Universidade de Santiago de Compostela
Departamento de Bioloxía Celular e Ecoloxía
Área de Bioloxía Celular

Development of Serotonergic and Catecholaminergic Systems in the Central Nervous System of the Sea Lamprey

Doctoral Thesis

Xesús Manoel Abalo Piñeiro

Santiago de Compostela, 2006

Cerebro, s. aparato con el que pensamos que pensamos. Lo que distingue al hombre que está satisfecho con ser algo, del que desea hacer algo. Un hombre de gran fortuna, o que se ha visto catapultado a un alto cargo, suele tener tanto cerebro en la cabeza que quienes le rodean no pueden llevar los sobreros puestos. En nuestra civilización y bajo nuestra forma de gobierno, se tiene en tan alta estima al cerebro que se le recompensa eximiéndole de ocupar cualquier cargo público.

Ambrose Bierce
(El diccionario del Diablo)

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DEPARTAMENTO DE BIOLOXÍA CELULAR E ECOLOXÍA

ÁREA DE BIOLOXÍA CELULAR

DEVELOPMENT OF SEROTONERGIC AND CATECHOLAMINERGIC SYSTEMS IN THE CENTRAL NERVOUS SYSTEM OF THE SEA LAMPREY

MEMORIA

Que para optar al Grado de Doctor en Biología presenta

Xesús Manoel Abalo Piñeiro

Santiago de Compostela, 2006

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 “Development of serotonergic and catecholaminergic systems in the central nervous system of the sea lamprey”, (Desarrollo de los sistemas serotoninérgico y catecolaminérgico en el sistema nervioso central de la lamprea de mar) que para optar al Grado de **Doctor en Biología** presenta D. XESÚS MANOEL ABALO PIÑEIRO, 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 22 de Mayo de 2006.

El Doctorando | La Directora de la Tesis

Fdo. Xesús Manoel Abalo Piñeiro | Fdo. María Celina Rodicio Rodicio

Esta Tesis Doctoral forma parte del Proyecto de Investigación titulado “Estudio de la proliferación, migración, muerte celular y desarrollo de las poblaciones neuronales en el sistema nervioso central de la lamprea de mar”, financiado por el Ministerio de Ciencia y Tecnología (BCM2000-0283; 12/01/2001 - 31/12/2003) y por la Secretaría Xeral de Investigación e Desenvolvemento de la Xunta de Galicia (PGIDT01PXI20006PN; 20/07/2001 - 19/07/2004).

Su realización ha sido posible gracias a la concesión de una Bolsa Predoutoral de la Xunta de Galicia a D. Xesús Manoel Abalo Piñeiro durante el período 01/10/2002 al 31/3/2005.

Un lustro e quinto. Seis anos larvarios e unha metamorfose de corenta minutos.

Co cambio de milenio convidoume o oráculo a acepta-la oferta de Dédalo. Unha empresa de seguro difícil, un castigo de Sísifo, pero con fin, ¿?. Esperaba esa resposta, esa e non outra. Neste tipo de viaxes dirixidas cómpre sempre chegar ó destino. O punto de partida. Así comeza"rá" outro tipo de travesía, quizais coma Cándido, quizais coma Zorba... unha post-metamorfose, na que se esixe berrar escritos, e se debe tentar proba-la Ambrosía, e obrigar a que Caronte nunca nos chegue a levar de todo.

Fixéronme compaña neste divagar moitas prosas ... e un verso entre tanta liña aliñada. TI.

Destaco na singradura a QUEN a fixo posible, quen me decidiron.

Destaco a QUEN dous. Déronme o fío para fuxir de Creta. QUEN tres, cos que desembarquei en Ítaca, na Cólquide, na Lacio ..... no Asgard.

Destaco o LUGAR. Na gamela Potemkin comecei o que quería. Jasón nos fixo isto.

Destaco a EXPERIENCIA. Nel convebín con Argonautas, cheguei a formar parte da Garda Pretoriana, e ata me re-partín. Permitiume coñece-lo meu desacougo polo "Fernweh", e apoucalo en parte coñecendo outros "Heimweh". Banquetes en Trimalción´s, moitos epicúreos, algún tartufo, chegar ó Nirvana con *Byldo*, Galician Oberture, The River Aras ou co tío Vania. Antoine Roquentin me quixo facer protagonista nalgunha ocasión que Sileno e os íncubos botaron pola borda.

Destaco o FINAL; a freza. Inda que non é alá abaixo, no río, e non son anádromo, unha cinta de Moebius nunca cambia.

Destácoteme a CHUS. Ela fai que o que é sexa, e o que non é o sexa, atc...

Ós agradecidos?!

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Biology of lampreys

Lampreys belong to the most ancient group of the extant vertebrates; the Agnathans, a superclass of aquatic vertebrates whose appearance goes back to the Paleozoic. The common ancestors of living lampreys are the ostracoderms, primitive jawless fishes that lived in the Cambrian and Devonian periods. These primitive jawless fishes were devoid of axial bony skeleton, jaws and even limbs, but possessed a shell of bony scales that covered the rostral part of the body, with openings for the eyes, the pineal eye, and the nasal opening (Forey and Janvier, 1994; Butler and Hodos, 1996).

The first fossil recognized as a lamprey is *Mayomyzon* (Bardack and Zangerl, 1971), from the Pennsylvanian period (280 million years ago), and it presents similarities with extant lampreys in the location and configuration of the cartilaginous elements of the head. They had no obvious teeth, and the general structure of the mouth indicated a way of feeding similar to the extant lampreys.

The evolution of lampreys from these primitive agnathans implied the acquisition of a sucking mouth, and the loss of the exo-skeleton (Ritchie, 1968) and the even fins. Therefore, the extant lampreys have an eel-shape body with 1.3 m in length at most, and their skin is covered with mucus, instead of scales (Fig. 1). They have 7 gill clefts, a nasal opening (Fig. 1) that ends in a blind duct that runs under the brain (the naso-hypophysary duct), and a cartilaginous skeleton with a notochord even in the adult phase, and have neither a bony skull nor even fins.

Figure 1. Lateral view of an adult sea lamprey.

The name agnathans is due to their lack of jaws. Instead, they posses a circular mouth with corneous teeth to hold on their preys (Fig. 2), to pierce the skin and to feed on the inner fluids (Hubbs and Potter, 1971). Most of the species, but not all, are parasitic of several species of fishes (Hubbs and Potter, 1971; Nieuwenhuys and Nicholson, 1998).

Figure 2. Detail of an adult sea lamprey mouth.

The habitat of lampreys is restricted to the tempered zones of both hemispheres, and there are species that live in both salt and fresh water (Hubbs and Potter, 1971; Nieuwenhuys and Nicholson, 1998). At present, 6 genuses with 41 species of lampreys have been identified so far. We have used the sea lamprey (*Petromyzon marinus*, Linnaeus, 1758) as the object of study in this work. Its taxonomic classification is noted in table 1.

Kingdom	*Animalia*
Phylum	*Chordata*
Subphylum	*Vertebrata*
Superclass	*Agnatha*
Class	*Cephalaspidomorphi*
Subclass	*Cyclostomata*
Order	*Petromyzoniformes*
Family	*Petromyzontidae*
Genus	*Petromyzon*
Species	*Petromyzon marinus*

Table 1. Taxonomic classification of sea lamprey.

Life cycle of sea lamprey

Sea lamprey is an anadromous species with an exceptional life cycle. The breeding season begins between May and July, when the water temperature in the rivers reaches about 16ºC, which triggers the sexual maturation of adults. Males reach first the breeding zone and start to build up the nest piling up rocks with their oral disc, and removing stones of the bottom of the river, with the purpose of making a circular depression in the bed of the river. Females arrive later and help males to finish building up the nest. Once the nest is built, the male twists around the female to fertilize the eggs, which will lay in the bed of the river. The diameter of the eggs is around 1 mm, and every female can lay about several hundreds of thousands. After spawning both progenitors die (Fig. 3).

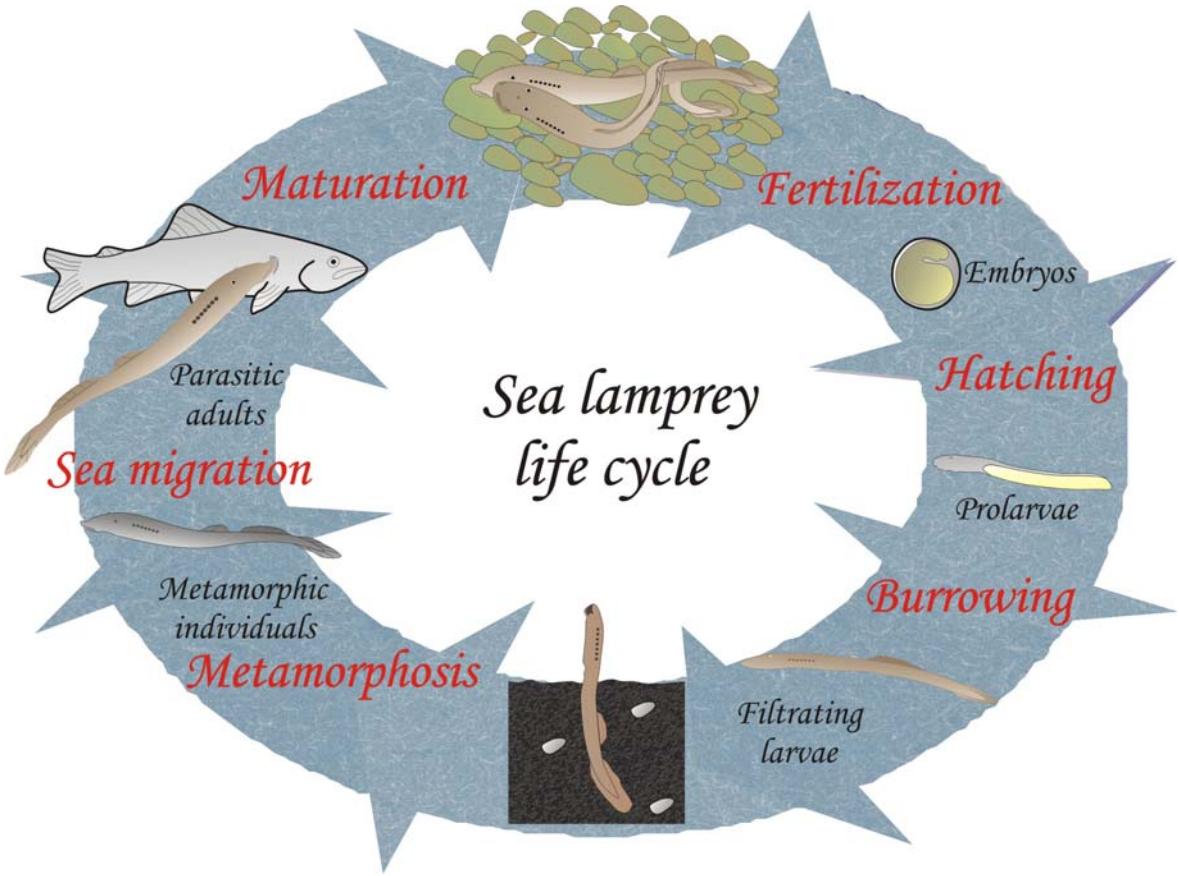


Figure 3. Life cycle of sea lamprey.

Fertilization is followed by an embryonic period and a prolarval stage that begins around 11 days after fertilization, when the eggs hatch. This stage lasts between 22 and 25 days, and the prolarvae feed on their yolk. It is in this period when they must learn to feed by filtration before the end of this stage, which is when they become larvae. The larval stage lasts between 4 and 7 years, and larvae feed on diatoms, protozoans and organic matter obtained by filtration. Due to the difference in structure and way of life between larvae (filtrating individuals) and adults (parasites), both were initially classified as separate species: *Ammocoetes branchialis* was the name given to the larvae, whereas *Petromyzon marinus* was the name given to the adults (Nieuwenhuys and Nicholson, 1998).

Larvae move downstream until they find a calm zone of water, and they bury in the mud. After this long stage sea lamprey larvae undergo a metamorphosis, which begins between July and September, and lasts for about 3 to 5 months.

Metamorphosis implies drastic changes in the morphology and the physiology of lampreys such as the acquisition of functional eyes, the development of the oral disc, the formation of the rough tongue, and changes in the body coloration and osmoregulation mechanisms (Youson, 1980; Nieuwenhuys and Nicholson, 1998).

After metamorphosis young lampreys migrate to the sea, favored by floods, from November to the end of March (Hardisty and Potter, 1971a). Once in the sea, they can already be called adults and remain in the sea from 2 to 4 years feeding as parasites on any class of fish. At the end of the adult stage lampreys stop feeding and return to the rivers. They migrate upstream, and undergo drastic changes again with the only aim of to get a better spawn. They undergo morphologic and physiological modifications, such as the atrophy of the intestine or the degeneration of the eyes (Hardisty and Potter, 1971b). The cycle finishes with the spawn and the death of the progenitors (Fig. 3).

Previous studies about lamprey development

In spite of having a relatively high number of species, of be widely distributed in the planet, and of be the most primitive vertebrates, there are few studies about the development of lampreys, due to the difficulty to obtain embryos *in vitro*, and to get correctly developed prolarvae and small larvae. The studies of Damas (1944) in *Lampetra fluviatilis*, Piavis (1971) in *Petromyzon marinus*, and Tahara (1988) in *Lampetra reissneri*, are the main references for studies in lampreys about developmental neuroanatomy or general embryonic development. Piavis (1971) described 18 stages in the development of *Petromyzon marinus* from fertilization to the burrowing stage, while Tahara (1988) described 31 stages in the development of *Lampetra reissneri*, without including the burrowing stage. Since lamprey development is not completed until post-metamorphic stages, the stages of metamorphosis in *P. marinus* described by Youson and Potter (1979) were also useful for our study. These authors described 7 metamorphic stages on the basis of morphological features, such as the appearance of the eye, the structure of the oral aperture with the tongue-like piston and teeth, and the body color. On what follows I will describe each stage of development of *Petromyzon marinus* based on the easily distinguishable morphologic features observed by Piavis (1971) and Youson and Potter (1979), and comparing with our own observations (Table 2).

The first stage defined by Piavis is stage 0 or non-fertilized egg, while stage 1 corresponds with the fertilized egg (the zygote), with the characteristic fertilization membrane. Subsequently, successive divisions result in the formation of the blastula, gastrula, and neural tube at stage 11, approximately six days after fertilization. The head appears at stage 12, between 6 and 8 days, and hatching occurs at stage 14, between 10 and 13 days post-fertilization. At hatching, individuals appear crooked and they do not begin to stretch until the following stage, stage 15, between days 13 and 16, when pigmentation begins to be visible in the dorsal part of the prolarvae. Stage 16 is defined by the appearance of the gill clefts, and finalizes with the appearance of the two eye spots. The burrowing stage begins between days 17 and 20 after fertilization, and its end means also the end of the prolarval stage.

The last stage described by Piavis (stage 18) corresponds with the beginning of the larval stage, in which the individuals are approximately 10 mm in length. For the metamorphic stages we follow the observations and stages described by Youson and Potter (1979).

Animals in stage 1 of metamorphosis are very similar to ammocoetes, but small differences can be detected in the size of the eye and shape of the oral hood. Stage 2 is characterized by prominent papilla-like projections on the inner dorsal surface of the oral hood, while the main characteristic of stage 3 is the differentiation into the eye of a dark inner pupil and a lighter outer iris. At stage 4 the oral disc is surrounded by an even continuous ring of tissue, whereas at stage 5 the precursors of the teeth are already visible. At stage 6 the prominent eyes protrude laterally and for the first time the lamprey is able to use the oral disc for attachment. Finally at stage 7 the teeth are yellow and the lingual lamina have fine serrate edges (Youson and Potter, 1979).

To use an easier nomenclature, embryos will be named with an "E" and the number of days after fertilization (e.g. E7). Prolarvae will be named with a "P" and the number of days after hatching (e.g. P12), while the metamorphic stages will be staged with an "M" and the number of the stage (e.g. M5).

Phase	Piavis (1971)	Stage	Days	Size
Embryos	0 - 13	Embryonic	E0 - E12	1 mm
Prolarvae	14	Hatching	P0 - P1	3.5 - 4.5 mm
	15	Pigmented	P2 - P3	5 mm
	16	Gill cleft	P4 - P7	5.5 - 7 mm
	17	Burrowing	P8 - P23	7 - 9/10 mm
Larvae	18	Larval	P24 - metamorphosis	10 - 90/160 mm
Metamorphosis	-	M1-M7	3 -5 months	160 - 200 mm
Adults	-	Adult	2 - 4 years	200 mm - 1.3 m

Table 2. Comparison between Piavis's and our stages and size of each prolarval stage of sea lamprey. The size of metamorphic (according Youson and Potter) and adult animals was also included.

Development of the central nervous system in lampreys

The development of the central nervous system (CNS) of lampreys follows the general pattern of vertebrates. The CNS develops from the neural plate, which progressively separates from the surface of the ectoderm and forms two folds with a cleft in its midline (the neural groove). The neural groove becomes deeper and the folds rise and approximate until they merge in the dorsal part, forming a hollow tube (the neural tube) that contains a cavity full of liquid. All these processes were called neurulation (Fig. 4).

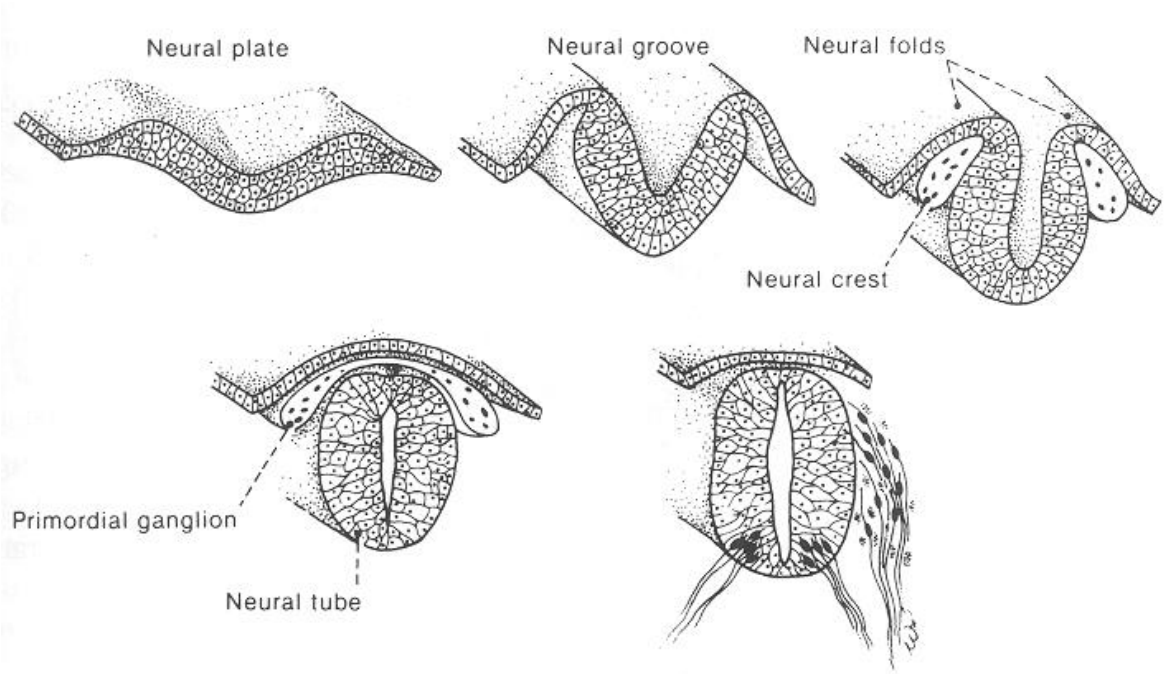


Figure 4. Scheme of the vertebrate neural tube formation; the neurulation.

The neural tube consists of a pair of lateral plates that are dorsally interconnected by the roof plate, and ventrally by the floor plate (Nieuwenhuys and Nicholson, 1998). The lateral plates become the neuronal parts of the brain and the spinal cord. The formation of the neural crests occurs at the same time, and their cells subsequently migrate to lead different structures of the head or the nervous system; e.g. the cerebral ganglia (Horigome et al., 1999).

The cylindrical and immature neural tube further develops several vesicles in the rostral zone (the presumptive brain), and a dorso-ventrally flattened zone (e.g. the spinal cord). The first observed subdivisions of the brain are the prosencephalon (forebrain), the mesencephalon (midbrain), and the rhombencephalon (hindbrain), that begin to be distinguished at the end of the embryonic stage (Tahara, 1988; Piavis, 1971). The prosencephalon will be divided in the telencephalon and the diencephalon, while the mesencephalon and rhombencephalon remain undivided in lampreys, contrary to that pointed in mammals, where the rhombencephalon is subdivided into metencephalon and myelencephalon (Fig. 5). Several limits in the rhombencephalon have been observed in late prolarvae and early larvae (Villar-Cheda, 2005), which mark the rhombomeres out, in agree with the position of several group of cells originated in the neural crests (Horigome et al., 1999), and with the appearance of the first cranial nerves (Kuratani et al., 1998; Horigome et al., 1999).

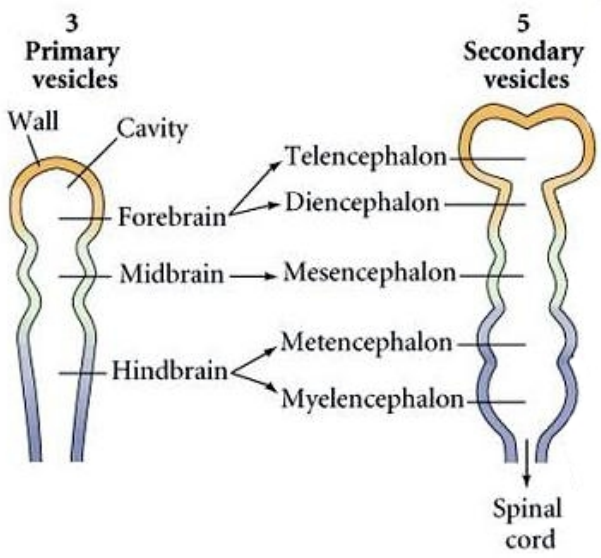


Figure 5. Three- and five-vesicle stages in the development of the central nervous system.

Lampreys have a laminate brain organization pattern, which means that most of the cell bodies remain in the periventricular region (gray matter), near the ventricular zone where the neuroblasts proliferate (Butler and Hodos, 1996), and neuronal migration towards the marginal zone (white matter) is scarce. The periventricular cell layer is not homogeneous, and it has a lot of neuronal groups. Some of them are accumulations of small cells, while other can be defined by bigger elements; e.g. motoneurons, or the giant Müller and Mauthner cells (Nieuwenhuys and Nicholson, 1998). The cells of the gray matter extend their dendrites to the white matter. The periventricular arrangements of most somata, as well as the absence of myelin, are considered ancestral features of central nervous systems of vertebrates.

The central nervous system of sea lamprey

The CNS of lampreys has raised great interest due to its combination of primitive features with others that display the basic organization of the structural plan common to the rest of vertebrates. Other interesting feature leading to the study of the lamprey CNS is its relative simplicity. Lampreys were considered as the initial point for some "evolutionary tendencies", such as neuronal migration, differentiation, segregation or specialization.

The CNS of lampreys comprises a rostral vesicular part (the brain), a spinal cord that runs longitudinally through the back, and the retina. The brain is approximately 12 mm in length, and 3 mm in height and width in adults, although it presents the major subdivisions of the vertebrate brain: telencephalon, diencephalon, mesencephalon and rhombencephalon (Fig. 6).

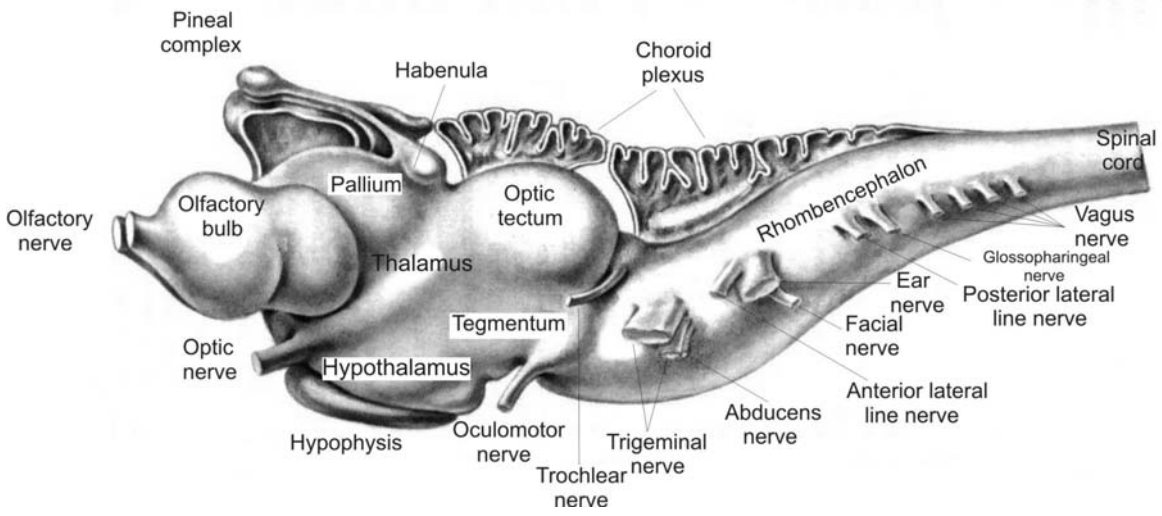


Figure 6. Sagittal drawing of a classic model of the adult sea lamprey brain. Image modified from Nieuwenhuys and Nicholson, 1998.

Two main models exist to explain the organization of the CNS in lampreys. One of them follows a columnar or dorso-ventral scheme (Johnston, 1902; Herrick, 1913; Heier, 1948; Schöber, 1964), while the other follows a neuromeric model (von Kupffer, 1906; Bergquist, 1932; Bergquist and Källén, 1953b; Pombal and Puelles, 1999; Puelles and Rubenstein, 2003).

Nieuwenhuys made in 1998 an historical revision over these two models of organization of the CNS. The columnar model supposes that the brain is organized in four dorso-ventral longitudinal columns, which are clearly observed in the rhombencephalon and the spinal cord. It was first based on its functional roles, and it was also supported by its developmental pattern, with dorso-ventral or ventro-dorsal developmental gradients that force cell groups to settle in a certain column. According to this model, these four columns would be structural and functional entities, mainly recognizable in each region of the brain, which are separated by ventricular sulci. The principal of these sulci is the sulcus limitans of His that separates the lateral plate into a dorsal and a basal plate. The dorsal plate is mainly sensory, whereas the basal plate is mainly motor. The main problem of this model is the location of the rostral limit of the limitans sulcus of His, because this sulcus is not visible in the prosencephalon, which makes it hard to identify the dorsal and basal parts in the whole prosencephalon.

The neuromeric model was postulated at the end of the 19th century. It is based on the existence of transverse segments in the embryonic brain which are called neuromeres. According to Orr (1887) the neuromeres are separated by external vertical constrictions which correspond to internal ventricular ridges and are correlative with segments in the head and body of vertebrates. Cells in these ridges are oriented radially to the inner surface of the neuromeres and cells of one neuromere do no extend into another neuromere. By the mid 20th century Bergquist and Källén (1953b, 1954) showed that the neuromeres represent centers of proliferation, early differentiation and migration. Recent studies have confirmed that neuromeres represent units of cell lineage restriction (Fraser et al., 1990; Figdor and Stern, 1993), that many of them have fixed relations with peripheral structures (Lumsden and Keynes, 1989; Keynes and Lumsden, 1990), that their boundaries may be marked by early-developing fiber systems (Mendelson, 1986; Metcalfe et al., 1986; Kimmel, 1993; Lumsden and Keynes, 1989; Figdor and Stern, 1993), and that their limits often correlate with the boundaries of the domains the expression of regulatory genes (Kimmel, 1993; Bulfone et al., 1993; Puelles and Rubenstein, 1993).

