Universidade de Santiago de Compostela

DEPARTAMENTO DE BIOLOXÍA CELULAR E ECOLOXÍA

ÁREA DE BIOLOXÍA CELULAR

"THE SEA LAMPREY MONOAMINERGIC SYSTEM: PERIPHERAL ORGANIZATION, ORIGIN OF THE AFFERENTS TO THE SPINAL CORD AND RELATIONSHIP WITH OTHER NEUROTRANSMITTER SYSTEMS"

MEMORIA

Que para optar al grado de Doctor en Biología presenta

Antón Barreiro Iglesias

Santiago de Compostela, 2010

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

CERTIFICA:

Que la presente memoria titulada "The sea lamprey monoaminergic system: peripheral organization, origin of the afferents to the spinal cord and relationship with other neurotransmitter systems", (El sistema monoaminérgico de la lamprea de mar: organización periférica, origen de las aferencias a la médula espinal y relación con otros sistemas de neurotransmisores) que para optar al Grado de **Doctor en Biología** presenta D. ANTÓN BARREIRO IGLESIAS, ha sido realizada bajo mi dirección, y considerando que constituye trabajo de TESIS DOCTORAL, autorizo su presentación al Consejo de Departamento correspondiente.

Y para que así conste, expido el presente certificado en Santiago de Compostela, a de de 2010.

El Doctorando La Directora de la Tesis

Fdo. Antón Barreiro Iglesias Fdo. María Celina Rodicio Rodicio

Esta Tesis Doctoral forma parte de los Proyectos de Investigación titulados:

- "Estudio hodológico y neuroquímico de los sistemas catecolaminérgico y serotoninérgico en el sistema nervioso central de la lamprea de mar, Petromyzon marinus (Cyclostomata)" del Ministerio de Educación y Ciencia (BFU2004-01080)
- "Nuevas aportaciones al estudio neuroquímico del desarrollo y organización del sistema nervioso central de la lamprea de mar" de la Xunta de Galicia (PGIDIT04PXID20003PR; 2004-2007).

Su realización ha sido posible gracias a la concesión de una Beca Predoctoral de la Xunta de Galicia, Consellería de Educación e Ordenación Universitaria y de un Contrato de Investigador en Prácticas "María Barbeito" de la Xunta de Galicia, Consellería de Innovación e Industria, a D. Antón Barreiro Iglesias durante los periodos del 1/10/2005 a 30/9/2007 y del 19/12/2007 a 31/3/2010 respectivamente.

Por fin…buffff, aquí estoy delante del papel en blanco tratando de recordar los buenos momentos que me han traido hasta este punto…y seguido. HABEMUS DOCTOR!!! (la fumata blanca no se si procede)… y, aunque yo he puesto de mi parte xD, me gustaría dar las gracias a la gente que ha puesto su grano o tonelada de arena para que este momento haya sido posible.

En primer lugar quiero agradecerle a mi jefa Celina que me diera la oportunidad de haberme adentrado en el maravillosos mundo de la neurociencia y por haber soportado a este cabezota durante casi 5 años, gracias de corazón. Y agradecerte también que siempre hayas escuchado mis ideas y opiniones y por no haber dudado en meterte conmigo en este asuntillo que tanto me gusta de la regeneración del SN. También un agradecimiento para el "boss" de la neuroanatomía comparada, Ramón, que siempre ha puesto su sabiduría a mi disposición. Also to Mickey, Catherina/Thomas and the people of their labs in Philly and Edinburgh for gave me the oportunity to work and learn with them.

En segundo lugar a mis compañeros departamentiles, especialmente a algunos que ya no están: Chusiño, "Ismailove", Clara, Ana, Sergio alias "el mister" e Iván por los buenos ratos que hemos pasado dentro y fuera (sobre todo fuera…jeje) del laboratorio. Y a los que se han incorporado hace más o menos tiempo: los tiburones Idoia, Sol y Gabriel o mis lampreas de reciente adquisición: Maru, Blanca y Silvia. Un abrazo también para Sole y Miguel que son los profes de Celular más simpáticos. Y en este apartado departamentil se merece una mención a parte, y muy especial, Verona "mi lampreyña" que ha sido la mejor compañera que me podía a ver tocado (si me la hubieran dejado diseñar a mi no habría salido mejor!!)…. gracias porque el trabajo ha sido muy fácil contigo al lado (miss you!!).

Llega el turno de los amiguetes. Otro pedazo de está tesis, aunque no entendaís nada de lo que pone ni falta que os hace, es para vosotros. Para los santiagueiros que siempre han estado a mi lado y con los que he vivido mis mejores momentos: Jitín, Gusiño, Manoleitor y Jose (no me olvido de tus padres, Teresa y Jose que siempre han sido unos buenos amigos). Al "peligro" de matarrosa, Adrián (buen fichaje gus!). Para los madrileños: Suker, Rodri, Pincho, Muñoz y Onru (gracias mallorca!!). Y para los americanos que me hicieron vivir una experiencia inolvidable: sobre todo Italo, Juan, Jona y Liana. Si se me olvida alguien, que seguro que sí, que imagine su nombre escrito en esta línea ____ que el/ella seguro que sabrá que tb. es una persona importante para mi.

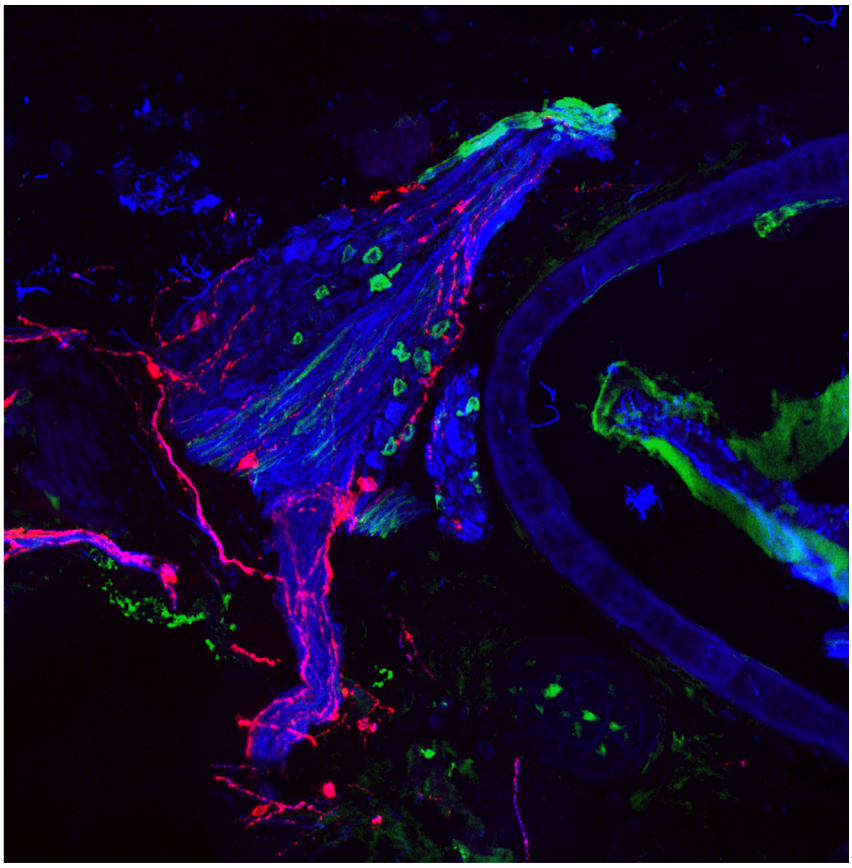
Y llega el turno de las personas más importantes de mi vida: mi familia. Un pedacito de tesis para mis tios pontevedreses (Carmen y Jose). Gracias a mis GRANDES hermanos, que son los mejores: Borja y Lucía (que para mí siempre será mi "pochi"), ya que os tengo siempre conmigo. Y sobre todo a mis padres que son lo más importante, daros las gracias por haber criado a este proyecto de científico y por haberos tenido siempre mi a lado (no os lo digo nunca pero a los 4 sabeis es os quiero mucho…).

Finalmente en este párrafo y como ordenan los canones llegaría el momento de la churri y/o parienta, como en estos momentos no dispongo de ese complemento sólo decir que si alguna chica de buena familia está interesada en un apuesto neuroscientist y futuro doctor que pregunte por Antón en la planta 3 del CIBUS.

Y ahora, allá vamos con lo que nos ocupa: el sistema monoaminérgico de este simpático bichejo….

Leave an impression. . .

CONTENTS



- **General introduction** 15
- **Chapter 1: Peripheral organization of the sea lamprey monoaminergic system**
  - **1.1 Extensive presence of serotonergic cells and fibers in the peripheral nervous system of lampreys.** 31
  - **1.2 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.** 81
- **Chapter 2: Origin of the descending monoaminergic innervation to the sea lamprey spinal cord**
  - **2.1 Descending brain-spinal cord projections in a primitive vertebrate, the lamprey: cerebrospinal fluid-contacting and dopaminergic neurons.** 127
  - **2.2 Development and organization of the descending serotonergic brainstem-spinal projections in the sea lamprey.** 161
- **Chapter 3: Relationship with other neurotransmitter systems**
  - **3.1 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.** 187
  - **3.2 Dopamine and GABA are colocalized in restricted groups of neurons in the sea lamprey brain: insights into the early evolution of neurotransmitter colocalization in**

vertebrates. **211**

- **Summary and Conclusions**

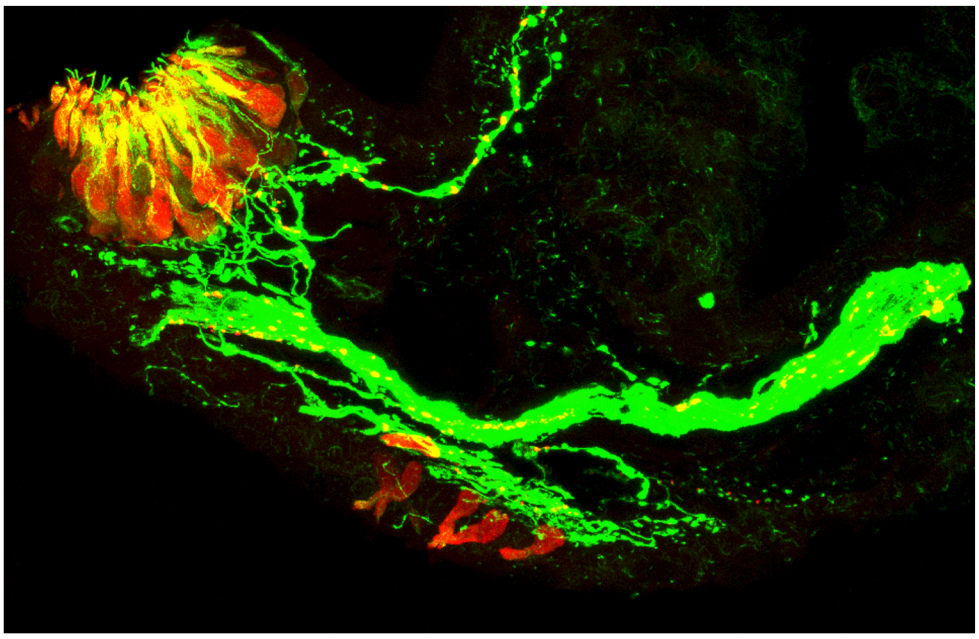
Summary **241**

Conclusions **249**

Resumen **251**

Conclusiones **259**

General Introduction



General introduction

Comparative neuroanatomical studies during the last decades have shown that many of the cell populations and their interconnections that were know to be present in mammals (including primates) were also present in nonmammalian vertebrates. The evolution of the vertebrate central nervous system (CNS) appears far more conservative than earlier investigators had realized. However, large differences in the organization of the brain exist between animals that are adapted to different modes of life. The study of these differences is that makes comparative neurobiology really interesting.

The lamprey as an animal model for neuroscience research

A search of the term "lamprey" on the PubMed (the NCBI database for biomedical literature citations and abstracts) retrieves more than 2650 results. Most of these studies pertain to the field of neuroscience research. Why lampreys are suitable and useful models for conducting basic research in neurobiology?

1) Lampreys are jawless vertebrates that belong to the most ancient group of extant vertebrates, the agnathans or cyclostomes (Hardisty and Potter, 1971). They hold a unique phylogenetic position between *Amphioxus* (protochordates) and gnathostomes (jawed vertebrates). The position occupied by lampreys near the root of the vertebrate tree makes them an important animal model for comparative studies of the nervous system. Lampreys 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 (for a revision see Osório and Rétaux, 2008; see also Murakami and Watanabe, 2009).

2) Comparative neuroanatomical studies of lampreys have a literature that start at middle 1800s, with the study of the gross anatomy of their nervous system (e.g. Reissner, 1860; Johnston, 1905). Even the first assignment of Sigmund Freud, before he had developed the psychoanalysis, was the investigation of the spinal cord of lamprey larvae (Freud, 1877; Freud, 1878). 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).

3) The lamprey nervous system has been used as an animal model to study the neuronal circuitry underlying locomotion in vertebrates (see Dubuc et al., 2008; see Grillner

et al., 2008). In some species that habitually swim, the excitability of the circuits for the coordination of propulsive locomotion movements is so high. The central nervous system (CNS) of the lamprey has all the main regions of the mammalian CNS, but much fewer neurons than in mammals. The isolated lamprey brainstem-spinal cord can be maintained alive in vitro for several days. 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).

The first Kavli Prize (http://www.kavliprize.no) in neuroscience (which is considered as equivalent to the Nobel Prize in this research area) went to Sten Grillner of the Karolinska Institute in Sweden. He was awarded because, as the prize's committee stated, "*his lamprey model is seen as the first and so far only vertebrate neuronal system controlling an integrated function that is understood at a molecular and cellular level*", which reflects the importance of the lamprey as a vertebrate model to study the neuronal networks that underlie locomotion in vertebrates.

4) The lamprey has been also established as a model organism for studies of spinal cord regeneration (Cohen et al., 1988). Lampreys recover locomotion almost completely after transection of their spinal cords (Rovainen, 1976; Selzer, 1978). 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). No vertebrate other than lamprey has 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).

5) In the last years, lampreys have been also used as an animal model to study the citotoxic effects in neurons of proteins involved in Alzheimer's disease. The lamprey brain has large identifiable neurons that can be microinjected with plasmids encoding human proteins. This allows to the direct investigation in vivo of the causes and consequences of the expression of different proteins that are thought to play a critical pathogenic role. One example is the tau protein, whose accumulation or aggregation is the intracellular hallmark of neurofibrillary degenerative diseases such as

Alzheimer's disease (Hall et al., 1999; Hall et al., 2000).

Aim of the present investigation

In our laboratory, we previously reported the development and organization of the serotonergic and dopaminergic immunoreactive systems in the CNS of the sea lamprey, *Petromyzon marinus* L., (Abalo et al., 2005, 2007). As a continuation of these previous studies, the goal of the present investigation was to advance on the knowledge of the monoaminergic system of lampreys.

The aims proposed to be developed in this Thesis project were:

I. To determine the possible presence of monoaminergic cells and fibres in the peripheral nervous system (PNS) of lampreys. Our experiments revealed a complex and extensive presence of serotonergic elements in the peripheral nervous system of the sea lamprey head. The organization of the serotonergic structures and their innervation patterns were studied. Special attention was aid on the neurochemical characterization of the sea lamprey taste buds in which a serotonergic cell type was observed.

II. To determine the cells of origin of the dopaminergic and serotonergic descending innervation of the spinal cord in lampreys by means of combined tract-tracing and immunohistochemical experiments. As an unexpected result, tract-tracing experiments also revealed neurons not previously reported as being spinal cord-projecting populations in lampreys.

III. To study the possible coexistence and colocalization of serotonin (5-HT) or dopamine (DA) and γ-aminobutiric acid (GABA) in the same neuron of the nervous system of the sea lamprey. Colocalization of these monoamines and GABA was observed in specific locations of the sea lamprey brain. Our experiments revealed that monoaminergic lamprey neurons are more neurochemically heterogeneous than previously thought.

Organization of the thesis

The present study has been organized in 3 chapters corresponding to the 3 main objectives proposed in the present investigation.

In the first chapter, a description of the distribution and organization of serotonergic cells and fibres in the PNS of the lamprey head is exposed. Tract-tracing techniques and immunohistochemistry against acetylated α-tubulin were used, in combination with the immunohistochemical detection of 5-HT, to study the innervation patterns of the serotonergic peripheral cells and as markers of nervous system structures. High performance liquid chromatography (HPLC) experiments were also performed in order to confirm biochemically the presence of 5-HT in the lamprey head after removal of brain/spinal cord. Dopamine was not detected in the

lamprey head and noradrenaline (NA) was detected in low levels (unpublished results). Immunohistochemical experiments with antibodies raised against tyrosine hydroxylase (TH; the rate-limiting enzyme for the catecholamine synthesis) or DA did not reveal any labelling in the peripheral tissues of the lamprey head (unpublished results). In addition, we focused on the neurochemical characterization of the sea lamprey taste buds; in these chemoreceptive structures a main serotonergic cell type was observed.

In the second chapter, we present the results of the investigation of the origin of the descending monoaminergic innervation of the spinal cord. Tract-tracing experiments in combination with DA and 5-HT immunofluorescence revealed monoaminergic cell nuclei of the brain with cells that project to the spinal cord. New cell nuclei not previously reported as spinal cord-projecting were also observed in our preparations of the sea lamprey brain.

Finally, in the third chapter, the results of the study of the possible colocalization of 5-HT or DA with GABA in neurons of the lamprey CNS is presented. Co-existence of monoamines and GABA was observed in neurons of different brain nuclei.

In the next sections, a brief introduction to the neurotransmitters systems here studied and to the material and methods used to develop the present investigation is presented.

The monoamines

This section is mainly based on the revision of the neurochemical systems made by Siegel et al. (2006). Monoamines are neurotransmitters and neuromodulators that contain one amino group, which is connected to an aromatic ring by a two-carbon chain (-CH2-CH2-). All monoamines are derived from aromatic amino acids like phenylalanine, tyrosine and tryptophan by the action of aromatic amino acid decarboxylase enzymes.

Serotonin

The indolealkylamine 5-hydroxytryptamine was initially identified because of its effects on smooth muscle. Since the mid-19th century, scientists have known that after blood clots, a substance in the resulting serum constricts vascular smooth muscle. Platelets were identified as the source of this substance. It was not until 1948 that Raport, Green and Page isolated and identified the serum vasoconstrictor factor as the 5-HT (Rapport et al., 1948). Twarog and Page, in 1953, detected 5-HT in extracts of brain.

The aminoacid L-tryptophan is the precursor for 5-HT synthesis. The synthesis and primary metabolic pathways of 5-HT are shown in Fig. 1.

In addition to classical synaptic transmission, 5-HT may relay information by volume transmission or paracrine neurotransmission. Serotonergic terminals make the usual specialized synaptic contacts

with target neurons and release 5-HT following nerve stimulation. However, in some brain areas, there are sites where 5-HT is released and there is no evidence for synaptic specializations. The lack of synaptic specialization implies a dynamic and perharps less specific interaction with target neurons. Neurotransmitter is released to the intercellular space and then diffuses over some distance; this is termed volume transmission. It is also seen in dopaminergic target areas (see below).

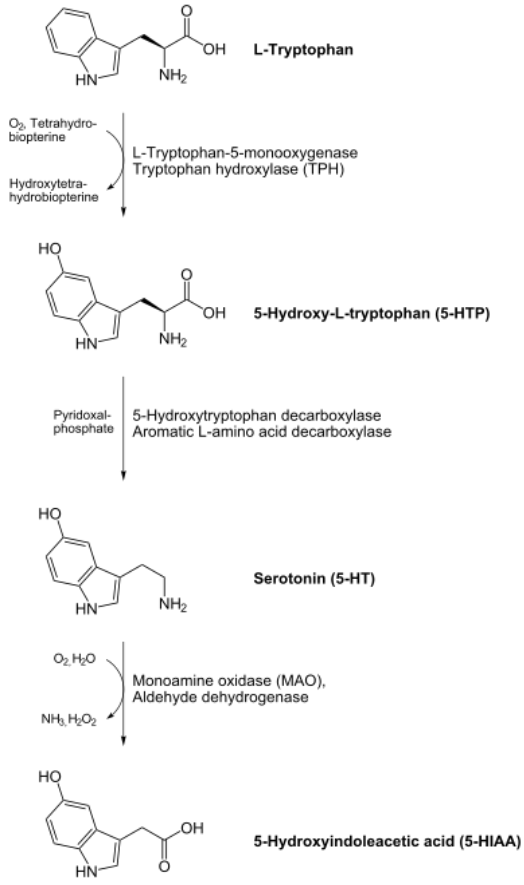


Fig. 1. Biosynthesis and catabolism of 5-HT.

Serotonin has been implicated in practically every type of behaviour, e.g. apetite, emotional, motoric, cognitive or autonomic. Serotonin has a general effect on behaviour by modulating the tone of nervous system activity, neuroendocrine function, circadian rhythmicity or feeding behaviour. In addition, recent studies support a role for 5-HT in development and in neuroplastic events that create, repair and degenerate the nervous system (see Sodhi and Sanders-Bush, 2004). In lampreys, 5-HT has been shown to participate in generation and modulation of locomotion (see Wallén et al., 1989) and contributes significantly to the rhythm generation (Zhang and Grillner, 2000).

Dopamine

Dopamine is one of the members of the neurotransmitters/hormones group called catecholamines together with NA and adrenaline (A). Dopamine, the precursor of NA and A, serves as a neurotransmitter in several important pathways of the nervous system.

The enzymatic processes involved in the formation of catecholamines have been characterized. The aminoacid L-tyrosine is the precursor for the synthesis of catecholamines. Tyrosine hydroxylase is the rate-limiting enzyme for the biosynthesis of catecholamines. The synthesis and primary metabolic pathways of catecholamines are shown in Fig. 2.

Dopamine was classically associated with mood and locomotive activity control, and their absence in humans produces several symptoms related to schizophrenia or Parkinson's disease. Dopamine has also an important role during development. An important role for DA in teleosts is also to

regulate the secretion of several hypophysial hormones (Kah et al., 1986, 1987). In lampreys, DA participates in the control of locomotion and body orientation (see Grillner et al., 1995) and its forebrain depletion has been shown to cause motor deficits similar to those observed in mammals (Thompson et al., 2008).

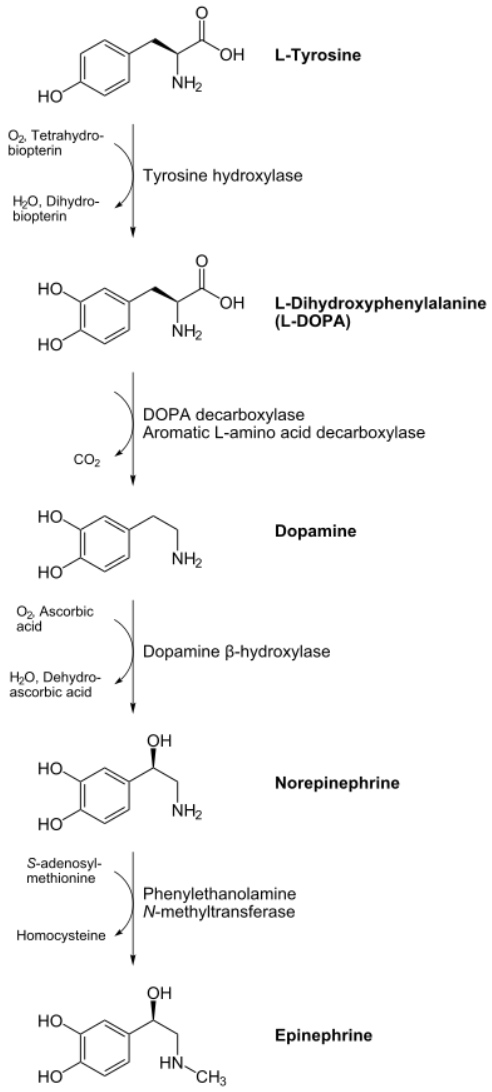


Fig. 2. Biosynthesis of catecholamines.

Organization of the monoaminergic system of the sea lamprey

In this section, we present a brief description of the organization of the serotonergic and dopaminergic systems in the CNS of the sea lamprey based on the results of Abalo and coworkers (DA: 2005; 5-HT: 2007).

The 5-HT immunoreactive system of the larval and adult sea lamprey

A schematic drawing showing the organization of the 5-HT-immunoreactive (-ir) system in the CNS of the larval and adult sea lamprey is shown in Fig. 3 [which is based on the results of Abalo et al. (2007) with slight modifications]. Serotonin-ir cell neurons are present in the sea lamprey spinal cord, rhombencephalon and diencephalon; no 5-HT-ir cells are present in the mesencephalon and telencephalon of the sea lamprey. The different 5-HT-ir brain populations observed in the adult sea lamprey are already observed at the end of the larval stage.

Eight 5-HT-ir neuronal populations are present in the CNS of the sea lamprey (Abalo et al., 2007): 5-HT-ir cells of the ventral margin of the spinal cord, vagal reticular group (caudal rhombencephalon), cells of the isthmic population (rostral rhombencephalon) in which 3 subgroups of 5-HT-ir cells are distinguishable (dorsal, medial and ventral isthmic subgroups), pretectal population, the population of the zona limitans intrathalamica, the populations of 5.HT-ir cerebrospinal fluid-

contacting (CSF-c) cells of the mammillary nucleus and tuberal nucleus of the hypothalamus, and 5-HT-ir cells of the pineal and parapineal organs.

The DA immunoreactive system of the larval and adult sea lamprey

A schematic drawing showing the organization of the DA-ir system of the CNS of the larval and adult sea lamprey is shown in Fig. 4 [which is based on the results of Abalo et al. (2005) with slight modifications]. Dopamine-ir cell nuclei are observed in the spinal cord, rhombencephalon, diencephalon

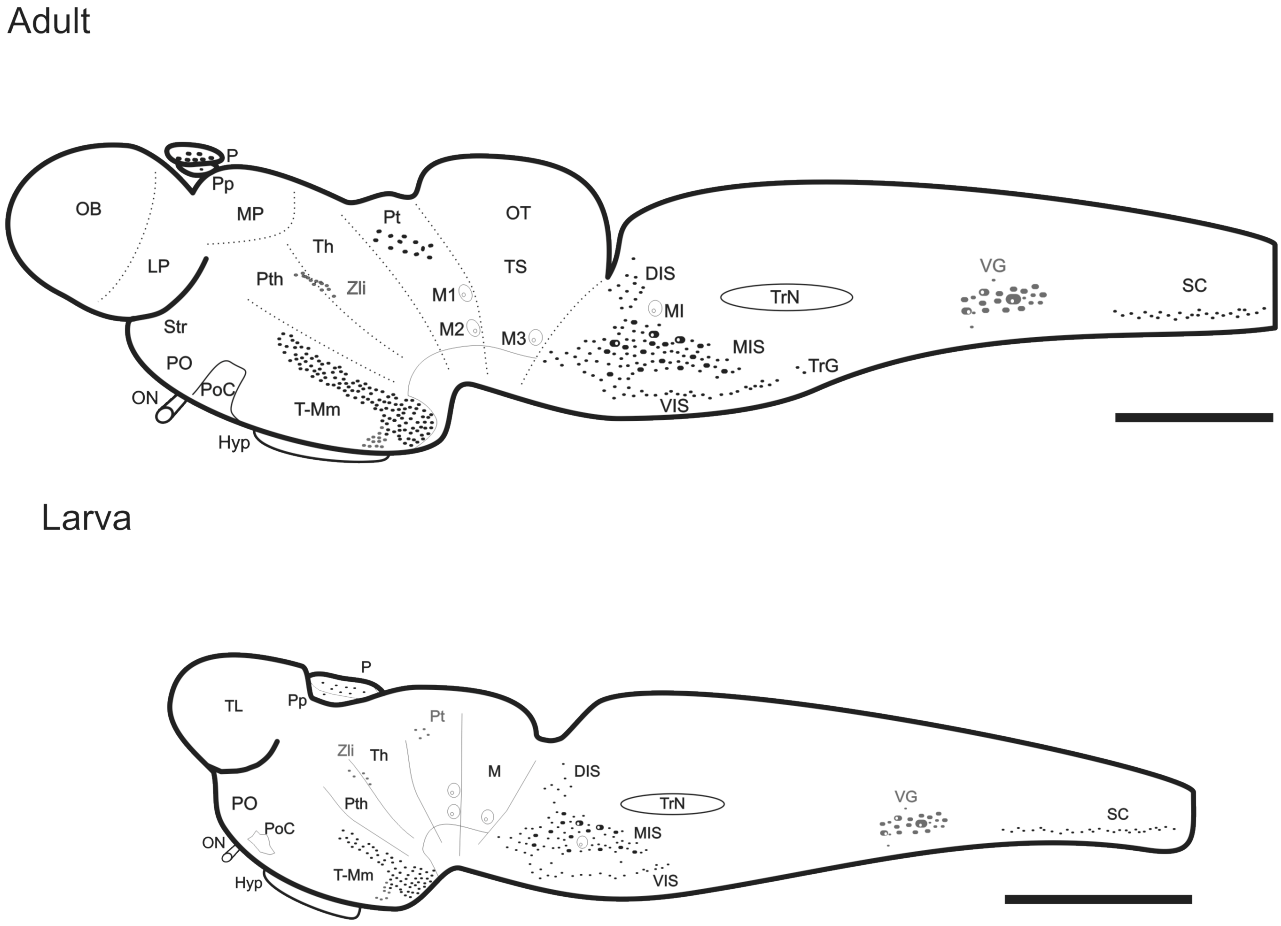


Fig. 3. Sagittal drawings of adult and larval brains of sea lamprey showing the 5-HT-ir cell groups and some segmental and regional limits in the brain. For abbreviations see list. Scale bars: adult:1 mm; larva: 500 mm.

and telencephalon; no DA-ir cells are observed in the mesencephalon of the sea lamprey. All the DA-ir cell nuclei observed in the adult sea lamprey CNS are also observed at the end of the larval stage with the exception of the ventral hypothalamic nucleus, which develops during the metamorphosis.

Ten DA-ir neuronal populations are observed in the CNS of the adult sea lamprey (Abalo et al., 2005): DA-ir cells of the spinal

cord, the group of CSF-c cells located under the ventricle of the caudal rhombencephalon, the small population of DA-ir cells of the ventral isthmus, numerous DA-ir CSF-c cells in the mammillary nucleus [postinfundibular commissure nucleus of Abalo et al. (2005)], paratubercular nucleus [posterior tubercle nucleus of Abalo et al. (2005)] and the dorsal and ventral hypothalamic nuclei, the DA-ir cells of the postoptic commissure nucleus, cells of the preoptic nucleus and the DA-ir population of the olfactory bulbs.

Materials and methods

The sea lamprey has a complex and long life cycle (Fig. 5). Embryonic, prolarval, larval, metamorphic and adult stages are distinguishable during its life cycle (see Hardisty and Potter, 1971). In the present investigation larval and adult samples were used. For an indirect estimation of the larval age the body length of the larvae was used.

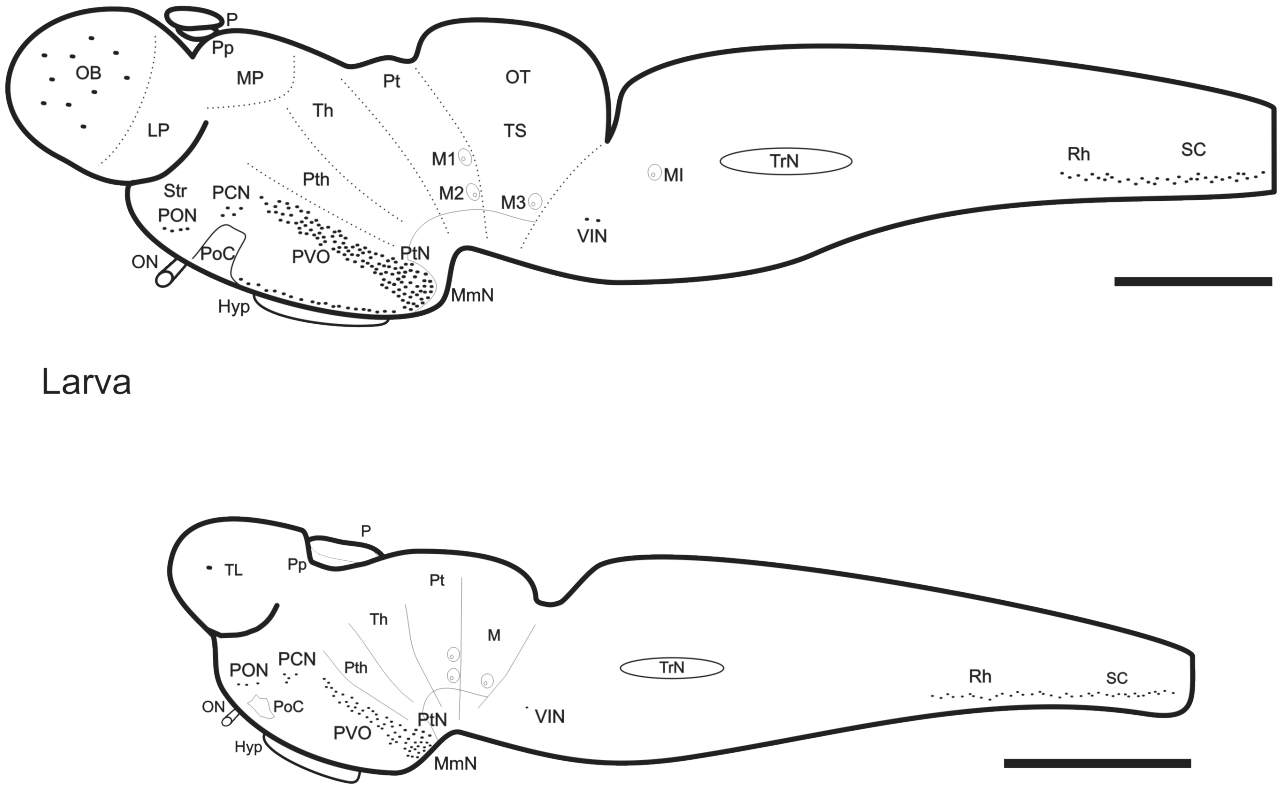


Fig. 4. Sagittal drawings of adult and larval brains of sea lamprey showing the DA-ir cell groups and some segmental and regional limits in the brain. For abbreviations see list. Scale bars: adult: 1 mm; larva: 500 μm.

Both postmetamorphic young adults and upstream migrating adults were used. Larval and postmetamorphic lampreys were collected from the River Ulla with permission from the Xunta de Galicia. Upstream migrating adults were purchased from commercial

supplier. Larvae were maintained in well aerated aquaria with a bed of river sediment, whereas postmetamorphic and adult lampreys were used immediately after capture. All experimental procedures were performed under deep anesthesia with benzocaine.

For the characterization of 5-HT-ir, DA-ir, TH-ir and GABA-ir cells and fibers we performed double immunofluorescence protocols using polyclonal and monoclonal primary antibodies. Immunofluorescence detection of other neurotransmitters and neuronal markers (calretinin, acetylated tubulin, glutamate, calcitonin gene-related peptide, PCNA, BrdU) was also used to characterize some of the neuronal types. For more details see Material and Methods sections of chapters 1.1, 1.2, 2.1, 2.2, 3.1, 3.2 and 3.3.

In order to determine the origin of the descending monoaminergic innervation of the sea lamprey spinal cord, we performed a combination of 5-HT or DA immunofluorescence and tract-tracing experiments using biotinylated dextranamines (BDA) or neurobiotin (NB) as tracers. For more details see Material and Methods sections of chapters 2.1, 2.2 and 3.3.

To confirm biochemically the presence of monoamines in the central or peripheral nervous systems of the sea lamprey, HPLC experiments were also performed. For more details see Material and Methods sections of chapters 1.1 and 3.3.

Brain/spinal cord sections were analyzed and photographed with a spectral confocal microscope TCS-SP2 (Leica, Wetzlar, Germany), which allowed to best analyze the colocalization of two neurotransmitters/ neuronal markers in a cell or of a neurotransmitter and BDA or NB in retrogradely labelled neurons.

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

Abbreviations

DIS: dorsal isthmic subgroup, Hyp: hypophysis, LP: lateral pallium, M: mesencephalon, M1-MI: Müller cells, MIS: medial isthmic subgroup, MmN: mammillary nucleus, MP: medial pallium, OB: olfactory bulbs, ON, optic nerve, OT: optic tectum, P: pineal organ, PCN: postoptic commissure nucleus, PO: preoptic region, PoC: postoptic commissure, Pp: parapineal organ, Pt: pretectum, Pth: prethalamus, PtN: paratubercular nucleus, PVO: paraventricular organ, Rh: rhombencephalon, SC: spinal cord, Str: striatum, Th: thalamus, T-Mm: tuberal-mammillary nucleus, TL: telencephalon, TrG: trigeminal group, TrN: trigeminal nucleus, TS: torus semicircularis, VG: vagal group, VIN: ventral isthmic nucleus, VIS: ventral isthmic subgroup, Zli: zona limitans intrathalamica.

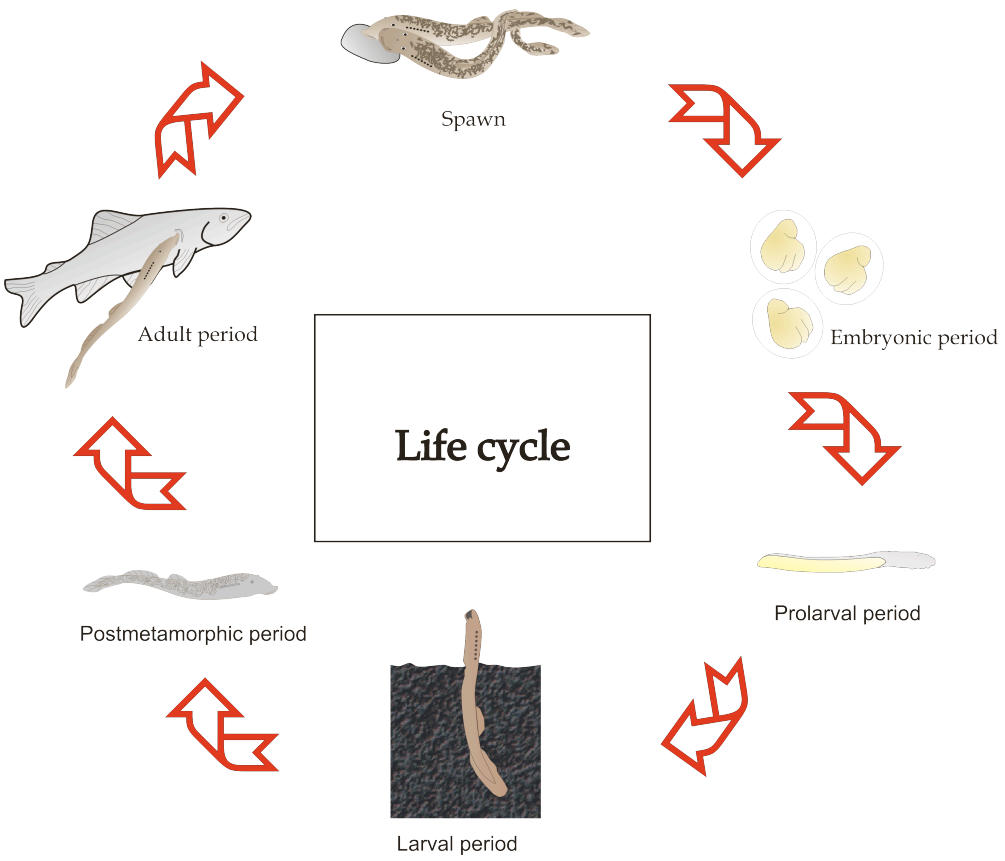


Fig. 5. Schematic drawing showing the sea lamprey life cycle.

References

Abalo XM, Villar-Cheda B, Anadón R, Rodicio MC. 2005. Development of the dopamine-immunoreactive system in the central nervous system of the sea lamprey. Brain Res Bull. 66:560-564.

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.

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

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.

Freud S. 1878. Uber Spinalganglien und

Ruckenmark des Petromyzon. Sitzungsb Acad Wiss Wien (Math-Naturwiss Cl) 77:81-167.

Freud S. 1887. Uber den Ursprung der hinteren Nervenwurzeln im Ruckenmark von Amnoceoetes (*Petromyzon planeri*). Sher Acad Wiss Wien (Math-Naturwiss Cl) 75:15-27.

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.

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.

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

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, 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.

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.

Hardisty MW, Potter IC. 1971. The general biology of adult lampreys. In: Hardisty MW, Potter IC, editors. The biology of lampreys, vol 1. London: Academic Press. p 127–206.

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

Kah O, Dubourg P, Onteniente B, Geffard M and Calas A. 1986. The dopaminergic innervation of the goldfish pituitary. An immunocytochemical study at the electron-microscope level using antibodies against dopamine. Cell Tissue Res. 244:577-582.

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE. 1987. Neuroanatomical substrate for the inhibition of gonadotropin secretion on goldfish: existence of a dopamienrgic preoptic-hypophyseal pathway. Neuroendocin. 45:451-458.

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

Nieuwenhuys R, Nicholson C. 1998. Lampreys, Petromyzontoidea. In: Nieuwenhuys R, ten Donkelaar HJ, Nicholson C, editors. The central nervous system of vertebrates, vol 1. Berlin:

Springer Verlag. p 397-486.

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

Raport MM, Green AA, Page IH. 1948. Serum vasoconstrictor (serotonin). IV. Isolation and characterization. J Biol Chem. 176:1243-1251.

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

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 (Lond.). 277:395-408.

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

Siegel GJ, Albers RW, Brady ST, Price DL, editors. 2006. Basic neurochemistry. Molecular, cellular and medical aspects. London: Elsevier Academic Press.

Sodhi MSK and Sander-Bush E. 2004. Serotonin and brain development. Int Rev Neurobiol. 59:111-174.

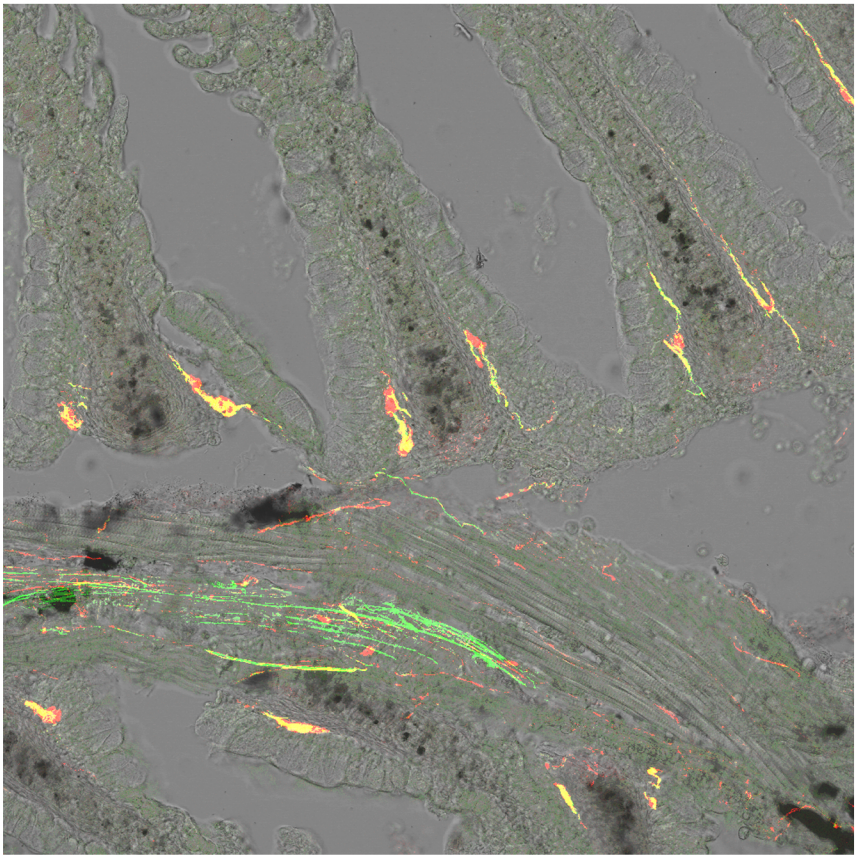
Thompson RH, Ménard A, Pombal M, Grillner S. 2008. Forebrain dopamine depletion impairs motor behavior in lamprey. Eur J Neurosci. 27:1452-1460.

Twarog BM, Page IH. 1953. Serotonin content of some mammalian tissues and urine and a method for its determination. Am J Physiol. 175:157-161.

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.

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

PERIPHERAL ORGANIZATION OF THE SEA LAMPREY MONOAMINERGIC SYSTEM



Extensive presence of serotonergic cells and fibers in the peripheral nervous system of lampreys

Introduction

The serotonergic system was one of the first neurotransmitter systems to be studied by histofluorescence methods in the vertebrate central nervous system (see Parent, 1981, 1984; Parent and Northcutt, 1982). The distribution of serotonergic cells and fibers in the central nervous system has also been studied by immunohistochemical methods in different vertebrates, including lampreys (Antri et al., 2006; Abalo et al., 2007), but fewer studies have been made with regard to the presence and distribution of serotonergic elements in the peripheral nervous system.

The intestine of lampreys (*Lampetra*) contains a loose network of nerve fibers and irregularly shaped multipolar neurons. Most of these neurons contain serotonin (5-HT), as shown by fluorescence histochemistry and microspectrofluorimetric studies (Baumgarten et al., 1973; Sakharov and Salimova, 1980). The latter authors also described a widespread system of yellow fluorescent fibers in the pharynx and the velum in larval *Lampetra planeri*. In addition, a study with the sea lamprey revealed the presence of serotonin-immunoreactive (5-HT-ir) nerve fibers and neurons in the olfactory nerve of larval specimens (Zielinski et al., 2000). However, there are no comprehensive studies of the distribution of 5-HT in the peripheral tissues of the head of lampreys.

The life cycle of the sea lamprey is very complex and includes clearly differentiated larval and adult stages. The larval stage takes place in rivers and lasts between 4 and 7 years. The larvae then undergo a complex metamorphosis to transform into young adults (postmetamorphic lampreys). The young sea lampreys descend to the sea and feed as parasites on teleost fishes. After 2 years, the large prespawning adults migrate to rivers to breed and die (reviewed by Hardisty and Potter, 1971).

The anatomy of the head differs greatly in larval and adult lampreys, reflecting their different lifestyles (see Hardisty and Potter, 1971). The larvae feed exclusively on microorganisms in suspension, which become embedded in mucus in the pharynx (Baatrup, 1985). Schematic drawings showing the anatomy of the larval samples are shown in Figure 1. The mouth of the larval sea lamprey is divided into the buccal and oral cavities. The anterior opening of the mouth leads into a large buccal cavity through a narrow oral aperture. Posterior oral cirri form a ring of branched projections around and across the oral aperture,

and prevent the passage of objects greater than 340 μm into the oral cavity (reviewed by Hardisty and Potter, 1971). The muscles used for feeding are innervated by the trigeminal nerve (Vth) (Homma, 1975).

In larvae, a muscular velum that is also innervated by the Vth nerve pumps water through the pharynx and gills. The current of water, used for respiration and feeding, flows into the pharynx by the coordinated action of the velum movements and by the sequential contraction and expansion of the branchial region. The water comes out through the seven pairs of branchiopores. The pharyngeal region is also the site where food particles are trapped before passing into the esophagus. Tracts of ciliated cells present in the pharynx help to move the food cord, which is formed by mucus secretion, toward the esophagus. These tracts are prominent in the bilateral pseudobranchial grooves, in the hypobranchial groove, and in the anterolateral surfaces of the gill seams (Mallat, 1979).

The larvae have seven pairs of branchiopores that open directly from the pharyngeal cavity, which is supported by an unjointed cartilaginous framework, not identical but similar to that of the adult. The branchiomeric muscles of the lamprey gills are innervated by the facial (VIIth), glossopharyngeal (IXth), and vagal (Xth) cranial nerves and act to compress the gills. The muscles of the first gill are innervated by the VIIth and IXth nerves, those of the second gill by nerve IXth and first branchial branch of nerve Xth; the other gills are supplied by the other branchial branches of the Xth nerve (Guimond et al., 2003). Each gill has a central septum, the interbranchial septum, which supports the gill filaments (principal lamellae), which project to the pharyngeal lumen like the pages of a book. Each filament has a series of secondary lamella on which the respiratory capillaries are situated. The lamellae are absent in the lateral portion of the filaments. The water passes through the side of these lamellae and the blood circulates through the capillaries in the opposite direction, to facilitate gas exchange. The first and last (eighth) gills are hemibranchs; the former bears filaments only on its posterior side and is also called the pseudobranch, the latter is composed exclusively of anterior filaments (Mallat, 1979).

The anatomy of the adults differs considerably from that of the larvae because of the changes that take place during metamorphosis. In the adult, the esophagus begins as a dorsal orifice of the oral cavity at the level of the velum and communicates directly with the nasopharyngeal pouch. It is separated from the more ventrally located water tube by cartilaginous processes (reviewed by Hardisty and Potter, 1971).

One of the crucial factors that affect the distribution of lamprey larvae is the amount of dissolved oxygen (Potter et al., 1970). Accordingly, lamprey larvae must possess

oxygen chemoreceptors to detect the oxygen levels in the respiratory water as well as in the blood. In aquatic vertebrates, hypoxia induces physiological changes that arise principally from O_2 chemoreceptors in the gills. In the sea lamprey, hypoxia leads to dilation of pre-gill arteries (ventral aorta, branchial afferents) and contraction of systemic arteries (dorsal aorta, branchial efferents, and celiacomesenteric) (Olson et al., 2001). Similar processes take place in other vertebrates, e.g., teleosts (Sundin et al., 2000) and mammals (Fukuda et al., 1989); the latter react to a hypoxic ambient or arterial condition with different responses such as vasoconstriction by the vascular smooth muscle, as well as changes in ventilation or in the heart rate. In 1948, Rapport et al. isolated and identified the serum tonic factor that causes constriction of the vascular smooth muscle and increased vascular tone, i.e., 5-HT. In aquatic vertebrates, the oxygen chemoreceptors (neuroepithelial or neuroendocrine cells) were initially identified by detection of the fluorescing biogenic amines resulting from formaldehyde treatment, and subsequently by immunohistochemistry to 5-HT (Dunel-Erb et al., 1982). One of the aims of the present study was to determine if lampreys have serotonergic cells in the gills that could play a similar role.

The structure of lamprey epidermal Merkel cells was described by Whitear and Lane (1981) and also by Takahashi-Iwanaga and Abe (2001) in transmission electron microscopy studies, but the neurochemistry of the Merkel cells of lampreys remains unknown. In different vertebrate groups, the Merkel cells are 5-HT-ir (Whitear, 1989; García-Caballero et al., 1989; English et al., 1992; Zaccone et al., 1994). An additional aim of the present study was thus to determine if 5-HT-ir is present in the Merkel cells of lampreys.

An extensive presence of serotonergic neurons and fibers has been observed in the peripheral nervous system of numerous invertebrate species; because lampreys belong to the earliest extant vertebrate group, we attempted to discover if the sea lamprey has a similar presence of serotonergic elements in its peripheral system.

In the present study we show for the first time the extensive distribution of serotonergic cells and fibers in the head musculature, pharynx, and gills of the sea lamprey using confocal immunofluorescence and high-performance liquid chromatography tography (HPLC) methods. We propose the possible roles of these peripheral serotonergic structures in chemoreception, behavioral and physiological control, neuromodulatory, and/or developmental processes.

Material and Methods

Larval (length 90–140 mm; n=25) and downstream migrating postmetamorphic (n=4) sea lampreys (*Petromyzon marinus* L.) were caught in the River Ulla (Galicia, Spain) and maintained in an aerated aquarium with river

sediment with appropriate conditions of ventilation and temperature (16°C) until processing. The larval and postmetamorphic sea lampreys were killed by an overdose of benzocaine (0.5 mg/L) and decapitated. All experiments were performed according to European Union (86/609/EEC) and Spanish (Royal Decree 223/1998) regulations for the care and handling of animals in research.

Immunofluorescence

Heads of larvae (from the snout to the last gill) and the branchial region (with the oral disc removed) of the postmetamorphic lampreys were fixed in 4% paraformaldehyde in 0.05 M Tris-buffered saline, pH 7.4 (TBS) by immersion for 5–6 hours. The tissue was rinsed in TBS, cryoprotected by immersion in 30% sucrose in TBS overnight, and then embedded in Tissue Tek (Sakura Fine Tek, Torrance, CA), frozen, and cut in sagittal or transverse planes on a cryostat (20–25 μm thick).

For 5-HT immunofluorescence the sections were incubated with a rabbit polyclonal antibody against 5-HT (Incstar, Stillwater, MN; code no. 20080; lot. no. 200493; dilution 1:2,500; immunogen: serotonin-formaldehyde-BSA conjugate), overnight at room temperature. A swine anti-rabbit immunoglobulin antibody (Dako, Glostrup, Denmark; diluted 1:30) coupled to rhodamine isothiocyanate (RITC) or fluorescein isothiocyanate (FITC) was then applied for 1 hour. The RITC emits red fluorescence (>590 nm) after excitation with green light at a maximum wavelength of 545 nm and FITC emits green fluorescence (519 nm) after excitation with blue light at a maximum wavelength of 495 nm. The antibody dilutions were carried out in TBS containing 10% normal swine serum and 0.2% Triton X-100.

Some samples were used for double immunostaining against 5-HT and acetylated α-tubulin. The anti-acetylated α-tubulin antibody serves as a marker of the peripheral nervous system (fibers and cells) and cilia. The sections were first incubated overnight with a cocktail of the anti-5-HT antibody and the mouse anti-acetylated α-tubulin antibody (Sigma, St. Louis MO; code no. T6793; lot. no. 6-11B-1; diluted 1:500; immunogen: acetylated tubulin from the outer arm of *Strongylocentrotus purpuratus*). The secondary antibodies used for the anti-tubulin antibody were: goat antimouse IgG antibody coupled to Alexa 546 (Interchim, Montluc¸om, France; diluted 1:100) or the goat anti-mouse IgG antibody coupled to FITC (Chemicon, Temecula, CA; diluted 1:50) combined with anti-rabbit antibodies (see above); the antibodies were applied for 1 hour. Alexa 546 emits red fluorescence (590 nm) after excitation with green light at a maximum wavelength of 545 nm and Cy5 emits red fluorescence (670 nm) after excitation at a maximum wavelength of 650 nm. The

antibodies were diluted with TBS containing 15% normal goat serum, 10% normal swine serum, and 0.2% Triton X-100.

Some larvae were prepared for double labeling combining immunofluorescence with 5-HT and with the application of a tract tracer in five regions of the head: the snout, velum, and the first, second, and fourth gills, to reveal the presence of labeled cells in the Vth, VIIth, IXth, and Xth cranial nerve ganglia. The tracer used was Neurobiotin (NB; Vector Laboratories, Burlingame, CA). NB crystals were applied with a minute pin (00) to larvae deeply anesthetized with benzocaine. After injection of the tracer the larvae were maintained for 1.5 days at 4°C in lamprey Ringer solution with the following composition: 137 mM NaCl, 2.9 mM KCl, 2.1 mM CaCl, 2.2 mM HEPES. After immunostaining sections with the antibody against 5-HT the presence of the tracer in ganglion cells was revealed with avidin-D coupled to FITC (Vector; diluted 1:1,000) in TBS with 0.3% Triton X-100, applied to sections for 4 hours. Sections were finally rinsed in distilled water and then coverslipped with mounting medium for fluorescence microscopy (Vectashield; Vector).

Photomicrographs

Confocal photomicrographs were taken with a spectral laser confocal microscope (Leica TCS-SP2). In order to visualize the anatomical structures of the larval head, light microscopy was also used in combination with confocal microscopy. For presentation, some fluorescence photomicrographs were inverted and converted to grayscale; photomicrographs were adjusted for brightness and contrast with Photoshop 7.0 (Adobe, San Jose, CA). The schematic drawings were made with Corel Draw 12 (Corel, Ottawa, Canada).

Measurements of the different cell types

The 5-HT-ir cells were measured with LITE (Leica confocal software). To estimate cell size we measured the long and short cell axes and calculated the mean of the two measurements.

For flask-shaped cells we measured only their long axis. The measurements were made in three larvae, and a minimum of 30 cells, of each 5-HT-ir cell type, were measured.

Antibody characterization and controls

The specificity of the anti-5-HT antibody has been tested by the supplier. The supplier reported no detectable crossreactivity with tryptamine, 5-methoxytryptamine, L-tryptophan, 5-hydroxytryptophan, dopamine, norepinephrine, or adrenaline. In the sea lamprey tissues, 5-HT staining was completely abolished by pretreatment of the diluted anti-5-HT antibody with 5-HT-BSA conjugate. Pretreatment of the diluted antibody with BSA (Sigma) did not affect the pattern of staining on lamprey tissues. In addition, the 5-HT antibody was tested in lamprey protein extracts by

Western blotting in our laboratory (Villar-Cerviño et al., 2006). No protein band was stained by the 5-HT antiserum in these blots.

The primary anti-tubulin antibody has been used as a ciliary marker (Piperno and Fuller, 1985) and has been shown to label specifically neurons and their processes in the nervous system of teleosts (Wilson et al., 1990; Chitnis and Kuwada, 1990; Doldán et al., 2000). Previous studies revealed a similar pattern of staining of neuronal elements in different lamprey species (Kuratani et al., 1997, 1998; Barreiro-Iglesias et al., 2008a).

As a control, the primary antibody was omitted from some sections during the immunohistochemical procedure. These control sections never exhibited positive staining. As a positive control for 5-HT and acetylated α-tubulin, larval brain sections were used. The typical serotonergic pattern reported in the brain of the larval sea lamprey (Abalo et al., 2007) was observed in these brain sections. Some sections of control larval heads were used to assess the absence of endogen biotin, which could react with fluorescein isothiocyanate (FITC)-labeled avidin-D; no staining was observed in these sections.

HPLC analytical procedure

In order to confirm biochemically the presence of 5-HT and 5-HT metabolites in the peripheral nervous system, larval sea lampreys were anesthetized with benzocaine and then killed by decapitation. Brains and spinal cord were removed from the heads, which were frozen and stored at -80°C. Tissue 5-HT and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) were extracted following homogenization of the heads without brain and spinal cord in 2 mL of cold 0.1 N perchloric acid containing 0.1 mM EDTA and 2.5 mM sodium metabisulfite. The homogenates were centrifuged in cold (2°C) for 20 minutes at 20,000g. The supernatants were filtered through Nylon Acrodisc 13 (0.20 μm pore diameter). After adequate dilutions, aliquots (20 μL) of the supernatants were analyzed by HPLC as previously described (Soengas and Aldegunde, 2004; Aldegunde and Mancebo, 2006) with slight modifications.

The chromatographic system consisted of an electrochemical detector (ESA Coulochem 5100A) with a 5011 dual analytical cell set at +50 mV (first, screening cell) and +350 mV (second, analytical cell). Chromatographic separation was done at room temperature using a security guard cartridge (C18, ODS, Phenomenex) and an Extrasil ODS2 C18 analytical column (150 mm x 4 mm, 5-μm diameter; Teknocroma, Barcelona, Spain). The mobile phase consisted of an acetate buffer (40 mM sodium acetate and 8 mMcitric acid) containing 0.134 mM EDTA, 1.30 mM octanesulfonic acid, and 10% (v/v) acetonitrile, adjusted to pH 3.65 with orthophosphoric acid.

Results

HPLC analysis

Analysis of extracts of larval lamprey heads (mainly consisting of muscles and connective tissues) by HPLC indicated the presence of high amounts of 5-HT as well as the presence of its metabolite 5-HIAA in peripheral tissues (Table 1). These biochemical results support the results of immunohistochemistry indicating the presence of a large number of cells and processes immunoreactive to 5-HT (see below).

	5-HT	**5-HIAA**
Larval heads without brain/spinal cord (n=4)	69 ± 16 pg/mg	3 ± 0.5 pg/mg

Table 1. Amounts of 5-HT and its metabolite 5-HIAA in larval lamprey heads without brain/spinal cord. Values are expressed as pg per mg of tissue. The mean and standard deviation of the four individuals analyzed are given.

General organization of the serotonin immunoreactive system

This study provides a complete description of the organization and distribution of 5-HT-ir structures in the head of the sea lamprey. The present results are based on immunohistochemistry against 5-HT and acetylated α-tubulin. Tubulin labelling revealed both peripheral neuronal elements and ciliary cells. All the serotonergic neuronal structures observed in the lamprey head were also stained with the anti-tubulin antibody. Comparison of 5-HT and tubulin immunostaining revealed that the serotonergic innervation represented a large percentage of the entire innervation of the head. The peripheral serotonergic cells were classified on the basis of their morphology, size, location, and innervation. Seven 5-HT-ir cell types were observed in the lamprey head: cells located in the cranial nerve ganglia, cells in the head musculature, putative oxygen chemoreceptors in the gills, flask-shaped cells located in pharyngeal regions that are covered by ciliary and mucus cells, cells in the taste buds, putative solitary bipolar chemoreceptors, and the tentatively named chain neurons. Double immunofluorescence, reveals that cell types positive for tubulin in the head tissues of the sea lamprey were also 5-HT-ir except most cells of the cranial nerve ganglia.

The organization and distribution of serotonergic elements in the larval head are shown in Figures 2-10. The presence of serotonergic cells and fibers in the trigeminal, facial, glossopharyngeal, and vagal cranial nerve ganglia and nerve branches are shown in Figures 2 and 3. The distribution of 5-HT-ir cells and fibers in the different head regions are shown in Figures 4 and 5. Finally, the double-immunostaining with anti-5-HT and anti-tubulin antibodies of the different serotonergic peripheral nervous system structures is shown in Figures 6-10.

Serotonergic cells and fibers in the trigeminal, facial, glossopharyngeal, and vagal cranial ganglia and nerves

The snout, the muscles used for feeding, and the velum are innervated by the Vth nerve. The gills are innervated by the VIIth, IXth, and Xth cranial nerves. The rostral portion of the first gill is innervated by the hyomandibular branch of the VIIth nerve, the caudal portion of the first gill, and the second gill is innervated by the IXth nerve and the last five gills by the Xth nerve. A few 5-HT-ir cells of ganglionic appearance were observed in the Vth, IXth, and Xth cranial nerve ganglia (Figs. 2, 3), but not in the VIIth ganglion (Fig. 2B–B"'). In contrast, a large number of 5-HT-ir fibers were observed in the ganglia and coursing in the different nerve branches that innervate the different head regions (Figs. 2, 3).

The application of Neurobiotin to the larval snout, velum, and gills (see Materials and Methods) revealed the primary sensory neurons that form the Vth, VIIth, IXth, and Xth cranial nerve ganglia. In addition, tubulin immunohistochemistry was used to label the different branches of the cranial nerves (not shown). A brief description of the different nerve branches is provided to better illustrate the results corresponding to the 5-HT immunostaining in the cranial nerves (a detailed description of cranial nerves is beyond the aim of the present study).

The large Vth ganglion of larvae is located between the orbit and the otic capsule (Figs. 2A-A"', 3A,B). This ganglion consists of two portions, a dorsolateral portion that corresponds to the ophthalmic division (V1), and another ventromedial portion that corresponds to the maxilo-mandibular division (V2).

The ophthalmic branch of the Vth nerve innervates the snout. A large number of serotonergic fibers were observed coursing in this branch. The principal maxilo-mandibular branch innervates the buccal and velar regions. Serotonergic fibers were observed in the different ramifications of this principal branch (Figs. 2A-A"', 3A). A rich serotonergic innervation was observed in the velum of the larvae (Fig. 4C); the innervation of this muscular structure originated from the mandibular branch of the Vth nerve, which entered through its dorsal margin; a thick bundle of serotonergic fibers of this origin was observed in the velum as well as a collection of scattered serotonergic fibers (Fig. 4C). A few 5-HT-ir bipolar cells were observed in the two portions of the trigeminal ganglion; these cells appeared to possess both central and peripheral processes (Figs. 2A-A"', 3A,B). The 5-HT-ir cells of the V1 ganglion ranged between 11 and 18 μm in size. The serotonergic cells observed in the V2 portion were smaller than those in the V1 portion: between 8 and 12 μm. Although after the injection of NB in the snout serotonergic cells pertaining to the V1 portion of the trigeminal ganglion were labeled with the tracer (Fig. 2A-A"'), the large number of

serotonergic fibers observed in the different branches of the Vth nerve indicated that most of these fibers represent high-order branches of the 5-HT-ir cells of the ganglia, not primary fibers (i.e., they do not originate in the areas).

