación de los embriones y prolarvas, y se añadió un tratamiento con ARNasa A a los lavados posthibridación. El anticuerpo de oveja anti-DIG conjugado con fosfatasa alcalina se incubó toda la noche a 4˚C en una solución bloqueadora. La coloración se realizó con BM Purple a 37ºC hasta que la señal fue claramente visible. Para cuantificar el ARNm durante la regeneración de la médula espinal, se empleó el programa Feature J Hessian para Image J. Para los análisis estadísticos, se usó el programa Prism.

Se usaron métodos de trazado neuronal retrógrado (usando neurobiotina o fluoro-gold como trazadores) en combinación con inmunofluorescencia para serotonina para determinar la habilidad de la inervación serotonérgica descendente de regenerar tras una lesión en la médula espinal. La médula espinal se expuso desde la línea media dorsal a nivel de la quinta branquia y se seccionó por completo con un bisturí. Los extremos cortados de la médula fueron visualizados bajo una lupa. Los animales con la médula seccionada se examinaron a las 24h de la cirugía para confirmar que no hubiese ningún movimiento caudal al lugar de la lesión, y se mantuvieron para su recuperación en tanques con agua fresca a 19-20ºC. La médula espinal se volvió a exponer tras el periodo de recuperación para aplicar cristales del trazador neurobiotina con la ayuda de

una minucia (00). Los animales se mantuvieron a 4ºC con la aireación adecuada en solución Ringer para lampreas para permitir el transporte del trazador. Para la detección indirecta del trazador, las secciones se incubaron a temperature ambiente durante 4h con avidina unida a fluoresceína isotiocianato (FITC). El trazador fluoro-gold se aplicó en el momento de la lesión con una minucia (00) y se observó directamente en el microscopio confocal con el láser ultravioleta. La inmunofluorescencia de serotonina se realizó utilizando un anticuerpo policlonal de conejo anti-5-HT a 4ºC durante 72h. Para la detección indirecta por inmunofluorescencia de la serotonina se usó un anticuerpo de cabra anti-conejo conjugado con Cy3.

Todos los experimentos fueron aprobados por el comité de ética de la Universidad de Santiago de Compostela y cumplieron las normas de la Unión Europea sobre cuidado y experimentación animal para minimizar el dolor y la molestia.

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Conclusiones

El análisis de los resultados en esta Tesis nos permite extraer las siguientes conclusiones:

1. Los análisis filogenéticos de las secuencias de *Tph* sugieren que la *Tph* de la lamprea de mar es ortóloga de las secuencias *Tph1* de vertebrados gnatóstomos.
2. La expresión del transcrito *Tph* en los núcleos diencefálicos en la lamprea de mar sugiere que ésta es una condición primitiva en vertebrados, mientras que su expresión en poblaciones rombencefálicas es una característica de lampreas que podría ser un carácter primitivo o uno recientemente desarrollado.
3. La ausencia de expresión del transcrito *Tph* en los tejidos periféricos sugiere la existencia de una segunda enzima con la capacidad de hidroxilar el triptófano en el sistema nervioso periférico.
4. La expresión del transcrito Tph en la retina sugiere que podría sintetizar serotonina en las células amacrinas y melatonina en los fotorreceptores, mientras que su presencia en neuroblastos sugiere que la serotonina podría tener otra función aparte de neurotransmisor en el desarrollo de la retina.
5. Los análisis filogenéticos de las secuencias 5-HT_{1A} y de su región de unión G_i/G_o altamente conservada sugieren que el receptor 5-HT_{1A} apareció antes de la separación entre agnatos y gnatóstomos.
6. La expresión del ARNm 5-ht1a en lamprea se extiende a regiones con neuronas serotonérgicas pero también con otros tipos neuronales, indicando que el 5-HT_{1A} puede actuar como como heteroreceptor y autoreceptor, y sugiriendo que las funciones pre- y post-sinápticas son un carácter común a los vertebrados.
7. La expresión del transcrito 5-ht1a comienza después de eclosionar en P1 y continúa a lo largo del desarrollo sin evidencias de poblaciones transitorias que expresen el receptor.

8. La expresión del transcrito 5-ht1a en los somas, dendritas basales y células gliales parece una característica compartida entre mamíferos y lampreas indicando que podría ser un carácter primitivo de vertebrados.

9. La expresión del transcrito 5-ht1a en la retina durante los diferentes estadios de desarrollo de lamprea sugiere que, además de ser auto- y heteroreceptor, podría tener una función en el desarrollo de la misma.

10. La expresión de 5-ht1a se incrementa drásticamente 1 semana después de la lesión tras una sección completa de la médula espinal tanto rostral como caudalmente al lugar de la lesión, y regresa a niveles similares al de animales sin lesionar entre 3 y 7 semanas después de la lesión, similar a lo que se ha observado en mamíferos.

11. La sobre-expresión de 5-ht1a puede ser una respuesta aguda compensatoria debida a la degeneración de fibras serotonérgicas tras una lesión de médula espinal.

12. Al menos el 20% de las neuronas serotonérgicas del rombencéfalo con inervación descendente regeneran sus axones hasta niveles caudales a la lesión, mientras que el crecimiento de los axones no lesionados puede compensar la pérdida de la inervación serotonérgica descendente.

13. El tamaño de las células serotonérgicas con inervación descendente después de una lesión medular es significativamente más pequeño que en los controles, sugiriendo que la sección del axón afecta a viabilidad de las neuronas serotonérgicas y que sólo las neuronas capaces de "escapar" a los procesos de muerte celular regenerarían sus axones.

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