At the beginning of the 20th century, von Kupffer (1906) exposed his neuromeric theory of the development of the lamprey CNS, based on the observation of rhombencephalic structures that he called rhombomeres, and more widely neuromeres for all the brain. Later in the century, Bergqvist and Källén (1953b) observed the presence of these neuromeres in all the brain of the lamprey, naming them according to their brain region: prosomeres in the prosencephalon, mesomeres in the mesencephalon, and rhombomeres in the rhombencephalon. More recently, von Kupffer's theory was confirmed by the observation of the presence of rhombomeres in *P. marinus* larvae, by their relationship with the arrangement of the motor nuclei of the cranial nerves and the giant reticular cells (Gilland and Baker, 1995), and with the study of the patterns of proliferation and cell differentiation in the caudal brain (Villar-Cheda, 2005).

Finally, Pombal and Puelles (1999) tried to apply the segmental prosomeric model of organization of the prosencephalon of gnathostomes (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Rubenstein et al., 1994) to the lamprey prosencephalon, to verify whether the pattern of organization of the CNS of agnathans could also be applied to this species. The segmental prosomeric model was proposed as a theory to explain also the inner subdivisions of the rostral brain of fishes (Pombal and Puelles, 1999; Wullimann and Puelles, 1999) based on gene expression domains, and proposed the existence of 6 prosomeres (p1- p6) in the prosencephalon. However, recent studies in other vertebrates have shown that only prosomeres p1-p3 could be considered as subdivisions of the caudal diencephalon, while prosomeres p4-p6 are today considered as the secondary prosencephalon (i.e. telencephalon plus rostral diencephalon or hypothalamus), a complex protosegment not subdivided into prosomeres (Puelles and Rubenstein, 2003).

Recent studies in the lamprey about axonal bundle formation (Kuratani et al., 1998), cell proliferation (Villar-Cheda, 2005; Villar-Cheda et al., 2006b), the ontogeny of the GABAergic system (Meléndez-Ferro et al., 2002b, 2003), or gene expression patterns (Murakami et al., 2002; Osorio et al., 2005) support the segmental organization of the brain. The following description of the lamprey CNS is based on the Nieuwenhuys and Nicholson (1998) revision, with the modifications advised by the neuromeric model.

- *Telencephalon*

The telencephalon is the more rostral part of the brain, and it is mainly related with the olfactory function. It caudally limits with the diencephalon. The telencephalon has an inner ventricle: the telencephalic ventricle, that rostrally extends into the paired telencephalic hemispheres (lateral ventricles), and caudally joins the third ventricle of the diencephalon. The telencephalon has an inner layer of gray matter of variable thickness where most of the neuronal somas are located. The fibers are normally thin and scattered in the marginal white matter.

The whole telencephalon can be divided in three parts: the olfactory bulbs, the telencephalic hemispheres and the mid-telencephalon. The olfactory bulbs and the telencephalic hemispheres are even structures, produced by evagination of the telencephalon during ontogeny. The telencephalic hemispheres are caudally joined to the mid-telencephalon that do not undergo evagination. The olfactory bulbs extend rostrally to the telencephalic hemispheres and are separated from them by a deep external groove called fissura circularis (Fig. 6). The mid-telencephalon ends rostrally in the lamina terminalis that dorsally and ventrally swells to give the dorsal (interbulbaris) commissure, and anterior commissure, respectively.

There are four layers in the olfactory bulbs: the olfactory fiber layer, the glomerular layer, the mitral cell layer, and the inner cell layer. On the other hand, the telencephalic hemispheres and the mid-telencephalon are divided into a dorsal part called pallium, and a ventral part called subpallium. The pallium comprises three regions: the medial pallium or primordium hippocampi, the dorsal pallium or subhippocampalis lobe, and the lateral pallium which comprises a dorsal part (the "lateral pallium primordium"), and a ventral part (the "piriform pallium primordium"). In the subpallium the septum and the striatum can be distinguished rostrally, and the preoptic nucleus caudo-ventrally. From a prosomeric model point of view, the whole telencephalon is included in the secondary prosencephalon (Puelles and Rubenstein, 2003).

The olfactory bulbs are connected by numerous thin axons to each other through the dorsal and anterior commissures, and ipsilaterally with the rest of telencephalon and the diencephalon (Northcutt and Puzdrowsky, 1988). The olfactory nerve is the first pair of cranial nerves, and reaches the telencephalon in the most rostral part of the olfactory bulbs.

- *Diencephalon*

The diencephalon is a brain region derived from the prosencephalon, and is sited between the telencephalon and the mesencephalon. Most of the diencephalic cells are sited in the periventricular zone with a diffuse differentiation between the diverse nuclei. That is the reason why it is usual to define the nucleus position as regards the intraencephalic grooves. The diencephalon was classically divided in four parts: epithalamus (dorsal zone), dorsal thalamus, ventral thalamus and hypothalamus (ventral zone) (Herrick, 1910).

The epithalamus includes the pineal complex and the habenula. The pineal complex consists of the pineal and parapineal (parietal) organs that are originated from evaginations of the diencephalic roof during ontogeny, both being photoreceptive organs. The pineal organ consists of a rostral terminal vesicle, an atrium and a long pineal stalk that joins to the posterior commissure. The parapineal organ consists of a rostral terminal vesicle and a medial ganglion that is connected to the left habenula (Yáñez et al., 1999). The habenula is clearly an asymmetric structure with right (noticeably bigger) and left parts. Both habenular ganglia mainly receive telencephalic afferents, and send efferents via the asymmetric retroflexus fascicle towards the mesencephalic interpeduncular nucleus that caudally continues in the rhombencephalon (Yáñez and Anadón, 1994).

The dorsal thalamus contains a compact group of periventricular neurons, and other scattered laterally located cells, which compose the lateral geniculate corpus that according to Heier (1948) can be divided in anterior geniculate corpus, posterior geniculate corpus, and nucleus of Bellonci. Heier also considered that the dorsal thalamus is a "sensory correlation center" since it receives inputs from the olfactory epithelium, the eyes, the parietal organs, and the sensory mesencephalic and rhombencephalic nuclei, processing the information and leading it toward coordination centers of the ventral part of the brain (Heier, 1948).

The ventral thalamus was considered as an important "motor correlation center". It receives afferents from the telencephalon, the rest of the diencephalon or the mesencephalic tectum, and it sends motor efferents to the hypothalamus and other motor coordination centers (Heier, 1948).

The hypothalamus is the largest diencephalic region. According to Heier (1948) it can be divided in a dorsal zone, that includes the nucleus of the postoptic commissure and

the dorsal hypothalamic commissure nucleus, and a ventral zone, formed by the preinfundibular, ventral hypothalamic (e.g. tuberal area), and postinfundibular nuclei (e.g. mammillary area). The ventral part of the hypothalamus contains four commissures: postoptic, preinfundibular, postinfundibular, and posterior tubercle commissures. The hypothalamus receives afferents from the telencephalon, the dorsal thalamus, and the mesencephalic tegmentum and tectum. The afferents from the thalamus and mesencephalon decussate partially in the postoptic commissure. The afferents from the telencephalon are more scattered. The more important efferents of the hypothalamus are the tracts that reach the mesencephalic tegmentum and the basal plate of the rhombencephalon (hypothalamus-tegmental tract), the tract of the pallium, and the hypothalamus-olfactory one, which partially decussate in the postoptic commissure.

The transition zone between the diencephalon and the mesencephalon was initially called sinencephalon (Bergqvist and Källén, 1953a, b). Its dorsal part is the pretectum, whereas its ventral part contains the nucleus of the medial longitudinal fascicle and the "mesencephalic" first and second pairs of giant Müller cells. The roof plate of the pretectum is considerably thickened and contains the posterior commissure. There is a bilateral organ below the posterior commissure that is denominated the subcommissural organ that secretes several substances to the cerebrospinal fluid, which condense and form the Reissner's fiber.

According to the prosomeric model (Pombal and Puelles, 1999; Wullimann and Puelles, 1999; Puelles and Rubenstein, 2003), the pretectum would correspond to the alar region of P1, the epithalamus plus the dorsal thalamus would correspond to the alar region of prosomere p2, and the ventral thalamus plus the eminentia thalami would correspond to the alar region of the prosomere p3. The limit between p2 and p3 would be marked by the zona limitans intrathalamica. The hypothalamus (rostral diencephalon), although Pombal and Puelles (1999) described it as the basal part of the P3-P6 prosomeres, is today considered as indivisible in prosomeres, in spite of a rostral and a caudal parts, with several regions in each one of them, could be observed (Puelles and Rubenstein, 2003).

The hypophysis, or pituitary gland, is a gland that controls the feed behavior and comprises a neural portion, the neurohypophysis, and a portion of glandular nature, the adenohypophysis. The hypophysis is located ventral to the hypothalamus, and is related to

it by means of the hypothalamo-hypophyseal tract. The optic nerve, the second pair of cranial nerves, reaches the diencephalon just rostral to the hypophysis, in the optic chiasm zone.

- *Mesencephalon*

The mesencephalon can be divided dorsoventrally in two main regions: a dorsal region that includes the mesencephalic tectum, or optic tectum, plus the torus semicircularis, and a ventral region called the mesencephalic tegmentum. The optic tectum has several neuronal and fiber layers. It receives afferents from the retina, from the spinal cord, the lateral line, vestibular centers, and the nucleus of the trigeminus, through the ascending medial and bulbar lemnisci. Optic tectum efferents go caudally towards the rhombencephalon and the spinal cord, and rostrally to the mesencephalic reticular area, the ventral thalamus and the hypothalamus.

The torus semicircularis is a periventricular gray substance zone that is the ventral continuation of the gray substance layer of the optic tectum. The afferents are similar to those of the optic tectum, but contrary to what happens in the optic tectum, the lateral line and vestibular fibers predominate over the visual ones. The torus semicircularis sends efferents from the telencephalon to the spinal cord (González et al., 1999).

The mesencephalic tegmentum is basically made up of the oculomotor nerve nucleus (third pair of cranial nerves) and the reticular formation (that includes the third pair of giant Müller cells). Although most authors include the interpeduncular nucleus in the mesencephalon, it is found in the isthmus. The tegmentum is mainly a region of fibers that ascend from the rhombencephalon and the spinal cord, or descend from the diencephalon and the telencephalon. The dorsal part of the lamprey mesencephalon is characteristically covered by a folded choroid plexus, which is originated from the roof plate.

- *Rhombencephalon*

The rhombencephalon comprises roughly a half of the total length of the brain. It is the brain region between the isthmus (that separates it from mesencephalon) and the obex (that caudally joins it to the spinal cord). The dorsal isthmic (cerebellar) commissure is the dorsal limit between the mesencephalon and the rhombencephalon (von Kupffer, 1906).

Whether or not lampreys have a real cerebellum is still unclear. Recent studies suggest that the cerebellar plate is fundamentally a dorsal commissural region (Pombal et al., 1996). The alar plates are separated dorsally and the fourth ventricle is covered by the choroid plexus.

According classic studies, the rhombencephalon contains bilaterally a basal plate ventrally and an alar plate dorsally, separated longitudinally by the sulcus limitans of His. Each of the plates can be subdivided in two longitudinal zones by the intermediodorsal (alar) and intermedioventral (basal) sulci (Nieuwenhuys and Nicholson, 1998). Therefore, four longitudinal columns with different functional meaning can be distinguished (Herrick, 1899; 1905), which correspond to four longitudinal proliferation zones (Bergquist and Källén, 1953a, b; 1954):

- **Somatosensory (dorsal) column**, mainly related to ending zones of sensory fibers from trigeminal, eighth, and lateral line nerves.

- **Viscerosensory (intermediate-dorsal) column**, which receives sensory fibers from the facial, glossopharyngeal, and vagal nerves.

- **Visceromotor (intermediate-ventral) column**, which contains the motor nuclei of the trigeminal, facial, glossopharyngeal, and vagal nerves. There is a pair of giant cells, called Mauthner cells, in this area.

- **Somatomotor (ventral) column**, which contains the motor nuclei of the abducens and hypoglossal nerves, and the rhombencephalic reticular nuclei. Four pairs of Müller neurons are located in this area.

According with the neuromeric theory, the rhombencephalon of the lamprey is organized in repetitive elements, called rhombomeres, which serve as centers for cell proliferation, migration and neuronal differentiation during development (Murakami et al., 2004; Villar-Cheda, 2005). Their segmental identity is linked to the expression patterns of the homeobox genes in the lineal order of that they occur in the chromosomes (Allman, 1998). On the other hand, the adult rhombencephalon of vertebrates lacks an overall segmented appearance and is composed of columnar arrays of neuronal nuclei with no obvious traces of segmental organization (Marín and Puelles, 1995).

- *Spinal cord*

The spinal cord is the part of the CNS that is caudal to the obex. In lamprey, it is characteristically flattened and transparent, lacks blood vessels and the gray matter consists of lateral horns that extend from the surrounding of the small central canal, which is the caudal continuation of the fourth ventricle. The flattened gray matter contains neurons very variable in form and size, such as the big motoneurons, dorsal cells, edge cells and several types of interneurons. The surrounding fiber zone is the "white" matter, which is devoid of myelin. Large axons running in the spinal cord originate in reticular cells and giant interneurons of the brain. Axons in the dorsal columns are sensory, but the majority of the spinal axons probably arise from interneurons.

Spinal motoneurons form a lateral column of large cells on each sides of the spinal cord. The dorsal cells are fusiform, big (more than 60 μm of diameter), numerous, and form two longitudinal rows in both sides of the midline. Dorsal cells are intraspinal first order sensory neurons and can be subdivided in three types, according to their response to touch, pressure or pain. Their central processes run in the dorsal funiculus.

Neurons whose processes do not leave the CNS are called interneurons; they are very abundant in the spinal cord. Examples of lamprey interneurons are the giant interneurons, lateral interneurons, edge cells, caudal crossed interneurons, exciting interneurons and the small ipsilateral inhibitory interneurons. There are different types of spinal interneurons according to the neurotransmitter that these neurons use [GABAergic (Meléndez-Ferro et al., 2003), dopaminergic (Abalo et al., 2005), cholinergic (Pombal et al., 2001) or serotonergic (Harris-Warrick et al., 1985)].

The spinal white matter can be subdivided in dorsal, lateral and ventral funiculi, which are separated by the dorsal and ventral nerve roots, which in lampreys do not exit at the same spinal level. Ascending and descending fiber tracts run in these funiculi.

Descending fibers. The midbrain and rhombencephalic reticular formation sends axons to the spinal cord that form the main descending fiber system, the reticulospinal pathways (Ronan, 1989). Müller cells (seven pairs according to Nieuwenhuys, 1972) contribute with their thick axons to those descending tracts that join the medial longitudinal fascicle in the midbrain, and remain ipsilaterally in the ventral funiculus of the spinal cord.

The lateral funiculus contains the Mauthner axon which decussates just at the beginning from the Mauthner cell.

Ascending fibers. There are two large ascending systems from the spinal cord to the midbrain: the dorsal funiculus and the spinal lemniscus. The dorsal column fibers, which ascend in the dorsal funiculus, reach the obex and continue ipsilaterally in the lateral rhombencephalic alar plate (Ronan and Northcutt, 1990). There are primary spinal afferents and axons from the dorsal cells in this column. Spinal lemniscus fibers ascend in the lateral funiculus carrying tactile, pain and temperature stimuli to the brain. Spinal lemniscus fibers innervate the octavolateral area, the optic tectum, and even the dorsal thalamus.

The Reissner's fiber, originated in the subcommissural organ, extends caudally until the caudal ampulla, at the tip of the spinal cord. This caudal ampulla is spherical and about 2.5 times the diameter of the central canal. Reissner's fiber is continuously secreted and its material accumulates in the caudal ampulla, where part of the components are degraded and can reach the bloodstream. The function of the Reissner's fiber is unknown.

The lamprey visual system

The general organization of the lamprey retina is roughly similar to the retina of gnathostome vertebrates, although with some specific structural and developmental variations, such as the number and distribution of layers, or the special developmental timing. Therefore, the retina of adult lampreys is a layered structure that contains neural cells of different types: photoreceptors, bipolar, horizontal, amacrine, and ganglion cells, and glial cells like the Müller cells (Rubinson and Cain 1989).

- The adult retina

The adult lamprey retina is a layered structure somewhat different to that observed in jawed vertebrates (Öhman, 1976). Its general structure and components are summarized in figure 7. The outer part is the pigment epithelium whose fine structure was first described by Öhman, (1974), which in addition to epithelial cells, shows wandering phagocytes (de Miguel et al., 1992). The photoreceptor layer consists of short and long photoreceptors, with their nuclei located in the outer nuclear layer (ONL). It is thought that the short and long photoreceptor cells described in the retina of lampreys (de Miguel and Anadón, 1987) correspond to rods and cones, respectively (Meyer-Rochow and Stewart, 1996; Villar-Cheda et al., 2006a). These two kinds of photoreceptors are associated with two different classes of bipolar cells, which differ in their location (Teranishi et al., 1982; Villar-Cheda et al., 2006a). However, the retina of the Austral lamprey *Mordacia mordax* contains a single morphological type of retinal photoreceptor, which possesses ultrastructural features of both rods and cones (Collin et al., 2004), whereas that of *Geotria australis* has a rod-like and two cone-like photoreceptor types (Collin et al., 1999).

Below the ONL there is a thin outer plexiform layer (OPL), where photoreceptors and bipolar and horizontal cell processes establish contacts. The inner nuclear layer (INL) is thick and complex and consists of two layers of horizontal cells and an inner region with small bipolar cells, amacrine cells and displaced ganglion cells. Large bipolar cells are located in the outer part of the INL close to the OPL. The innermost retina is composed of a thick inner plexiform layer (IPL) that, in addition to typical bipolar cell terminals and processes of amacrine and ganglion cells, also contains bundles of optic fibers in its outer region and ganglion cells and displaced amacrine cells. Therefore, the ganglion cell and

optic fiber layers typical of the jawed vertebrate retinae are not distinguishable from the IPL in lampreys (Fritzsch and Collin, 1990; Rio et al., 1998). This is observable since larval stages (de Miguel et al., 1989). Whereas most ganglion cell dendrites branch in the IPL and are contacted by amacrine and bipolar cell processes, some ganglion cells located in the INL have dendrites ascending through the OPL that establish direct contacts with photoreceptors (de Miguel et al., 1989; Fritzsch and Collin, 1990; Rio et al., 1998). This cell type corresponds to the biplexiform ganglion cells already described in some gnathostomes, and could provide a fast-forward signal from photoreceptors to ganglion cells, bypassing the usual bipolar cell interneuron (de Miguel et al., 1989; Rio et al., 1998). The glial Müller cells are located in the INL, and their processes form the outer and the inner limiting membranes.

- *Retinal development*

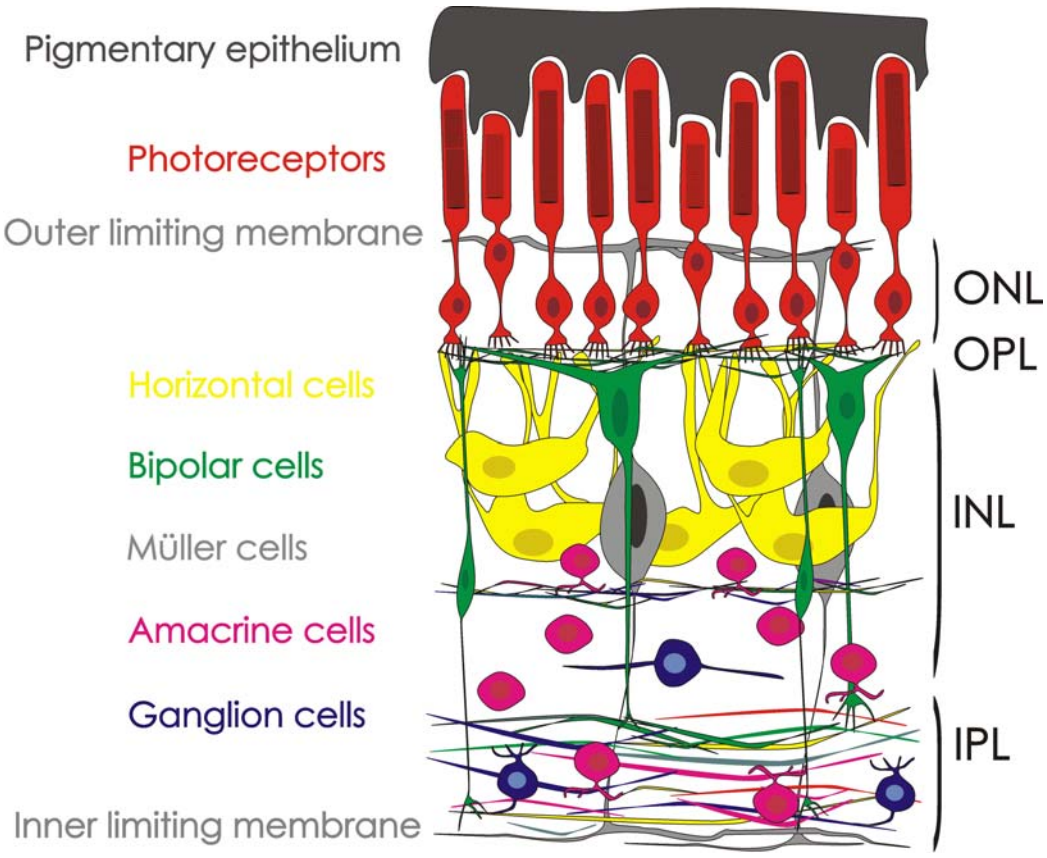


Figure 7. Schema of the adult sea lamprey retina showing the layers (ONL, OPL, INL, and IPL) and the cell types (photoreceptors, bipolar, horizontal, amacrine, ganglion and Müller cells).

Development and differentiation of the lamprey retina is unique in vertebrates (Rubinson and Cain, 1989; Rubinson, 1990; de Miguel and Anadón, 1987; de Miguel et al., 1990). Development begins with the formation of the optic vesicles in the embryonic stage and the posterior development into a rudimentary eye in prolarval stages, when cell differentiation in the retina begins, as shown by the appearance of both PCNA-negative cells and opsin positive photoreceptors (Meléndez-Ferro et al., 2002a). Cell differentiation occurs only in a small zone surrounding the optic nerve head of larval retina (central retina) that remains almost unchanged from early larvae until early metamorphosis (de Miguel and Anadón, 1987). In larvae more than 55-60 mm, a lateral retina consisting mainly of a thick neuroblastic layer develops around the early retina (Fig. 8).

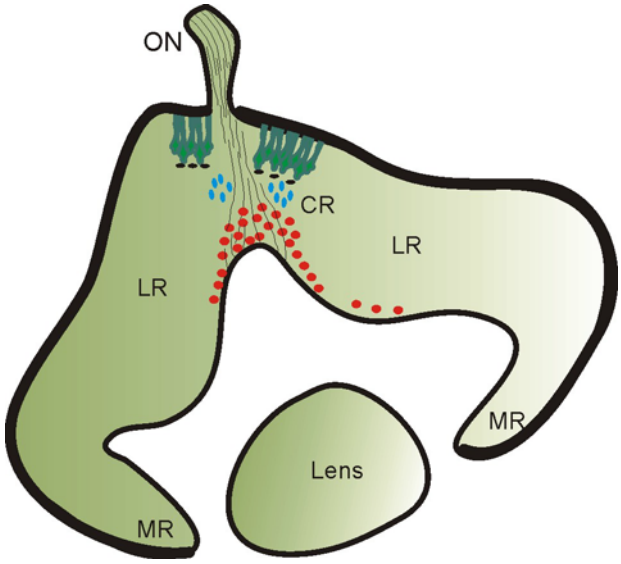


Figure 8. Scheme of a larval retina showing the retinal zones (central retina, CR; lateral retina, LR; and the marginal retina, MR). Note in the differentiated central retina the presence of photoreceptors (green), horizontal (black), bipolar (blue), and ganglion (red) cells. ON points the optic nerve.

The larval central retina contains a type of opsin-immunoreactive photoreceptors (Meléndez-Ferro et al., 2002a), bipolar cells and ganglion, while in the lateral retina only few ganglion cells can be observed (de Miguel et al., 1989; Villar-Cheda et al., 2006a; Villar-Cerviño et al., accepted) (Fig. 8). From 60 mm onwards, the larval retina grows by extension of the lateral undifferentiated retina, which in large larvae may be subdivided into a lateral germinal zone, and a more thickened intermediate zone with already differentiated ganglion cells and optic fibers (de Miguel and Anadón, 1987; de Miguel et al., 1989). The

retinal development is completed during metamorphosis with the differentiation of adult photoreceptors, horizontal, bipolar and amacrine cells. The ultrastructural changes in the retinal pigment epithelium of the sea lamprey during metamorphosis, to acquire the adult organization, have been studied in the sea lamprey (de Miguel et al., 1992).

Characterization of the adult retina and the two larval retinal regions was also done in the Southern hemisphere *Geotria australis* by electron microscopy (Meyer-Rochow and Stewart, 1996).

Tectal development spans the larval period but a spurt in tectal growth and differentiation are correlated with the completion of the retinal circuitry in metamorphosis (Rubinson, 1990). From the metamorphic M3 stage the optic tectum has the same lamination than in adults: a periventricular cell layer that is subdivided by fiber bands, and in the lateral region a stratum cellulare centralis and a stratum cellulare et fibrosum externum (de Miguel and Anadón, 1987).

- *Retinal projections*

The retina receives from the brain retinopetal or centrifugal fibers and sends to it retinofugal or centripetal fibers. The lamprey retinopetal fibers were first demonstrated by Vesselkin et al. (1989) in adults and de Miguel et al. (1989) in larvae. These are thin and varicose fibers originated in bilateral neurons of the tegmental midbrain, that run parallel to the ganglion cell axons of the optic nerve, to reach the IPL (Fritzsch and Collin, 1990), and contact directly with ganglion and amacrine cells (Vesselkin et al., 1989, 1996; de Miguel et al., 1989). The mesencephalic neuronal population that gives rise to the retinopetal fibers increases throughout the larval period (de Miguel et al., 1989; Rodicio et al., 1995).

The retinofugal fibers in adults leave the retina via the optic nerve to reach the thalamus and the optic tectum. Contralateral retinal projections to superficial layers of the pretectum, the optic tectum and the conspicuous “lateral geniculate nucleus” have been observed, while ipsilateral retinal projections are restricted to a small zone at the ventrolateral margins of the pretectum and optic tectum (Kennedy and Rubinson, 1977).

In larvae, the retinofugal system has two different patterns. In small larvae (less than 60 mm in length) only a single tract, the axial optic tract, projects to the contralateral diencephalon, pretectum, and mesencephalic tegmentum. In larvae longer than 60-70 mm,

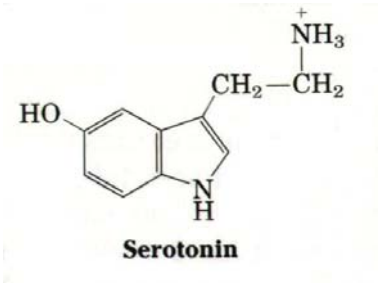
there is an additional contralateral tract, the lateral optic tract, which extends to the whole tectal surface. In addition, a small ipsilateral retinofugal projection is found in both small and large larvae. Initially, the ipsilateral projection is restricted to the thalamus-pretectum, but it reaches the optic tectum in late larvae (de Miguel et al., 1990).