Serotonergic fibers were also observed in the anterior lateral line nerve and ganglion, but no 5-HT-ir cells were observed in this ganglion (Fig. 3B). Serotonergic fibers were also observed in the different branches of the ocular motor cranial nerves that supply the eye musculature, coursing among the extraocular muscle fibers and extending to the anterior region of the larval eye (Fig. 6C).

The VIIth ganglion is situated in the rostral portion of the facial/auditory complex, which is located within the otic capsule (Fig. 2B). Application of the tracer to the rostral portion of the first gill resulted in labeling of cell bodies in the VIIth ganglion (Fig. 2B,B',B"'). However, no 5-HT-ir cells were observed in this ganglion (Fig. 2B"). The VIIth cranial nerve consists of three principal branches: a buccal branch that crosses the floor of the otic capsule and courses ventral to the larval eye, an ophthalmic superficial branch that crosses the otic capsule dorsally, and the hyomandibular branch that crosses the otic capsule and courses to the ventral region of the head to innervate the first gill. The serotonergic immunofluorescence revealed the presence of fibers in the facial ganglion which leave/reach the ganglion and that innervate the rostral portion of the first gill. These fibers were observed leaving/reaching the ganglion by the hyomandibular branch. Serotonergic fibers were also observed in the ophthalmic and buccal branches of this nerve (not shown). No 5-HT-ir cells were observed in the octaval ganglion (VIIIth; not shown).

Neurobiotin injected in the first and second gills labeled cell bodies in the IXth ganglion (not shown), which is located caudal to the otic capsule (Fig. 3C-D). This ganglion is part of the metaotic complex and is situated in a ventrolateral position. A few serotonergic bipolar spherical cells (between 16 and 19 μm in diameter) were observed in this ganglion (Fig. 3D), but no double-labeled 5-HT-ir cells were observed in the larvae in which the NB was injected in the first and second gills. Serotonin immunoreactive fibers and cells were observed both in the dorsal and ventral branches of the IXth cranial nerve (Fig. 3C). The dorsal branch innervates the musculature of the dorsal region of the head, whereas the ventral branch innervates the first and second gills (Fig. 3C).

Primary sensory neurons of the Xth ganglion were labelled by injection of NB in the fourth gill (not shown). The vagal ganglion is located in the metaotic complex in a dorsomedial position relative to the IXth ganglion (Fig. 3C,D). The peripheral branches of the Xth nerve are formed by the diverse branches, which leave it laterally and ventrally to the Xth ganglion to innervate the last five gills. A large number of serotonergic fibers

were observed in a thick bundle that ran caudally from the ganglion (Fig. 3D), and at the level of each one of the last five gills gave rise to perpendicular collaterals to innervate the gills (three of these branches are shown in Fig. 6A-A"). As in the case of the IXth ganglion, a few 5-HT-ir cells were observed in the Xth ganglion (Fig. 3C,D) but none of them were labeled by injection of the tracer. The 5-HT-ir cells of the Xth ganglion were between 14 and 15 μm in size.

Distribution of serotonergic fibers in the larval head

The connective and muscular tissues of the head are richly endowed with serotonergic fibers, which form extensive networks in most head tissues. No 5-HT-ir structures were observed in the epidermis of the skin (Fig. 4A). The larval epidermis received rich nonserotonergic innervation, which was revealed by tubulin immunohistochemistry (not shown). These non-5-HT-ir fibers reached the larval epidermis at right angles relative to its surface, and formed fine free intraepithelial nerve endings.

In the larval dermis, fairly numerous 5-HT-ir nerve fibers were observed interspersed among collagen lamellae (Fig. 4A). The subcutaneous layer situated between the dermis and the musculature showed long 5-HT-ir fibers that coursed parallel to the body surface and others coursing perpendicularly and directed to the dermis (Fig. 4A).

The larval musculature presented the densest serotonergic fiber networks (Figs. 4A-D, 6A-D'). In the head muscles the 5-HT-ir fibers are thin (less than 1 μm in diameter) and finely beaded, branching occasionally. Most of the thin 5-HT-ir fibers coursed very close and parallel to the muscle fibers, but fibers were also observed coursing in other directions. In the muscle, 5-HT-ir fibers were coextensive with αT-ir/5-HTnegative fibers, which also innervated this tissue (Fig. 6A-B"). The serotonergic innervation of the head musculature appears to represent a high percentage of the total innervation. Our observations suggest that the majority of these 5-HT-ir fibers have a peripheral origin from serotonergic cells located in the muscles (Fig. 4D; see next section). Numerous serotonergic fibers were observed surrounding the larval eye (Fig. 6C). The extraocular muscles received rich serotonergic innervation. The absence of serotonergic cells from the ocular motor nerves suggests that these serotonergic fibers originate in the serotonergic cells present in neighboring muscles. The lamprey retina did not exhibit 5-HT immunoreactive structures until the adult period (see Villar-Cerviño et al., 2006).

The oral cirri showed a small bundle of serotonergic fibers coursing along their axes and reaching the top of the cirri (Figs. 4B, 7A-A"'). These serotonergic fibers were interspersed among αT-ir/5-HT-negative fibers that also innervated the cirri (Fig. 7A-A"'). Unlike the larval skin, the epidermis of the

buccal and oral cavities presented free serotonergic nerve endings (Fig. 4B).

A large number of serotonergic fibers were also observed in the pharyngeal and branchial regions. The interbranchial septum of each gill arch presented numerous longitudinal 5-HT-ir fibers coursing dorsoventrally (Figs. 4E,F, 5E, 7C-C"). Serotonin immunoreactive fibers were also observed at the sides of the gill filaments but were not observed in the respiratory lamellae or in the central portion of each gill filament at levels where lamellae were present (Figs. 4E, 7B-B", 9A-A"). Only a few serotonergic fibers were observed reaching the filament or interbranchial septal epithelia that are exposed to water flow. Rather, tubulin immunohistochemistry revealed the presence of a large number of non-5-HT-ir fibers that innervated the entire filament, the respiratory lamellae, and the gill epithelium (Fig. 7B-C"). The distribution of serotonergic fibers in the gills appeared to be closely associated with the principal afferent and efferent filamentary blood vessels, and were absent from regions with respiratory capillaries. Some of these 5-HT-ir fibers course close to the arterial smooth muscle cells, although the existence of direct contacts with the muscle could not be assessed. In addition, serotonergic fibers were observed in the gill seam surrounding the efferent branchial artery (Fig. 9C-C", E-E"). These fibers coursed longitudinally in a dorsoventral direction and appeared to be intermingled with the αT-ir/5-HT-negative descending fibers that innervated this region.

Serotonin immunoreactive cells

Cells immunoreactive to 5-HT were observed in the muscles of the head (MuCs), with their processes distributed among muscle fibers or directed toward superficial tissues (Fig. 4A, C, D, 5F, 6B-D' and 7A'-A"', 9D-D"). These cells were spindle-shaped or spherical and gave rise to fibers branching in the muscles, where they formed a rich network of serotonergic fibers intermingled with other fiber types revealed by αT-ir (Fig. 6B-B"). Some 5-HT-ir cells were observed intermingled in the thick bundles of αT-ir fibers. Some αT-ir/5-HT-negative fibers appeared to be closely associated with the MuCs, and also some MuCs processes appeared to be in contact with other MuCs, but whether they form synaptic contacts could not be assessed. The MuCs observed in larval muscles were between 11 and 17 μm in size.

The MuCs were observed in the snout, buccal, velar, pharyngeal, and body wall musculature but they were more abundant in the muscles of the velum (Fig. 4C). Some of these cells were located among the principal branches of the trigeminal nerve that innervate the velum (Fig. 4C). The processes of the velar serotonergic neurons were distributed in the velar musculature, forming an extensive network of serotonergic fibers. In addition, as

in the rest of the muscles, αT-ir/5-HT-negative fibers were observed intermingled with 5-HT-ir cells and fibers. The serotonergic cells of the velar musculature were larger than in other head muscles: between 12 and 19 μm. In addition, a few 5-HT-ir cells were observed in the cirri of the oral cavity (Fig. 7A-A"'), which also contain muscular structures. Most of these cells were observed at the base of the cirri but 5-HT-ir cells were also occasionally observed in the cirrus axis.

A continuous band of numerous 5-HT-ir flask-shaped cells (FSCs) was observed extending along the bilateral pseudobranchial groves (Figs. 5A, 8A-B"), the hypobranchial groove (Fig. 9D-D"), and in the gill seam (Fig. 9C-C"). These FSCs were closely arranged in superficial layers in the epithelium of the grooves. The thick apical tip of the FSCs pointed toward the lumen of the pharynx. These pharyngeal epithelia also contained numerous ciliated cells, with their cilia directed to the lumen of the groove, as revealed by immunohistochemistry against αT (Figs. 8B-B", 10B-B"). There appears to be a close relation between the FSCs and the ciliated cells of the grooves as well as with the mucus-secreting cells that were present in these pharyngeal regions (Mallat, 1979). No serotonergic fibers were observed leaving the FSCs, but the base of the FSCs was contacted by αT-ir fibers, which coursed perpendicularly from the core of the filament (Figs. 8A-B", 9C-D", 10B-B"). In addition, some longitudinal serotonergic fibers were observed interspersed with these αT-ir/5-HTnegative fibers. The FSCs were between 18 and 27 μm in size. The FSCs were much more abundant in the pseudobranchial grooves (Fig. 8A-B") than in the hypobranchial groove or in the gill seam.

The gills of the larvae showed another type of 5-HT-ir cells (serotonergic gill cells; GiCs), located in subepithelial regions in the interbranchial septum, at the filament base as well as in the lateral and apical portions of the filaments, i.e., near the afferent and efferent filamentary blood vessels (Figs. 4E,F, 5B-E, 7B-B", 9A-A"). Most of these cells were bipolar and were observed in the seven gill arches but not in the respiratory lamellae or in the inner portion of the filaments where the respiratory lamellae are located. The GiCs were between 11 and 24 μm in size. The beaded processes of the GiCs were distributed in neighboring regions where the GiC was situated, coursing close to the gill blood vessels. However, whether these processes contacted directly with vascular smooth muscles could not be assessed.

In the middle branchial edge, small 5-HT-ir cells (4.5- 6.5 μm in size) were observed positioned as dorsoventral chains parallel to the branchial afferent artery (Fig 10).

These cells were tentatively termed chain neurons, like the cells described in the

branchial region of zebrafish by Jonz and Nurse (2003).

Other cell types

In lampreys, the first six pairs of gill arches bear volcano like papillae that face the water current (Mallat, 1979; Baatrup, 1983), these structures have been considered as taste buds because of their similarities with taste buds of gnathostomes (Baatrup, 1983). The lamprey taste buds are composed of two main types of elongated cells, bi-ciliated cells and supporting cells; the bi-ciliated cells were considered taste receptors (Baatrup, 1985). The bi-ciliated taste bud cells of the sea lamprey are 5-HT-ir and αT-ir cells (for a complete description of these chemoreceptive structures, see chapter 1.2).

Solitary 5-HT-ir/αT-ir cells were occasionally observed in the epithelium, close to the larval taste buds (Fig. 9B-B"). These flask-shaped cells have a thin apical portion directed to the pharyngeal lumen and a thicker basal portion, which ultimately gives rise to a basal process which run out from the surface.

Adult sea lamprey

In adult lampreys, only the region between the velum and the end of the pharyngeal region was studied. Results corresponding to the organization and distribution of 5-HT-ir structures in adults were very similar to those observed in the larval sea lamprey. Accordingly, only a brief description of the different serotonergic structures is reported here. Figure 11 shows some of the 5-HT-ir cells and fibers observed in the postmetamorphic samples studied.

As in the larvae, the epidermis of the adult head lacked serotonergic elements. The musculature of the pharyngeal walls contained a large number of 5-HT-ir MuCs and fibers forming extensive networks (Fig. 11A,B). Two rows of 5-HT-ir presence of 5-HT-ir FSCs located in epithelium of the pharyngeal grooves and the gill seam was also observed in adults.

Serotonergic bi-ciliated cells were also observed in taste buds of the adult sea lamprey (see chapter 1.2).

Discussion

In this study we report for the first time the widespread and complex distribution of serotonergic elements in the head of the sea lamprey, which has not previously been described in any other vertebrate species. The present results, together with previous report on the presence of serotonergic fibers in the olfactory nerve (Zielinski et al., 2000) and of serotonergic cells in taste buds (Chapter 1.2), provide a general view of the organization of serotonergic elements in the peripheral tissues of the sea lamprey head. The comparison between larvae and adults revealed that the organization of this peripheral system does not change significantly during metamorphosis.

Serotonergic cells in the cranial nerve ganglia

The presence of serotonergic cells in the Vth ganglion, as well as the presence of serotonergic fibers in the trigeminal nerve, has been reported in two lampreys (*Ichthyomyzon unicuspis* and *Lampetra fluviatilis*; Di Prisco et al., 1994). Our results in the sea lamprey extend the presence of serotonergic elements to the IXth and Xth cranial nerve ganglia and nerves. The presence of serotonergic cells in cranial nerve ganglia was not reported in other vertebrate groups, although 5-HT-ir fibers have been reported in the Gasserian ganglion of cats (Chouchkov et al., 1988). Serotonin may act as a modulator of ganglion cells transmitting somatosensory and/or viscerosensory stimuli from the lamprey head, but the actual function of these 5-HT-ir cells remains to be determined.

5-HT in the head skin

Serotonin immunohistochemistry revealed serotonergic structures in the dermis of the sea lamprey head, but not in the epidermis. In the skin of mammals, serotonin has been implicated in enhancing inflammatory reactions by attracting mast cells at the site of tissue lesion (Kushnir-Sukhov et al., 2006), and a similar role for 5-HT in the dermis of lampreys may occur. The absence of serotonergic structures in the epidermis contrasts with their presence in the epidermis of different invertebrates (Myhrberg, 1971; Licata et al., 1998; McKenzie et al., 1998; Faccioni-Heuser et al., 2004) and vertebrates (Zaccone, 1986; García-Caballero et al., 1989; Zaccone et al., 1990, 1994; Fasulo et al., 1993). The presence of serotonin in epidermal Merkel cells has been reported in teleosts, amphibians, dipnoans, and mammals (Whitear, 1989; García-Caballero et al., 1989; English et al., 1992; Zaccone et al., 1994). Although epidermal Merkel cells have been described in lampreys (Whitear and Lane, 1981; Takahashi-Iwanaga and Abe, 2001), the absence of 5-HT in the lamprey epidermis indicates that they are not 5-HT-ir.

Presence of serotonergic structures in the sea lamprey musculature

The widespread presence of serotonergic fibers innervating the sea lamprey head musculature and the presence of 5-HT-ir neuron-like cells in this tissue appear to be unique features among vertebrates. This also indicates that serotonin may play important roles in the muscular activity of the sea lamprey.

The presence of serotonergic structures in the musculature has principally been reported in invertebrates, including nematodes (Dernovici et al., 2007), cycliophora (Wanninger, 2005), entoprocta (Fuchs et al., 2006), platyhelminths (Stewart et al., 2003; Sebelova et al., 2004), annelids (Reglödi et al., 1997; Csoknya et al., 2005), and mollusks (Faccioni-Heuser et al., 2004). In these invertebrates the number of serotonergic peripheral neurons far exceeds those found in

the central nervous system (Faccioni-Heuser, 2004). Therefore, the extensive presence of neurons in the peripheral nervous system of the sea lamprey and the preponderance of serotonergic elements in its musculature, as well as in other peripheral tissues, could be interpreted as a primitive character that appeared before divergence of vertebrates from invertebrates. However, studies in invertebrate chordates (tunicates, amphioxus) did not reveal serotonergic fibers in the muscles (amphioxus: Holland and Holland, 1993; Candiani et al., 2001; tunicates: Pennati et al., 2001; Stach, 2005). Interestingly, the presence of preserved fossilized neurons in the musculature has been reported in a Late Devonian placoderm fish (Trinajstic et al., 2007). These neurons are similar to the MuCs reported in the present study. These early, extinct gnathostomes display muscle structures such as those observed in lampreys (Trinajstic et al., 2007). Because of these similarities between early extinct gnathostomes and the sea lamprey, the peripheral nervous system in the latter might be said to be characteristic of primitive vertebrates.

One larval lamprey muscular structure that has been studied in great detail is the velum, owing to its crucial role for unidirectional water flow and suspension feeding (reviewed by Rovainen, 1996). Previous studies described two types of neuromuscular junctions in the larval velum: one which was probably cholinergic, as the usual innervation of the vertebrate skeletal musculature, and the other that was described as probably serotonergic, as revealed by the Fälck-Hillarp formaldehyde-induced fluorescence (FIF) method for the detection of biogenic amines (see Hardisty and Rovainen, 1982). Our findings confirm the presence of serotonergic neuromuscular innervation in the velum of the sea lamprey larvae and show that velar muscles contain a richer serotonergic plexus than other muscles. Most of the αT-ir/5-HT-ir-negative fibers observed in the velum are probably cholinergic. We suggest that the velar muscle and the remaining musculature of the sea lamprey head are excited by typical cholinergic synapses and that the 5-HT terminals may act to modulate the muscular activity elicited by cholinergic transmission. The possible modulatory effect of 5-HT in the nicotinic transmission was previously considered in other vertebrates (Koketsu et al., 1982). A modulatory effect elicited by 5-HT has been demonstrated in central cholinergic synapses (Consolo et al., 1994a,b), as well as its role in the central control of the muscular activity (Bazarevitch et al., 1978; Harris-Warrick and Cohen, 1985; Van Dongen et al., 1986; Buchanan and Grillner, 1991). In addition, since αT-ir/5-HT-negative fibers are closely associated with the 5-HT-ir MuCs, a modulation of these MuCs by these fibers should not be ruled out.

Some physiological studies reported different effects elicited by 5-HT in the

musculature of different mollusk species (Weiss et al., 1978; Satterlie and Norekian, 1995; Gainey et al., 2003). In vertebrates, 5-HT has effects on the visceral musculature: in frogs it provokes a decrease in the tone of lung smooth muscle (Schnizer et al., 1969) and in the goat ruminal musculature, in vivo application of 5-HT inhibits the contraction sequences (Veenendaal et al., 1980). On the basis of present results, we suggest the acquisition of a modulatory role of 5-HT in the control of striated muscles in the earliest vertebrates, the agnathans. In jawed vertebrates, no effects of serotonin on this musculature have been reported, suggesting the possibility that the serotonergic innervation of muscles was secondarily acquired in lampreys. Physiological studies are required to investigate the actual role of 5-HT in the striated musculature of the sea lamprey.

Possible developmental role in lampreys

During lamprey metamorphosis the larval head undergoes important changes; many of the muscles of the larval head degenerate and are replaced by the characteristic muscles of the buccal funnel, the piston, and the pharynx of the adult (reviewed by Hardisty and Potter, 1971). In the mouse, 5-HT has been proposed as playing a crucial role in the development of the nervous system (Sillar et al., 1995; Rubenstein, 1998; Gaspar et al., 2003; Brustein et al., 2003) and in craniofacial morphogenesis (Shuey et al., 1992, 1993; see Moiseiwitsch, 2000; Levin et al., 2006). A possible developmental role for 5-HT in the transformation of the larval head structures during metamorphosis cannot be ruled out owing to the extensive presence of serotonergic elements in the different tissues (present results).

Serotonin and oxygen sensing

The oxygen level in water is one of the principal factors that affect the distribution of lamprey larvae (Potter et al., 1970). Respiration in lamprey larvae is stimulated by hypoxia (revised by Rovainen, 1996) and responses to it probably arise from oxygen chemoreceptors in the gills. Hypoxia causes vasoconstriction of the lamprey systemic vessels (Olson et al., 2001) and 5-HT is known to cause vasoconstriction of the vascular musculature through activation of 5-HT2A receptors. The presence of oxygen chemoreceptive cells in vertebrates was determined by detection of the biogenic amines by FIF and by immunohistochemistry to 5-HT (Dunel-Erb et al., 1982). The position that the GiCs occupy in the lamprey gills, in close relation to the water flow and the blood vessels, would enable detection of the oxygen levels and initiate the response to hypoxia by degranulation of vesicles containing 5-HT, in a similar process to that described in jawed fishes (Dunel-Erb et al., 1982), and the cells may correspond to serotonergic oxygen chemoreceptors. The presence of this type of cell in the gills has

been described in elasmobranchs (Bailly et al., 1992; Sundin, 1995; Burleson et al., 2006), teleosts (Bailly et al., 1992; Sundin, 1995; Jonz and Nurse, 2003; Saltys et al., 2006), and in amphibian larvae (Saltys et al., 2006).

Hypoxic vasoconstriction is an intrinsic feature of systemic vascular smooth muscle in cyclostomes and is similar to the process that occurs in mammals (Olson et al., 2001). In mammals, specialized cells sensitive to changes in O_2 tension (pO_2) have been described: neuroepithelial bodies in the lung (Youngson et al., 1993; Fu et al., 2002), type I cells in the carotid body (González et al., 1994), 5-HT-ir glomus cells in the aortic body (Dvorakova and Kummer, 2005), peripheral neurons (Campanucci et al., 2003), and vascular smooth muscle cells (Michelakis et al., 1995). In teleosts, hypoxia also induces physiological changes that arise principally from O_2 chemoreceptors in the gills (Jonz et al., 2004). The present results suggest that the GiCs of lampreys are comparable to the gill serotonergic cells of teleosts and amphibian larvae and to mammalian O_2 chemoreceptors, which suggests that they may play a similar role in initiating the response to hypoxia.

Relation between serotonergic cells and ciliary activity and mucus secretion

The sea lamprey pharyngeal grooves are surrounded by tracts of ciliated and mucus cells that help to move the water and the food cord toward the pharynx. The present results show that there is a close relation between these cells and the FSCs of the pharyngeal groves and gill seam. Studies carried out in echinoderms (Wada et al., 1997), gastropods (Diefenbach et al., 1991; McKenzie et al., 1998; Uhler et al., 2000; Doran et al., 2004; Braubach et al., 2006), mollusks (Pavlova et al., 1999; Kuang and Goldberg, 2001; Koss et al., 2003), and amphibians (Maruyama et al., 1984a,b; Naruse et al., 2005) showed that 5-HT plays a key role in the control of mucus secretion and ciliary beat frequency. The close relation between 5-HT-ir FSCs and the ciliated and mucous epithelia observed in lamprey may indicate that these cells are also involved in the control of the ciliary beat and mucus secretion. An increase in the ciliary beat frequency in response to a hypoxic situation elicited by these serotonergic cells to accelerate the water flow cannot be ruled out, as in gastropods (Koss et al., 2003). In addition, the presence of αT-ir/5- HT-negative fibers in the base of the FSC may also provide this cell type with a sensory innervation by afferent fibers. The specific effect of the 5-HT in these processes must be investigated in more detail in physiological studies.

Other 5-HT-ir cell types

Aquatic anamniote vertebrates, including hagfishes, possess dispersed epidermal chemosensory cells that could be phylogenetically related to taste buds (Finger, 1997). In the present study we describe for the

first time 5-HT-ir flaskshaped cells that present a basal process running to the dermis were occasionally observed in the vicinity of the taste buds. The actual role of these solitary cells observed in the sea lamprey remains to be determined, but they appear to be a unique type of cell in vertebrates, because in fishes the solitary chemoreceptors are similar to the taste receptors of the taste buds, which are elongate cells that synapse onto the distal process of remote ganglion cells (Sbartati and Oscultati, 2003). The lamprey taste buds will be discussed in detail in next 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.

Aldegunde M, Mancebo M. 2006. Effects of neuropeptide Y on food intake and brain biogenic amines in the rainbow trout (*Oncorhynchus mykiss*). Peptides 27:719-727.

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.

Baatrup E. 1983. Terminal buds in the branchial tube of the brook lamprey (*Lampetra planeri* (Bloch) - putative respiratory monitors. Acta Zool 64:139-147.

Baatrup E. 1985. Physiological studies on the pharyngeal terminal buds in the larval brook lamprey, *Lampetra planeri* (Bloch). Chem Senses 10:549-558.

Bailly Y, Dunel-Erb S, Laurent P. 1992. The neuroepithelial cells of the fish gill filament: indolamine-immunocytochemistry and innervation. Anat Rec 233:143-161.

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.

Baumgarten HG, Björklund A, Lachenmayer L, Nobin A, Rosengren E. 1973. Evidence for the existence of serotonin, dopamine-, and noradrenaline-containing neurons in the gut of *Lampetra fluviatilis*. Z Zellforsch Mikrosk Anat 141:33-54.

Bazarevitch G, Sadekov M, Tereschenko V, Devjataev A, Markarova L. 1978. Role of serotonin in the regulation of respiratory system function. Acta Physiol Pol 29:131-138.

Braubach OR, Dickinson AJ, Evans CC, Croll RP. 2006. Neural control of the velum in larvae of the gastropod, *Ilyanassa obsoleta*. J Exp Biol 209:4676 -4689.

Brustein E, Chong M, Holmqvist B, Drapeau P. 2003. Serotonin patterns locomotor

network activity in the developing zebrafish by modulating quiescent periods. J Neurobiol 57:303-322.

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

Burleson ML, Mercer SE, Wilk-Blaszczak MA. 2006. Isolation and characterization of putative O2 chemoreceptor cells from the gills of channel catfish (*Ictalurus punctatus*). Brain Res 1092:100-107.

Campanucci VA, Fearon IM, Nurse CA. 2003. O2-sensing mechanisms in efferent neurons to the rat carotid body. Adv Exp Med Biol 536:179-185.

Candiani S, Augello A, Oliveri D, Passalacqua M, Pennati R, De Bernardi F, Pestarino M. 2001. Immunocytochemical localization of serotonin in embryos, larvae and adults of the lancelet, *Branchiostoma floridae*. Histochem J 33:413-420.

Chitnis AB, Kuwada JY. 1990. Axonogenesis in the brain of zebrafish embryos. J Neurosci 10:1892-1905.

Chouchkov C, Lazarov N, Davidoff M. 1988. Serotonin-like immunoreactivity in the cat trigeminal ganglion. Histochemistry 88:637-639.

Consolo S, Arnaboldi S, Giorgi S, Russi G, Ladinsky H. 1994a. 5-HT4 receptor stimulation facilitates acetylcholine release in rat frontal cortex. Neuroreport 5:1230-1232.

Consolo S, Bertorelli R, Russi G, Zambelli M, Ladinsky H. 1994b. Serotonergic facilitation of acetylcholine release in vivo from rat dorsal hippocampus via serotonin 5-HT3 receptors. J Neurochem 62:2254-2261.

Csoknya M, Takács B, Koza A, De´ nes V, Wilhelm M, Hiripi L, Kaslin J, Elekes K. 2005. Neurochemical characterization of nervous elements innervating the body wall of earthworms (Lumbricus, Eisenia): immunohistochemical and pharmacological studies. Cell Tissue Res 321:479-490.

Dernovici S, Starc T, Dent JA, Ribeiro P. 2007. The serotonin receptor SER-1 (5HT2ce) contributes to the regulation of locomotion in *Caenorhabditis elegans*. Dev Neurobiol 67:189-204.

Diefenbach TJ, Koehncke NK, Goldberg JI. 1991. Characterization and development of rotational behavior in *Helisoma* embryos: role of endogenous serotonin. J Neurobiol 22:922-934.

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.

Doldán MJ, Prego B, Holmqvist B, Helvik JV, de Miguel E. 2005. Emergence of

axonal tracts in the developing brain of the turbot (*Psetta maxima*). Brain Behav Evol 56:300-309.

Doran SA, Koss R, Tran CH, Christopher KJ, Gallin WJ, Goldberg JI. 2004. Effect of serotonin on ciliary beating and intracellular calcium concentration in identified populations of embryonic ciliary cells. J Exp Biol 207:1415-1429.

Dunel-Erb S, Bailly Y, Laurent P. 1982. Neuroepithelial cells in fish gill primary lamellae. J Appl Physiol Respir Environ Exerc Physiol 53:1342-1353.

Dvorakova MC, Kummer W. 2005. Immunohistochemical evidence for species-specific coexistence of catecholamines, serotonin, acetylcholine and nitric oxide in glomus cells of rat and guinea pig aortic bodies. Ann Anat 187:323-331.

English KB, Wang ZZ, Stayner N, Stensaas LJ, Martin H, Tuckett RP. 1992. Serotonin-like immunoreactivity in Merkel cells and their afferent neurons in touch domes from the hairy skin of rats. Anat Rec 232:112-120.

Faccioni-Heuser MC, Zancan DM, Achaval M. 2004. Monoamines in the pedal plexus of the land snail *Megalobulimus oblongus* (Gastropoda, Pulmonata). Braz J Med Biol Res 37:1043-1053.

Fasulo S, Tagliafierro G, Contini A, Ainis L, Ricca MB, Yanaihara N, Zaccone G. 1993. Ectopic expression of bioactive peptides and serotonin in the sacciform gland cells of teleost skin. Arch Histol Cytol 56:117-125.

Finger TE. 1997. Evolution of taste and solitary chemoreceptor cell systems. Brain Behav Evol 50:234 -243.

Fu XW, Nurse CA, Wong V, Cutz E. 2002. Hypoxia-induced secretion of serotonin from intact pulmonary neuroepithelial bodies in neonatal rabbit. J Physiol 539:503-510.

Fuchs J, Bright M, Funch P, Wanninger A. 2006. Immunocytochemistry of the neuromuscular systems of *Loxosomella vivipara* and *L. parguerensis* (Entoprocta: Loxosomatidae). J Morphol 267:866 -883.

Fukuda Y, Sato A, Suzuki A, Trzebski A. 1989. Autonomic nerve and cardiovascular responses to changing blood oxygen and carbon dioxide levels in the rat. J Auton Nerv Syst 28:61-74.

Gainey LF Jr, Walton JC, Greenberg MJ. 2003. Branchial musculature of a venerid clam: pharmacology, distribution, and innervation. Biol Bull 204:81-95.

Garcia-Caballero T, Gallego R, Roson E, Basanta D, Morel G, Beiras A. 1989. Localization of serotonin-like immunoreactivity in the Merkel cells of pig snout skin. Anat Rec 225:267-271.

Gaspar P, Cases O, Maroteaux L. 2003. The developmental role of serotonin: news

from mouse molecular genetics. Nat Rev Neurosci 4:1002-1012.

Gonzalez C, Almaraz L, Obeso A, Rigual R. 1994. Carotid body chemoreceptors: from natural stimuli to sensory discharges. Physiol Rev 74:829-898.

Guimond JC, Auclair F, Lund JP, Dubuc R. 2003. Anatomical and physiological study of respiratory motor innervation in lampreys. Neuroscience 122:259-266.

Hardisty MW, Potter IC. 1971. The general biology of adult lampreys. In: Hardisty MW, Potter IC, editors. The biology of lampreys, vol 1. London: Academic Press. p 127-206.

Hardisty MW, Rovainen CM. 1982. In: Hardisty MW, Potter IC, editors. The biology of lampreys, vol 4A. London: Academic Press.

Harris-Warrick RM, Cohen AH. 1985. Serotonin modulates the central pattern generator for locomotion in the isolated lamprey spinal cord. J Exp Biol 116:27-46.

Holland ND, Holland LZ. 1993. Serotonin-containing cells in the nervous system and other tissues during ontogeny of a lancelet, *Branchiostoma floridae*. Acta Zool 74:195-204.

Homma S. 1975. Velar motoneurons of lamprey larvae. J Comp Physiol A 104:175-183.

Jonz MG, Nurse CA. 2003. Neuroepithelial cells and associated innervation of the zebrafish gill: a confocal immunofluorescence study. J Comp Neurol 461:1-17.

Jonz MG, Fearon IM, Nurse CA. 2004. Neuroepithelial oxygen chemoreceptors of the zebrafish gill. J Physiol 560:737-752.

Koketsu K, Akasu T, Miyagawa M, Hirai K. 1982. Modulation of nicotinic transmission by biogenic amines in bullfrog sympathetic ganglia. J Auton Nerv Syst 6:47-53.

Koss R, Diefenbach TJ, Kuang S, Doran SA, Goldberg JI. 2003. Coordinated development of identified serotonergic neurons and their target ciliary cells in *Helisoma trivolvis* embryos. J Comp Neurol 457:313-325.

Kuang S, Goldberg JI. 2001. Laser ablation reveals regulation of ciliary activity by serotonergic neurons in molluscan embryos. J Neurobiol 47:1-15.

Kuratani S, Ueki T, Aizawa S, Hirano S. 1997. Peripheral development of cranial nerves in a cyclostome, Lampetra japonica: morphological distribution of nerve branches and the vertebrate body plan. J Comp Neurol 384:483-500.

Kuratani S, Horigome N, Ueki T, Aizawa S, Hirano S. 1998. Stereotyped axonal bundle formation and neuromeric patterns in embryos of a cyclostome,

Lampetra japonica. J Comp Neurol 391:99-114.

Kushnir-Sukhov NM, Gilfillan AM, Coleman JW, Brown JM, Bruening S, Toth M, Metcalfe DD. 2006. 5-Hydroxytryptamine induces mast cell adhesion and migration. J Immunol 177:6422-6432.

Levin M, Buznikov GA, Lauder JM. 2006. Of minds and embryos: left-right asymmetry and the serotonergic controls of pre-neural morphogenesis. Dev Neurosci 28:171-185.

Licata A, Mauceri A, Ainis L, Martella S, Ricca MB, Lauriano ER, Amato A. 1998. Immunohistochemical localization of bioactive substances in the epidermis of the earthworm, *Lumbricus terrestris* (Annelida, oligochaeta). Eur J Histochem 42:303-308.

Mallat J. 1979. Surface morphology and functions of pharyngeal structures in the larval lamprey *Petromyzon marinus*. J Morphol 162:249-274.

Maruyama I, Inagaki M, Momose K. 1984a. The role of serotonin in mucociliary transport system in the ciliated epithelium of frog palatine mucosa. Eur J Pharmacol 106:499-506.

Maruyama I, Shioda S, Nakai Y. 1984b. Immunocytochemical localization of serotonin-like immunoreactivities in the ciliated epithelium of the frog palatine mucosa. Acta Anat (Basel) 120:160-163.

McKenzie JD, Caunce M, Hetherington MS, Winlow W. 1998. Serotonergic innervation of the foot of the pond snail *Lymnaea stagnalis* (L.). J Neurocytol 27:459-470.

Michelakis ED, Archer SL, Weir EK. 1995. Acute hypoxic pulmonary vasoconstriction: a model of oxygen sensing. Physiol Res 44:361-367.

Moiseiwitsch JR. 2000. The role of serotonin and neurotransmitters during craniofacial development. Crit Rev Oral Biol Med 11:230-239.

Myhrberg HE. 1971. Ulstructural localization of monoamines in the epidermis of *Lumbricus terrestris* (L.). Z Zellforsch Mikrosk Anat 117:139-154.

Naruse H, Gomi T, Kimura A, Adriaensen D, Timmermans JP. 2005. Structure of the respiratory tract of the red-bellied newt *Cynops pyrrhogaster*, with reference to serotonin-positive neuroepithelial endocrine cells. Anat Sci Int 80:97-104.

Olson KR, Russell MJ, Forster ME. 2001. Hypoxic vasoconstriction of cyclostome systemic vessels: the antecedent of hypoxic pulmonary vasoconstriction? Am J Physiol Regul Integr Comp Physiol 280:198-206.

Parent A. 1981. The anatomy of serotonin-containing neurons across phylogeny. In: Jacobs BL, Gelperin A, editors.

Serotonin neurotransmission and behavior. Cambridge, MA: MIT Press. p 3-34.

Parent A. 1984. Functional anatomy and evolution of monoaminergic systems. Am 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.

Pavlova GA, Willows AO, Gaston MR. 1999. Serotonin inhibits ciliary transport in esophagus of the nudibranch mollusk *Tritonia diomedea*. Acta Biol Hung 50:175-184.

Pennati R, Groppelli S, Sotgia C, Candiani S, Pestarino M, De Bernardi F. 2001. Serotonin localization in *Phallusia mammillata* larvae and effects of 5-HT antagonists during larval development. Dev Growth Differ 43:647-656.

Piperno G, Fuller MT. 1985. Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. J Cell Biol 10:2085-2094.

Potter IC, Hill BJ, Gentleman S. 1970. Survival and behaviour of ammocoetes at low oxygen tensions. J Exp Biol 53:59-73.

Rapport MM, Green AA, Page IH. 1948. Serum vasoconstrictor (serotonin). IV. Isolation and characterization. J Biol Chem 176:1243-1251.

Reglödi D, Lubics A, Szelier M, Lengvári I. 1997. Serotonin immunoreactivity in the peripheral nervous system of oligochaeta. Acta Biol Hung 48:439-451.

Rovainen CM. 1996. Feeding and breathing in lampreys. Brain Behav Evol 48:297-305.

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

Sakharov DA, Salimova NB. 1980. Serotonin neurons in the peripheral nervous system of the larval lamprey, *Lampetra planeri*. A histochemical, microspectrofluorometric and ultrastructural study. Zool Jb Physiol 84:231-239.

Saltys HA, Jonz MG, Nurse CA. 2006. Comparative study of gill neuroepithelial cells and their innervation in teleosts and *Xenopus tadpoles*. Cell Tissue Res 323:1-10.

Satterlie RA, Norekian TP. 1995. Serotonergic modulation of swimming speed in the pteropod mollusc *Clione limacina*. III. Cerebral neurons. J Exp Biol 198:917-930.

Sbartati A, Oscultati F. 2003. Solitary chemosensory cells in mammals. Cell Tissue Organs 175:51-55.

Schnizer W. 1969. Biological determination of 5-hydroxytryptamine in the frog lung preparation. Z Biol 116:211-215.

Sebelova S, Stewart MT, Mousley A, Fried B, Marks NJ, Halton DW. 2004. The musculature and associated innervation of adult and intramolluscan stages of *Echinostoma caproni* (Trematoda) visualised by confocal microscopy. Parasitol Res 93:196-206.

Shuey DL, Sadler TW, Lauder JM. 1992. Serotonin as a regulator of craniofacial morphogenesis: site specific malformations following exposure to serotonin uptake inhibitors. Teratology 46:367-378.

Shuey DL, Sadler TW, Tamir H, Lauder JM. 1993. Serotonin and morphogenesis. Transient expression of serotonin uptake and binding protein during craniofacial morphogenesis in the mouse. Anat Embryol 187: 75-85.

Sillar KT, Woolston AM, Wedderburn JF. 1995. Involvement of brainstem serotonergic interneurons in the development of a vertebrate spinal locomotor circuit. Proc Biol Sci 259:65-70.

Soengas JL, Aldegunde M. 2004. Brain glucose and insulin: effects on food intake and brain biogenic amines of rainbow trout. J Comp Physiol A 190:641-649.

Stach T. 2005. Comparison of the serotonergic nervous system among Tunicata: implications for its evolution within Chordata. Organ Divers Evol 5:5-24.

Stewart MT, Marks NJ, Halton DW. 2003. Neuroactive substances and associated major muscle systems in *Bucephaloides gracilescens* (Trematoda: Digenea) metacercaria and adult. Parasitol Res 91:12-21.

Sundin L. 1995. Serotonergic vasomotor control in fish gills. Braz J Med Biol Res 28:1217-1221.

Sundin L, Reid SG, Rantin FT, Milson WK. 2000. Branchial receptors and cardiorespiratory reflexes in a neotropical fish, the tambaqui (*Colossoma macropomum*). J Exp Biol 203:1225-1239.

Takahashi-Iwanaga H, Abe K. 2001. Scanning electron microscopic observation of Merkel cells in the lamprey epidermis. Kaibogaku Zasshi 76:375-380.

Trinajstic K, Marshall C, Long J, Bifield K. 2007. Exceptional preservation of nerve and muscle tissues in Late Devonian placoderm fish and their evolutionary implications. Biol Lett 3:197-200.

Uhler GC, Huminski PT, Les FT, Fong PP. 2000. Cilia-driven rotational behavior in gastropod (*Physa elliptica*) embryos induced by serotonin and putative serotonin reuptake inhibitors (SSRIs). J Exp Zool 286: 414-421.

Van Dongen PA, Grillner S, Hökfelt T. 1986. 5-Hydroxytryptamine (serotonin) causes a reduction in the afterhyperpolarization following the action potential in lamprey motoneurons and premotor interneurons. Brain Res 366:320-325.

Veenendaal GH, Woutersen-van Nijnanten FM, van Miert AS. 1980. Responses of goat ruminal musculature to bradykinin and serotonin in vitro and in vivo. Am J Vet Res 41:479-483.

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.

Wada Y, Mogami Y, Baba S. 1997. Modification of ciliary beating in sea urchin larvae induced by neurotransmitters: beat-plane rotation and control of frequency fluctuation. J Exp Biol 200:9-18.

Wanninger A. 2005. Immunocytochemistry of the nervous system and the musculature of the chordoid larva of *Symbion pandora* (Cycliophora). J Morphol 265:237-243.

Weiss KR, Cohen JL, Kupfermann I. 1978. Modulatory control of buccal musculature by a serotonergic neuron (metacerebral cell) in *Aplysia*. J Neurophysiol 41:181-203.

Whitear M. 1989. Merkel cells in lower vertebrates. Arch Histol Cytol 52:415-422.

Whitear M, Lane EB. 1981. Fine structure of Merkel cells in lampreys. Cell Tissue Res 220:139-151.

Wilson SW, Ross LS, Parrett T, Easter SS. 1990. The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio*. Development 108:121-145.

Youngson C, Nurse C, Yeger H, Cutz E. 1993. Oxygen sensing in airway chemoreceptors. Nature 365:153-155.

Zaccone G. 1986. Neuron-specific enolase and serotonin in the Merkel cells of conger-eel (*Conger conger*) epidermis. An immunohistochemical study. Histochemistry 85:29-34

Zaccone G, Tagliafierro G, Fasulo S, Contini A, Ainis L, Ricca MB. 1990. Serotonin-like immunoreactivity in the epidermal club cells of teleost fishes. Histochemistry 93:355-357.

Zaccone G, Fasulo S, Ainis L. 1994. Distribution patterns of the paraneuronal endocrine cells in the skin, gills and the airways of fishes as determined by immunohistochemical

and histological methods. Histochem J 26:609-629.

Zielinski BS, Moretti N, Hua HN, Zaidi AU, Bisaillon AD. 2000. Serotonergic nerve fibers in the primary olfactory pathway of the larval sea lamprey, *Petromyzon marinus*. J Comp Neurol 420:324-334.

Abbreviations

ALLN	Anterior lateral line nerve
BC	Buccal cavity
C	Cirri
ChN	Chain neurons
F	Filaments
FSC	Serotonergic flask-shaped cell
GiC	Serotonergic gill cell
GS	Gill seam
HBG	Hypobranchial groove
IS	Interbranchial septum
IXth	Glossopharyngeal nerve
L	Lamellae
M	Musculature
MBE	Middle branchial edge
MuC	Serotonergic cells of the musculature
Oc	Oral cavity
OC	Otic capsule
PB	Pseudobranch
PBG	Pseudobranchial groove
V	Velum
V1	Ophthalmic division of the trigeminal nerve
V2	Maxilo-mandibular division of the trigeminal nerve
VIIIth	Octaval nerve
VIIth	Facial nerve
Xth	Vagal nerve

FIGURES
CHAPTER 1.1

Figure 1. A, B: Diagram of the larval sea lamprey mouth and pharynx in frontal hemisection (modified form Mallat, 1979). Dorsal half in A and ventral half in B. **C:** Schematic drawing of a horizontal section of a single gill. The rostral region is at the top in all figures. In C, medial is on the right.

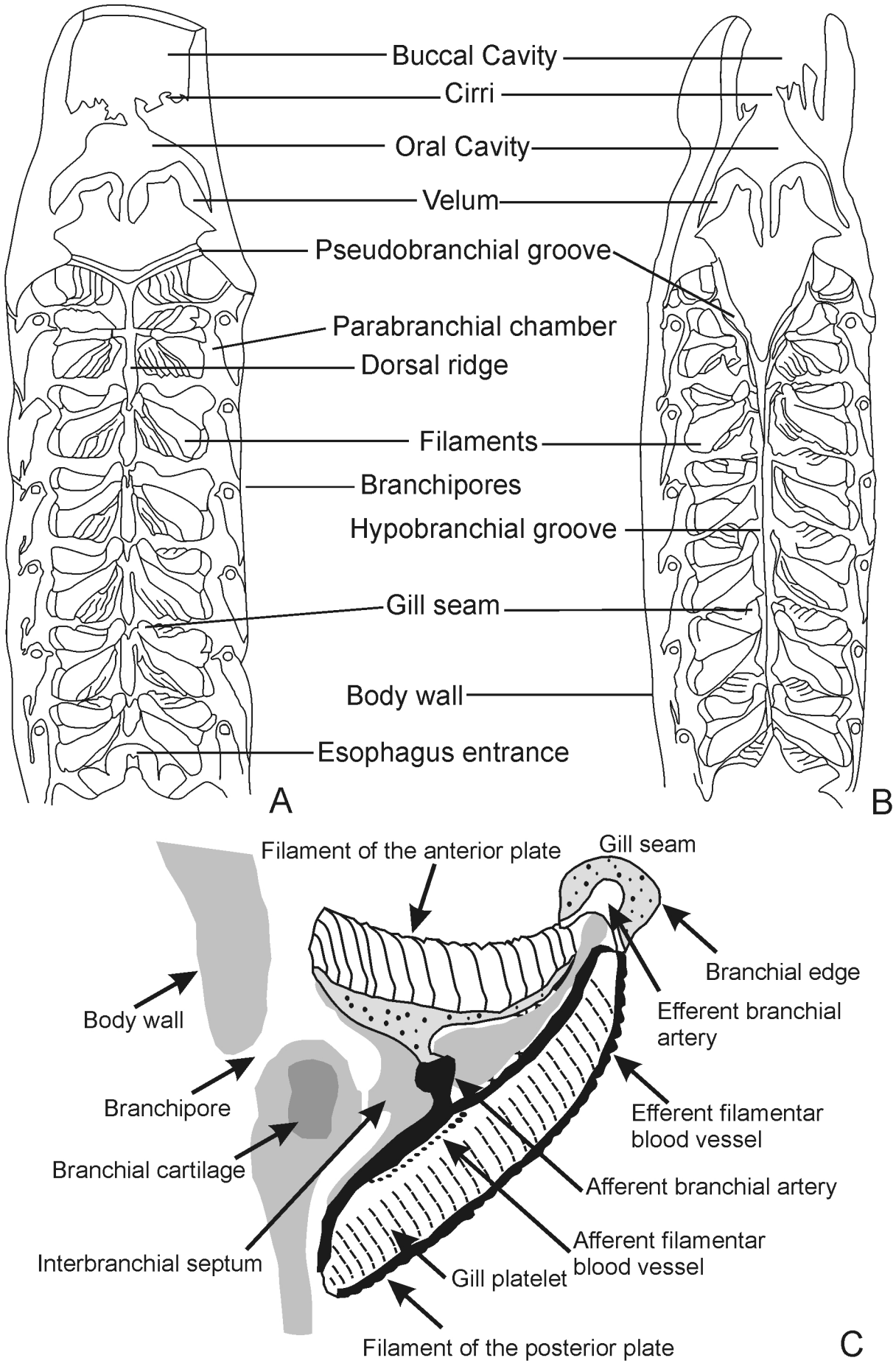

Buccal Cavity
Cirri
Oral Cavity
Velum
Pseudobranchial groove
Parabranchial chamber
Dorsal ridge
Filaments
Branchipores
Hypobranchial groove
Gill seam
Body wall
Esophagus entrance
A
B
Filament of the anterior plate
Gill seam
Branchial edge
Efferent branchial artery
Body wall
Branchipore
Efferent filamentar blood vessel
Branchial cartilage
Afferent branchial artery
Afferent filamentar blood vessel
Interbranchial septum
Gill platelet
Filament of the posterior plate
C

FIGURE 1

Figure 2. Photomicrographs of larval head sections showing the Vth and VIIth cranial nerve ganglia cells labeled with the tracer neurobiotin (green channel) and with anti-5-HT (magenta channel) antibodies. Figures A and B are merged photomicrographs from light and fluorescence microscopy showing the anatomical organization of the regions. **A-A''':** Photomicrographs of a sagittal section of the head showing the presence of serotonergic cells and fibers in the V1 and V2 cranial nerve ganglia. Note the presence of a double labeled cell in the V1 ganglion after injection of neurobiotin in the larval snout (see a detail of this double labeled cell in the right-hand-side corner of plates A'-A'''). White arrows indicate serotonergic cells located in the cranial nerve ganglia. **B-B''':** Photomicrographs of a sagittal section of the VIIth cranial nerve ganglion showing cells and fibers labeled with NB after injection of the tracer in the first gill. Note the absence of 5-HTir cells and the presence of serotonergic fibers in this ganglion. Dorsal is at the top and rostral on the right in all figures. For abbreviations, see list. Scale bars: 100 μm in A-A''' and 50 μm in B-B'''.

.

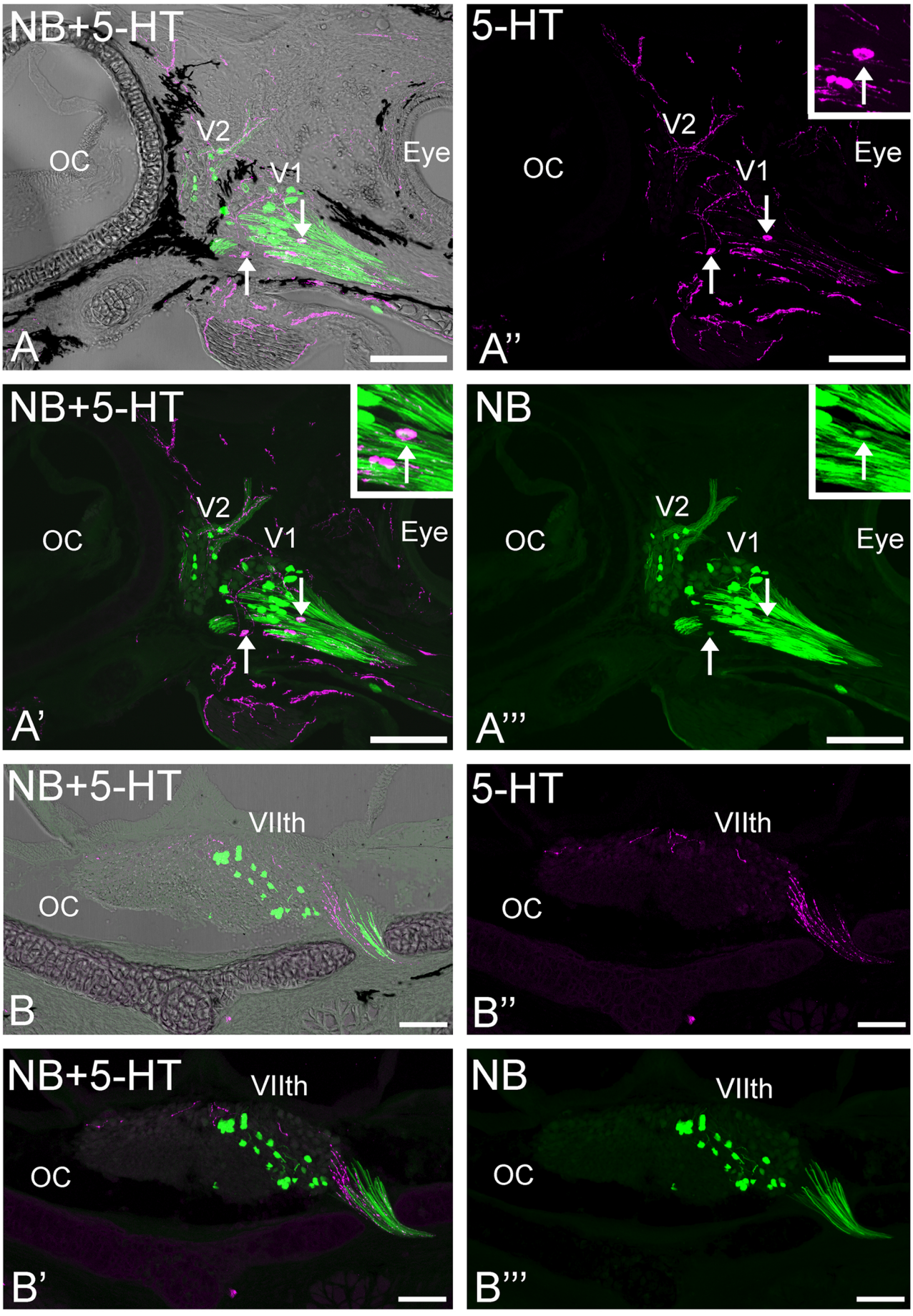

NB+5-HT
OC
V2
V1
Eye
A
5-HT
OC
V2
V1
Eye
A''
NB+5-HT
OC
V2
V1
Eye
A'
NB
OC
V2
V1
Eye
A'''
NB+5-HT
VIIth
OC
B
5-HT
VIIth
OC
B''
NB+5-HT
VIIth
OC
B'
NB
VIIth
OC
B'''

FIGURE 2

Figure 3. Photomicrographs of larval head sections showing the presence of serotonergic cells and fibers in the V1, V2, ALLN, IXth and Xth cranial nerve ganglia. **A:** Photomicrograph showing several 5-HTir fibers and some cells in the V2 ganglion. **B:** Photomicrograph of a sagittal head section showing the presence of 5-HTir fibers in the ALLN ganglion, which is located under the otic capsule. Note the absence of labeled cells in this ganglion and the presence of a serotonergic cell in the V1 ganglion. The V2 cranial nerve ganglion is also shown. **C:** Photomicrograph of the region caudal to the otic capsule showing the presence of serotonergic cells and fibers in the Xth cranial nerve ganglion. The arrowheads indicate the ventral branch of the IXth cranial nerve. Note the presence of a 5-HTir cell (thick arrow) and some fibers in the ventral branch of the IXth nerve. **D:** Photomicrograph showing the presence of 5-HTir cells in the IXth and Xth cranial nerve ganglia. Black arrows indicate 5-HTir cells of the cranial nerve ganglia. Dorsal is at the top and rostral is on the right in all figures. For abbreviations, see list. Scale bars: 100 μm in A/B/D 75 μm in C.

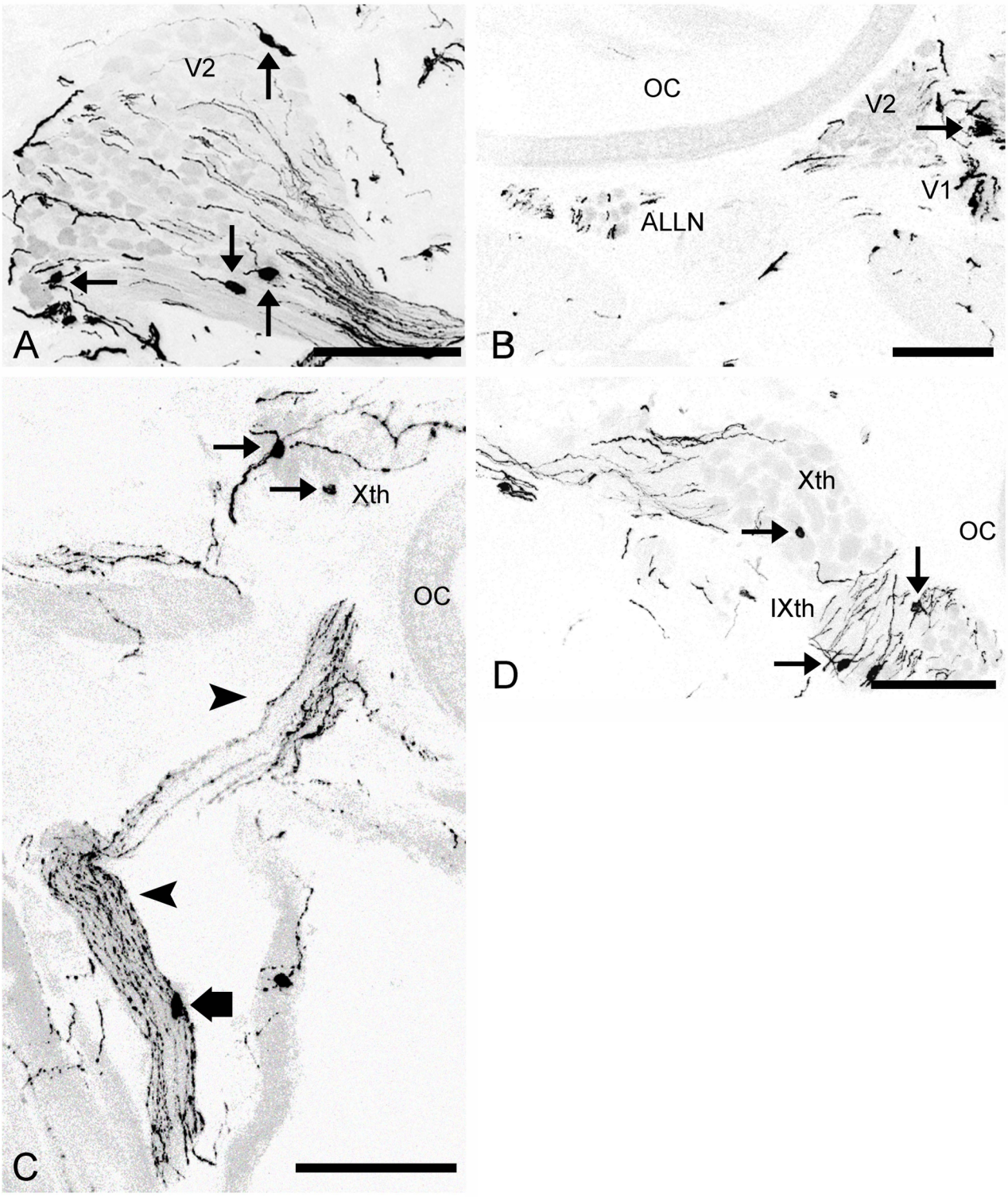


Figure 3

Figure 4. Photomicrographs showing the distribution of serotonergic fibers in larval sea lamprey head sections. **A:** Photomicrograph of a sagittal section of the ventral body wall of the larval head showing the extensive presence of serotonergic fibers in the musculature. Note the absence of serotonergic fibers in larval skin (black star). **B:** Photomicrograph of a transverse section of the buccal cavity showing the presence of serotonergic fibers in the mouth musculature. Note the presence of serotonergic fibers at the central edge of the cirri (arrowheads). **C:** Photomicrograph of the larval velum showing the numerous serotonergic fibers in this muscular structure. Note the presence of a thick bundle of 5-HTir cells (arrows) and fibers of the Vth cranial nerve in the rostral part of the velum. **D:** Photomicrograph of the larval body wall musculature showing a network of serotonergic fibers originated from intrinsic MuCs. **E:** Photomicrograph showing the presence of serotonergic fibers distributed along the IS. Note the presence of GiCs at the base of the filaments and in the IS close to the blood vessels (outlined stars). Note the absence of 5-HTir cells or fibers in the gill lamellae. **F:** Detail of the GiCs (curved arrows) in Figure E. Black arrows indicate MuCs. Dorsal is at the top in figures A-D and on the left in figures E-F. Rostral is on the right in figures A, C and D and at the bottom in figures E and F. For abbreviations, see list. Scale bars: 125 μm in A, 150 μm in B and C, 50 μm in D, 200 μm in E and 25 μm in F.

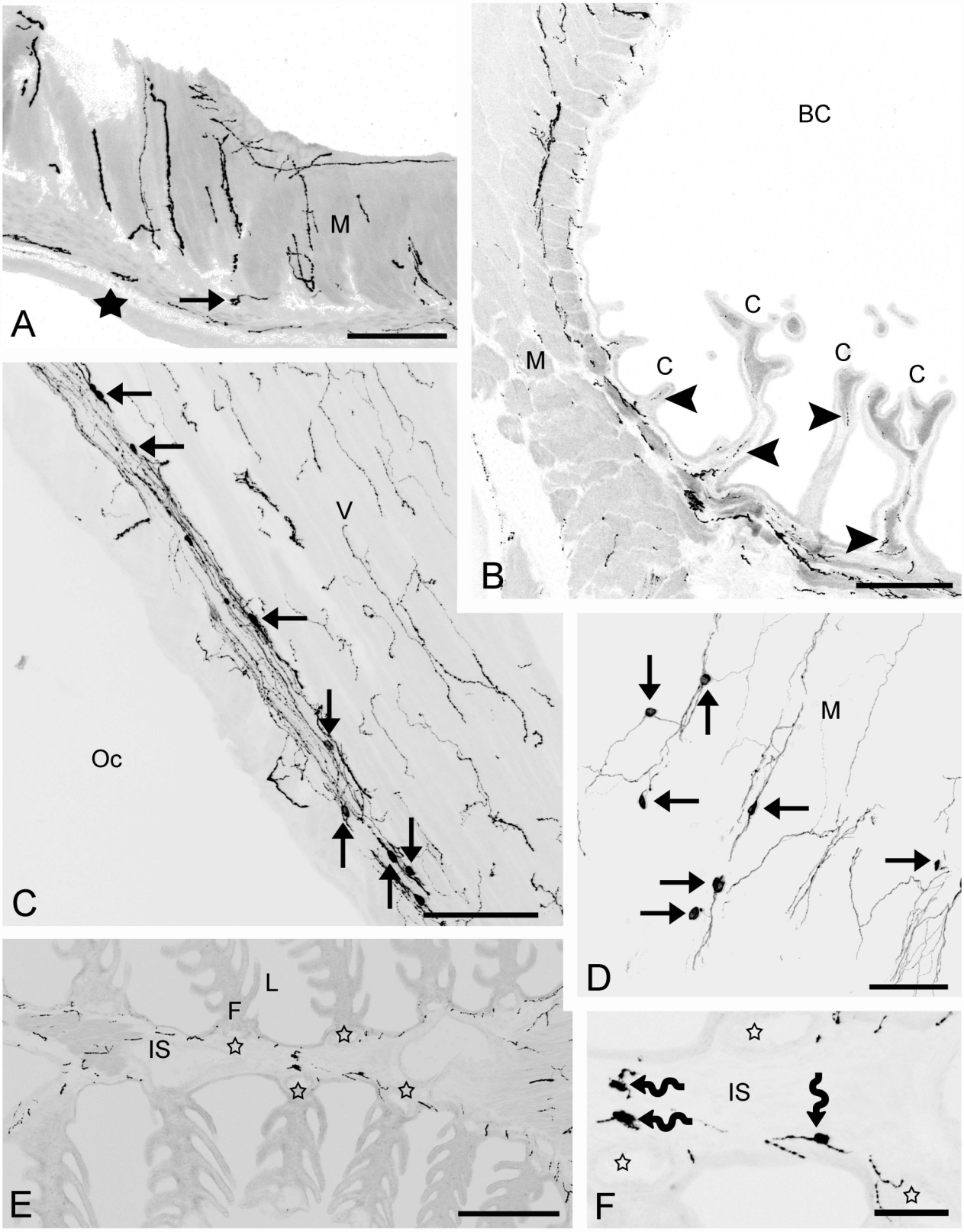


Figure 4

Figure 5. Photomicrographs showing different 5-HTir cell types of the larval sea lamprey head. **A:** Photomicrograph showing FSCs of the pseudobranchial groove (open arrowhead). Note the presence of serotonergic fibers in the first gill and the absence of serotonergic fibers arising form the FSCs. Dorsal is on the right and rostral is at the top. **B/C:** Photomicrographs of the PB showing 5-HTir GiCs. Note the processes of this cells distributed in the gill. **D:** Photomicrograph of a GiC located at the base of a gill filament. Note the distribution of the GiC processes around the gill filament. **E:** Photomicrograph of a serotonergic bipolar GiC located in the interbranchial septum. Note that the cell is located close to the afferent filamentar blood vessel (outlined stars). **F:** Photomicrograph showing the presence of a multipolar MuC (black arrow) in the myotomal musculature of the head. Note the presence of a long process arising from this cell and innervating the musculature. Dorsal is at the top and rostral is on the right in figures B-F. Curved arrows indicate GiCs. For abbreviations, see list. Scale bars: 100 μm in A, 75 μm in C, 50 μm in B, D, E and F.