The early appearance of a neural circuitry in the larval central retina (e.g. photoreceptors, bipolar and ganglion cells) supports the idea that functional retinofugal pathways are established in larvae. These fibers arising from the central retina run through the axial optic tract and reach early the pretectal area. The larval retinopetal fibers are hypothesized to provide some survival factors to the ganglion cells, until the complete development of the whole retina. Therefore, visual projections are organized during the blind larval period, and their development is largely independent of visual function (de Miguel et al., 1990).

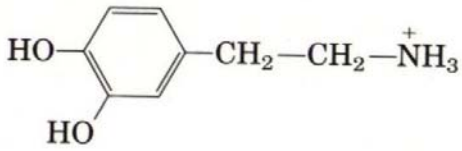
Whole metamorphic changes in the retina, the optic tectum and their connections, complete the functional development of the visual system and provide for the adult lamprey's predatory and reproductive behavior (Rubinson, 1990).

Monoaminergic systems

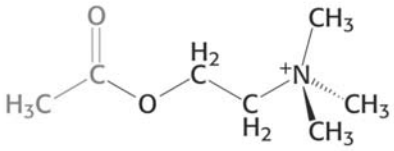
In monoaminergic systems the neuronal communication is mediated by biogenic monoamines of the CNS. They are classified in serotonergic and catecholaminergic, according the use of the indoleamine serotonin (5-hydroxytryptamine) or of catecholamines: dopamine (DA), noradrenaline (NA), and adrenaline (A) (Fig. 9). The most abundant catecholamine in the CNS is DA, while NA and A have less abundance. Monoamines have wide distribution in the Animal Kingdom, including vertebrate and invertebrate species, and have an ancient phylogenetic history (Parent, 1981). A common feature of monoamines is that all of them can work as neurotransmitters and neurohormones as well.



Serotonin



Dopamine



Acetylcholine

Figure 9. Structure of serotonin, dopamine and acetylcholine neurotransmitters.

* *Serotonergic system*

Serotonin is a biogenic monoamine synthesized from the essential amino acid tryptophan, which is captured from the bloodstream. Serotonin was identified as a neurotransmitter about 50 years ago. Synthesis of serotonin begins with the initial hydroxylation of L-tryptophan to 5-hydroxytryptophan carried out by the enzyme tryptophan-5-hydroxylase, which is the rate-limiting enzyme of serotonin synthesis. 5-hydroxytryptophan is rapidly decarboxylated by the enzyme aromatic amino acid decarboxylase (AADC, also acting in catecholamine synthesis) to produce serotonin (Fig. 10). Serotonin is actively stored in vesicles and released through a calcium-dependent process of exocytosis (Fig. 11). Once in the synaptic cleft, serotonin binds to pre- and/or postsynaptic serotonin receptors to produce a chemical signaling; in the presynaptic cell the feedback signaling is mediated by autoreceptors (Fig. 11).

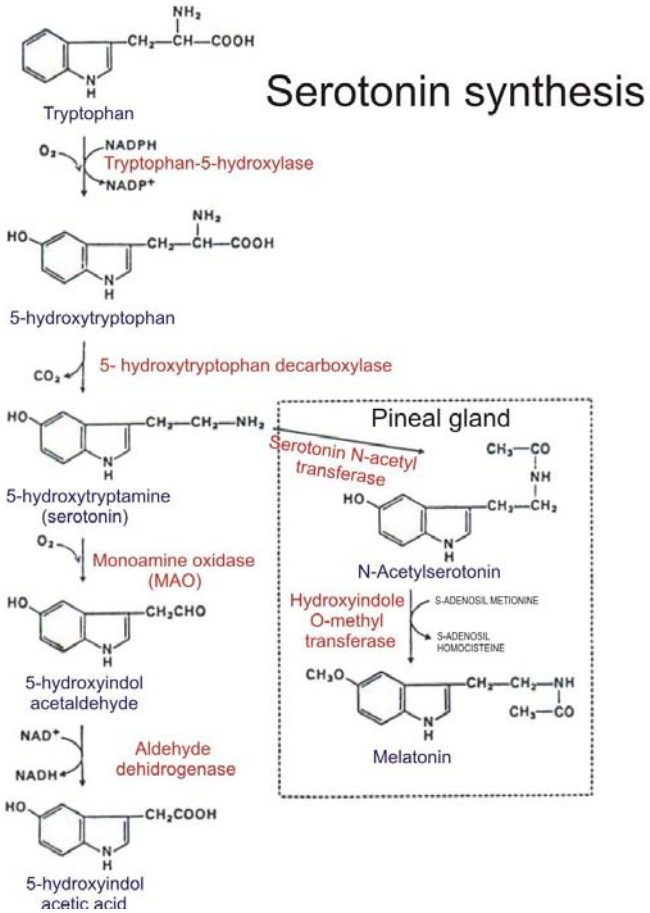


Figure 10. Schematic indicating the molecular structure of the serotonin metabolites and the identity of the enzymes involved in its synthesis and degradation. It is also shown the melatonin synthesis pathway in the pineal gland.

Once in the synaptic cleft, serotonin is recycled by reuptake via membrane transporters and restored in vesicles, or degraded by the mitochondrial monoamine oxidase enzyme (MAO, also acting in catecholamine degradation) (Figs. 10, 11). In the pineal organ serotonin is a melatonin precursor (Fig. 10). There are at least seven types of serotonin receptors, and each one has a different distribution pattern and biological responses (Burt, 1993). Most serotonergic synapses are inhibitory, although some are excitatory.

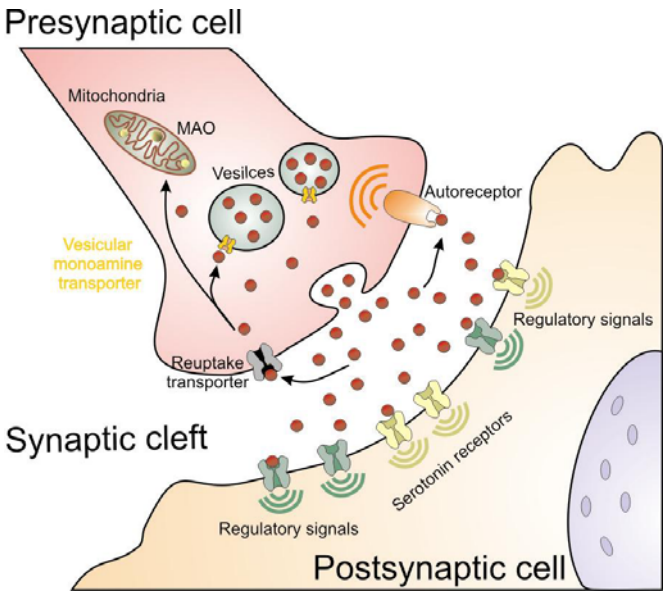


Figure 11. Diagram of a serotonin synapse. It shows serotonin synthesis, storage, liberation, receptors union, and degradation.

Serotonin acts both as a neurotransmitter and a modulator of neurotransmission, and is involved in regulation of neuronal development. The amount of serotonin is higher during development than in the adult, which implies an important role for this monoamine in the early development of the CNS. Whole actions of serotonergic neurons in the CNS are complex, and functional generalizations are difficult to make. However, the developmental and neurochemical pattern in the serotonergic system of all species studied is similar (Azmitia and Whitaker-Azmitia, 1997). The serotonergic system is associated with the regulation of body temperature, blood pressure, rapid eye movement during sleep, circadian rhythms, and pain perception (Burt, 1993).

To investigate the distribution of serotonin in the CNS of vertebrates, either FIF (formaldehyde-induced fluorescence) or autoradiographic techniques were first used. Currently more specific techniques are used, such as the immunohistochemical detection using specific antibodies against serotonin or its synthesis enzyme, or *in situ* hybridization of mRNA of its synthesis or degradation enzymes.

* *Catecholaminergic system*

Catecholamines are a family of neurotransmitters synthesized in nervous tissue from a common substrate: tyrosine, which is captured from the bloodstream. The synthesis of catecholamines is summarized in figure 12. Tyrosine hydroxylase (TH) is the initial and rate-limiting enzyme in the catecholamine pathways. It is a soluble enzyme concentrated in the axon terminals of all catecholaminergic neurons that transforms tyrosine into L-DOPA. L-DOPA is rapidly decarboxylated to DA by the cytosolic enzyme DOPA decarboxylase, and DA is stored in vesicles of dopaminergic, noradrenergic and adrenergic neurons. Because DOPA decarboxylase is not specific to L-DOPA and can also decarboxylate other aromatic amino acids, the preferred name for this enzyme is aromatic amino acid decarboxylase. Dopamine β-hydroxylase (DBH) is an soluble intravesicular enzyme that hydroxylates DA to get NA in noradrenergic and adrenergic neurons, which is released during the synaptic transmission (Burt, 1993; Deutch and Roth, 1999; Schwartz, 2000). NA is converted in A by the phenylethanolamine N-methyltransferase (PNMT) enzyme

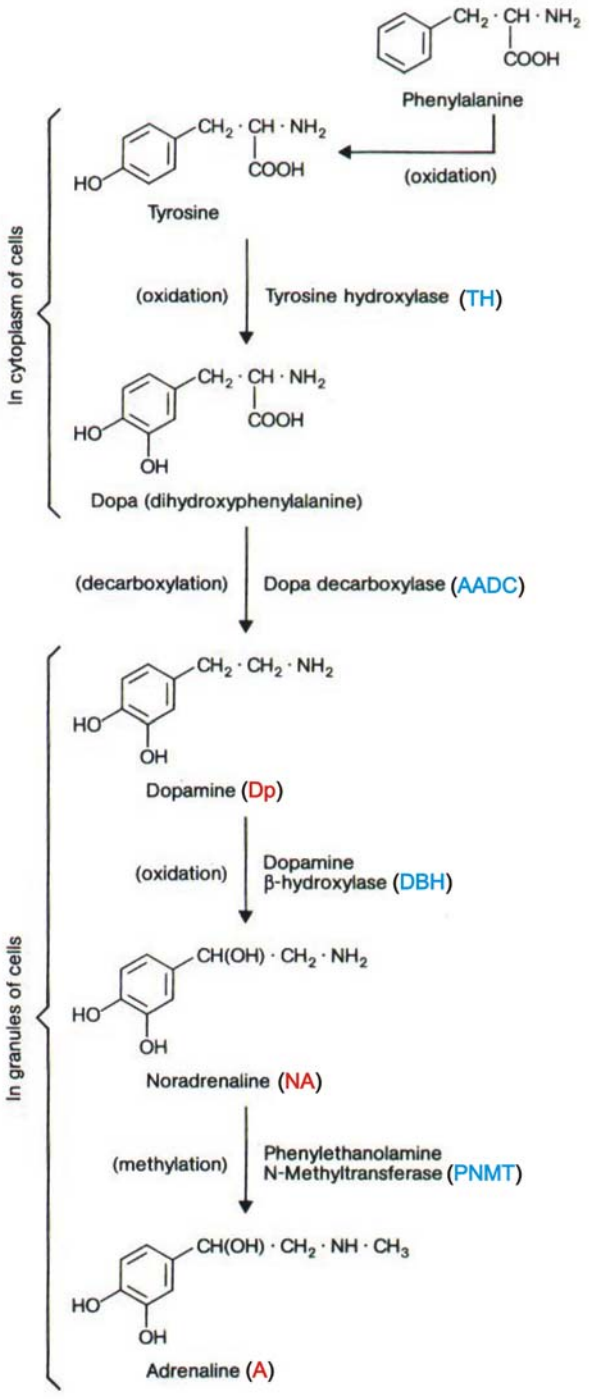


Figure 12. Schematic indicating the molecular structure of the different catecholamines (in red) and the identity of the enzymes involved in their serial transformation (in blue), beginning with the non-catecholamine precursor tyrosine.

present only in adrenergic neurons.

Once in the synaptic cleft, the neurotransmitter binds to one of the group of catecholamine receptors, which comprises also autoreceptors in the presynaptic membrane that regulate catecholamine synthesis (Fig. 13). 80% of catecholamines are removed from the synaptic cleft by a reuptake transporter. Once in the presynaptic cell, catecholamines are restored in vesicles or degraded by several enzymes, including MAO and catechol-O-methyl transferase (Burt, 1993; Deutch and Roth, 1999; Schwartz, 2000).

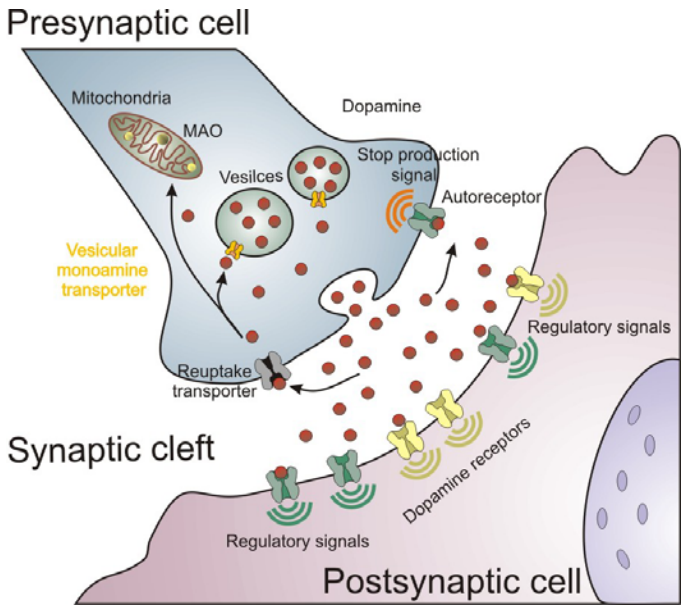


Figure 13. Diagram of a dopamine synapse. It shows dopamine synthesis, storage, liberation, receptors union, and degradation.

Catecholamines were classically associated with mood and locomotive activity control, and their absence produces several symptoms related to schizophrenia or Parkinson's disease. Catecholamines have also an important role during development. An important role for DA in teleosts is to regulate the activity of several hypophyseal hormones (Kah et al., 1986, 1987).

As in the case of serotonin, two major anatomical methodologies have been used for catecholamine detection: FIF and immunohistochemistry, although *in situ* hybridization is also currently used, due to its higher specificity.

Cholinergic system

Acetylcholine was the first neurotransmitter discovered. It is the only accepted low-molecular-weight amine transmitter substance that is not an amino acid or derived directly from one (Deutch and Roth, 1999; Schwartz, 2000). Acetylcholine is synthesized in axon terminals from choline and acetyl-coenzyme A by the enzyme choline acetyltransferase (ChAT) (Fig. 14). Neurons do not synthesize choline, and since choline cannot cross the blood-brain barrier, the main source choline in the brain comes from the breakdown of phosphatidylcholine. ChAT, although synthesized in the soma, is transported to the nerve terminal. Acetylcholine molecules are stored in vesicles (2000-10000 molecules per vesicle), and are released after depolarization to the synaptic cleft. In the synaptic cleft, acetylcholine binds to post- and presynaptic receptors and is degraded by acetylcholinesterase (AChE) (Fig. 15).

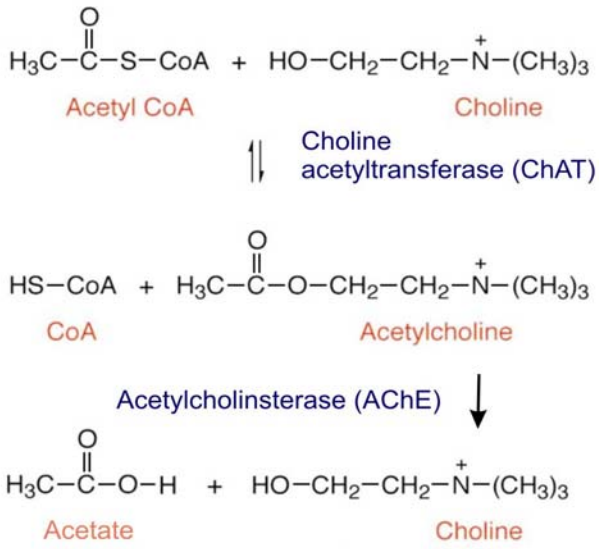


Figure 14. Schematic indicating the acetylcholine synthesis and degradation, and the identity of the enzymes in it implied.

There are two families of acetylcholine receptors: nicotinic and muscarinic, and there are soluble and transmembrane AChE forms in the pre- and postsynaptic membranes. Once acetylcholine is degraded, choline can be transported to the presynaptic neuron to be used again. Cholinergic synapses are quite conservative and recycle over 50% of choline (Burt, 1993; Deutch and Roth, 1999; Schwartz, 2000).

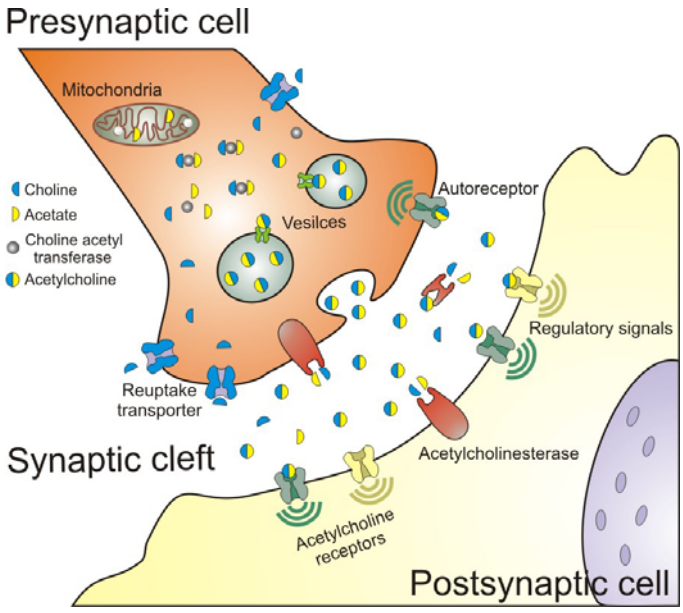


Figure 15. Diagram of a cholinergic synapse and acetylcholine synthesis, storage, release, union to 0receptors, and degradation.

As for the detection of cholinergic systems, immunohistochemistry with antibodies against ChAT and AChE, and *in situ* hybridization are the most used techniques to detect cholinergic neurons, or fibers. Acetylcholine is the main transmitter used by motoneurons and therefore is released at all vertebrate neuromuscular junctions. In the autonomic nervous system it is the transmitter in all preganglionic neurons and in parasympathetic postganglionic neurons as well (Burt, 1993). As in other vertebrates, there are also several non-motor cholinergic populations of the brain (Pombal et al., 2001) and retina (Pombal et al., 2003) of lampreys.

Animals used

The adult lampreys used in experiments (Fig. 17A) were bought to a local supplier, while postmetamorphic individuals (Fig. 17B) and larvae (Fig. 17C) were obtained by digging the edges of the river Ulla (Galicia, Northwest Spain). The embryos (Fig. 17E) and prolarvae (Fig. 17D), were obtained in the laboratory by in vitro fertilization using sperm and ovules from mature spawning lampreys. We let some embryos to develop in the lab to get prolarvae and early larvae, but progression to mid-sizez larvae was not possible in the lab. All experiments were conducted in accordance with European Community guidelines on animal care and experimentation to minimize pain and discomfort.

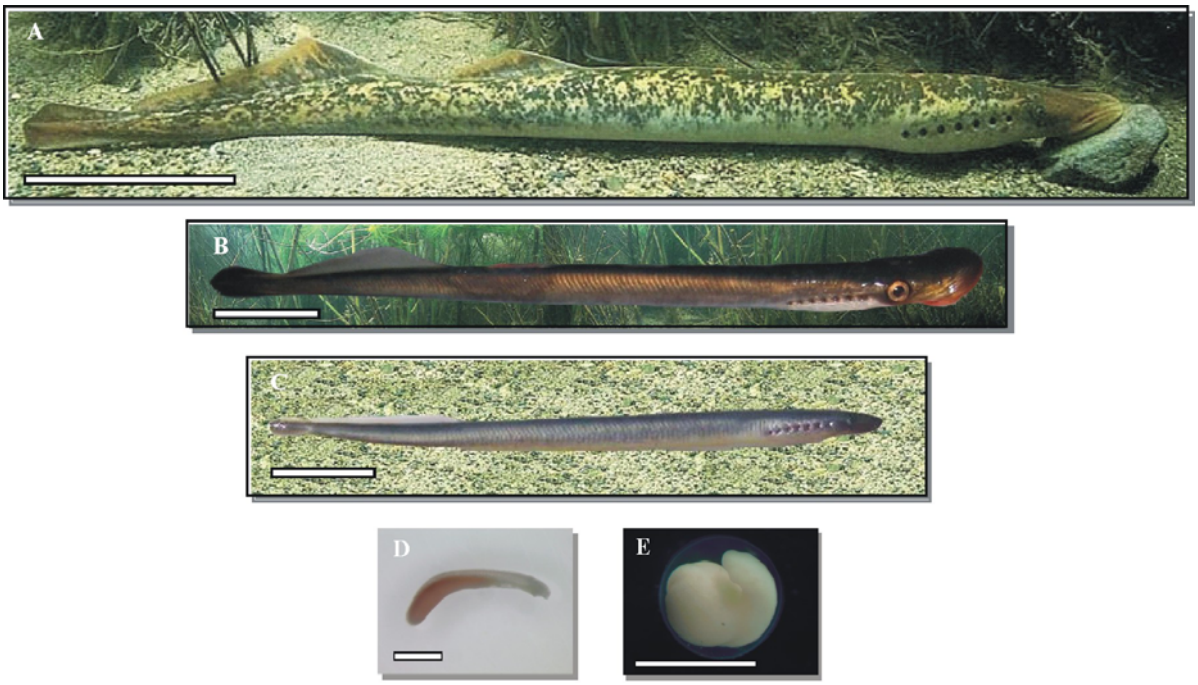


Figure 17. Lateral view of an adult (A), a postmetamorphic individual (B), a large larva (C), a prolarva (D), and an embryo (E) of sea lamprey. Scale bars = 20 cm in A, 20 mm in B and C, and 1 mm in D and E.

Brief introduction to the used techniques

Immunohistochemistry was the main technique used in our study. Immunohistochemistry is based on the specific union of an antibody to its antigen in the desired tissue. Three immunohistochemical methods were used: the peroxidase-antiperoxidase (PAP) method (Sternberger et al., 1970), the avidin-biotin complex (ABC) method (Hsu et al., 1981a, b), and the indirect immunofluorescence method.

The PAP method uses a preformed peroxidase-antiperoxidase complex (PAP) made up of three horseradish peroxidase enzyme molecules and two molecules of the antiperoxidase antibody (Fig. 16A). The method uses three layers: a primary antibody directed against the antigen, a secondary antibody against the primary antibody, that is also a bridge between the primary antibody and the third layer, the PAP complex (Fig. 16A). The visualization of the immune complex is achieved by means of subsequent incubation in hydrogen peroxide and diaminobenzidine (DAB), when peroxidase produces an insoluble brown precipitate of DAB.

Avidin is a glycoprotein with a great affinity for the vitamin biotin. Many molecules of biotin can be attached to a molecule of antibody. The biotinylated antibody thus produced can later bind to many molecules of avidin. In the ABC method the primary antibody is followed by a biotinylated secondary antibody. The third step is the application of the avidin-biotin preformed complex, labeled with peroxidase, which attaches to the biotin of the secondary antibody (Fig. 16B). The visualization process is similar to the PAP method. This procedure is usually more sensitive than the PAP method.

Immunofluorescence is a similar methodology with multiple variations. Thus, the secondary antibody can be coupled to a fluorescent molecule for an easy observation with a fluorescence microscope or a spectral confocal microscope. It makes double labeling possible by using primary antibodies raised in different species followed by two secondary antibodies that are coupled to different fluorophores, which are excited by and emit in different wavelengths, for the detection of two different antigens in the same sample.

It is also possible to combine immunofluorescence with neuronal tracers. In order to do this, the tracer is first injected in the desired CNS region and allowed to be transported retrogradely or anterogradely, depending on the tracer. The tracer should be coupled to a fluorescent or a biotinylated compound.

The immunohistochemical technique is carried out afterwards to detect the double fluorescent labeling of the tracer and the antibody against the desired substance. This technique reveals the neurochemical phenotype of certain neuronal populations at the same time that allows determining the projections of these groups.

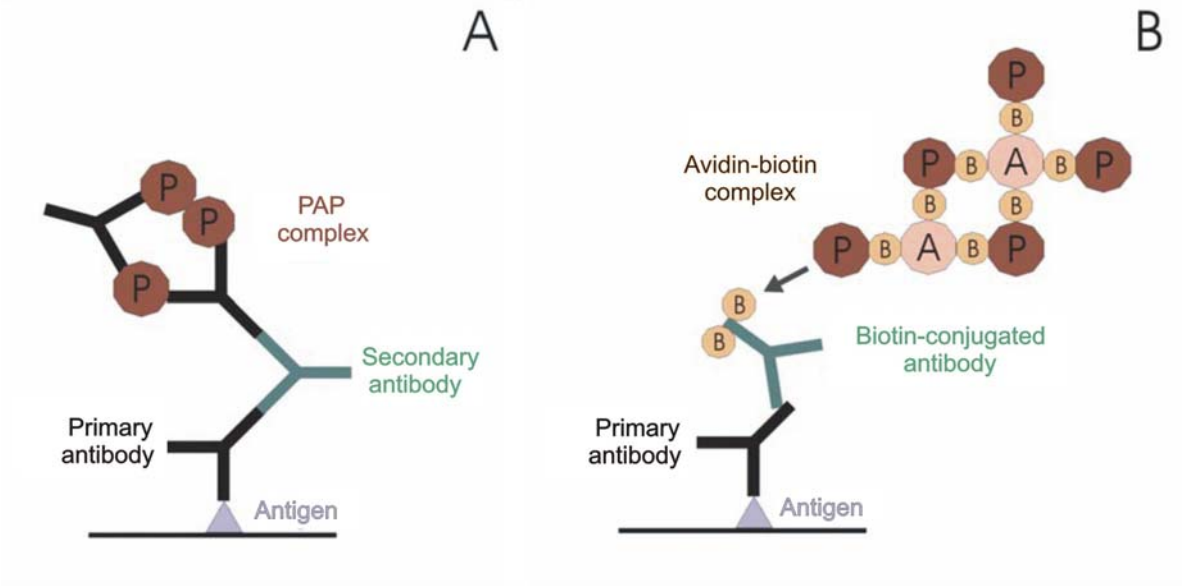


Figure 16. PAP (A) and ABC (B) immunohistochemical method diagrams for the detection of antigens in tissue.

- *Immunohistochemical used procedures*

I will briefly describe the general immunohistochemical considerations, nevertheless in each chapter the used technique will be developed more in detail. Detection of monoaminergic neurons was done by using polyclonal antibodies against serotonin (Incstar, Stillwater, MN), DA (Steinbusch, The Netherlands), TH (Chemicon, Temecula, CA), and DBH (Chemicon). Acetylcholine-synthesizing structures were demonstrated by using a polyclonal antibody against ChAT (Chemicon).

Whole specimens were fixed, after deep anesthesia with 0.05% benzocaine (Sigma, St. Louis, MO), in 4% paraformaldehyde in 0.1 M Tris-buffered saline pH 7.4 (TBS) for serotonin, TH, DBH, and ChAT detection, or in cold (4°C), freshly prepared 5% glutaraldehyde in TBS containing 1% sodium metabisulfite, for dopamine detection, overnight. Whole samples were cryoprotected with 30% sucrose in TBS, after rinsing in TBS, subsequently embedded in OCT Tissue Tek (Sakura, Torrance, CA), and frozen using

liquid nitrogen-cooled isopentane. Transverse, sagittal, or horizontal serial sections (12-18 µm thick) were cut on a cryostat, and mounted on charged slides.