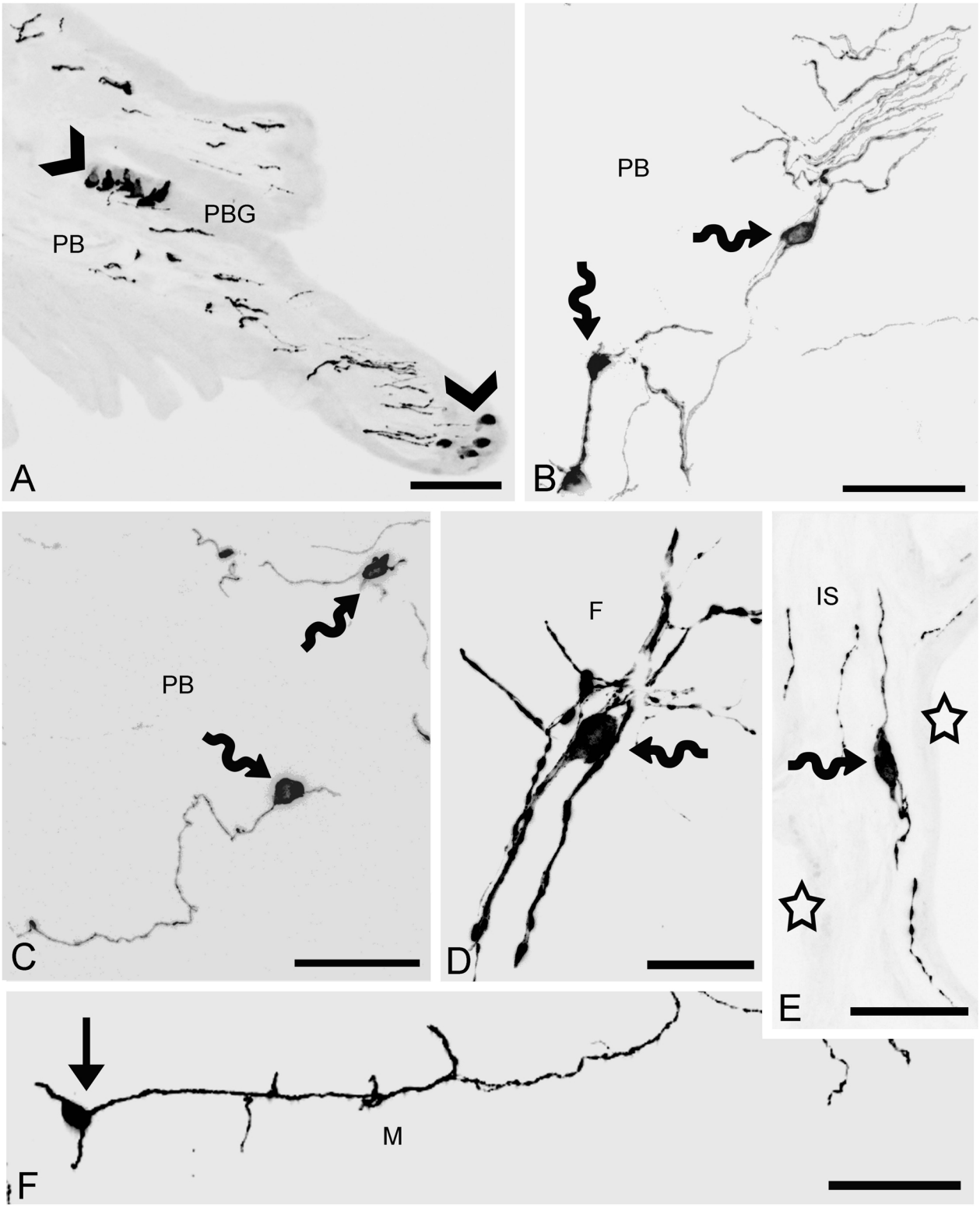


Figure 5

Figure 6. Photomicrographs showing the distribution of serotonergic fibers innervating different larval head muscular structures. **A-A'':** Photomicrograph of a sagittal section of the larval head showing three branches of the Xth nerve (arrowheads). Immunoreactivity to α-tubulin (green channel) reveals the presence of the entire innervation. Note that a large percentage of the fibers are 5-HTir fibers (magenta channel). **B-B'':** Photomicrograph showing the presence of two MuCs labeled with antibodies against α-tubulin (magenta channel) and 5-HT (green channel). Note that the processes of the serotonergic cells are intermingled with other non-5-HTir fibers. **C:** Photomicrograph showing the extensive presence of serotonergic fibers (magenta channel) surrounding the larval eye and innervating the eye musculature. White arrows indicate MuCs. Dorsal is at the top and rostral is at the right in all figures. For abbreviations, see list. Scale bars: 60 μm in B-B'' and 100 μm in A-A'' and C.

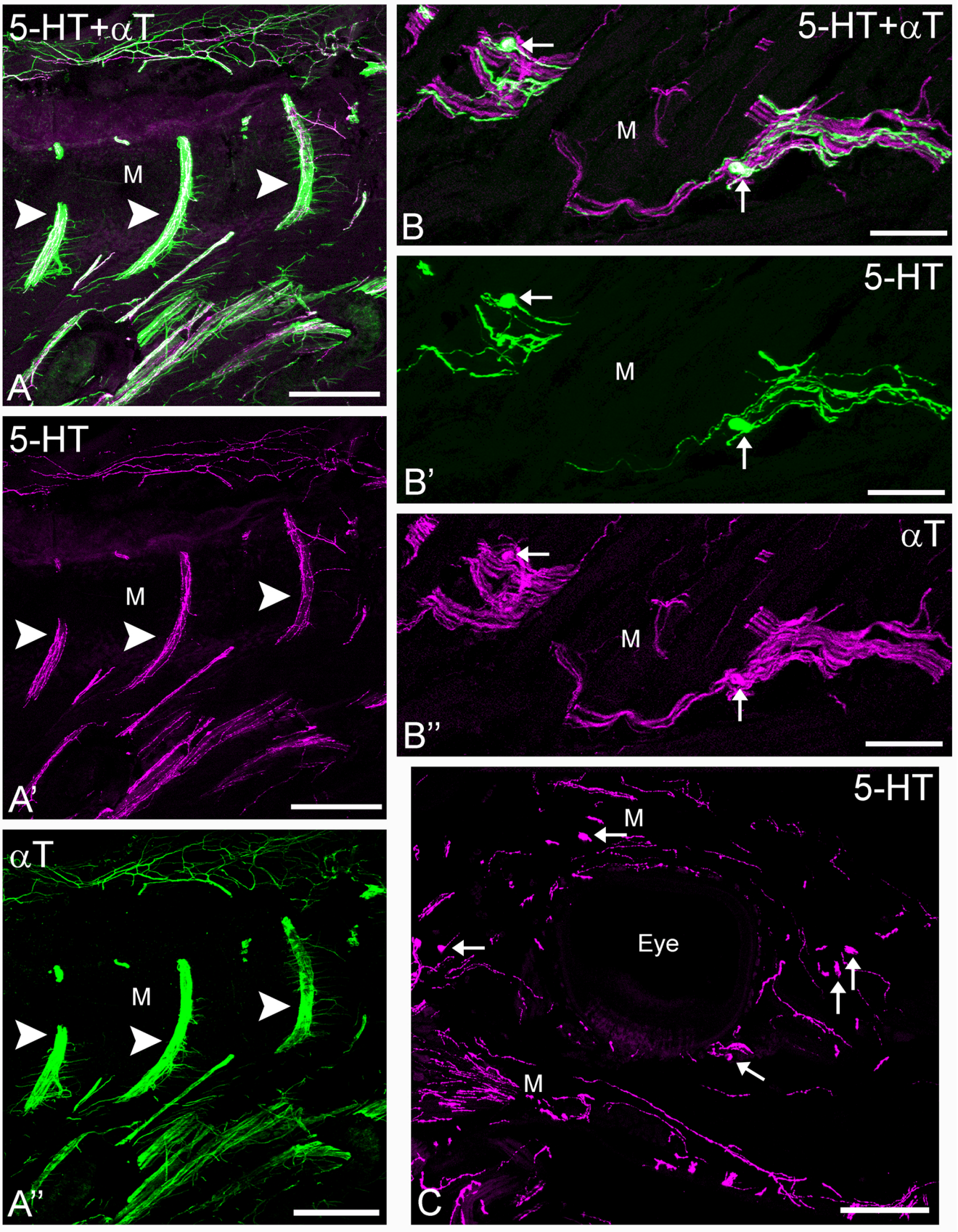


FIGURE 6

Figure 7. Photomicrographs of sagittal sections showing the distribution of fibers labeled with antibodies against 5-HT (magenta channel) and α-tubulin (green channel) in different structures of the larval head. **A-A''':** Photomicrographs showing the presence of bundles of tubulin-ir fibers that innervate the oral cirri. Note that only a few of the fibers in this muscular structure are 5-HTir (arrowheads). Note the presence of a 5-HTir/tubulin-ir cell in one of the cirri (white arrow). See a detail of this double labeled cell in the right-hand-side corner of plates A'-A'''. Figure A is a light and fluorescence merged photomicrograph. **B-B'':** Photomicrographs showing the innervation of the lateral portion of the gill filament. Note that only a small percentage of the entire innervation corresponds to the serotonergic innervation. Observe the presence of a GiC in the filament (curved arrow). **C-C'':** Photomicrographs showing the innervation of the interbranchial septum. Note that some of the tubulin-ir longitudinal fibers that innervate this region are 5-HTir. Most of the 5-HTir fibers are located close to the afferent filamentar blood vessels (outlined stars). Dorsal is at the top and rostral is on the right in all figures. For abbreviations, see list. Scale bars: 100 μm in A-C''.

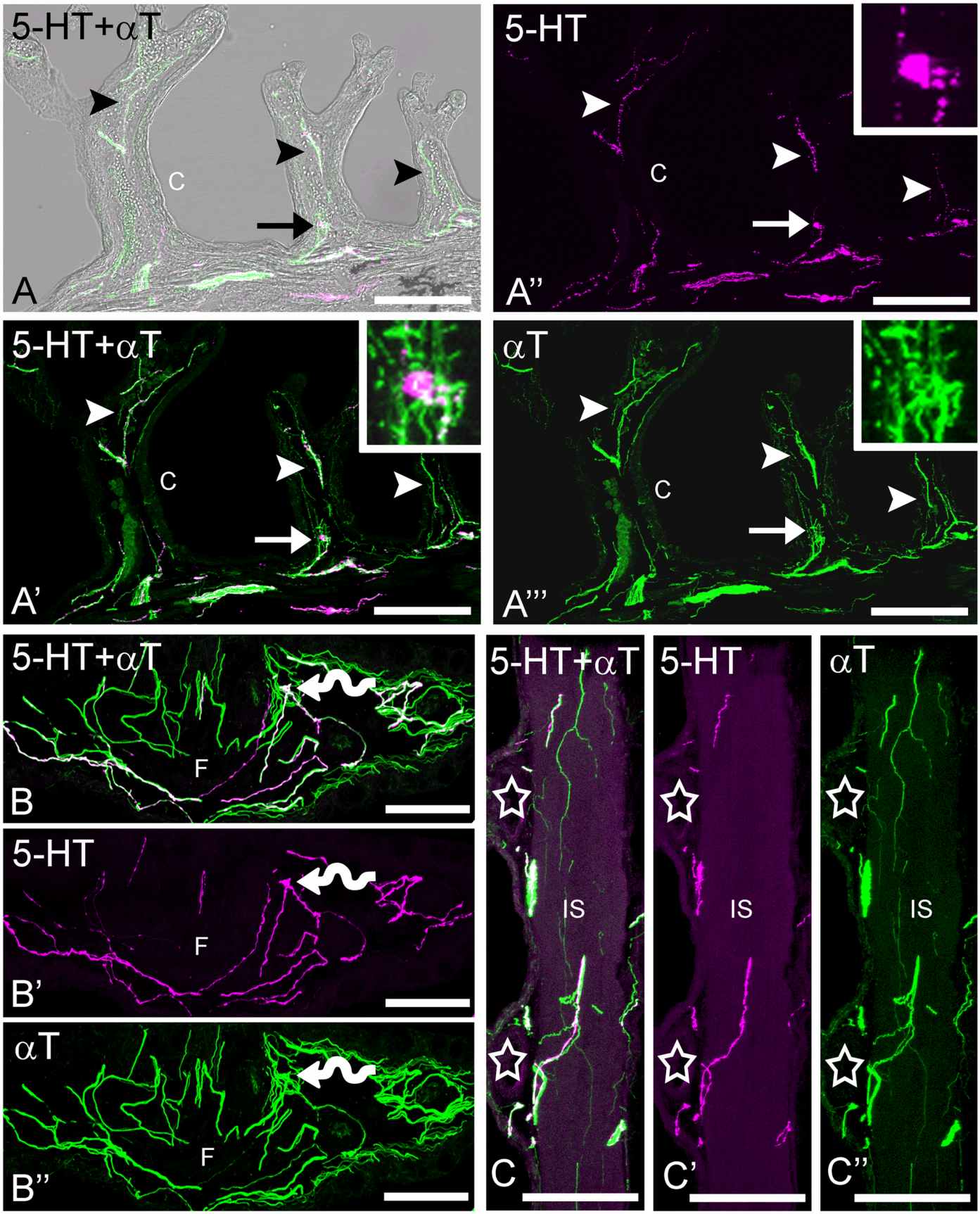


FIGURE 7

Figure 8. Photomicrographs of sections of the pseudobranch labeled with antibodies against tubulin (green channel in A-A'' and magenta channel in B-B'') and 5-HT (magenta channel in A-A'' and green channel in B-B''). **A-A'':** Photomicrographs of a transverse section of the pseudobranch showing the presence of FSCs with the apical region pointing towards the pseduobranchial groove. Note the presence of afferent tubulin-ir fibers that reach the base of the FSCs. Dorsal is at the top. **B-B'':** Photomicrographs of a sagittal section of the head at the level of the pseudobranch showing the presence of 5-HTir FSCs in close association with the ciliary cells of the pseduobranchial groove (the tubulin-ir reveals the presence of the ciliary cells; double arrow). Note the presence of afferent tubulin-ir fibers associated with the base of the FSCs. Dorsal is on the right and rostral is at the top. Open arrowheads indicate the 5-HTir FSCs. Arrowheads indicate the afferent tubulin-ir fibers. For abbreviations, see list. Scale bars: 50 μm in A-A'' and 150 μm in B-B''.

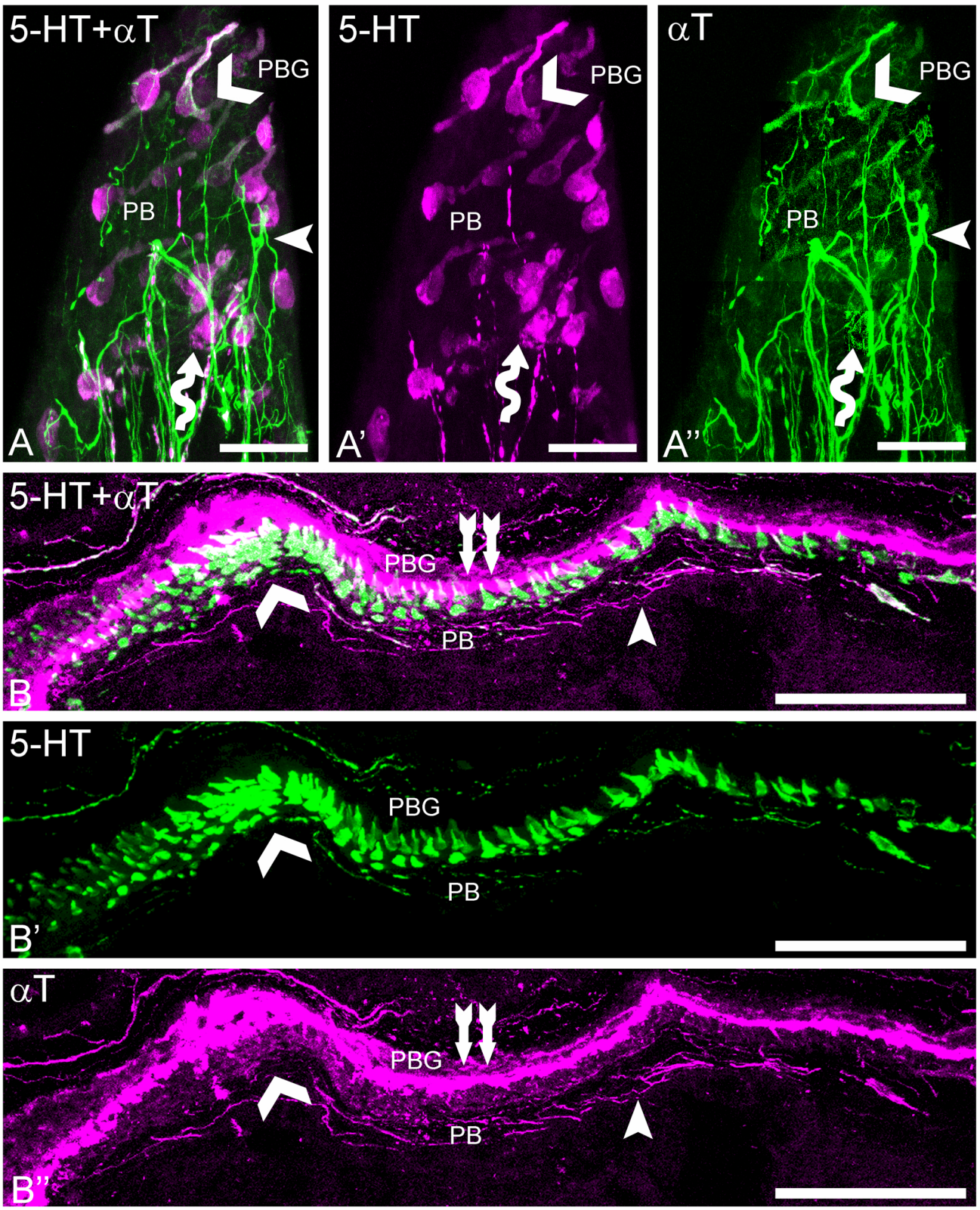

5-HT+αT
PBG
PB
A
5-HT
PBG
PB
A'
αT
PBG
PB
A''
5-HT+αT
PBG
PB
B
5-HT
PBG
PB
B'
αT
PBG
PB
B''

FIGURE 8

Figure 9. Photomicrographs of the larval head showing different types of cells labeled with antibodies against 5-HT (magenta channel) and tubulin (green channel). **A-A'':** Photomicrographs of a transverse section of a gill filament showing the presence of a bipolar 5-HTir/tubuli-ir GiC in subepithelial location (curved arrow). **B-B'':** Photomicrographs of a transverse section showing a solitary bipolar 5-HTir/tubulin-ir cell. Note the presence of a thick basal process extending from the cell. **C-C'':** Photomicrographs of a sagittal section of the head showing FSCs of the gill seam contacted by tubulin-ir afferent fibers (arrowhead). **D-D'':** Photomicrographs of a sagittal section of the ventral surface of the pharynx showing 5-HTir/tubulin-ir FSCs of the hypobranchial groove contacted by afferent tubulin-ir afferent fibers. Note the presence of 5-HTir/tubulin-ir MuCs and their processes in the ventral musculature. **E-E'':** Photomicrographs of a sagittal section of the middle branchial edge showing the presence of the tentatively named 5-HTir/tubulin-ir chain neurons. Note the longitudinal positioning of these cells at the branchial edge. Open arrowheads indicate the FSCs. White arrows indicate MuCs. Dorsal is on the right in Figures A-C'' and at the top in Figures D-E''. For abbreviations, see list. Scale bars: 75 μm in A-A'' and E-E'', 15 μm in B-B'', 100 μm in C-C'' and 50 μm in D-D''.

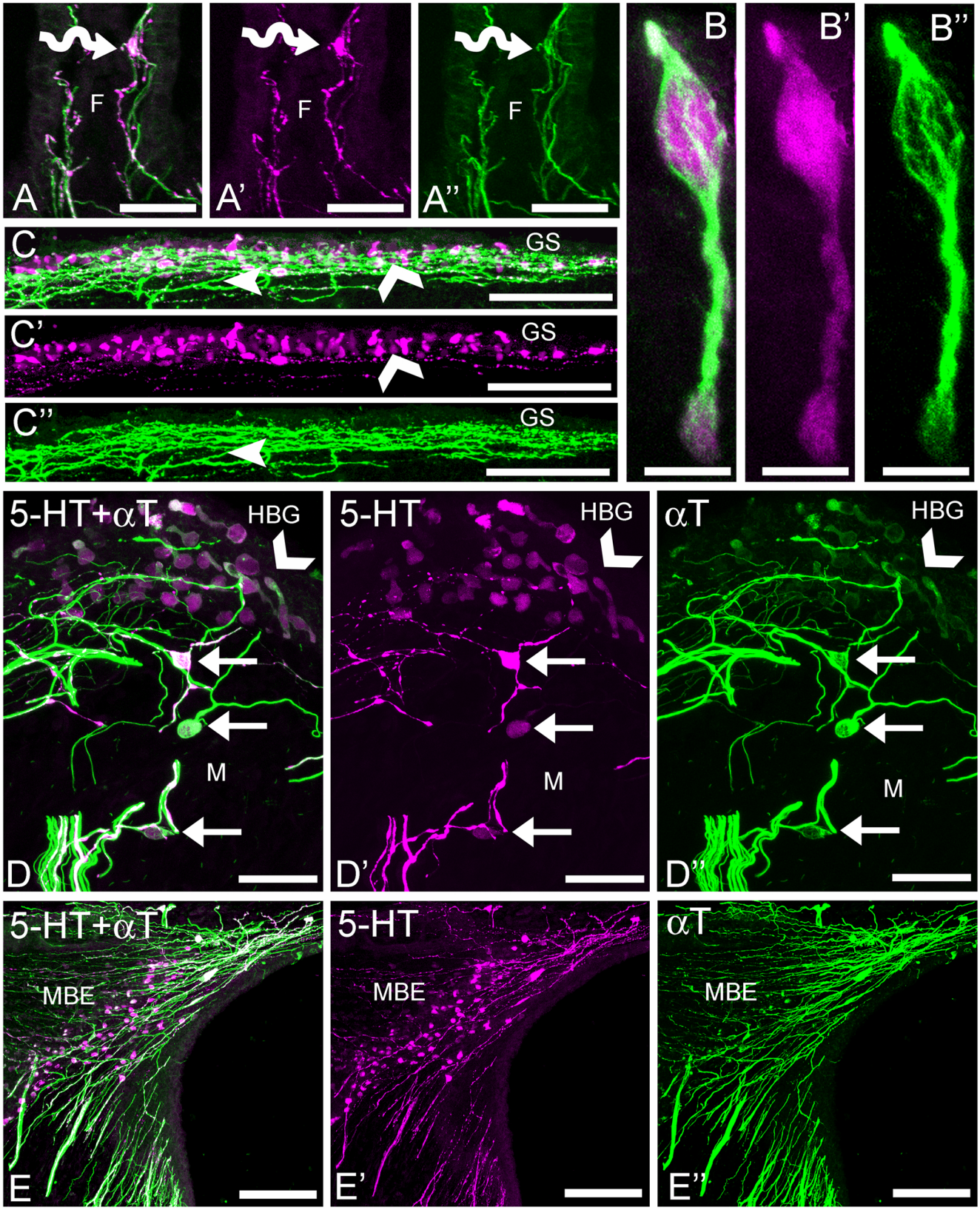

A
A'
A''
F
F
F
B
B'
B''
C
C'
C''
GS
GS
GS
5-HT+αT
5-HT
αT
HBG
HBG
HBG
M
M
M
D
D'
D''
5-HT+αT
5-HT
αT
MBE
MBE
MBE
E
E'
E''

FIGURE 9

Figure 10. A-A'': Photomicrographs of a sagittal section of a gill arch at the level of the middle branchial edge showing the presence of 5-HTir/tubulin-ir structures tentatively named chain neurons. **B-B''**: Photomicrographs of a semi-sagittal section of the head showing the middle branchial edge and the gill seam of a gill arch. Note the presence in the middle branchial edge of the chain neurons and also the presence of a FSC (open arrowhead) in the gill seam in close relation with a ciliary cell (see the presence of the tubulin-ir cilia; arrow). Note the presence of the cilia covering the gill seam (double arrow). Dorsal is at the top and rostral is on the right in all figures. For abbreviations, see list. Scale bars: 50 μm in A-A'' and 75 μm in B-B''.

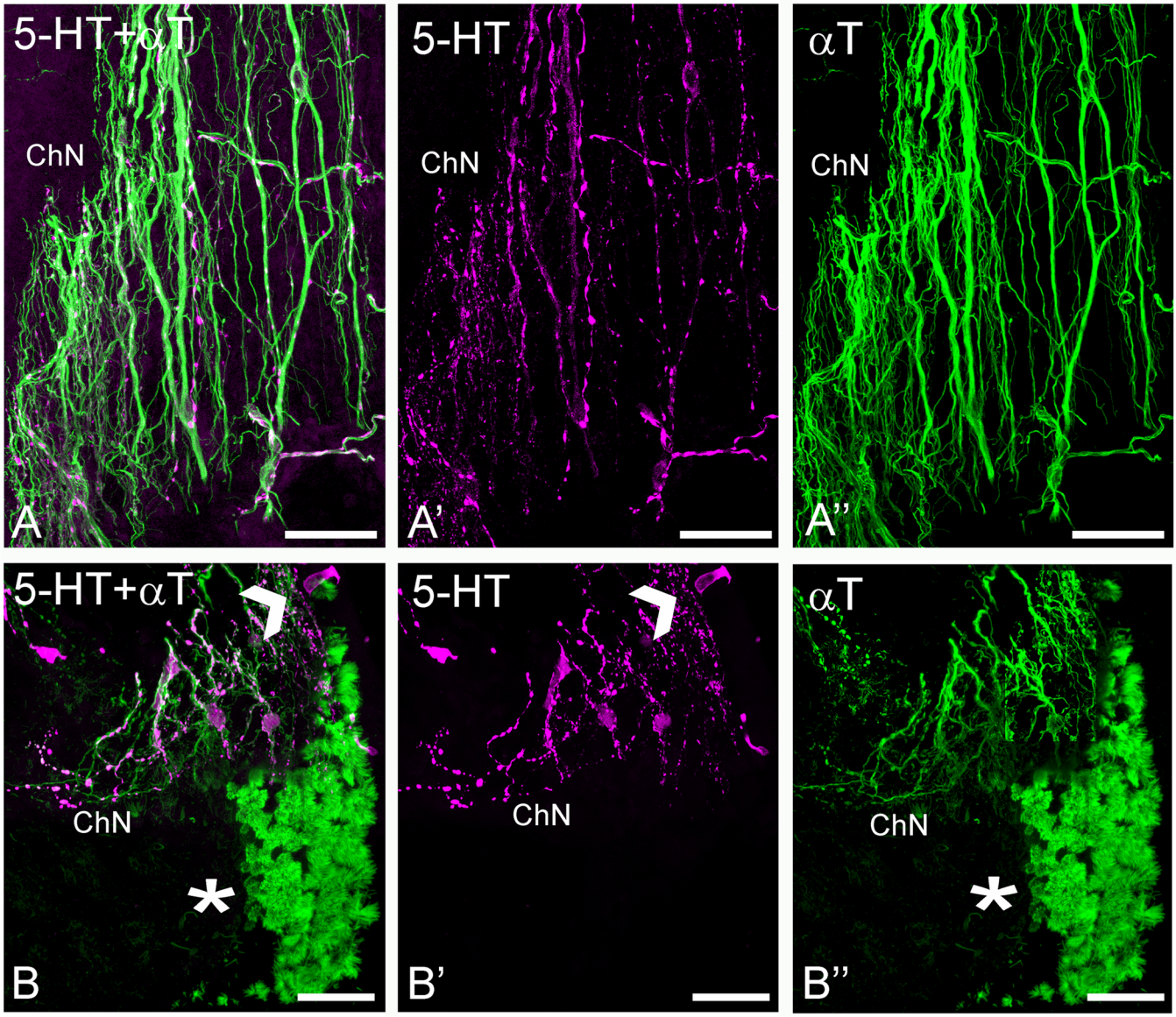


FIGURE 10

Figure 11. Photomicrographs of adult sea lamprey head sections showing the presence of different serotonergic cells and fibers. **A:** Photomicrograph showing an extensive network of serotonergic MuCs and their processes in the musculature of the adult sea lamprey body wall. **B:** Detail of multipolar MuCs. **C/D:** Photomicrographs of sagittal sections of different branchial arches showing the presence of 5-HTir GiCs and fibers in the gill filaments and in the interbranchial septum. Note the presence of longitudinal fibers in the interbranchial septum (arrowhead) and observe the location of the GiCs in the basal portion of the filaments (curved arrows). Black arrows indicate the MuCs. Dorsal is at the top and rostral is on the right in Figures A-C. Dorsal is on the right and rostral is at the bottom in Figure D. For abbreviations see list. Scale bars: 150 μm in A, 100 μm in B-D.

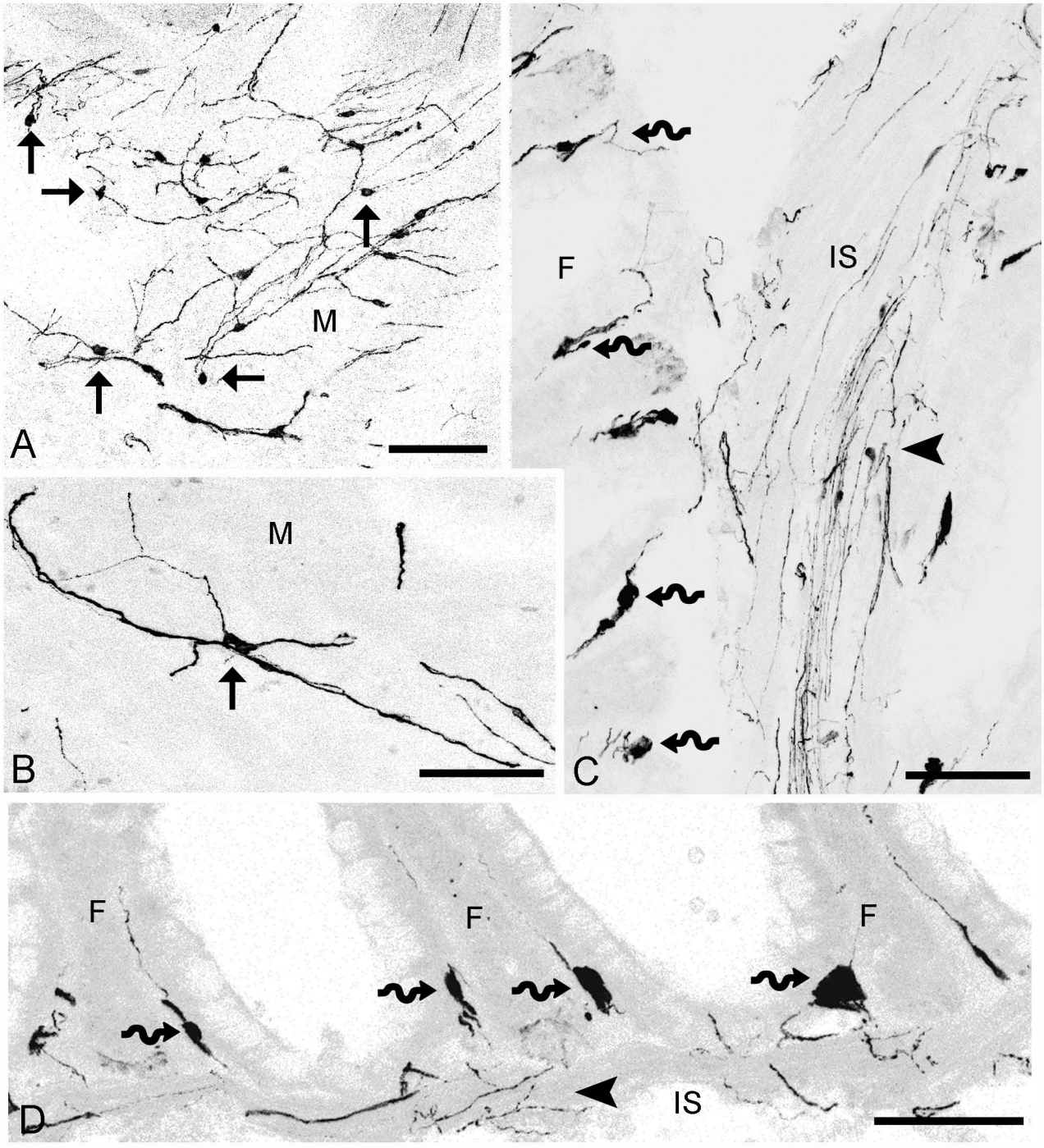


Figure 11

Neurochemical characterization of the sea lamprey taste buds and afferent gustatory fibres. Presence of serotonin, calretinin and CGRP immunoreactivities in taste bud bi-ciliated cells of the earliest vertebrates

Introduction

Lampreys are the most primitive vertebrates that possess taste buds (TBs) (Northcutt, 2004). The sea lamprey, *Petromyzon marinus* L., has a complex life cycle that begins with eggs laid in rivers. The embryonic period is followed by a short prolarval stage and a microphagous larval stage (ammocoete), which lasts for several years before complex metamorphosis occurs, and transformation into young (postmetamorphic) adults takes place. Young adults descend to the sea and feed as parasites on teleost fishes before migrating to rivers to breed and die (see Hardisty and Potter, 1971). Downstream and upstream migrating adult lampreys do not feed. The larvae feed exclusively on suspended microorganisms that become embedded in mucus in the pharynx (Baatrup, 1985a). Water for respiration and feeding enters the mouth and flows out through the branchial openings by the coordinated action of velum and sequential contraction and expansion of the branchial region. The larvae have seven gill sacs supported by cartilaginous arches that open directly from the pharyngeal cavity.

The first six pairs of gill arches bear volcano-like papillae that face the water current (Mallat, 1979; Baatrup, 1983a). These papillae have been considered as TBs because of their similarities to the TBs of gnathostomes (Retzius, 1893; Fahrenholz, 1936). Ultrastructural studies have revealed the presence of bi-ciliated sensory cells and microvillar supporting cells in the TBs of larval and adult lamprey, defined by Baatrup (1983a, b, 1985b) as light and dark cells, respectively, supporting the idea that there is only a single type of taste receptor cell in lamprey. However, no neurochemical studies have been performed on these TBs, and whether there are present neurochemically different kinds of sensory cells is not known. In lampreys, the facial, glossopharyngeal and vagal nerves innervate these taste structures, as in jawed fishes (Alcock, 1899; Johnson, 1905). Nerve fibre recordings obtained from the gill arch failed to demonstrate mechanical responsiveness, but the buds were found to be most sensitive to chemical stimulants (Baatrup, 1985a). The branchial region in adult lampreys differs considerably from that in larvae; the branchial sacs are connected with the pharyngeal cavity

and the outer head surface via water tubes (see Hardisty and Potter, 1971). In adults, the buds are present in the water tube, near the inner gill openings, presenting the same cell types and organization as those observed in the larval lamprey (Retzius, 1893; Fahrenholz, 1936; Baatrup, 1983b, 1985b).

In mammals, taste buds (TBs) consist of at least of three types of elongated cells (type I, II and III) and well as basal cells. The use of immunocytochemical methods has revealed the presence of serotonin (5-HT) and/or its synthesizing enzymes in type III cells of mammals (Fujimoto et al., 1987; Kim and Roper, 1995; Yee et al., 2001; Huang et al., 2005a; Dvoryanchikov et al., 2007) and serotonin in Merkel-like basal cells of teleost fishes, frog and mudpuppy (Jain and Roper, 1991; Delay et al., 1993; Zaccone et al., 1994; Kim and Roper, 1995). Type II and III cells are receptor cells in mammalian TBs (see Roper, 2007), and recent studies have revealed that they probably use purinergic transmission (Finger et al., 2005; Roper, 2007). Serotonin release from type III cells was observed when TBs were exposed to sweet and bitter taste stimuli (Huang et al., 2005a,b,c), suggesting that 5-HT may be a neuromodulator or neurotransmitter (NT) in TBs (Kim and Roper, 1995; Huang et al., 2005a,b,c).

The presence of epinephrine and norepinephrine has also been reported in TBs of frog, rat and mouse (Zancanaro et al., 1995; Herness et al., 2002; Dvoryanchikov et al., 2007). Neuropeptide Y (NPY) immunoreactivity occurs in some TB cells in rat (Zhao et al., 2005) and in afferent fibres of amphibians and mammals (Montavon et al., 1991; Yamamoto et al., 1997; Kawakami et al. 1998; Kusakabe, 1998). GABA and glutamate immunoreactivity has been observed in afferent fibres of mudpuppy TBs (Jain and Roper, 1991) and GABA immunoreactivity has also observed in TB cells in fishes (Eram and Michel, 2005) and mammals (Obata et al., 1997). Calcitonin gen-related peptide (CGRP) immunoreactive afferent fibres were observed in amphibians and mammals (Kawakami et al., 1998; Witt and Reutter, 1998). Calretinin (CR; an E-F hand cytoplasmic calcium binding protein) was also reported in taste cells of teleosts (Díaz-Regueira et al., 2005; Northcutt, 2005; Germanà et al., 2007) and in fibres innervating TBs of teleosts (Díaz-Regueira et al., 2005; Germanà et al., 2007) and rat (Ichikawa et al., 1992).

The aim of the present study was to investigate the presence of neuroactive substances (5-HT, catecholamines, GABA, glutamate, NPY and CGRP) in TBs of the sea lamprey, to improve our knowledge of neurotransmitters potentially involved in the transmission of the gustatory sensation in lampreys and, hence, on the evolution of neurotransmission in vertebrate TBs. Other immunohistochemical markers (tubulin and CR) were used to investigate the taste cells and their innervation. We also investigate the

location of proliferating cells in the larval lamprey TBs by means of two methods involving proliferation markers, BrdU labelling and proliferating cell nuclear antigen (PCNA) immunohistochemistry, markers that were previously used in lamprey brain (Tobet et al., 1996; Vidal-Pizarro et al., 2004; Villar-Cheda et al., 2005, 2006b). The present results reveal for the first time the presence of 5-HT, CGRP and CR in bi-ciliated cells in lamprey TBs, and demonstrate the non-proliferative status of the vast majority of cells in the TBs.

Material and Methods

Animals

Sea lamprey, *Petromyzon marinus* L., larvae (n=40), of between 15 and 150 mm in body length, were caught in the River Ulla (Galicia, Spain) and maintained at 16ºC in an aerated aquarium containing river sediment, until processing. Adult migrating sea lampreys (n=3) were bought from local fishermen. All the experiments were performed according to the regulations of the European Union (86/609/EEC) and Spain (Royal Decree 223/1998) for the care and handling of animals in research.

Immunofluorescence

The animals were killed by overdose of benzocaine (0.5 mg/L) and decapitated. The larval heads were fixed in 4% paraformaldehyde in 0.05 M tris buffered pH 7.4 (TBS) by immersion for 6 hours. The branchial region of the adult lampreys was extracted and fixed by immersion for 12 hours in the same fixative. After fixation and posterior rinsing in TBS, the tissue was cryoprotected in 30% sucrose in TBS overnight, embedded in Tissue Tek (Sakura), frozen and cut on a cryostat (20 to 25 µm thick).

Sections were incubated with a combination of one of the following polyclonal antibodies (see table 1): rabbit polyclonal anti-serotonin antibody (dilution 1:2,500; Incstar, Stillwater, MN), rabbit polyclonal anti-TH antibody (dilution 1:100; Chemicon, Temecula, CA), rabbit polyclonal anti-calretinin antibody (dilution 1:500; SWant, Bellinzona, Switzerland) or rabbit polyclonal anti-NPY antibody (dilution 1:600; Sigma, St. Louis, MO) with a mouse monoclonal anti-acetylated α-tubulin antibody (dilution 1:500; Sigma) or a mouse monoclonal anti-CGRP antibody (dilution 1:300; Sigma) for 72h at 4ºC. For GABA and glutamate immunofluorescence larval heads were fixed in 5% glutaraldehyde and 1% sodium metabisulfite in 0.05 M TBS during 17 hours and sectioned as above. Sections were pre-treated with 0.2% NaBH4 in distilled water for 45 minutes at room temperature. Then, the sections were incubated with a mouse monoclonal anti-GABA antibody (dilution 1:1,200; Sigma) and a rabbit polyclonal anti-glutamate antibody (dilution 1:4,500; Inmunosolution, Jesmond, Australia)

for 72h at 4ºC. Sections were rinsed and then incubated with a cocktail of rhodamine isothiocyanate (RICT) or fluorescein isothiocyanate (FICT)-conjugated swine anti-rabbit antibody (diluted 1:30; Dako, Glostrup, Denmark) and fluorescein isothiocyanate (FICT)-coupled goat anti-mouse antibody (diluted 1:50; Chemicon) or with Alexa 546-coupled goat anti-mouse antibody (diluted 1:100; Interchim, Montlucom, France) for 1 hour. All antibody dilutions were carried out in TBS containing 15% normal goat serum (NGS), 10% normal swine serum (NSS) and 0.2% Triton X-100. Sections were rinsed in distilled water and then coverslipped with mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA).

Antibody characterization and specificity controls

The specificity of all primary antibodies has been well characterized by the suppliers (see Table 1 showing information about the different antibodies). Western blot experiments performed in the present study were done following experimental procedures detailed in a previous study carried out in our laboratory (Villar-Cerviño et al., 2006). Calretinin was used as positive control in these western blot experiments.

The anti-tubulin antibody has been already used as a cilia marker (Piperno and Fuller, 1985) and has also been shown to label neurons and their processes in the nervous system (Wilson et al., 1990; Chitnis and Kuwada, 1990). This antibody was used as neuron and receptor cell marker of the lamprey both central and peripheral nervous system (Kuratani et al., 1997; Barreiro-Iglesias et al., 2008).

The specificity of the anti-5-HT antibody was tested by the supplier, who reported no detectable cross-reactivity with tryptamine, 5-methoxytryptamine, L-tryptophan, 5-hydroxytryptophan, dopamine, norepinephrine or adrenaline. This antibody has been used in recent immunohistochemical studies in the sea lamprey brain and retina (Antri et al., 2006; Villar-Cerviño et al., 2006; Abalo et al., 2007) and was previously tested by western blot in lamprey brain protein extracts in our laboratory (Villar-Cerviño et al., 2006). No protein band was detected in these blots. Moreover, we previously tested the specificity of this antibody in lamprey in pre-adsorption experiments (Abalo et al., 2007); immunostaining was completely abolished after pre-adsorption of the diluted anti-serotonin antibody with 5-HT-BSA conjugates.

The monoclonal anti-CGRP antibody was raised against a synthetic C-terminal fragment of rat CGRP conjugated with glutaraldehyde to bovine serum albumin (Szabat et al., 1994; see Table 1). It was purified from the exhausted supernatant by chromatography. The sequence of this CGRP fragment is well conserved between rat and

fish (9 out of 10 identical aa in rat and pufferfish; accession nº of pufferfish sequence taken from GenBank: AJ309015). We tested this antibody by western blot in lamprey protein extracts; no protein band was detected in these blots (Fig. 1). Moreover, we tested the specificity of this antibody in lamprey in pre-adsorption experiments; immunostaining was completely abolished after pre-adsorption of the diluted anti-CGRP antibody with CGRP from rat (Sigma).

The anti-CR antibody was tested in western blots of lamprey extracts in our laboratory (Villar-Cheda et al., 2006a; present results). A protein band of 29 kDa was observed in these blots (Fig. 1), which is exactly the same size of protein observed in blots for rat CR (Villar-Cheda et al., 2006a). In teleosts, this antibody specifically stained many TB sensory cells (Northcutt, 2005; Díaz-Regueira et al., 2005).

The specificity of the anti-TH antibody was tested by the supplier. This antibody has been used in immunohistochemical studies of lamprey brain and retina (Pierre et al., 1997; Villar-Cerviño et al., 2006), and it was tested by western blot in lamprey and rat brain protein extracts in our laboratory, which revealed similar sized bands for the lamprey and rat TH enzymes (not shown).

The anti-NPY antibody reacts with NPY conjugated to BSA in dot blot. The supplier reported that cross reactivity is observed by dot blot with NPY (human), and NPY fragments including NPY (18-36), NPY (13-36) and peptide YY (PYY), (human) conjugated to BSA. The antiserum shows no cross-reactivity in dot blot with substance P, neurokinin A, neurokinin B, vasoactive intestinal peptide (VIP), CGRP (rat), calcitonin and somatostatin conjugated to BSA, or BSA. Moreover, we tested the specificity of this antibody in lamprey in pre-adsorption experiments; immunostaining was completely abolished after pre-adsorption of the diluted anti-NPY antibody with 10 mM NPY (porcine).

The monoclonal anti-GABA antibody was evaluated for activity and specificity by dot-blot immunoassay by the supplier. No cross-reaction was observed with BSA, L-a-aminobutyric acid, L-glutamic acid, L-aspartic acid, glycine, d-aminovaleric acid, L-threonine, L-glutamine, taurine, putrescine, L-alanine, and carnosine. The antibody showed weak cross-reaction with b-alanine. Furthermore, the sections of the brain and retina of sea lamprey incubated with this antibody revealed the same pattern of immunostaining revealed in studies with other anti-GABA antibodies (Meléndez-Ferro et al., 2001, 2002, 2003; Villar-Cerviño et al., 2006). In addition, the antibody was previously tested by western blotting with lamprey brain protein extracts in our laboratory (Villar-Cerviño et al., 2008). No protein band was stained in these blots. Immunostaining was completely abolished after pre-adsorption of

the diluted anti-GABA antibody with GABA-BSA conjugates (Sigma).

The polyclonal anti-glutamate antibody was evaluated by the supplier. Dot blots against a variety of other amino acid conjugates, including aspartate, do not yield significant immunoreaction. In addition, we tested the anti-glutamate antibody by western blotting with lamprey brain protein extracts. No protein band was stained in these blots (Fig. 1). Furthermore, the sections of the retina of sea lamprey incubated with this antibody revealed the same pattern of immunostaining revealed in studies with other anti-GLU antibody generated against GLU coupled to KLH (Sigma; Villar-Cerviño et al., 2006). Glutamate was detected at the EM level in the appropriate terminals in our laboratory (Villar-Cerviño et al., 2006). Immunostaining with the Sigma antibody was completely abolished after pre-adsorption of the diluted anti-GLU antibody with Glutamate-KLH conjugates (Sigma).

As a control for secondary antibodies, the primary antibodies were omitted from the immunohistochemical procedure. No staining was observed in these sections.

PCNA immunocytochemistry

The lamprey larvae were deeply anaesthetized with benzocaine (Sigma, St. Louis, MO), (n = 3), then fixed in Bouin's fluid and embedded in paraffin wax. Sections (8-10-µm thick) were cut on a rotary microtome. Dewaxed sections were processed by sequential incubation with 1) 10% NGS for 1 hour, 2) mouse monoclonal anti-PCNA antibody (diluted 1:400; PC10 clone, Sigma) overnight, 3) a goat anti-mouse immunoglobulin (diluted 1:50; Dako) for 1 hour and 4) mouse peroxidase-anti-peroxidase complex (PAP, Sigma; diluted 1:500). All antibodies were diluted with TBS containing 0.2% Triton X-100 as detergent and 3% NGS. The immunostaining was developed with 0.6 mg/ml 3-3'-diaminobenzidine (DAB; Sigma) and 0.003% H2O2. PCNA antibody specificity was tested by the supplier and it was shown that in the lamprey brain it only stains cells in the proliferating ventricular zone (Villar-Cheda et al., 2005, 2006b). Furthermore, no immunostaining was observed in sections processed without the primary antibody.

BrdU labelling

Lamprey larvae (n = 3) were placed in 1% BrdU (Sigma) in freshwater for 6 days following previous studies in the larval brain (Villar-Cheda et al., 2006b), in order to label a substantial proportion of cells in these slow-proliferating vertebrates. They were then anaesthetized with benzocaine and fixed by immersion in Clarke's fixative (75% ethanol, 25% acetic acid), embedded in paraffin and cut (10 µ m thickness) on a rotary microtome. Sections were dewaxed and pretreated sequentially with 0.1 M glycine (30 minutes), 0.5% sodium borohydride in TBS (15 minutes), 1% Triton and 1% DMSO in TBS (20

minutes), and 2N HCl (60 minutes), and rinsed with TBS between steps. Sections were then incubated sequentially with 1) 10% NGS, 2) monoclonal anti-BrdU antibody (diluted 1:500; clone BU 33, Sigma; overnight), 3) biotinylated goat anti-mouse antibody (diluted 1:500; Vector), and 4) avidin-biotin complex (Vectastain Elite ABC reagent, Vector). The sections were developed with 0.6 mg/ml DAB (Sigma) and 0.003% H_2O_2, as above. BrdU antibody specificity was tested by the supplier. No immunostaining was observed in sections processed without prior HCl hydrolysis or without primary antibody.

Additional Material

Series of larval lamprey heads stained with haematoxylin-eosin (H-E) were at our disposal.

Photography and measurements

Photomicrographs were taken with a spectral confocal laser microscope (Leica TCS-SP2), or with an Olympus photomicroscope (AX-70; Provis) equipped with a colour digital camera (Olympus DP70). The TB cells were measured on confocal micrographs with LCS Leica confocal software, or on light photomicrographs, with ImageJ software (NIH); the cells of 3 buds of 3 larvae were counted. Photomicrographs were adjusted for brightness and contrast with Adobe Photoshop 7.0 software. Some photomicrographs were converted to grey scale.

Results

Present results were mainly obtained in larval lampreys because they are easily accessible and the experimental procedure is simpler than in adult samples. Taste bud cellular organization in adult lampreys does not differ from that observed in larvae (Baatrup, 1983b, 1985b; present results). To test if neurochemical composition of larval and adult TBs is also the same, 5-HT and aT immunohistochemistries were used in 3 adults. Since no appreciable differences were observed with respect to the larval lampreys, the present description is centred on larval TBs. A schematic drawing showing the location of TBs in relation to the gill support elements in the larval lamprey is provided (Fig. 2).

Taste buds in larval lamprey mostly consist of non-proliferating cells

The first six pairs of gills of larval sea lampreys possessed between 7 and 10 TBs, which were situated in the posterior medial gill seam with their taste pores directed to the gill surface environment (the water flow), in accordance with the results of Mallat (1979). As observed with H-E staining or with Nomarski's differential interference contrast (DIC), these TBs comprised of tall columnar cells, with nuclei in the lower half, and which converged towards the apical taste pore (Fig. 3). The TBs were surrounded by a stratified branchial epithelium, the border of which contained small cells that partially covered the

Antisera used						
Antisera	**Host**	**Source and code**	**Dilution**	**Fixative**	**Lot**	**Inmunogen**
TH	Rabbit	Chemicon code AB152	1:100	P4%	0509011790	Denatured TH from cat pheochromocytoma
5-HT	Rabbit	Incstar code 200800	1:2,500	P4%	050017	Serotonin-formaldehyde-BSA conjugate
CR	Rabbit	SWant code 7699/4	1:500	P4%	18299	Recombinant human calretinin
NPY	Rabbit	Sigma code N 9528	1:600	P4%	057K4869	Synthetic neuropeptide Y (porcine) conjugated to KLH
GABA	Mouse	Sigma clone GB-69	1:1,200	G5%	075K4795	Purified GABA conjugated to BSA
Alpha-tubulin	Mouse	Sigma code 6-11B-1	1:500	P4%	054K4835	Aceylated tubulin from the outer arm of *Strongylocentrotus purpuratus*
GLU	Rabbit	Immunosolution code IG1007	1:4,500	G5%	*	Glutamate conjugated to porcine thyroglobin using glutaraldehyde
CGRP	Mouse	Sigma code CD8	1:300	P4%	086K1608	Synthetic fragment of C-terminal rat α-CGRP (VPTNVGSKAF) glutaraldehyde conjugated to BSA
PCNA	Mouse	Sigma code P-8825 clone PC10	1: 400	Bouin's fluid	034K4789	Proliferating cell nuclear antigen-Protein A fusion protein
BrdU	Mouse	Sigma code B2531 clone BU 33	1:500	Clarke's fixative	074H4814	BromodeoxyUridine conjugated to KLH

Table 1. Antibodies used in the immunohistochemical experiments in this study. P4%: the antibody requires paraformaldehyde fixation; G5%: the antibody requires glutaraldehyde fixation. For other abbreviations, see list. *No information about the lot was provided by the supplier.

bud. As indicated by both PCNA immunohistochemistry and BrdU labelling, the tall cells (bi-ciliated and supporting cells) of the taste bud were not proliferating, i.e. they were both PCNA- and BrdU-negative (Fig. 3). This was also observed in TBs of the smallest larvae studied (15-25 mm in length). In contrast, flattened and spherical epithelial cells surrounding the bud showed both incorporation of BrdU (Fig. 3D and E) and PCNA immunostaining (Fig. 3A, B and C). These labelled cells do not pertain to the taste bud and were located in the basal cell layer of the epithelium. Moreover, only occasional cells with BrdU- (Fig. 3D and E) or PCNA-ir (Fig. 3A, B and C) nuclei were observed in a few sections of TBs, which were located above the basal lamina (Fig. 3C and D) or just below the basal lamina (Fig. 3B and E). In addition, few PCNA-ir cells were observed among the tall cells in the basal region of the bud (Fig. 3A).

Bi-ciliated TBs cells of lamprey are revealed by α-tubulin immunocytochemistry

In the TBs of sea lamprey larva, α-tubulin immunoreactivity (αTir) enabled recognition of both the bi-ciliated TB cells and their afferent innervation by afferent (gustatory) fibres (Fig. 4A, 5A', B' and C'). In the TBs, numerous tall flask-shaped αTir cells were arranged in ovoid clusters (Fig. 4A, 5A', B' and C'), and exhibited a polarized structure with a narrow apical region. The immunofluorescence against α-tubulin indicated the presence of abundant microtubules in these cells, this immunoreactivity was much more intense in the apical region than in the basal region of the cell (Fig. 4A, 5A', B' and C'). In addition, the apical cell surface showed two strongly αT-immunostained cilia that protruded in the taste pore (Fig. 4A and 5B/B'). In ultrastructural studies of lamprey TBs, the microtubule- and cilia-bearing cells (light cells) have been identified as receptor cells (Baatrup, 1983a,b, 1985b). The long axis of the bi-ciliated cells varied between 30 and 34 μm (mean = 32.0 μm). On the other hand, the study of the TBs of adult lampreys (Fig. 6A') showed that the cellular organization of the bi-ciliated cells was similar to that observed in larval lampreys.

Fairly abundant immunolabelled αTir afferent gustatory fibres coursed in the basal lamina toward the TBs and appeared to contact the basal portion of the TB α Tir bi-ciliated cells through dilatations of the nerve fibres (Figs. 4A, 5A', B', C' and 6A'). The majority of these coarse fibres did not appear to enter into the TB (Figs. 4A, 5B''), although occasionally thin branches of a αTir fibre were observed among TB cells in marginal regions (Figs 4A).

Serotonin is co-localized with αT in bi-ciliated cells of lamprey TBs

Numerous flask-shaped cells of larval sea lamprey TBs were strongly 5-HTir (Fig. 4B, 5A'', B'', C''). The presence of 5-HTir

cells in the TBs was observed in all the larvae studied. Confocal microscopy of double labelled TBs revealed that 5-HT was co-located in all the cells of each taste bud that showed αT immunoreactivity (Fig. 5A-C). As in the larval sea lampreys, the bi-ciliated cells of adult TBs showed both α T and 5-HT immunoreactivity (Fig. 6A/A'/A''), but the intensity of the 5-HT immunoreactivity was clearly less than that observed in the larval TBs, principally in the apical portion of the cells.

In contrast to the observation made with αT immunocytochemistry, no 5-HTir fibres were observed contacting the basal lamina coursing to or from the TBs (Fig. 5A-C), although occasional thin 5-HTir fibres coursed near the base of TBs (Fig. 5B" and C"). On the other hand, numerous 5-HTir fibres coursed in branches of cranial nerves along the branchial arches (Fig. 5C"). This strongly suggests that all α Tir afferent gustatory fibres were 5-HT immunonegative.

Calretinin is co-localized with aTir in cells and fibres of TBs in larval lampreys

Calretinin immunofluorescence revealed the presence of CRir cells in TBs of sea lamprey larvae (Fig. 4C). Double immunofluorescence showed CR in almost all the aTir bi-ciliated cells (Fig. 6B/B'/B''). Calretinin immunoreactivity was more intense in the basal region of the cells than aT immunoreactivity (Fig. 6B/B'/B''). This made the observation of colocalization in TB cells difficult, but colocalization was clear in the apical region of taste cells. Since CR and tubulin were co-localized in TB cells, CR probably also co-localizes with 5-HT in these cells, although the combination of 5-HT and CR antibodies used here did not allowed to asses this by double immunofluorescence experiments. In addition, some of the αTir fibres which surround the TBs appeared to be also CRir, and some of these CRir fibres contacted the basal surface of TB cells (Fig. 6B/B'/B'').

CGRP immunoreactivity is co-localized with 5-HTir cells in lamprey TBs

Moderate CGRP immunoreactivity was observed in elongated cells of the sea lamprey TBs. Double immunofluorescence showed clearly that all 5-HTir cells of TBs were also CGRPir (Fig. 7A/A'/A''), and thus bi-ciliated lamprey taste cells appear to contain CGRP. Some serotonergic fibres coursing in the cranial nerve branches innervating the branchial arches also showed CGRP immunoreactivity (not showed), but no double labelled CGRPir/5-HTir fibres reached the basal surface of TB cells. As a positive control (not showed), the presence of 5-HTir structures that were also immunoreactive to CGRP was observed in spinal cord sections of the same lamprey larvae, in agreement with a previous report (Brodin et al., 1988) showing that 5-HTir fibres of the dorsal column system were also CGRPir.

Afferent fibres immunoreactive to CGRP were not observed in relation to lamprey TBs in all the larval samples studied.

Lamprey TBs do not show TH-immunoreactive (catecholaminergic) structures

No TH-immunoreactive (THir) cells were observed in TBs of larval lampreys (Fig. 8A/A'). In addition, the afferent aTir gustatory fibres which reach the TBs are also not THir (Fig. 8A/A'). The possibility that this was a false negative result was ruled out because the brain of the same head sections of larval lamprey showed the distribution of THir structures reported by other studies (Pierre et al., 1997). For instance, conspicuous THir cells can be observed in a photomicrograph of the diencephalic paratubercular nucleus (Fig. 8B).

Lamprey TBs and afferent gustatory fibres do not show GABA or GLU immunoreactivities

No GABA or GLU immunoreactive (GABAir, GLUir) cells or fibres were observed in lamprey TBs (not showed). Afferent gustatory fibres that surround the TBs were neither GABAir or GLUir. The possibility that this was a false negative result was ruled out because the brain and/or retina of the same larval head sections showed the characteristic distribution of GABAir and GLUir structures observed in previous studies (Meléndez-Ferro et al., 2002; Villar-Cerviño et al., 2006). For instance, GABAir cells are shown in the pretectal nucleus (Fig. 8C) and GLUir bipolar cells in the retina (Fig. 8D) of the same larvae.

Lamprey TB cells and afferent gustatory fibres do not exhibit NPY immunoreactivity

No NPY-immunoreactive (NPYir) cells were observed in TBs of sea lampreys (not showed). The afferent gustatory plexus surrounding each TB was also not NPYir. Positive controls showed that this is not a false negative result, because the brain of the same larvae showed the distribution of NPYir cells and fibres reported previously in the lamprey brain (Chiba, 1999). For instance, NPYir cells and fibres were observed in the characteristic population described by this author in the isthmic region of the rhombencephalon (Fig. 8E).

Discussion

The present results report for the first time the non-proliferating status and the presence of αT, 5-HT, CR and CGRP immunoreactivity in taste bi-ciliated cells and the innervation pattern in TBs of a representative of the earliest vertebrates, the sea lamprey, as determined by use of immunocytochemical markers. Baatrup (1983a) reported the presence of light (bi-ciliated receptor) and dark (sustentacular) cells in lamprey TBs, and observed occasional Merkel cells, although the latter cells were not described in a more recent study by Baatrup (1985b). Our results suggest the presence of 3

cell types in the lamprey TBs: bi-ciliated cells (which are mostly immunoreactive to αT, 5-HT, CR and CGRP), undifferentiated basal cells (labelled by PCNA and BrdU immunocytochemistry) and sustentacular cells (they were not labelled by any of the antisera used in the present study). The present study with different cell markers does not allow characterization of the Merkel cells of sea lamprey TBs.

Proliferating cells were observed, in both PCNA and BrdU experiments, above and below the basal lamina of the lamprey TBs. The cells above the basal lamina may be tentatively considered as progenitors of the tall TB cells, as described in other vertebrates (see Finger, 2006). Few PCNA-ir cells were observed among the tall TB cells, but cells in this location were not observed with BrdU, which labelled mitotic cells passing through the S-phase. However, PCNA is considered a marker of cycling cells or of cells that have recently left the cell cycle (Kurki et al., 1986; Bravo and McDonald-Bravo, 1987; Yu et al., 1992). Such differences between PCNA and BrdU may account for the differences in the labelling pattern.

It has been debated for a long time whether dark, intermediate and light cells of mammalian TBs are different types of cells or whether they represent different states of a single cell type (see Delay et al., 1986; Pumplin et al., 1997). Transmission electron microscopic studies in lamprey have revealed two clearly different cell morphologies in TBs: bi-ciliated light cells, which have been considered as the taste receptors, and microvillar dark cells, considered as sustentacular (Baatrup, 1983a, 1985b). Our results with two different proliferation markers revealed that all these cells were not proliferating cells.

The present results with neurochemical markers reveal the presence of a large number of 5-HTir bi-ciliated cells in TBs of the sea lamprey, as indicated by colocalization with tubulin immunoreactivity. Interestingly, although the presence of serotonergic cells in the TBs has been observed from teleost to mammals (see Finger, 2006), only a few cells were 5-HTir in each TB in these vertebrates (see Roper, 1989; Yoshie et al., 1990; Reutter and Witt, 1993; Yee et al., 2001). This is a striking difference between lampreys and other vertebrates. In lamprey, 5-HTir flask-shaped cells also exhibited CR and CGRP immunoreactivities. Judging from the dense and regular arrangement of these cells in TBs, which is strikingly reminiscent of the light (sensory) cells observed by transmission electron microscopy (Baatrup, 1983a, 1985b), the 5-HTir cells probably represent most of the cells of the TBs. These findings clearly depart from the distribution of 5-HT in TBs reported in other anamniotes, in which 5-HT was only observed in scarce Merkel-like basal cells (Toyoshima and Shimamura, 1987; Delay and Roper, 1988; Delay et al., 1993; Reutter and

Witt, 1993). Whether lamprey TBs also posses 5-HTir Merkel cells could not be assessed. A comparative scheme of the distribution of the 5-HTir cells in vertebrate TBs is shown in Figure 9.

Serotonin released by Merkel-like basal cells is thought to affect perception of taste via modulation of firing of taste receptor cells and gustatory nerve cells (Imendra et al., 2002). Such an indirect role for 5-HT in taste signal transduction appears rather improbable in lamprey owing to the abundance of 5-HTir cells observed in TBs. Like the 5-HTir type III taste cells in rat (Yee et al., 2001), the lamprey serotonergic taste cells span the entire height of the bud, although they are much more numerous than in rat TBs. Since tall serotonergic cells similar to those of lampreys were not observed in TBs of jawed fishes or amphibians (Toyoshima and Shimamura, 1987; Delay and Roper, 1988; Delay et al., 1993; Reutter and Witt, 1993), the morphological resemblance between lamprey 5-HTir cells and mammal 5-HTir type III taste cells may result from convergent evolution. Moreover, TBs of mammals show differentiated taste receptor cells (type II or light cells) that exhibit different neurochemical markers (α-gustducin, neuronal specific enolase) and that predominate in the buds (Yee et al., 2001). In light of the massive presence of 5-HTir cells in lamprey TBs, investigation should be made of whether cells similar to mammalian type II cells occur in lamprey TBs and/or whether these markers are expressed in the lamprey 5-HTir cells.