The procedure used for serotonin, TH, or DBH detection was similar, while the procedure for dopamine detection was appreciably different due to fixation of tissue in glutaraldehyde. Serial sections were processed for immunocytochemistry as follows. Non-specific binding sites were blocked by incubation with 10% normal serum (Vector Laboratories). The sections were then incubated in a humid chamber at room temperature with the primary antibody, overnight. There are several options for the secondary antibody depending on the method chosen;

a) For the PAP method, endogen peroxidase was blocked before incubation by pretreatment with 10% hydrogen peroxide in TBS. Subsequently, sections were incubated with the primary antibody, rinsed twice in TBS, incubated with the secondary antibody for 1 hour, rinsed twice in TBS, and incubated with PAP complex for an hour.

b) For the ABC method, before the incubation with the primary antibody endogen avidin and biotin were blocked with avidin and biotin solutions (Vector Laboratories). Subsequently, sections were incubated with the primary antibody solution, rinsed in TBS, incubated with the biotinylated secondary antibody for an hour, and later incubated with the avidin-biotin complex for half an hour. In both methods, the immunocomplexes were developed using 0.6 mg/ml DAB (Sigma) and 0.003% hydrogen peroxide. After development, the sections were rinsed in TBS, dehydrated, and coverslipped.

c) For immunofluorescence, after incubation with the primary antiserum and rinsing in TBS, the sections are incubated with a fluorescent-conjugated secondary antibody for an hour and subsequently rinsed in TBS and mounted with a mounting medium for fluorescence (Vector Laboratories). To minimize autofluorescence induced by glutaraldehyde fixation, the sections were incubated in a solution of $NaBH_4$ 0.2%, for 45 minutes, before the incubation with the primary antibody.

All antibody dilutions were made in TBS containing 0.2% Triton X-100 as detergent and 3% normal serum. For dopamine detection, 1% sodium metabisulfite was added to the primary antibody solution. For ChAT detection, the primary antibody solution contained 1% bovine sero-albumin. The specificity of primary antibodies has been tested

by the suppliers. As a further control, some sections were processed either omitting the primary antibody, or replacing the primary antiserum by normal serum. In these sections, no immunostaining was observed.

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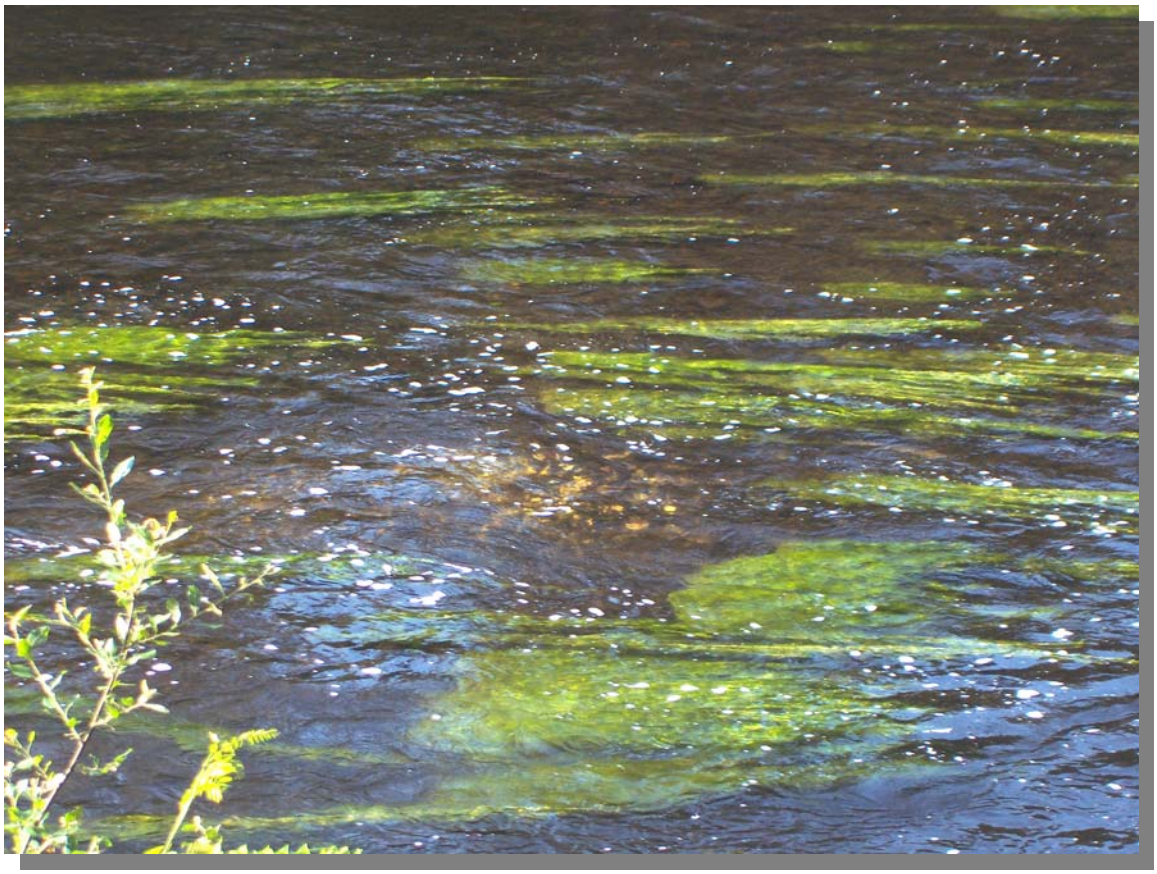


Aims of the Thesis

Aims of this Thesis

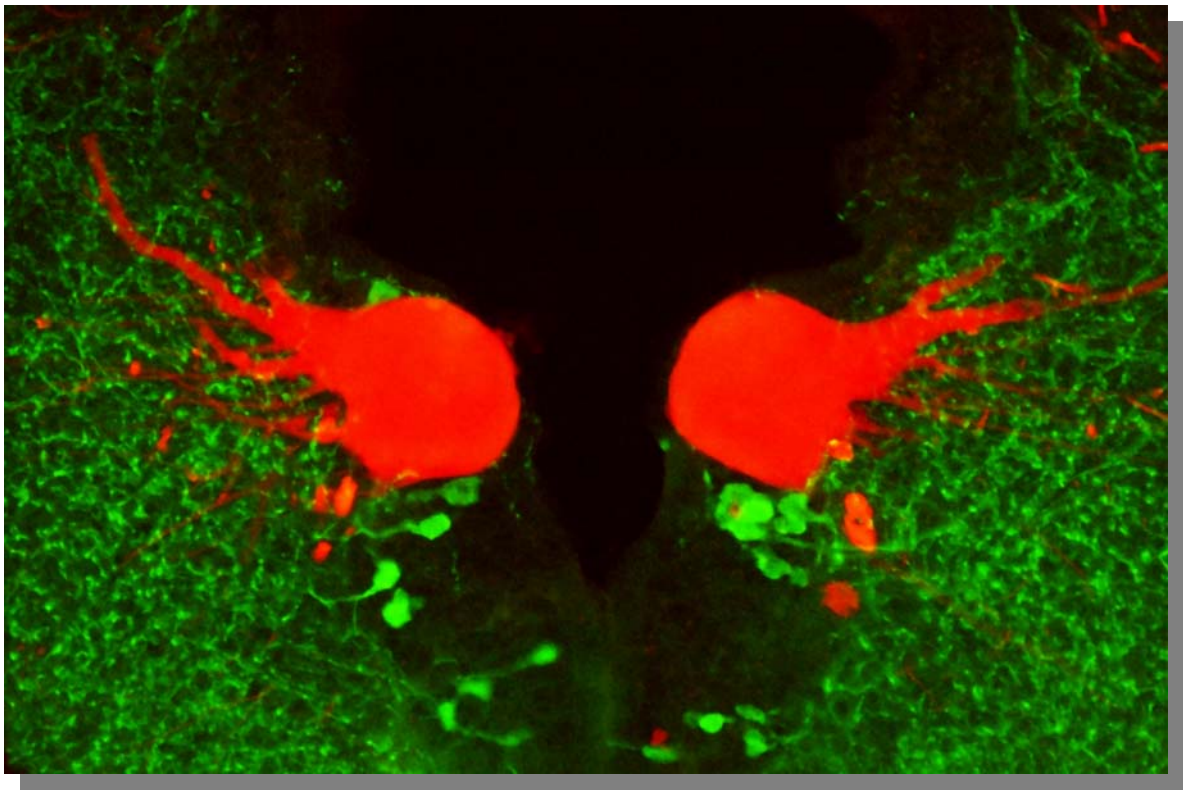
This work was included in the series of studies on the development of the central nervous system of the sea lamprey performed by our investigation group, in particular on the neurochemical maturation. The characterization of the chemically identified neuronal groups and their development in lamprey can provide important information for a better understanding of the development of the nervous system in early vertebrates. In this work we tried to shed light on the patterns of development of the serotonergic and catecholaminergic systems in the sea lamprey brain, spinal cord and retina. The specific aims were:

- To determine the adult distribution of the serotonergic and catecholaminergic cell groups in the central nervous system of the sea lamprey, and its comparison with that reported in other lamprey species.

- To establish the developmental pattern of these populations from the embryonic period to adulthood.

- To compare the spatio-temporal developmental patterns of these systems in the sea lamprey with those described in the other species of vertebrates in order to know the degree of conservation of these patterns.

- To shed light on the neurochemical differentiation of sea lamprey retina during development, reporting the appearance of immunoreactivities to serotonergic, dopaminergic and cholinergic markers.



Chapter 1

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Development of the Serotonergic System in the Central Nervous System of the Sea Lamprey

ABSTRACT

Lampreys belong to the most primitive extant group of Vertebrates, the Agnathans, which are considered the sister group of jawed vertebrates. Accordingly, characterization of neuronal groups and their development may provide useful information for understanding early evolution of the nervous system in vertebrates. Here, the development of the serotonergic system in the central nervous system of the sea lamprey was investigated immunocytochemically from embryos to adults. The appearance of the different serotonin-immunoreactive (5HT-ir) neuronal populations found in adults occurs between the embryonic and metamorphic stages. The earliest serotonergic neurons appear in the basal plate of the isthmus region of late embryos. In prolarvae, there is a progressive appearance of new serotonergic cell groups: first in the spinal cord, followed by those of the pineal organ, tuberal region, zona limitans intrathalamica, rostral isthmus, and the caudal part of the rhombencephalon. In early larvae, a new serotonergic cell group appears in the mammillary region, whereas in the pretectal region and the parapineal organ first serotonergic cells appear in middle and late larval stages, respectively. The first serotonergic fibers appear in early prolarvae, with fibers that ascend to and descend from the isthmic cell group, and the number of immunoreactive fibers progressively increases until the adult period. The present results are discussed in a comparative and developmental context.

INTRODUCTION

Serotonin is an important neurotransmitter in the brain of vertebrates. The serotonergic system has been one of the first components of the central nervous system (CNS) to be characterized histochemically (Parent, 1981; Parent and Northcutt, 1982; Parent, 1984). Evolution of the serotonergic system in vertebrates appears to involve a progressive disappearance of cells in rostral serotonergic populations and an increase in number of cells in more caudal serotonergic populations (Pierre et al., 1992). During development, serotonin appears early in the central nervous system (mammals: Frankfurt et al., 1981; Lidov and Molliver, 1982; Wallace and Lauder, 1983; Botchkina and Morin, 1993; chick: Wallace, 1985; anurans: van Mier et al., 1986; Zhao and Debski, 2005; teleosts: Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994). In addition to its role as neurotransmitter regulating basic behaviors, serotonin is involved in the regulation of neuronal development (Lauder and Krebs, 1978; Gromova et al., 1983; Haydon et al., 1984; Chubakov et al., 1986; Lauder, 1987; Goldberg and Kater, 1989; Lauder, 1993; Goldberg et al., 1991; Meier et al., 1991; Gaspar et al., 2003; Sodhi and Sanders-Busch, 2004). Recently, a role of serotonin in the regulation of programmed cell death during brain development has also been suggested (Persico et al., 2003).

Sea lampreys belong to the most ancient group of extant vertebrates and they have a complex life cycle that starts with eggs laid in the bed of the river. The embryonic period (about 12 days), is followed by a non-feeding prolarval period (about 24 days) and a microphagous larval period (several years long) in which the animals are blind and live buried in burrows in the river where they were born. Through a complex metamorphosis, larvae (ammocoetes) transform into young adult lampreys that descend

to the sea to feed parasitically on marine fishes, growing rapidly until they finally ascend the river to breed and subsequently die (Hardisty and Potter, 1971).

The serotonergic system in the brain of adult lampreys has been investigated by means of formaldehyde-induced fluorescence (Honma, 1969; Baumgarten, 1972) or immunocytochemistry (Steinbusch and Nieuwenhuys, 1979; Steinbusch et al., 1981; Pierre et al., 1992; Antri et al., 2006). The immunocytochemical methods have been particularly useful to define neuronal populations in the brain by their location and morphology (Pombal and Puelles, 1999; Weigle and Northcutt, 1999; Meléndez-Ferro et al., 2002b). Although there have been a few reports on the presence of serotonergic cells in larval lamprey (Yáñez, 1992; Hay-Schmidt, 2000; Meléndez-Ferro et al., 2002a, b; Antri et al., 2006), no systematic analysis of the development of this system has been done to date. Therefore, little is known on the time of appearance or the spatio-temporal developmental patterns of serotonergic cell groups and fibers. Knowledge of the early organization of this system in lampreys may provide important data on the phylogeny of the vertebrate serotonergic system, and the roles that serotonin may play in the developing nervous system. In this study, we describe the development of the serotonergic populations in the CNS of the sea lamprey from embryos to adults, and provide information on the formation of the serotonergic circuitry.

MATERIALS AND METHODS

Animals

Late embryos (E9-E11; n= 5), prolarvae (P0-P23; n= 15), larvae (9-170 mm in length; n= 20), metamorphic individuals (n= 10), and postmetamorphic (n= 10) and sexually mature adults (n= 5) of the sea lamprey (*Petromyzon marinus* L.) were used. Embryos and prolarvae were obtained in the laboratory from *in vitro* fertilized eggs

using eggs and sperm from sexually mature adult lampreys caught in the river Ulla (Galicia, Northwest Spain). Eggs and embryos were maintained under appropriate conditions of darkness, temperature (16ºC) and aeration. Larvae, metamorphic and postmetamorphic individuals were caught in the river Ulla, while adult individuals were purchased from a local supplier. All stages were kept in an aerated aquarium until processing. All experiments were conduced in accordance with European Community guidelines on animal experimentation.

Embryos and prolarvae were staged by age (e.g. E11 indicates 11 days post-fertilization embryos; P4 indicates 4 days post-hatching prolarvae, and so on). In our broods, hatching occurred 11-12 days after fertilization. In addition, to further classify prolarvae, we used the stages defined for the sea lamprey by Piavis (1971): hatching (P0-P1), pigmentation (P2-P3), gill cleft (P4-P7), and burrowing (P8-P23). Larvae were classified by total body length. Metamorphic individuals of *Petromyzon marinus* were staged as M1-M7 according to Youson and Potter (1979).

Serotonin immunocytochemistry

Before experiments, the animals were deeply anesthetized with 0.05% benzocaine (Sigma, St. Louis, MO) in fresh water. All embryos and prolarvae, heads of larvae or brain of adults were fixed by immersion in freshly prepared 4% paraformaldehyde in 0.1M phosphate saline, pH 7.4 (PB). Two sectioning methods were used. After rinsing in PB, some samples were cryoprotected with 30% sucrose in PB, embedded in Tissue Tek (Sakura, Torrance, CA), frozen using liquid nitrogen-cooled isopentane, and cut on a cryostat. The other samples were dehydrated in alcohols, cleared in xylene, embedded in paraffin wax and cut on a rotary microtome. In

both cases, transverse and sagittal sections (10-16 μm thickness) were mounted on gelatin-subbed slides.

For immunohistochemistry, non-specific binding sites in the sections were blocked by incubating with 10% normal goat serum (Vector Laboratories, Burlingame, CA). Then, the sections were incubated in a humid chamber at room temperature with a rabbit polyclonal anti-serotonin antibody (Incstar, code nº 20080; dilution 1:500-1:5000), overnight. The immune complex was visualized either by either the peroxidase-antiperoxidase (PAP) method or the indirect immunofluorescence method. In the first case, after rinsing in phosphate buffered saline (PBS; pH 7.4), the sections were incubated with a goat anti-rabbit antibody (Sigma, code nº R3382; diluted 1:100), for one hour at room temperature. The sections were incubated in peroxidase-antiperoxidase complex (PAP complex, Sigma, code nº P2026; diluted 1:400), rinsed and developed using 0.6 mg/ml 3-3´diaminobenzidine (Sigma) and 0.003% H_2O_2 in PBS. After developing, the sections were rinsed in distilled water, dehydrated, and coverslipped with Eukitt (Panreac, Barcelona, Spain). For indirect immunofluorescence detection of serotonin, a FICT-conjugated swine anti-rabbit antibody (Dako, code nº F0205; diluted 1:30) was used. After rinsing in distilled water, sections were coverslipped with mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA). All antibody dilutions were done in PBS containing 3% normal serum and 0.2% Triton X-100 as detergent.

The specificity of the anti-serotonin antibody has been tested by the supplier, who reported typical 5-HT pattern immunostaining in rat suprachiasmatic nucleus and spinal cord with this antibody. Staining was completely abolished by pretreatment of the diluted antibody with 5-HT-BSA conjugate. The supplier also reported no detectable cross-reactivity with tryptamine, 5-methoxytryptamine, L-tryptophan, 5-

hydroxytryptophan, dopamine, norepinephrine, or adrenaline. As a further control, some control sections were processed as above but either omitting the primary antibody or replacing the primary antiserum by the corresponding preimmune serum. In all of these cases, no immunostaining was observed in the sections. This antibody has been used in a recent study of serotonin in the sea lamprey brainstem (Antri et al., 2006).

Additional material

For a better characterization of the development of the CNS of the sea lamprey we also used our collection of embryonic, prolarval and larval brains immunostained for proliferating cell nuclear antigen (PCNA) (for methodological details, see Villar-Cheda et al., 2002, 2005, 2006). For topographical purposes, brain series stained with haematoxylin/eosin or cresyl violet were also used.

Measurements and cell counts

Cell counts and measurements were performed on serial transverse sections of adult and larval lampreys with the aid of a Visopan (Reichert, Wien), or on photomicrographs of sections. In order to avoid overestimation of values, only cells exhibiting the cell nucleus in the section were counted, and cell counts were corrected using Abercrombie's factor (Abercrombie, 1946). Due to the very heterogeneous size of serotonergic isthmic cell populations, for the purpose of counting, adult isthmic cells were classified into three size classes (small, 6-14 μm; medium, 15-24 μm and large, 25-30 μm in transverse diameter), and correction factors were calculated and applied for each of these size classes. Despite Abercrombie's factor may introduce a bias in cell counts, the scattered distribution of serotonergic cells in most populations gives calculations enough precision for a semiquantitative description.

Photography

Photomicrographs were taken with an Olympus AX-70 photomicroscope equipped with color digital cameras (Olympus DP-12, DP-70). The photographs were converted to gray scale and adjusted for brightness and contrast using Corel Photo-Paint 12 software (Corel, Ottawa, Canada). Photomontage and lettering were done using Corel Draw 12.

Nomenclature of brain structures

The nomenclature used for the brainstem nuclei and other brain regions of the adult sea lamprey follows in general that of Nieuwenhuys and Nicholson (1998), but for the adult prosencephalon and midbrain we adopted most limits and regions defined by Pombal and Puelles (1999). For prolarval and larval brain regions, we adopted the nomenclature used in previous studies from our laboratory (Meléndez-Ferro et al., 2002b, 2003; Abalo et al., 2005; Villar-Cheda et al., 2006).

RESULTS

In the central nervous system of the sea lamprey most serotonin-immunoreactive (5HT-ir) cell groups were observed in the diencephalon, rhombencephalon and spinal cord, although occasional 5HT-ir cells were also observed in the telencephalon and in the caudal mesencephalon. The distribution of 5HT-ir cell groups in the brain of adults, larvae and prolarvae of *Petromyzon marinus* is schematically illustrated in sagittal projections of the brain and rostral spinal cord shown in Figure 1, and in schematic drawings of transverse sections of Figure 2. All 5HT-ir cell groups present in the adult sea lamprey appeared between the embryonic

and the late larval stages (Fig. 2), and the stage of first appearance of these nuclei is summarized in Table 1.

Serotonin distribution in the adult sea lamprey

Cell bodies

Prosencephalon

In the telencephalon, occasional small 5HT-ir cells were observed in the olfactory bulbs (Fig. 3A). These cells were located in the inner granular layer. A few pear-shaped 5HT-ir cells were also observed in the caudal preoptic region in a cell layer separated from the ventricle by a layer of neuropil (Fig. 3B), probably corresponding to the paracommissural preoptic nucleus of Pombal and Puelles, 1999.

In the hypothalamus, a large population of small 5HT-ir bipolar cells (6-9 μm in diameter) was observed in the walls of the third ventricle extending from the tuberal to the mammillary region (Figs. 3C, E). Most these cells were of cerebrospinal fluid-contacting (CSF-c) type, with bipolar perikarya located close the ependymal layer showing a ventricular dendrite ending as a club on the ventricular surface (Figs. 3D, E), and an axonal process coursing to the lateral area (Fig. 3C). Attending to the topography and time of appearance of cells, this large hypothalamic group could be subdivided into three subpopulations: dorsal and ventral tuberal, and mammillary. The most conspicuous group was the dorsal tuberal subpopulation (dorsal hypothalamic nucleus of Pierre et al., 1992; hypothalamic periventricular organ of Pombal and Puelles, 1999), which showed a large number of strongly stained 5HT-ir bipolar cells disposed in two or three irregular rows parallel to the ventricle, being the innermost cells located close to or among the ependymocytes (Figs. 3C, D). The length of the apical dendrite was variable depending on the location of the perikaryon. The lateral processes of these neurons formed a conspicuous ventrolateral field of varicose 5HT-ir fibers. The ventral

tuberal subpopulation (ventral hypothalamic nucleus of Pierre et al., 1992; tuberal nucleus of Pombal and Puelles, 1999) was widely separated from the dorsal subpopulation at rostral levels, but caudally both subpopulations were confluent. The ventral tuberal subpopulation consists of faintly 5HT-ir small CSF-c cells preferentially located in the outer part of the ependymal layer and showing a short apical dendrite. The 5HT-ir mammillary CSF-c subpopulation fills the walls of the posterior infundibular recess (Fig. 3E), being continuous with the tuberal CFS-c subpopulation. The appearance and density of the mammillary 5HT-ir CSF-c subpopulation varied; thus in the ventral wall the cells were plump and short, but in lateral walls the cells were taller and slender. The highest cell density was observed in the ventrolateral corner of the recess, where most periventricular cells were 5HT-ir (Fig. 3E).

In a position intermediate between the dorsal and ventral thalamus, a small row (1-5 cells of about 8 μm in diameter per transverse section) of moderately stained 5HT-ir cells was located in the cell layer parallel to the ventricle (Fig. 3F). In previous studies, this row of 5HT-ir cells was characterized as pertaining to the zona limitans intrathalamica (Pombal and Puelles, 1999; Meléndez-Ferro et al., 2002b), and probably corresponds to the two dorsal thalamic 5HT-ir populations of Pierre et al. (1992). In the caudal diencephalon, a 5HT-ir population of small cells (7-10 μm in diameter) was observed in the pretectal region (Fig. 3G). It consists of a sparse subgroup of bipolar neurons, located in the cellular "periventricular stratum" of Pombal and Puelles (1999), which have axons that project laterally, and a lateral subgroup with fewer 5HT-ir cells scattered in the "intermediate stratum" of these authors that is located medial, dorsolateral and caudal to the posterior commissure tract (Fig. 3G). The 5HT-ir cells were mainly observed in the pretectum "dorsal tier" of Pombal and Puelles (1999), although some cells were also observed in their "medial tier". Together, these pretectal

cells correspond to the brainstem serotonergic group I recently described in sea lamprey by Antri et al. (2006).

The pineal complex (pineal and parapineal organs) contained abundant 5HT-ir CSF-c flask-shaped or polygonal cells, but a different distribution of 5HT-ir cells was observed in the pineal and parapineal organs (Fig. 3H). The pineal vesicle and stalk showed numerous strongly 5HT-ir cells, but only a few 5HT-ir cells were observed in the ventral wall of the parapineal vesicle (Fig. 3H).

Mesencephalon

Following Pombal and Puelles (1999), we adopted as the midbrain/hindbrain boundary the limit between the optic tectum and the cerebellar plate, dorsally, and the caudal limit of the giant M3 Müller cells, oculomotor nerve exit and chiasm and the rostral limit of the decussation of the anterior octavomotor tract, ventrally. In the sea lamprey mesencephalon, only very occasional small 5HT-ir cells (one or two per brain) were observed in the caudal optic tectum, while no 5HT-ir cells were found in the tegmentum. The most rostral 5HT-ir isthmic cells were observed at a level just caudal to the pair of M3 Müller cells.

Rhombencephalon

For the description of 5HT-ir populations of the rhombencephalon, we used topographically-defined territories based in the study of Pierre et al. (1992) in river lamprey and in our own observations on sea lamprey. For distinguishing some basal plate territories, it was useful to consider its location with respect to the fascicle of giant-large axons, the medial longitudinal fascicle (mlf), which can be easily assessed in sections by using Nomarski's differential interference contrast. From the level of the octaval nerve entrance to the spinal cord, this fascicle occupies a location close to the midline near the fourth ventricle, and cells of the middle and inferior reticular formation

are located just dorsal or lateral to it. In the trigeminal region the mlf becomes progressively displaced away the midline, and in the isthmus it becomes placed at an intermediate dorsoventral level separated from the ependymal layer by an intermediate layer of neurons. In the trigeminal region, but mainly in the isthmus, there appears a prominent neuronal population that lays ventromedially to the mlf. This population consists of small to medium-sized cell located ventromedial to the mlf, and correspond with the nucleus isthmi rhombencephali ventrales (those of the isthmus) and the nucleus rhombencephali ventrales anterior (those of the trigeminal region) of Pierre et al. (1992). The trigeminal population extends caudally till the group of giant Müller cells of the rostral octaval region. In the isthmus, the basal plate region dorsal and medial to the mlf corresponds to the nucleus isthmi rhombencephali dorsales of Pierre et al. (1992). Large reticular cells of the isthmus lay in this region just dorsal and/or dorsolateral to the mlf. In the lamprey isthmus, the most dorsal region of the basal plate lays near the trochlear nucleus, and this region has been referred to as nucleus isthmi by Pombal et al. (2001).

In the isthmus, an uneven band of 5HT-ir cells was observed extending from its most rostral part to rostral levels of the trigeminal motor nucleus. Neurons in this group were heterogeneous in size, with perikarya ranging from 6 to 30 μm in transverse diameter, and they occupied different dorso-ventral locations allowing to distinguish several subpopulations (Figs. 4A-E). At dorsal isthmic levels, close to the dorsal (cerebellar) isthmic commissure or even overlying the commissure, there is a subgroup consisting of a few small 5HT-ir neurons (8 to 13 μm), the dorsal isthmic 5HT-ir subgroup (Fig. 4C). Neurons of this subgroup were mostly located ventral to the trochlear nerve nucleus. The medial isthmic 5HT-ir subgroup was observed around the intermedioventral sulcus, and most of its cells were located in the outer part of the

periventricular cell mantle (Figs. 4A, B, D). This subgroup consisted of small (6 to 14 μm in diameter; 88% of the total 5HT-ir cells), medium sized (15 to 24 μm; 10% of the total) and large (25 to 30 μm; 2% of the total) perikarya. In the ventral periventricular cell mantle, small 5HT-ir neurons (6 to 10 μm) were abundant, forming a subgroup here referred to as the ventral isthmic 5HT-ir subgroup (Figs. 4A, D, E). In the ventrolateral trigeminal region caudal to the isthmus, a group of few small (6 to 10 μm) 5HT-ir cells was observed in the small celled mantle located ventromedial to the mlf, i.e. in the anterior ventral rhombencephalic nucleus (Fig. 4F). Occasional small 5HT-ir neurons were observed in the outer region of the prominent trigeminal motor nucleus, which mostly consists of big motoneurons with long ventrolateral dendrites. Together, these isthmic and trigeminal subgroups correspond to the brainstem group II described recently in the sea lamprey (Antri et al., 2006).