Lamprey bi-ciliated cells are also CGRPir (present results), and these cells also co-localize 5-HT immunoreactivity. In lamprey, the colocalization of 5-HT and CGRP was previously reported in other cells and fibres in the nervous system (Brodin et al., 1988). The existence of a similar taste cell type immunoreactive to CGRP was not reported in TBs of other vertebrates. On the contrary, the presence of CGRPir fibres afferent to TBs was reported in both amphibians (Kawakami et al., 1998) and mammals (Hisa et al., 1992; Shin et al., 1995; Astbäck et al., 1995; Wakisaka et al., 1996; Huang and Lu, 1996; Astbäck et al., 1997; Yamamoto et al., 1997; Witt and Reutter, 1998; Kusakabe et al., 1998; Huang et al., 2003; Crescimanno et al., 2004; Ogura et al., 2007), but was not observed in lamprey. Apparently, lampreys and jawed vertebrates followed different evolutionary pathways as regards the distribution of CGRP in the taste system. A role of CGRP in modulation of serotonergic neurotransmission was reported in the human trigemino-vascular system, where CGRP receptors are co-localized with 5-HT receptors (Oliber et al., 2002). Release of CGRP depends on activation of cells by calcium influx (Evans et al., 2000). This fact may be related with the presence of CR in lamprey taste bi-ciliated cells (see below).

Double immunofluorescence against acetylated α-tubulin and 5-HT also reveals that

5-HT was always co-located with αT immunoreactivity in the bi-ciliated cells of the sea lamprey TBs, and that these cells are contacted at their base by afferent nerve fibres which are also αTir but 5-HT negative. A very unusual TEM observation in larval lamprey (Baatrup, 1983a, 1985b) was that the afferent nerve fibres do not enter the TB, but have varicosities that directly contact the base of receptor cells through small holes in the basal lamina. Our observations in larvae also indicate that almost all the αTir afferent fibres contact the base of 5-HTir flask-shaped cells without entering among cells of the TB, unlike in mammals and other jawed vertebrates (see Finger, 2006). The presence of afferent fibres coursing among TB cells appears to be occasional. Although numerous 5-HTir fibres were observed in the branchial region, these fibres do not appear to come in direct contact with TBs, which suggests that they are not afferent fibres.

Present observations with immunofluorescence to CR and αT reveal the presence of CR immunoreactivity in almost all αTir bi-ciliated cells. Accordingly, CR is very probably co-localized with 5-HT in cells of the bud (this has not been directly tested because both the 5-HT and CR antibodies were raised in rabbit). This raises the possibility that 5-HTir taste cells use CR as a calcium buffering system. As regards the presence of CR, lamprey TB bi-ciliated cells appear similar to CRir putative taste receptor cells of newt and teleosts (Barlow et al., 1996; Díaz-Regueira et al., 2005; Northcutt, 2005; Germanà et al., 2007). In addition, CRir fibres were observed contacting the basal region of the bi-ciliated TB cells in lamprey (present results). The presence of CRir fibres of the nerve plexus innervating TBs is shared by teleosts (Díaz-Regueira et al., 2005; Germanà et al., 2007) and mammals (Ichikawa et al., 1992).

The presence of catecholamines such as epinephrine and norepinephrine has been reported in afferent fibres of TBs of some vertebrates (amphibians: Zancanaro, 1995; mammals: Herness et al., 2002; Dvoryanchikov et al., 2007). However, no detectable immunoreactivity to tyrosine hydroxylase - the rate-limiting common enzyme of the catecholamine biosynthesis - was observed in cells or fibres of lamprey TBs. This was not due to methodological limitations, because well-stained positive cells and/or fibres were observed in the sectioned brain contained in the same larval sections.

Present results revealed the absence of NPY immunoreactivity in the lamprey TBs or afferent gustatory plexus. The presence of NPY immunoreactivity was observed in a subset of rat cells within the TB (Zhao et al., 2005). Neuropeptide Y-ir afferent fibres were observed in some vertebrate species investigated, from amphibians to mammals: salamander (Kawakami et al., 1998), dog (Yamamoto et al., 1997), pig (Montavon et al., 1991) or human (Kusakabe, 1998). The

presence of NPYir structures in taste bud cells or afferent fibres appears to be a derived condition in vertebrates.

GABA and GLU immunoreactivities were observed in *Necturus maculosus* fibres that innervate TBs (Jain and Roper, 1991), and GABAir cells were also observed in TBs of rat (Obata et al., 1997) and channel catfish (Eram and Michel, 2005). Our results do not reveal any of these immunoreactivities in lamprey TBs. The absence of GABA and GLU immunoreactivity in sea lamprey TBs suggests that GABA and GLU do not play a role in the transmission of the gustatory stimulus in lampreys (present results).

Final considerations: A hypothesis on the early evolution of TBs

Since different vertebrates present different types of 5-HT-containing cells in their TBs, it is not surprising that the roles attributed to 5-HT in TBs are diverse. 5-HTir cells were proposed to act as interneurons between the taste receptor cells and the afferent fibres (Delay et al., 1993) or as neuromodulatory cells (Ewald and Roper, 1994; Kim and Roper, 1995; Nagai et al., 1996; Delay et al., 1997). In studies showing an increase in the 5-HT expression in the Merkel-like basal cells after denervation of the afferent gustatory fibres, a trophic or developmental role was also attributed to 5-HT (Hamasaki et al., 1998; Toyoshima et al. 1999). Recent behavioural tests of wild-type versus null mice lacking the 5-HT3A subunit showed no significant differences in their responses to stimuli representative of each of the different types of taste qualities (Finger et al., 2005). In mammal TBs, recent studies indicate that ATP is a major neurotransmitter, as purinergic receptors are expressed in afferent taste fibres (Finger et al., 2005; Huang et al., 2007), but whether this also applies to other vertebrates is not known. These data strongly argue against the possibility that primary taste information is encoded in the form of 5-HT quanta and suggest another role for serotoninergic signalling in TB physiology in mammals (Romanov et al., 2007).

The present results suggest that 5-HT may be a major NT in lamprey TBs, and also suggest the possibility of early evolution of TBs in vertebrates. The first true TBs in vertebrates, the lamprey TBs, would contain a single type of receptor cell in accordance with their neurochemistry. This putative “taste receptor” was serotonergic and contacted primary afferent fibres, as seen in TBs of extant lampreys. However, lamprey TBs appear to be responsive to a variety of classical taste substances (sodium chloride, quinine, some amino acids) and show thresholds similar to those of catfish TBs (Baatrup, 1985a). The presence in lampreys of elongate receptor cells expressing different taste receptors, as reported in teleosts (Ishimaru et al., 2005), should be tested in future studies. Although amphioxus has secondary sensory cells bearing a cilium

surrounded by a collar of microvilli and contacting nerve fibres, located either singly or in groups in buccal cirri (see Lacalli, 1999), whether they are mechanosensory or chemosensory is at present unknown. Moreover, no immunoreactivity to 5-HT was observed in amphioxus oral structures (Holland and Holland, 1993). Sensory organs resembling TBs, the Schreiner organs, are found in the epidermis of hagfishes, but they are not homologous to TBs (Braun, 1998). This sensory modality of hagfishes has no direct homologue in vertebrates and appears to be a specialization of hagfishes (Braun, 1998). The presence of taste organs in cartilaginous fishes also vary: true taste buds have been described in *Scyliorhinus* (Reutter 1994; Whitear and Moate 1994a), but are lacking in *Raja* (Whitear and Moate 1994b). Previous and present results suggest that the basic TB characteristics evolved in lampreys (the earliest jawless vertebrates).

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.

Alcock R. 1899. The peripheral distribution of the craneal nerves of ammocetes. J Anat Physiol 33: 133-135.

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.

Astbäck J, Arvidson K, Johansson O. 1995. Neurochemical markers of human fungiform papillae and taste buds. Regul Pept 59: 389-398.

Astbäck J, Arvidson K, Johansson O. 1997. An immunohistochemical screening of neurochemical markers in fungiform papillae and taste buds of the anterior rat tongue. Arch Oral Biol 42: 137-147.

Baatrup E. 1983a. Ciliated receptors in the pharyngeal terminal buds of larval *Lampetra planeri* (Bloch) (Cyclostomata). Acta Zool 64: 67-75.

Baatrup E. 1983b. Terminal buds in the branchial tube of the brook lamprey (*Lampetra planeri* (Bloch) – Putative respiratory monitors. Acta Zool 64: 139-147.

Baatrup E. 1985a. Physiological studies on the pharyngeal terminal buds in the larval brook lamprey, *Lampetra planeri* (Bloch). Chem Senses 10: 549-558.

Baatrup E. 1985b. Sensory terminal buds of larval and adult lampreys. Fortschritte der Zoologie

Barlow LA, Chien CB, Northcutt RG. 1996. Embryonic taste buds develop in the absence of innervation. Development 122: 1103-1111.

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.

Bravo R, McDonald-Bravo H. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. J Cell Biol 105: 1549-1554.

Braun CB. 1998. Schreiner organs: a new craniate chemosensory modality in hagfishes. J Comp Neurol 392: 135-63.

Brodin L, Buchanan JT, Hökfelt T, Grillner S, Rehfeld JF, Frey P, Verhofstad AAJ, Dockray GJ, Walsh JH. 1988. Immunohistochemical studies of cholecystokininlike peptides and their relation to 5-HT, CGRP, and bombesin immunoreactivities in the brainstem and spinal cord of lampreys. J Comp Neurol 211: 1-18.

Chiba A. 1999. Immunohistochemical distribution of neuropeptide Y-related substance in the brain and hypophysis of the arctic lamprey, *Lethenteron japonica*. Brain Behav Evol 53: 102-109.

Chitnis AB, Kuwada JY. 1990. Axonogenesis in the brain of zebrafish embryos. J. Neurosci 10: 1892-1905.

Crescimanno C, Merigo F, Bernardi P, Osculati F, Sbarbati A. 2004. Neurochemistry of the gustatory subgemmal plexus. Chem Senses 29: 537-546.

Delay RJ, Roper SD. 1988. Ultrastructure of taste cells and synapses in the mudpuppy *Necturus maculosus*. J Comp Neurol 277: 268-280.

Delay RJ, Kinnamon JC, Roper SD. 1986. Ultrastructure of mouse vallate taste buds: II. Cell types and cell lineage. J Comp Neurol 253:242-252.

Delay RJ, Taylor R, Roper SD. 1993. Merkel-like basal cells in *Necturus* taste buds contain serotonin. J Comp Neurol 335: 606-613.

Delay RJ, Kinnamon SC, Roper SD. 1997. Serotonin modulates voltage-dependent calcium current in *Necturus* taste cells. J Neurophysiol 77: 2515-2524.

Díaz-Regueira SM, Lamas I, Anadón R. 2005. Calretinin immunoreactivity in taste buds and afferent fibres of the grey mullet *Chelon labrosus*. Brain Res 1031: 297-301.

Dvoryanchikov G, Tomchik SM, Chaudhari N. 2007. Biogenic amine synthesis and uptake in rodent taste buds. J Comp Neurol 505: 302-313.

Eram M, Michel WC. 2005. Morphological and biochemical heterogeneity in facial and vagal nerve innervated taste buds of the channel catfish, *Ictalurus punctatus*. J Comp Neurol 486: 132-144.

Evans BN, Rosenblatt MI, Mnayer LO, Oliver KR, Dickerson IM. 2000. CGRP-RCP,

a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. J Biol Chem 275: 31438-31443.

Ewald DA, Roper SD. 1994. Bidirectional synaptic transmission in *Necturus* taste buds. J Neurosci 14: 3791-3804.

Fahrenholz C. 1936. Die sensiblen Einrichtungen der Neunaugenhaut. Z mikrosk-anat Forsch 40: 323-380.

Finger TE. 2006. Evolution of taste. In Kaas J, Bullock TH, editors. The Evolution of Nervous Systems, vol 2. Oxford: Academic Press. p. 424-438.

Finger TE, Danilova V, Barrows J, Bartel DL, Vigers AJ, Stone L, Hellekant G, Kinnamon SC. 2005. ATP signaling is crucial for communication from taste buds to gustatory nerves. Science 310: 1495-1499.

Fujimoto S, Ueda H, Kagawa H. 1987. Immunocytochemistry on the localization of 5-hydroxytryptamine in monkey and rabbit taste buds. Acta Anat 128: 80-83.

Germanà A, Paruta S, Germanà GP, Ochoa-Erena FJ, Montalbano G, Cobo J, Vega JA. 2007. Differential distribution of S100 protein and calretinin in mechanosensory and chemosensory cells of adult zebrafish (*Danio rerio*). Brain Res 1162: 48-55.

Hamasaki K, Seta Y, Yamada K, Toyoshima K. 1998. Possible role of serotonin in Merkel-like basal cells of the taste buds of the frog, *Rana nigromaculata*. J Anat 193: 599-610.

Hardisty MW, Potter IC. 1971. The general biology of adult lampreys. In Hardisty MW, Potter IC, editors. The Biology of Lampreys, vol 1. London: Academic Press. p. 127-206.

Herness S, Zhao FL, Kaya N, Lu SG, Shen T, Sun XD. 2002. Adrenergic signalling between rat taste receptor cells. J Physiol 543: 601-614.

Hisa Y, Uno T, Tadaki N, Murakami Y, Okamura H, Ibata Y. 1992. Distribution of calcitonin gene-related peptide nerve fibers in the canine larynx. Eur Arch Otorhinolaryngol 249: 52-55.

Holland ND, Holland LZ. 1993. Serotonin-containing cells in the nervous system and other tissues during ontogeny of a lancelet, *Branchiostoma floridae*. Acta Zool 74: 195-204.

Huang YJ, Lu KS. 1996. Immunohistochemical studies on protein gene product 9.5, serotonin and neuropeptides in vallate taste buds and related nerves of the guinea pig. Arch Histol Cytol 59: 433-441.

Huang YJ, Wu YH, Lu KS. 2003. Immunoelectron microscopic studies on protein gene product 9.5 and calcitonin gene-related peptide in vallate taste cells and related nerves in the guinea pig. Microsc Res Tech 62: 383-395.

Huang YJ, Maruyama Y, Lu KS, Pereira E, Plonsky I, Baur JE, Wu D, Roper SD. 2005a. Mouse taste buds use serotonin as a neurotransmitter. J Neurosci 26: 843-847.

Huang YJ, Maruyama Y, Lu KS, Pereira E, Plonsky I, Baur JE, Wu D, Roper SD. 2005b. Using biosensors to detect the release of serotonin from taste buds during taste stimulation. Arch Ital Biol 143: 87-96.

Huang YJ, Maruyama Y, Lu KS, Pereira E, Roper SD. 2005c. Mouse taste buds release serotonin in response to taste stimuli. Chem Senses 30: 39-40.

Huang YJ, Maruyama Y, Dvoryanchikov G, Pereira E, Chaudhari N, Roper SD. 2007. The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. Proc Natl Acad Sci USA 104: 6436-6441.

Ichikawa H, Jacobowitz DM, Sugimoto T. 1992. Calretinin-immunoreactivity in the oro-facial and pharyngeal regions of the rat. Neurosci Lett 146:155-158.

Imendra KG, Miyamoto T, Okada Y, Toda K. 2002. Serotonin differentially modulates the electrical properties of different subsets of taste receptor cells in bullfrog. Eur J Neurosci 16: 629-640.

Ishimaru Y, Okada S, Naito H, Nagai T, Yasuoka A, Matsumoto I, Abe K. 2005. Two families of candidate taste receptors in fishes. Mech Dev 122: 1310-1321.

Jain S, Roper SD. 1991. Immunocytochemistry of gamma-aminobutyric acid, glutamate, serotonin, and histamine in *Necturus* taste buds. J Comp Neurol 307: 675-682.

Johnson JB. 1905. The cranial nerve components of *Petromyzon*. Morphol Jahrb 34: 149-205.

Kawakami T, Kusakabe T, Matsuda H, Hiruma H, Gono Y, Kurihara K, Takenaka T. 1998. Distribution of neuropeptide-containing nerve fibers in the salamander taste organs. Histol Histopathol 13: 115-119.

Kim DJ, Roper SD. 1995. Localization of serotonin in taste buds: a comparative study in four vertebrates. J Comp Neurol 353: 364-370.

Kuratani S, Ueki T, Aizawa S, Hirano S. 1997. Peripheral development of cranial nerves in a cyclostome, *Lampetra japonica*: morphological distribution of nerve branches and the vertebrate body plan. J Comp Neurol 384: 483-500.

Kurki P, Vanderlaan N, Dolbeare F, Gray J, Tan EM. 1986. Expression of proliferating cell nuclear antigen (PCNA)/cyclin during the cell cycle. Exp Cell Res 166: 209-219.

Kusakabe T, Matsuda H, Gono Y, Furukawa M, Hiruma H, Kawakami T, Tsukuda M, Takenaka T. 1998.

Immunohistochemical localisation of regulatory neuropeptides in human circumvallate papillae. J Anat 192: 557-564.

Lacalli TC. 1999. The oral nerve plexus in amphioxus larvae: function, cell types and phylogenetic significance. Proc R Soc Lond B 266: 1461-1470.

Mallat J. 1979. Surface morphology and functions of pharyngeal structures in the larval lamprey *Petromyzon marinus*. J Morphol 162: 249-274.

Meléndez-Ferro M, Pérez-Costas E, Rodríguez-Muñoz R, Gómez-López MP, Anadón R, Rodicio MC. 2001. GABA immunoreactivity in the olfactory bulbs of the adult sea lamprey *Petromyzon marinus* L. Brain Res 893: 253-260.

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.

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B, Rodríguez-Muñoz R, Anadón R, Rodicio MC. 2003. Ontogeny of gamma-aminobutyric acid-immunoreactive neurons in the rhombencephalon and spinal cord of the sea lamprey. J Comp Neurol. 464:17-35.

Montavon P, Lindstrand K, Luts A, Sundler F. 1991. Peptide-containing nerve fibers in the fungiform papillae of pigs and rats. Regul Pept 32:141-150.

Nagai T, Kim DJ, Delay RJ, Roper SD. 1996. Neuromodulation of transduction and signal processing in the end organs of taste. Chem Senses 21: 353-365.

Northcutt RG. 2004. Taste buds: development and evolution. Brain Behav Evol 64: 198-206.

Northcutt RG. 2005. Taste bud development in the channel catfish. J Comp Neurol. 482: 1-16.

Obata H, Shimada K, Sakai N, Saito N. 1997. GABAergic neurotransmission in rat taste buds: immunocytochemical study for GABA and GABA transporter subtypes. Mol Brain Res 49: 29-36.

Ogura T, Margolskee RF, Tallini YN, Shui B, Kotlikoff MI, Lin W. 2007. Immuno-localization of vesicular acetylcholine transporter in mouse taste cells and adjacent nerve fibers: indication of acetylcholine release. Cell Tissue Res 330: 17-28.

Oliber KR, Wainwright A, Edvinsson L, Pichard JD, Hill RG. 2002. Immunohistochemical localization of calcitonin receptor-like receptor and receptor activity-modifying proteins in the human cerebral vasculature. J Cereb Blood Flow Metab 22:620-629.

Pierre J, Mahouche M, Suderevskaya EI, Repérant J, Ward R. 1997. Immunocytochemical localization of dopamine and its synthetic enzymes in the central nervous system of the lamprey *Lampetra fluviatilis*. J Comp Neurol 380: 119-135.

Piperno G, Fuller MT. 1985. Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. J Cell Biol 10: 2085-2094.

Pumplin DW, Yu C, Smith DV. 1997. Light and dark cells of rat vallate taste buds are morphologically distinct cell types. J Comp Neurol 378: 389-410.

Retzius G. 1893. Über Geschmacksknospen bei *Petromyzon*. Biol Untersuchungen V: 69-70.

Reutter, K. 1994. Ultrastructure of taste buds in the spotted dogfish *Scyliorhinus caniculus* (Selachii). In Kurihara K, Suzuki N, Ogawa H (eds.) Olfaction and taste, vol. 11. Tokyo, Springer. p 754.

Reutter K, Witt M. 1993. Morphology of vertebrate taste organs and their nerve supply. In Simon SA, Roper SD (eds.) Mechanisms of taste transduction. Boca Raton, CRC Press, pp 29-82.

Romanov RA, Rogachevskaja OA, Bystrova MF, Jiang P, Margolskee RF, Stanislav S Kolesnikov. 2007. Afferent neurotransmission mediated by hemichannels in mammalian taste cells. EMBO J 26: 657-667.

Roper SD. 1989. The cell biology of vertebrate taste receptors. Annu Rev Neurosci 12: 329-353.

Roper SD. 2007. Signal transduction and information processing in mammalian taste buds. Pflugers Arch 454:759-776.

Shin T, Nahm I, Maeyama T, Miyazaki J, Matsuo H, Yu Y. 1995. Morphologic study of the laryngeal taste buds in the cat. Laryngoscope 105: 1315-1321.

Szabat E, Salo A, Virtanen I, Uusitalo H, Soinila S. 1994. Production and characterization of a monoclonal antibody against human calcitonin gene-related peptide (CGRP) and its immunohistochemical application to salivary glands. Histochem J 26:317-326.

Tobet SA, Chickering TW, Sower SA. 1996. Relationship of gonadotropin-releasing hormone (GnRH) neurons to the olfactory system in developing lamprey (*Petromyzon marinus*). J Comp Neurol 376: 97-111.

Toyoshima K, Shimamura A. 1987. Monoamine-containing basal cells in the taste buds of the newt *Triturus pyrrhogaster*. Arch Oral Biol 32: 619-621.

Toyoshima K, Seta Y, Toyono T, Takeda S. 1999. Merkel cells are responsible for

the initiation of taste organ morphogenesis in the frog. J Comp Neurol 406: 129-140.

Vidal-Pizarro I, Swain GP, Selzer ME. 2004. Cell proliferation in the lamprey central nervous system. J Comp Neurol 469: 298-310.

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.

Villar-Cerviño V, Barreiro-Iglesias A, Anadón R, Rodicio MC. 2008. Distribution of glycine immunoreactivity in the brain of adult sea lamprey. (*Petromyzon marinus*). Comparison with gamma-aminobutyric acid. J Comp Neurol. 2008 507: 1441-1463.

Villar-Cheda B, Abalo XM, Anadón R, Rodicio MC. 2005. The tegmental proliferation region in the sea lamprey. Brain Res Bull 66: 431-435.

Villar-Cheda B, Abalo XM, Anadón R, Rodicio MC. 2006a. Calretinin and calbindin immunoreactivity in the retina of adult and larval sea lamprey. Brain Res 1068: 118-130.

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

Wakisaka S, Miyawaki Y, Youn SH, Kato J, Kurisu K. 1996. Protein gene-product 9.5 in developing mouse circumvallate papilla: comparison with neuron-specific enolase and calcitonin gene-related peptide. Anat Embryol 194: 365-372.

Whitear M, Moate RM. 1994a. Microanatomy of taste buds in the dogfish, *Scyliorhinus canicula*. J Submicr Cytol Pathol 26: 357-367.

Whitear M, Moate RM. 1994b. Chemosensory cells in the oral epithelium of *Raja clavata* (Chondrichthyes). J Zool 232: 295-312.

Wilson SW, Ross LS, Parrett T, Easter SS. 1990. The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio*. Development 108: 121-145.

Witt M, Reutter K. 1998. Innervation of developing human taste buds. An immunohistochemical study. Histochem Cell Biol 109: 281-291.

Yamamoto Y, Atoji Y, Suzuki Y. 1997. Innervation of taste buds in the canine larynx as revealed by

immunohistochemistry for the various neurochemical markers. Tissue Cell 29: 339-346.

Yee CL, Yang R, Bottger B, Finger TE, Kinnamon JC. 2001. "Type III" cells of rat taste buds: immunohistochemical and ultrastructural studies of neuron-specific enolase, protein gene product 9.5, and serotonin. J Comp Neurol 440: 97-108.

Yoshie S, Wakasugi C, Teraki Y, Fujita T. 1990. Fine structure of the taste bud in guinea pigs. I. Cell characterization and innervation patterns. Arch Histol Cytol 53: 103-119.

Yu CC, Woods AL, Levison DA. 1992. The assessment of cellular proliferation by immunohistochemistry: a review of currently available methods and their applications. Histochem J 24: 121-131.

Zaccone G, Fasulo S, Ainis L. 1994. Distribution patterns of the paraneuronal endocrine cells in the skin, gills and the airways of fishes as determined by immunohistochemical and histological methods. Histochem J 26: 609-629.

Zancanaro C, Sbarbati A, Bolner A, Accordini C, Piemonte G, Osculati F. 1995. Biogenic amines in the taste organ. Chem Senses 20: 329-335.

Zhao FL, Shen T, Kaya N, Lu SG, Cao Y, Herness S. 2005. Expression, physiological action, and coexpression patterns of neuropeptide Y in rat taste-bud cells. Proc Natl Acad Sci USA 102:11100-11105.

Abbreviations

5-HT	Serotonin
αT	Acetylated alpha-tubulin
b1-b5	Branchial arches 1 to 5
BrdU	Bromodeoxyuridine
CGRP	Calcitonin gene-related peptide
CR	Calretinin
GABA	γ-aminobutiric acid

GLU	Glutamate
IX	Glossopharingeal nerve
NPY	Neuropeptide Y
PCNA	Proliferating cell nuclear antigen
TB	Taste bud
TH	Tyrosine hydroxylase
X	Vagal nerve
VII	Facial nerve

FIGURES
CHAPTER 1.2

Figure 1. Western blotting of lamprey protein extracts immunostained with antisera to calretinin (used as positive control), glutamate and CGRP used here. Note the protein band stained in the calretinin lane and the absence of labelling in the glutamate and CGRP lanes.

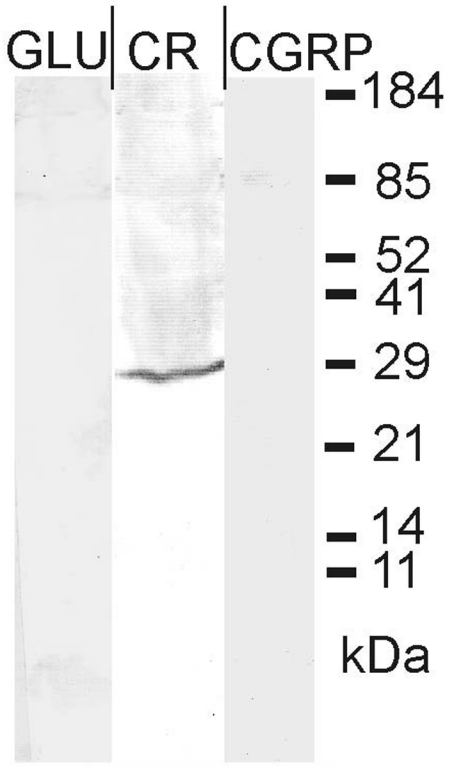


FIGURE 1

Figure 2. A: Schematic drawing showing the location of taste buds in relation to the gill support elements and the afferent innervation (at the left). The upper part of the drawing represents a transversal section at the level of the caudal rhombencephalon while the lower part represents an oblique more caudal section of the branchial region in which rostral is at the top [modified from Baatrup (1985a)].

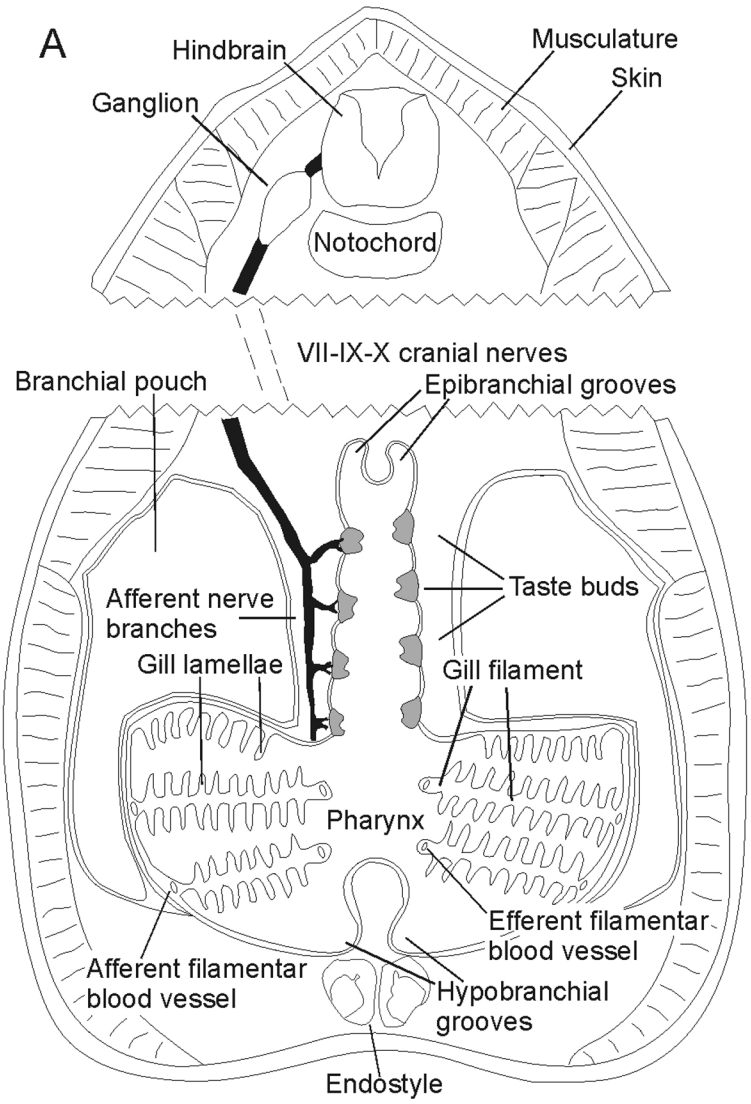

A
Hindbrain
Musculature
Skin
Ganglion
Notochord
VII-IX-X cranial nerves
Branchial pouch
Epibranchial grooves
Taste buds
Afferent nerve branches
Gill lamellae
Gill filament
Pharynx
Efferent filamentar blood vessel
Afferent filamentar blood vessel
Hypobranchial grooves
Endostyle

FIGURE 2

Figure 3. Photomicrographs of transverse sections of sea lamprey larvae showing proliferating cells of different TBs. **A:** Photomicrograph of a larva showing the presence of two PCNA immunoreactive spherical cells in the ventral part of a TB (arrowheads) intermingled with tall TB cells. Note the extensive presence of proliferating cells in the epithelia surrounding the bud. **B-C:** Photomicrographs of two larvae showing the presence of PCNA immunoreactive cells (black arrows) just below the basal lamina in B and above the basal lamina in C. **D-E:** Photomicrographs of two larvae labelled with BrdU. Note the presence of solitary proliferating cells (black arrows) just above the basal lamina in D and below the basal lamina in E. For abbreviations see list. Scale bars: 25 μm in A-E.

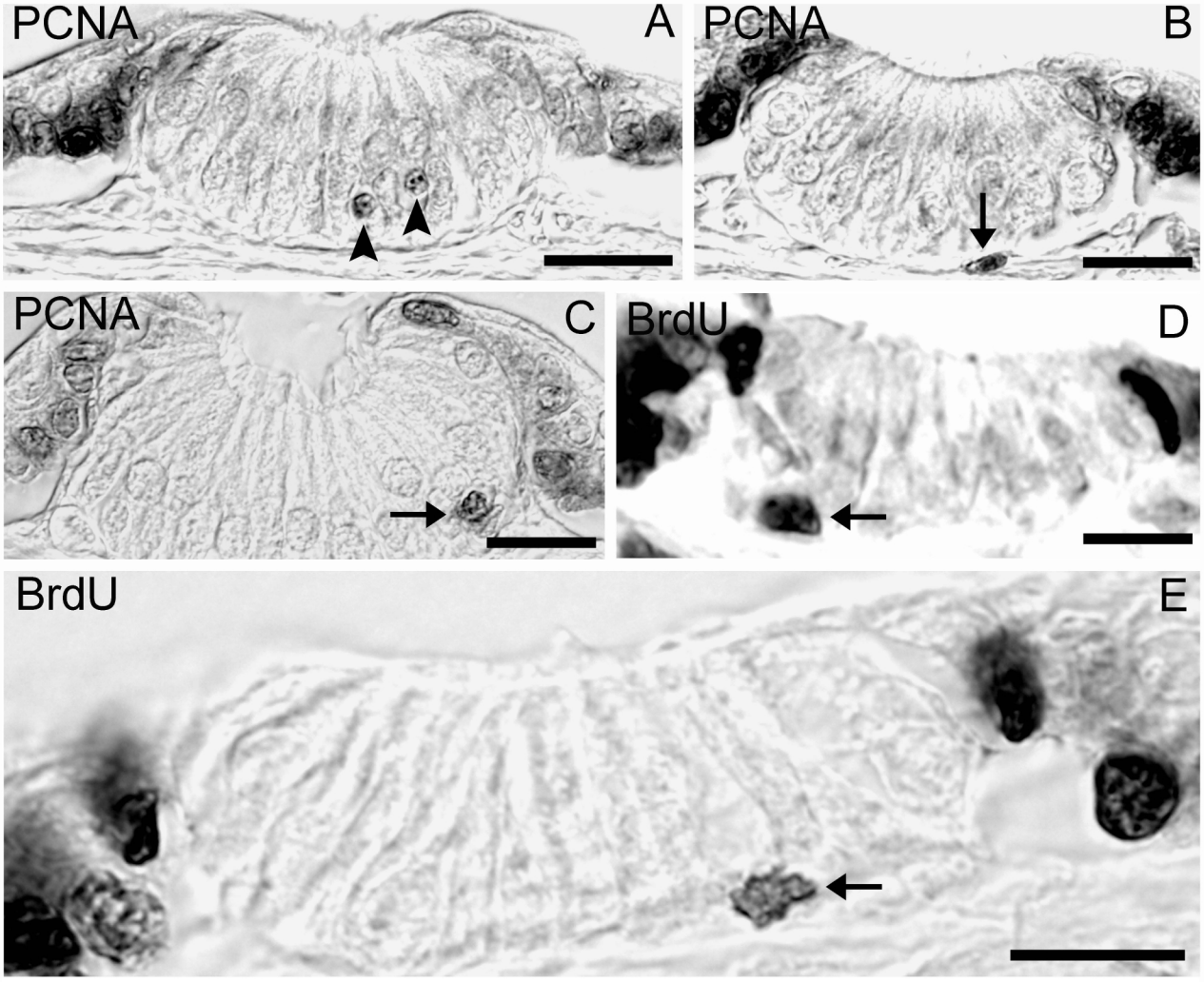


FIGURE 3

Figure 4. Taste bud bi-ciliated cells of larval sea lampreys labeled with antibodies against acetylated α-tubulin (A), 5-HT (B), and CR (C) in transversal sections. Note in A the presence of afferent gustatory fibers that contact the base of the α-tubulin-ir bi-ciliated cells. The arrow indicates the two cilia of a bi-ciliated cell. Branches of an tubulin-ir fiber were observed among TB cells (arrowhead). For abbreviations, see list. Scale bar: 25 μm in A-C.

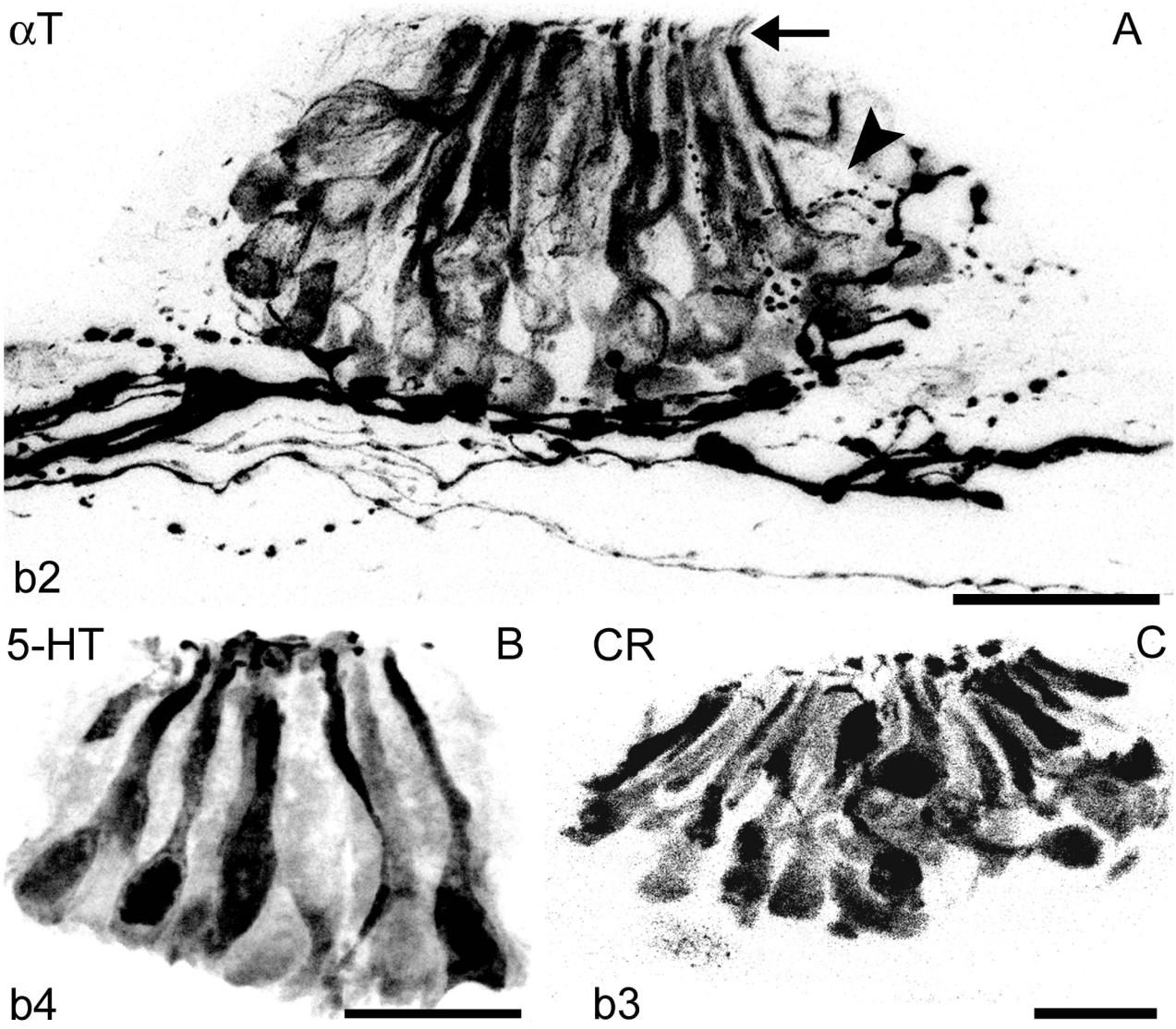


FIGURE 4

Figure 5. Taste buds of larval sea lampreys labelled with antibodies against acetylated α-tubulin (green channel) and 5-HT (magenta channel). A-A'': Photomicrographs showing the presence of a TB at the level of the first gill. Note that the bi-ciliated cells (αTir) of the bud are also 5-HTir. B-B'': Photomicrographs showing fibres of a branch of the IX nerve reaching the base of a TB. The white arrows point to the two cilia of each bi-ciliated cell. C-C'': Photomicrographs showing the presence of five TBs at the level of the fourth branchial arch. In figures B and C some fibres in the vicinity of the buds are 5-HTir but these fibres do not contact the base of the TBs (arrowheads). Figures A-C are from sagittal head sections. For abbreviations see list. Scale bars: 25 μm (A-B), 50 μm (C).

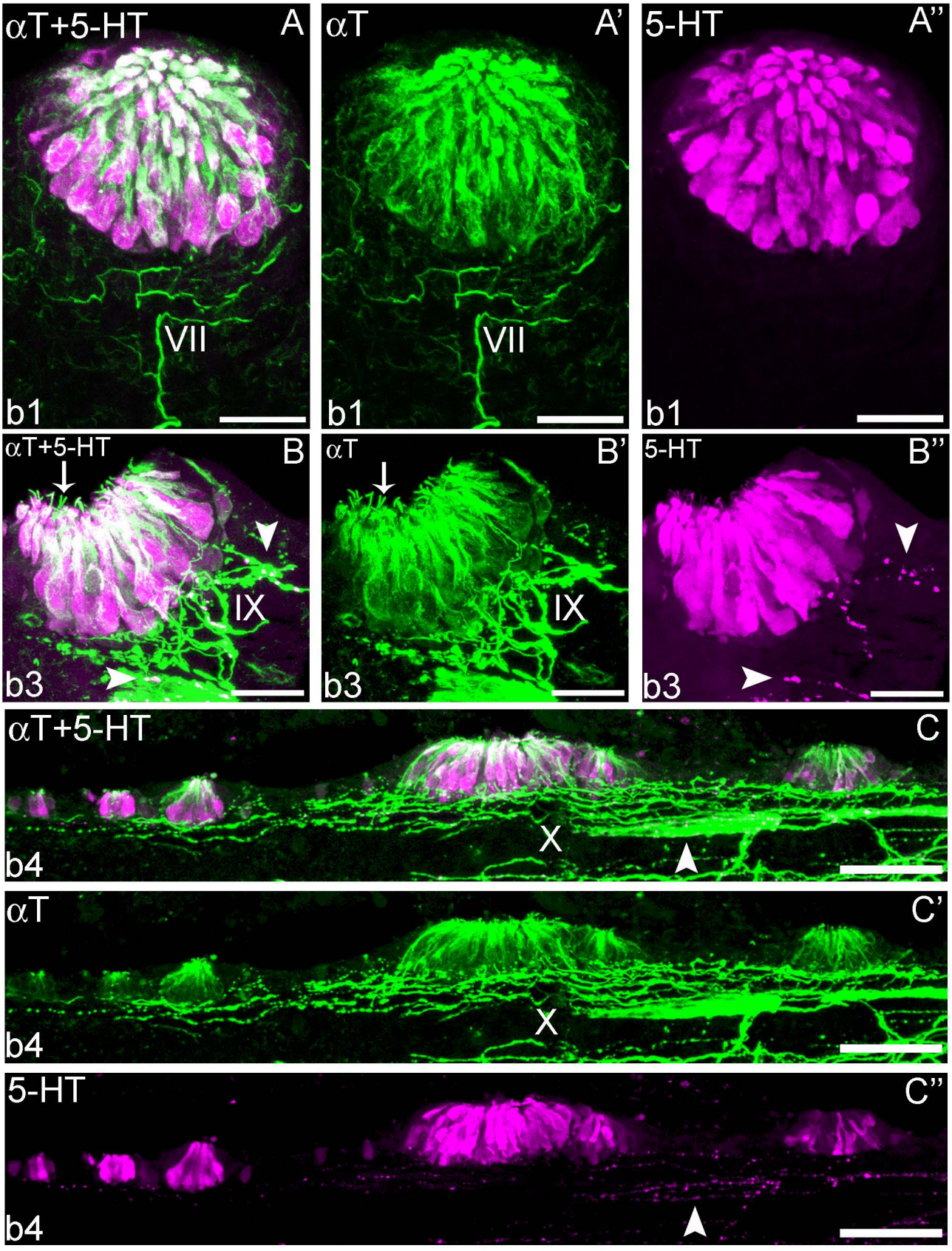

αT+5-HT
A
αT
A'
5-HT
A''
VII
VII
b1
b1
b1
αT+5-HT
B
αT
B'
5-HT
B''
IX
IX
b3
b3
b3
αT+5-HT
C
X
b4
αT
C'
X
b4
5-HT
C''
b4

FIGURE 5

Figure 6. A-A'': Photomicrographs of a transversal section showing the presence of 5-HTir (magenta channel) and αTir (green channel) bi-ciliated cells in TBs of upstream migrating adult sea lamprey at the level of the first branchial arch. Note the contacts between the afferent gustatory fibres and the bi-ciliated cells. **B-B'':** Photomicrographs of a sagittal section showing the colocalization of CR (green channel) and αT (magenta channel) immunoreactivities in the larval sea lamprey bi-ciliated cells. For abbreviations see list. Scale bars: 25 μm in A and 25 μm B.

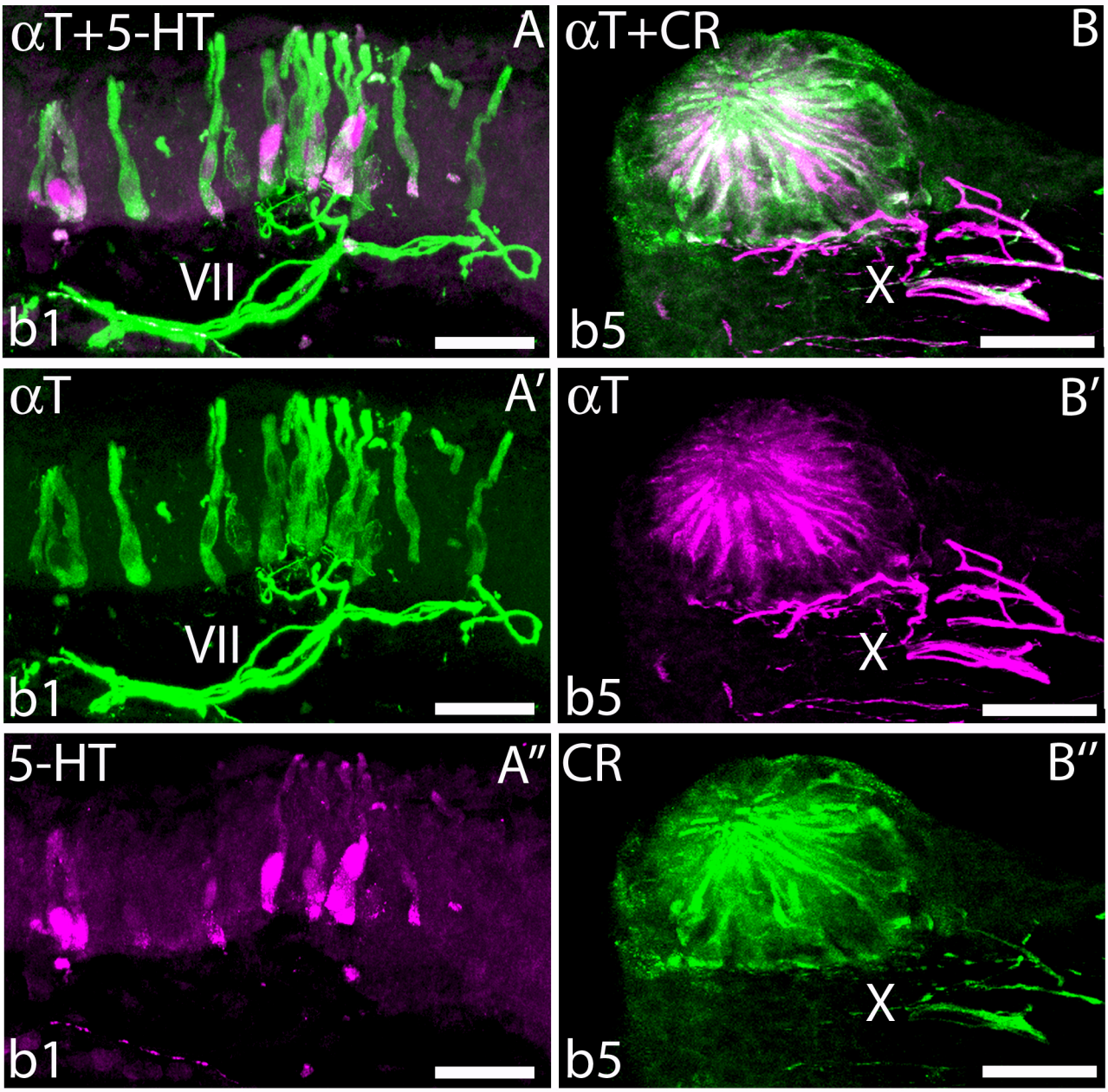


FIGURE 6

Figure 7. A-A'': Photomicrographs showing colocalization of 5-HT (magenta channel) and CGRP (green channel) immunoreactivities in the larval lamprey bi-ciliated cells. For abbreviations see list. Scale bars: 25 μm.

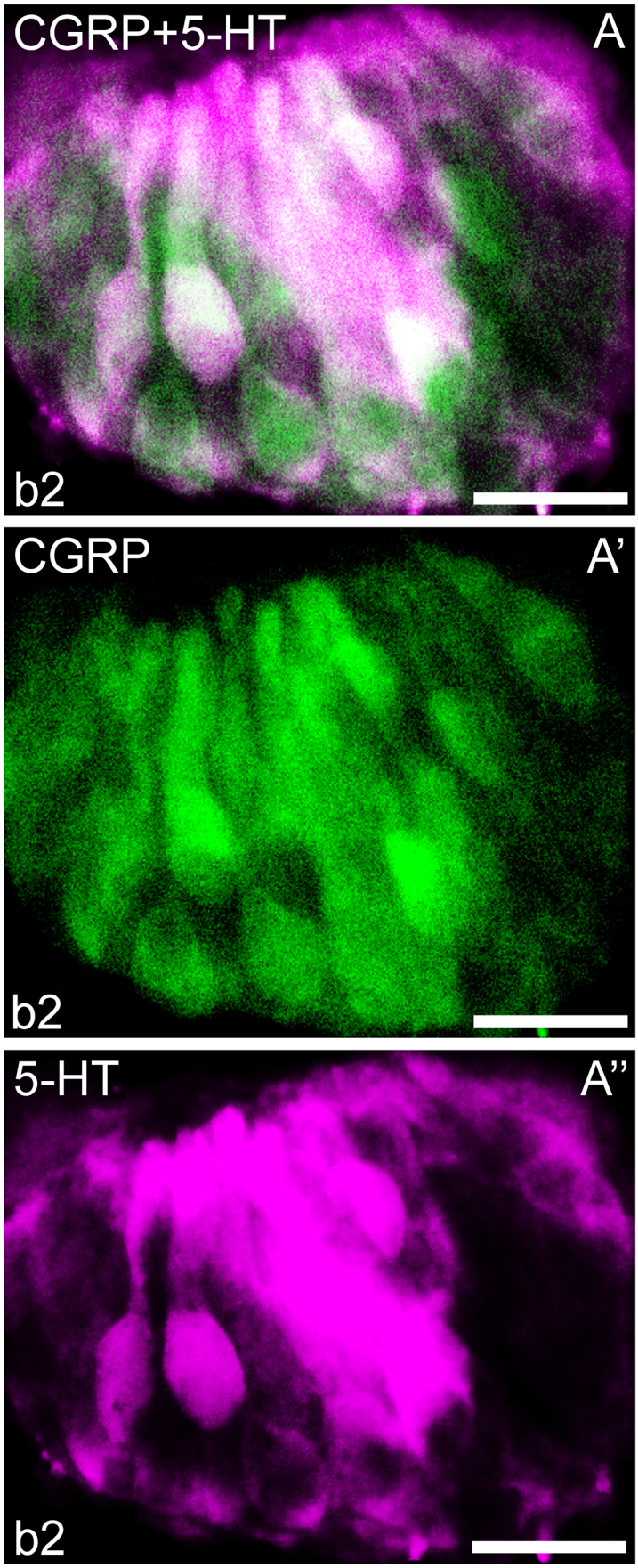


FIGURE 7

Figure 8. Photomicrographs showing positive controls for TH, GABA, NPY and GLU in the sections of the brain and retina of the same larval samples used in this study of the TB neurochemistry. **A/A':** Photomicrograph of a transversal section of a sea lamprey taste bud labelled with antibodies against αT (A) showing the absence of THir (A'). **B:** Photomicrograph of a sagittal section of the brain showing the presence of the TH immunoreactivity in paratubercular cells. **C:** Photomicrograph of a transversal section of the brain showing the presence of the GABAergic cells in the pretectal nucleus. **D:** Photomicrograph of a transversal section of the larval retina showing GLUir bipolar cells. **E:** Photomicrograph of a transversal section of the rostral rombencephalon showing NPYir cells of the isthmic region. See list for abbreviations. Scale bars: 25 μm in, B, C and D, 50 μm and E.

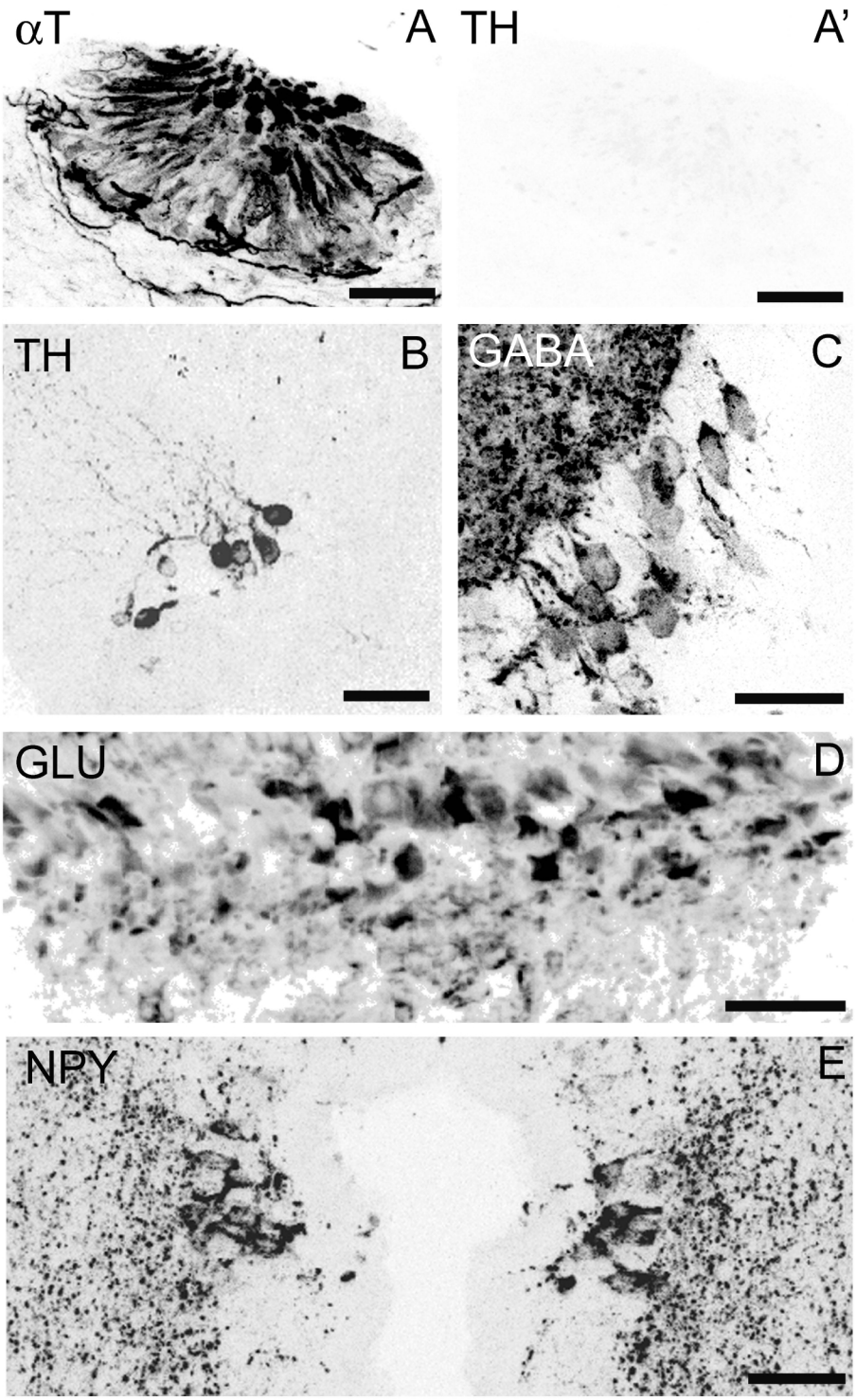


FIGURE 8

Figure 9. Schematic drawing showing the organization of the TBs from representative species of lampreys [based in figure 1 by Baatrup (1985b) and present results], fishes, amphibians and mammals [modified from Finger (2006)]. White cells in the larval sea lamprey bud represent sustentacular microvillar cells. See colour and symbol code.

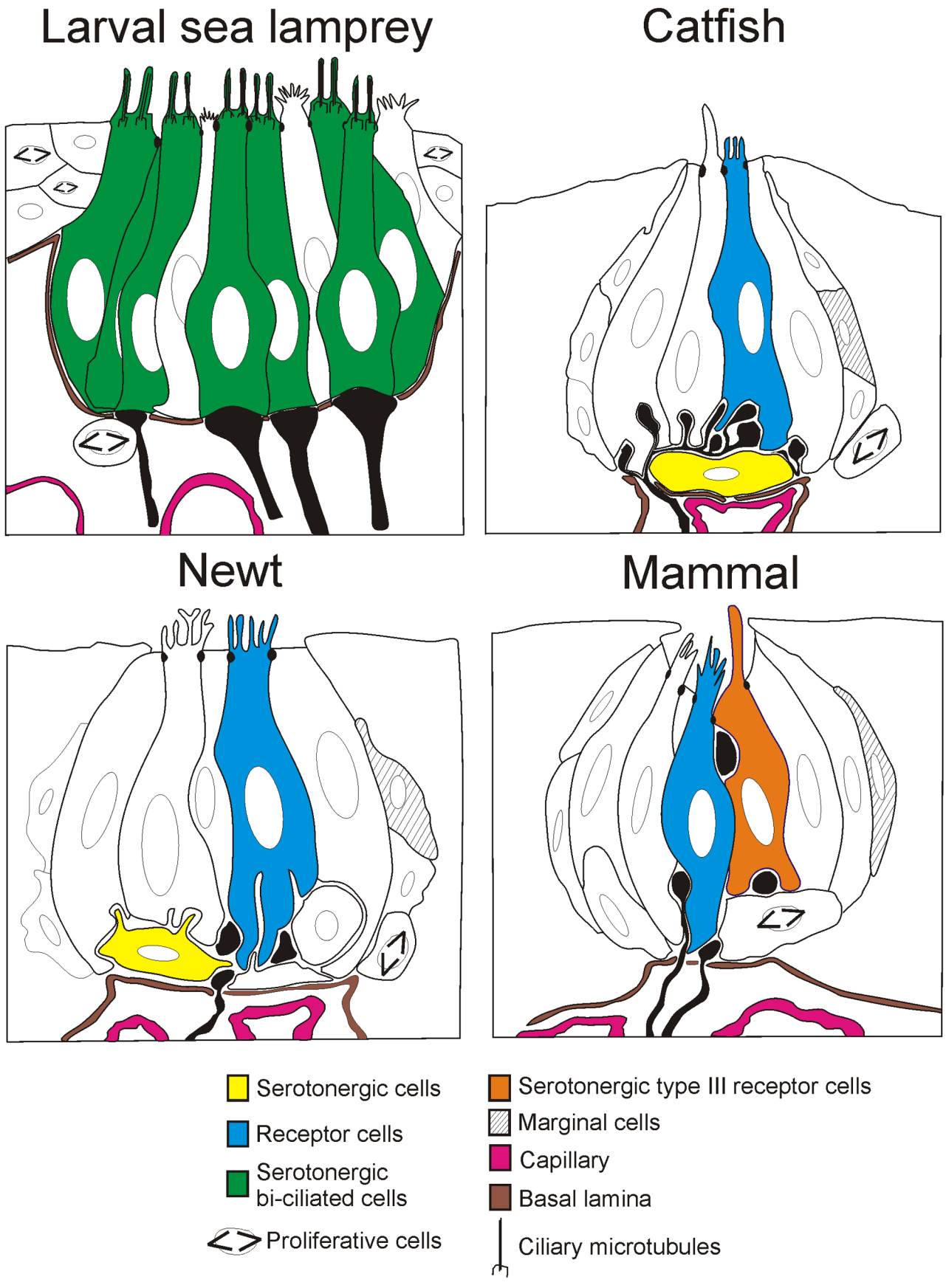
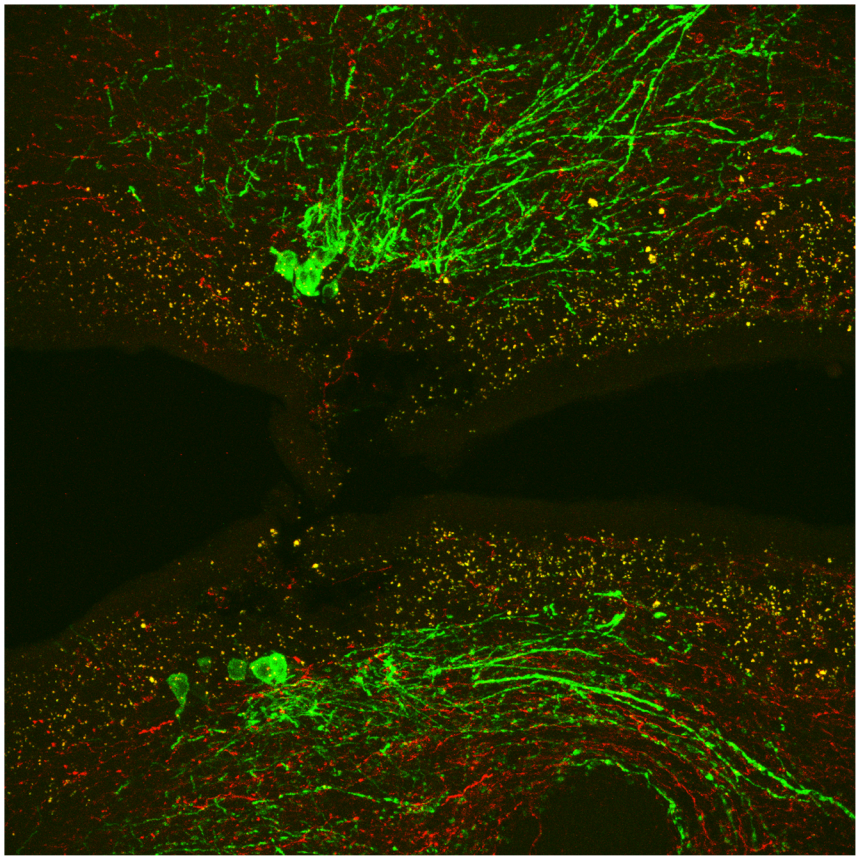

Larval sea lamprey
Catfish
Newt
Mammal
Serotonergic cells
Receptor cells
Serotonergic bi-ciliated cells
Proliferative cells
Serotonergic type III receptor cells
Marginal cells
Capillary
Basal lamina
Ciliary microtubules

FIGURE 9

Origin of the descending monoaminergic innervation to the sea lamprey spinal cord



Descending brain-spinal cord projections in a primitive vertebrate, the lamprey: Cerebrospinal fluid-contacting and dopaminergic neurons

Introduction

Lampreys are important models in analyzing the structure and function of the central nervous system because they are living agnathans, the most primitive group of vertebrates. The lamprey spinal cord has been fundamental for revealing the neural mechanisms underlying the control of locomotion (see Grillner and Wallén, 2002). In addition, lampreys have been an important model in studies of regeneration in the nervous system (Wood and Cohen, 1979; Davis and McClellan, 1994; Zhang et al., 2005). Knowledge about the descending spinal projections from the brain is therefore essential in understanding both locomotion pattern generation and axonal regeneration. Experimental studies with horseradish peroxidase (HRP) revealed that various neuronal populations of the reticular formation, descending trigeminal tract, intermediate and posterior octavomotor nuclei and the nucleus of the posterior tubercle project to the lamprey spinal cord (Ronan, 1989; Swain et al., 1993; Davis and McClellan, 1994). Although most of these descending projections appear to be glutamatergic, a spinal projection from serotonin-immunoreactive neurons of the brainstem has been observed in lamprey by use of combined retrograde tracing and immunohistochemistry (Brodin et al., 1986).