At the level of the vagal motor nucleus in the caudal rhombencephalon , the ventral periventricular cell mantle (medial inferior reticular region) showed some faint to moderate 5HT-ir cells of different size (15 to 44 μm in diameter) and shape (fusiform, triangular) that form the vagal 5HT-ir cell group (Figs. 5A, B). These cells are intermingled with larger reticular cells (Fig. 5A) and disappear at the transition to the spinal cord, recognizable by the exit of first spino-occipital nerves (see Pombal et al., 2001). This caudal rhombencephalic group corresponds to the brainstem group III described in the sea lamprey (Antri et al., 2006).

Rostral spinal cord

The 5HT-ir cells and fiber plexuses of the spinal cord of adult sea lamprey have been previously described by Zhang et al. (1996) and therefore they will not be considered in detail here, except for a brief general description allowing comparison with developmental stages. Numerous 5HT-ir small cells (10 to 14 μm in diameter)

were observed below the central canal of the spinal cord (Figs. 5C, D). These fusiform cells send processes towards a very dense plexus of strongly-stained medio-ventral fibers (Figs. 5C, D). Some dorsal dendrites of these cells contact the walls of the central canal.

Fibers

Beaded 5HT-ir fibers were widely distributed in the CNS of the adult sea lamprey (Fig. 2). 5HT-ir fibers arising from more caudal levels reached the telencephalon through two diffuse tracts, ventrolateral and mediodorsal, and were distributed throughout all the telencephalic lobes. The greatest density of 5HT-ir fibers in the telencephalon was observed in the periventricular region of the striatum (Fig. 6A), and in the lateral area of the dorsal pallium (Fig. 6B), between the medial and lateral pallia, whereas periventricular pallial regions and the nucleus of the terminal lamina showed very scarce 5HT-ir fibers. Some 5HT-ir fibers decussated in the dorsal telencephalic commissure (Fig. 6B). In the olfactory bulbs, fairly abundant beaded 5HT-ir fibers were observed in the inner granular layer, being scarce in the mitral cell layer and absent in olfactory glomeruli (Fig. 3A).

In the diencephalon, many beaded 5HT-ir fibers appeared to ascend from the isthmic 5HT-ir groups, with high fiber density in superficial regions of the pretectum and dorsal thalamus (Fig. 3F). Ascending axons from tuberal 5HT-ir cells join to these pathways, coursing together to the postoptic commissure and the telencephalon, where some fibers ascend dorsally, and some others course among cells around the postoptic recess. Numerous 5HT-ir axons coursed from the tuberal group to the ventrolateral hypothalamic area (Fig. 3C) and infundibulum, showing the highest fiber density of the diencephalon. In the thalamus, the lateral area contained abundant beaded 5HT-ir fibers,

but only a few of these fibers reached the habenula, running parallel to the fasciculus retroflexus (Fig. 3E). Beaded 5HT-ir fibers were also observed in the posterior commissure.

The lateral neuropil and fiber area ("white matter") of the mesencephalic tegmentum showed numerous 5HT-ir fibers, and a high density of these fibers was observed just dorsal to the interpeduncular nucleus. Fairly abundant 5HT-ir fibers were also observed in the subventricular neuropil, whereas the periventricular cell layers showed scarce fibers. In the optic tectum, 5HT-ir fibers were fairly abundant in most strata except in the outer half of the stratum fibrosum et griseum superficiale (stratum marginale of Kennedy and Rubinson, 1977), and numerous 5HT-ir fibers formed a well-defined layer in the inner part of the stratum fibrosum et griseum superficiale (stratum opticum; Heier, 1948) (Fig. 6C). Abundant 5HT-ir fibers crossed in the intertectal commissure.

In the rhombencephalon, 5HT-ir fibers ran in the basolateral area of most regions, and some of them decussated in the isthmic dorsal (cerebellar) and ventral commissures (Figs. 4C, E), and then coursed either rostrally to the optic tectum and torus semicircularis or caudally in the octavolateral area of the rhombencephalon. 5HT-ir innervation was scarce in the dorsal octavolateralis nucleus and in the caudal octaval nucleus, and among the perikarya of the trigeminal motor nucleus. Few commissural 5HT-ir fibers were also observed at the level of the caudal rhombencephalic 5HT-ir cell group. In the spinal cord, numerous fibers were scattered in the dorsal (Fig. 5D) and lateral funiculi, in addition to the aforementioned rich serotonergic plexus of the medioventral surface of the spinal cord (Figs. 5C, D). Numerous 5HT-ir fibers were found around motoneurons perikarya, but they are more scarce in other parts of the lateral horns.

Serotonin immunoreactivity during development

In late embryos and early prolarvae, the brain is a thickened neuroepithelial tube with scarce regional differentiation. The brain is bent ventrally at the level of the rostral end of the notochord, and its ventricular surface shows two characteristic sulci, the anterior and posterior intraencephalic sulci. These sulci represent the telencephalic/diencephalic and the midbrain/hindbrain boundaries, respectively. In late prolarvae and early larvae, most neuroanatomical regions become clearly distinguishable, but the evagination of the telencephalic hemispheres occurs in small larvae (15-30 mm) (Meléndez-Ferro et al., 2002b, 2003; Villar-Cheda et al., 2002, 2005, 2006). Some regions are late developing. In the medial pallium and the optic tectum the layered pattern becomes outlined in the largest larvae (de Miguel and Anadón, 1987; present results).

First 5HT-ir cells appear in the isthmus of late embryos, and most of the 5HT-ir cell groups reported in the adult brain appear during prolarval or early larval stages. Table 1 summarizes the time of appearance of the different serotonergic populations, which for more clear description were grouped in tuberal, mammillary, zona limitans intrathalamica, pretectal, pineal, parapineal, isthmic, trigeminal, caudal rhombencephalic, and spinal populations. All 5HT-ir cell groups observed in embryos and prolarvae were also present in larval stages, although some changes were observed. No neuronal groups transiently expressing serotonin during development were found in the sea lamprey.

Hypothalamus

In the hypothalamus, the earliest 5HT-ir cells appear in the tuberal region of late prolarvae (P12) and soon after dorsal and ventral subpopulations become distinguishable (Fig. 7A). The neurons of the dorsal subgroup were more numerous and more intensely stained than those of the ventral subgroup, exhibiting ventricular dendrites ending in small bulbs on the ventricular surface and lateral processes coursing laterally (Fig. 7A). The sparse, faintly 5HT-ir cells of the ventral subgroup were located in the lateral region of the neuronal mantle (Fig. 7A) and their processes were hardly visible. 5HT-ir cells increased in number in both subgroups, which became confluent in small larvae. However, differences in the location of 5HT-ir perikarya, periventricularly in the dorsal tuberal subgroup and more lateral in the ventral subgroup, were maintained. In the caudal hypothalamus, a few strongly stained CSF-c 5HT-ir cells appeared in the mammillary recess of larvae of about 30 mm in length. In these larvae, this mammillary subgroup was clearly separated from the 5HT-ir tuberal subgroups (Fig. 7B), but in later larval stages it grows considerably and becomes continuous with the tuberal populations (Fig. 7C). Acquisition of the adult pattern of the tuberal and mammillary 5HT-ir subpopulations occurs during metamorphosis; the dorsal tuberal subgroup became clearly stratified, with perikarya located in two-three lines parallel to the ependymal surface (Fig. 7D). This subgroup also extended rostrally, some cells reaching the supracommissural postoptic region. The 5HT-ir mammillary subpopulation increased markedly in cell number in transforming stages, covering the walls of the postinfundibular recess.

Zona limitans intrathalamica

First zona limitans intrathalamica 5HT-ir cells (Pombal and Puelles, 1999; Meléndez-Ferro et al., 2002b) appeared below the habenula in the boundary region

between prosomeres 2 and 3 of late prolarvae (P11), (Fig. 8A). These cells are small and show no clear processes. In transverse sections, they form a line of cells perpendicular to the ependyma (Figs. 8A, B), whereas in sagittal sections it appears as a short oblique band that follows the interprosomeric boundary (Fig. 8C). In larval stages, this population is maintained with little changes (Fig. 8B, C), the most obvious being the progressive increase in distance to the habenula owing to the asymmetrical growth of dorsal thalamic regions. In transforming stages these cells increase in size and lateral processes become conspicuous.

Pretectal region

No 5HT-ir cells were observed in the pretectum of prolarvae or early larval stages. Occasional 5HT-ir pretectal neurons were first observed in a 52 mm larva, and the number of cells increased considerably in late larval stages (80 -136 mm in length), which showed very faint 5HT-ir neurons mainly located in the periventricular cell layer (Fig. 8C). The pretectal 5HT-ir population changes during transformation stages, showing both cells that remained in the periventricular layer and cells migrated to more superficial pretectal regions and showing a stronger staining (Fig. 8D). The distribution of these cells in the pretectum at the end of the metamorphosis is similar to that observed in adults, though cell diameter is considerably smaller.

Pineal complex

In pineal organ, first 5HT-ir cells were observed in P9 prolarvae in the vesicle primordium. At prolarval stages, they were scarce but cell number increased progressively in larval stages (Figs. 8E, F). In larval stages, the pineal organ consists of a pineal vesicle and a pineal stalk. CSF-c 5HT-ir cells were numerous in the pineal vesicle although observation was obscured by the dense pigmentation of its proximal

wall. 5HT-ir cells appeared in the pineal stalk since early larvae, and were numerous in large larvae. Seemingly, these cells do not form nerve tracts entering the brain. In the parapineal vesicle, a few 5HT-ir CSF-c cells were first observed in a 62 mm larva. These cells were found in ventrocaudal and rostral regions of the parapineal vesicle (Fig. 8F).

Isthmus

A few 5HT-ir cells were observed for the first time in the isthmic tegmentum of the E11 embryos, being located at the outer margin of the thick ventricular zone (Fig. 9A). Characteristically, these cells are yolk-filled (Fig. 9A), as other brain cells. The 5HT-ir cells give rise to lateral processes extending in the neighbor thin marginal layer (Fig. 9A). In the prolarval pigmentation stage (P2-P3), this isthmic 5HT-ir group is slightly more numerous and has extended rostrocaudally, as seen in sagittal sections. All 5HT-ir cells lay close but caudal to the mid/hindbrain boundary, which is clearly appreciated in these sections. In these prolarvae, fibers arising from the isthmic 5HT-ir group coursed rostrally for a short distance but caudally some 5HT-ir fibers reached the rostral spinal cord. In a gill cleft prolarva (P7), the number of cells of the isthmic 5HT-ir population was estimated in about 30-40 cells on each side of the brain, and they were located in the mantle layer of the ventral region, either close to the ventricular layer or in more lateral position (Fig. 9B). The descending 5HT-ir axons of the isthmic group coursed in the rhombencephalic and spinal marginal layer without forming a defined tract, and progressively reached more caudal spinal levels. Some ascending axons from this cell group coursed rostrally to the ventral midbrain, and some coursed dorsally and crossed in the dorsal isthmic (cerebellar) commissure.

In burrowing prolarvae (P16), a distinction between isthmic 5HT-ir subpopulations located in intermediate and ventral levels becomes appreciable (Fig.

9C). The intermediate 5HT-ir subpopulation (in the primordium of the medial isthmic rhombencephalic nucleus) is more numerous, consists of larger cells (6-8 μm) and extends throughout the isthmic region, whereas the ventral subpopulation (in the primordium of the ventral isthmic rhombencephalic nucleus) has smaller cells (5-6 μm) and is only present at middle isthmic levels. At the beginning of the burrowing stage, before the appearance of hypothalamic 5HT-ir cells, a few 5HT-ir fibers reached the region of the postoptic commissure from the isthmic cell group. These fibers coursed rostrally in the ventral marginal zone of the mesencephalic tegmentum to the hypothalamus; a few 5HT-ir axons reached the neurohypophysis primordium, others crossed in the postoptic commissure and some 5HT-ir axons reached the telencephalon. Some days later, strongly 5HT-ir axons were observed in the striatum.

During early larval stages (from 10 mm to 30 mm in length), the intermediate and ventral isthmic 5HT-ir subpopulations maintain the cell size differences noted in late prolarvae and progressively become displaced to more dorsolateral or dorsal regions by the increase of cells (mostly 5HT-negative) in the ventral isthmic rhombencephalic nucleus. Since 60 mm larvae, faint 5HT-ir small cells were observed in the region of the dorsal isthmic nucleus (Fig. 9D), whereas in the rostral trigeminal region of large larvae only occasional 5HT-ir cells were observed at the level of most rostral part of the trigeminal motor nucleus. In large larvae, the 5HT-ir subpopulations of the dorsal and ventral isthmic rhombencephalic nuclei occupy rather dorsal levels, long away of the midline raphe and the ventral surface, being scattered among the negative cells of these nuclei (Figs. 9E, F). A few cells of the dorsal nucleus migrate away of the cell mantle, settling in the dorsolateral reticular area. In transforming stages, the three isthmic 5HT-ir subpopulations (dorsal, medial and ventral) were similar to those found in young adults.

Caudal rhombencephalon

In the caudal rhombencephalon a few 5HT-ir small cells were first observed in P16 prolarvae (Fig. 10A). These faintly 5HT-ir cells were located in the cell mantle near the ventral region of the fourth ventricle at the level of the vagal nerve, forming the primordium of the 5HT-ir caudal rhombencephalic group. The staining intensity and size of these 5HT-ir cells increased as development proceeds, but in general they appear more faint than the isthmic population. In late larval stages, these caudal 5HT-ir cells became pyramidal or spindle-shaped (Fig. 10B), being located above the medial longitudinal fascicle.

Spinal cord

A few, yolk-filled 5HT-ir cells were observed in the spinal cord of P1 prolarvae (Fig. 10C), some of them contacting the central canal (CSF-contacting type). The number of CSF-c 5HT-ir spinal neurons increased moderately in early prolarvae and, at the end of the gill cleft stage (P7), a medio-ventral fascicle of thick varicose fibers originated from these cells was observed for the first time in the spinal cord. In larvae, most spinal 5HT-ir cells become progressively displaced ventrally or laterally a short distance away from the central canal; in large larvae, most 5HT-ir cells lacked a CSF-c process, but some CSF-c cells were observed even in metamorphic individuals (M4, M5). In the spinal cord of larvae, numerous 5HT-ir fibers seemingly arising from the spinal neurons were observed close to the ventral surface below the fascicle of giant Müller axons (medial longitudinal fascicle), but rather abundant 5HT-ir fibers also coursed in the ventral, dorsal and lateral funiculi (Fig. 10D). The origin of fibers coursing longitudinally in these funiculi was not established, but a proportion probably comes from rhombencephalic 5HT-ir populations.

DISCUSSION

In this study, we analyzed the development of the serotonergic cell groups and fibers in the central nervous system of the sea lamprey from embryos to adults. The development of serotonergic populations in the sea lamprey is progressive and no evidence of neuronal populations transiently expressing serotonin was obtained. In the chick spinal cord, the number of 5HT-ir cells does not diminish after hatching (Wallace, 1985), indicating persistence of this phenotype. In the frog, the number of cells in the different reticular 5HT-ir populations also increases from early tadpoles to adults (Zhao and Debski, 2005). This is in contrast with the transient serotonin immunoreactivity reported during development in some brain populations of rat (Frankfurt et al., 1981; Wallace, 1985; Gaspar et al., 2003) and some teleosts (Ekström and van Veen, 1984; Ekström et al., 1985; Ekström and Ebbesson, 1989; Bolliet and Ali, 1992). In adult rat and in *Gasterosteus aculeatus*, some serotonergic populations could only be immunohistochemically detected after pharmacological treatment with inhibitors of the monoamine oxidase (Frankfurt et al., 1981; Ekström et al., 1985). In mammals, some diencephalic populations have a partial serotonergic phenotype consisting of expression of the high-affinity 5HT transporter and the vesicular monoamine transporter, allowing them to capture and store 5HT, but not to synthesize it (Gaspar et al., 2003). However, diencephalic nuclei (preoptic, posterior tubercular, caudal hypothalamic and pineal), anterior and posterior raphe nuclei, and spinal 5HT-ir populations of larval zebrafish express tryptophan hydroxylase genes (Bellipanni et al., 2002; Teraoka et al., 2004), strongly suggesting that all of them synthesize serotonin. If all lamprey 5HT-ir cells synthesize serotonin is not known, but it appears quite probable based on these recent results in zebrafish.

Serotonergic cell groups in adult lampreys

The results of this study indicate that the distribution of serotonergic cell groups in the brain of the adult sea lamprey (present results) is roughly similar to that reported in the adult river lamprey (*Lampetra fluviatilis*: Baumgarten, 1972; Steinbusch and Nieuwenhuys, 1979; Steinbusch et al., 1981; Brodin et al., 1988; Pierre et al., 1992), though the number of cell groups differs among these authors. The possible equivalences between the serotonergic groups found in the sea lamprey and those described by Pierre et al. (1992) are indicated in Table 2. The equivalence between the groups described in the river lamprey by the different authors has been thoroughly discussed in Pierre et al. (1992) and will not be dealt here. Assignation of the serotonergic groups of the river lamprey to brain regions (Pierre et al., 1992) was done following non-segmental classical studies (Heier, 1948; Schöber, 1964), while for the description of cell groups in the sea lamprey we followed a segmental developmental viewpoint (Pombal and Puelles, 1999; Meléndez-Ferro et al., 2002b, 2003). Accordingly, some nomenclatural differences between present results and those of previous studies of river lamprey result from the ascription of the same group to a different major brain region, whereas other differences appear to be genuine. Thus, occasional cells were observed in the olfactory bulbs and the preoptic area of big larvae and adults of the sea lamprey (present results), but not in the river lamprey (Baumgarten, 1972; Steinbusch and Nieuwenhuys, 1979; Steinbusch et al., 1981; Pierre et al., 1992). The presence of 5HT-ir cells in the olfactory bulbs is not exclusive of the sea lamprey, since a serotonergic population was reported in the olfactory bulb of chondrosteans (Adrio et al., 1999). In chondrosteans, there are also 5HT-ir cells in the medioventral wall of the telencephalic ventricle, which has not been observed in lampreys.

Our results indicate that the serotonergic populations originated in major developmental transverse domains demonstrated in the sea lamprey, such as the isthmic and the pretectal groups, do not spread to adjacent regions in adults (i.e. to the midbrain and thalamus, respectively). This is in contrast with the regional assignation of these populations reported in adult river lamprey (Pierre et al., 1992) and sea lamprey (Antri et al., 2006). Moreover, although during development the serotonergic tuberal and mammillary groups are clearly separated populations, as development proceeds they become confluent, suggesting that they form part of a major hypothalamic compartment with scarcely differentiated regions. In this region, GABA immunocytochemistry, cell proliferation and early gene expression studies have not demonstrated clearly separated domains (Meléndez-Ferro et al., 2002b; Osorio et al., 2005; Villar-Cheda et al., 2006), which is in line with present observations. In zebrafish, the different regions of the paraventricular organ and the posterior recess organ consisting of 5HT-ir CSF-c cells are continuous in adult (Kaslin and Panula, 2001), although they appear as three separated loci in larvae (Teraoka et al., 2004), as it occurs in the sea lamprey.

A recent immunohistochemical study of the sea lamprey brainstem has reported a group of serotonergic cells (group I) extending between the posterior commissure and the oculomotor nucleus (Antri et al., 2006), which these authors assign to the dorsal midbrain. The segmental analysis of developmental stages and adults of sea lamprey (present results) clearly indicates that this group is actually pretectal, not mesencephalic. These authors have also described a midbrain-rhombencephalic serotonergic group (group II) extending between the oculomotor nucleus in the mesencephalon and the level of the rostral one-third of the trigeminal motor nucleus. Our segmental analysis indicates that the group II of these authors ends rostrally just caudal to the mid-hindbrain boundary, i.e. it is only rhombencephalic. Our results reveal a more complex

organization of the isthmic group, with distinguishable subgroups that become progressively settled during the larval period. The rhombencephalic group described by Antri et al. (2006) in the caudal bulbar region (group III) closely corresponds to our caudal group.

Since the serotonergic cells of the pineal complex were the subject of detailed studies in adult and developing lampreys (see Tamotsu et al., 1990, 1997; Pombal et al., 1999; Yáñez et al., 1999; Meléndez-Ferro et al., 2002a), they will not be discussed here.

The earliest serotonergic neurons

This study reveals that the earliest serotonergic neurons of the lamprey brain originate ventrally in the isthmic basal plate, although as the development proceeds they extend dorsally, and then ventral, medial and dorsal subgroups become distinguishable. In both chick and mammals, early serotonergic cell groups are located in the rhombencephalic basal plate ventral to the cranial motoneuron pools (Wallace and Lauder, 1983; Wallace, 1985). Studies in chick indicate that the notochord and floor plate induce differentiation of serotonergic cells (Yamada et al., 1991). In lampreys, some isthmic serotonergic neurons appear to be located very dorsally, though ventral to the trochlear nucleus motoneurons (present results). These motoneurons arise from a quite dorsal region of the isthmus (Pombal et al., 1994), which has led to suggest that the basal/alar plate boundary in the lamprey isthmus is shifted very dorsally as regards this limit in jawed vertebrates. One of the reasons of this striking shift may be the absence of a true cerebellum. The dorsal extension of the basal plate at the isthmus in lampreys might explain the dorsal distribution of some isthmic serotonergic populations observed here.

The early appearance of isthmic serotonergic neurons in the development of the lamprey CNS is stressed by the undifferentiated aspect of the neural tube, and because in that moment serotonergic cells are still full of yolk. An early appearance of serotonergic cells during development was reported in teleosts (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994; Doldán, 1999; Bellipanni et al., 2002; Teraoka et al., 2004), chicken (Wallace, 1985; Wallace and Lauder, 1992), and mammals (Lidov and Molliver, 1982; Wallace and Lauder, 1983, 1992). The early development of isthmic serotonergic cells that project to wide brain regions observed in this study indicates that, like in mammals (Gaspar et al., 2003), serotonin may play a relevant role in the brain development of lamprey.

The development of serotonergic system in sea lamprey begins at about the same time than the GABAergic system (Meléndez-Ferro et al., 2002b, 2003), but both systems precede in time to the dopaminergic system (Abalo et al., 2005). These results are consistent with those reported in teleosts (Ekström et al., 1985, 1992; Ekström and Ohlin, 1995).

Development of serotonergic cell groups

From the embryonic period to larval metamorphosis, the various serotonergic cell groups appear at different times in the lamprey CNS. These results probably indicate differences in the mechanisms of serotonergic cell specification between different regions. In zebrafish, differences between diencephalic and rhombencephalic serotonergic cells include the expression of different tryptophan hydroxylase genes (Bellipanni et al., 2002; Teraoka et al., 2004). In mammals, the transcriptional network that specifies the serotonergic phenotype in raphe nuclei (Pfaar et al., 2002) is also

different from the network that specifies this phenotype in neural crest derivatives (Gershon, 1997).

As regards the growth of the larval populations, in all brain serotonergic populations there was a notable increase in cell size between the earliest stages in which a population was observed and the adult. An increase in size and number of 5HT-ir cells has been recently reported in three brainstem populations between large larvae (135-155 mm), and postmetamorphic and reproductive adults (Antri et al., 2006). These authors have also reported changes in morphology of 5HT-ir cells in adult as regards that found in larvae, generally monopolar or bipolar in larvae, which is similar to that observed here.

Diencephalic groups

In late prolarvae, three diencephalic serotonergic cell groups were observed: the hypothalamic tuberal region, the zona limitans intrathalamica, and the pineal organ. From larvae around 30 mm in length onwards, a 5HT-ir group was observed in the mammillary region, and in larger larvae, serotonergic cells appeared in the pretectum and the parapineal organ. Extra-hypothalamic serotonergic populations have been observed in the diencephalon of some teleosts during early development (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994; Doldán, 1999) and in adult zebrafish (Kaslin and Panula, 2001). In the chicken, all diencephalic serotonergic neurons were located in the hypothalamus (Wallace, 1985; Wallace and Lauder, 1992). The rat diencephalon does not present 5HT-ir cells (Lidov and Molliver, 1982; Wallace and Lauder, 1983, 1992), although cells that capture and store serotonin were transiently observed in the thalamus and hypothalamus of the mouse (Gaspar et al., 2003).

The tuberal serotonergic cell group is the first hypothalamic population to appear in the sea lamprey. It consists of CSF-c cells and probably corresponds with the

primordium of the lateral recess nucleus of teleost embryos (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994; Doldán, 1999), and the chicken paraventricular organ (Wallace, 1985), whose cells also are CSF-c. Its early development in lamprey is similar to that reported in fishes (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994; Doldán, 1999), but is in contrast to that pointed out in the chick paraventricular organ, which is late appearing (Wallace, 1985). This tuberal group becomes confluent with the mammillary serotonergic group only in adults. The mammillary serotonergic group of lamprey seems to correspond to the posterior recess nucleus of teleosts (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994). In both teleosts and lamprey, the appearance of the posterior recess/mammillary group is delayed with respect to that of the tuberal/lateral recess group (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994; present results). Two groups of non-CSF-c 5HT-ir cells were observed during early development in the hypothalamus of *Gasterosteus aculeatus* (Ekström et al., 1985) and *Scomber scombrus* (Bolliet et al., 1994), but there was no evidence of similar populations in lampreys, either adult or larval (present results).

The serotonergic population of the lamprey zona limitans intrathalamica does not seem to have an equivalent in gnathostomes. Only in *Scomber scombrus*, a few serotonergic cells that could be equivalent to the lamprey zona limitans intrathalamica have been described dorsal to the lateral recess nucleus (Bolliet et al., 1994). This early serotonergic population has been characterized in studies of the prosomeric organization of the adult and larval brain of lampreys (Pombal and Puelles, 1999; Meléndez-Ferro et al., 2002a).

The pretectal serotonergic group of the adult sea lamprey appears to correspond topologically to that of the pretectal area of teleosts (Ekström et al., 1985; Bolliet and

Ali, 1992; Bolliet et al., 1994; Kaslin and Panula, 2001). While in teleosts the pretectal 5HT-ir group arises during the embryonic period, the appearance of the pretectal 5HT-ir population of the lamprey is delayed until rather late larval periods (present results). In addition, 5HT-ir amacrine cells appear in the lamprey retina during metamorphosis (unpublished observations), whereas in teleosts occur after hatching (van Veen et al., 1984; Negishi and Wagner, 1995). These differences between teleosts and lamprey might be a result of the different developmental pattern of their visual systems. In adult lampreys, eyes are well developed and the dorsal thalamus, the pretectal region and the optic tectum receive most retinal projections (Kennedy and Rubinson, 1977; Vesselkin et al., 1980). However, these visual pathways are poorly differentiated during early-middle larval stages (de Miguel et al., 1990): the image-forming eye and layering of the retina and optic tectum are acquired during metamorphosis several years after the onset of larval stage (Rovainen, 1979; de Miguel and Anadón, 1987). Instead, the visual system of teleosts matures at early developmental stages (for references, see Candal et al., 2005). These observations suggest that the serotonergic pretectal populations of sea lamprey are directly involved in visual functions.