In lampreys, dopamine-immunoreactive (DA-ir) cells were observed in the spinal cord and some brain regions (McPherson and Kemnitz, 1994; Pierre et al., 1997; Pombal et al., 1997; Abalo et al., 2005). In the brain, most dopaminergic neurons are located in the hypothalamus and are of cerebrospinal fluid-contacting (CSF-c) type (Pierre et al., 1997; Pombal et al., 1997; Abalo et al., 2005). In the spinal cord, small DA-ir neurons located slightly ventral to the central canal give rise to a sparse plexus of DA-ir fibers (McPherson and Kemnitz, 1994; Abalo et al., 2005; Rodicio et al., 2008). Several studies have addressed the functions of dopamine in the spinal cord of lampreys, and have shown that it is involved in the modulation of spinal neurons (McPherson and Kemnitz, 1994; Schotland et al., 1996; Kemnitz, 1997). Dopamine is also involved in the function of higher brain centers (Thompson et al., 2008). On the other hand,

descending catecholaminergic projections to the spinal cord have been reported in some vertebrates (Skagerberg and Lindvall, 1985; Cechetto and Saper, 1988; Sánchez-Camacho et al., 2001; Qu et al., 2006). However, it is not known if dopaminergic populations of the lamprey brain contribute to the dopaminergic innervation of the spinal cord.

In the present study, we used neurobiotin and low molecular weight dextran amine in order to re-evaluate the descending projections from the brain. We report here previously unidentified cerebrospinal fluid-contacting (CSF-c) cell populations that give rise to descending spinal projections in lampreys. Combination of tract-tracing with immunohistochemistry against dopamine was also used with the aim of searching for spinal cord-projecting DA-ir neurons, which are reported here for the first time. We also compare this descending system in lampreys with those reported in other vertebrates in order to obtain further information about the evolution of the dopaminergic descending projections.

Material and Methods

Animals

Larval (n= 60; body length between 50 and 150 mm) and upstream migrating adult (n=12) sea lampreys (*Petromyzon marinus*) were used. All experiments conformed to the European Community guidelines on animal care and experimentation. Before experiments, the animals were deeply anesthetized with 0.05% benzocaine (Sigma, St. Louis, MO) in fresh water.

Retrograde tract tracing

In the larvae, the spinal cord was exposed by a transverse incision made in the dorsal region of the body. Crystals of neurobiotin (NB); (Vector Laboratories, Burlingame, CA) were applied with a minute pin (00) at one of three different levels of the spinal cord: the level of the 2nd branchiopore (8.7% of the body length), the level of the 4th branchiopore (15% of the body length) and just caudal to the 7th branchiopore (20% of the body length). The incision was closed with Histoacryl tissue glue (B. Braun Surgical). The larvae were maintained at 4ºC, with appropriate aeration conditions, in lamprey Ringer solution of the following composition (in mM): 137 NaCl, 2.9 KCl, 2.1 $CaCl_2$, 2 HEPES. In six larvae, the tracer was applied to only one side of the spinal cord to assess the laterality of the projections, and the cord was sealed with tissue glue. After a period ranging between 1 and 5 days, the larvae were deeply anesthetized and the heads were fixed by immersion in one of the following fixatives: 1) 4% paraformaldehyde in 0.4 M Tris buffer pH 7.4 (TBS) for 5 to 6 hours, or 2) 5% glutaraldehyde/1% sodium metabisulfite in 0.05 M TBS for 17 hours.

The heads were rinsed in TBS, then cryoprotected with 30% sucrose in TBS, embedded in Tissue Tek (Sakura, Torrance, CA), frozen using liquid nitrogen-cooled isopentane, and cut in the transverse or sagittal planes (20 μ m thickness) on a cryostat.

For application of tracer to the spinal cord of adults, NB crystals were inserted with a minute pin (00) in the rostral spinal cord at the level of the 2nd branchiopore of previously dissected brain/spinal cord wholemounts (n= 6). The brain/spinal cord wholemounts were maintained in the same conditions as larval samples for 3 days, and then processed as above.

Indirect detection of NB

For detection of NB, the sections were incubated at room temperature with fluorescein isothiocyanate (FITC)-labeled avidin D (Vector Laboratories, Burlingame, CA) diluted 1:1000 in TBS containing 0.3% Triton X-100, for 4 hrs. The sections were rinsed in distilled water and coverslipped with mounting medium for fluorescence (Vectashield; Vector Laboratories). In addition, some sections of heads of untreated larvae fixed as above were treated with FITC-labeled avidin-D to rule out the possibility that staining was due to endogenous brain biotin. No FITC staining was observed in these sections (not shown).

Dopamine immunofluorescence combined with tract tracing

After the application of NB in the rostral spinal cord, as above, and the corresponding survival times, some larval heads (80 to 150 mm in body length, n=15) and adult brains (n=3) were fixed by immersion in freshly prepared 5% glutaraldehyde/1% sodium metabisulfite in 0.05 M TBS for 17 hrs, and prepared for sectioning as above.

Sections were pre-treated with 0.2% $NaBH_4$ in distilled water for 45 minutes at room temperature. The sections were then incubated with a rabbit polyclonal anti-dopamine antibody (dilution 1:750; H.W.M. Steinbusch, Maastricht, The Netherlands). This anti-dopamine antibody was raised against dopamine-glutaraldehyde-BSA conjugates. For detection of the immunocomplex, a rhodamine isothiocyanate (RICT)-conjugated swine anti-rabbit antibody (dilution 1:30; Dako, Glostrup, Denmark) was used (incubation time: 1 h at room temperature). All antibodies were diluted with TBS containing 1% metabisulfite, 10% normal swine serum and 0.2% Triton X-100 as detergent. The NB was then revealed with FITC-labeled avidin D as above.

Control sections for the dopamine antibody were processed as above, except for the omission of the primary antiserum. No staining was observed in these controls. We tested the specificity of this antibody in

lamprey with pre-adsorption experiments; immunostaining was completely abolished after pre-adsorption of the diluted anti-dopamine antibody with dopamine-glutaraldehyde-BSA conjugates. However, staining was not modified after pre-adsorption of the primary antiserum with BSA (Sigma). Western blotting of lamprey brain protein extracts with this anti-dopamine antibody showed that it does not recognize native proteins (Fig. 1A). As a positive control for western blot we used an anti-calretinin antibody (dilution 1:500; SWant, Bellinzona, Switzerland; code 7699/4; lot 18299; immunogen: recombinant human calretinin), which recognizes a band of 29 kDa corresponding to lamprey calretinin (Fig. 1A; Villar-Cheda et al., 2006). This antibody was used previously to demonstrate specific populations of cells in the spinal cord (Megías et al., 2003) and retina (Villar-Cheda et al., 2006) of the sea lamprey.

Additional experiments

Most of the new spinal-projecting cells described in the present study were CSF-c cells. The following controls were included to ensure that the tracer was taken up via the axons, and not from the cerebrospinal fluid, blood or other routes:

1) NB was injected into the fourth ventricle of 3 larval and 3 adult lampreys (dissected brains plus the rostral spinal cord) under deep anesthesia, either with a minute pin through the choroid plexus, or by injection with a hypodermic syringe (NB diluted 10% in distilled water). No CSF-c cells were labeled in the brain or spinal cord in these experiments (not shown).

2) Adult lamprey brain show blood capillaries that have an endothelial blood-brain barrier to macromolecules like HRP or microperoxidase (Bundgaard, 1982). Instead, larval lamprey brains lack blood vessels (Rovainen, 1979; present results) and uptake of tracer from the blood during experiments therefore appears unlikely. However, to preclude this route of entry of tracer, NB crystals were inserted intraperitoneally into 3 larvae and after 3 days, the larvae were processed as above. No cells were labeled in the brain and spinal cord, although meningeal vessels were labeled (not shown).

3) Larvae (n=3) were fixed with 4% paraformaldehyde in TBS. Small crystals of a lipophilic carbocyanine dye (DiI; Molecular Probes, Eugene, OR) were applied with a minute pin to the rostral spinal cord. The larvae were maintained in darkness in frequently renewed fresh fixative for 3 months at 37°C. The brains were then dissected out, embedded in 3% agarose and sectioned on a Vibratome (100 μm thickness).

4) Neurobiotin may also be a transneuronal tract-tracer (Huang et al., 1992). To prevent CSF-c cells from being labeled transneuronally, 3 larvae were injected in the rostral spinal cord following

the same protocol described above but with biotinylated dextran amine (BDA, 3,000 Da; Molecular Probes, Eugene, OR) instead of NB, and with an incubation time of 5 days. BDA was revealed with FITC-labeled avidin-D, as for NB.

5) In previous studies of the lamprey spinal cord, HRP was used to reveal the brain-spinal projecting neurons (Ronan, 1989; Swain et al., 1993; Davis and McClellan, 1994), and thus the differences observed in the present results may be due to different efficiency of NB. For direct comparison of the transport efficiency of NB and HRP (Serva, Heidelberg, Deutschland), a mixture of both tracers was applied in the rostral spinal cord of 3 larvae. The larvae were maintained in the same conditions as above for 1 day, and then fixed and sectioned as above. HRP was revealed by sequential incubation with a mouse monoclonal anti-HRP (dilution 1:1,000 in 0.2% TST and 15% normal goat serum; Sigma, St. Louis, MO; code P6-38; Lot. 102K4812) and a secondary goat anti-mouse antibody coupled to Alexa 546 (dilution 1:100; Interchim, Montlucon, France). The NB was then revealed as above. Omission of the anti-HRP antibody did not result in immunostaining in any sections.

Photography

Photomicrographs were taken with a spectral confocal laser microscope (Leica TCS-SP2). The photomicrographs were adjusted for brightness and contrast with Adobe Photoshop 7.0 software. For presentation in plates, some photomicrographs were inverted and converted to grey scale.

Cell measurements and counts

The sizes of the cells in the larval brains were measured with LCS (free Leica confocal software) on confocal projections. Five well-labeled larvae were used, and 30 cells of each nucleus were measured (either the long axis or the diameter in spherical cells). The total number of NB-labeled cells was counted in the spinal-cord projecting nuclei reported here for the first time in the two brain sides in these larvae. Counts were made on photomicrographs of all the transverse sections in which each nucleus was present. Neurobiotin labeled elements with neuronal appearance (more than 9 mm in diameter) were included in the counts. To avoid double count of cells, series of photographs were aligned with ImageJ (free NIH software) and the position of labeled cells in pairs of consecutive sections was compared. This unbiased approach and the low number of labeled cells encountered make unnecessary to introduce any correction factor for possible overestimations of cell numbers. Since high variations were found in the number of labeled cells in each of the nuclei, the range of labeled cells is provided for each nucleus. In addition, the percentage

of retrogradely labeled cells that were DA-ir in these nuclei was also calculated.

Nomenclature of brain structures

For the brain nuclei previously reported to project to the spinal cord, we adopted the nomenclature used by Ronan (1989), although the correspondence of the spinal cord-projecting nuclei to brain regions was made in the context of the lamprey prosomeric model (Pombal and Puelles (1999) and the revision of Puelles and Rubenstein (2003). The nomenclature for the new positive cells was adopted from that used in previous studies carried out in our laboratory (Meléndez-Ferro et al., 2002, 2003; Abalo et al., 2007).

Results

A schematic drawings showing a dorsal view of the lamprey brain is presented in Figure 1B. Schematic drawings showing the organization of the spinal cord-projecting cells in the brain are presented in Figure 2. Figures 3 and 4 show the new spinal cord-projecting cells reported here for the first time. Figures 5 to 7 show the double labeling experiments made with the combination of HRP and NB or DA and NB.

Technical comments

In the present study, we evaluated the labeling of spinal projections in larvae maintained for between 1 and 5 days after NB application. All the new spinal cord-projecting nuclei reported here were observed after 1 day of incubation (Fig. 5B), but optimal labeling was achieved after two days. An increase in the survival time did not improve the labeling or reveal more cell groups. In addition, when the tracer was applied to one side of the spinal cord, the diencephalo-spinal projecting groups identified here for the first time were not labeled; these cells were only labeled when NB application involved the medial region of the spinal cord. This is probably because fibers arising from these groups ran in the ventral midline of the brain and spinal cord (see Figure 4A-B).

Control experiments

The neuronal populations revealed with NB tracing were also labeled in experiments in which BDA was used as a neuronal tracer, although fewer cells were labeled in the new populations reported here (i.e. those of the ventral hypothalamus; Fig. 1C). Moreover, DiI experiments in paraformaldehyde-fixed larval lampreys (n=3) labeled cells in all the populations that showed labeled cells in NB experiments (i.e. those of the ventral hypothalamus; Fig. 1D). Tract-tracing experiments with a mixture of HRP and NB demonstrated that with a short incubation period, NB labeled many more

cells and fibers and reached more rostral brain regions (Fig. 5A-B).

Distribution of cells projecting to the spinal cord in the brain of lamprey

The present study was mainly performed in larvae of sea lampreys (n=60), and therefore we mainly describe the spinal cord-projecting nuclei of this stage. Analysis of the adult brains (n=12), used as controls, showed that the organization of their nuclei projecting to the spinal cord is similar to that found in larvae. Spinal-projecting nuclei were observed in the diencephalon, mesencephalon and rhombencephalon. The pattern of neuron labeling in the mesencephalon and rhombencephalon revealed by NB and BDA tracing was similar to that previously revealed with HRP as a tracer (Swain et al., 1993) and these well-known spinal-projecting cells will not be described here. However, our experiments revealed further groups of labeled cells situated in the caudal rhombencephalon and the diencephalon. Remarkably, all the new cell groups reported in larvae were also labeled in adults, e.g. the mammillary population (Fig. 7B).

Caudal rhombencephalon

The experiments revealed a group of between 40 and 51 CSF-c cells situated in the caudal rhombencephalon around the central canal that projects caudally (Fig. 6A, A', B and B'). The CSF-c cells labeled in the caudal rhombencephalon, were small-sized bipolar cells of about 18 to 21 μm in their major axis, with an apical dendrite that ended in a small intraventricular protrusion (Fig. 6A-A'). Cells of this type were also labeled in the spinal cord rostral to the site where the tracer was applied. In addition, occasional non-CSF-c cells of about 10-11 μm in diameter were also labeled in the caudal rhombencephalon (Fig. 6A-A'). Most of the CSF-c cells were located ventromedially in the central canal wall at levels just rostral to the site of application of the tracer; a few of the CSF-c cells have a ventrolateral location in the ependymal region of the caudal rhombencephalon. These cells were only retrogradely labeled when the tracer was applied at the level of the 2nd branchiopore, i.e. their caudal projections only reached spinal cord levels close to the obex.

Spinal-projecting nuclei in the sea lamprey diencephalon

The application of NB in the rostral spinal cord labeled a number of small neurons in the ventral hypothalamic, mammillary and paratubercular nuclei that were not previously reported as spinal-projecting by other authors. In addition, our experiments labeled medium-sized cells in the nucleus of the medial longitudinal fasciculus, posterior tubercle and ventral thalamus/dorsal hypothalamus (medial diencephalic nucleus) as previously reported with other tracers (Ronan, 1989).

The location of the labeled cells in the sea lamprey diencephalon is shown schematically in Figures 2A-C.

Paratubercular nucleus

Between 10 and 16 labeled cells were observed in the paratubercular nucleus of the larvae studied (Fig. 3A, 4A and 6C-C'). These were small, round cells that ranged between 10 and 13 μm in diameter and showed both rostral and caudal processes. These cells were only labeled when the neurobiotin was applied at rostral spinal cord levels (2nd branchiopore).

Mammillary nucleus

Between 30 and 42 labeled CSF-c cells were observed in the so-called mammillary population (Mm) in the walls of the posterior infundibular recess (Fig. 3C, 3D, 4C, 5B and 7A-B') when NB was applied at the level of the 2nd branchiopore. They were small oval or pear-shaped cells, ranging from 9 to 10.5 μm in size. Their soma gave rise to an apical dendrite that protruded in the ventricle as a club and a basal process arising from the opposite cell pole. The density of cells in this Mm population varied; the highest density was observed in the ventrolateral corner of the recess and the lowest in its dorsal wall, where only very few labeled cells were observed. The caudodorsal region of this population partially overlaps with the paratubercular nucleus labeled cells, but the cell morphology and size allowed clear differentiation between these two spinal-projecting cell populations.

Ventral hypothalamic population

A large population of bipolar cells was labeled in the ventral hypothalamus (VHyp) in the walls of the infundibular recess (Fig. 2A, B, 3B, 4D and 5C). As regards the size and location of cells, and the lengths of their projections, this hypothalamic group can be subdivided into two subpopulations: dorsal (VHypD) and ventral (VHypV). Dopaminergic immunohistochemistry allowed assessment of the location of these cells in the diencephalon in comparison with that of the dopaminergic paraventricular organ, which is situated in the dorsal hypothalamus (Fig. 5C). The spinal cord-projecting cells of the VHyp were located ventral to this organ. The dorsal subpopulation was formed by scattered medium-sized cells (between 10 and 20) of diameter between 13 and 16 μm, and cells of this subpopulation were labeled when the NB was applied rostrally (2nd branchiopore level) or more caudally (at the level of 4th or 7th branchiopores), but the number of labeled cells decreased when the point of application of tracer was more caudal. Most, if not all, of the dorsal subpopulation cells were not CSF-contacting. The ventral subpopulation was formed by a compact group of numerous (between 40 and 60) small, CSF-c round cells

(10-12 μm in diameter). Cells of the ventral subpopulation were only labeled when the tracer was applied at the level of the 2nd branchiopore. Most of the labeled VHypV cells were of CSF-c type, with bipolar perikarya showing a ventricular dendrite and a basal process coursing to the lateral area. The VHyp is situated rostral to the Mm; these nuclei were separated by a gap where no labeled cells were observed in any experiment (Fig. 3C).

Dopaminergic neurons projecting to the spinal cord

Combined experiments revealed that some spinal cord-projecting CSF-c cells of the caudal rhombencephalon/rostral spinal cord (Fig. 6A-B'') and mammillary nucleus (Fig. 7) and some cells of the paratubercular nucleus (Figs. 6C-C''), were also DA-ir. These spinal cord-projecting DA-ir cells were observed both in larval and adult lampreys, and appeared well developed in 80 mm larvae.

Caudal rhombencephalic DA-ir CSF-c cells

In the caudal rhombencephalon and rostral spinal cord, DA-ir CSF-c cells formed a longitudinal cell column extending in the ventral margin of the central canal (Fig. 6A, A'', B and B''). A high proportion of the NB-labeled cells in this region were DA-ir CSF-c cells (between 45% and 50% of the cells). Very few non-CSF-c DA-ir cells located in the ventral midline were also double-labeled from the rostral spinal cord (Fig. 6A-A''). Ventral processes of these cells coursed towards a ventromedial DA-ir plexus of the caudal rhombencephalon and spinal cord.

Paratubercular nucleus

After application of the tracer at rostral spinal cord levels, occasional rounded or pear-shaped DA-ir paratubercular cells were double-labeled (Fig. 6C-C''). Neurons of this nucleus showed processes ascending in a dorsorostral direction or coursing in a caudal direction, as was observed by immunofluorescence in sagittal brain sections. However, the low number of double-labeled DA-ir neurons observed in this nucleus indicates that most cells do not project to the spinal cord.

Mammillary nucleus

A periventricular DA-ir cell group, the mammillary nucleus, was observed around the posterior infundibular recess. The majority, if not all, of the DA-ir double-labeled cells of the mammillary nucleus were CSF-c neurons (Fig. 7). After application of the tracer in the rostral spinal cord, between 30% and 35% of NB-labeled cells of the mammillary population were DA-ir. Fibres arising from the ventral pole of these cells formed a ventromedial dopaminergic plexus that coursed caudally (Fig. 7A-A''). Most of the double-labeled DA-ir cells were situated

in the caudoventral walls of the mammillary recess.

Discussion

Methodological considerations

The present study increases the number of known brain populations that project to the spinal cord in lampreys by four, three of which consist of CSF-c neurons. The present results showed that application of NB (322.8 Da) to the rostral spinal cord led to the retrograde labeling of a greater size range of cells than in previous studies with HRP (molecular weight, 40,000 Da) (Ronan, 1989; Swain et al., 1993; Davis and McClellan, 1994), and demonstrated a number of small CSF-c spinal-projecting neurons in regions not mentioned by these authors. Differences between present and previous studies in lampreys may be explained in part for the different molecular weight/chemical nature of the tracers used and the different point of tracer application [8.7% of the body length (present results); 3rd muscle segment (Ronan, 1989; level of the 2nd branchiopore?); 10-20% of the body length (Swain et al., 1993; Davis and McClellan, 1994)]. The various additional experiments performed, especially the positive staining of these newly populations by alternative tracers such as BDA applied in vivo or DiI applied to fixed tissue (see Material and Methods), clearly indicate that this was not due to transneuronal labeling or to diffusion of NB through the CSF or the blood.

Some hypothalamic CSF-c cells project to the spinal cord in lampreys

The most remarkable new finding from the present investigation was the direct demonstration of a prominent hypothalamo-spinal projection arising from CSF-c cells of the walls of the infundibular and mammillary recesses, which has not previously been described in lampreys or other vertebrates. Hypothalamic CSF-c cells were first described in the late 1960s in most vertebrates, including lampreys (Nakai et al., 1979), although they decrease in number from fishes to mammals (see Vigh et al., 2004). In lampreys, some hypothalamic CSF-c neurons were shown to project to the striatum (Pombal et al., 1997), torus semicircularis (González et al., 1999) and optic tectum (de Arriba and Pombal, 2007), but as far as we are aware no projections from diencephalic CSF-c neurons to the spinal cord have been reported. A study in lungfish, in which cobalt-lysine was applied intraocularly, revealed a number of brain targets attributed to projections of CSF-c cells of the paraventricular organ, including fibers that extend to the spinal cord (von Bartheld and Meyer, 1990). No projections from CSF-c cells of similar circumventricular regions were reported in zebrafish (McLean and Fetcho, 2004a; b) or amphibians (Sánchez-

Camacho et al., 2001). It is not clear why similar spinal connections have not been reported in other vertebrates, because a paraventricular organ is present in all non-mammalian vertebrates (see Vigh et al., 2004). It is possible that most tracers used in studies of spinal connections were taken up in very low amounts by fibers of these cells, or were transported very slowly.

In the lamprey, hypothalamic CSF-c cells are neurochemically heterogeneous, including cells immunoreactive to dopamine (Pierre et al., 1997; Pombal et al., 1997; Abalo et al., 2005), noradrenaline (Steinbusch et al., 1981), serotonin (Steinbusch et al., 1981; Abalo et al., 2007), histamine (Pombal and Puelles, 1999), GABA (Meléndez-Ferro et al., 2002; Robertson et al., 2007) and various neuropeptides, but their projections are largely unknown. Present results with combined tracing and immunofluorescence methods indicate that axons from some dopaminergic CSF-c neurons reach the spinal cord. These cells may indicate changes in the CSF composition (see Vigh et al. 2004), and en-passant or terminal release of dopamine by their fibers may modulate the activity of the regions through which axons course, including the rostral spinal cord, in response to variations in the third ventricle.

Dopaminergic projections to the spinal cord in vertebrates

The present results showed that the sea lamprey dopaminergic brain neurons that project to the spinal cord are located in the diencephalon (paratubercular nucleus and mammillary nucleus) and the caudal rhombencephalon. Some experimental studies of descending dopaminergic projections performed in other vertebrates were carried out with tyrosine hydroxylase (TH) immunohistochemistry (Skagerberg and Lindvall, 1985; Cechetto and Saper, 1988; Sánchez-Camacho et al., 2001; Qu et al., 2006). These studies reported the presence of diencephalic catecholaminergic cells that project to the spinal cord in some mammals and amphibians (zona incerta: Blessing and Chalmers, 1979; Sánchez-Camacho et al., 2001; dorsal hypothalamus: Skagerberg and Lindvall, 1985; Cechetto and Saper, 1988; Sánchez-Camacho et al., 2001; Qu et al., 2006), which show no DA-ir spinal projecting cells in lamprey. Since TH is the common enzyme of synthesis of catecholamines, the presence of this enzyme does not identify unequivocally the catecholamine contained by these cells. Immunohistochemical studies in whole mount developing zebrafish also reveal the origin in the brain of early catecholaminergic fibers observed in the spinal cord, and dopaminergic fibers (THir and dopamine-β-hydroxylase negative) putatively originated from cells of the posterior tubercle, whereas noradrenergic fibers (dopamine-β-hydroxylase

immunoreactive) arise from the locus coeruleus (McLean and Fetcho 2004a). The most conserved catecholaminergic projection to the spinal cord in lower vertebrates appears to be the projection from cells of the posterior tubercle nucleus (larval zebrafish: McLean and Fetcho, 2004a, b; amphibians: Sánchez-Camacho et al. 2001). This nucleus was proposed to be equivalent to the paratubercular nucleus of lampreys (Abalo et al, 2005). The dopaminergic projection to the spinal cord from the paratubercular nucleus appears to be less developed in lampreys than in other vertebrates studied, since only very few DA-ir cells of this nucleus became double-labeled after the application of NB. The paratubercular region has been considered by Baumgarten (1972) and Pombal et al. (1997) to be homologous to the substantia nigra of mammals on the basis of the cytoarchitectural organization and striatal projections respectively. Although in mice the substantia nigra does not contain spinal cord-projecting cells, some catecholaminergic neurons of the midbrain ventral tegmental area (A10 group) project to the spinal cord (Qu et al., 2006). As regards the locus coeruleus projections observed in other vertebrates, their projections are noradrenergic (McLean and Fetcho, 2004a, b). Moreover, no DA-ir cells were observed in the rostral rhombencephalon of larval sea lamprey (Abalo et al., 2007; present results).

Projections from catecholaminergic cells of the nucleus of the solitary tract were observed in some amphibians (Sánchez-Camacho et al., 2002) and in mammals (Pindzola et al., 1988; Maisky and Dorosenko, 1991) but a similar DA-ir population projecting to the spinal cord was not observed in lamprey.

Functional implications of the dopaminergic projections to the spinal cord

Dopamine is involved in a variety of functions in the brain of vertebrates, including locomotion, emotion, learning, and autonomic and neuroendocrine modulation (Riederer et al., 1985; Kobayashi and Sano, 2000). In the lamprey spinal cord, dopamine modulates the cycle period of fictive swimming and motoneuron physiology (McPherson and Kemnitz, 1994). However, owing to the abundance of intraspinal DA-ir neuronal populations (Schotland et al., 1996; Pombal et al., 1997; Abalo et al., 2007; Rodicio et al., 2008), it is not possible to infer any possible contribution of the descending dopaminergic projections to the modulation of locomotor circuitry. It has also been suggested that the hypothalamic-spinal cord dopaminergic projection plays a role in the regulation of the sympathetic outflow in mammals (Qu et al., 2006). Lampreys do not possess sympathetic ganglia, and it has even been suggested that they lack a sympathetic nervous system (Gibbins, 1994; Funakoshi

and Nakano, 2007). Accordingly, any relation between dopaminergic descending spinal innervation from hypothalamic cells in lampreys and autonomic functions appears unlikely. At the present, any functional consideration of this descending system in lampreys remains speculative.

Conclusions

The most important novel finding of the present study was the existence in lampreys of a spinal cord projection system arising from CSF-c cells of the ventral hypothalamus and caudal rhombencephalon, not previously reported in any vertebrate species. Important differences between the sea lamprey and other previously studied vertebrates were also observed in the organization of the dopaminergic projection system to the spinal cord.

Literature cited

Abalo XM, Villar-Cheda B, Anadón R, Rodicio MC. 2005. Development of the dopamine-immunoreactive system in the central nervous system of the sea lamprey. Brain Res Bull 66: 560-564.

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.

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

Blessing WW, Chalmers JP. 1979. Direct projection of catecholamine (presumably dopamine)-containing neurons from hypothalamus to spinal cord. Neurosci Lett 11: 35-40.

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.

Bundgaard M. 1982. Brain barrier systems in the lamprey. I. Ultrastructure and permeability of cerebral blood vessels. Brain Res 240: 55-64.

Cechetto DF, Saper CB. 1988. Neurochemical organization of the hypothalamic projection to the spinal cord in the rat. J Comp Neurol 272:579-604.

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

de Arriba MC, Pombal MA. 2007. Afferent connections of the optic tectum in lampreys: an experimental study. Brain Behav Evol 69: 37-68.

Funakoshi K, Nakano M. 2007. The sympathetic nervous system of anamniotes. Brain Behav Evol 69: 105-113.

Gibbins I. 1994. Comparative anatomy and evolution of the autonomic nervous system. In Nilsson S, Holmgren S (eds), The autonomic nervous system, vol 4: Comparative physiology and evolution of the autonomic nervous system. Chur, Switzerland: Harwood Academic. p 1-67.

González MJ, Yáñez J, Anadón R. 1999. Afferent and efferent connections of the torus semicircularis in the sea lamprey: an experimental study. Brain Res 826: 83-94.

Grillner S, Wallén P. 2002. Cellular bases of a vertebrate locomotor system-steering, intersegmental and segmental co-ordination and sensory control. Brain Res Rev 40: 92-106.

Huang Q, Zhou D, DiFiglia M. 1992. Neurobiotin, a useful neuroanatomical tracer for in vivo anterograde, retrograde and transneuronal tract-tracing and for in vitro labeling of neurons. J Neurosci Methods 41: 31-43.

Kemnitz CP. 1997. Dopaminergic modulation of spinal neurons and synaptic potentials in the lamprey spinal cord. J Neurophysiol 77:289-298.

Kobayashi K, Sano H. 2000. Dopamine deficiency in mice. Brain Dev 22: S54-60.

Maisky VA, Doroshenko NZ. 1991. Catecholamine projections to the spinal cord in the rat and their relationship to central cardiovascular neurons. J Auton Nerv Syst 34: 119-28.

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.

McPherson DR, Kemnitz CP. 1994. Modulation of lamprey fictive swimming and motoneuron physiology by dopamine, and its immunocytochemical localization in the spinal cord. Neurosci Lett 166: 23-26.

Megías M, Álvarez-Otero R, Pombal MA. 2003. Calbindin and calretinin immunoreactivities identify different types of neurons in the adult lamprey spinal cord. J Comp Neurol 455: 72-85.

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B, Abalo XM, Rodríguez-

Muñoz R, Anadón R, Rodicio MC. 2003. Ontogeny of γ -aminobutyric acid-immunoreactive neurons in the rhombencephalon and spinal cord of the sea lamprey. J Comp Neurol. 464: 17-35.

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.

Nakai Y, Shioda S, Tashiro N, Honma Y. 1979. Fine structures of the cerebrospinal fluid-contacting neurons in the hypothalamus of the lamprey, *Lampetra japonica*. Arch Histol Jpn 42: 337-353.

Pierre J, Mahouche M, Suderevskaya EI, Repérant J, Ward R. 1997. Immunocytochemical localization of dopamine and its synthetic enzymes in the central nervous system of the lamprey *Lampetra fluviatilis*. J Comp Neurol 380: 119-135.

Pindzola RR, Ho RH, Martin GF. 1988. Catecholaminergic innervation of the spinal cord in the North American opossum, *Didelphis virginiana*. Brain Behav Evol 32: 281-292.

Pombal MA, Puelles L. 1999. Prosomeric map of the lamprey forebrain based on calretinin immunocytochemistry, Nissl stain, and ancillary markers. J Comp Neurol 414: 391-422.

Pombal MA, El Manira A, Grillner S. 1997. Afferents of the lamprey striatum with special reference to the dopaminergic system: a combined tracing and immunohistochemical study. J Comp Neurol 386:71-91.

Puelles L, Rubenstein JL. 2003. Forebrain gene expression domains and the evolving prosomeric model. Trends Neurosci 26: 469-476.

Qu S, Ondo WG, Zhang X, Xie WJ, Pan TH, Le WD. 2006. Projections of diencephalic dopamine neurons into the spinal cord in mice. Exp Brain Res 168:152-156.

Riederer P, Beckmann H, Brücke T. 1985. Current biochemical hypotheses of endogenous depression. Wien Klin Wochenschr 97: 190-196.

Robertson B, Auclair F, Ménard A, Grillner S, Dubuc R. 2007. GABA distribution in lamprey is phylogenetically conserved. J Comp Neurol 2007 503: 47-63.

Rodicio MC, Villar-Cerviño V, Barreiro-Iglesias A, Anadón R. 2008. Colocalization of dopamine and GABA in spinal cord neurones in the sea lamprey. Brain Res Bull 76:45-49.

Ronan M. 1989. Origins of the descending spinal projections in petromyzontid

and myxinoid agnathans. J Comp Neurol 281: 54-68.

Rovainen CM. 1979. Neurobiology of lampreys. Physiol Rev 59: 1007-1077.

Sánchez-Camacho C, Marín O, Smeets WJ, Ten Donkelaar HJ, González A. 2001. Descending supraspinal pathways in amphibians. II. Distribution and origin of the catecholaminergic innervation of the spinal cord. J Comp Neurol 434: 209-232.

Sánchez-Camacho C, Marón O, López JM, Moreno N, Smeets WJ, ten Donkelaar HJ, González A. 2002. Origin and development of descending catecholaminergic pathways to the spinal cord in amphibians. Brain Res Bull 57: 325-330.

Schotland JL, Shupliakov O, Grillner S, Brodin L. 1996. Synaptic and nonsynaptic monoaminergic neuron systems in the lamprey spinal cord. J Comp Neurol 372:229-244.

Skagerberg G, Lindvall O. 1985. Organization of diencephalic dopamine neurones projecting to the spinal cord in the rat. Brain Res 342: 340-351.

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 Suppl 24:107-122.

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.

Thompson RH, Ménard A, Pombal M, Grillner S. 2008. Forebrain dopamine depletion impairs motor behavior in lamprey. Eur J Neurosci 27:1452-1460.

Vigh B, Manzano e Silva MJ, Frank CL, Vincze C, Czirok SJ, Szabó, 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, Abalo XM, Anadón R, Rodicio MC. 2006. Calretinin and calbindin immunoreactivity in the retina of adult and larval sea lamprey. Brain Res 1068: 118-130.

von Bartheld CS, Meyer DL. 1990. Paraventricular organ of the lungfish *Protopterus dolloi*: morphology and projections of CSF-contacting neurons. J Comp Neurol 297: 410-434.

Wood MR, Cohen MJ. 1979. Synaptic regeneration in identified neurons of

the lamprey spinal cords. Science 206: 344-347.

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

Abbreviations

BDA	Biotinylated dextran amine
CSF-c	Cerebrospinal fluid-contacting cell
Di	Diencephalon
DHyp	Dorsal hypothalamus
DiI	1,1'-dioctadecyl 3,3,3',3' tetramethylindocarbocyanine perchlorate
DTh	Dorsal thalamus
HRP	Horseradish peroxidase
HY	Hypophysis
Ith	Olfactory nerve
IVth	Trochlear nerve
IXth	Glossopharyngeal nerve
M	Mesencephalon
M1-M2	Müller cells 1 and 2
MD	Medial diencephalic
Mm	Mammillary population
NB	Neurobiotin
nMLF	Nucleus of the medial longitudinal fasciculus
OB	Olfactory bulb
PT	Posterior tubercle
Pt	Paratubercular nucleus
PVO	Paraventricular organ
Rh	Rhombencephalon
T	Telencephalon
VHyp	Ventral Hypothalamus
VHyp D	Dorsal subpopulation of the ventral hypothalamus
VHyp V	Ventral subpopulation of the ventral hypothalamus
VIIth	Facial nerve
VIIIth	Octaval nerve

Vth	Trigeminal nerve
VTh	Ventral thalamus
Xth	Vagal nerve

FIGURES
CHAPTER 2.1

Figure 1. A: Western blot of lamprey protein extracts immunostained with antisera to calretinin (used as positive control) and DA. Note the protein band of 29 kDa stained in the calretinin lane and the absence of labeling in the DA lane. **B:** Schematic drawing showing a dorsal view of the sea lamprey brain. The black lines indicate the approximate levels of the transverse sections C and D. **C:** Photomicrograph of transverse section of the ventral hypothalamus showing cells labeled with BDA applied in the rostral spinal cord. The arrow indicates the dendrite of a CSF-c cell. **D:** Photomicrograph of transverse section of the ventral hypothalamus showing cells labeled with DiI applied in the rostral spinal cord. Scale bars: 50 μm in C and 75 μm in D.

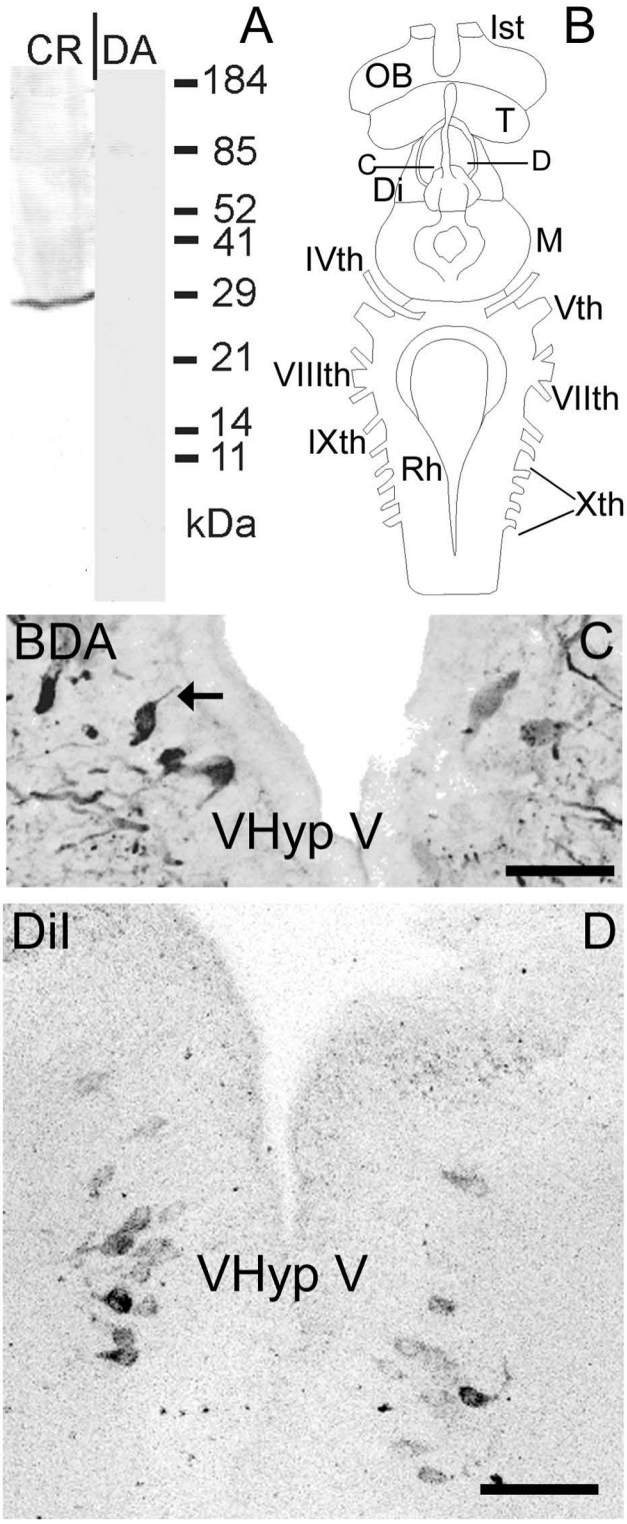

CR
DA
A
184
85
52
41
29
21
14
11
kDa
Ist
B
OB
T
C
D
Di
M
IVth
Vth
VIIIth
VIIth
IXth
Rh
Xth
BDA
C
VHyp V
DiI
D
VHyp V

FIGURE 1

Figure 2. A: Schematic drawing representing the distribution and organization of the spinal cord projecting populations in the larval sea lamprey diencephalon in lateral view. Oblique bars at the top indicate the approximate levels of the transverse schematic drawings B and C. **B and C:** Schematic drawings of transverse sections showing the location of the diencephalic spinal cord projecting cells. For abbreviations see list.

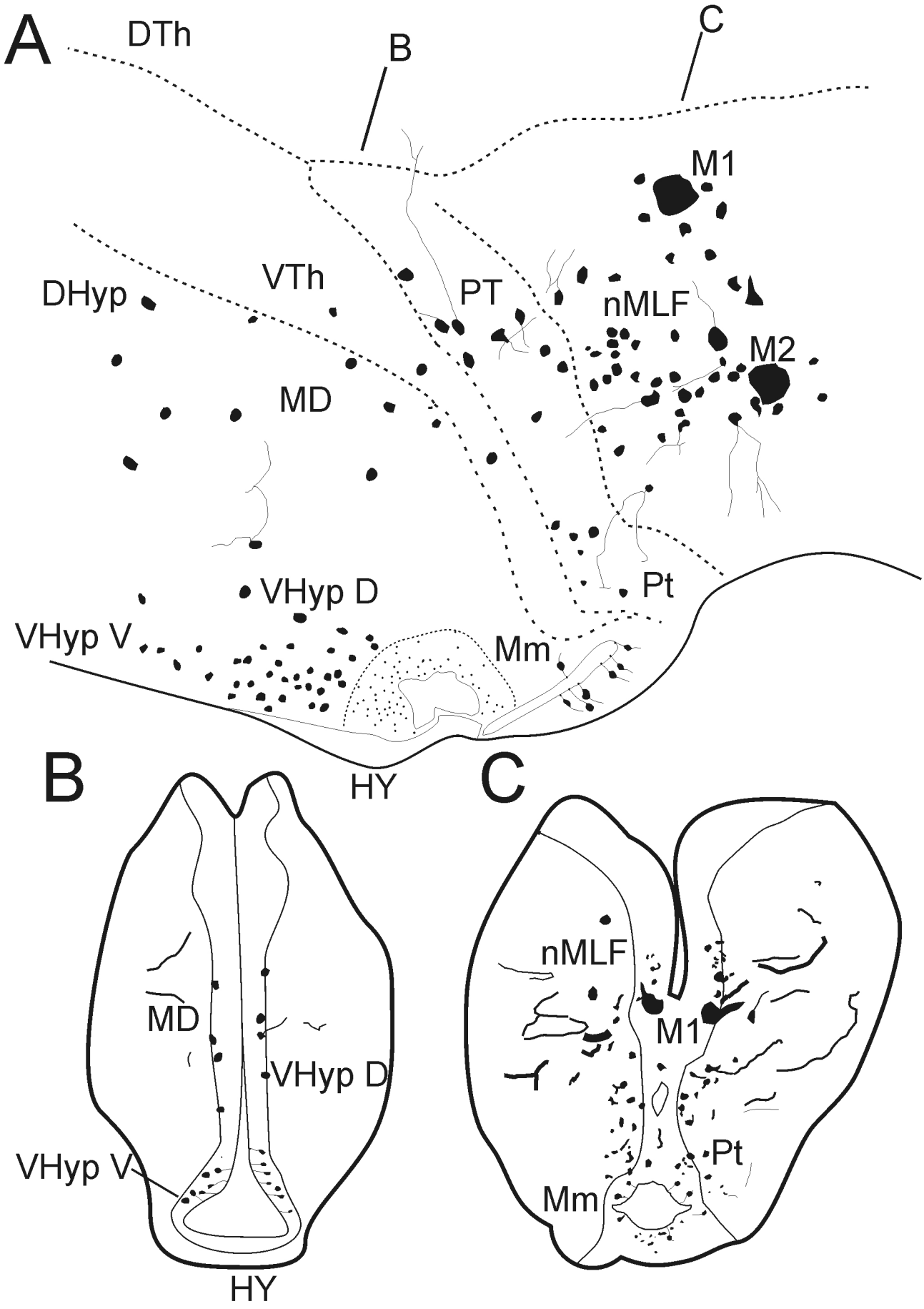

A
DTh
B
C
M1
DHyp
VTh
PT
nMLF
M2
MD
VHyp D
Pt
VHyp V
Mm
HY
B
MD
VHyp D
VHyp V
HY
C
nMLF
M1
Pt
Mm

FIGURE 2

Figure 3. Photomicrographs of sagittal sections of larval brains and spinal cord showing the cell populations labeled after application of NB to the rostral spinal cord. **A:** Photomicrograph of a sagittal section of the paratubercular nucleus of a larva showing the presence of small labeled cells. **B**: Photomicrograph of a sagittal section of the hypothalamic region of a larva showing numerous spinal cord-projecting cells in the VHyp population. **C**: Photomicrograph of a sagittal section of the hypothalamic region of a larva showing labeled CSF-c cells of the Mm and Vhyp populations. **D**: Detail of a CSF-c cell spinal cord-projecting cell situated in the ventral part of the mammillary recess. The arrows indicate the ventricular dendrite. For abbreviations see list. In all photomicrographs, rostral is on the left. The outlined stars (B, C and D) indicate the ventricle. Scale bars: 50 μm in D-E, 75 μm in C and 12.5 μm in D.

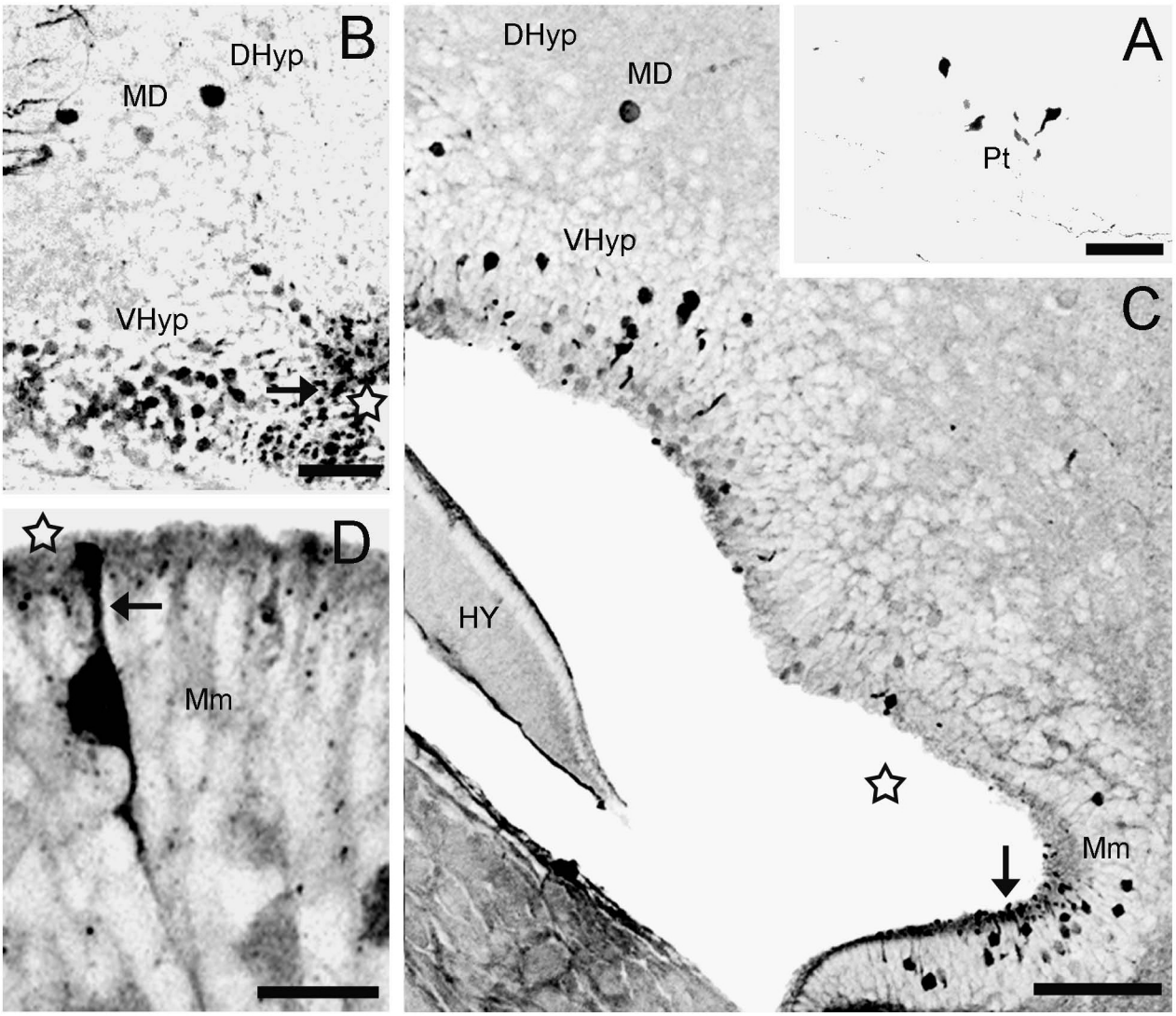


FIGURE 3

Figure 4. Photomicrographs of transverse sections of the larval diencephalon after application of NB to the rostral spinal cord. **A**: Photomicrograph of a transverse section of the caudal diencephalon showing labeled cells of the MLF (including the M1 giant neuron), posterior tubercle and paratubercular nucleus. Note the presence of descending fibers in the ventral midline of the hypothalamus. **B**: Detail of Figure 5A showing thin labeled fibers running in the ventral hypothalamus. **C**: Photomicrograph of a section of the mammillary nucleus showing labeled CSF-c cells. **D**: Photomicrograph of a transverse section of the rostral diencephalon showing CSF-c cells of the VHyp V. The arrowheads in A and B indicate thin labeled fibers coursing in the ventral midline of the hypothalamus. The outlined stars indicate the ventricle. Small dots in C-D are autofluorescent lipidic droplets present in ependymal cells of lampreys. For abbreviations see list. Scale bars: 150 μm in A, 100 μm in B and D and 50 μm in C.

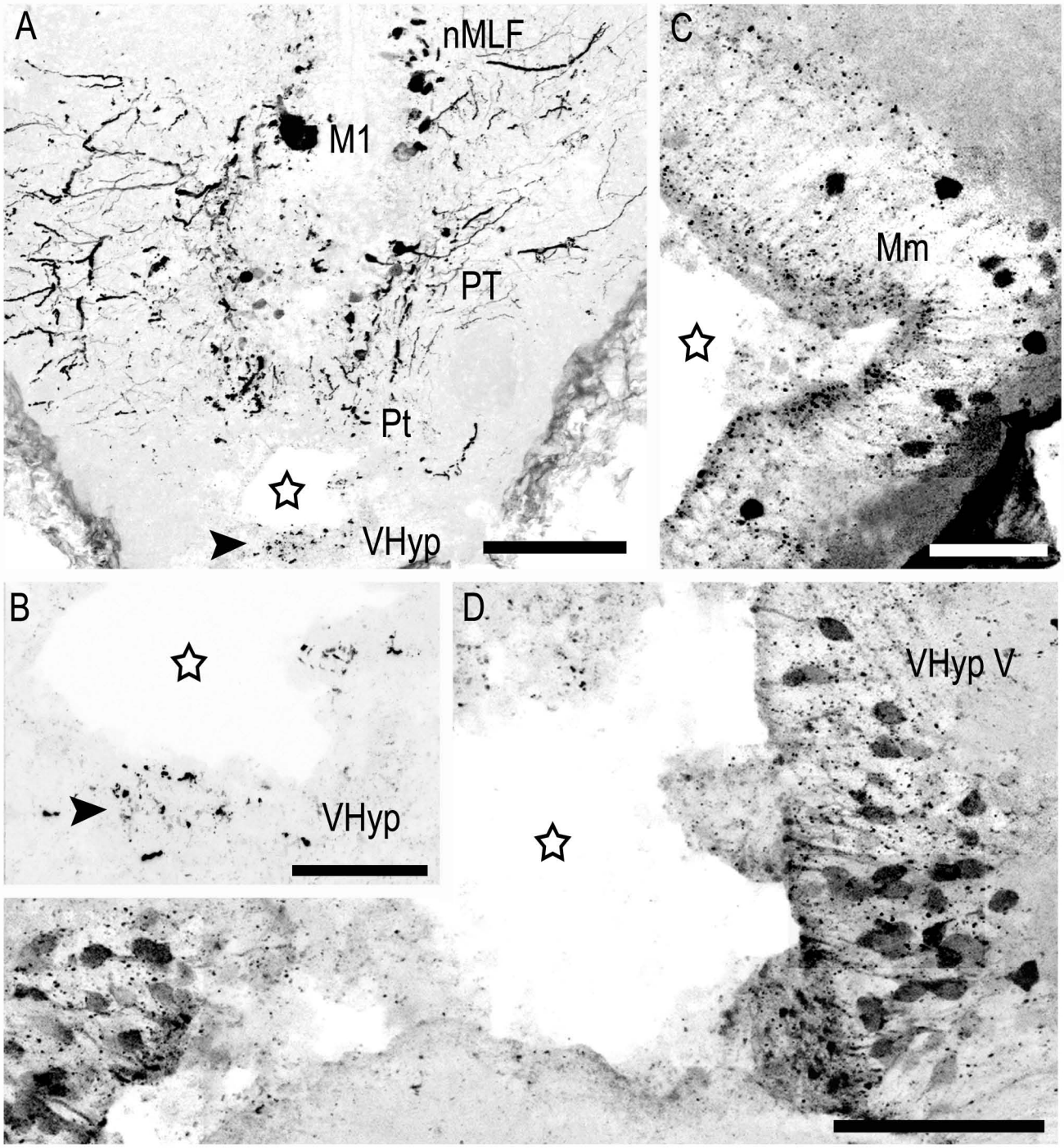


FIGURE 4

Figure 5. A-A': Photomicrographs of a sagittal section of the caudal diencephalon showing cells and descending fibers labeled with NB (green channel) applied in the spinal cord after 1 day of incubation, only the large fibers were labeled with HRP (magenta channel). **B**: Photomicrograph of a sagittal section of the diencephalon of the same larval sample of figure A-A' showing NB labeled cells in the mammillary and ventral hypothalamic nuclei. Note that HRP (magenta channel) did not label any cell or fiber in the lamprey diencephalon. **C**: Photomicrographs of a transverse section of the rostral diencephalon showing the location of spinal-projecting cells of the medial diencephalic and ventral hypothalamic nuclei labeled with NB (green channel) with respect to the dopaminergic (magenta channel) paraventricular organ, which is located in the dorsal hypothalamus. The arrows indicate a large fiber labeled with NB and HRP in the spinal cord. The arrowheads indicate cells labeled with NB in the ventral hypothalamus. For abbreviations see list. Scale bars: 100 μm in A/A' and B, 50 μm in C.

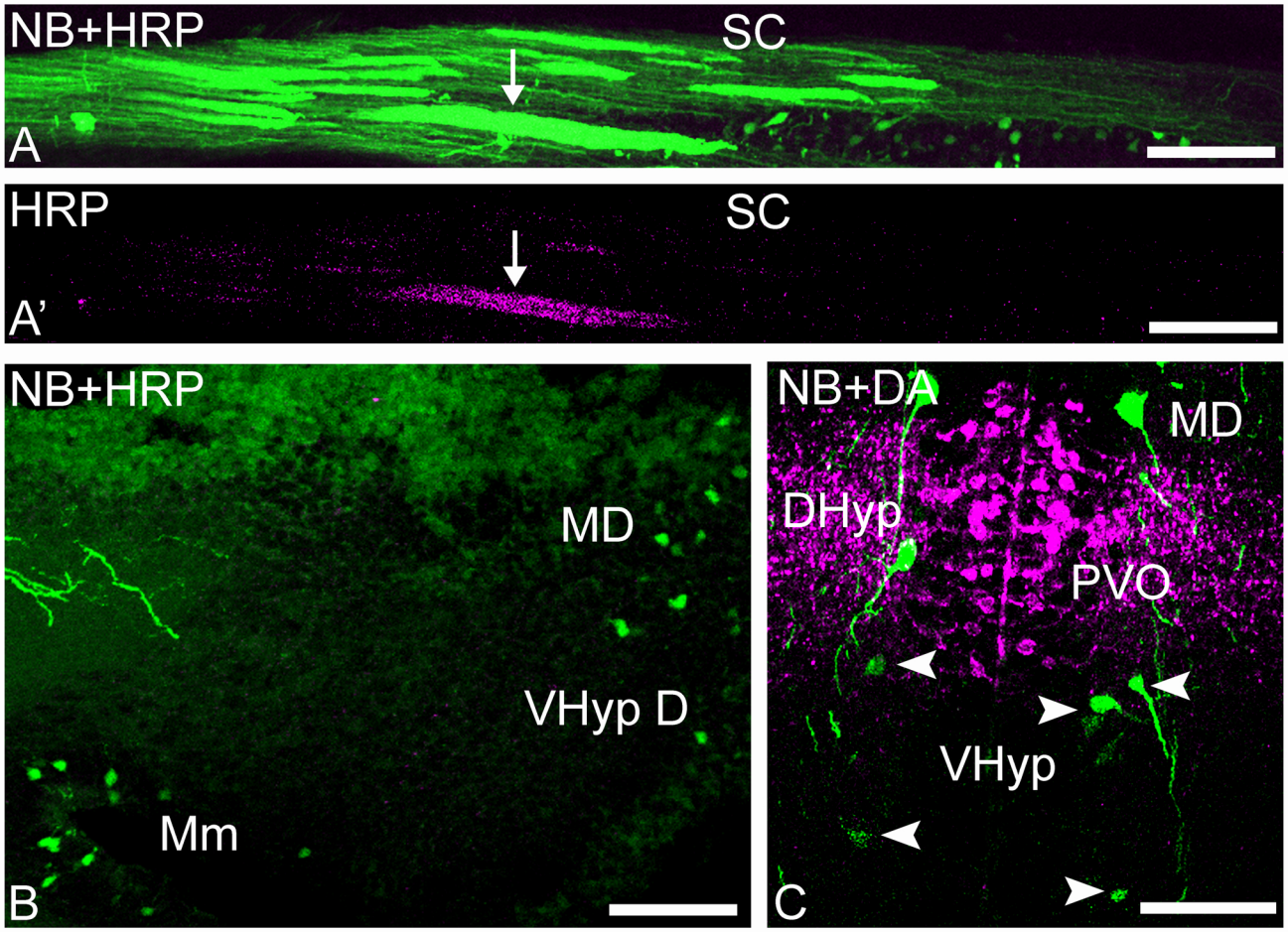


FIGURE 5

Figure 6. A-A'' and **B-B''**: Photomicrographs of sagittal sections showing dopaminergic cells (magenta channel) located in the ventral midline of the rostral spinal cord (A) and caudal rhombencephalon (B) labeled with NB (green channel) applied in the rostral spinal cord. **C-C''**: Photomicrographs of sagittal brain sections showing dopaminergic spinal cord projecting cells of the paratubercular nucleus. The arrows indicate double labeled neurons**.** Curved arrows indicate non-CSF-cells of the ventral midline of the spinal cord or caudal rhombencephalon. The outlined stars indicate the ventricle (B) and the central canal (A). For abbreviations see list. Scale bars: 75 μm in A-A'', 50 μm in B-B'' and 25 μm in C-C''.

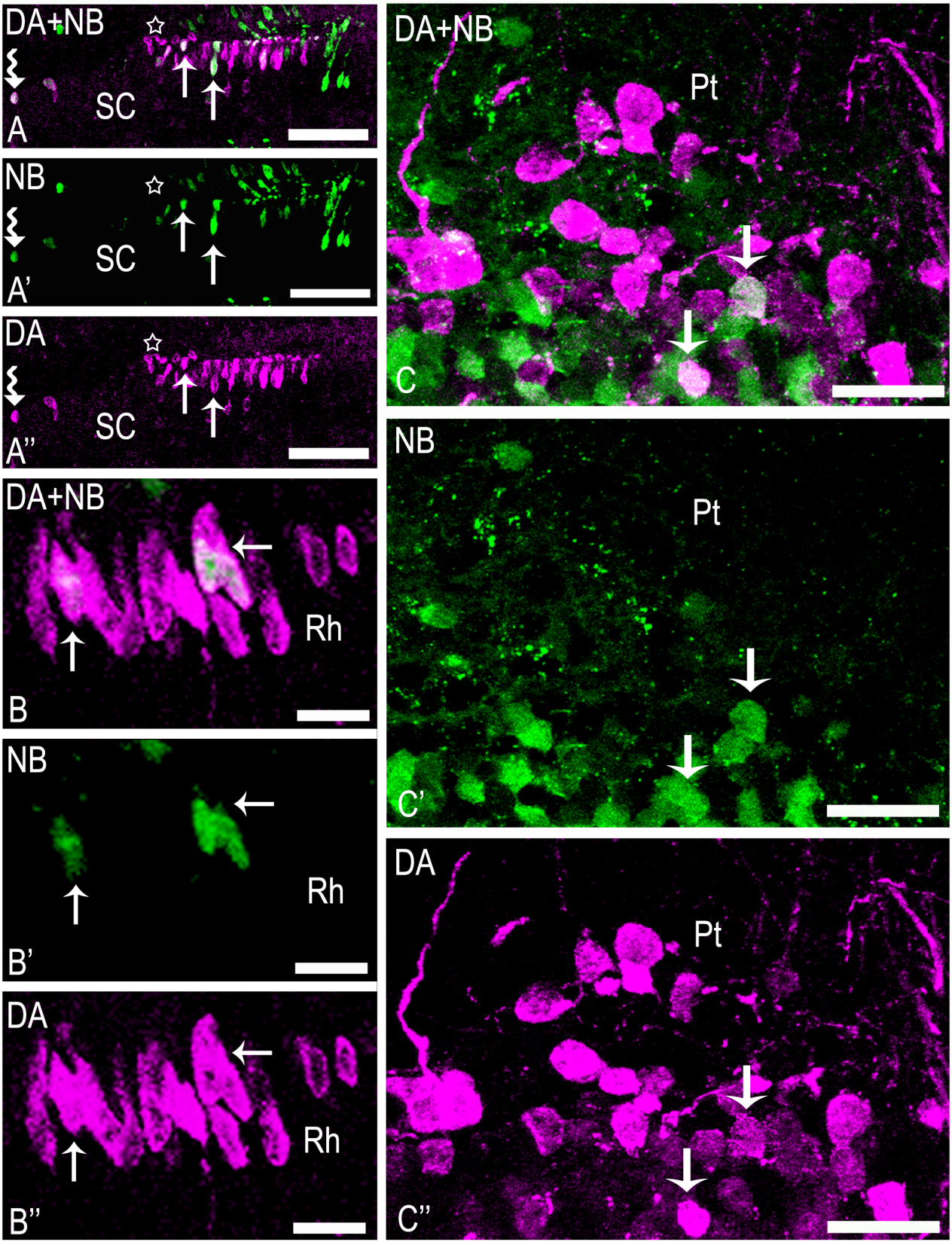

DA+NB
SC
A
NB
SC
A'
DA
SC
A''
DA+NB
Rh
B
NB
Rh
B'
DA
Rh
B''
DA+NB
Pt
C
NB
Pt
C'
DA
Pt
C''

FIGURE 6

Figure 7. Photomicrographs of sagittal sections of the caudal hypothalamus showing the presence of dopaminergic cells (magenta channel) of the mammillary nucleus labeled with NB (green channel) applied in the rostral spinal cord in larval (A) and adult (B) lampreys. The arrows indicate double labeled neurons. The arrowheads indicate the descending dopaminergic plexus in the caudal hypothalamus, which probably arises from the mammillary nucleus. The outlined stars indicate the ventricle. For abbreviations see list. Scale bars: 50 μm in A and B.

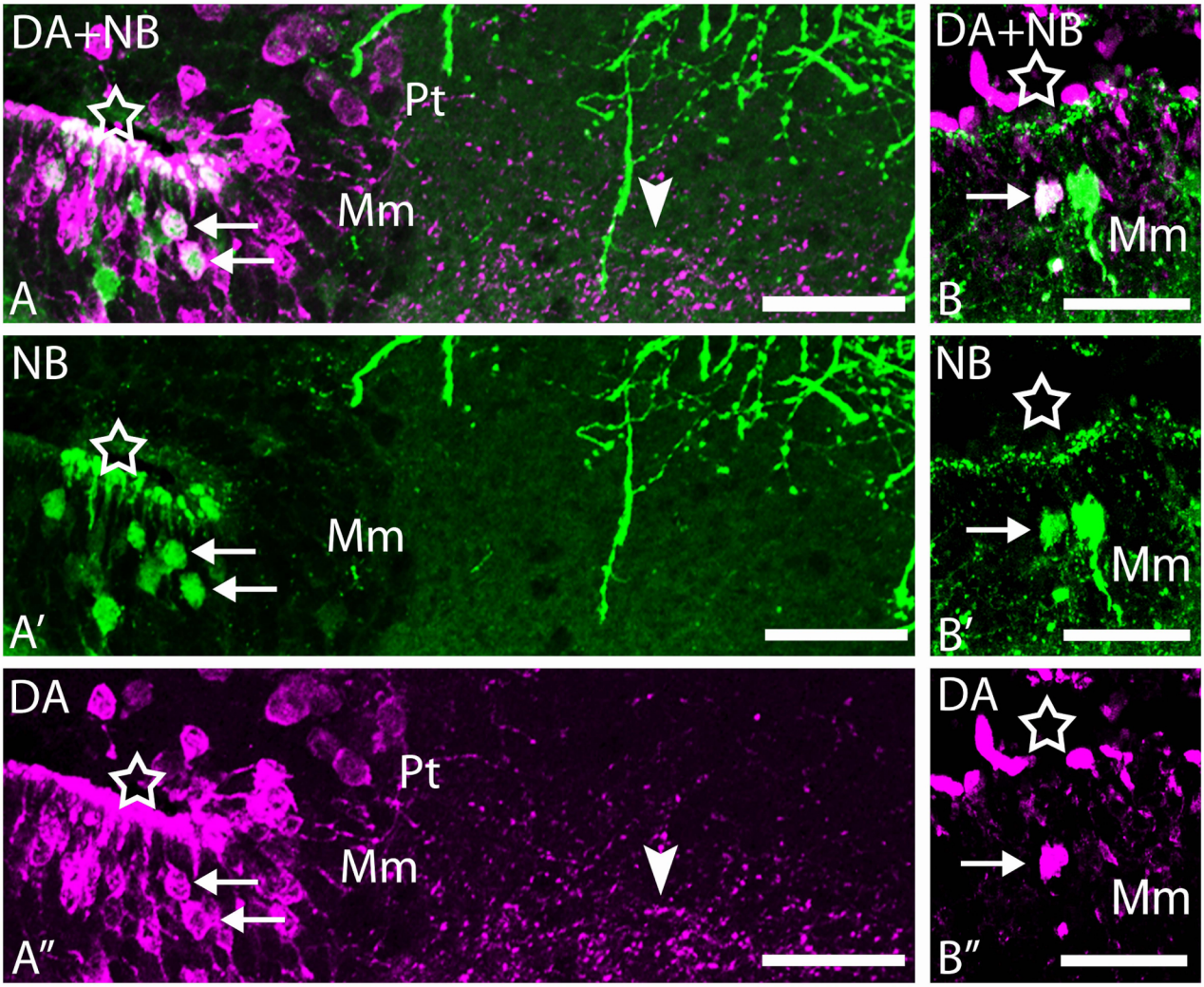


FIGURE 7

Development and organization of the descending serotonergic brainstem-spinal projections in the sea lamprey

Introduction

Serotonin plays a role in the production and modulation of locomotory rhythms in vertebrates (Barbeau and Rossignol, 1991; Cazalets et al., 1992; Schmidt and Jordan, 2000; Gordon and Whelan, 2006). In lamprey, 5-HT is also known to modulate the locomotion generator network (Harris-Warrick and Cohen, 1985; Schotland and Grillner, 1993; Zhang et al., 1996). Intrinsic serotonergic neurons are present in the lamprey spinal cord (Zhang et al., 1996; Abalo et al., 2007), and two rhombencephalic nuclei (one within the posterior reticular nucleus of the rhombencephalon and another rostral to the trigeminal motor nucleus) provide serotonergic innervation to the spinal cord in the adult river lamprey, *Lampetra fluviatilis* (Brodin et al., 1986). Furthermore, these serotonergic nuclei have been observed in early developmental stages of the sea lamprey life cycle (Abalo et al., 2007). However, no studies have been made of the development and organization of these descending serotonergic projections in larval lampreys. The lifestyles of larval and adult lampreys differ greatly (see Hardisty and Potter, 1971), and therefore it may be interesting to discover whether the organization of the descending projections differs between these two stages. Moreover, after the pioneering study by Brodin et al. (1986), the serotonergic isthmic group was shown to be far more complex than previously thought (Pierre et al., 1992; Abalo et al., 2007). Study of the projection patterns of this serotonergic nucleus may therefore provide further information about the organization of this nucleus.

Accordingly, the aims of the present investigation were: 1) To identify the subgroups of the serotonergic isthmic group from which spinal projections originate, 2) to compare the pattern of serotonergic projections in larval and adult sea lampreys, in order to determine when the adult pattern of projections becomes established, and 3) to compare the spinal serotonergic projections of the sea lamprey and other vertebrates.

Material and Methods

Animals

Larval (n=35) and upstream migrating adult (n=3; between 90 and 100 cm in body length) sea lampreys, *Petromyzon marinus* L., were used. The larvae were collected from the

river Ulla (Galicia, Spain) and the adult specimens were bought from local fishermen. Since the age of larval samples was not known, total larval length was used as an indirect estimation of the larval age (Beamish and Medland, 1988; Quintella et al., 2003). The larvae were classified in terms of their body length into three groups: small (10-30 mm; first year), medium-sized (45-90 mm; first-2nd year) and large (110-135 mm; 3rd year), in accordance with the results of a previous study of the serotonergic system development by our group (Abalo et al., 2007). Before all experiments, the animals were deeply anesthetized with 0.05% benzocaine (Sigma, St. Louis, MO) in fresh water. The experiments were approved by the Ethics committee of the University of Santiago de Compostela and comply with European Community rules on animal care and experimentation.

Retrograde tracing

Retrograde tracers used were: biotinylated dextran amine (BDA, of 3,000 Da molecular weight; Molecular Probes, Eugene, OR) or neurobiotin (NB, of 322.8 Da molecular weight; Vector Laboratories, Burlingame, CA). The larval spinal cord was exposed by a transverse incision made in the dorsal region of the body at the level of the third gill. The recrystallized tracer was applied in the rostral spinal cord (at the level of the second branchial arch) with the aid of a minute pin (00). In three larvae the tracer was applied only on the right side of the spinal cord in order to determine the laterality of the descending projections. To avoid diffusion of the tracer when it was applied to hemisectioned spinal cords, the left side was covered with tissue glue (Histoacryl, B. Braun Surgical). The incision was also closed with tissue glue. The animals were maintained at 4ºC with appropriate ventilation, in lamprey Ringer solution of the following composition: 137 mM NaCl, 2.9 mM KCl, 2.1 mM $CaCl_2$, 2 mM HEPES, for 4 days in the case of the application of BDA and 2 days in the case of the application of NB, to allow transport of the tracer from the injection point to the neuronal somata. The transport times used were obtained on the basis of the results of control experiments performed to test the optimal transport time for both tracers in larval and adult lampreys.

The adult lampreys were deeply anesthetized before their brains and rostral spinal cords were dissected out, and BDA or NB crystals were inserted with the aid of a minute pin (00) in the rostral spinal cord at the level of the second branchial arch. In the case of adult animals the brain was dissected out because of the complexity of maintaining mature adult migrating lampreys alive in the laboratory. The brains were maintained at 4ºC in lamprey Ringer solution, for 5 days in the case of application of BDA, and 3 days for NB, to allow transport of the tracer from the

injection point to the neuronal somata. The adult brain/spinal cord is longer than in the larvae and the transport of tracers takes longer.

Immunofluorescence

After the incubation period, the larval heads and the adult brains were fixed by immersion in freshly prepared 4% paraformaldehyde in 0.4 M Tris buffer pH 7.4 (TBS) for 6 hours. The samples were rinsed in TBS, then cryoprotected with 30% sucrose in TBS, embedded in Tissue Tek (Sakura, Torrance, CA), frozen in liquid nitrogen-cooled isopentane, and cut serially on a cryostat (20 µm thick) in either transverse or sagittal planes. Sections were mounted on subbed glass slides.

Sections were incubated with a rabbit polyclonal anti-serotonin antibody (dilution 1:2,500; Incstar, Still Water, MN; code 200800; lot. 050017) at 4ºC for 72h. For indirect immunofluorescence detection of serotonin, rhodamine isothiocyanate (RITC)-conjugated swine anti-rabbit antibody (dilution 1:30; Dako, Glostrup, Denmark) was used (incubation time: 1h at room temperature). All antibodies were diluted in TBS containing 10% normal swine serum and 0.2% Triton X-100 as detergent.

For indirect detection of the tracer the sections were incubated at room temperature for 4h with fluorescein isothiocyanate (FITC)-labeled avidin D (Vector Laboratories, Burlingame, CA) previously diluted to 1:1000 with TBS containing 0.3% Triton X-100. The sections were rinsed in distilled water then coverslipped with mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA).

Photography, cell counts and measurements

Photomicrographs were taken with a spectral confocal laser microscope (Leica TCS-SP2). Data was acquired in the spectral confocal microscope by use of a narrow window tuned to the specific emission wavelength of each fluorescent marker (FICT, RICT). The photomicrographs were adjusted for brightness and contrast with Adobe Photoshop 7.0 software.

To estimate size of serotonergic spinal-cord projecting cells the minor axes of 20 perikarya from each cell group in each larval group were measured with the aid of the Leica confocal software (LCS) in transversal sections. Mean values and SD are given for each cell group.

Controls

The specificity of the anti-serotonin antibody was tested by the supplier, who reported typical 5-HT pattern immunostaining in rat suprachiasmatic nucleus and spinal cord with this antibody. The supplier also reported no detectable cross-reactivity with tryptamine, 5-methoxytryptamine, L-

tryptophan, 5-hydroxytryptophan, dopamine, norepinephrine or adrenaline. Staining was completely abolished after pre-adsorption of the diluted antibody with a 5-HT-BSA conjugate, whereas Western blotting of lamprey brain protein extracts with this anti-serotonin antibody indicated that it does not recognize native brain proteins (Villar-Cerviño et al., 2006). This anti-serotonin antibody has also been used in recent studies of the sea lamprey brain (Antri et al., 2006; Abalo et al., 2007). As a further control, some sections were processed as above but with either the primary antibody omitted or the primary rabbit anti-serotonin antiserum replaced by the corresponding rabbit preimmune serum (Incstar). No immunostaining was observed in any of the corresponding sections.

Some sections of control larval heads were used to confirm the absence of detectable endogenous brain biotin, which may display affinity for FITC-labeled avidin-D. No immunostaining was observed in these controls.

Nomenclature

The nomenclature adopted for the various brainstem serotonergic nuclei was that used in a previous developmental study carried out in the same laboratory (Abalo et al., 2007). For rhombencephalic reticular nuclei the nomenclature of Ronan (1989) and Davis and McClellan (1994) was adopted. For brainstem regions the nomenclature of Villar-Cerviño et al. (2008) was adopted.

Results

Cytoarchitecture and development of the serotonergic projecting nuclei to the spinal cord in larval lamprey

After application of the tracer in the larval spinal cord, double labeled 5-HT-immunoreactive (5-HT-ir) cells (5-HT-ir neurons which project to the spinal cord) were observed in two serotonergic brain nuclei: the isthmic (IS) (Figs. 1, 3A and 3B) and the vagal groups (VG) (Figs. 2 and 3C) described by Abalo et al. (2007). These serotonergic nuclei correspond to brainstem groups II and III, as described by Antri et al. (2006). Schematic drawings showing the organization of the IS and VG nuclei are shown in Figures 1A and 4. No differences in the organization of double labeled cells in each nucleus were observed after the application of BDA or NB. High variability in the number of serotonergic reticulospinal cells labeled with the tracers was observed among the different larvae examined (e.g. in the VG, between 25% and 53% of cells were labeled in the different samples studied). In spite of this high variability, the number of serotonergic double labeled cells in the VG was always higher than in the IS in all the larvae studied. From early larval stages (10 mm in body length) onwards, 5-HT-ir spinal cord-projecting cells were observed in both nuclei.

The sizes of serotonergic reticulospinal and non-reticulospinal cells of the MIS and VG for each larval group and adult lampreys are shown in Table 1.

		MIS	VG
Small larvae	Reticulospinal cells	15.16 ± 1.53	13.77 ± 1.60
	Non-reticulospinal cells	10.60 ± 1.34	14.07 ± 1.60
Medium-sized larvae	Reticulospinal cells	15.08 ± 1.61	13.95 ± 1.54
	Non-reticulospinal cells	11.66 ± 1.78	14.10 ± 1.18
Large larvae	Reticulospinal cells	16.14 ± 1.58	15.74 ± 1.87
	Non-reticulospinal cells	12.22 ± 1.36	15.83 ± 2.77
Adult lamprey	Reticulospinal cells	16.24 ± 2.07	15.62 ± 1.90
	Non-reticulospinal cells	13.83 ± 2.08	16.76 ± 1.55

Table 1. The table shows the sizes (in μm) of the serotonergic reticulospinal and non-reticulospinal cells of the MIS and VG during the larval period and in adult lampreys. Mean and SD is given for each cell group.

The immunofluorescence staining revealed the same larval organization of the IS and VG 5-HT-ir nuclei, as previously reported (Abalo et al., 2007). The IS consists of three subgroups: dorsal (DIS), medial (MIS) and ventral (VIS). The DIS is situated near the dorsal isthmic commissure and is mainly formed by small 5-HT-ir neurons; this subgroup was first observed in medium sized larvae (about 65 mm). The MIS consists of neurons of various sizes, located around the intermedioventral ventricular sulcus. The VIS consists of small neurons located in the ventral periventricular cell mantle. Both MIS and VIS are recognized from the beginning of the larval period. The VG serotonergic nucleus varies in size and is situated in the ventral periventricular cell mantle at the same transversal level as the vagal motor nucleus. This nucleus is also recognizable in the earliest larvae.

After injection of tracer in the spinal cord, only two of the three subdivisions of the IS, the MIS and VIS, displayed double labeled cells (5-HT-ir reticulospinal cells). No double labeled 5-HT-ir cells were observed in the dorsal subgroup. Most 5-HT-ir reticulospinal cells in the isthmus were observed in the MIS (Figs. 1B, 1C, 1D, 3A and 3B). The largest isthmic Müller reticulospinal cell (I1) served as a reference to describe the MIS since this cell was labeled in all tracer applications to the spinal cord. Most of the 5-HT-ir reticulospinal cells of the MIS were observed caudal to the I1 (Figs. 1A, 1B and 3A), but a few double labeled cells were observed just rostral to it (Figs. 1C, 1D and 3B). In the VIS, only occasional 5-HT-ir cells were double labeled (Fig. 1D), indicating that most of these cells do not have spinal projections or that they only project to spinal cord regions rostral to the tracer application site. The descending 5-HT-ir axons of the IS coursed in the marginal layer of the rhombencephalon and spinal cord without forming any defined tract (Fig. 3B). This organization of the spinal projecting 5-HT-ir

cells of the IS was observed from the beginning of the larval period.

The 5-HT-ir VG is situated in the caudal rhombencephalon among a rather large amount of reticulospinal cells. Double labeling experiments showed that only a portion of the 5-HT-ir were labeled from the spinal cord, and that the majority of these reticulospinal cells were situated in the caudal portion of the nucleus (Figs. 1A, 2A, 2B and 3C). The processes of these cells appeared to form a diffuse tract situated ventrolaterally to the cell soma (Figs. 2B and 3C). This organization of the spinal cord projecting cells in the VG was observed from the beginning of the larval period.

As regards the laterality of the serotonergic reticulospinal projections, in experiments involving a spinal hemisection, most of the labeled cells were contralateral to the side of tracer injection, both in the MIS and VG. These groups of 5-HT-ir reticulospinal cells appeared to form part of two larger groups of reticulospinal cells (Fig. 1B-3). These tracer-labeled cell groups probably correspond to the anterior rhombencephalic reticular nucleus (ARRN) (Fig. 4C) and posterior rhombencephalic reticular nucleus (Fig. 4C), respectively, as described by Ronan (1989) and Davis and McClellan (1994) in the brain of larval sea lamprey.

Adult serotonergic projecting nuclei

In upstream migrating adult sea lampreys, both the MIS (Fig. 1E) and VG (Fig. 2C) contained serotonergic cells projecting to the spinal cord (double labeled cells). Study of these adult individuals revealed that the organization of the 5-HT-ir reticulospinal cell groups in the isthmic and vagal regions is the same as observed in the larval stages.

Discussion

Combined tract-tracing and serotonin immunofluorescence was used in the sea lamprey to reveal the organization of the serotonergic cells that project to the spinal cord throughout the larval period. The present results showed that the general organization of the spinal cord-projecting cells in the serotonergic nuclei does not differ in the early larvae and the upstream migrating adults.

Methodological considerations

High variability in the percentage of double labeled 5-HT-ir reticulospinal cells was observed among the different larvae examined. Similar variability in the number of labeled cells in the various neuronal populations projecting to the spinal cord was observed in a previous experimental study in the larval sea lamprey (Swain et al., 1993). The variability in experimental results following application of tracers to the spinal cord suggests that these procedures do not entirely demonstrate the

actual population of afferent neurons, which also implies that not all the serotonergic reticulospinal fibres have taken up the tracers. This probably contributed to the variations in the number of double labeled cells observed in larvae. Despite this, the present results can be considered representative of the organization of the serotonergic reticulospinal neurons in the brain of the larval sea lamprey.

Two serotonergic reticulospinal groups are present in the sea lamprey

The present study reveals that two serotonergic hindbrain nuclei project to the spinal cord in both larval and adult sea lampreys, i.e., the isthmic and the vagal reticular nuclei, which correspond to the "nucleus rostral to the trigeminal motor nucleus" and the "reticular nucleus of the rhombencephalon", respectively, as reported in the adult river lamprey (Brodin et al., 1986). There are similarities and differences between adults of the river lamprey and the sea lamprey as regards the 5-HT-ir reticulospinal populations. No 5-HT-ir reticulospinal cells were observed in the ventral isthmus region of the river lamprey (Brodin et al., 1986), which is in contrast with the present results in the sea lamprey. As in the river lamprey (Brodin et al., 1986), the majority of the serotonergic reticulospinal cells in the sea lamprey were labeled contralaterally with respect to the tracer injection side (present results). These results reveal a similar pattern in lampreys. On the other hand, the present results show that most of the serotonergic MIS reticulospinal cells are located caudal to the I1 isthmic Müller cell, which suggests a previously unreported differential arrangement of the cells in the isthmic nucleus as regards the spinal and brain projections. These results also reveal that the MIS and VG populations consist of reticulospinal and non-reticulospinal serotonergic cells that may form functional groups, owing to the probable similarity of their afferents. As regards the non-reticulospinal serotonergic cells of these nuclei, some of them would give rise to projections ascending to the telencephalon, diencephalon and mesencephalon, as suggested by the findings of Abalo et al. (2007) in developing lamprey. The non-reticulospinal cells in the MIS appear to be smaller on average than the reticulospinal cells, which may be related to the different length of their axons (shorter in those projecting on brain centers), although such a difference was not observed in the VG neurons.

The presence of serotonergic cells with descending projections has been reported in the hindbrain of other vertebrates, but these cells are mainly located in the raphe. In larval zebrafish, a few serotonergic neurons of the inferior raphe region project to the spinal cord, as demonstrated by retrograde labeling combined with immunohistochemistry (McLean and Fetcho, 2004a, b). Spinal projections originated from serotonergic raphe cells have been reported by

immunohistochemistry in some developing amphibians (larval *Xenopus*: van Mier et al., 1986; embryos of *Rana*: Woolston et al., 1994), although the precise arrangement of these cells relative to the other serotonergic raphe cells was not established. Serotonergic descending projections to the spinal cord of turtle were assumed to originate predominantly from the inferior raphe nucleus in an immunohistochemical study (Ueda et al., 1983). Whole mount serotonin immunostaining of rat embryos indicated that descending serotonergic projections to the spinal cord mainly arise from the caudal hindbrain raphe group (Aitken and Törk, 1988). Combined retrograde transport and immunohistochemistry in the adult rat, monkey and cat indicate that large numbers of 5-HT-ir neurons in the medullary raphe nuclear complex (raphe pallidus, raphe obscurus, raphe magnus and adjacent reticular region) project to the spinal cord (Bowker et al., 1981, 1982, 1987; Bowker and Abbott, 1990), whereas those in the central grey and midbrain reticular formation project only to the cervical cord (rat; Bowker et al., 1981) or caudal hindbrain (monkey; Yezierski et al., 1982). This suggests that serotonergic raphe-spinal projections from the caudal medulla are well conserved during the evolution of jawed vertebrates.

In jawed vertebrates (from bony fishes to mammals) hindbrain serotonergic neurons are mainly located in the midline (raphe nuclei), although a number of serotonergic cells that vary between species migrate in the reticular region ventrolateral to the raphe nuclei. However, all of them have the same origin in the hindbrain basal plate close to the floor plate (Wallace and Lauder, 1992). In lamprey, hindbrain serotonergic cells have a similar origin close to the floor plate, but migrate laterally to the ventral midline, lacking raphe nuclei (Abalo et al., 2007). This suggests that raphe nuclei are a shared feature of jawed vertebrates acquired after divergence from agnathans. In spite of the different final location of the hindbrain serotonergic cells in lampreys, the embryonic origin and rostrocaudal organization of these serotonergic cells in two rhombencephalic cell groups (caudal and rostral), together with the presence of descending projections to the spinal cord suggest that serotonergic reticular nuclei of lampreys are homologues of the caudal and rostral parts of the serotonergic raphe/reticular complex of jawed vertebrates, respectively. However, lampreys exhibit more conspicuous serotonergic spinal projections from the rostral (isthmus) populations than jawed vertebrates.

Development of the serotonergic projections to the spinal cord

The results of the present study show for the first time that the serotonergic spinal projections from the IS and VG were already established at the beginning of the larval period. The early development of the spinal cord projections from brainstem cells has also

been reported in different vertebrate groups: teleosts (Brustein et al., 2003; McLean and Fetcho, 2004a, b), amphibians (van Mier et al., 1986; Woolston et al., 1994), birds (Okado et al., 1992) and mammals (Rajaofetra et al., 1989; Ballion et al., 2002). Comparison between different vertebrate species shows that the pattern of development of the serotonergic descending projection is established very early on in both agnathans and gnathostomes.

The early appearance of the descending serotonergic projections suggests that 5-HT plays an early role in the developing sea lamprey spinal cord. Further investigation is required to establish whether this role is developmental, as suggested in other vertebrates (Sillar et al., 1995; Rubenstein, 1998; Gaspar et al., 2003; Brustein et al., 2003), or whether serotonin plays a role in early modulation of locomotion, as reported in adult lampreys (Buchanan and Grillner, 1991; Zhang et al., 1996; El Manira et al., 1997; Schwartz et al., 2005), or whether both of these roles are involved.

Conclusions

The results of the present experimental study in the sea lamprey showed that the pattern of serotonergic projections from caudal and rostral reticulospinal nuclei is already established in the earliest larvae studied. The cells in each serotonergic nucleus appear to be topographically organized in function of their projection target. Comparison of present results in lampreys with those previously reported in other vertebrates suggests that the hindbrain serotonergic nuclei show a roughly similar developmental pattern, with rostral and caudal groups probably appearing before the separation of agnathans and gnathostomes. However, the early migration behavior of these nuclei (tangentially towards the periventricular reticular regions or ventrally close to the raphe) differs greatly in lampreys and jawed vertebrates. Moreover, the lateroventral migration from the embryonic raphe region of the serotonergic reticular nuclei observed in many jawed vertebrates is probably a secondary feature divergent from that observed in lampreys. In addition, proportionally many more rostral hindbrain serotonergic cells appear to contribute to the spinal serotonergic projections in the sea lamprey than in the jawed vertebrates.

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.

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.

Ballion B, Branchereau P, Chapron J, Viala D. 2002. Ontogeny of descending serotonergic innervation and evidence for intraspinal 5-HT neurons in the mouse spinal cord. Dev Brain Res 137: 81-88.

Barbeau H, Rossignol S. 1991. Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic and dopaminergic drugs. Brain Res 546: 250-260.

Beamish FWH, Medland TE. 1988. Age determination for lampreys. Trans Amer Fish Soc 117: 63-71.

Bowker RM, Abbott LC. 1990. Quantitative re-evaluation of descending serotonergic and non-serotonergic projections from the medulla of the rodent: evidence for extensive co-existence of serotonin and peptides in the same spinally projecting neurons, but not from the nucleus raphe magnus. Brain Res 512: 15-25.

Bowker RM, Reddy VK, Fung SJ, Chan JY, Barnes CD. 1987. Serotonergic and non-serotonergic raphe neurons projecting to the feline lumbar and cervical spinal cord: a quantitative horseradish peroxidase-immunocytochemical study. Neurosci Lett 75: 31-37.

Bowker RM, Westlund KN, Coulter JD. 1981. Origins of serotonergic projections to the spinal cord in rat: an immunocytochemical-retrograde transport study. Brain Res 226: 187-199.

Bowker RM, Westlund KN, Coulter JD. 1982. Origins of serotonergic projections to the lumbar spinal cord in the monkey using a combined retrograde transport and immunocytochemical technique. Brain Res Bull 9: 271-278.

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.

Brustein E, Chong M, Holmqvist B, Drapeau P. 2003. Serotonin patterns locomotor network activity in the developing zebrafish by modulating quiescent periods. J Neurobiol 57: 303-322.

Buchanan JT, Grillner S. 1991. 5-Hydroxytryptamine depresses reticulospinal excitatory postsynaptic potentials in motoneurons of the lamprey. Neurosci Lett. 122 : 71-74.

Cazalets JR, Sqalli-Houssaini Y, Clarac F. 1992. Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. J Physiol 455 : 187-204.

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.

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

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.

Harris-Warrick RM, Cohen AH. 1985. Serotonin modulates the central pattern generator for locomotion in the isolated lamprey spinal cord. J Exp Biol 116: 27-46.

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

Gordon IT, Whelan PJ. 2006. Monoaminergic control of cauda-equina-evoked locomotion in the neonatal mouse spinal cord. J Neurophysiol 96: 3122-3129.

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.

Okado N, Sako H, Homma S, Ishikawa K. 1992. Development of serotoninergic system in the brain and spinal cord of the chick. Prog Neurobiol 38: 93-123.

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.

Quintella BR, Andrade NO, Almeida PR. 2003. Distribution, larval stage duration and growth of the sea lamprey ammocoetes, *Petromyzon marinus* L., in a highly modified river basin. Ecol Freshwater Fish 12：286-293．

Rajaofetra N, Sandillon F, Geffard M, Privat A. 1989. Pre- and post-natal ontogeny of serotonergic projections to the rat spinal cord. J Neurosci Res 22: 305-321.

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

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

Schmidt BJ, Jordan LM. 2000. The role of serotonin in reflex modulation and

locomotor rhythm production in the mammalian spinal cord. Brain Res Bull 53: 689-710.

Schotland JL, Grillner S. 1993. Effects of serotonin on fictive locomotion coordinated by a neural network deprived of NMDA receptor-mediated cellular properties. Exp Brain Res 93: 391-398.

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.

Sillar KT, Woolston AM, Wedderburn JF. 1995. Involvement of brainstem serotonergic interneurons in the development of a vertebrate spinal locomotor circuit. Proc R Soc Lond B 259: 65-70.

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.

Ueda S, Takeuchi Y, Sano Y. 1983. Immunohistochemical demonstration of serotonin neurons in the central nervous system of the turtle (*Clemmys japonica*). Anat Embryol 168: 1-19.

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.

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.

Villar-Cerviño V, Barreiro-Iglesias A, Anadón R, Rodicio MC. 2008. Distribution of glycine immunoreactivity in the brain of adult sea lamprey (*Petromyzon marinus*). Comparison with gamma-aminobutyric acid. J Comp Neurol 507: 1441-1463.

Wallace JA, Lauder JM. 1992. Development of the serotonergic system in rat and chick embryos. In: Björklund A, Hökfelt T, Toyama M. (Eds.), Handbook of Chemical Neuroanatomy, vol. 10: Ontogeny of Transmitters and Peptides in the CNS. Elsevier, Amsterdam, pp. 619-645.

Woolston AM, Wedderburn JF, Sillar KT. 1994. Descending serotonergic spinal projections and modulation of locomotor rhythmicity in *Rana temporaria* embryos. Proc R Soc Lond B 255: 73-79.

Yezierski RP, Bowker RM, Kevetter GA, Westlund KN, Coulter JD, Willis WD. 1982. Serotonergic projections to the

caudal brain stem: a double label study using horseradish peroxidase and serotonin immunocytochemistry. Brain Res 239: 258-264.

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.

Abbreviations

5-HT-ir	Serotonin immunoreactive
ARRN	Anterior rhombencephalic reticular nucleus
BDA	Biotinylated dextran amine
D	Diencephalon
DCN	Dorsal column nucleus
DIS	Dorsal isthmic subgroup
DN	Dorsal nucleus of the octavolateral area
Hyp	Hypophysis
III	Oculomotor nucleus
I1	Largest isthmic Müller reticulospinal cell
IS	Isthmic group
Is	Isthmus
IV	Trochlear nucleus
IXm	Glossopharingeal motor nucleus
IP	Interpeduncular nucleus
M	Mesencephalon
MIS	Medial isthmic subgroup
MN	Medial nucleus of the octavolateral area
MRRN	Medial rhombencephalic reticular nucleus
Mth	Mauthner cell
NB	Neurobiotin
ON	Olfactory nerve
P	Pineal organ
Pp	Parapineal organ
PRRN	Posterior rhombencephalic reticular nucleus
Rh	Rhombencephalon

SC	Spinal cord
Sl	Sulcus limitans
SOC	Spinooccipital motor column
TL	Telencephalon
TrN	Trigeminal nucleus
VG	Vagal group
VIIm	Facial motor nucleus
VIS	Ventral isthmic subgroup
VN	Ventral nucleus of the octavolateral area
Vm	Trigeminal motor nucleus
Xm	Vagal motor nucleus

FIGURES
CHAPTER 2.2

Figure 1. The isthmic (IS) serotonergic reticulospinal group of the sea lamprey, as revealed by combined retrograde tract-tracing and immunofluorescence. In all figures rostral is on the left and dorsal is at the top. **A**: Schematic drawing of the brain of a larval sea lamprey in lateral view showing the distribution of serotonergic reticulospinal cell cells in the IS and the vagal region (VG). Green: Müller cells. Red: Double labeled cells. Black: serotonergic cells**. B, C-D**: Sagittal sections through the rostral rhombencephalon of larvae showing double labeled serotonergic cells in the IS after the application of the tracer (BDA in B, NB in C-D) to the spinal cord (red: 5-HT, green: tracer). Note in B the presence of a double labeled cell just rostral to the I1 cell, and the small size of serotonergic cells of the MIS relative to the non-serotonergic reticulospinal cells (green only). In D, note a double labeled cell in the VIS. White arrows indicate double labeled cells. The asterisks indicate the I1 cell. **E**: Sagittal section through the MIS of an adult lamprey showing 5-HT-ir reticulospinal cells (arrow). Scale bars: 100 μm (1B, 1C), 50 μm (1D, 1E). For abbreviations, see list.

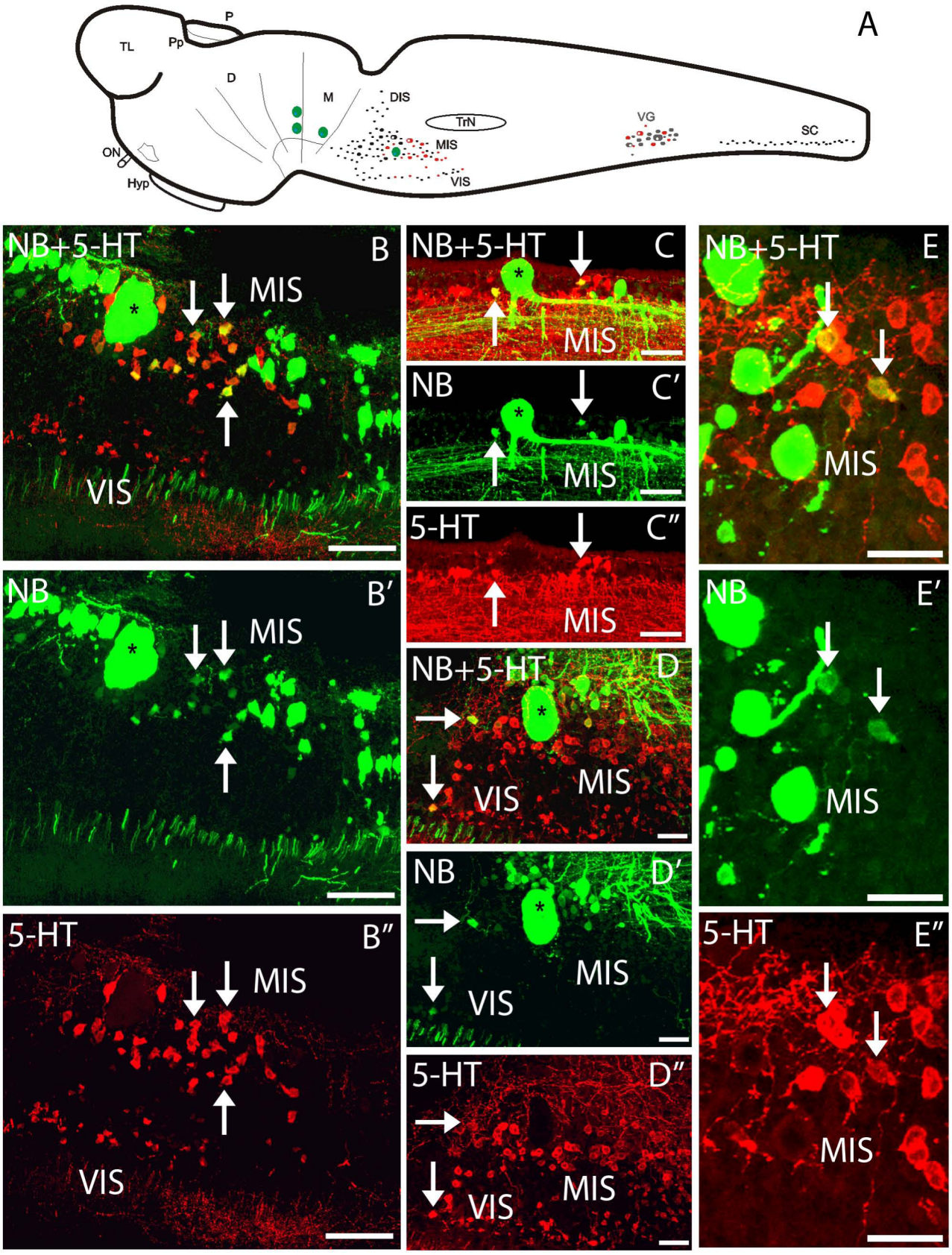

A
P
TL
Pp
D
M
DIS
TrN
VG
SC
MIS
VIS
ON
Hyp
NB+5-HT
B
MIS
VIS
NB
B′
5-HT
B″
C
C′
C″
D
D′
D″
E
E′
E″

FIGURE 1

Figure 2. The serotonergic reticulospinal group of the vagal region, as revealed by combined retrograde tract-tracing and immunofluorescence. **A-B**: Sagittal section of the caudal rhombencephalon showing double labeled serotonergic cells in the VG after application of the tracer (NB in A and BDA in B) in the larval spinal cord (red: 5-HT, green: tracer). White arrows indicate double labeled cells. **C**: Sagittal section showing 5-HT-ir reticulospinal cells in the VG of an adult lamprey. In all figures rostral is on the left and dorsal is at the top. Scale bars: 100 μ m (2A, 2C), 125 μm (2B). For abbreviations, see list.

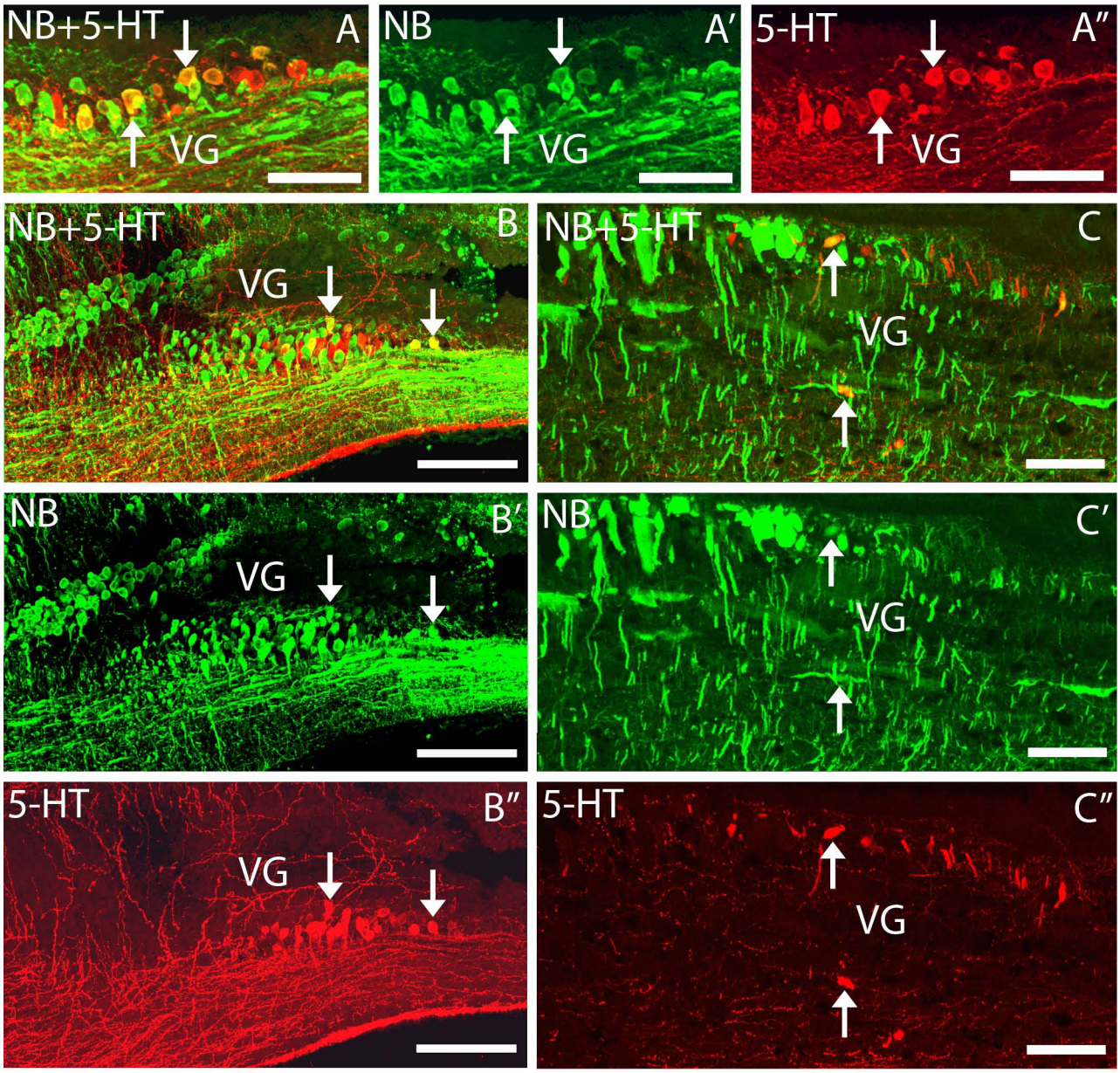

NB+5-HT
A
VG
NB
A′
VG
5-HT
A″
VG
NB+5-HT
B
VG
NB+5-HT
C
VG
NB
B′
VG
NB
C′
VG
5-HT
B″
VG
5-HT
C″
VG

FIGURE 2

Figure 3. The serotonergic reticulospinal groups of the isthmic and vagal regions showed in brain transversal sections. **A-B**: Transversal sections of the rostral rhombencephalon showing double labeled serotonergic cells in the MIS after application of NB in the larval spinal cord (red: 5-HT, green: tracer). The asterisks indicate the I1 Müller cell. **C:** Transversal section of the caudal rhombencephalon showing double labeled serotonergic cells in the VG. White arrows indicate double labeled cells. Scale bars: 50 μm (3A, 3B), 25 μm (3C). For abbreviations, see list.

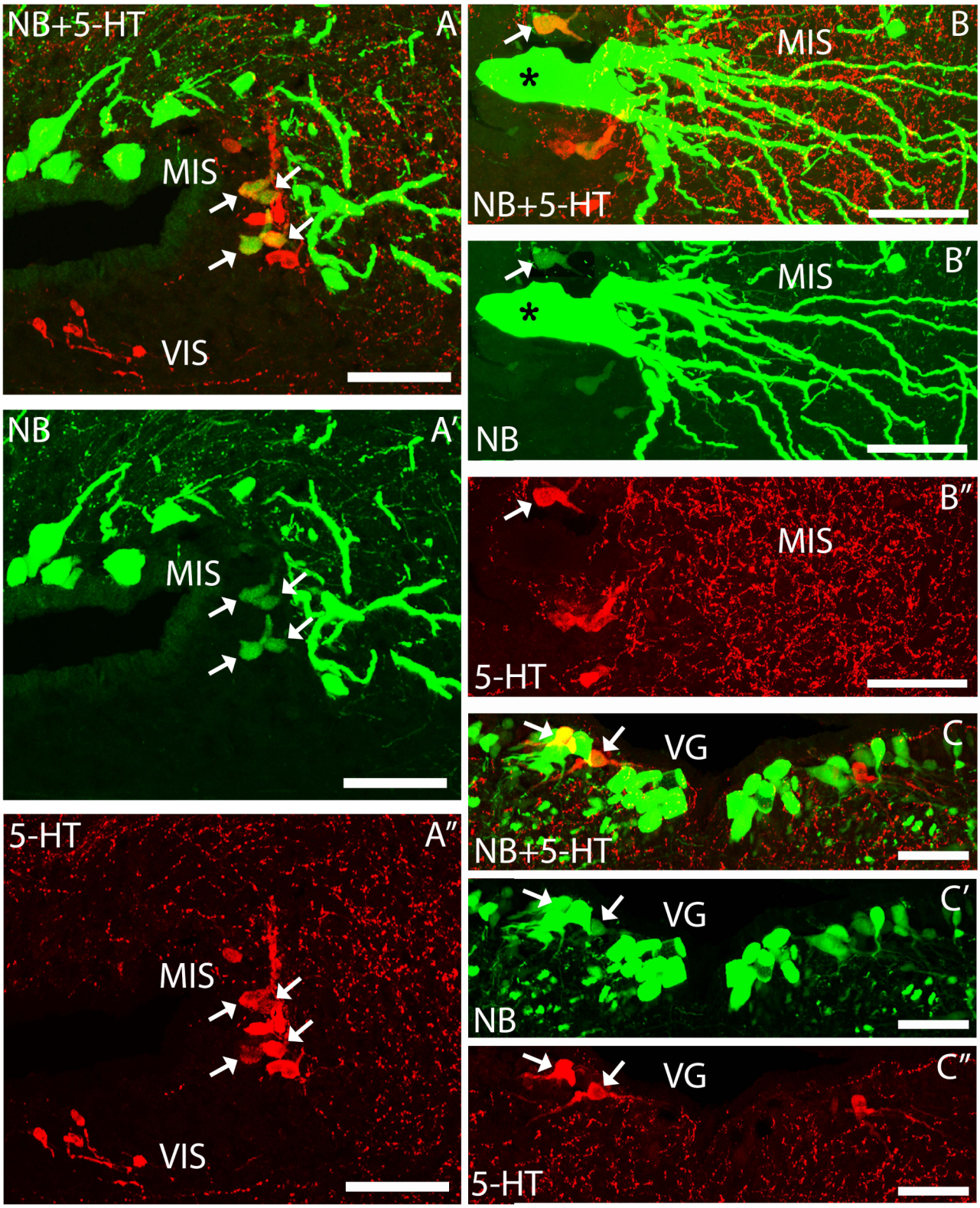

NB+5-HT
A
MIS
VIS
B
MIS
NB+5-HT
B′
MIS
NB
NB
A′
MIS
B″
MIS
5-HT
C
VG
NB+5-HT
5-HT
A″
MIS
VIS
C′
VG
NB
C″
VG
5-HT

FIGURE 3

Figure 4. Schematic drawings of transversal (A and B) and horizontal (C) sections of the brain of a larval sea lamprey showing the distribution of serotonergic reticular cells in the isthmic and the vagal regions. In A and B: black: serotonergic spinal cord projecting cells, grey: serotonergic cells. In C: grey: reticulospinal nuclei, black: reticulospinal serotonergic cells of the MIS and VG. The black lines in C correspond to the cutting point of schematic drawings A and B. For abbreviations, see list.

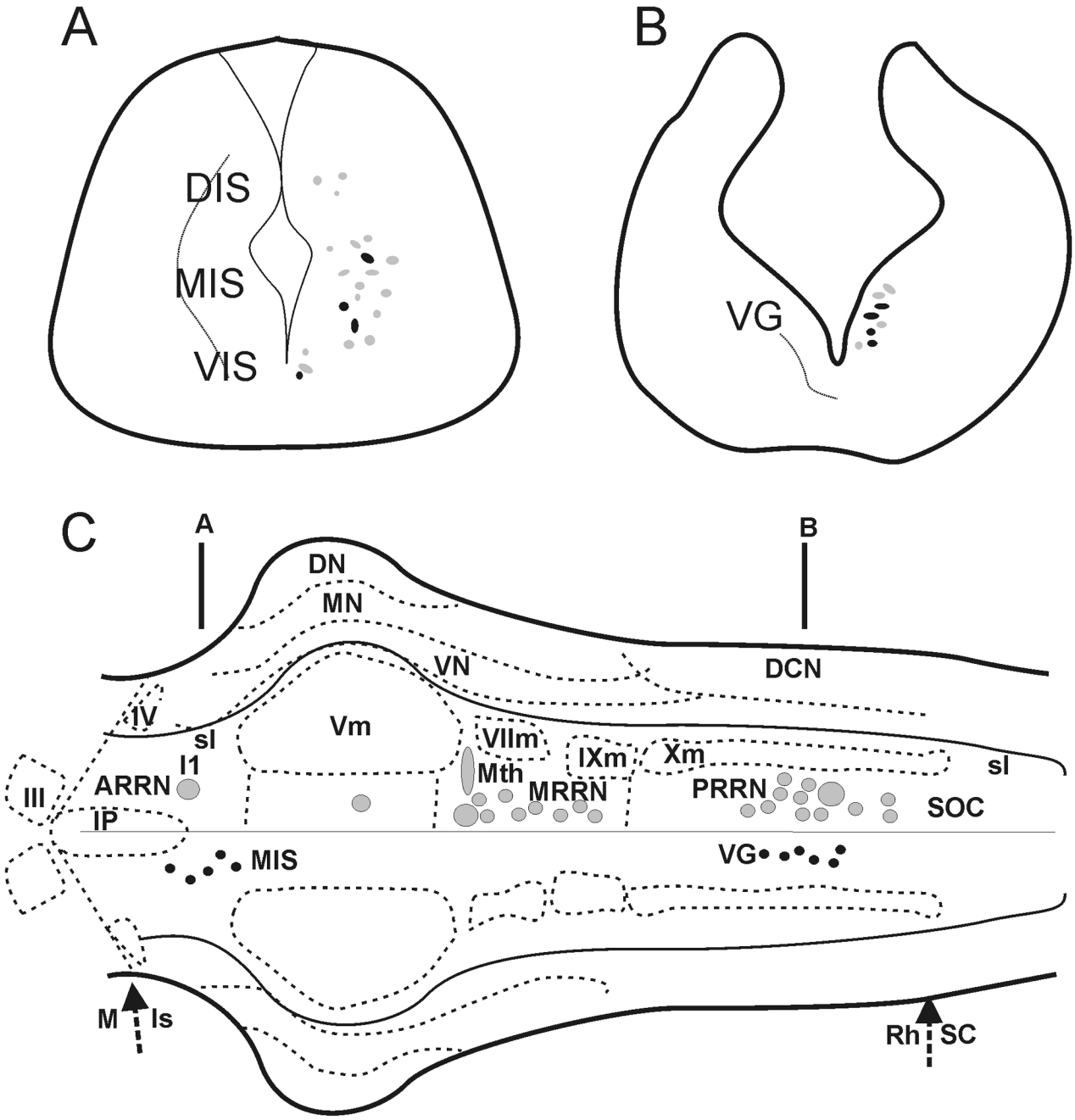
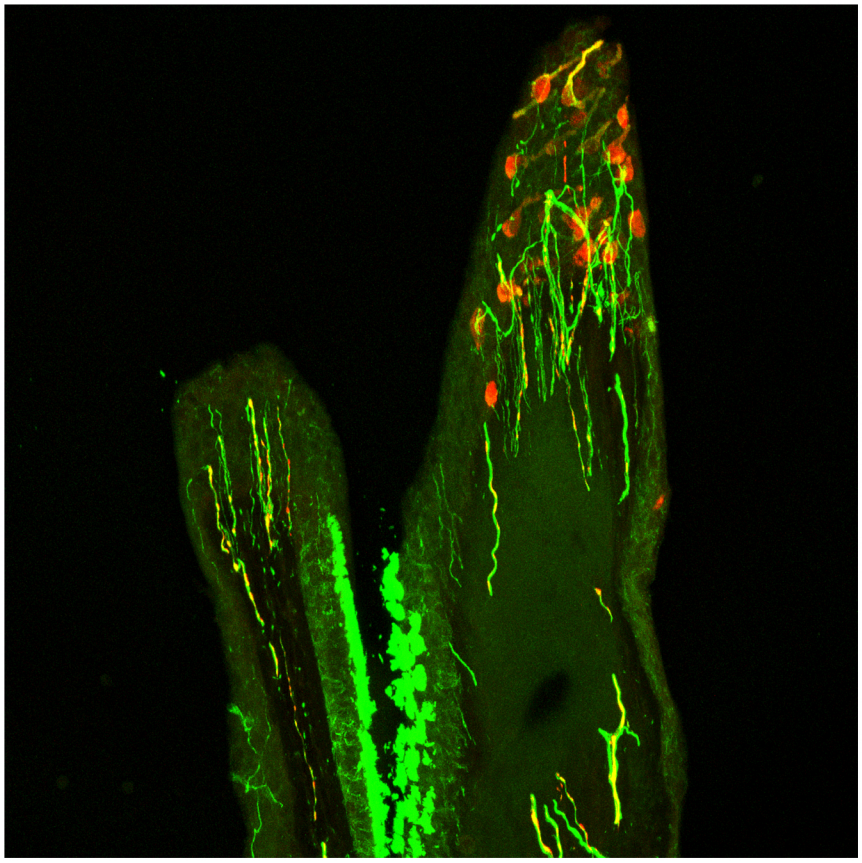

A
DIS
MIS
VIS
B
VG
C
A
B
DN
MN
VN
DCN
IV
sl
Vm
VIIm
IXm
Xm
sl
I1
ARRN
III
IP
Mth
MRRN
PRRN
SOC
MIS
VG
M
Is
Rh
SC

FIGURE 4

RELATIONSHIP WITH OTHER NEUROTRANSMITTER SYSTEMS



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

---

Introduction

The presence of neurons showing immunoreactivity for both serotonin (5-HT) and γ-aminobutyric acid (GABA) [or the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD)] has been reported in a few cells of some raphe nuclei (raphe dorsalis, raphe magnus, raphe obscurus, pontine and supralemniscal nuclei) of the rat brain (Belin et al., 1983; Millhorn et al., 1987; Millhorn et al., 1988; Stamp and Semba, 1995). Uptake of ^{3}H-GABA by some raphe 5-HT-immunoreactive (5-HT-ir) cells has also been demonstrated (Gamrani et al., 1984). Both GABA and 5-HT can be accumulated by fibres of serotonergic systems such as supra- and subependymal fibre plexuses (Harandi et al., 1986). Such reports represented one of the first indications of the coexistence of "canonical neurotransmitters" in neuronal subsystems and contributed to the general acceptance of the concept of cotransmission (reviewed by Hökfelt et al., 2000; Vergé and Calas, 2000). On the other hand, several studies have indicated that 5-HT exerts a modulatory effect on GABAergic neurotransmission by potentiation of the inhibitory response of neurons to GABA (Xu et al., 1998; Wang et al., 1999; Li et al., 2000; An et al., 2005).

The neuroanatomical organization of the GABAergic and serotonergic systems has been studied in the sea lamprey (serotonin: Antri et al., 2006; Abalo et al., 2007; GABA: Meléndez-Ferro et al., 2001, 2002, 2003; Robertson et al., 2007). However, as far as we are aware there are no studies concerning the possible colocalization of GABA and 5-HT in neurons of the lamprey brain. There is also a notable lack of information on the presence of this type of neurochemical cell (serotonergic/GABAergic neurons) in the brain of non-mammalian vertebrates. The phylogenetic position occupied by lampreys between invertebrate chordates and gnathostome vertebrates makes these animals key subjects for understanding the early evolution of the different neurotransmitter systems.

In the present study, double immunofluorescence methods and confocal microscopy were used to investigate the possible co-existence of 5-HT and GABA in

sea lamprey brain neurons. The results indicate for the first time the colocalization of these classical neurotransmitters in lamprey neurons, which were restricted to a few serotonergic populations. Understanding the neuroanatomical organization of serotonergic/GABAergic neurons will provide insights into the co-functions of the neurotransmitters and their possible co-release in lamprey circuits.

Material and Methods

Animals

Larval sea lampreys (n=11; 90-140 mm in total body length) were used. The larvae were collected from the river Ulla (Galicia, Spain). Before all experiments, the animals were deeply anaesthetized with 0.05% benzocaine (Sigma, St. Louis, MO) in fresh water. The experiments were approved by the Ethics Committee of the University of Santiago de Compostela and comply with European Community rules on animal care and experimentation.

Immunofluorescence

The larval heads were fixed by immersion in freshly prepared 2% paraformaldehyde, 2.5% glutaraldehyde and 0.5% sodium metabisulphite in 0.05 M tris buffered saline pH 7.4 (TBS) for 20 hours. The samples were rinsed in TBS containing 1% sodium metabisulphite, then cryoprotected with 30% sucrose in TBS, 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.

Sections were pre-treated with 0.2% $NaBH_4$ in distilled water for 45 minutes at room temperature. The sections were then incubated with a cocktail of a rabbit polyclonal anti-5-HT antibody (dilution 1:2,500; Incstar, Still Water, MN; code 200800; lot. 050017; immunogen: 5-HT-formaldehyde-BSA conjugate) and a mouse monoclonal anti-GABA antibody (dilution 1:1,200; Sigma; clone GB-69; lot. 075K4795; immunogen: purified GABA conjugated to BSA) and for 3 days at 4ºC. Sections were rinsed in TBS and then incubated with a cocktail of rhodamine isothiocyanate (RICT)-conjugated swine anti-rabbit antibody (diluted 1:30; Dako, Glostrup, Denmark) and fluorescein isothiocyanate (FICT)-coupled goat anti-mouse antibody (diluted 1:50; Chemicon, Temecula, CA) for 1 hour at room temperature. All antibody dilutions were prepared in TBS containing 1% sodium metabisulphite, 15% normal goat serum (NGS), 10% normal swine serum (NSS) and 0.2% Triton X-100. Sections were rinsed in distilled water and then coverslipped with mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA).

Controls

The specificity of the anti-5-HT antibody was tested by the supplier, who reported no detectable cross-reactivity with tryptamine, 5-methoxytryptamine, L-tryptophan, 5-hydroxytryptophan, dopamine, norepinephrine or adrenaline. This antibody has been used in recent immunohistochemical studies in the sea lamprey brain and retina (Villar-Cerviño et al., 2006; Abalo et al., 2007) and was previously tested by western blot in lamprey brain protein extracts in our laboratory (Villar-Cerviño et al., 2006). No protein band was detected in these blots. Moreover, the specificity of this antibody was tested in lamprey in pre-adsorption experiments (Abalo et al., 2007); immunostaining was completely abolished after pre-adsorption of the diluted anti-5-HT antibody with 5-HT-BSA conjugates.

The monoclonal anti-GABA antibody was evaluated for activity and specificity by dot-blot immunoassay, by the supplier. No cross-reaction was observed with BSA, L-α-aminobutyric acid, L-glutamic acid, L-aspartic acid, glycine, δ-aminovaleric acid, L-threonine, L-glutamine, taurine, putrescine, L-alanine or carnosine. The antibody showed weak cross-reaction with β-alanine. Furthermore, the sections of the brain and retina of sea lamprey incubated with this antibody revealed the same pattern of immunostaining revealed in studies with other anti-GABA antibodies (Meléndez-Ferro et al., 2001, 2002, 2003; Villar-Cerviño et al., 2006; Robertson et al., 2007). In addition, the antibody was previously tested in our laboratory by western blotting with lamprey brain protein extracts (Villar-Cerviño et al., 2008a). No protein band was stained in these blots. Immunostaining was completely abolished after pre-adsorption of the diluted anti-GABA antibody with a GABA-BSA conjugate (Sigma).

As a further control, some sections were processed as above but without the primary antibody. No immunostaining was observed in any of the corresponding sections.

Photography and cell measurements

Photomicrographs were taken with a laser spectral confocal microscope (Leica TCS-SP2). Data was acquired in the spectral confocal microscope by use of a narrow window tuned to the specific emission wavelength of each fluorescent marker (FICT, RICT). For a review about advantages of spectral confocal microscopy see Zimmermann et al., (2002). Photomicrographs were adjusted for brightness and contrast with Adobe Photoshop CS (8.0.1) software. Schematic drawings were made with Corel Draw 12 software.

To estimate the size of the 5-HT-ir/GABA-ir cells, the minor axes of 15 perikarya from each cell group were measured with the aid of the Leica confocal software (LCS) in transverse sections. Mean values and standard deviations (SD) are given for each cell

group. In the case of the tuberal and pretectal populations, the mean is not given because very few double-labelled cells were observed in the samples. The percentage of 5-HT-ir cells showing GABA immunoreactivity was calculated for those nuclei that showed a high degree of colocalization (the thalamic and ventral isthmic groups) in 3 samples. For quantitative analysis, only clearly stained neurons were counted. To avoid counting cells twice, series of photographs were aligned with ImageJ (free NIH software), and the positions of 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.

Nomenclature

The nomenclature adopted for the various serotonergic nuclei of the sea lamprey was that used in a previous developmental study carried out in our laboratory, with slight modifications (Abalo et al., 2007).

Results

General organization of the serotonergic and GABAergic systems

Numerous serotonin-ir neuronal bodies are located in discrete groups of cells distributed in the diencephalon, rhombencephalon and the spinal cord of the sea lamprey (Antri et al., 2006; Abalo et al., 2007). GABAergic cells are much more numerous and widely distributed in the sea lamprey brain (Meléndez-Ferro et al., 2001, 2002, 2003; Robertson et al., 2007). Accordingly, the present results on GABA/5-HT colocalization will be described in relation to the 5-HT-ir nuclei. A detailed description of the organization of the serotonergic and GABAergic immunoreactive systems is outside the scope of the present investigation. The topographical organization of the serotonergic populations in large sea lamprey larvae and the location neurons double labelled for GABA/5-HT is schematically illustrated in Figure 1, which is based on the results of Abalo et al., (2007).

Serotonin-ir cells were observed in the diencephalon, rhombencephalon and the spinal cord. Present observations in paraformaldehyde/glutaraldehyde fixed samples did not reveal any differences in the 5-HT-ir system respect to previous reports in paraformaldehyde fixed samples (Abalo et al., 2007). In the diencephalon, 5-HT-ir cells were observed in 4 nuclei (Figs. 1a and 1b): the pretectal area [group I of Antri et al., (2006)], the thalamus [zona limitans intrathalamica nucleus of Abalo et al., (2007)] and the tuberal and mammillary regions of the hypothalamus. Two cell nuclei were observed in the rhombencephalon (Figs. 1a and c): one in the caudal rhombencephalon [group III of Antri et al., (2006); vagal group of Chapter 2.2] and another in the rostral rhombencephalon, the isthmic nucleus [group II of Antri et al.,

(2006)]. Serotonin-ir cells were also observed in the ventral margin of the spinal cord (Fig. 1a). The telencephalon and mesencephalon of the larvae studied did not exhibit 5-HT-ir cells. The general pattern of GABA-ir cell groups observed in the brain of large larvae of the sea lamprey coincided with that previously shown in smaller larvae, by other authors (Meléndez-Ferro et al.,, 2002, 2003), and will not be described.

Colocalization of 5-HT and GABA in lamprey neurons

Double immunofluorescence experiments in tissue fixed in a paraformaldehyde/glutaraldehyde mixture revealed the presence of GABA-ir neurons in addition to 5-HT-ir neurons in most regions of the sea lamprey brain containing serotonergic cells. However, colocalization of GABA and 5-HT immunoreactivities in cells was only observed in a few serotonergic groups (Figs. 2-4). Double-labelled cells were mainly observed in the thalamic (Fig. 2) and isthmic serotonergic nuclei (Fig. 3). Occasional double-labelled cells were observed in another two diencephalic nuclei, the tuberal and pretectal nuclei (Fig. 4). No double-labelled cells were observed in the serotonergic nucleus of the mammillary recess, in the vagal 5-HT-ir reticular nucleus or in the ventromedial region of the spinal cord (i.e. those of the spinal cord; Fig. 4c).

In the alar diencephalon, numerous GABAergic cells were observed in nuclei of the prethalamus, thalamus and pretectum, whereas serotonergic cells were only observed in a thalamic nucleus [the 5-HT-ir nucleus of the zona limitans intrathalamica of Abalo et al., (2007)] and in the pretectum. A number of serotonergic cells in the thalamus were double labelled with the anti-5-HT and anti-GABA antibodies. Serotonin was mainly colocalized with GABA in a row of cells of the thalamus (prosomere 2) (Fig. 2) close to its rostral limit with the prethalamus (prosomere 3). 87% of the 5-HT-ir cells of this thalamic nucleus were also GABA-ir. The perikarya of these 5-HT-ir/GABA-ir cells were rounded or polygonal, and 10.04 ± 2.57 μm in size. The region devoid of any GABA-labelled cells that is intercalated between the prethalamic and thalamic GABA-ir populations probably corresponds to the zona limitans intrathalamica separating both prosomeres (Fig. 2). The GABA-ir population of the rostral part of the thalamus was far more abundant than that of 5-HT-ir cells, so that the GABA-ir cells outside the zona limitans were 5-HT negative. A nucleus of faint 5-HT-ir round cells was observed in the pretectum of large larvae (i.e. prosomere 1). Occasional cells of this pretectal nucleus (between 7 and 9 μm in size) were double labelled for 5-HT and GABA (Fig. 4b-b´´).

Scarce 5-HT -ir/GABA-ir cells were observed in nuclei of the basal diencephalon. In the hypothalamus, 5-HT-ir bipolar cells were

observed in the tuberal region, where dorsal and ventral serotonergic subpopulations were recognizable. The neurons of the dorsal hypothalamic subgroup were more numerous and more intensely stained than those of the ventral subgroup; most of the cells in the dorsal subgroup exhibited intraventricular dendrites with a club-shaped end and processes coursing laterally, and are therefore cerebrospinal fluid contacting (CSF-c) cells (paraventricular organ). GABA-ir CSF-c cells of similar appearance were also observed in the periventricular hypothalamus, including the regions containing 5-HT-ir neurons. Occasional CSF-c cells in the dorsal hypothalamic subpopulation (between 7 and 8 μm in minor diameter) showed immunoreactivity for both 5-HT and GABA (Fig. 4a-a´´). Occasional bipolar cells of the ventral subpopulation (between 8 and 9 μm in size) were also 5-HT-ir/GABA-ir (not shown).

In the isthmus (rostral rhombencephalon), a complex group of serotonergic cells was observed. Three subpopulations of 5-HT-ir cells were recognized in this region, the dorsal, medial and ventral isthmic subpopulations (cf. Abalo et al.,, 2007). Experiments using immunofluorescence methods revealed numerous GABA-ir neurons intermingled with the medial and ventral subpopulations, whereas such cells were absent from the area occupied by the dorsal serotonergic subpopulation. Double-labelled cells were observed in the ventral subpopulation (Fig. 3). These cells were rounded or fusiform and small (8.56 ± 1.09 μm), and were located in the periventricular cell layer. They represent about the 61% of the total serotonergic cells of the ventral subgroup. By comparison, the larger 5-HT-ir cells of the medial isthmic subgroup were not GABA-ir, although numerous GABA-ir cells were intermingled with these serotonergic cells.