Rhombencephalic groups

From prolarval stages onwards, two widely separated serotonergic populations were observed in the rhombencephalic tegmentum of the sea lamprey, a rostral isthmo-trigeminal group and a caudal rhombencephalic group. The appearance of rostral and caudal serotonergic groups in the brainstem at early developmental stages has also been reported in teleosts (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994; Doldán, 1999; Teraoka et al., 2004), chicken (Wallace, 1985) and rat (Wallace and Lauder, 1983). The isthmo-trigeminal group of sea lamprey may roughly correspond to the anterior (superior) raphe group and its derivatives in teleosts (Ekström et al., 1985;

Bolliet and Ali, 1992; Bolliet et al., 1994; Doldán, 1999; Teraoka et al., 2004), *Xenopus* (van Mier et al., 1986), chicken (Wallace, 1985; Wallace and Lauder, 1992) and rat (Lidov and Molliver, 1982; Wallace and Lauder, 1983, 1992). The rhombencephalic caudal group of lampreys may roughly correspond to the posterior (inferior) raphe group and its derivatives in teleosts (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994; Doldán, 1999), *Xenopus* (van Mier et al., 1986), chicken (Wallace, 1985; Wallace and Lauder, 1992) and rat (Lidov and Molliver, 1982; Wallace and Lauder, 1983, 1992). In the sea lamprey, the isthmo-trigeminal group appears earlier than the caudal group, as in other vertebrates (Lidov and Molliver, 1982; Wallace and Lauder, 1983; Ekström et al., 1985; Wallace, 1985; van Mier et al., 1986; Bolliet and Ali, 1992; Wallace and Lauder, 1992; Bolliet et al., 1994; Doldán, 1999), suggesting a conserved developmental pattern.

Interestingly, in the sea lamprey rhombencephalic serotonergic cells do not migrate toward the ventral midline raphe during development (present results) or locate in that position in adults (Baumgarten, 1972; Steinbusch and Nieuwenhuys, 1979; Brodin et al., 1988, Pierre et al., 1992; present results). Instead, some serotonergic cells appear distributed in rather dorsal positions close to the fourth ventricle roof, and lateral migration is only observed in an intermediate isthmic subpopulation. The behavior during development of serotonergic populations in the lamprey hindbrain is in marked contrast with that reported in embryos of gnathostomes. In embryos of *Gasterosteus aculeatus* (Ekström et al., 1985), chicken (Wallace, 1985) and rat (Lidov and Molliver, 1982; Wallace and Lauder, 1983), the serotonergic cells arise in the ventricular zone adjacent to the floor plate, and many of them migrate ventrally forming vertical cell bands located close to or in the ventral midline raphe, the raphe nuclei. In the rat, some cells migrate from here ventrolaterally (for instance those of the B9 group), something

which is not observed in lamprey, either during development or in adults (present results). Since the raphe nuclei are present in adults of jawed fishes (*elasmobranchs*: Stuesse et al., 1991, 1995; Stuesse and Cruce, 1992; *chondrosteans*: Adrio et al., 1999; *teleosts*: Ekström, 1994), this indicates either that rhombencephalic serotonergic populations use different migration clues in lampreys and gnathostomes, or that key molecules directing migration have a different expression pattern. Our results suggest that the migration pattern of raphe nuclei cells observed in jawed vertebrates was established after divergence from lines leading to lampreys.

Spinal cord

The spinal population of serotonergic cells located ventrally to the central canal is know from previous studies of adult lamprey (Steinbusch and Nieuwenhuys, 1979; Steinbusch et al., 1981; Pierre et al., 1992; Zhang et al., 1996). Here we report its early appearance in the sea lamprey prolarvae. The presence of 5HT-ir spinal cells, and its early appearance, has also been reported in teleosts (Ekström et al., 1985; Bolliet and Ali, 1992; Bolliet et al., 1994; Bellipanni et al., 2002; Teraoka et al., 2004), chicken (Wallace, 1985) and rat (Wallace and Lauder, 1983), indicating that these cells have been conserved during evolution. The CSF-contacting morphology of some spinal serotonergic cells is shared by the sea lamprey, chondrosteans (Adrio et al., 1999), elasmobranchs (Stuesse et al., 1991), and embryonic chick (Wallace and Lauder, 1992). Present results also show that this early serotonergic population gives rise rather early in larvae to a rich ventromedial serotonergic plexus, as reported in adults (Zhang et al., 1996). In adult lampreys, experimental studies indicate that this intraspinal population exerts a modulatory and a stabilizing role in the spinal locomotor network (Zhang et al., 1996; Zhang and Grillner, 2000), which probably applies to developing stages.

Development of serotonergic projections

The adult sea lamprey brain is richly innervated by serotonergic fibers (present results). The distribution of these fibers in sea lamprey is similar to that observed in adult *Lampetra fluviatilis* and *Ichthyomyzon unicuspis* (Pierre et al., 1992; Viana di Prisco et al., 1994). Our results in lamprey reveal that development of 5HT-ir innervation follows closely the appearance of first serotonergic population. The first serotonergic fibers appear in the primordial marginal zone adjacent to the first isthmic serotonergic neurons in late embryos/early prolarvae, and soon after ascending and descending serotonergic fibers were observed growing in the primordial marginal area. At these developmental stages, the developing brain and spinal cord show a thick proliferating ventricular zone and some postmitotic cells located in a primordial mantle zone (Meléndez-Ferro et al., 2002b, 2003; Villar-Cheda et al., 2005, 2006). In early stages, serotonergic axons from the isthmic group also coursed dorsally to the optic tectum and octavolateral area. In developing mammals, serotonin is released by growing axons before conventional synapses are established, and modulate events such as cell division, neuronal migration and cell differentiation (see Gaspar et al., 2003). The first lamprey serotonergic fibers might accomplish similar functions during development.

The main longitudinal ascending and descending serotonergic pathways are formed early in lamprey development, and then some axons or axon collaterals leave the main pathways to reach different targets and branch, as reported in rat embryos (Lidov and Molliver, 1982). The main serotonergic ascending pathway of prolarvae arises from the isthmic group. When diencephalic groups appear, axons from these groups intermingle with those from the isthmic group, making it further impossible to distinguish between axons of hypothalamic or isthmic origin. The mix of axons

originated from the rhombencephalon and diencephalon in ascending serotonergic pathways was also reported in teleosts (Ekström et al., 1985).

Serotonergic fibers reach the prolarval telencephalon at the beginning of the pigmentation stage, when the telencephalon is still very poorly differentiated (Meléndez-Ferro et al., 2002b; Villar-Cheda et al., 2006). An early serotonergic innervation of the telencephalon also occurs in mammal embryos (Lidov and Molliver, 1982; Wallace and Lauder, 1983; Botchkina and Morin, 1993). The arrival of first serotonergic fibers to striatum (burrowing stage) coincides in time with the appearance of the striatal GABAergic population (Mélendez-Ferro et al., 2002b), and precedes that of incoming dopaminergic fibers (Abalo et al., 2005). The early arrival of serotonergic fibers to the lamprey striatum, just before dopaminergic fibers, is compatible with regulation by serotonin of the ingrowth and terminal development of these fibers, as it was suggested in the rat cortex (Benes et al., 2000). From middle-sized larvae, when most telencephalic regions are easily recognizable (Villar-Cheda et al., 2006), serotonergic fibers are especially abundant in the pallial marginal region. A notable exception is the medial cortex, which in adults is very richly innervated by 5HT-ir fibers but that lacks serotonergic innervation until late larval stages. Proliferation studies in sea lamprey indicate that this is a late-developing pallial region (Villar-Cheda et al., 2006), which may explain the late maturation of its serotonergic innervation. In mammals, serotonin appears to be involved in regulation of the development of the cortex (Vitalis and Parnavelas, 2003; Janusonis et al., 2004), but possible developmental roles of serotonin in the larval lamprey are speculative.

From prolarval stages onwards, serotonergic fibers reach the infrachiasmatic part of the nucleus of the postoptic commissure, which in lampreys has been suggested to be the homologous of the suprachiasmatic nucleus of other vertebrates (Weigle et al.,

1996). In mammals, the circadian pacemaker, the suprachiasmatic nucleus, receives strong serotonergic innervation that modulates its rhythmicity, and its innervation occurs postnatally (Botchkina and Morin, 1993).

The serotonergic isthmic group of lampreys projects rostrally, dorsally and caudally. Although in the adult rat the superior raphe nuclei project rostrally and the posterior raphe nuclei project caudally (Wallace and Lauder, 1983), most of the first serotonergic cells appear to be monopolar and initially the same cells show both descending and ascending processes (Wallace and Lauder, 1983). These data suggest that the presence of ascending and descending projections from the isthmic group (superior raphe) represent a primitive condition of vertebrates. As in the rat (Wallace and Lauder, 1983), rhombencephalic serotonergic nuclei of lamprey also send contralateral processes crossing the midline beneath the floor plate. In the developing rat, growth of the contralateral 5HT-ir processes precedes migration movements leading to fusion of 5HT-ir cell groups in the midline, but this does not occur in lamprey.

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TABLES

Table 1

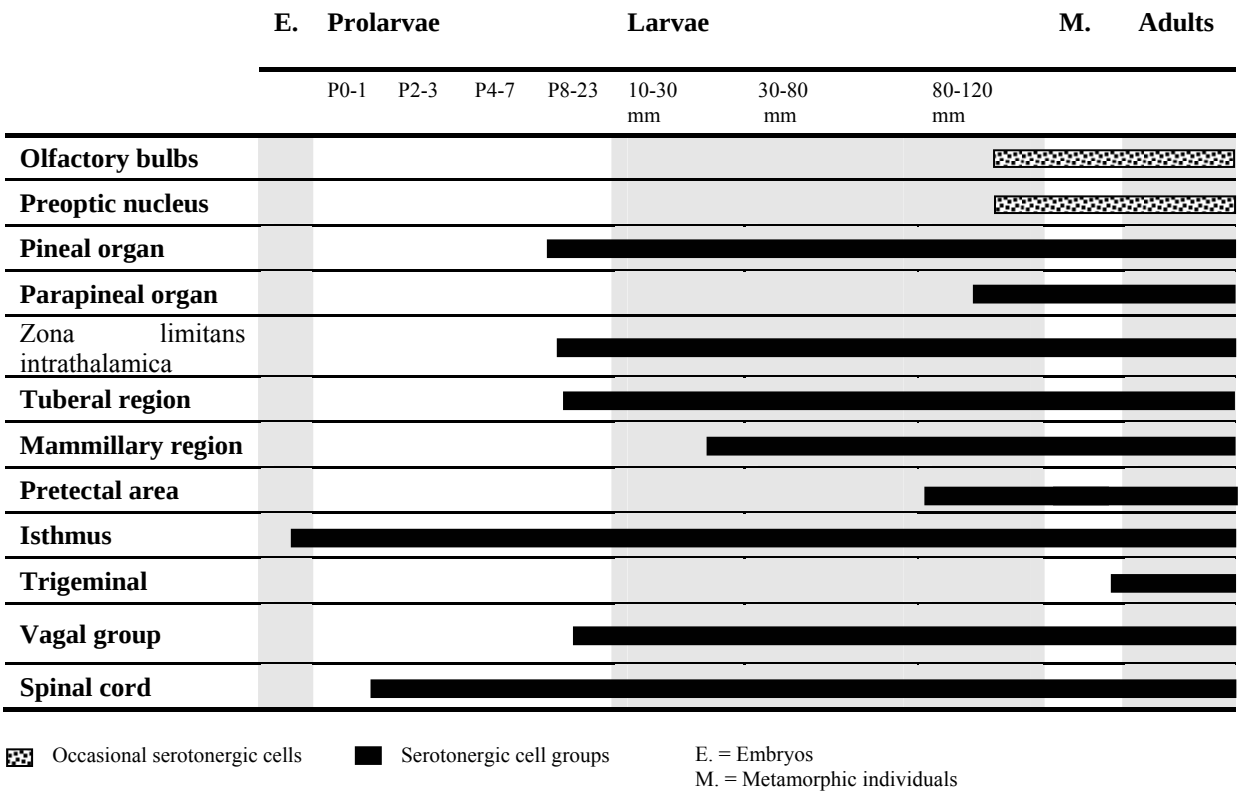


Table 2

Petromyzon marinus	*Lampetra fluviatilis* **(Pierre et al., 1992)**
Pineal complex	
Zona limitans intrathalamica	**NDTh (6)**
Tuberal region	**NDH (1), NVH (4), NVT (5)**
Mammillary region	**NCPid (2), NCPiv (3)**
Pretectal area group	**NDTc (7), NCPm (8), CGLpe (9), NCPl (10)**
-------	**SFCP (11), NTM (12), RMA (13)**
Medial isthmic subgroup	**NIRva (14), vm (15), vp (16); NIRdm (17), dpi (18)**
Ventral isthmic subgroup	**-------**
Dorsal isthmic subgroup	**NIRdps (19), dorsal isthmic comm. (20)**
Trigeminal group	**-------**
-------	**Lateral to Vº motor nucleus (23)**
Vagal group	**NRva (21), NRvp (22)**
Spinal cord	

FIGURE LEGENDS

Table 1: Timetable of the appearance of each serotonergic cell group in the central nervous system of sea lamprey.

Table 2: Equivalences between serotonergic cell groups in the CNS of the sea and river lampreys. Abbreviations for *L. fluviatilis*: CGLpe; external part of the *corpus geniculatus laterale*. NCPid, NCPiv; dorsal and ventral parts of the postinfundibular commissure nucleus. NCPl, NCPm; lateral and medial parts of the posterior commissure nucleus. NDH; hypothalamic dorsal nucleus. NDTc, NDTh; caudal and subhabenular parts of the dorsal thalamic nucleus. NIRdm, dpi, dps, va, vm, vp, dorso-medial, inferior and superior parts of dorsal posterior, ventro -anterior, -medial and -posterior parts of the rhombencephalic isthmus nuclei. NRva, vp; ventral anterior and posterior parts of the rhombencephalic nucleus. NVH, NVT; hypothalamic and thalamic ventral nuclei. NTM; mesencephalic tegmental nucleus. RMA; reticular mesencephalic area. SFCP; periventricular and cellular fibrosum strata. The numbers between brackets indicate those of serotonergic cell groups in Pierre et al. (1992).

Fig. 1. Sagittal drawings of adult, larval, and prolarval brains of sea lamprey showing the serotonergic cell groups, and some putative limits into the brain. Vertical bars in adult, prolarva and prolarva indicate the approximate levels of the transversal sections drawn in figure 2. For abbreviations, see list. Scale bars = 250 μm (prolarva), 500 μm (larva), 1 mm (adult).

Fig. 2. Schematic drawings of transverse sections through the brain and rostral spinal cord of adult, larval and prolarval brain of sea lamprey, showing at the right the

distribution of serotonin-immunoreactive perikarya and fibers. For abbreviations, see list. Scale bars = 0.1 mm (prolarva), 0.5 mm (larva and adult).

Fig. 3. Photomicrographs of sections of the prosencephalon of adult sea lamprey showing serotonergic cells and fibers. **A**: Transverse section of the prosencephalon showing two 5HT-ir cells and numerous fibers in the olfactory bulbs (arrows). Note the absence of these fibers in olfactory glomeruli. **B**: Transverse section of the preoptic area showing a 5HT-ir cell (arrow). **C, D**: Transverse sections of the hypothalamus showing 5HT-ir cells (black arrows) of the dorsal tuberal subgroup, the fibers of this group that project laterally (asterisks), and the projections to the ventricle (white arrows). **E**: Transverse section of the mammillary region showing 5HT-ir perikarya (black arrows) and their ventricular dendrites (white arrow). **F**: Sagittal section of the prosencephalon showing 5HT-ir cells in the zona limitans intrathalamica (arrowhead). **G**: Transverse section of the pretectum showing 5HT-ir cells (arrow) and 5HT-ir fibers running to the posterior commissure (white asterisk). Left side points the rostral part. **H**: Transverse section of the pineal complex showing 5HT-ir cells in the pineal (black arrow) and parapineal (white arrow) organs. For abbreviations, see list. Scale bars = 50 μm (A, B, C, E, G, H), 25 μm (D), 100 μm (F).

Fig. 4. Photomicrographs of sections of the rostral rhombencephalon of an adult sea lamprey showing serotonergic subgroups and fibers. Black arrows show medial subgroup; arrowheads, ventral subgroup; white arrows, dorsal subgroup; and big-headed arrow, trigeminal subgroup. In sagittal sections, left side points the rostral part. **A**: Sagittal section of the isthmus region showing the location of the mid-hindbrain boundary (dotted line), the mesencephalic Müller 3 cell, and the three 5HT-ir isthmic

subgroups. **B**: Transverse section showing a detail of 5HT-ir neurons of the medial isthmic subgroup. **C**: Sagittal section showing a detail of 5HT-ir neurons of the dorsal isthmic subgroup and 5HT-ir fibers coursing to the dorsal isthmic commissure. The curved arrow shows the immunonegative big reticular cells. **D**: Transverse section through the isthmus showing perikarya of the three 5HT-ir subgroups. The curved arrow shows the immunonegative big reticular cells. **E**: Transverse section of the caudal isthmus showing 5HT-ir cells of the ventral subgroup and 5HT-ir fibers of the ventral isthmic commissure. **F**: Transverse section of the trigeminal region showing neurons of the 5HT-ir trigeminal group. For abbreviations, see list. Scale bars = 50 μm (A, D, E, F), 25 μm (B, C).

Fig. 5. Photomicrographs of sections of the caudal rhombencephalon (A-B) and spinal cord (C-D) of adult sea lamprey showing 5HT-ir cells and fibers. In sagittal sections, left side points the rostral part. **A**: Sagittal section showing 5HT-ir cells of vagal group (white arrows). Note the orientation of dendrites in the transverse plane. Arrowheads point to 5HT-negative large reticular rhombencephalic neurons. **B**: Transverse section showing the bipolar or multipolar appearance of 5HT-ir cells. **C**: Sagittal sections of the rostral spinal cord showing the longitudinal row of 5HT-ir cells (arrow) and the strongly stained ventral 5HT-ir plexus (asterisk). **D**: Transverse section showing 5HT-ir ventral to the central canal. The black stars indicate the giant Müller axons. For abbreviations, see list. Scale bars = 50 μm (A, C, D), 100 μm (B).

Fig. 6. Photomicrographs of sections of adult lamprey brain showing fields of 5HT-ir fibers. **A**: Transverse section of the telencephalon showing the rich 5HT-ir innervation of the striatum (asterisk). **B**: Transverse section of the telencephalon showing the rich

plexus of 5HT-ir fibers of the dorsal pallium (black asterisk), and an immunoreactive fiber croursing in the dorsal telencephalic commissure (white asterisk). **C**: Transverse section of the optic tectum showing the rich plexus of 5HT-ir fibers of the stratum opticum (black arrow) and the absence of positive fibers in the outer part of the stratum fibrosum et griseum superficiale (white arrow). Note accumulation of 5HT-ir fibers in the medial margin of the optic tectum, near the choroid tela covering the midbrain ventricle. For abbreviations, see list. Scale bars = 50 μm.

Fig. 7. Photomicrographs of sections of the hypothalamus of developing sea lamprey showing 5HT-ir cells and fibers. **A**: Transverse section of a P16 prolarva showing the dorsal (white arrow) and ventral (black arrow) tuberal subgroups. Asterisks indicate the lateral field with axons of the dorsal tuberal subgroup. **B**: Transverse section of a 62 mm larva showing the 5HT-ir cells of the mammillary group (black arrows). **C**: Sagittal section of a 115 mm larva showing the continuity of the dorsal tuberal (white arrow) and the mammillary (big-headed arrow) 5HT-ir cell groups. The thick black arrow points to the ventral tuberal subgroup. Arrowhead, zona limitans intrathalamica. Left side points the rostral part. **D**: Transverse section of a metamorphic lamprey (M7) showing the stratification of the 5HT-ir perikarya (white arrow) in the dorsal tuberal subgroup, as well as their ventricular and lateral processes (asterisk). For abbreviations, see list. Figures C-D are inverted prints of fluorescence micrographs. Scale bars = 25 μm (A, D), 50 μm (B), 100 μm (C).

Fig. 8. Photomicrographs of sections of the diencephalon of developing sea lamprey showing 5HT-ir cells and fibers. **A**: Transverse section of a P16 prolarva showing 5HT-ir cells in the zona *limitans intrathalamica* (arrowheads) and the dorsal tuberal subgroup

(black arrow). Note 5HT-ir fibers of the tuberal neurons in the lateral area (asterisk). **B**: Transverse section of a 62 mm larva showing 5HT-ir cells in the zona limitans intrathalamica (arrowheads). **C**: Sagittal section of a 115 mm larva showing 5HT-ir cells in the zona limitans intrathalamica (arrowheads) and very faint 5HT-ir cells in the pretectal area (white arrow). Note the immunonegativity of the fasciculus retroflexus. Asterisk, field of lateral processes of tuberal 5HT-ir neuons. Left side points the rostral part. **D**: Transverse section of a metamorphic lamprey (M7) showing 5HT-ir cells in the medial (white arrow) and lateral (thin arrow) pretectal subgroups. Asterisk, 5HT-ir fibers coursing to the posterior commissure. **E**: Transverse section of a 53 mm larva showing 5HT-ir cells in the pineal organ (white arrows). Note the absence of 5HT-ir structures in the parapineal organ. **F**: Transverse section of a 115 mm larva showing 5HT-ir cells in the pineal (white arrow) and parapineal (black arrow) organs. For abbreviations, see list. Figures C, E and F are inverted prints of fluorescence micrographs. Background observed in the pineal organ is due to autofluorescence of the pineal pigment cells. Scale bars = 50 μm (A, B, D, E, F), 100 μm (C).

Fig. 9. Photomicrographs of sections of the isthmus region of developing sea lamprey showing 5HT-ir cells and fibers. **A**: Horizontal section of a E11 embryo showing the first 5HT-ir cells in the isthmus (black arrows). Note the neuroepithelial appearance of the brain. Asterisk, fibers in the very thin marginal zone. **B**: Transverse section a P7 prolarva showing 5HT-ir cells in the isthmus (black arrows), and numerous 5HT-ir processes coursing in the marginal zone (asterisk). **C:** Transverse section of the isthmus of a P16 prolarva showing cells of the intermediate (black arrow) and ventral (white arrow) 5HT-ir subgroups. **D-E**: Transverse sections through the isthmus of 84 mm (D), and 62 mm (E) larvae showing cells of the dorsal (arrowhead), medial (black arrows),

and ventral (white arrow) 5HT-ir subgroups. The asterisk in D indicates the dorsal isthmic commissure. **F**: Sagittal section of the rostral rhombencephalon of a 115 mm larva showing the location of 5HT-ir neuronal populations caudal to mid-hindbrain boundary (dotted line). Note the different cell size of cells in the medial (black arrows) and ventral (white arrows) isthmic subgroups. Left side points the rostral part. For abbreviations, see list. Figures D and F are inverted prints of fluorescence micrographs. Scale bars = 25 μm (A), 50 μm (B, C, D, E), 100 μm (F).

Fig. 10. Photomicrographs of sections of the caudal rhombencephalon (A-B) and spinal cord (C-D) of developing sea lamprey showing 5HT-ir cells and fibers. **A, B**: Transverse sections of a P16 prolarva (A) and a 115 mm larva (B) showing 5HT-ir cells (white arrows) in the vagal group, and fibers running in the lateral region (white asterisks). **C:** Transverse section of a P3 prolarva showing 5HT-ir cells (arrows) close to the ventral midline. Note the neuroepithelial appearance of the spinal cord. **D**: Transverse section of a 90 mm larva showing 5HT-ir cells close to the central canal (arrow) and cells migrated lateral and ventral to it. Note the ventral 5HT-ir plexus close to the meninges (asterisk). Figure B is an inverted print of a fluorescence micrograph. For abbreviations, see list. Scale bars = 50 μm (A, B), 15 μm (C), 25 μm (D).