In the caudal rhombencephalon, the 5-HT-ir cells of the vagal nucleus were intermingled with GABA-ir cells but no double-labelled cells were observed in this region (not shown). Double-labelled cells were not observed in the spinal cord; 5-HT-ir cells were observed in a ventromedial position away from the spinal cord central canal, whereas the nearest GABA-ir cells were of CSF-c type and were observed around the central canal (Fig. 4c).

Discussion

Organization of the serotonergic/GABAergic cells in lampreys: comparison with mammals

The present investigation is the first demonstration of the colocalization of 5-HT and GABA immunoreactivities in neurons of the larval lamprey brain. Although the different 5-HT-ir neuronal populations observed at the end of the larval stage are the same as in adults (Abalo et al., 2007), studies in adult lampreys will be necessary to know if the pattern of colocalization of these neurotransmitters change during metamorphosis. As far as we are

aware, colocalization of 5-HT and GABA has not been reported in the brain of other non-mammalian vertebrates. The absence of studies of this cell type in non-mammalian species may be related to the different fixatives required for immunostaining with the anti-5-HT and anti-GABA antibodies (paraformaldehyde-based and glutaraldehyde-based fixatives, respectively). In the present study, fixation with a paraformaldehyde and glutaraldehyde mixture was developed in order to carry out the double immunofluorescence experiments. Moreover, the results obtained with the different controls (western blotting and preadsorption with neurotransmitter-BSA conjugates; see Material and Methods section) indicate that the antibodies were highly specific for 5-HT and GABA, respectively.

Studies in the rat have reported that neurons double labelled with 5-HT-ir/GABA-ir or with 5-HT-ir/GAD-ir represent a small or very small percentage of the 5-HT-ir cells in the brainstem raphe nuclei (Belin et al., 1983; Millhorn et al., 1987; Millhorn et al., 1988; Stamp and Semba, 1995). These scarce serotonergic/GABAergic cells were mainly observed in the raphe dorsalis nucleus (Belin et al., 1983), or in the raphe magnus nucleus and raphe obscurus nucleus (Millhorn et al., 1987; Millhorn et al., 1988; Stamp and Semba, 1995); very few cells double labelled with 5-HT-ir and GAD-ir were reported in the pontine raphe nuclei and supralemniscal nucleus (Stamp and Semba, 1995). In all these rat raphe nuclei a mixture of GABA-ir neurons and 5-HT-ir neurons was observed (Stamp and Semba, 1995). In the lamprey brain, the 5-HT-ir populations were largely intermingled with GABA-ir neurons (with the exception of the dorsal isthmic subpopulation), suggesting a conserved pattern. As regards the possible equivalences between the serotonergic reticular groups of the lamprey rhombencephalon with the raphe-reticular populations of mammals, considerable uncertainty is introduced by the non migration of 5-HT-ir cells toward the raphe during lamprey development (Abalo et al.,, 2007), which is unlike that observed in all jawed vertebrates, including cartilaginous fishes (Carrera et al., 2008). In the sea lamprey rhombencephalon, 5-HT-ir/GABA-ir neurons were observed in the ventral isthmic subgroup (present results), which extends between the isthmus and trigeminal levels. We previously suggested that the serotonergic reticular nuclei of lampreys are homologues of the caudal and rostral parts of the serotonergic raphe/reticular complex of jawed vertebrates (Chapter 2.2). This suggests a level equivalence with the neurochemically similar neurons of the rat raphe dorsalis/raphe magnus, although possible homology between the 5-HT-ir/GABA-ir cells of the lamprey ventral isthmus and those of the rat brainstem raphe is uncertain. In the medial isthmic and the caudal (vagal) reticular serotonergic populations, GABA is not colocalized with serotonin. These nuclei contain spinal-projecting serotonergic cells

(Chapter 2.2). Occasional serotonergic cells of the ventral isthmic subnucleus were found to project to the rostral spinal cord (Chapter 2.2), but whether any of them colocalize GABA has not been investigated.

The rat diencephalon does not contain serotonergic cells (Lidov and Molliver, 1982; Wallace and Lauder, 1983, 1992), which precludes comparison with diencephalic serotonergic groups of lampreys. Like in lampreys, the diencephalon of other fishes contains abundant CSF-c serotonergic cells in the paraventricular organ, and transient or permanent pretectal-thalamic serotonergic populations have been reported in some teleosts and elasmobranchs (see Ekström et al., 1985; Kaslin and Panula, 2001; Carrera et al., 2008). However, the absence of data on GABA/serotonin colocalization in other fish species prevents comparison with lampreys. Thus, it is not clear whether or not the presence of 5-HT-ir/GABA-ir cells in the diencephalon is a characteristic exclusive of lampreys.

Previous studies of the serotonergic system of the developing and adult sea lamprey described the presence of a 5-HT-ir nucleus in or close to the zona limitans intrathalamica (Zli; Pombal and Puelles, 1999; Meléndez-Ferro et al., 2002; Abalo et al., 2007), which is considered a stretched basal-analogous territory separating the prosomeres p2 and p3. The serotonergic zona limitans population appears early on in lamprey development (Meléndez-Ferro et al., 2002; Abalo et al., 2007). This cell population was apparently located at the rostral limit of the band of GABA-ir cells of the dorsal thalamus (Melendez-Ferro et al., 2002), but codistribution or colocalization of these substances was not investigated. Here we show that most of the zona limitans 5-HT-ir cells were also GABA immunoreactive and that double-labelled cells are located at the rostral border of the thalamic GABA-ir cell band. Accordingly, this appears to be a unique characteristic of this lamprey population.

Several double immunofluorescence studies of the sea lamprey have shown that GABA is colocalized with different neurotransmitters in cells of different brain/spinal cord nuclei (5-HT: present results; dopamine: Rodicio et al., 2008; glycine: Villar-Cerviño et al., 2008a; aspartate: Villar-Cerviño et al., 2008b). This indicates that GABAergic populations of lamprey do not represent morphologically and neurochemically homogeneous groups and reveals a complex relationship between the GABAergic system and other neurotransmitter systems in the most primitive line of vertebrates, the agnathans. Furthermore, 5-HT is colocalized with dopamine in ventromedial cells of the lamprey spinal cord (Schotland et al., 1995), again indicating that serotonergic populations in lampreys are neurochemically heterogeneous as regards the presence of other classical neurotransmitters in the cell.

Functional implications of the colocalization of 5-HT and GABA in lamprey neurons

Both 5-HT and GABA are stored in synaptic vesicles of neurons (revised by Siegel et al., 2006). The presence of both neurotransmitters in the here described lamprey neurons suggests that both neurotransmitters may be coreleased from these neurons at the synaptic cleft. GABA is a major inhibitory neurotransmitter of the central nervous system, and acts on GABA(A) receptors to elicit inward Cl^- currents (Li et al., 2000). Some studies have shown that 5-HT changes the response of rat neurons to GABA by postsynaptic potentiation of the GABA-induced Cl^- current (Xu et al., 1998; Wang et al., 1999; Li et al., 2000). This effect appears to be mediated by the activation of 5-HT2 receptors and subsequent activation of the PKC-dependent phosphorylation of the GABA(A) receptor (An et al., 2005).

If these results also apply to lamprey, the present colocalization data would suggest that the double-labelled 5-HT/GABA-ir neurons found mainly in the lamprey isthmus and thalamus are a specialized type of neurons that may potentiate the GABA inhibitory effects on postsynaptic neurons via co-release of serotonin, possibly contrasting with effects produced by GABA-only or serotonin-only synapses. However, the targets of these 5-HT/GABA-ir lamprey neurons are not known, which precludes further speculation.

Conclusions

The present investigation is the first demonstration of the presence of both 5-HT and GABA in neurons of the brain of a non-mammalian vertebrate. Serotonergic /GABAergic cells of the lamprey ventral isthmus may be homologues of serotonergic /GABAergic raphe neurons of the rat. However, the thalamic serotonergic /GABAergic cells appear to be characteristic of lampreys. Neurochemical and anatomical data from the present study suggest that the colocalization of 5-HT and GABA in neurons appeared in evolution before the separation of agnathans and gnathostomes.

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.

An J, Chen CH, Guan BC, Tang M, Yu CG, Li ZW. 2005 5-HT2 receptor mediated the potentiation of GABA-activated current in the membrane of the dorsal root ganglion neurons of rat. Yao Xue Xue Bao 40: 1-7.

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.

Belin MF, Nanopoulos D, Didier M, et al. 1983. Immunohistochemical evidence for the presence of gamma-aminobutyric acid and serotonin in one nerve cell. A study on the raphe nuclei of the rat using antibodies to glutamate decarboxylase and serotonin. Brain Res 275: 329-339.

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.

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.

Gamrani H, Harandi M, Belin MF, Dubois MP, Calas A. 1984. Direct electron microscopic evidence for the coexistence of GABA uptake and endogenous serotonin in the same rat central neurons by coupled radioautographic and immunocytochemical procedures. Neurosci Lett 48: 25-30.

Harandi M, Didier M, Aguera M, Calas A, Belin MF. 1986. GABA and serotonin (5-HT) pattern in the supraependymal fibers of the rat epithalamus: combined radioautographic and immunocytochemical studies. Effect of 5-HT content on [3H]GABA accumulation. Brain Res 370: 241-249.

Hökfelt T, Arvidsson U, Cullheim S, et al. 2000. Multiple messengers in descending serotonin neurons: localization and functional implications. J Chem Neuroanat 18: 75-86.

Kaslin J, Panula P. 2001. Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). J Comp Neurol 440: 342-377.

Li H, Lang B, Kang JF, Li YQ. 2000. Serotonin potentiates the response of neurons of the superficial laminae of the rat spinal dorsal horn to gamma-aminobutyric acid. Brain Res Bull 52: 559-565.

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

Meléndez-Ferro M, Pérez-Costas E, Rodríguez-Muñoz R, Gómez-López MP, Anadón R, Rodicio MC. 2001. GABA immunoreactivity in the olfactory bulbs of the adult sea lamprey *Petromyzon marinus* L. Brain Res 893: 253-260.

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B, et al. 2002. Ontogeny of gamma-aminobutyric acid-immunoreactive neuronal populations

in the forebrain and midbrain of the sea lamprey. J Comp Neurol 446: 360-376.

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B, Rodríguez-Muñoz R, Anadón R, Rodicio MC. 2003. Ontogeny of gamma-aminobutyric acid-immunoreactive neurons in the rhombencephalon and spinal cord of the sea lamprey. J Comp Neurol 464: 17-35.

Millhorn DE, Hökfelt T, Seroogy K, Oertel W, Verhofstad AA, Wu JY. 1987. Immunohistochemical evidence for colocalization of gamma-aminobutyric acid and serotonin in neurons of the ventral medulla oblongata projecting to the spinal cord. Brain Res 410: 179-185.

Millhorn DE, Hökfelt T, Seroogy K, Verhofstad AA. 1988. Extent of colocalization of serotonin and GABA in neurons of the ventral medulla oblongata in rat. Brain Res 461: 169-174.

Pombal MA, Puelles L. 1999. Prosomeric map of the lamprey forebrain based on calretinin immunocytochemistry, Nissl stain, and ancillary markers. J Comp Neurol 414 : 391-422.

Robertson B, Auclair F, Ménard A, Grillner S, Dubuc R. 2007. GABA distribution in lamprey is phylogenetically conserved. J Comp Neurol 503: 47-63.

Rodicio MC, Villar-Cerviño V, Barreiro-Iglesias A, Anadón R. 2008. Colocalization of dopamine and GABA in spinal cord neurones in the sea lamprey. Brain Res Bull 76: 45-49.

Schotland J, Shupliakov O, Wikstrom M et al. 1995. Control of lamprey locomotor neurons by colocalized monoamine transmitters. Nature 374: 266-268.

Siegel GJ, Albers RW, Brady ST, Price DL. 2006. *Basic Neurochemistry. Molecular, Cellular, and Medical Aspects*. San Diego: Elsevier Academic Press.

Stamp JA, Semba K. 1995. Extent of colocalization of serotonin and GABA in the neurons of the rat raphe nuclei. Brain Res 677: 39-49.

Vergé D, Calas A. 2000. Serotoninergic neurons and serotonin receptors: gains from cytochemical approaches. J Chem Neuroanat 18: 41-56.

Villar-Cerviño V, Abalo XM, Villar-Cheda B, et al. 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.

Villar-Cerviño V, Barreiro-Iglesias A, Anadón R, Rodicio MC. 2008ª. Distribution of glycine immunoreactivity in the brain of adult sea lamprey (*Petromyzon*

marinus). Comparison with gamma-aminobutyric acid. J Comp Neurol 507: 1441-1463.

Villar-Cerviño V, Barreiro-Iglesias A, Anadón R, Rodicio MC. 2008b. Aspartate immunoreactivity in the telencephalon of the adult sea lamprey: comparison with GABA immunoreactivity. Brain Res Bull 75: 246-250.

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

Wallace JA, Lauder JM. 1992. Development of the serotoninergic systems in rat and chick embryos. In: Björklund A, Hökfelt T, Tohyama M. (Eds.), Handbook of Chemical Neuroanatomy. Ontogeny of transmitters and peptides in the CNS, vol. 10. Elsevier, Amsterdam, pp. 619-645.

Wang DS, Xu TL, Li JS. 1999. 5-HT potentiates GABA- and glycine-activated chloride currents on the same neurons in rat spinal cord. J Hirnforsch 39: 531-537.

Xu TL, Pang ZP, Li JS, Akaike N. 1998. 5-HT potentiation of the GABA(A) response in the rat sacral dorsal commissural neurones. Br J Pharmacol 124: 779-787.

Zimmermann T, Rietdorf J, Girod A, Georget V, Pepperkok R. 2002. Spectral imaging and linear un-mixing enables improved FRET efficiency with a novel GFP2-YFP FRET pair. FEBS Lett 531: 245-249.

Abbreviations

H Habenula
Hyp Hypophysis
I1 Müller isthmic giant cell
M Mesencephalon
ON Optic nerve
P Pineal organ
PO Preoptic region
PoC Postoptic commissure
Pp Parapineal organ
Pt Pretectum
PTh Prethalamus

Th Thalamus

TL Telencephalon

T-Mm Tuberal-mammillary nuclei

TrN Trigeminal nucleus

VG Vagal nucleus

Zli Zona limitans intrathalamica

FIGURES
CHAPTER 3.1

Figure 1. Schematic drawings of sagittal (A) and transverse (B and C) sections of the brain of a larval sea lamprey showing the distribution of double-labelled serotonin/GABA immunoreactive cells (black) in comparison with the distribution of serotonergic cells (grey). (A) Sagittal section of the larval brain/spinal cord (SC) showing the organization of the serotonergic nuclei and the location of the 5-HT-ir/GABA-ir cells within these nuclei. The black lines indicate approximately the positions of the transverse sections shown in Figs. 1B and 1C. (B) Transverse section of the diencephalon showing the location of the 5-HT-ir/GABA-ir cells of the thalamic nucleus (ThN) and the dorsal and ventral tuberal subpopulation (DTS and VTS respectively). (C) Transverse section of the rostral rhombencephalon showing the location of the 5-HT-ir/GABA-ir cells in the lamprey isthmus. Note the presence of double-labelled cells only in the ventral isthmic subpopulation (VIS) and the absence of this type of cell in the medial (MIS) and dorsal (DIS) subpopulations. For abbreviations, see list.

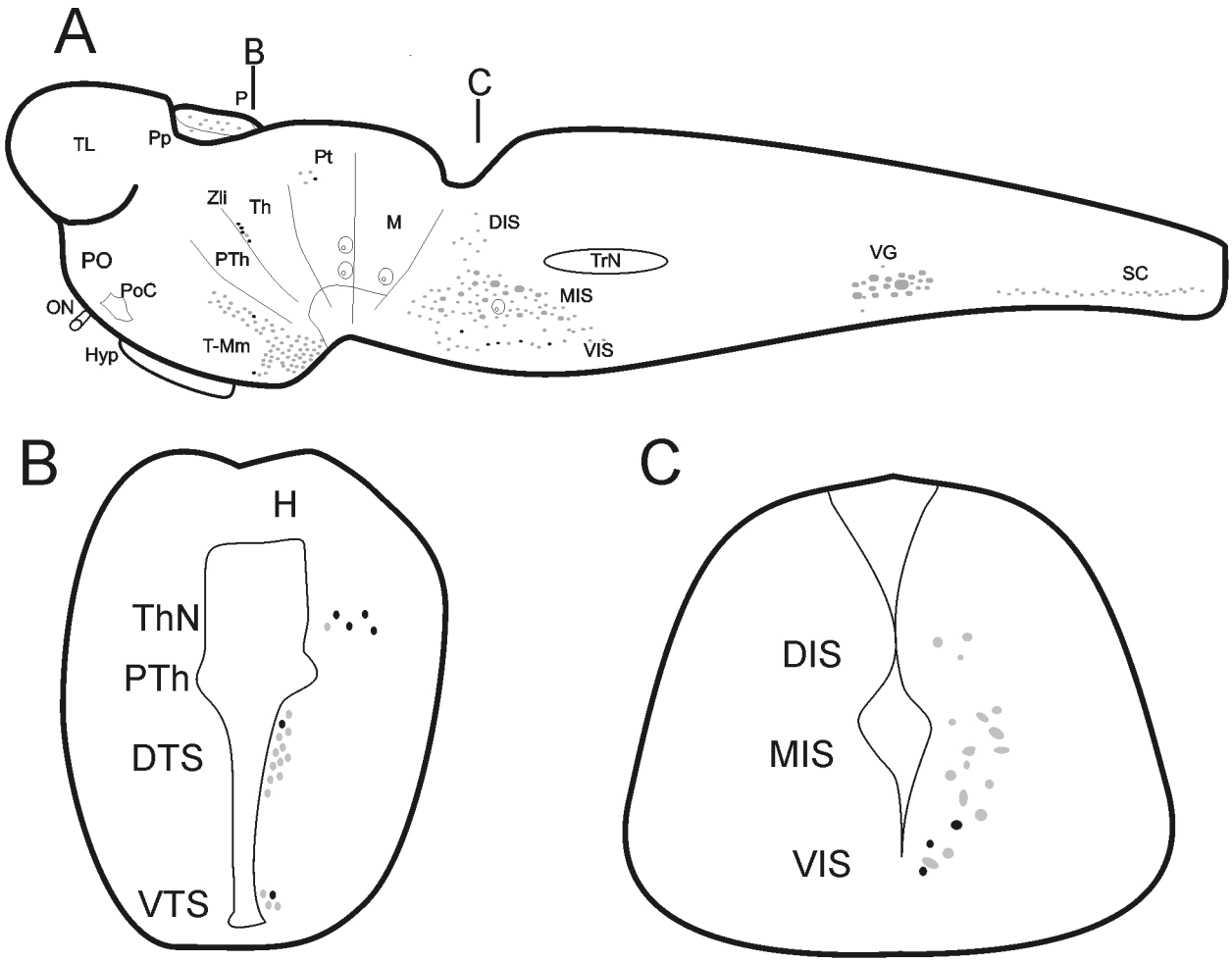

A
B
C
P
TL
Pp
Pt
Zli
Th
M
DIS
PO
PTh
TrN
VG
SC
PoC
ON
MIS
T-Mm
Hyp
VIS
B
H
ThN
PTh
DTS
VTS
C
DIS
MIS
VIS

FIGURE 1

Figure 2. Transverse sections through the thalamic and tuberal nuclei of the diencephalon. (A-A´´) Photomicrographs of a transverse section of the diencephalon showing double-labelled cells (5-HT in red, GABA in green) in the thalamus (white arrows). Note the presence of a gap without labelled cells between the thalamus and the prethalamus that may correspond to the zona limitans intrathalamica (Zli). Note the absence of double-labelled cells in the dorsal tuberal subgroup of the hypothalamus (arrowheads). (B-B´´) Photomicrographs of a transverse section of the dorsal diencephalon showing double-labelled cells in the thalamus (white arrows). Stars indicate the ventricle. All photomicrographs are Z-stack projections from confocal images. Scale bars: 40 μm.

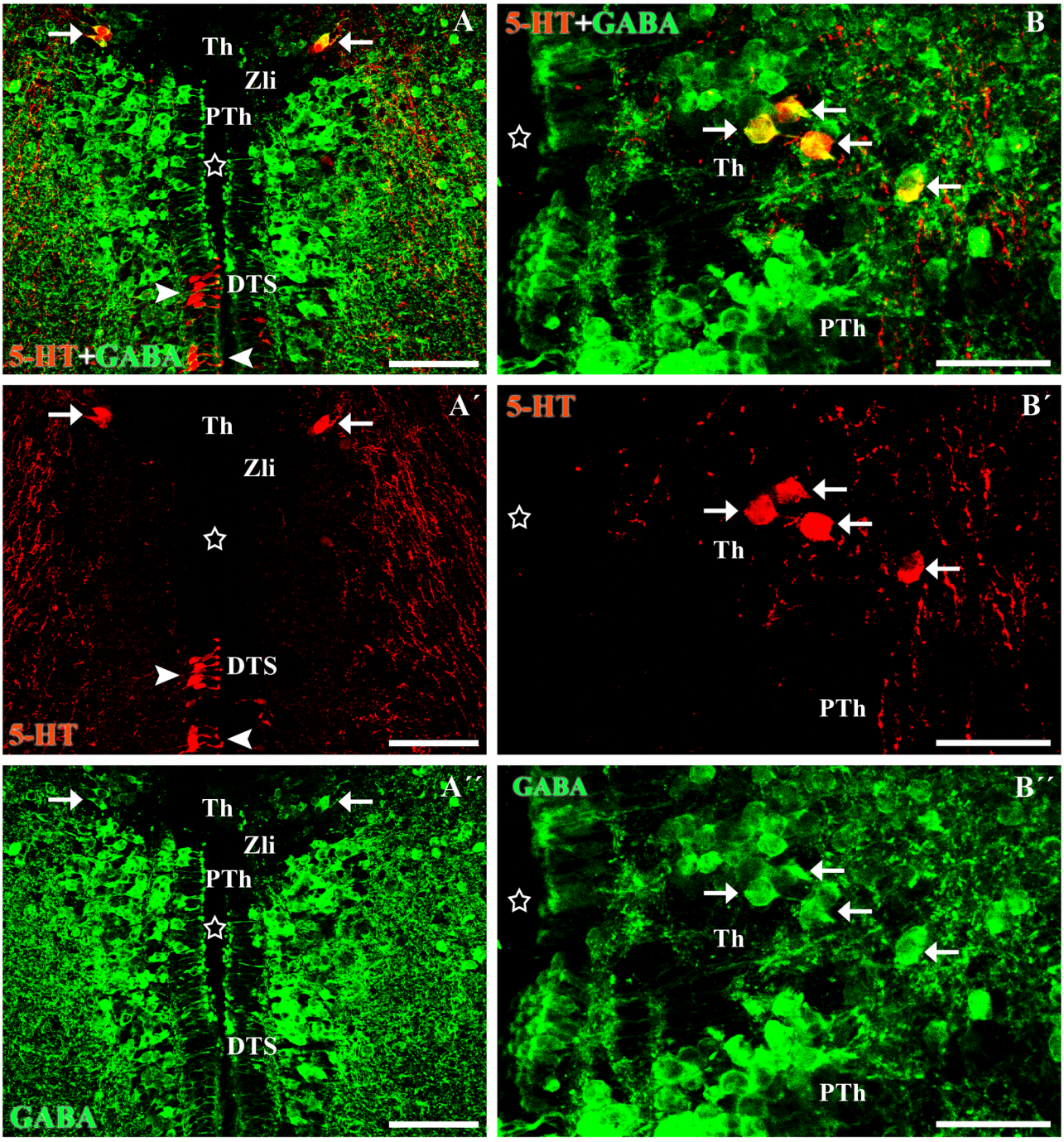

A
Th
Zli
PTh
DTS
5-HT+GABA
5-HT+GABA
B
Th
PTh
A´
Th
Zli
DTS
5-HT
5-HT
B´
Th
PTh
A´´
Th
Zli
PTh
DTS
GABA
GABA
B´´
Th
PTh

FIGURE 2

Figure 3. Transverse sections through the isthmic region of the rhombencephalon. (A-A´´) Photomicrographs of a transverse section of the rostral rhombencephalon showing 5-HT-ir (red) and GABA-ir (green) cells. Note the presence of double-labelled cells in the ventral isthmus (white arrows) and the absence of double-labelled cells in the medial isthmic subgroup (arrowheads). (B-B´) Enlargements of Figs. 2A-A´´. Stars indicate the ventricle. Note that the anti-5-HT immunofluorescence labelling fills the somas, whereas the GABA labelling is more concentrated at the border of the neuronal somas. All photomicrographs are Z-stack projections from confocal images. Scale bars: 50 μm.

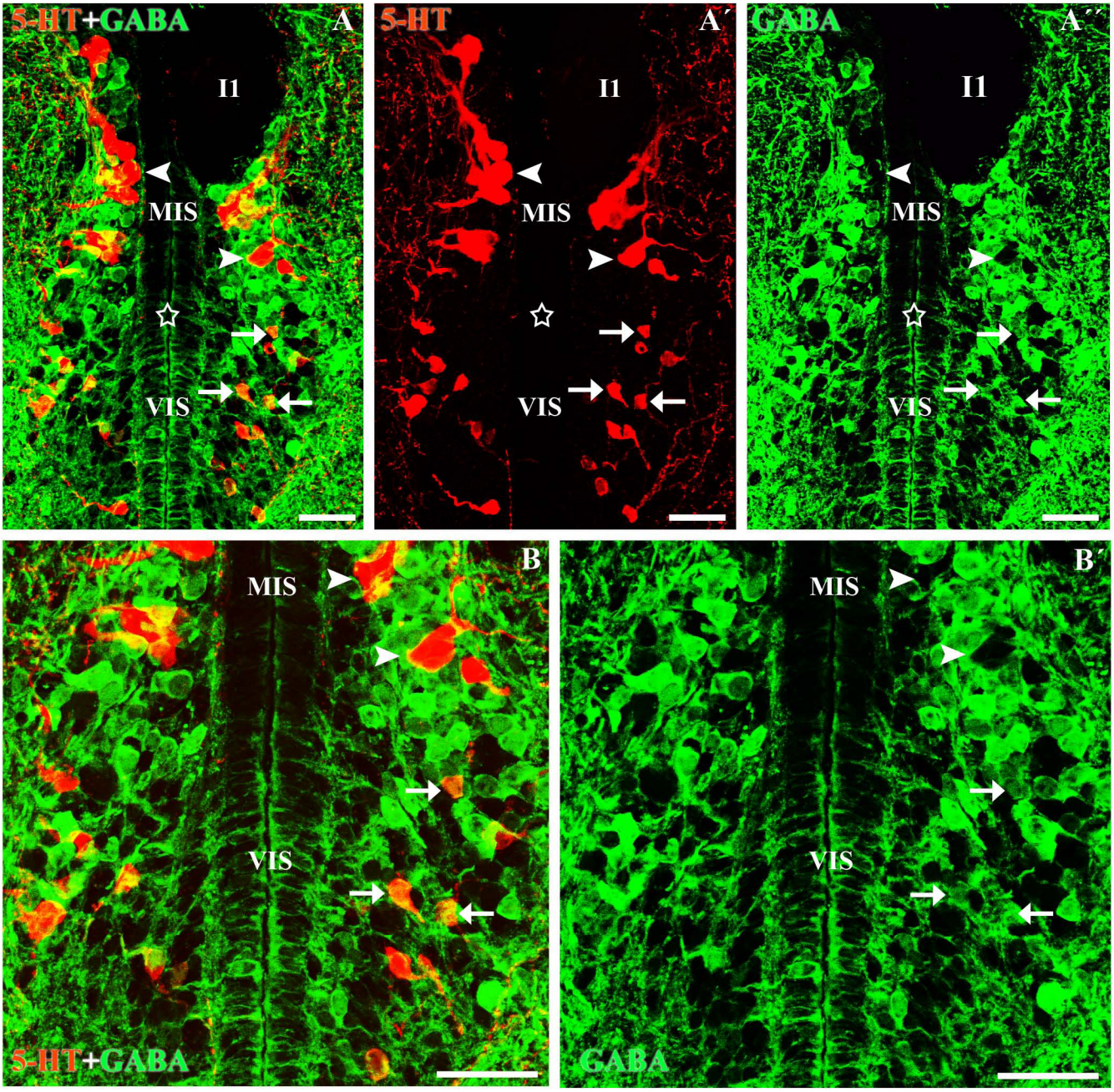

5-HT+GABA
A
I1
MIS
VIS
5-HT
A´
I1
MIS
VIS
GABA
A´´
I1
MIS
VIS
B
MIS
VIS
5-HT+GABA
B´
MIS
VIS
GABA

Figure 3

Figure 4. Transverse sections through the dorsal tuberal subgroup and the pretectal nucleus of the diencephalon and the spinal cord. (A-A´´) Photomicrographs of a transverse section of the hypothalamus showing a 5-HT-ir (red)/GABA-ir (green) cerebrospinal fluid-contacting cell of the dorsal tuberal subgroup (white arrow). (B-B´´) Photomicrographs of a transverse section of the caudal diencephalon showing a couple of double-labelled cells in the pretectal nucleus (white arrows). Note that the cells of the pretectum were faintly immunoreactive for the anti-5-HT antibody (red). (C) Photomicrograph of a transverse section of the spinal cord illustrating the absence of double-labelled cells (the thick arrow indicates 5-HT-ir cells and the arrowheads indicate GABA-ir cells). Stars indicate the ventricle. All photomicrographs are Z-stack projections from confocal images. Scale bars: 20 μm in A, 25 μm in B and 40 μm in C.

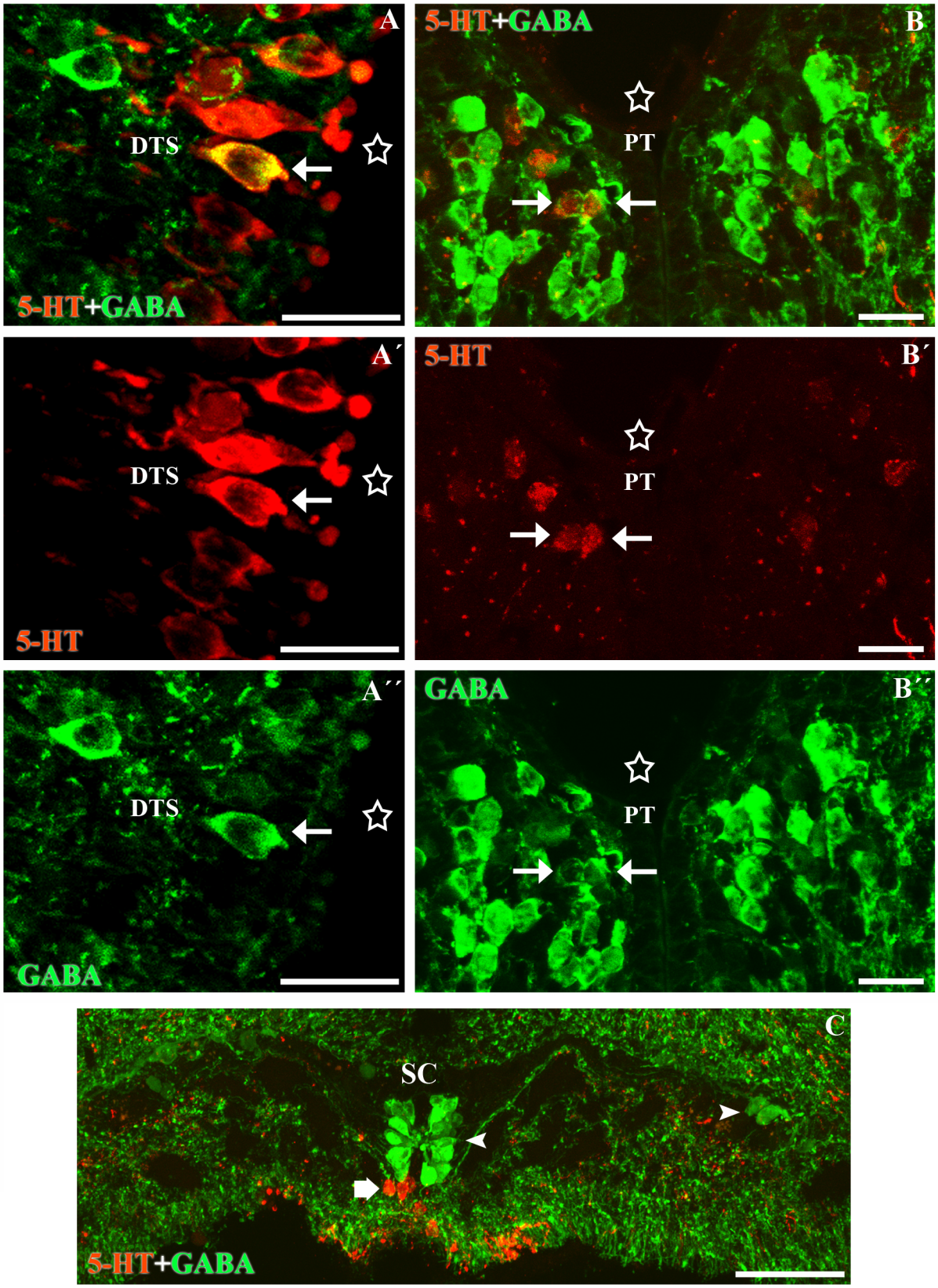

A
DTS
5-HT+GABA
5-HT+GABA
B
PT
A´
DTS
5-HT
5-HT
B´
PT
A´´
DTS
GABA
GABA
B´´
PT
C
SC
5-HT+GABA

FIGURE 4

Dopamine and GABA are colocalized in restricted groups of neurons in the sea lamprey brain: Insights into the early evolution of neurotransmitter colocalization in vertebrates

Introduction

Experimental evidence refuting Dale's principle (i.e. the notion that each neuron synthesizes and releases only one neurotransmitter) has been accumulated in the past decades (see Strata and Harvey, 1999; Trudeau and Gutiérrez, 2007). Cotransmission by multiple neurotransmitters from the same neuron or axon terminal (and even from the same vesicle) is now well documented (see Hökfelt et al., 1992; Trudeau and Gutiérrez, 2007), and the postulate "one neuron, one neurotransmitter" is probably the exception rather than the rule. The corelease of "classical" neurotransmitters and a peptide, nucleotide, nitric oxide (NO), neurotrophic factors or other molecules is well documented. However, the coexistence and corelease of two "classical" neurotransmitters in a specific neuron has been less widely studied. Studies in recent years have focused on the cotransmission and corelease of γ-aminobutyric acid (GABA) and glycine (Lu et al., 2008), GABA and glutamate (Sandler and Smith, 1991; Gutiérrez, 2005), and glutamate and dopamine (DA) or serotonin (5-HT) (reviewed in Trudeau, 2004; and in Descarries et al., 2008). On the other hand, the coexistence of monoamines and GABA in the same cell and possible cofunctions of these neurotransmitters have been less well studied.

The coexistence of both DA and GABA, or their synthesizing enzymes, in the same cell has been previously reported in specific neuronal populations of a few vertebrate species. Colocalization of tyrosine hydroxylase (TH; the rate-limiting enzyme of catecholamine synthesis) and GAD or GABA has been reported in some neurons in mammalian brains: in the olfactory bulb, diencephalon, mesencephalic central grey and cerebral cortex (Chronwall, 1985; Kosaka et al., 1985; 1987). Immunohistochemical and in situ hybridization studies in the rat have also shown that a subset of DA neurons in the substantia nigra compacta contains glutamic acid decarboxylase (GAD; the enzyme that catalyzes GABA synthesis) (Hédou et al.,, 2000; González-Hernández et al., 2001). However, these results remain controversial since no immunoreactivity for GABA has been observed in these dopaminergic neurons (Kosaka et al., 1987; Sulzer et al., 1998). Unlike in mammals, the coexistence of DA or

TH and GAD or GABA in central neurons in other vertebrate groups has been reported for only a few locations and species (*Xenopus* pituitary axons: de Rijt et al., 1992; dogfish spinal cord: Sueiro et al., 2004; snake olfactory bulb: Kosaka et al., 1991). Neurons containing both DA and GABA have also been reported in some invertebrates (*Periplaneta*: Distler, 1990; *Aplysia*: Díaz-Ríos et al., 2002; Díaz-Ríos and Miller, 2005).

Lampreys are important model organisms for the study of the neuronal basis of vertebrate locomotion (see Dubuc et al., 2008; Grillner et al., 2008). Neurotransmitter co-release may be important in the modulation and plasticity of neuronal networks at synaptic levels to enable interaction with various sets of receptors. We previously reported that in lampreys, GABA was colocalized with DA in some spinal cord neurons (Rodicio et al., 2008). Other studies carried out in our laboratory on the sea lamprey brain also reported the colocalization of GABA and aspartate (Villar-Cerviño et al., 2008a), GABA and glycine (Villar-Cerviño et al., 2008b), and GABA and serotonin (Chapter 3.1) in neurons of different nuclei. These studies suggest that the colocalization of "classical" neurotransmitters is rather extended in extant jawless vertebrates. Accordingly, knowledge of colocalization of neurotransmitters in the different neuronal populations of lampreys appears important for a better understanding of the neurochemical basis of circuits underlying locomotion control and other brain functions.

To provide some new insight into the co-distribution of different neurotransmitters in the sea lamprey brain, we studied the possible colocalization of DA and GABA in neurons by means of double immunofluorescence methods. Results from the present investigation showed that DA and GABA are colocalized in subsets of neurons in different nuclei of the sea lamprey brain previously characterized as dopaminergic. Present and previous studies in our laboratory indicate that lampreys are very useful vertebrate models for studies of the corelease and cotransmission of "classical" neurotransmitters.

Material and Methods

Animals

Upstream migrating adult sea lampreys (*Petromyzon marinus* L.; n = 5; about 75 cm in total body length) caught in the river Ulla (northwest Spain) and provided by a commercial supplier were used in this study. The lampreys were deeply anaesthetized with benzocaine, killed by decapitation and their brains dissected out and fixed immediately by immersion in 5% glutaraldehyde and 1% sodium metabisulphite in Tris buffer (TBS) at pH 7.4 for 17 h. The brains were then embedded in Tissue Tek, frozen in liquid nitrogen-cooled isopentane and transversely sectioned on a cryostat (16 μm thick). All experiments conformed to the European

Community guidelines on animal care and experimentation and were approved by the Ethics Committee of the University of Santiago de Compostela.

Immunofluorescence

Double immunofluorescence experiments to study the colocalization of DA and GABA in sea lamprey neurons were performed as previously described (Rodicio et al., 2008). Briefly, sections were pre-treated with 0.2% $NaBH_4$ for 45 min and subsequently incubated for 3 days at 4 °C with a mixture of rabbit polyclonal anti-dopamine antiserum (H.W.M. Steinbusch, Maastricht, The Netherlands; 1:900) and mouse monoclonal anti-GABA antibody (Sigma, clone GB-69; 1:1200) in Tris-buffered saline (TBS) containing 1% sodium metabisulfite, 15% normal goat serum and 0.2% Triton as detergent. After being rinsed in TBS, the samples were incubated for 1 h with a cocktail of Cy3 conjugated goat anti-rabbit immunoglobulin (Chemicon; 1:200) and fluorescein conjugated goat anti-mouse immunoglobulin (Chemicon; 1:50) in TBS, rinsed again and mounted with fluorescence anti-fade mounting medium (Vectashield, Vector).

Controls

The specificities of the GABA and DA antibodies have been well characterized by the suppliers. The monoclonal anti-GABA antibody does not cross-react with BSA, l-α-aminobutyric acid, l-glutamic acid, l-aspartic acid, glycine, δ-aminovaleric acid, l-threonine, l-glutamine, taurine, putrescine, l-alanine or carnosine. The dopamine antiserum was raised against a DA-BSA conjugate and does cross-react with noradrenaline (less than 10% cross reaction) and with other monoamines (less than 1% cross reaction). No immunoreactivity was detected when the primary antibodies were omitted from the immunohistochemical processing. Control experiments (pre-adsorption with the corresponding antigens, GABA-BSA and DA-BSA conjugates, respectively, and Western blots of lamprey brain protein extracts) previously carried out in our laboratory confirmed the specificity of these antibodies (anti-DA: Chapter 2.1; anti-GABA: Villar-Cerviño et al., 2008b) in lamprey tissue.

Image acquisition and processing

Photomicrographs were taken with a spectral confocal laser scanning microscope (Leica TCS-SP2). Except when specified, photomicrographs are z-stack projections from confocal images. Data was acquired in the spectral confocal microscope by use of a narrow wavelength window tuned to the specific emission of each fluorescent marker (FITC or Cy3). The photomicrographs were adjusted for brightness and contrast with Adobe Photoshop C.S.4 software.

Cell counts and measurements

The percentage of DA-immunoreactive (DA-ir) cells that also were GABA-ir was calculated (n=3). For quantitative analysis, only clearly stained neurons were counted in one of every three of the serial sections of each brain nucleus. As the size of DA cells did not vary greatly within the same population (although it may vary among different populations) and as determination of the total number of these cells was not the aim of the study, no correction factor was introduced. The percentage of DA-ir cells that also were GABA-ir was calculated for the brain nuclei that showed the highest degree of colocalization. To estimate cell size, the minor axis of 15 DA-ir/GABA-ir neurons was measured in each cell population in transverse sections with the aid of Leica Confocal Software (LCS). The mean and the standard deviation (SD) are given for each DA-ir brain nuclei.

Nomenclature

The nomenclature used in the present study for dopaminergic neuronal nuclei of the sea lamprey followed that used in a developmental study of the dopaminergic system carried out in our laboratory (Abalo et al., 2005), with slight modifications.

Results

General organization of the dopaminergic and GABAergic systems

Numerous DA-ir neuronal bodies are located in discrete groups distributed in the telencephalon, diencephalon and rhombencephalon of the sea lamprey (Abalo et al., 2005; Chapter 2.1). Double DA/GABA immunolabelling revealed that GABAergic cell populations are much more numerous and widely distributed in the sea lamprey brain and most of them do not display DA-immunoreactive (DA-ir) cells. Accordingly, colocalization of DA/GABA will be described on the basis of the DA-ir nuclei. The general organization of the dopaminergic and GABAergic immunoreactive systems in the sea lamprey has already been studied (DA: Abalo et al., 2005; GABA: Robertson et al., 2007) and its detailed study is beyond the scope of the present investigation. The topological organization of the dopaminergic populations in the adult sea lamprey and the location of DA/GABA double immunolabelled neurons are schematically illustrated in Figure 1. The following neuronal populations were considered: A) the group of DA-ir cerebrospinal fluid-contacting (CSF-c) cells in the caudal rhombencephalon, B) the few DA-ir cells located in the ventral isthmus, C) the numerous DA-ir CSF-c cells of the mammillary nucleus [postinfundibular commissure nucleus of Abalo et al., (2005)], D) the paratubercular nucleus [posterior tubercle nucleus of Abalo et al., (2005)], E) the numerous DA-ir CSF-c cells of the dorsal and ventral hypothalamic nuclei, F) the DA-ir cells

of the postoptic commissure nucleus, G) the DA-ir cells of the preoptic nucleus, H) DA-ir neurons located in the striatum and I) the population of DA-ir granule-like cells of the olfactory bulbs.

Colocalization of DA and GABA in lamprey neurons

Double immunofluorescence experiments revealed that DA and GABA immunoreactivities are colocalized in some neurons of most dopaminergic populations of the sea lamprey brain (Figs. 2, 3 and 4). The proportion of cells showing DA and GABA colocalization varied among the different dopaminergic populations. The dopaminergic populations of the brain that showed the highest degree of colocalization of both immunoreactivities (more than 25% of dopaminergic cells) were: the caudal rhombencephalic CSF-contacting population, the ventral isthmus, the postoptic commissure nucleus, the preoptic nucleus, the striatum and the granule-like cells of the olfactory bulbs. In the mammillary and the dorsal and ventral hypothalamic nuclei, very few of the DA-ir cells were also GABA-ir. Double labelled cells were not observed in the paratubercular nucleus of any of the lampreys studied.

The group of DA-ir CSF-c cells of the caudal rhombencephalon was observed in the subependyma near the ventral midline of the fourth ventricle (Figs. 2C and D). This group represents a rostral extension of the spinal DA-ir CSF-c population described by Rodicio et al., (2008) beyond the transition between the spinal cord and the rhombencephalon (Figs. 2A-A'' and B). These small cells (9.47 ± 1.43 μm in diameter) displayed an apical dendrite ending as a club on the ventricular surface and some of them also showed an axonal-like basal process arising from the opposite pole of the cell. All the DA-ir cells (100%) in this nucleus were also labelled by the anti-GABA antibody. The axonal processes of these DA-ir/GABA-ir neurons extended ventrally and double labelling of these processes was often appreciable (Figs. 2A, B, C and D).

In the ventral isthmus (rostral rhombencephalon), a small population of DA-ir cells was observed (Figs. 2E-E''). Approximately 58% of DA-ir cells in this region were also GABA-ir. The perikarya of these double-labelled cells were rounded or fusiform and 14.55 ± 1.49 μm in diameter. In some cells the axonal process was observed coursing laterally.

In the hypothalamus, three conspicuous DA-ir populations mostly consisting of small CSF-c cells were distinguishable: the mammillary nucleus located around the posterior infundibular recess and the ventral and the dorsal hypothalamic nuclei. Double-labelled (DA-ir/GABA-ir) cells were scarce in these hypothalamic nuclei. In the mammillary nucleus very few DA-ir cells were GABA-ir (not shown); these were 12.62 ± 0.42 μm in diameter. In the dorsal hypothalamus most, if

not all, of the DA-ir cells (8.97 ± 2.29 μm) were of CSF-c type. Occasional DA-ir cells in this nucleus displayed GABA immunoreactivity (not shown). Strongly stained DA-ir cells, mostly of CSF-c type, were observed in the ventral hypothalamic nucleus extending between the postoptic and infundibular recesses (Figs. 2F-G'). About 18% of the DA-ir cells of the ventral hypothalamic nucleus were also GABA-ir. The double-labelled perikarya of this nucleus were usually observed at some distance from the ventricle in the second or third parallel row of cells located away the ependyma and most of them were not CSF-c type (Figs. 2F-G'). These cells were spherical or fusiform in shape (11.54 ± 1.66 μm in diameter).

In the postoptic commissure nucleus, ventral (around the postoptic recess) and dorsal (Figs. 3A-A'') DA-ir parts are recognizable (Abalo et al., 2005; present results). Colocalization of DA and GABA was only observed in DA-ir cells (about 23% of cells) in the dorsal zone (Figs. 3A-A'') and no double-labelled cells were observed in the ventral zone (not shown). Double-labelled perikarya were fusiform and small (10.46 ± 2.50 μm), some were CSF-c type and most of them had an axon arising from the basal pole.

A small group of DA-ir cells (about 40 cells for specimen) was observed in the striatum. Two types of DA-ir cells were distinguished on the basis of the appearance and location of their perikarya. The perikarya of the first type were located at some distance from the ventricle and were intermingled with GABAergic cells of the cell row characteristic of the lamprey striatum (Figs. 3B-B''). Some DA-ir cells of this type were also GABA-ir (about the 25% of the DA-ir cells of this type), and their perikarya were fusiform or polygonal (12.51 ± 1.3 μm). The neurons of the second type were CSF-c cells (Figs. 3C-C'') and all displayed colocalization of DA and GABA immunoreactivities. These scarce, oval or polygonal cells presented an apical dendrite protruding into the ventricle, and perikarya were 7.95 ± 0.56 μm in diameter.

In the preoptic nucleus, the DA-ir cells occupied the caudal wall of the preoptic recess and were mostly observed in a periventricular location (Figs. 3D-E'') over the optic chiasm, but some were located in a more lateral position in the optic stalks (not shown). About 82 % of the DA-ir cells located over the optic chiasm were also GABA-ir (Figs. 3D-E''), whereas those in the optic stalks were not GABA-ir (not shown), which allowed us to differentiate two subpopulations of dopaminergic cells in this nucleus. The DA-ir/GABA-ir cells of the preoptic nucleus were 13.63 ± 2.47 μm in size, and fusiform or polygonal in shape.

In the olfactory bulb, a population of DA-ir granule-like cells was observed in the inner cellular layer (Figs. 4A-A''). About 26% of the DA-ir granule-like cells also exhibited GABA immunoreactivity (Figs. 4A-A'').

These DA-ir/GABA-ir cells contained rounded perikarya (11.47 ± 1.85 μm in size).

Colocalization of DA and GABA immunoreactivities in fibres and terminals

Representative brain regions were checked in order to observe the presence of fibres or terminals that were double labelled by the anti-DA and anti-GABA antibodies. To ensure the existence of double labelled terminals, confocal photomicrographs (stacks 0.5 μm thick) were obtained at high magnification. In addition to the DA-ir/GABA-ir axonal processes arising from the CSF-c cells of the caudal rhombencephalon (see above; Figs. 2C, D and 5A), double-labelled fibers or terminals were observed in the dorsal isthmic commissure, mesencephalic tegmentum (Figs. 5B-B''), mammillary region, dorsal and ventral tuberal regions, preoptic region (Fig. 5C), striatum (Fig. 5D), lateral pallium, medial pallium (Fig. 5E) and in the olfactory bulb. In these regions, these fibers and terminals only represent a small proportion and most of the DA-ir fibres did not show double labelling (Fig. 5). Most double labelled fibres and terminals were observed in lateral areas, however, commissural fibres were observed in the preoptic region (Fig. 5D).

Discussion

The coexistence of DA and GABA immunoreactivities in neurons of several nuclei of the sea lamprey brain is reported here for the first time. In addition, the results revealed the presence of two new types of DA-ir neurons in the striatum, one consisting of a few cells located away from the ventricle and the other of few periventricular CSF-c cells. Cerebrospinal fluid-contacting cells, including DA-ir (Pierre et al., 1997; Pombal et al.,1997; Abalo et al., 2005; Chapter 2.1) and GABA-ir (Meléndez-Ferro et al., 2002, 2003; Robertson et al., 2007) cells, are very abundant in the lamprey brain. It has been proposed that this type of cells modulates the activity of their target neurons in response to chemical variations in the CSF (Vigh et al., 2004). The presence of striatal dopaminergic cells has not previously been reported in lampreys (river lamprey: Pierre et al., 1997; Pombal et al., 1997; sea lamprey: Abalo et al., 2005). The different results in sea lamprey may be attributed to the use of large, mature animals in the present study. It is not clear whether the absence of similar cells in the river lamprey is due to interspecific differences or to the use of animals in earlier stages of sexual maturation. The appearance of populations immunoreactive to galanin specifically associated with latter stages of sexual maturation has been reported in the trout telencephalon (Rodríguez et al.,, 2003), and something similar may also occur with dopaminergic populations during lamprey maturation.

Colocalization of DA and GABA was observed in most DA-ir nuclei of the lamprey brain

The present results showed colocalization of DA and GABA in neurons of most of the dopaminergic nuclei of the lamprey brain, and in some of these nuclei colocalization was observed in all or most of the dopaminergic cells. Previous studies have reported the coexistence of DA and GABA (or their synthesizing enzymes) in neurons of some vertebrates (mammals: Kosaka et al., 1987; Gall et al., 1987; Betarbet et al., 1997; Hédou et al., 2000; González-Hernández et al., 2001; a snake: Kosaka et al., 1991; *Xenopus*: de Rijt et al., 1992; dogfish shark: Sueiro et al., 2004) and invertebrates (*Periplaneta*: Distler, 1990; *Aplysia*: Díaz-Ríos et al., 2002; Díaz-Ríos and Miller, 2005). Together with the results of these studies, the present results indicate that colocalization of DA and GABA in neurons appeared early on during vertebrate evolution.

In the sea lamprey rhombencephalon, all the dopaminergic cells of the caudal rhombencephalic nucleus and about a half of the scarce cells located in the ventral isthmic region were also GABAergic. The presence of dopaminergic descending afferents to the spinal cord from the caudal rhombencephalic nucleus has previously been reported by our group (Chapter 2.1). Accordingly, these cells also appear to be a source of GABAergic descending projections to the spinal cord in lampreys, although this hypothesis must be tested experimentally. The presence of both dopaminergic and DA-ir/GABA-ir cells in the ventral isthmus appears to be characteristic of lampreys, since the dopaminergic cells of the isthmus in lampreys do not correspond to the locus coeruleus of other vertebrates, which is noradrenergic (Abalo et al., 2005).

A few DA-ir/GABA-ir neurons were observed in the dopaminergic populations of the lamprey hypothalamus. In mammals, scarce dopaminergic neurons of the arcuate nucleus also colocalize DA and GABA (review in Chronwall, 1985) and they appeared to be the source of the DA-ir/GABA-ir tubero-hypophyseal axons that innervate the pituitary neurointermediate lobe (Vuillez et al., 1987; Schimchowitsch et al., 1991). Colocalization of DA and GABA has been also reported in amphibian pituitary terminals originated in the suprachiasmatic nucleus (de Rijk et al., 1992), although GABA could not be detected in the neuronal bodies (Tuinhof et al., 1994). Although these results suggest the existence of similar patterns of DA/GABA colocalization in hypothalamic neurons of different vertebrates, no studies have been carried out to investigate whether or not the hypothalamic DA-ir/GABAir neurons innervate the lamprey pituitary. On the other hand, a high degree of DA and GABA colocalization was observed in dopaminergic cells of the sea lamprey preoptic nucleus, and about a quarter of the dopaminergic neurons in the dorsal part of the postoptic commissure nucleus also express GABA. As far as we are aware, colocalization of DA and GABA has not been reported in cells of the preoptic region of other vertebrates.

Although the presence of these preoptic cells may represent a specialization characteristic of lampreys, other hypotheses cannot be ruled out as similar studies have not been carried out with other vertebrates.

The present report is the first description of the presence of DA-ir/GABA-ir cells in the olfactory bulb of lampreys. The olfactory bulb of sea lampreys contains five types of GABAergic neurons (Meléndez-Ferro et al., 2001), but colocalization of dopamine and GABA was only observed in granule-like cells of inner cellular layer. In mammals, TH and GAD or GABA immunoreactivities have been reported to colocalize in some neurons of the olfactory bulb (rat: Kosaka et al., 1985; 1987; Gall et al., 1987; hamster: Kosaka et al., 1988). The presence of TH-ir/GABA-ir cells has been also reported in the olfactory bulb of a snake (Kosaka et al., 1991). Accordingly, these studies suggest that the local circuit dopaminergic/GABAergic cells of the olfactory bulbs appeared early on in phylogeny, before the separation of agnathans and gnathostomes. However, it is not clear whether the dopaminergic/GABAergic cell types (granule-like cells) found in lamprey olfactory bulbs correspond to those reported in land vertebrates. In mammals, the two types of double labelled cells were mainly located in the periglomerular region and the external plexiform layer (rat: Kosaka et. al, 1985; Gall et al., 1987; hamster: Kosaka et al., 1988), whereas no double labelled cells were observed in the periglomerular region of lamprey. In a snake, two types of double labelled (GABA/TH) cells of the olfactory bulbs were mostly located in periglomerular regions but also in mitral/plexiform layers (Kosaka et al., 1991). Thus, the type of dopaminergic/GABAergic cell present in the olfactory bulb of the sea lamprey appears to differ from those observed in mammals and snakes.

DA and GABA are colocalized in cells of the striatum of the adult lamprey brain

The present results revealed the presence of DA-ir cells in the striatum, where about a quarter of those non-CSF-c cells and all of those CSF-c cells were also GABA-ir. In mammals, the striatum also harbours a population of dopaminergic cells, all of non-CSF-c type, and the vast majority of which are similar to medium-sized spiny striatal interneurons and express the GAD65 enzyme (reviewed by Huot and Parent, 2007). The striatum is the largest integrative component of the basal ganglia. The presence of a dopaminergic/GABAergic neuronal population in the lamprey striatum (present results) suggests that the neurochemical organization of the basal ganglia is quite well conserved between lampreys and mammals. In mammals, the major source of dopamine in the striatum is the substantia nigra, whereas there is no similar dopaminergic midbrain nucleus in lampreys. The dopaminergic input to the lamprey

striatum originates from the posterior tubercle nucleus (i.e. paratubercular nucleus), which has been suggested to be homologous to the mammalian substantia nigra (Pombal et al., 1997). The motor deficits arising after striatal depletion of dopamine are qualitatively similar in lampreys and mammals, and the role of the dopaminergic innervation of the striatum appears to be conserved throughout vertebrate evolution (Thompson et al., 2008). However, our study shows no GABA immunoreactivity in the DA-ir cells of the sea lamprey paratubercular nucleus, whereas the coexistence of TH and GAD (Hédou et al., 2000; González-Hernández et al., 2001) and DA and GABA (González-Hernández et al., 2001) have been reported in some substantia nigra neurons of mammals.

Functional considerations on the colocalization of DA and GABA in brain neurons

The presence of DA and GABA immunoreactivities in fibres or terminals in different regions of the lamprey brain suggests the co-release of both neurotransmitters by some lamprey neurons distributed through various brain regions. Although several studies have shown colocalization of GABAergic and catecholaminergic markers (see above), functional studies on these cells are scant. Functional evidence of the co-release of the fast-acting inhibitory neurotransmitter GABA and DA by periglomerular cells of the rat olfactory bulb has recently been reported (Maher and Westbrook, 2008). The functional significance of the coexistence of DA, GABA and neuropeptide Y in axon terminals for the regulation of alpha-MSH release has also been investigated in *Xenopus* pituitary, where all three neurotransmitters appear to act as fast inhibitory neurotransmitters in an additive way, but with differential actions (Leenders et al., 1993). The results of these studies may also apply to other cells that display colocalization of GABA and dopamine, including those of lampreys, although this must be tested in further physiological studies.

It is now well established that axons of dopaminergic neurons can exhibit varicosities or terminals with synaptic membrane specialization and terminals without junctional complexes, which are morphologically distinguishable by electron microscopy (see Descarries et al., 2008). The transmitter released from these non-junctional or non-synaptic terminals (asynaptic) may diffuse into the extracellular space and reach remote targets, allowing for effects at a distance or volume transmission. The finding that a proportion of monoaminergic neurons of rat were immunoreactive for glutamate provided indirect evidence that CNS monoamine neurons may use glutamate as a cotransmitter, a hypothesis also supported by physiological evidence on the nigrostriatal system in vivo and in culture (Sulzer et al., 1998). For those dopaminergic neurons expressing glutamate it

has been proposed that the non-synaptic terminals may be specialized for the release of DA, while the terminals with synaptic junctions may constitute the site of glutamate release (Trudeau and Gutiérrez, 2007; Descarries et al., 2008). Another possibility is the co-release of both neurotransmitters at the same synaptic terminals (Descarries et al., 2008). Similarly, one or both of these mechanisms of neurotransmitter release may apply to neurons containing DA and GABA. The extensive colocalization of DA and GABA observed in neurons of different brain nuclei makes sea lampreys a potential vertebrate model for testing these hypotheses and studying cotransmission by "classical neurotransmitters".

Conclusions

Colocalization of dopamine and GABA occurs in dopaminergic cells and fibers of various CNS regions of lampreys. Comparison of the present results in the sea lamprey with results of studies in other animals suggests that colocalization of these two neurotransmitters is an ancient characteristic of dopaminergic cells. Together with previous reports on colocalization of GABA and DA in the spinal cord (Rodicio et al., 2008) and of GABA and serotonin in the brain (Chapter 3.1), the present results show that the coexpression of classical neurotransmitters in monoaminergic CNS populations in lampreys is far more extensive and complex than previously reported. Lampreys potentially constitute a very interesting animal model for exploring cotransmission and the functions of dual release of classical neurotransmitters in the brain of vertebrates.

Literature cited

Abalo XM, Villar-Cheda B, Anadón R, Rodicio MC. 2005. Development of the dopamine-immunoreactive system in the central nervous system of the sea lamprey. Brain Res Bull 66: 560-564.

Betarbet R, Turner R, Chockkan V et al. 1997. Dopaminergic neurons intrinsic to the primate striatum. J Neurosci 17: 6761-6768.

Chronwall BM. 1985. Anatomy and physiology of the neuroendocrine arcuate nucleus. Peptides 6: 1-11.

de Rijk EP, van Strien FJ, Roubos EW. 1992. Demonstration of coexisting catecholamine (dopamine), amino acid (GABA), and peptide (NPY) involved in inhibition of melanotrope cell activity in *Xenopus laevis*: a quantitative ultrastructural, freeze-substitution immunocytochemical study. J Neurosci 12: 864-871.

Descarries L, Bérubé-Carrière N, Riad M, Bo GD, Mendez JA, Trudeau LE. 2008. Glutamate in dopamine neurons: synaptic versus diffuse transmission. Brain Res Rev 58: 290-302.

Díaz-Ríos M and Miller MW. 2005. Rapid dopaminergic signaling by interneurons that contain markers for catecholamines and GABA in the feeding circuitry of *Aplysia*. J Neurophysiol 93: 2142-2156.

Díaz-Ríos M, Oyola E, Miller MW. 2002. Colocalization of gamma-aminobutyric acid-like immunoreactivity and catecholamines in the feeding network of *Aplysia californica*. J Comp Neurol 445: 29-46.

Distler P. 1990. Synaptic connections of dopamine-immunoreactive neurons in the antennal lobes of *Periplaneta americana*. Colocalization with GABA-like immunoreactivity. Histochemistry 93: 401-408.

Dubuc R, Brocard F, Antri M et al. 2008. Initiation of locomotion in lampreys. Brain Res Rev 57: 172-182.

Gall CM, Hendry SH, Seroogy KB, Jones EG, Haycock JW. 1987. Evidence for coexistence of GABA and dopamine in neurons of the rat olfactory bulb. J Comp Neurol 266: 307-318.

González-Hernández T, Barroso-Chinea P, Acevedo A, Salido E, Rodríguez M. 2001. Colocalization of tyrosine hydroxylase and GAD65 mRNA in mesostriatal neurons. Eur J Neurosci 13: 57-67.

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.

Gutiérrez R. 2005. The dual glutamatergic-GABAergic phenotype of hippocampal granule cells. Trends Neurosci 28: 297-303.

Hédou G, Chasserot-Golaz S, Kemmel V et al. 2000. Immunohistochemical studies of the localization of neurons containing the enzyme that synthesizes dopamine, GABA, or gamma-hydroxybutyrate in the rat substantia nigra and striatum. J Comp Neurol 426: 549-560.

Hökfelt T, Arvidsson U, Bean A et al. 1992. Colocalization of neuropeptides and classical neurotransmitters -- functional significance. Clin Neuropharmacol 15 Suppl 1 Pt A: 309A-310A.

Huot P and Parent A. 2007. Dopaminergic neurons intrinsic to the striatum. J Neurochem 101, 1441-1447.

Kosaka T, Hataguchi Y, Hama K, Nagatsu I, Wu JY. 1985. Coexistence of immunoreactivities for glutamate decarboxylase and tyrosine hydroxylase in some neurons in the periglomerular region of the rat main olfactory bulb: possible coexistence of gamma-aminobutyric acid (GABA) and dopamine. Brain Res 343: 166-171.

Kosaka T, Kosaka K, Hataguchi Y et al. 1987. Catecholaminergic neurons containing GABA-like and/or glutamic acid decarboxylase-like immunoreactivities

in various brain regions of the rat. Exp Brain Res 66: 191-210.

Kosaka K, Hama K, Nagatsu I, Wu JY, Kosaka T. 1988. Possible coexistence of amino acid (gamma-aminobutyric acid), amine (dopamine) and peptide (substance P); neurons containing immunoreactivities for glutamic acid decarboxylase, tyrosine hydroxylase and substance P in the hamster main olfactory bulb. Exp Brain Res 71: 633-642.

Kosaka T, Kosaka K, Nagatsu I. 1991. Tyrosine hydroxylase-like immunoreactive neurons in the olfactory bulb of the snake, *Elaphe quadrivirgata*, with special reference to the colocalization of tyrosine hydroxylase- and GABA-like immunoreactivities. Exp Brain Res 87: 353-362.

Leenders HJ, de Koning HP, Ponten SP, Jenks BG, Roubos EW. 1993. Differential effects of coexisting dopamine, GABA and NPY on alpha-MSH secretion from melanotrope cells of *Xenopus laevis*. Life Sci 52: 1969-1975.

Lu T, Rubio ME, Trussell LO. 2008. Glycinergic transmission shaped by the corelease of GABA in a mammalian auditory synapse. Neuron 57: 524-535.