ABBREVIATIONS

CC: central canal

Ch: optic chiasm

DF: dorsal funiculus

DIC: dorsal isthmic commissure

DIS: dorsal isthmic subgroup

DP: Dorsal pallium

DTS: dorsal tuberal subgroup

DTh: dorsal thalamus

DV: diencephalic ventricle

Fr: fasciculus retroflexus

Gl: olfactory glomeruli

H: hypothalamus

Hb: habenula

Hyp: hypophysis

IG: isthmic group

IN: interpeduncular nucleus

IR: infundibular recess

LF: lateral funiculus

LP: lateral pallium

M: mesencephalon

M1-3: Müller cells

MI: isthmic Müller cell

MIS: medial isthmic subgroup

Mm: mammillary region

MP: medial pallium

MT: mesencephalic tegmentum

MV: mesencephalic ventricle

OB: olfactory bulbs

ON: optic nerve

OT: optic tectum

P: pineal organ

PC: posterior commissure

PO: preoptic area

PoC: postoptic commissure

Pp: parapineal organ

PR: posterior recess

Pt: pretectum

PTl: primordium telencephali

RV: rhombencephalic ventricle

SC: spinal Cord

ScO: subcommissural organ

SFGS: stratum fibrosum et griseum superficiale

SOP: stratum opticum

Str: striatum

T: tuberal region

TL: telencephalic lobe

T-Mm: tubero-mammillary region

TrG: caudal isthmic subgroup

TrN: trigeminal motor nuclei

TS: torus semicircularis

TV: telencephalic ventricle

VF: ventral funiculus

VG: vagal group

VIC: ventral isthmic commissure

VIS: ventral isthmic subgroup

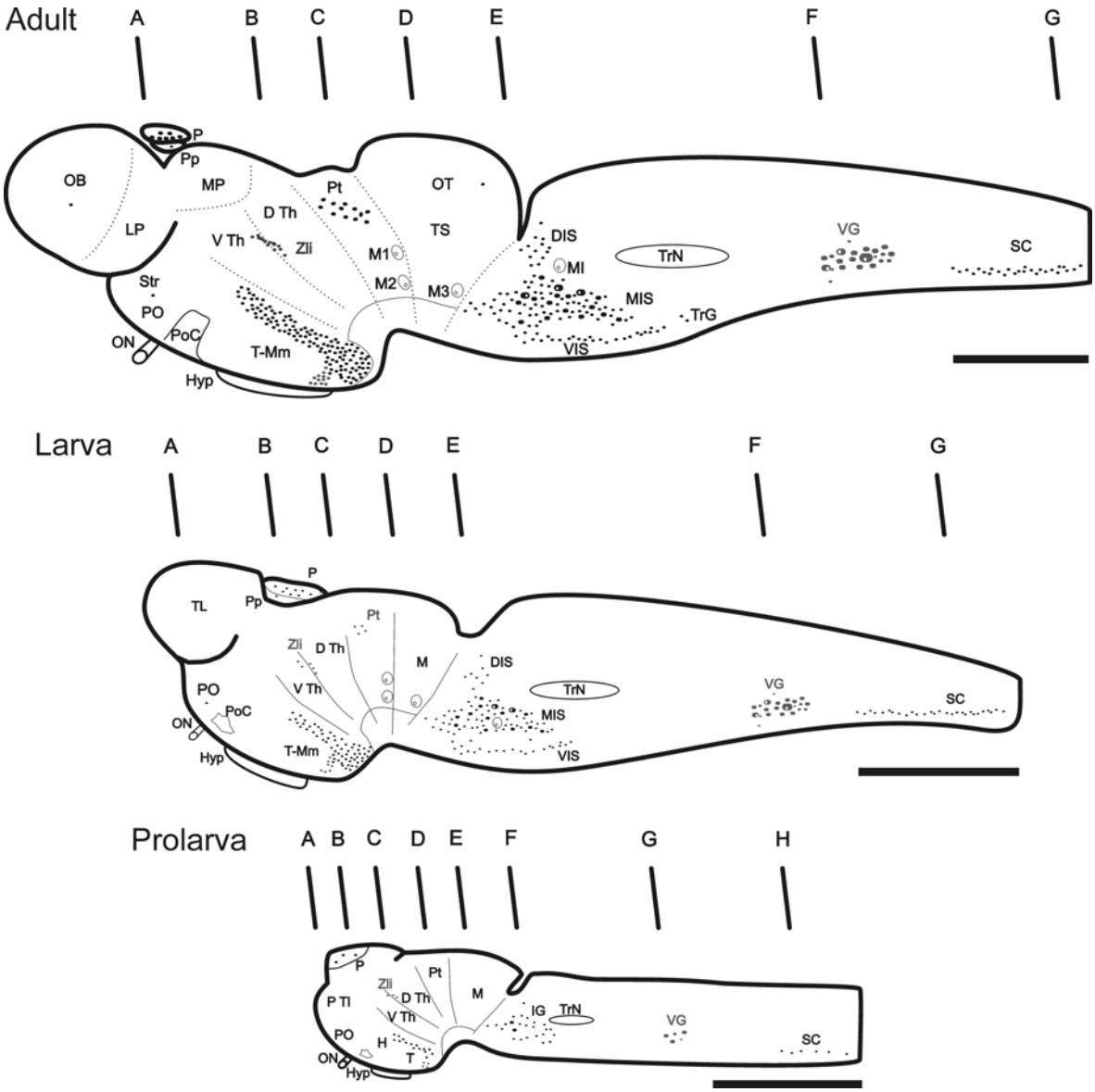
VTS: ventral tuberal subgroup

VTh: ventral thalamus

Zli: zona limitans intrathalamica

FIGURES

Figure 1



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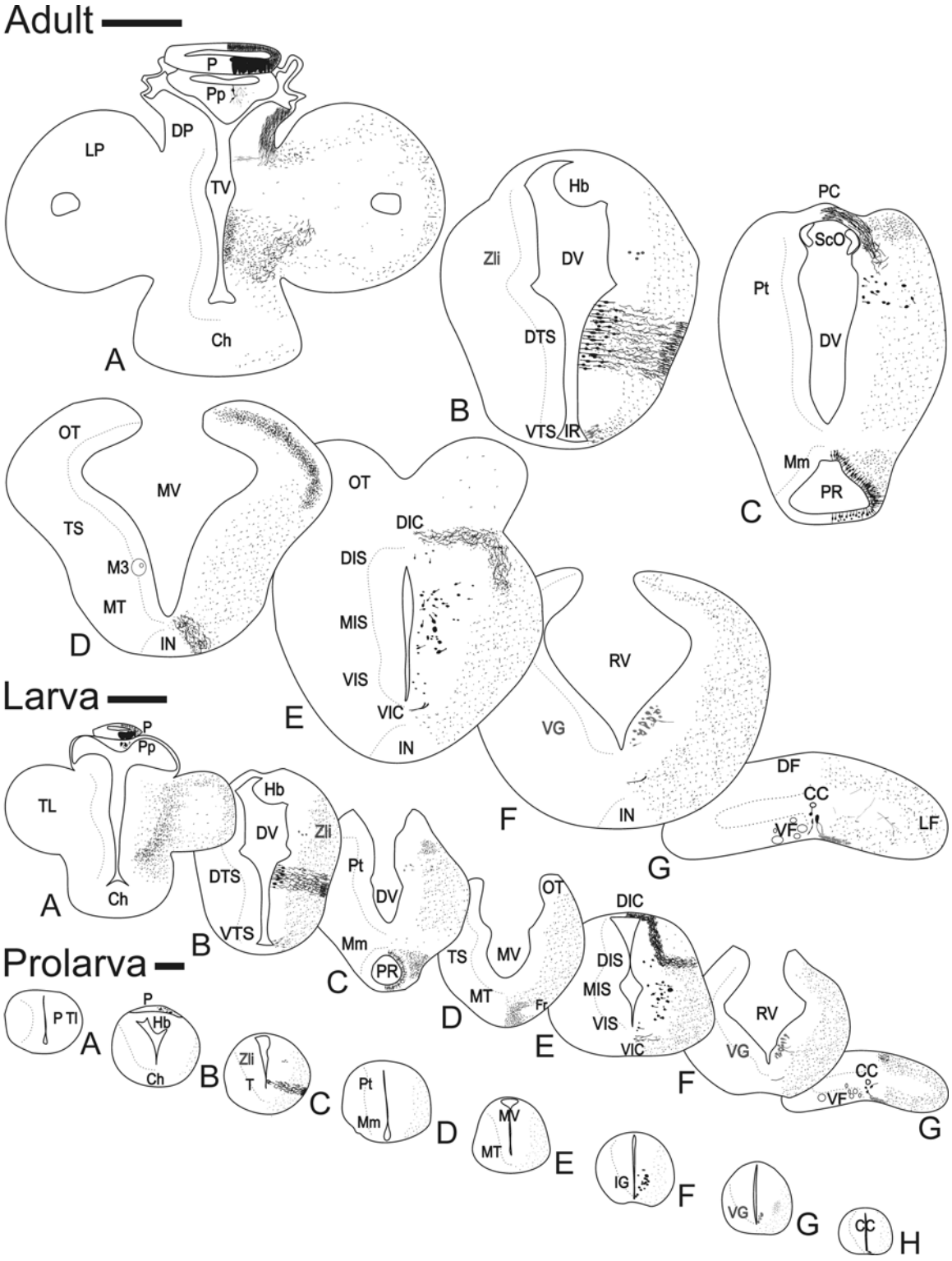
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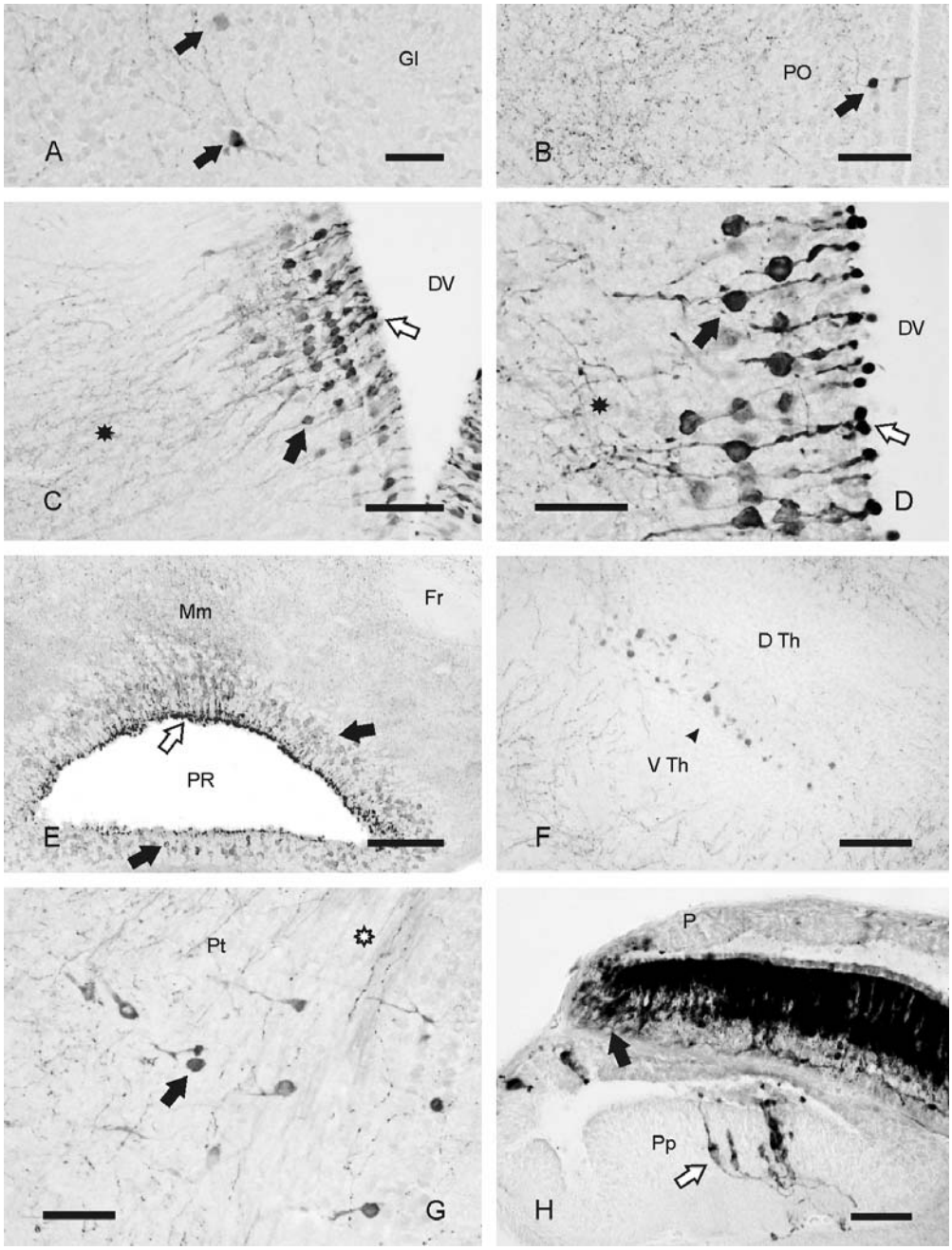
Figure 3



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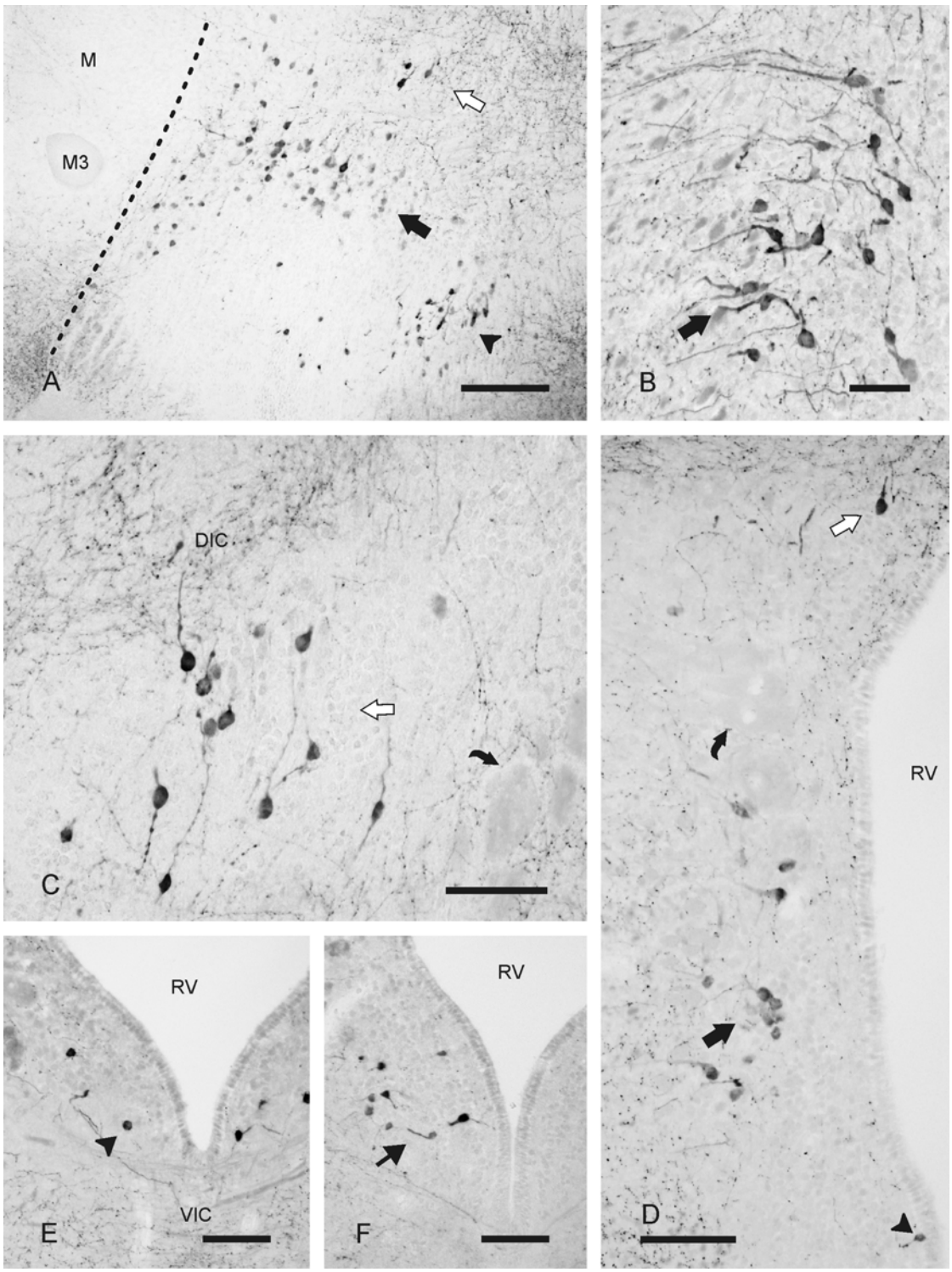

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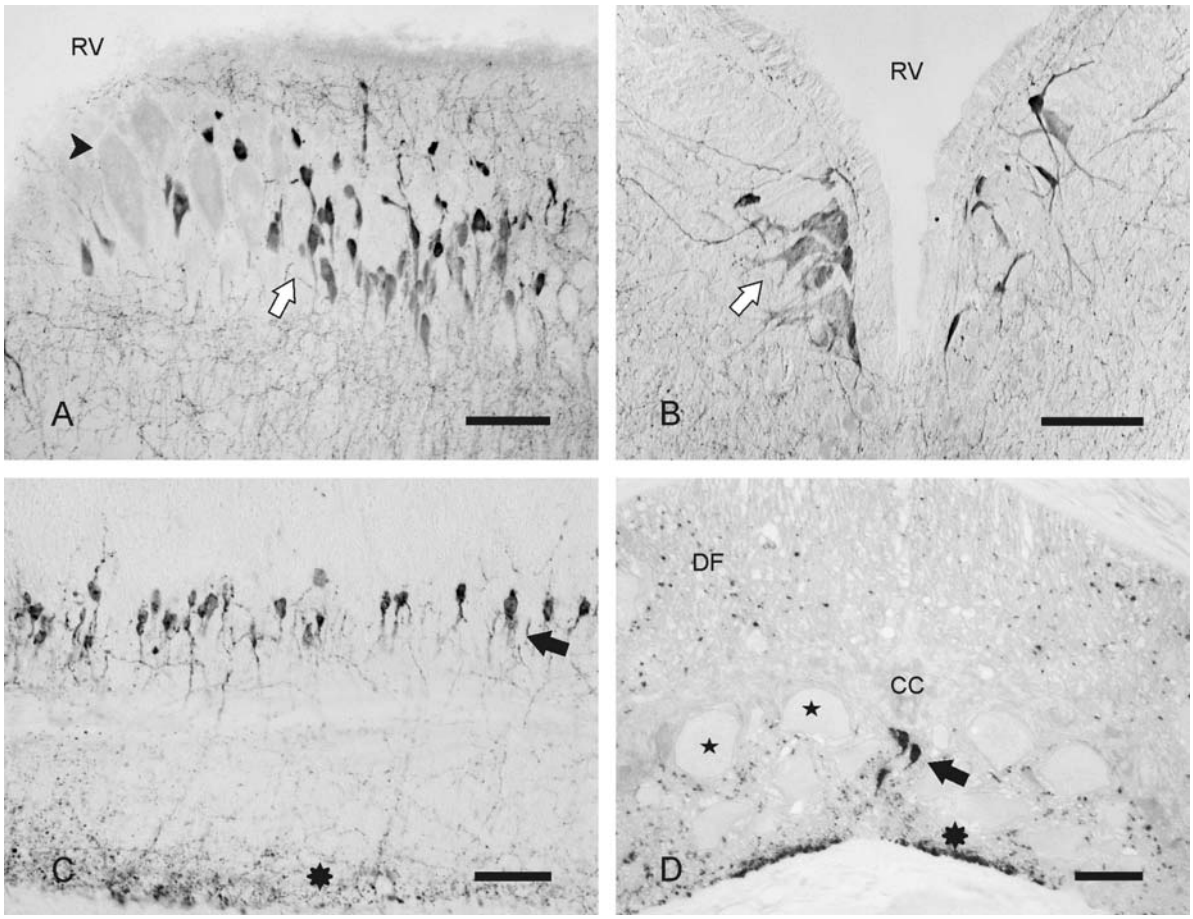

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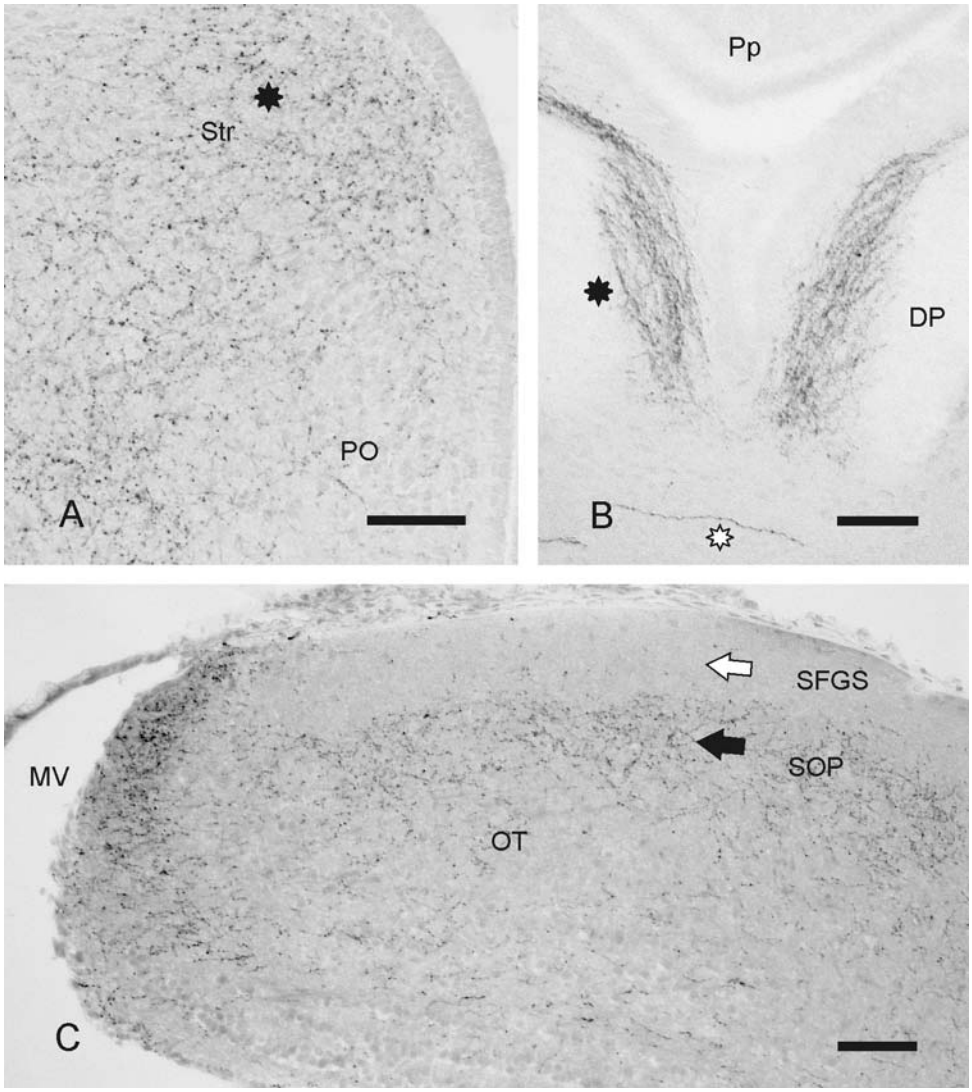

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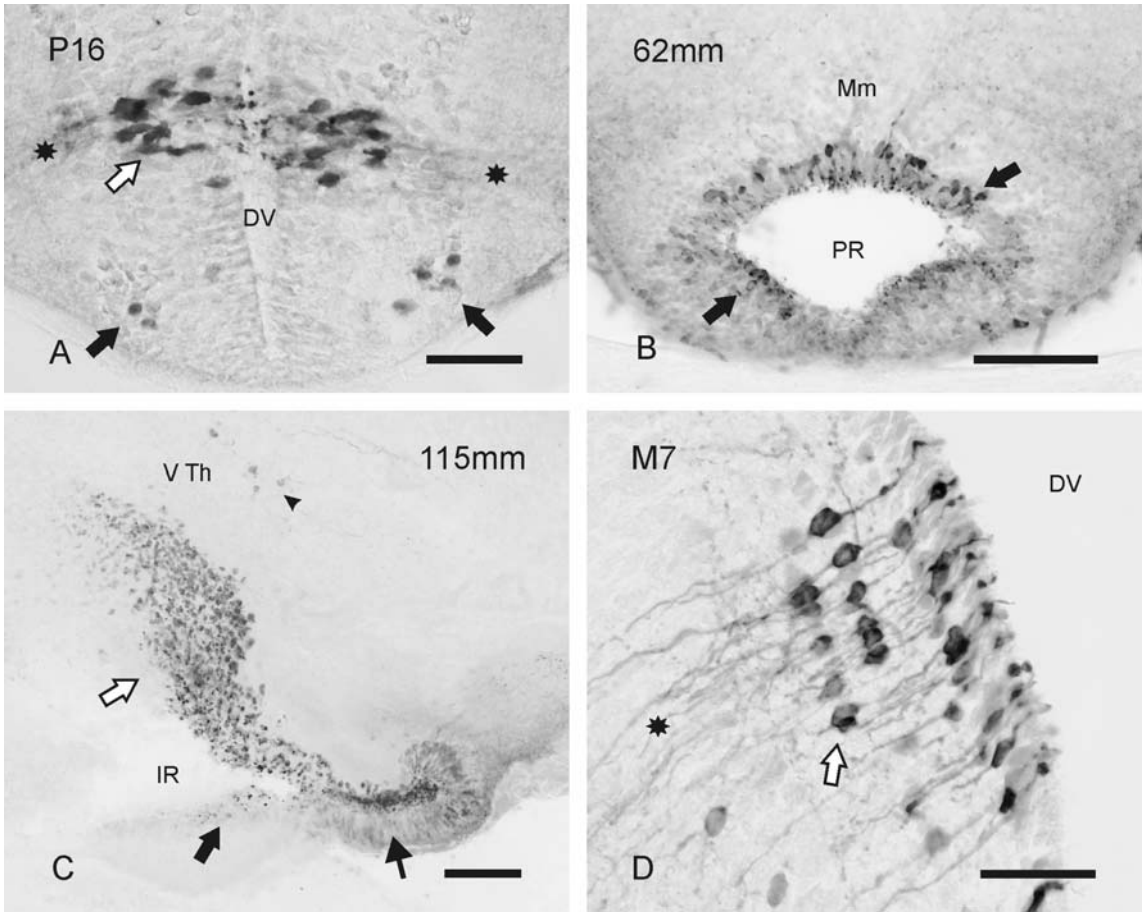

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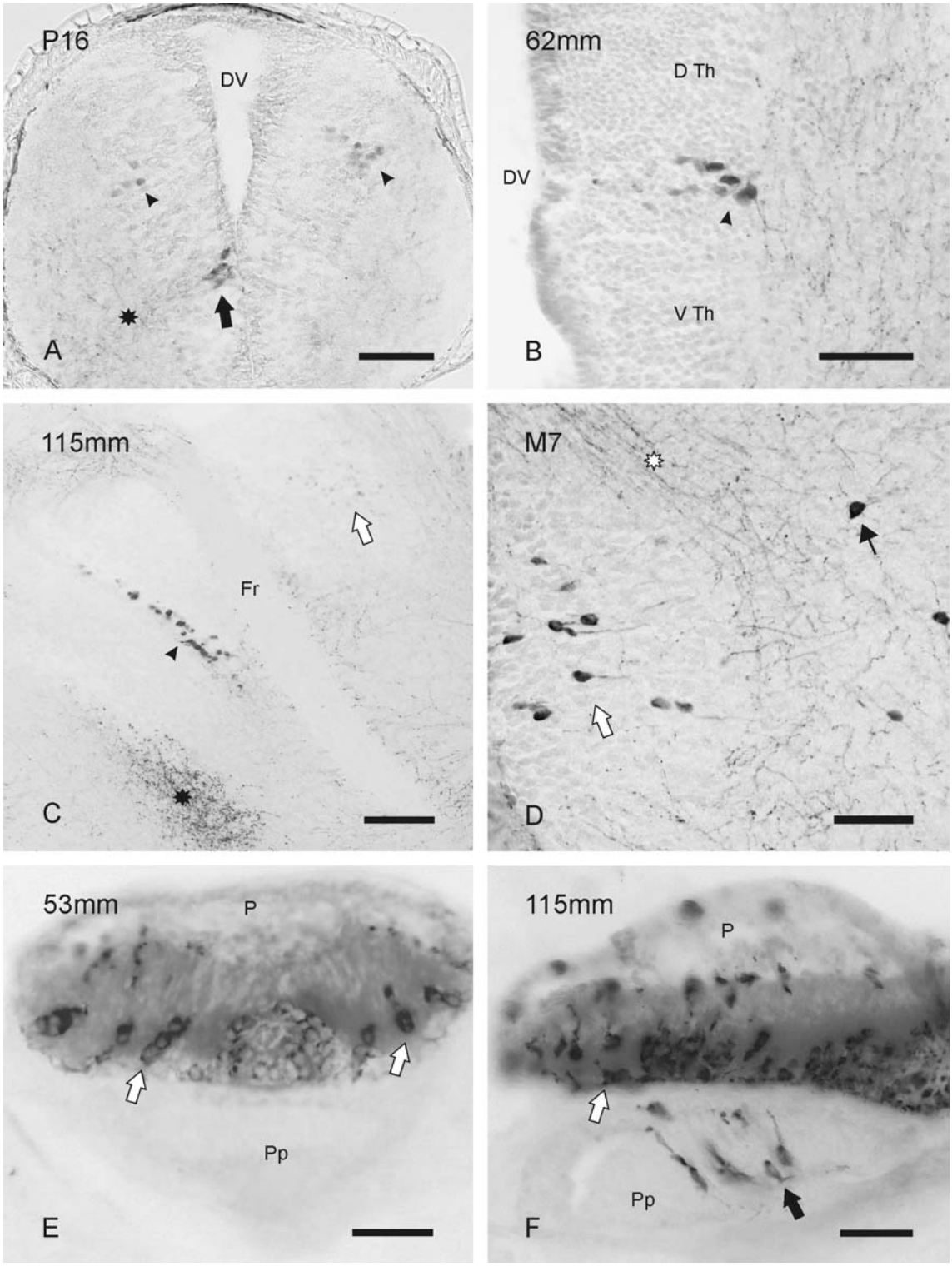