Maher BJ and Westbrook GL. 2008. Co-transmission of dopamine and GABA in periglomerular cells. J Neurophysiol 99: 1559-1564.

Meléndez-Ferro M, Pérez-Costas E, Rodríguez-Muñoz R, Gómez-López MP, Anadón, R, Rodicio MC. 2001 GABA immunoreactivity in the olfactory bulbs of the adult sea lamprey *Petromyzon marinus* L. Brain Res 893: 253-260.

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B et al. 2002. Ontogeny of gamma-aminobutyric acid-immunoreactive neuronal populations in the forebrain and midbrain of the sea lamprey. J Comp Neurol 446: 360-376.

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B, Rodríguez-Muñoz R, Anadón R, Rodicio MC. 2003. Ontogeny of gamma-aminobutyric acid-immunoreactive neurons in the rhombencephalon and spinal cord of the sea lamprey. J Comp Neurol 464: 17-35.

Pierre J, Mahouche M, Suderevskaya EI, Repérant J, Ward R. 1997. Immunocytochemical localization of dopamine and its synthetic enzymes in the central nervous system of the lamprey *Lampetra fluviatilis*. J Comp Neurol 380: 119-135.

Pombal MA, El Manira A, Grillner S. 1997. Afferents of the lamprey striatum with special reference to the dopaminergic system: a combined tracing and immunohistochemical study. J Comp Neurol 386: 71-91.

Robertson B, Auclair F, Ménard A, Grillner S, Dubuc R. 2007. GABA distribution in lamprey is phylogenetically conserved. J Comp Neurol 503: 47-63.

Rodicio MC, Villar-Cerviño V, Barreiro-Iglesias A, Anadón R. 2008. Colocalization of dopamine and GABA in spinal cord neurones in the sea lamprey. Brain Res Bull 76: 45-49.

Rodríguez MA, Anadón R, Rodríguez-Moldes I. 2003. Development of galanin-like immunoreactivity in the brain of the brown trout (*Salmo trutta fario*), with some observations on sexual dimorphism. J Comp Neurol 465: 263-285.

Sandler R and Smith AD. 1991. Coexistence of GABA and glutamate in mossy fiber terminals of the primate hippocampus: an ultrastructural study. J Comp Neurol 8: 177-192.

Schimchowitsch S, Vuillez P, Tappaz ML, Klein MJ, Stoeckel ME. 1991. Systematic presence of GABA-immunoreactivity in the tubero-infundibular and tubero-hypophyseal dopaminergic axonal systems: an ultrastructural immunogold study on several mammals. Exp Brain Res 83: 575-586.

Strata P and Harvey R. 1999. Dale's principle. Brain Res Bull 50: 349-350.

Sueiro C, Carrera I, Molist P, Rodríguez-Moldes I, Anadón R. 2004. Distribution and development of glutamic acid decarboxylase immunoreactivity in the spinal cord of the dogfish *Scyliorhinus canicula* (elasmobranchs). J Comp Neurol 478: 189-206.

Sulzer D, Joyce MP, Lin L et al. 1998. Dopamine neurons make glutamatergic synapses in vitro. J Neurosci 18: 4588-4602.

Thompson RH, Ménard A, Pombal M, Grillner S. 2008. Forebrain dopamine depletion impairs motor behavior in lamprey. Eur J Neurosci 27: 1452-1460.

Trudeau LE. 2004. Glutamate co-transmission as an emerging concept in monoamine neuron function. J Psychiatry Neurosci 29: 296-310.

Trudeau LE and Gutiérrez R (2007) On cotransmission & neurotransmitter phenotype plasticity. Mol Interv 7: 138-146.

Tuinhof R, González A, Smeets WJ, Scheenen WJ, Roubos EW. 1994. Central control of melanotrope cells of *Xenopus laevis*. Eur J Morphol 32: 307-310.

Vigh B, Manzano e Silva MJ, Frank CL et al. 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-Cerviño V, Barreiro-Iglesias A, Anadón R, Rodicio MC. 2008a. Aspartate immunoreactivity in the telencephalon

of the adult sea lamprey: comparison with GABA immunoreactivity. Brain Res Bull 75: 246-250.

Villar-Cerviño V, Barreiro-Iglesias A, Anadón R, Rodicio MC. 2008b. Distribution of glycine immunoreactivity in the brain of adult sea lamprey (*Petromyzon marinus*). Comparison with gamma-aminobutyric acid. J Comp Neurol 507: 1441-1463.

Vuillez P, Pérez SC, Stoeckel ME. 1987. Colocalization of GABA and tyrosine hydroxylase immunoreactivities in the axons innervating the neurointermediate lobe of the rat pituitary: an ultrastructural immunogold study. Neurosci Lett 18: 53-58.

Abbreviations

CRh	Caudal rhombencephalon
DHyp	Dorsal hypothalamus
Hyp	Hypophysis
LP	Lateral pallium
Mm	Mammillary nucleus
MP	Medial pallium
MT	Mesencephalic tegmentum
OB	Olfactory bulbs
ON	Optic nerve
OT	Optic tectum
P	Pineal organ
PCN	Postoptic commissure nucleus,
PON	Preoptic nucleus
PO	Preoptic region
PoC	Postoptic commissure
Pp	Parapineal organ
Pt	Pretectum
PTh	Prethalamus
PtN	Paratubercular nucleus
Rh	Rhombencephalon
SC	Spinal cord
ST	Striatum

Th	Thalamus
TrN	Trigeminal nucleus
TS	Torus semicircularis
VIS	Ventral isthmus
Vhyp	Ventral hypothalamus

FIGURES
CHAPTER 3.2

Figure 1. Schematic drawing of a lateral view of the brain of the sea lamprey showing the distribution of double labelled DA/GABA immunoreactive cells (black dots) in comparison with the distribution of DA-ir cells (grey dots). M1-3 and MI represent giant Müller cells. Scale bar: 1 mm.

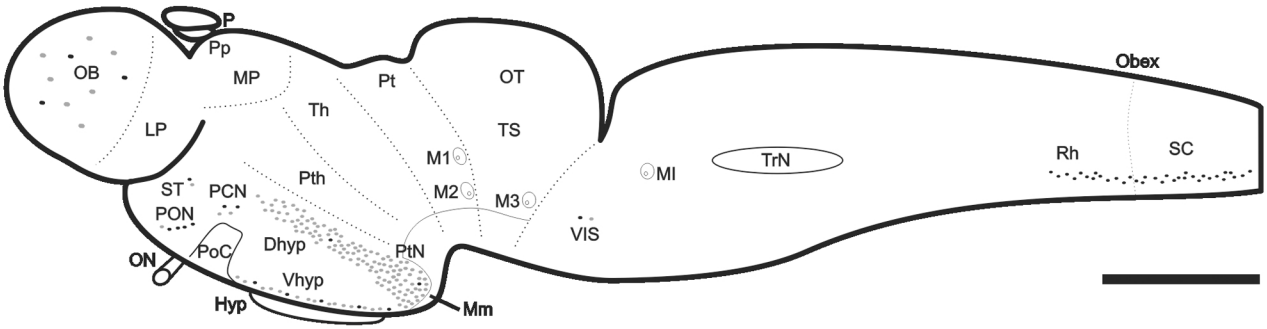

P
Pp
OB
MP
Pt
OT
Th
TS
LP
M1
Pth
M2
M3
MI
TrN
Obex
Rh
SC
ST
PCN
PON
VIS
PoC
Dhyp
PtN
ON
Vhyp
Hyp
Mm

Figure 1

Figure 2. Transverse sections of the brain showing DA (red) and GABA (green) immunoreactivities. (A-A´´) Photomicrographs of a transverse section at the transition between the spinal cord and the rhombencephalon (obex) showing double labelled CSF-c cells in the ventral midline, close to the fourth ventricle. Note the presence of a DA-ir/GABA-ir cell ventral to the ependyma (arrow). Note also the presence of double labelled thin fibers coursing in the raphe (arrowheads). (B) Detail of the double labelled fibers of Fig. 2A. (C) Photomicrograph of a transverse section of the caudal rhombencephalon showing double labelled CSF-c cells. Note the presence of a double labelled process arising from a DA-ir/GABA-ir CSF-c cell (arrowhead). (D) Detail of Fig. 2C. (E-E'') Photomicrographs of a transverse section of the rostral rhombencephalon showing double labelled cells in the ventral isthmus (arrows). Note the presence of a dopaminergic cell not labelled by the anti-GABA antibody (curved arrow). Also note the presence of a double labelled axonal like process arising from one of the DA-ir/GABA-ir cells (arrowhead). (F) Photomicrograph of a transverse section of the ventral hypothalamus showing double-labelled cells (arrows) in addition to a number of singly GABA-ir and DA-ir cells. (G-G') Detail of a double labelled cell from Fig. 2F. Stars indicate the ventricle. Dorsal is up in figures 2A-E' and right in figures 2F-G'. For other abbreviations, see list. Scale bars: 50 µm in A-A'', C and F, 25 µm in E-E'' and 12.5 µm in B, D and G-G'.

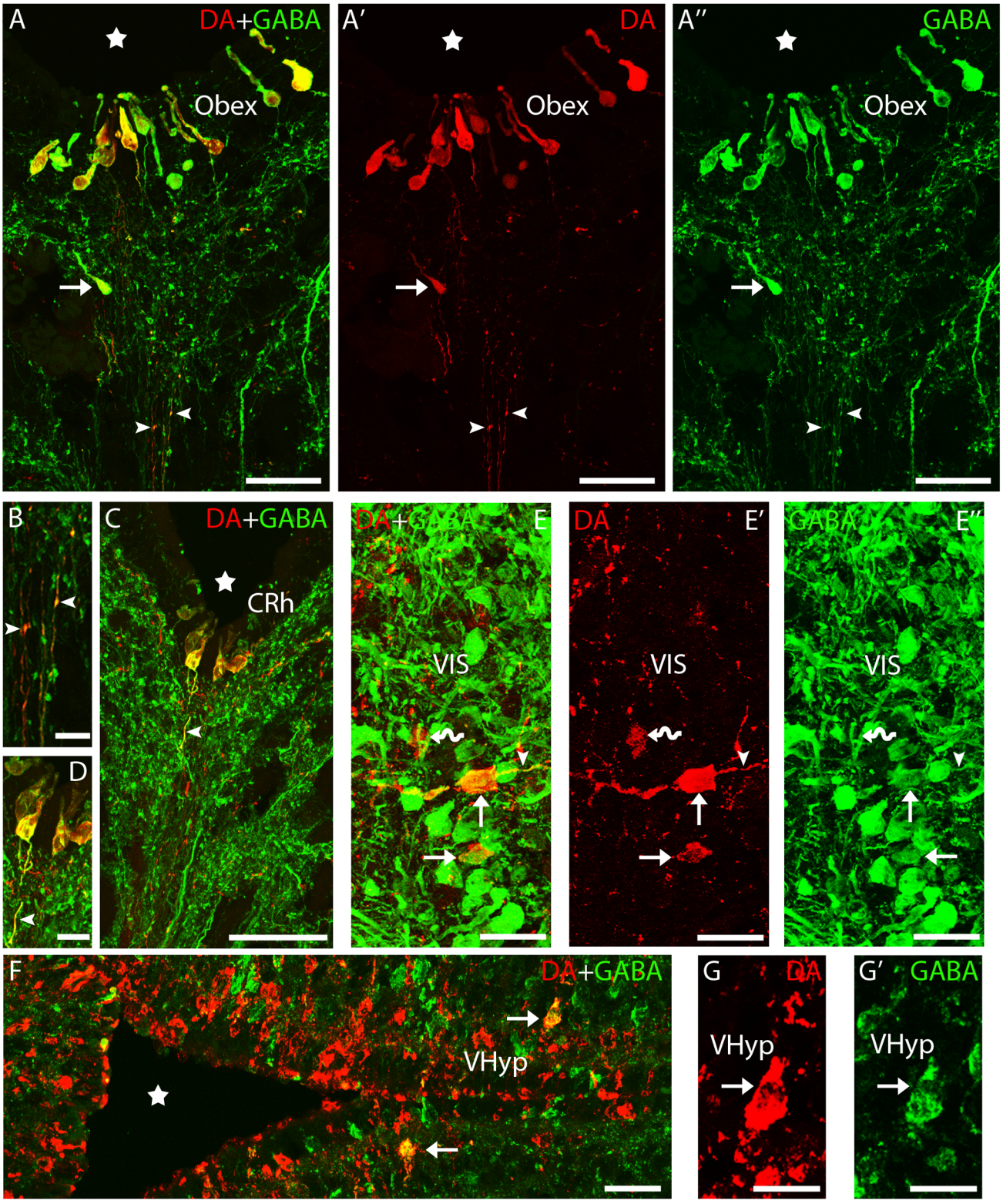

A
DA+GABA
Obex
A′
DA
Obex
A″
GABA
Obex
B
C
DA+GABA
CRh
D
DA+GABA
E
VIS
DA
E′
VIS
GABA
E″
VIS
F
DA+GABA
VHyp
G
DA
VHyp
G′
GABA
VHyp

FIGURE 2

Figure 3. Transverse sections of the rostral diencephalon and telencephalon showing DA (red) and GABA (green) immunoreactivities. (A-A'') Photomicrographs of a transverse section of the rostral diencephalon showing a double labelled cell in the dorsal part of the postoptic commissure nucleus (arrows). (B-B'') Photomicrographs of a transverse section of the striatum showing a double labelled non-CSF-c cell of this region. (C-C'') Photomicrographs of a transverse section of the striatum showing a double labelled CSF-c cell. (D) Photomicrograph of a section of the preoptic region showing the high proportion of double labelled (GABA/DA) dopaminergic cells. (E-E'') Details of a double labelled cell of the preoptic nucleus. Note the presence of a double labelled terminal (arrowhead). Arrows indicate a double labelled cells. Stars in figures indicate the impar telencephalic ventricle (A-C) and the preoptic recess (D). Photomicrographs B-C'' and E-E'' are Z-stack projections from 8 confocal optical sections (0.5 μm thick). Dorsal is up in all figures. For abbreviations, see list. Scale bars: 50 μm in A-A'' and D, 12.5 μm in B-B'', 15 μm in C-C'' and 20 in E-E''.

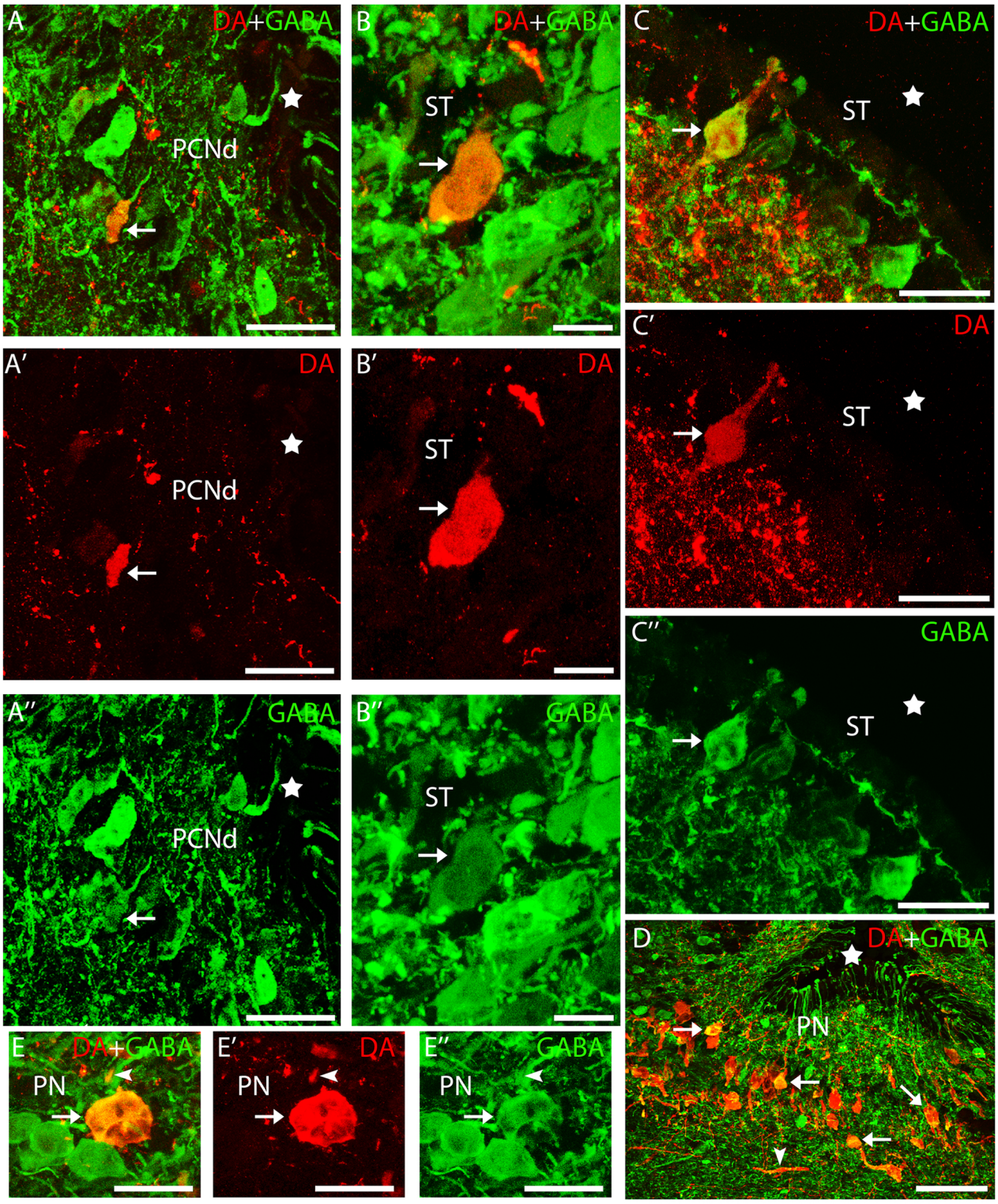

A
DA+GABA
PCNd
B
DA+GABA
ST
C
DA+GABA
ST
C′
DA
ST
A′
DA
PCNd
B′
DA
ST
C″
GABA
ST
A″
GABA
PCNd
B″
GABA
ST
D
DA+GABA
PN
E
DA+GABA
PN
E′
DA
PN
E″
GABA
PN

FIGURE 3

Figure 4. Transverse section of the sea lamprey olfactory bulb showing DA (red) and GABA (green) immunoreactivities. (A-A'') Photomicrographs of a transverse section of the olfactory bulb showing a double labelled granule-like cell (arrowhead). Dorsal is up in the figure. For abbreviations, see list. Scale bars: 25 μm in A and 50 μm in B-B''.

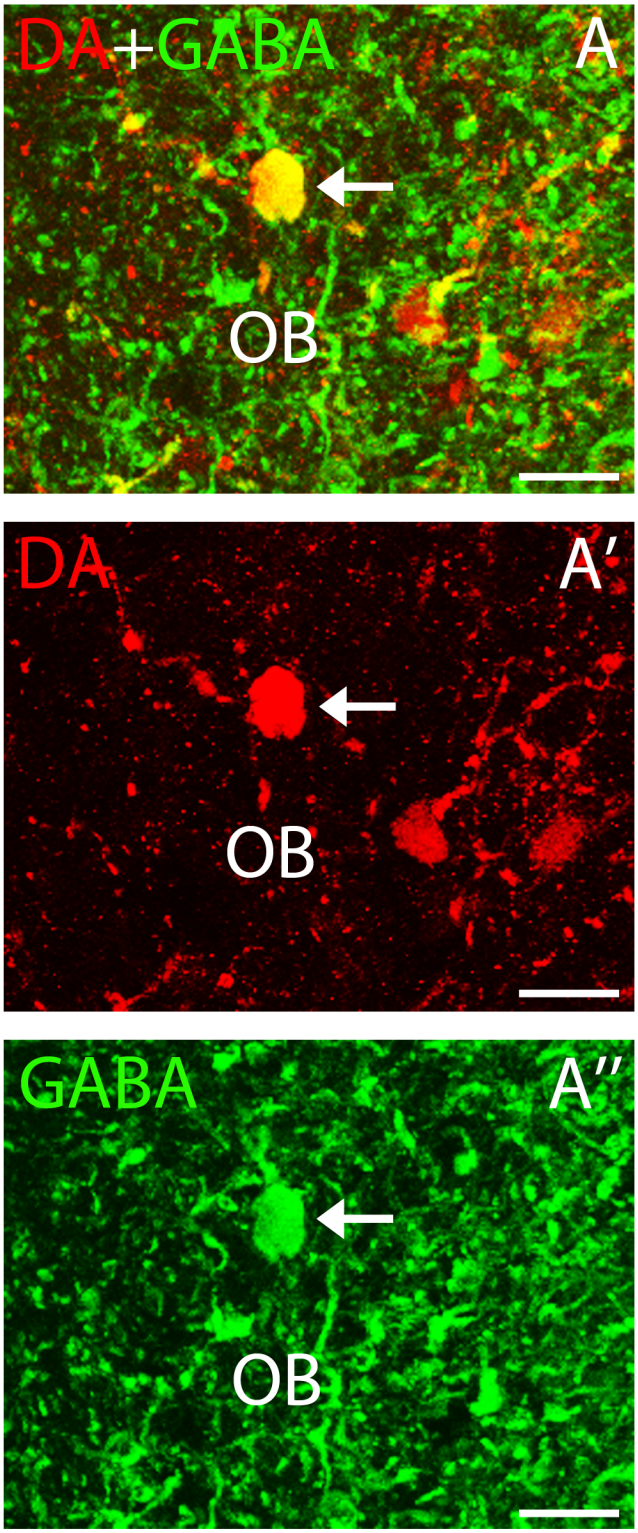


FIGURE 4

Figure 5. Photomicrographs of transverse sections of the sea lamprey brain showing DA (red) and GABA (green) immunoreactivities in fibres or terminals (arrow heads). (A) Caudal rhombencephalon. (B-B'') Mesencephalic tegmentum. (C) Preoptic region. (D) Striatum. (E) Medial pallium. Photomicrographs A and C are Z-stack projections from confocal images. Photomicrographs B-B'' and E are Z-stack projections from 4 confocal optical sections (0.5 μm thick). Photomicrograph D is a 0.5 μm thick confocal optical section. Dorsal is up in all figures. For abbreviations, see list. Scale bars: 12 μm in A, C-E and 18 μm in B-B''.

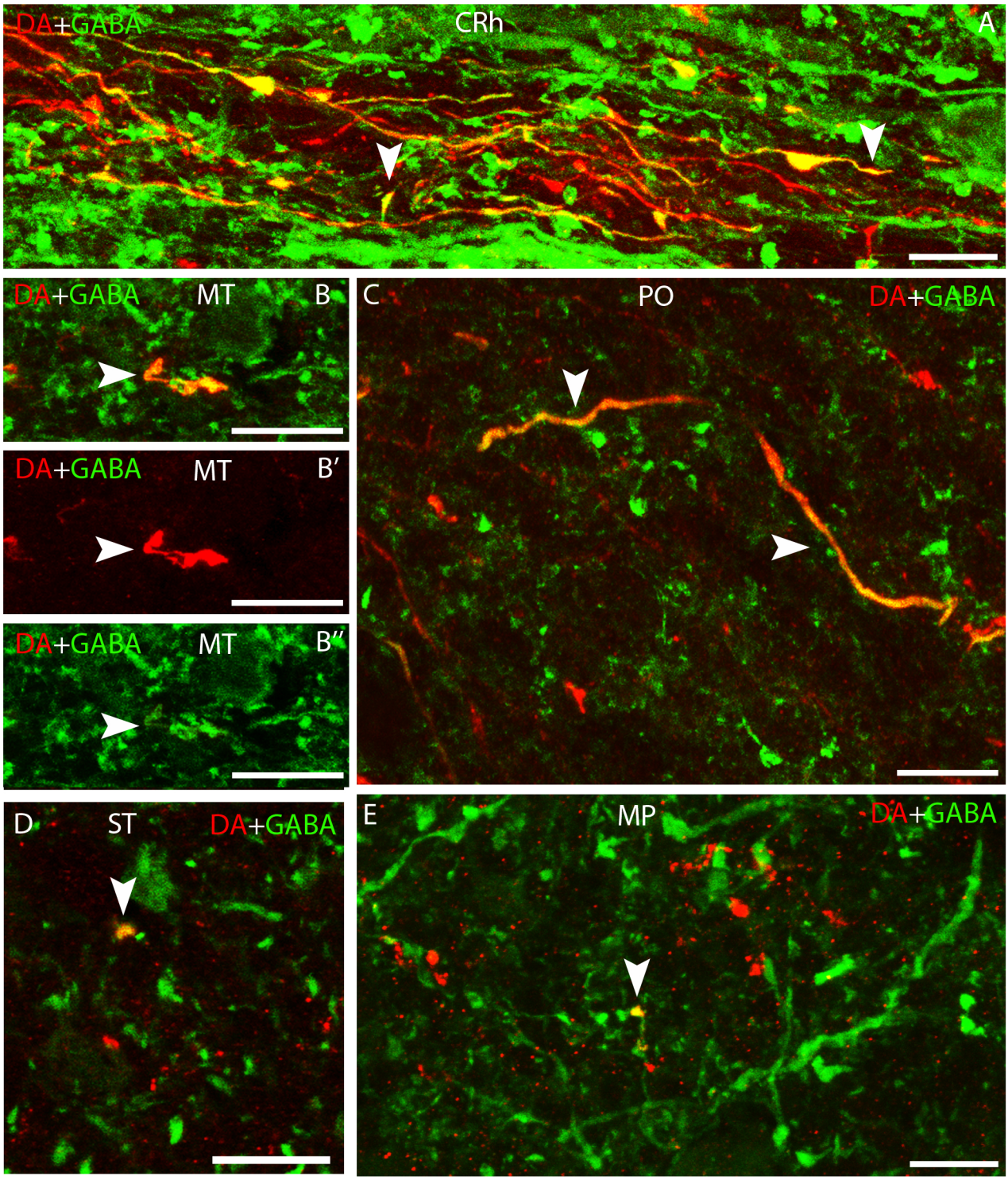
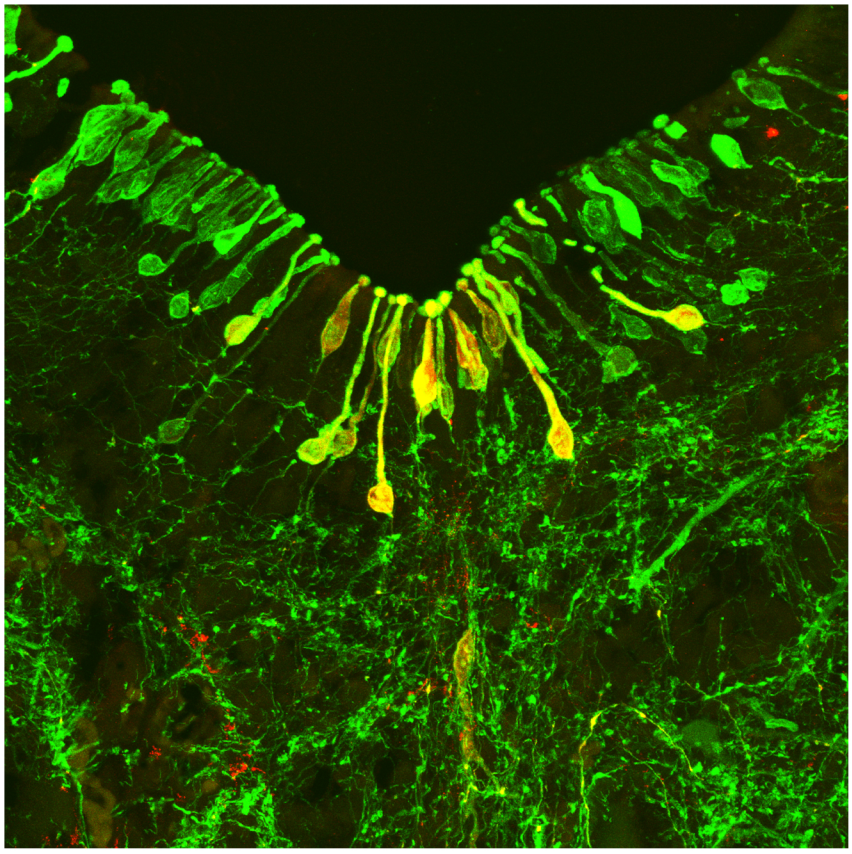

DA+GABA
CRh
A
DA+GABA
MT
B
C
PO
DA+GABA
DA+GABA
MT
B′
DA+GABA
MT
B″
D
ST
DA+GABA
E
MP
DA+GABA

FIGURE 5

Summary and conclusions



SUMMARY

The monoamines serotonin and dopamine are neurotransmitters in the nervous system of vertebrates that modulate a number of neuronal functions. Lampreys belong to the most primitive group of extant vertebrates, the jawless vertebrates or agnathans, considerer as the sister group of jawed vertebrates (gnathostomes). So, they occupy a key phylogenetic position at the root of the vertebrate group, which make them an interesting animal model to study the evolution of the nervous system. In addition, lampreys have emerged as an interesting animal model to study mechanisms of nervous system regeneration, the neuronal circuitry that underlies locomotion in vertebrates or to be used to test the role of different molecules in nervous system diseases. Basic neuroanatomical research in lampreys has provided important information about the organization and development of the nervous system to further conduct other type of neurobiological studies. In the present thesis project, we aimed to advance on the knowledge about the organization of neurotransmitters systems of lampreys focusing in the organization of the monoaminergic system.

Previous studies of our group reported the development and organization of the serotonin and dopamine immunoreactive systems of the central nervous system of the sea lamprey (Abalo et al., 2005, 2007). In the present thesis project, we studied new aspects of these systems: the peripheral organization of the serotonergic system, the origin of the descending monoaminergic innervation of the sea lamprey spinal cord and the coexistence of serotonin or dopamine and GABA in sea lamprey brain neurons.

In the first chapter of the thesis the organization of the serotonergic peripheral nervous system of the sea lamprey head is described. Different serotonergic cells types and a complex network of serotonergic fibers were observed in the different head tissues. Among the different structures containing serotonergic cells we focused on the neurochemical characterization of the sea lamprey taste buds. No catecholaminergic structures appear to be present in the sea lamprey head outside the central nervous system. In the second chapter, results concerning to the investigation of the origin of the descending serotonergic and dopaminergic innervation of the sea lamprey spinal cord are presented. In addition, new cell nuclei, mainly formed by CSF-c cells, of the brain not previously reported as bearing descending neurons in lampreys are described here for the first time. Finally, results about the investigation of the colocalization of GABA in restricted serotonergic and dopaminergic populations of the lamprey brain are presented. All chapters of this Doctoral Thesis were previously published in the form of

research articles with the following references:

- Chapter 1.1: J Comp Neurol (2009) 512: 478-499.
- Chapter 1.2: J Comp Neurol (2008) 511: 438-453.
- Chapter 2.1: J Comp Neurol (2008) 511: 711-723.
- Chapter 2.2: J Chem Neuroanat (2008) 36: 77-84.
- Chapter 3.1: J Anat (2009) 215: 435-443.
- Chapter 3.2: J Anat (2009) 215: 601-610.

For this thesis project immunofluorescence methods were used for the characterization of monoaminergic structures, some other neuronal types and their innervation patterns by using antibodies generated against different neuronal markers (serotonin, dopamine, GABA, glutamate, glycine, tyrosine hydroxylase, NPY, calretinin or acetylated α-tubulin). High performance liquid chromatography methods were used to biochemically confirm the presence of monoamines in the lamprey head without the brain and spinal cord. Tract-tracing methods (using neurobiotin or biotinylated dextranamines as retrograde tracers) in combination with serotonin or dopamine immunofluorescence were used to determine the origin of the descending monoaminergic innervation of the spinal cord.

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

CHAPTER 1.1. EXTENSIVE PRESENCE OF SEROTONERGIC CELLS AND FIBERS IN THE PERIPHERAL NERVOUS SYSTEM OF LAMPREYS.

Lampreys are suitable animal models for studying the evolution of the vertebrate nervous system because they belong to the earliest group of extant vertebrates, the agnathans. The general organization of the serotonergic central nervous system is well known in lampreys, but information about its peripheral organization is lacking. In the present study, high performance liquid chromatography and immunohistochemistry with an antibody against serotonin were used to study the presence and peripheral distribution of serotonergic elements in the head of larval sea lampreys. Adult lampreys were also analyzed in order to compare the organization of the system in larval and postmetamorphic stages. Serotonergic cells were observed in the cranial nerve ganglia, the musculature, the taste buds and in the gills. Serotonergic fibers were extensive in all tissues except the epidermis. Possible roles were proposed for the different cell types observed in relation to their location, structure and innervation patterns. The serotonergic cells appear to be involved in the oxygen and taste chemoreception processes, the modulation of musculature contraction and in the regulation of cilia beat frequency and mucus secretion in lampreys. The extensive presence of serotonergic elements in the peripheral nervous system of lampreys suggests a close relationship with the organization of this system in invertebrate groups.

CHAPTER 1.2. NEUROCHEMICAL CHARACTERIZATION OF THE SEA LAMPREY TASTE BUDS AND AFFERENT GUSTATORY FIBRES. PRESENCE OF SEROTONIN, CALRETININ AND CGRP IMMUNOREACTIVITIES IN TASTE BUD BI-CILIATED CELLS OF THE EARLIEST VERTEBRATES.

Neuroactive substances such as serotonin and other monoamines have been suggested to be involved in the transmission of gustatory signals from taste bud cells to afferent fibres. Lampreys are the earliest vertebrates that possess taste buds, although these differ in structure from taste buds in jawed vertebrates, and their neurochemistry remains unknown. We used immunofluorescence methods with antibodies raised against serotonin, tyrosine hydroxylase (TH), GABA, glutamate, calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), calretinin and acetylated α -tubulin to characterize the neurochemistry and innervation of taste buds in the sea lamprey, *Petromyzon marinus* L. For localization of proliferative cells in taste buds we used bromodeoxyuridine (BrdU) labeling and proliferating cell nuclear antigen immunohistochemistry. Results with both markers indicate that proliferating cells are restricted to a few basal cells, and that almost all cells in taste buds are non-proliferating since early developmental stages. A large number of serotonin, calretinin and CGRP immunoreactive bi-ciliated cells were revealed in lamprey taste buds. This suggests that serotonin participates in the transmission of gustatory signals and indicates that this substance appeared early in this system in vertebrate evolution. The basal surface of the bi-ciliated taste bud cells was contacted by tubulin-ir fibres. Some of the fibres surrounding the taste bud were calretinin immunoreactive. Lamprey taste bud cells or afferent fibres did not exhibit TH, GABA, glutamate or NPY immunoreactivity, which suggests that expression of these substances evolved in taste buds of some gnathostomes lines after the separation of gnathostomes and lampreys.

CHAPTER 2.1. DESCENDING BRAIN-SPINAL CORD PROJECTIONS IN A PRIMITIVE VERTEBRATE, THE LAMPREY: CEREBROSPINAL FLUID-CONTACTING AND DOPAMINERGIC NEURONS.

We used neurobiotin as a retrograde tract-tracer in both larval and adult sea lampreys, and observed a number of neuronal brainstem populations (mainly reticular and octaval populations and some diencephalic nuclei) that project to the spinal cord, in agreement with the results of previous tracer studies. We also observed small labeled neurons in the ventral hypothalamus, the mammillary region and the paratubercular nucleus, nuclei that were not reported as spinal projecting. Notably, most of the labeled cells of the mammillary region and some of the ventral hypothalamus were cerebrospinal fluid-contacting (CSF-c) neurons. Combined tract-tracing and immunocytochemistry showed that some of the labeled neurons of the mammillary and paratubercular nuclei were dopamine immunoreactive. In addition, some CSF-c cells were labeled in the caudal rhombencephalon and rostral spinal cord, and many were also dopamine immunoreactive. Results with other tracers (biotinylated dextran amines, horseradish peroxidase and the carbocyanine dye DiI) also demonstrated that the molecular weight or the molecular nature of the tracer was determinant in revealing diencephalic cells with very thin axons. The results show that descending systems afferent to the spinal cord in lampreys are more varied than previously reported, and reveal a descending projection from CSF-c cells, which was unknown in vertebrates. The present results also reveal the existence of large differences between agnathans and gnathostomes in the organization of the dopaminergic cells that project to the spinal cord.

CHAPTER 2.2. DEVELOPMENT AND ORGANIZATION OF THE DESCENDING SEROTONERGIC BRAINSTEM-SPINAL PROJECTIONS IN THE SEA LAMPREY.

The organization and development of the descending spinal projections from serotonergic rhombencephalic neurons in the larval sea lamprey were investigated by double labeling, tract-tracing methods and immunocytochemistry against serotonin. The results showed that two serotonergic populations of the isthmic and vagal reticular regions present reticulospinal neurons from the beginning of the larval period. Of the three serotonergic subpopulations recognized in the isthmic reticular group, only two - the medial and ventral subpopulations - project to the spinal cord, with most of the projecting cells in the caudal part of the medial isthmic subpopulation. Occasional cells projecting to the spinal cord were observed in the ventral subpopulation. The vagal reticular serotonergic nucleus situated in the caudal rhombencephalon also presents cells with descending projections. The early development of the brainstem serotonergic projections to the spinal cord appears to be a conserved trait in all vertebrates studied. Although a serotonergic hindbrain-spinal projection system appears to have been present before the divergence of agnathans and gnathostomes, no serotonergic cells were observed in the raphe region in lamprey. Moreover, proportionally more rostral hindbrain serotonergic cells contribute to the spinal serotonergic projections in the sea lamprey than in jawed vertebrates.

CHAPTER 3.1. 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.

Colocalization of the classic neurotransmitters serotonin (5-HT) and γ-aminobutyric acid (GABA) (or the enzyme that synthesizes the latter, glutamate decarboxylase) has been reported in a few neurons of the rat raphe magnus-obscurus nuclei. However, there are no data on the presence of neurochemically similar neurons in the brain of non-mammalian vertebrates. Lampreys are the oldest extant vertebrates and may provide important data on the phylogeny of neurochemical systems. The colocalization of 5-HT and GABA in neurons of the sea lamprey brain was studied using antibodies directed against 5-HT and GABA and confocal microscopy. Colocalization of the neurotransmitters was observed in the diencephalon and the isthmus. In the diencephalon, about 87% of the serotonergic cells of the rostral tier of the dorsal thalamus (close to the zona limitans) exhibited GABA immunoreactivity. In addition, occasional cells double labelled for GABA and 5-HT were observed in the hypothalamic tuberal nucleus and the pretectum. Of the three serotonergic isthmic subgroups already recognized in the sea lamprey isthmus (dorsal, medial and ventral), such double-labelled cells were only observed in the ventral subgroup (about 61% of the serotonergic cells in the ventral subgroup exhibited GABA immunoreactivity). An equivalence between these lamprey isthmic cells and the serotonergic/GABAergic raphe cells of mammals is suggested. Present findings suggest that serotonergic/GABAergic neurons are more extensive in lampreys than in the rat and probably appeared before the separation of agnathans and gnathostomes. Cotransmission by release of 5-HT and GABA by the here described lamprey brain neurons is proposed.

CHAPTER 3.2. DOPAMINE AND GABA ARE COLOCALIZED IN RESTRICTED GROUPS OF NEURONS IN THE SEA LAMPREY BRAIN: INSIGHTS INTO THE EARLY EVOLUTION OF NEUROTRANSMITTER COLOCALIZATION IN VERTEBRATES.

In recent years, the possible co-release of classic neurotransmitters from neurons has received much attention. Colocalization of monoamines and amino acidergic neurotransmitters (mainly glutamate and dopamine (DA) or serotonin) in mammalian neurons has been reported. However, few studies have dealt with the colocalization of DA and γ-aminobutyric acid (GABA) in neurons. With the aim of providing some insight into the colocalization of neurotransmitters during early vertebrate phylogeny, we studied GABA expression in dopaminergic neurons in the sea lamprey brain by using double immunofluorescence methods with anti-DA and anti-GABA antibodies. Different degrees of colocalization of DA and GABA were observed in different dopaminergic brain nuclei. A high degree of colocalization (GABA in at least 25% of DA-immunoreactive neurons) was observed in populations of the caudal rhombencephalon, the ventral isthmus, the postoptic commissure nucleus, the preoptic nucleus and in granule-like cells of the olfactory bulb. A new DA-immunoreactive striatal population that showed colocalization with GABA in about a quarter of its neurons was observed. In the periventricular hypothalamus, colocalization was observed in only a few cells, despite the abundance of DA- and GABA-immunoreactive neurons. No double labelled cells were observed in the paratubercular nucleus. The frequent colocalization of DA and GABA reveals that the dopaminergic populations of lampreys are more complex than previously reported. Double-labelled fibres or terminals were observed in different brain regions, which suggest possible co-release of DA and GABA by these lamprey neurons. The present results suggest that colocalization of DA and GABA in neurons appeared early on in vertebrate evolution.

CONCLUSIONS

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

1. Lampreys have a complex network of serotonergic cells and fibers in the peripheral nervous system not previously described in any other vertebrate species. The extensive presence of serotonergic elements in the peripheral nervous system of lampreys suggests a close relationship with the organization of this system in invertebrate groups.
2. The organization, morphology and innervation patterns of the serotonergic cells of the sea lamprey peripheral nervous system indicate that they could be involved in oxygen and taste chemoreception, the modulation of muscles contraction and in the regulation of cilia beat frequency and mucus secretion in lampreys.
3. The neurotransmitter serotonin appears to have a similar or common role in the transmission and/or response to the hypoxia situation in all vertebrates studied including lampreys.
4. The preponderance of serotonergic elements in the head and the complex metamorphosis of head structures in lampreys suggest a role of serotonin in the development and morphogenesis of the head as previously suggested in mammals.
5. Serotonin appears as the most probable neurotransmitter used by the bi-ciliated taste receptors for the transmission of the gustatory information in lampreys, whereas in mammals it acts as a neuromodulator. In addition, CGRP may act as a neuromodulator during the transmission of the gustatory information in lampreys.
6. Tract-tracing experiments demonstrated that small neurons, in the ventral hypothalamus, the mammillary region (being most of them cerebrospinal fluid-contacting cells) and the paratubercular nucleus project to the spinal cord in lampreys. The presence of cerebrospinal fluid-contacting cells projecting from the brain to the spinal cord probably y is a unique characteristic of lampreys.
7. Present results reveal the existence of large differences between agnathans and gnathostomes in the organization of the dopaminergic cells that project to the spinal cord. However, combination of hodological and immunohistochemical experiments confirms the equivalence between the paratubercular nucleus of lampreys and the posterior tubercle nucleus of other vertebrates.

8. Although a serotonergic hindbrain-spinal projection system appears to have been present before the divergence of agnathans and gnathostomes, no serotonergic cells were observed in the raphe region in the lamprey. The early development of the brainstem serotonergic projections to the spinal cord appears to be a conserved trait in all vertebrates studied.
9. Proportionally more rostral hindbrain serotonergic cells contribute to the spinal projections in the sea lamprey than in jawed vertebrates.
10. Present findings indicate that serotonergic/GABAergic neurons are more extensive in the sea lamprey than in the rat and that this cell type appeared before the separation of agnathans and gnathostomes. Present hodological and colocalization results also suggest equivalence between the sea lamprey serotonergic isthmic cells and the superior raphe cells of mammals.
11. Cotransmission by release of serotonin and GABA by diencephalic and isthmic lamprey neurons is proposed.
12. The presence of double-labelled fibers or terminals with antibodies against DA and GABA in different brain regions suggests possible co-release of these neurotransmitters by several lamprey neurons. Comparison of these systems in vertebrates suggests that colocalization of DA and GABA in neurons appeared early on during vertebrate evolution.
13. Present and previous results indicate that the GABAergic system of lampreys is more complex than previously reported and that colocalization/cotransmission of GABA with other classic neurotransmitters in neurons of the vertebrate brain is probably the rule rather than the exception.

RESUMEN

Las monoaminas serotonina y dopamina son neurotransmisores que modulan diversas funciones en el sistema nervioso de los vertebrados. Las lampreas pertenecen al grupo de vertebrados actuales más primitivo, los vertebrados sin mandíbula o agnatos. Por lo tanto, ocupan un lugar filogenético clave en la raíz de los vertebrados, lo cual hace que sean animales modelo interesantes para estudiar la evolución del sistema nervioso. Además, las lampreas también resultan animales modelo muy interesantes para el estudio de mecanismos de regeneración del sistema nervioso, para estudiar los circuitos neuronales que subyacen al proceso de locomoción en vertebrados o para ser usadas para determinar el papel patogénico que juegan diferentes moléculas en enfermedades del sistema nervioso. Por lo tanto, la investigación de tipo neuroanatómica en lampreas proporcionará información acerca de la organización y el desarrollo del sistema nervioso que servirá de base para llevar a cabo otro tipo de estudios neurobiológicos. En esta tesis, hemos intentado avanzar en el conocimiento y organización de los sistemas de neurotransmisores de las lampreas centrándonos en la organización del sistema monoaminérgico.

En estudios previos de nuestro grupo hemos publicado el desarrollo y organización de los sistemas inmunorreactivos a serotonina y dopamina en el sistema nervioso central de la lamprea de mar (Abalo y col., 2005, 2007). En esta tesis hemos estudiado nuevos aspectos de estos sistemas: la organización periférica del sistema serotonérgico, el origen de la inervación monoaminérgica descendente a la médula espinal de la lamprea de mar y la coexistencia de serotonina o dopamina y GABA en neuronas del cerebro de la lamprea de mar.

En el primer capítulo de la tesis se describe la organización del sistema serotonérgico periférico de la cabeza de larvas y adultos de lamprea de mar. Se observó la presencia de diferentes tipos celulares serotonérgicos y una compleja red de fibras serotonérgicas en los diferentes tejidos de la cabeza. Entre las diferentes estructuras conteniendo células serotonérgicas nos hemos centrado en la caracterización neuroquímica de las papilas gustativas de la lamprea de mar. No se observó la presencia de estructuras catecolaminérgicas en la cabeza de la lamprea de mar a excepción de en su sistema nervioso central. En el segundo capítulo se presentan los resultados relacionados con la investigación del origen de la inervación serotonérgica y dopaminérgica descendente a la médula espinal de la lamprea de mar. Además, como fruto de esta investigación se han encontrado nuevos núcleos cerebrales, principalmente

formados por células de tipo CSF-c, que no se habían descrito previamente como de innervación descendente a la médula. Finalmente los resultados relacionados con la investigación de la presencia de colocalización de serotonina o dopamina y GABA en determinados grupos de neuronas del cerebro de la lamprea se presentan en el último capítulo. Todos los capítulos de esta Tesis Doctoral han sido previamente publicados en forma de artículos de investigación con la siguientes referencias:

- Capítulo 1.1: J Comp Neurol (2009) 512: 478-499.
- Capítulo 1.2: J Comp Neurol (2008) 511: 438-453.
- Capítulo 2.1: J Comp Neurol (2008) 511: 711-723.
- Capítulo 2.2: J Chem Neuroanat (2008) 36: 77-84.
- Capítulo 3.1: J Anat (2009) 215: 435-443.
- Capítulo 3.2: J Anat (2009) 215: 601-610.

Para la realización de este proyecto de tesis se han empleado técnicas de inmunofluorescencia para la caracterización de las neuronas y fibras monoaminérgicas u otros tipos celulares; para ello se usaron anticuerpos generados contra diferentes marcadores neuronales (serotonina, dopamina, GABA, glutamato, tirosina hidroxilasa, NPY, calretinina o tubulina acetilada). La técnica de cromatografía líquida de alta precisión se usó para confirmar bioquímicamente la presencia de monoaminas en la cabeza de la lamprea de mar sin el cerebro ni la médula espinal. Anticuerpos contra diferentes marcadores neuronales (calretinina o tubulina acetilada) se emplearon para la caracterización de las estructuras monoaminérgicas y sus patrones de innervación. Métodos de trazado neuronal (usando neurobiotina o dextranaminas biotiniladas como trazadores retrógrados) en combinación con inmunofluorescencia contra serotonina o dopamina se usaron para determinar el origen de la innervación monoaminérgica descendente a la médula espinal.

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.

CAPÍTULO 1.1. ELEVADA PRESENCIA DE CÉLULAS Y FIBRAS SEROTONÉRGICAS EN EL SISTEMA NERVIOSO PERIFÉRICO DE LAS LAMPREAS.

Las lampreas son animales modelo muy adecuados para el estudio de la evolución del sistema nervioso de los vertebrados porque pertenecen al grupo de vertebrados actuales más temprano, los agnatos. La organización general del sistema nervioso central serotonérgico se conoce muy bien en las lampreas, pero falta información acerca de su organización periférica. En este estudio se emplearon técnicas de cromatografía líquida de alta precisión y técnicas de inmunohistoquímica, usando un anticuerpo contra serotonina, para determinar la presencia de elementos serotonérgicos en la cabeza de larvas de lamprea de mar. Lampreas adultas también fueron analizadas con el objetivo de comparar la organización del sistema entre los estadios larvario y postmetamórfico. Se observó la presencia de células serotonérgicas en los ganglios de los nervios craneales, la musculatura, las papilas gustativas y las branquias. En todos los tejidos a excepción de la epidermis se observó una amplia presencia de fibras serotonérgicas. Se propusieron diferentes papeles que podrían desempeñar los diferentes tipos celulares descritos en función de su localización, estructura o patrones de inervación. Las células serotonérgicas de la lamprea parecen estar implicadas en procesos de quimiorecepción de oxígeno y gusto, en la modulación de la contracción muscular y en la regulación del batido ciliar y la secreción de mucus. La amplia presencia de elementos serotonérgicos en el sistema nervioso periférico de las lampreas sugiere una relación muy cercana con la organización de este sistema en grupos de invertebrados.

CAPÍTULO 1.2. CARACTERIZACIÓN NEUROQUÍMICA DE LAS PAPILAS GUSTATIVAS Y DE LAS FIBRAS GUSTATIVAS AFERENTES DE LA LAMPREA DE MAR. PRESENCIA DE INMUNOREACTIVIDAD A SEROTONINA, CALRETININA Y CGRP EN CÉLULAS BI-CILIADAS DE LAS PAPILAS GUSTATIVAS DE LOS VERTEBRADOS MÁS TEMPRANOS.

Se ha sugerido que sustancias neuroactivas como la serotonina y otras monoaminas podrían estar involucradas en la transmisión de las señales del gusto desde las células que forman las papilas gustativas a las fibras aferentes. Las lampreas son los vertebrados más tempranos que poseen papilas gustativas, aunque la estrucutura de las mismas difiere con respecto a la de los vertebrados con mandíbula y su neuroquímica no es conocida. Hemos empleado métodos de inmunofluorescencia con anticuerpos generados contra serotonina, tirosina hidroxilasa (TH), GABA, glutamato, péptidos relacionado con el gen de la calcitonina (CGRP), neuropéptido Y (NPY), calretinina y tubulina acetilada para caracterizar la neuroquímica y la innervación de las papilas gustativas en la lamprea de mar, *Petromyzon marinus* L. Para determinar la localización de las células proliferativas en las papilas gustativas empleamos marcaje con bromodesoxiuridina (BrdU, detectado por inmunohistoquímica) e inmunohistoquímica contra el antígeno nuclear de proliferación celular. Los resultados con ambos marcadores indican que las células proliferantes se restringen a unas pocas células basales y que casi todas las células en las papilas gustativas no son proliferantes ya en estados tempranos de desarrollo. Se observó la presencia de un gran número de células bi-ciliadas inmunorreactivas a serotonina, calretinina y CGRP en las papilas gustativas de la lamprea de mar. Esto sugiere que la serotonina participa en la transmisión de las señales gustativas e indica que esta sustancia apareció en este sistema tempranamente durante la evolución de los vertebrados. Fibras inmunorreactivas a α-tubulina contactan la superficie basal de las células bi-ciliadas de las papilas gustativas. Varias de las fibras que rodean las papilas presentaron inmunorreactividad a calretinina. Las células de las papilas gustativas o las fibras aferentes no presentaron inmunorreactividad a TH, GABA, glutamato o NPY, lo que sugiere que la expresión de estas sustancias apareció en las papilas gustativas de diversas líneas de gnatóstomos después de la separación de gnatóstomos y lampreas.

CAPÍTULO 2.1. PROYECCIONES CEREBRO-ESPINALES DESCENDENTES EN UN VERTEBRADO PRIMITIVO, LA LAMPREA: NEURONAS CONTACTANTES DEL LÍQUIDO CEREBROESPINAL Y DOPAMINÉRGICAS.

Usamos la neurobiotina como trazador neuronal retrógrado tanto en larvas como en adultos de lamprea de mar y se observó la presencia de un alto número de poblaciones del tronco cerebral (principalmente núcleos reticulares, octavos y diencefálicos) que proyectan a la médula espinal, en concordancia con resultados publicados previamente con otros trazadores. También se observó la presencia de pequeñas neuronas en el hipotálamo ventral, la región mamilar y en el núcleo paratubercular; estos núcleos no fueron previamente reconocidos como de proyección espinal. La mayoría de las células de la región mamilar y varias del hipotálamo ventral son neuronas contactantes del líquido cerebroespinal (CSF-c). La inmunohistoquímica combinada con trazado neuronal mostró que parte de las neuronas marcadas en los núcleos mamilar y paratubercular son inmunorreactivas a dopamina. Además se marcaron varias células CSF-c del rombencéfalo caudal y la médula espinal rostral siendo parte de ellas inmunorreactivas a dopamina. Resultados con otros trazadores (dextranaminas biotiniladas, peroxidasa de rábano y DiI) también demostraron que el peso molecular o la naturaleza del trazador es determinante a la hora de revelar células diencefálicas con axones muy finos. Los resultados muestran que los sistemas descendentes aferentes a la médula espinal en lampreas son más variados que lo previamente descrito y revelan una proyección descendente desde células CSF-c, lo cual es desconocido en los vertebrados. Estos resultados también revelan la existencia de grandes diferencias entre agnatos y gnatóstomos en la organización de células dopaminérgicas que proyectan a la médula espinal.

CAPÍTULO 2.2. DESARROLLO Y ORGANIZACIÓN DE LAS PROYECCIONES DESCENDENTES SEROTONÉRGICAS DESDE EL TRONCO CEREBRAL A LA MÉDULA ESPINAL EN LA LAMPREA DE MAR.

Se emplearon técnicas de doble marcaje, mediante métodos de trazado neuronal e inmunocitoquímica contra serotonina, para investigar la organización y desarrollo de las proyecciones descendentes desde neuronas serotonérgicas rombencefálicas a la médula espinal. Los resultados obtenidos mostraron que dos poblaciones serotonérgicas de las regiones reticulares ístmica y vagal presentan neuronas reticuloespinales desde el inicio del periodo larvario. De las tres subpoblaciones del grupo reticular ístmico, sólo dos, las subpoblaciones medial y ventral proyectan a la médula espinal, con la mayoría de las células de proyección situadas en la parte caudal de la subpoblación ístmica medial. Sólo ocasionalmente se observó la presencia de células de proyección a la médula espinal en la subpoblación ventral. El núcleo reticular vagal serotonérgico, que está situado en el rombencéfalo caudal, también presenta células de proyección descendente. El desarrollo temprano de las proyecciones serotonérgicas desde el tronco cerebral a la médula espinal parece ser una característica conservada en todos los vertebrados estudiados. Aunque un sistema de proyección rombencéfalico-espinal parece haberse desarrollado antes de la divergencia de agnatos y gnatóstomos, no se observaron células serotonérgicas en la región rafeal en la lamprea. Además se observó que, proporcionalmente, más células serotonérgicas del rombencefálo rostral contribuyen a la proyección espinal en la lamprea de mar que en vertebrados con mandíbula.

CAPÍTULO 3.1. LA SEROTONINA Y EL GABA COLOCALIZAN EN DETERMINADOS GRUPOS DE NEURONAS DEL CEREBRO DE LARVAS DE LAMPREA DE MAR: EVOLUCIÓN TEMPRANA DE LA COLOCALIZACIÓN DE NEUROTRANSMISORES EN LOS VERTEBRADOS.

Se ha publicado previamente la existencia de colocalización de los neurotransmisores clásicos serotonina (5-HT) y ácido γ-aminobutírico (GABA) (o de la enzima que sintétiza este ultimo, descarboxilasa del glutamato) en unas pocas neuronas del núcleo del rafe magno-oscuro de la rata. Sin embargo no hay datos acerca de la presencia de neuronas neuroquímicamente similares en el cerebro de vertebrados no mamíferos. Las lampreas son los vertebrados actuales más antiguos y pueden proporcionar datos importantes a cerca de la filogenia de los sistemas neuroquímicos. La colocalización de 5-HT y GABA en neuronas del cerebro de la lamprea de mar se estudió con doble inmunofluorescencia usando anticuerpos contra serotonina y GABA y microscopia confocal. La colocalización de estos neurotransmisores se observó en el diencéfalo y el istmo. En el diencéfalo, alrededor del 87% de las células serotonérgicas de la capa rostral del tálamo dorsal (cerca de la zona limitans) mostraron inmunorreactividad a GABA. Además, ocasionalmente se observó la presencia de células doblemente marcadas para GABA y 5-HT en el núcleo hipotalámico tuberal y en el pretecho. De los 3 subgrupos serotonérgicos ístmicos previamente reconocidos en el istmo de la lamprea de mar (dorsal, medial y ventral), células doblemente marcadas se observaron únicamente en el subgrupo ventral (alrededor del 61% de las células serotonérgicas del subgrupo ventral mostraron inmunorreactividad a GABA). Estos resultados sugieren una equivalencia entre estas células ístmicas de la lamprea y las células rafeales serotonérgicas/GABAérgicas de los mamíferos. Nuestros resultados sugieren que las neuronas serotonérgicas/GABAérgicas son proporcionalmente más abundantes en lampreas que en la rata y que probablemente aparecieron en la evolución antes de la separación de agnatos y gnatóstomos. Proponemos la cotransmisión mediante la liberación de 5-HT y GABA por parte de las neuronas aquí descritas.

CAPÍTULO 3.2 LA DOPAMINA Y EL GABA COLOCALIZAN EN DETERMINADOS GRUPOS DE NEURONAS DEL CEREBRO DE LA LAMPREA DE MAR: EVOLUCIÓN TEMPRANA DE LA COLOCALIZACIÓN DE NEUROTRANSMISORES EN LOS VERTEBRADOS.

Durante los últimos años la posible coliberación de neurotransmisores desde una misma neurona ha recibido mucha atención. La colocalización de monoaminas y neurotrasmisores aminoacidérgicos (sobre todo glutamato y dopamina (DA) o serotonina) ha sido previamente observada en neuronas de mamíferos. Sin embargo, se ha prestado menos atención al estudio de la colocalización de la DA y el ácido γ-aminobutírico (GABA). Con el objetivo de arrojar luz sobre la colocalización de neurotransmisores durante la filogénica de los vertebrados tempranos estudiamos la expresión de GABA en neuronas dopaminérgicas en el cerebro de la lamprea de mar mediante métodos de doble inmunofluorescencia con anticuerpos anti-DA y anti-GABA. Se observaron diferentes grados de colocaliación de DA y GABA en los diferentes núcleos dopaminérgicos del cerebro. Se observó un alto grado de colocalización (el GABA está presente al menos en el 25% de la neuronas DA-inmunorreactivas) en poblaciones del rombencéfalo caudal, el istmo ventral, el núcleo de la comisura postóptica, el núcleo preóptico y en células grano de los bulbos olfativos. Se observó la presencia de una nueva población estriatal DA-inmunorreactiva que mostró colocalización con GABA en aproximadamente una cuarta parte de sus neuronas. A pesar de la abundacia de neuronas inmunorreactivas a DA y GABA en el hipotálamo periventricular, la colocalización se observó unicamente en unas pocas células. No se observó la presencia de células doblemente marcadas en el núcleo paratubercular. La frecuente colocalización de DA y GABA indica que las poblaciones dopaminérgicas de las lampreas son más complejas que lo previamente descrito. Se observó la presencia de fibras y terminales doblemente marcados en diferentes regiones del cerebro, lo que sugiere la posibilidad de coliberación de DA y GABA por parte de este tipo de neuronas de las lampreas. Estos resultados sugieren que la colocalización de DA y GABA en neuronas apareció tempranamente en la evolución de los vertebrados.

CONCLUSIONES

A partir de los resultados obtenidos en esta Tesis hemos extraído las siguientes conclusiones:

1. Las lampreas tiene una compleja red de células y fibras serotonérgicas en el sistema nervioso periférico que nunca había sido descrita en otras especies de vertebrados. La gran presencia de elementos serotonérgicos en el sistema nervioso periférico de las lampreas sugiere una relación muy próxima con respecto a la organización de este sistema en grupos de invertebrados.
2. La organización, la morfología y el patrón de innervación de las células serotonérgicas del sistema nervioso periférico de la lamprea de mar indica que podrían participar en procesos de quimiorecepción de oxígeno y gusto, en la modulación de la contracción muscular y en la regulación de la frecuencia del batido ciliar y secreción de mucus.
3. El neurotransmisor serotonina parece tener un papel similar en la transmisión y/o respuesta a situaciones de hipoxia en todos los vertebrados estudiados incluidas las lampreas.
4. La preponderancia de elementos serotonérgicos en la cabeza y la compleja metamorfosis de las diferentes estructuras de la cabeza en lampreas sugiere que la serotonina podría jugar un papel en el desarrollo y morfogénesis de la cabeza como se ha sugerido previamente en los mamíferos.
5. La serotonina aparece como el neurotransmisor que probablemente es empleado por las células bi-ciliadas receptoras del gusto para la transmisión de la información gustativa en lampreas, mientras que en mamíferos actúa como un neuromodulador. Además el CGRP podría actuar como un neuromodulador durante la transmisión de la información gustativa en lampreas.
6. Experimentos de trazado neuronal demostraron que pequeñas neuronas, en el hipotálamo ventral, la región mamilar (siendo la mayor parte de ellas células contactantes del líquido cerebroespinal) y en el núcleo paratubercular, proyectan a la médula espinal en las lampreas. La presencia de células contactantes del líquido cerebroespinal que proyectan desde el cerebro a la médula espinal probablemente es una característica única de la lampreas.
7. Los resultados actuales revelan la existencia de grandes diferencias entre agnatos y gnatóstomos en la organización de las células dopaminérgicas que proyectan a

la médula espinal. Por otro lado, la combinación de experimentos hodológicos e inmunohistoquímicos confirma la equivalencia entre el núcleo paratubercular de las lampreas y núcleo del tubérculo posterior de otros vertebrados.

8. Aunque un sistema serotonérgico de proyección tronco cerebral-médula espinal parece haber estado presente antes de la divergencia de agnatos y gnatóstomos, no se observó la presencia de células serotonérgicas en la región rafeal en la lamprea. El desarrollo temprano de las proyecciones serotonérgicas desde el tronco cerebral a la médula espinal parece ser una característica conservada en todos los vertebrados estudiados.
9. Proporcionalmente más células serotonérgicas del rombencéfalo rostral contribuyen a la proyección espinal en la lamprea de mar que en los vertebrados con mandíbula.
10. Los resultados de colocalización indican que las neuronas serotonérgicas/GABAérgicas son proporcionalmente más numerosas en la lamprea de mar que en la rata e indican también que este tipo celular apareció antes de la separación de agnatos y gnatóstomos. Los resultados hodológicos y de colocalización también sugieren una equivalencia entre la células serotonérgicas ístmicas de la lamprea de mar y las células del rafe superior de los mamíferos.
11. Se propone la cotransmisión mediante la liberación de serotonina y GABA por parte de las neuronas diencéfalicas e ístmicas de la lamprea que muestran colocalización.
12. La presencia de fibras y terminales doblemente marcados con anticuerpos contra DA y GABA en diferentes regiones del cerebro sugiere la posible coliberación de estos neurotransmisores por parte de diversas neuronas de la lamprea. La comparación de estos sistemas en vertebrados sugiere que la colocalización de DA y GABA en neuronas apareción tempranamente en la evolución de los vertebrados.
13. Los resultados de colocalización indican que el sistema GABAérgico de las lampreas es más complejo que lo anteriormente publicado y que la colocalización/cotransmisión de GABA con otros neurotransmisores clásicos en neuronas del cerebro de los vertebrados es probablemente la norma y no la excepción.