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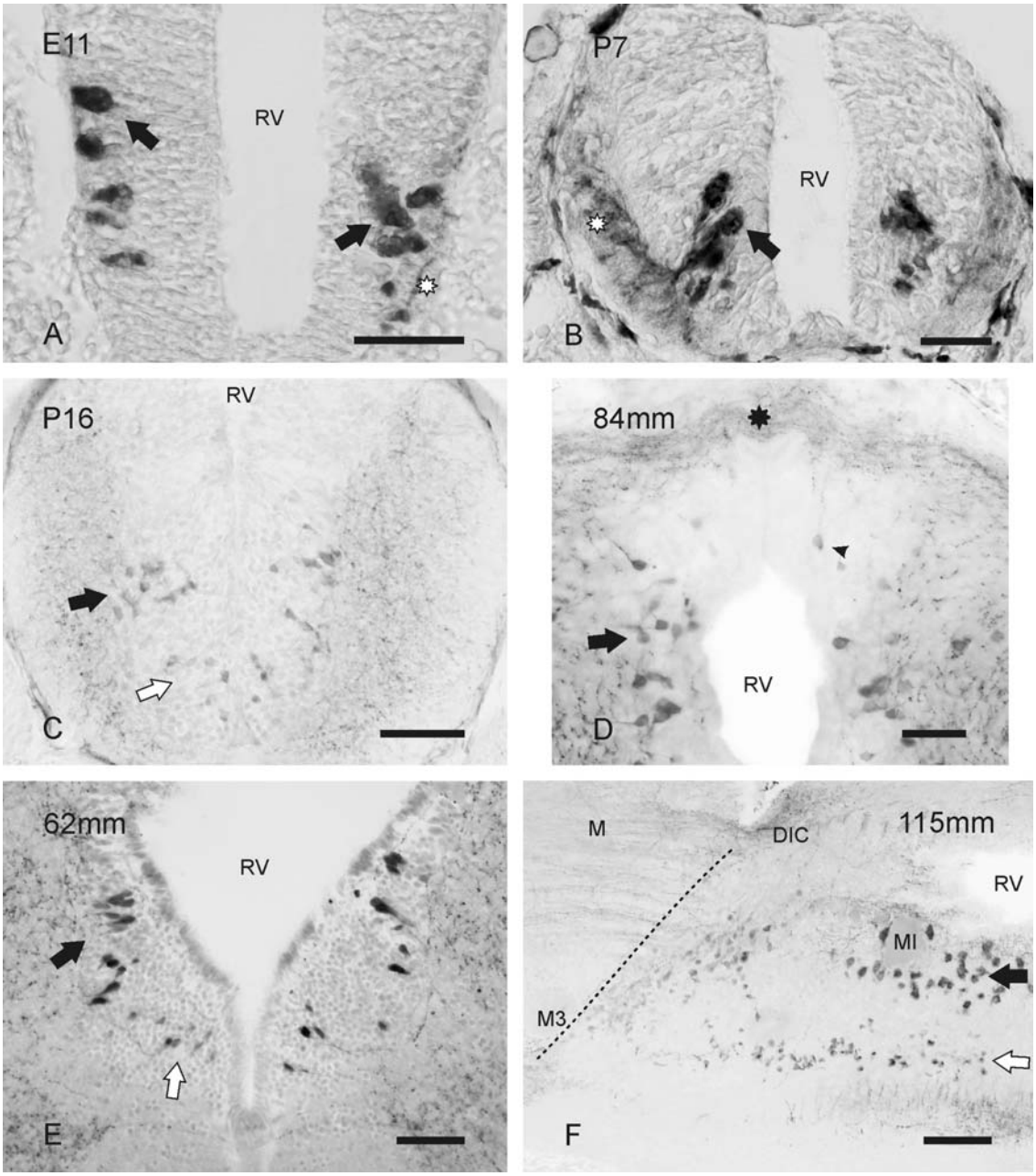
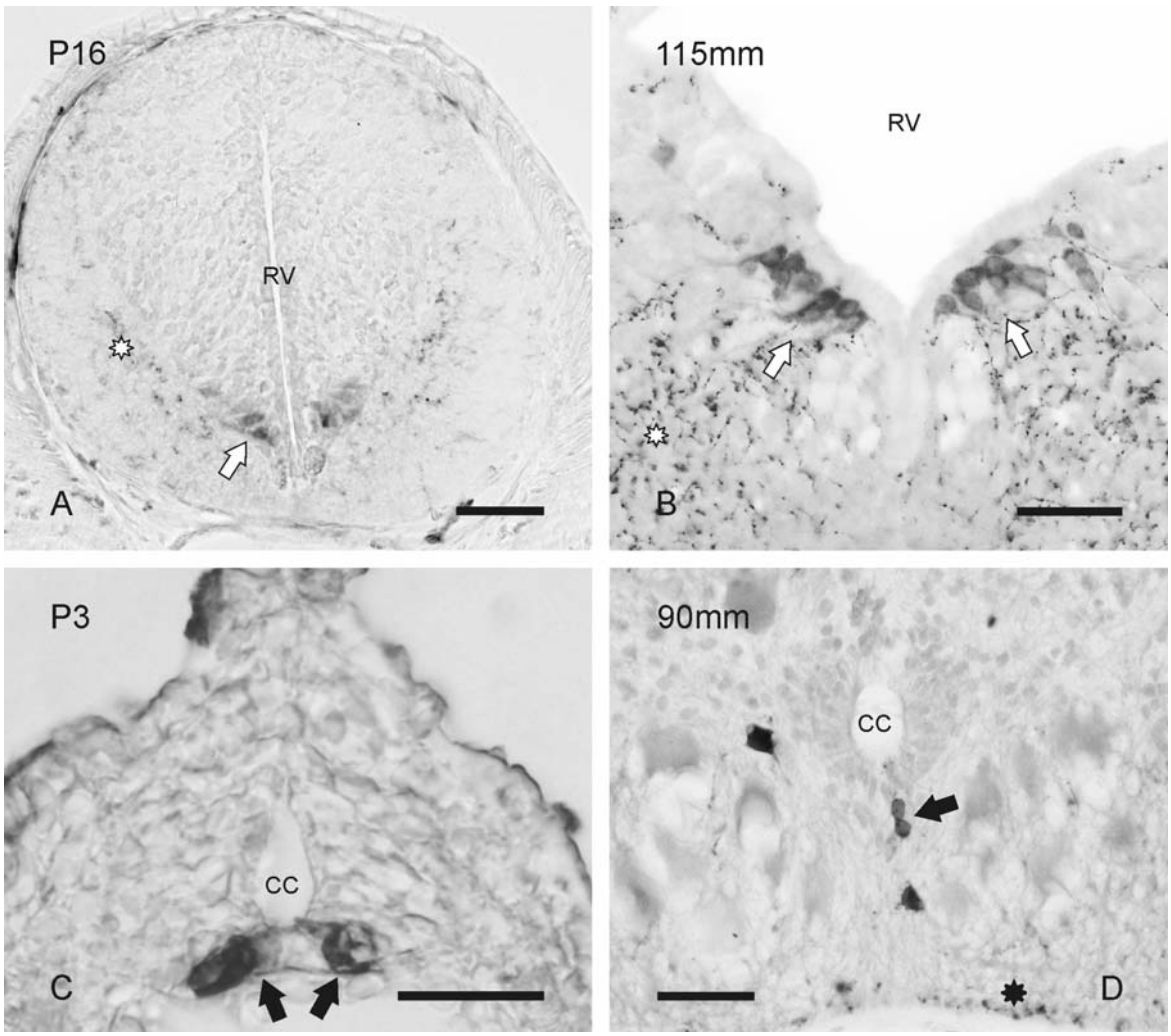
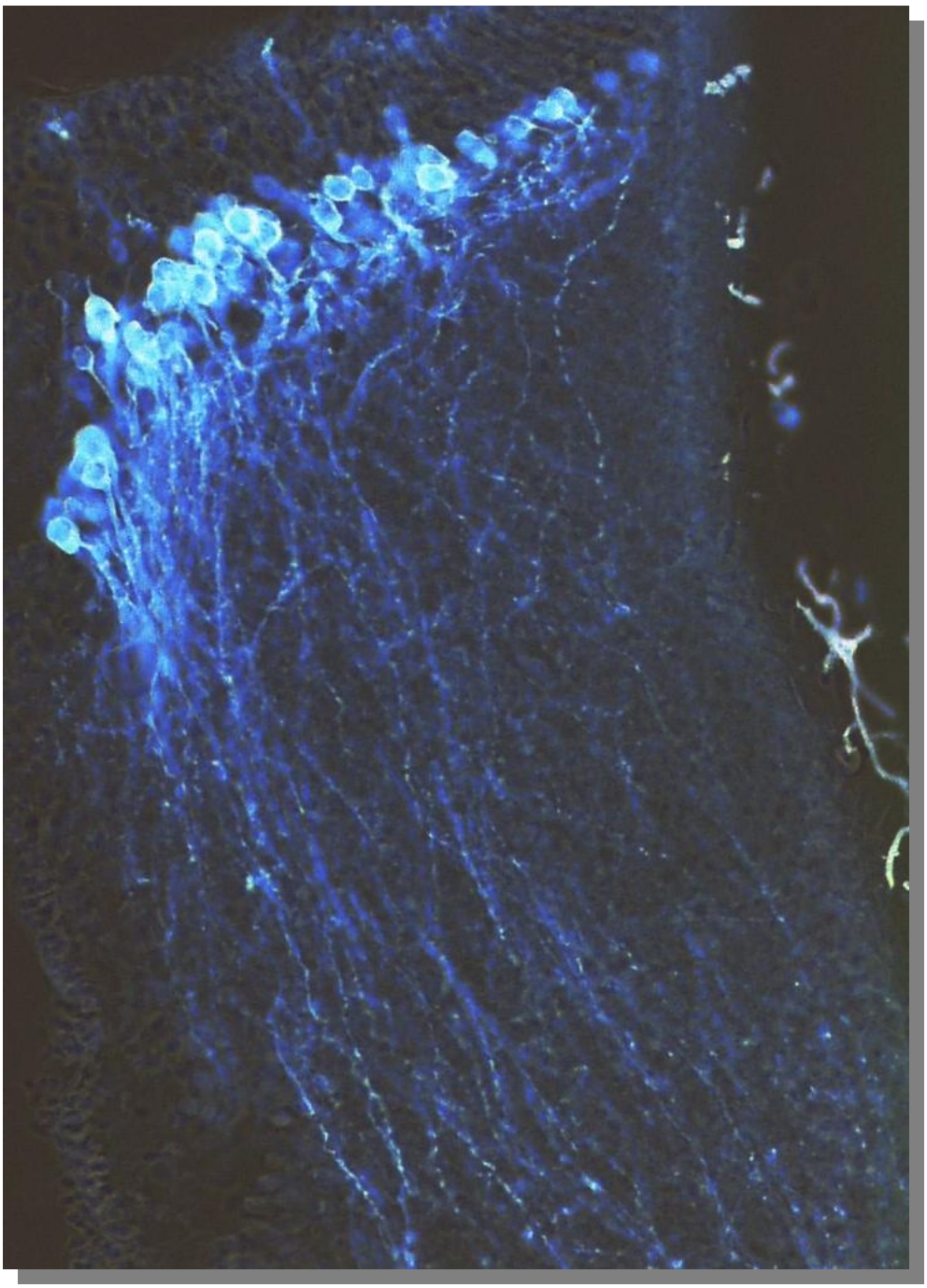


Figure 10





Chapter 2

Chapter 2

Development of the Catecholaminergic System in the Central Nervous System of the Sea Lamprey

ABSTRACT

The development of the catecholaminergic cell populations in the central nervous system of the sea lamprey, a modern representative of the earliest vertebrates, was studied using antibodies raised against tyrosine hydroxylase (TH), dopamine (DA) and dopamine-β-hydroxylase (DBH). The appearance of immunoreactivity to these substances begins in late prolarvae. The first TH-immunoreactive (TH-ir) cells appear in the paratubercular and preoptic nucleus of prolarvae. In larvae, new TH-ir cells appear progressively in the postinfundibular commissure nucleus, the postoptic commissure nucleus, the paraventricular organ, striatum, olfactory bulbs and spinal cord. In adult sea lamprey TH-ir cells are also present in the lamina terminalis and the ventral hypothalamic nucleus.

The distribution of TH and DA immunoreactivities matches rather well, although in the olfactory bulbs and lamina terminalis TH-ir cells were not DA-ir, and in some populations the time of appearance of these immunoreactivities is different. The first DA-ir cell groups appeared in the spinal cord, the posterior tubercle nucleus and the dorsal hypothalamic nucleus of prolarval stages. In larvae, new DA-ir cell groups were observed in the caudal preoptic nucleus, the postoptic commissure nucleus, the postinfundibular commissure nucleus and the caudal rhombencephalon.

All these DA-ir cell groups were also observed in adults, which showed an additional DA-ir cell group in the ventral hypothalamic nucleus. The distribution and development of DBH-ir structures in the lamprey brain is very different from TH immunoreactivity. The first DBH-ir cells appear in the paraventricular organ of prolarvae, and then new cells appeared in the caudal rhombencephalon and the synencephalic-mesencephalic tegmentum of larvae. There are striking differences between sea lampreys and other vertebrates as regards to the relative time of appearance of DA-ir cells, which is probably related with the complex life cycle of the sea lamprey. As in other vertebrates, development of the GABAergic and serotoninergic systems precedes that of catecholaminergic systems.

INTRODUCTION

Catecholamines are neuroregulatory substances that include dopamine (DA), noradrenaline (NA) and adrenaline (A), which are synthesized from the aromatic amino acid tyrosine by successive enzymatic steps requiring distinct enzymes. Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (DOPA), which is the first and rate-limiting step in catecholamine biosynthesis. The use of antibodies against TH and other enzymes of the synthesis of catecholamines [aromatic L-amino acid decarboxylase (AADC); dopamine-ß-hydroxylase (DBH); phenylethanolamine-N-methyltransferase (PNMT)] as immunohistochemical reagents enabled a detailed study of the catecholaminergic systems in the central nervous system of a number of vertebrates (for a comprehensive review of vertebrate catecholaminergic systems see Smeets and González, 2000). Although DA and NA appear to be widely distributed in the brain of vertebrates, in lamprey brains, A could not be detected via high-performance liquid chromatography and adrenergic neurons were not observed using antibodies against PNMT, the enzyme synthesizing adrenaline from noradrenaline (Pierre et al., 1997).

Developmental studies of catecholaminergic systems have been made in a few species of vertebrates (teleosts: Ekström et al., 1992; Manso et al., 1993; amphibians: González et al., 1994a, b; Sánchez-Camacho et al., 2002; reptiles: Medina et al., 1994a, b; birds: Puelles and Medina, 1994; mammals: Specht et al., 1981a, b; Foster et al., 1985; Tillet and Thibault, 1987; Kalsbeek et al., 1992; Jaeger and Teitelman, 1992; Foster, 1992, 1994). These studies have shown a number of similarities but also important differences among vertebrates in organization, time of appearance and regions of origin of catecholaminergic populations. From a comparative point of view,

these studies have been useful to define the territories of origin of the different catecholaminergic populations (Puelles and Medina, 1994).

Lampreys are extant representatives of Agnathans, the most ancient group of vertebrates. Distribution of catecholamines in adult lamprey brains was first studied with the formaldehyde-induced fluorescence methods (Baumgarten, 1972; Konstantinova, 1973; Ochi and Hosoya, 1974; Tsuneki, et al., 1975; Ochi et al., 1979). These studies revealed the presence of catecholamine-containing neurons in the diencephalon, brainstem and spinal cord, as well as numerous positive fibers in the brain and hypophysis. More recently, immunohistochemical techniques were used to study the distribution of catecholaminergic systems in adult river lamprey (Steinbusch et al., 1981; Schotland et al., 1996; Pierre et al., 1997; Pombal et al., 1997). Although previous studies have reported the development of TH-ir neurons in larval river lamprey (Pierre-Simons et al., 2002), and that of the dopaminergic system in the sea lamprey (Abalo et al., 2005), an integrative study of the catecholaminergic system in all phases of the life cycle of lampreys is lacking.

The complex life cycle of the sea lamprey includes a short embryonic period (about 12-13 days after fertilization), a three weeks prolarval period during which the mouth, pharynx and brain become functional, and a very long (four or more years long), blind, microphagous larval phase (ammocoete) in which animals live burrowing in the mud of the river and prepare for a complex metamorphosis giving rise to the adult (see Piavis, 1971). The parasitic, adult phase of sea lamprey occurs mostly in the sea, from which prespawning adults migrate upstream in the river to breed and die. The aim of the present paper is to study the catecholaminergic systems of the sea lamprey through the life cycle from a developmental and comparative point of view.

MATERIAL AND METHODS

Animals

Late embryos, prolarvae, larvae and adults of the sea lamprey (*Petromyzon marinus* L.) were used. Embryos and prolarvae were obtained from *in vitro* fertilized eggs reared in the laboratory under appropriate conditions of darkness, temperature (16ºC) and aeration. Larval lampreys were caught in the River Ulla (Galicia, northwest Spain) and kept in an aerated aquarium until processing. Adults were purchased to local suppliers at the River Ulla and used for comparison with larval stages. All experiments were conducted in accordance with European Community guidelines on animal care and experimentation to minimize pain and discomfort.

Stages of embryos, prolarvae, and early larvae are indicated by age (e.g., E12 indicates 12 days after fertilization; P3 indicates 3 days posthatching, and so on; in our broods hatching occurred 11-13 days after fertilization). In addition, to classify prolarvae, we used the stages defined for sea lamprey by Piavis (1971): hatching (P0-1), pigmentation (P2-3), gill-cleft (P4-7), and burrowing (P8-23) stages. Larval stages (with the exception of larvae from broods, in which age is known) are categorized simply by total body length, ranged from 12 to 145 mm in length and comprised individuals from the first year to the sixth year class of age according to the studies of Hardisty and Potter (1971) and Purvis (1979).

TH and DBH immunohistochemistry

After deep anaesthesia with benzocaine (0.5 ml/l; Sigma, St. Louis, MO), lamprey embryos (n = 3), prolarvae (n = 10), heads of larvae (n = 20 for TH and 15 for DBH), and removed brains postmetamorphic individuals (n = 4) and adults (n = 4) were fixed by immersion either in cold 4% paraformaldehyde in 0.1M phosphate buffer (PB)

at pH 7.4 or in the same fixative containing 0.1% picric acid. Samples were cryoprotected with 30% sucrose in 0.1M PB, embedded in Tissue Tek (Sakura, Torrance, CA) and cut on a cryostat (14-18 μm thick). Series of transversal and sagittal sections were processed by either the peroxidase-antiperoxidase (PAP) or streptavidin-biotin methods. Sections were washed in phosphate buffer saline (PBS) and incubated either in a polyclonal rabbit anti-TH (diluted 1:1000) or in a polyclonal rabbit anti-DBH antibody (diluted 1:250, both from Chemicon Temecula, CA), with PBS containing 0.3-0.5% Triton X-100 and 1% bovine serum albumin (BSA, Sigma) for 16-18 hours at 4°C. The sections were rinsed three times in PBS-Triton X-100, and incubated sequentially in a secondary goat anti-rabbit (GAR, 1:100) or GAR biotinylated (1:300, Dako) antibodies in PBS-Triton X-100, and in rabbit PAP complex (Dako; 1:600) or streptavidin-biotin complex (Dako), respectively, for 1 hour at room temperature. After successive rinses in PBS, sections were developed with 0.5 mg/ml 3,3'-diaminobenzidine (DAB; Sigma) and 0.01% H_2O_2 in PBS for 3-15 minutes. The sections were dehydrated and mounted on gelatin subbed slides with Eukitt medium. Some brains were fixed in Bouin's fluid without acetic acid, paraffin-embedded and sections were processed for immunohistochemistry following a similar schedule. In some cases alternate series of sections were processed for TH and DBH to compare these systems.

The specificity of primary antibodies has been well-characterized by the suppliers and used in a number of mammalian and non-mammalian species, including lampreys (Pierre-Simons et al., 2002). Control sections were processed identically, except for the omission of primary antiserum. No staining was observed in these controls.

DA immunohistochemistry

For DA detection animals were anaesthetized with benzocaine (0.5 ml/l). Embryos (n = 4) and prolarvae (n = 15; both staged as in Meléndez-Ferro et al., 2002), heads of larvae (n = 12; staged by body length) or brains of postmetamorphic (n = 5) and adults (n = 5) of the sea lamprey, were fixed by immersion in glutaraldehyde 5% in 0.1 M Tris-buffered saline pH 7.4 (TBS) containing 1% sodium metabisulfite overnight. After rinsing in TBS, specimens were cryoprotected in 30% sucrose overnight, embedded in Tissue Tek (Sakura, Torrance, CA), frozen and cut on a cryostat.

For immunocytochemistry, sections were processed by sequential incubation with a rabbit polyclonal antibody against DA (HWM Steinbusch, U Maastricht, Netherlands; 1:1000) overnight, a goat anti-rabbit antibody (Sigma; 1:100) for 1 hour and peroxidase-antiperoxidase complex (PAP; from Sigma; 1:400) for 1 hour, and the immunoreaction was developed with 0.6 mg/ml 3-3'-diaminobenzidine (DAB: Sigma) and 0.003% H_2O_2. All antibody dilutions were carried out in TBS containing 0.2% Triton X-100 and 3% normal goat serum.

Additional material

For a better characterization of the development of the CNS of the sea lamprey we also used our collection of embryonic, prolarval and larval brains immunostained for proliferating cell nuclear antigen (PCNA) (for methodological details, see Villar-Cheda et al., 2006). For topographical purposes, brain series stained with haematoxylin-eosin or cresyl violet were also used.

Photography

Photomicrographs were taken with an Olympus AX-70 photomicroscope equipped with color digital cameras (Olympus DP-12, DP-70). The photographs were converted to gray scale and adjusted for brightness and contrast using Corel Photo-Paint 12 software (Corel, Ottawa, Canada). Photomontage and lettering were done using Corel Draw 12.

Nomenclature of brain structures

The nomenclature used for the brainstem nuclei and other brain regions of the adult sea lamprey follows in general that of Nieuwenhuys and Nicholson (1998), but for the adult prosencephalon and midbrain we adopted most limits and regions defined by Pombal and Puelles (1999). For prolarval and larval brain regions, we adopted the nomenclature used in previous studies from our laboratory (Meléndez-Ferro et al., 2002, 2003; Abalo et al., 2005; Villar-Cheda et al., 2006).

RESULTS

Immunocytochemistry with TH and DA antibodies reveal numerous positive fibers distributed throughout the brain and perikarya mainly located in olfactory bulbs, diencephalic nuclei and along the caudal rhombencephalon and spinal cord. The distribution of TH- and DA-immunoreactive (TH-ir and DA-ir) fibers is quite similar, though there are some differences with respect to the localization of immunoreactive neuronal perikarya. The distribution of TH-ir and DBH-ir structures in the brain of the lamprey was different. Accordingly, we will first describe the distribution of TH-ir and DA-ir neurons and fibers, and finally the distribution of DBH-ir neurons and fibers.

Distribution of TH immunoreactivity in the sea lamprey CNS

The distribution of TH-ir cell groups in the adult *P. marinus* brain is schematically illustrated in a sagittal projection of the brain and rostral spinal cord in Figure 1.

Adult

Cell bodies

In the adult brain, TH-ir cell groups were observed in the prosencephalon, the most caudal part of the rhombencephalon and in the spinal cord. No TH-ir perikaryon was observed in the mesencephalon or in the rostral rhombencephalon.

Prosencephalon

Numerous TH-ir cells were observed in the olfactory bulbs. Many of them were weakly-stained small (5-7 μm), spindle-shaped or tripolar cells that located in the inner granular layer, between the olfactory glomerules and the telencephalic ventricle (Fig. 2A). These cells were tentatively considered as granule cells. Other TH-ir neurons were

observed among olfactory glomeruli to which they send a process, suggesting they were periglomerular cells (see Meléndez-Ferro et al., 2001, for topography of the olfactory bulbs in the sea lamprey adults). In the telencephalon proper, a few TH-ir cells were observed in nucleus of the lamina terminalis. They were bipolar or tripolar neurons and many of them showed a cerebrospinal fluid-contacting (CSF-c) dendrite (Fig. 2B). Some pale TH-ir cells extended dorsally and rostrally from the nucleus of the lamina terminalis towards the ventral telencephalon.

In the caudal telencephalon, a band of strongly stained TH-ir cells extended from the ventrolateral wall of the preoptic recess (parvocellular preoptic nucleus) (Fig. 2C), over the postoptic commissural plate (paracommissural preoptic nucleus of Pombal et al., 2001), to the lateral walls of the third ventricle (postoptic commissure nucleus, pars dorsalis hypothalami). A gradient of increasing cell size was observed from the preoptic recess to dorsal hypothalamic locations. In the preoptic region of the adult lamprey, TH-ir cells were observed mostly at the bottom of the preoptic recess over the optic chiasm (Fig. 2C), but a group of TH-ir cells was also observed in the preoptic area just lateral to the magnocellular preoptic nucleus, and a few cells were also observed among the magnocellular neurons, which formed a cell band parallel to but separated from the periventricular layers. Cells in the preoptic region exhibited thick dendrites extending to the lateral or ventral neuropil (Fig. 2C) and thick TH-ir fibers of these cells ran caudally along the ventral wall of the hypothalamus to the neurohypophysis. Most of the TH-ir neurons of the chiasma region are of CSF-c type, varying from small cells with short ventricular dendrites to larger cells with long ventricular processes. Dorsolateral processes of these cells extended to the neighbor areas, and thick TH-ir fibers of this nucleus also run along the ventral surface of the hypothalamus to the neurohypophysis.

Rostral and dorsal to the preoptic nucleus, some pale TH-ir cells were also observed in the ventral telencephalon (striatum), which showed occasional TH-ir CSF-c cells.

In the hypothalamus, a band of CSF-c TH-ir neurons extended along the lateral infundibular walls in the so-called paraventricular organ (pars ventralis thalami-nucleus dorsalis hypothalami). It consists of two different types of TH-ir cells, large and small, which were darkly and weakly stained, respectively. Large TH-ir cells (accompanying cells of the paraventricular organ) were located away the ventricle and sent a thick ventricular dendrite that was originated either from the perikaryon or a lateral dendrite. The small TH-ir bipolar neurons had a short ventricular dendrite and their ventrolateral process form a small marginal bundle. These cells formed a palisade-like subventricular group (nucleus ependymalis thalami et hypothalami of Baumgarten, 1972). Caudalwards, this TH-ir nucleus shifted gradually to a more ventral location (pars ventralis hypothalami).

The most conspicuous TH-ir nucleus was the paratubercular nucleus (nucleus paratubercularis: Baumgarten, 1972), located dorsal to and around the dorso-posterior infundibular recess (Fig. 2D). The paratubercular cells exhibited intense TH immunoreactivity, with long thick dendrites extending far from the perikarya dorsally, rostrally and laterally (Fig. 2D). Axons from these cells run rostrally towards the telencephalon, caudo-dorsally to the optic tectum or caudally to the rhombencephalon and spinal cord.

From the dorsal wall of the caudal region of the postoptic recess to the posterior infundibular (mammillary) recess, a population of weakly to moderately stained CSF-c neurons were present, the ventral hypothalamic TH-ir group. The number of cells was scarce in the dorsal wall of the postoptic recess but increase more caudally. This group

joins another TH-ir cell group in the postinfundibular commissure nucleus, around the infundibular recess (Fig. 2E), that appears as a ventral extension of the paraventricular organ and have numerous cells in the walls of the mammillary recess, in which dorsal wall intermingle with the caudal paratubercular cells.

Caudal rhombencephalon and rostral spinal cord

In the rostral spinal cord and caudal rhombencephalon (Fig. 2F), occasional small TH-ir cells formed a longitudinal column extending in the ventral margin of the central canal. These CSF-c bipolar neurons had a short ventricular dendrite and a thin process that arose from the opposite pole of the perikaryon. The TH-ir CSF-c neurons of the central canal of the spinal cord were very numerous.

Fibers

A large number of TH-ir fibers were observed throughout the brain and spinal cord, although there were regional variations in innervation density. In the telencephalon, numerous TH-ir fibers were distributed in the intermediate layers around the ventricles of the telencephalic lobes except in their anterior end, showing the striatum the densest innervation. They were scarce in the lobus subhippocampalis. The number of TH-ir axonal processes in most parts of the olfactory bulb was very scarce, despite the presence granule and periglomerular TH-ir cells (Fig. 2A).

In the diencephalon, the thalamus, hypothalamus and paratubercular nucleus showed numerous TH-ir fibers (Figs. 2D, E). From the periventricular nuclei many TH-ir fibers coursed to form lateroventral accumulations in the diencephalon. From the preoptic region, thick TH-ir fibers coursed towards the neurohypophysis, where they formed a rich terminal plexus. The innervation of the epithalamus by TH-ir fibers was scarce and only a few fibers coursed to the habenula and the parapineal ganglion.

Abundant TH-ir fibers coursed through the tegmental lateral region of the mesencephalon, and some fibers ran dorsally to the optic tectum where, in large larvae, they coursed either in the subventricular layer or in a fiber layer external to the stratum cellulare internum. As observed in sagittal sections, a proportion of these fibers were originated from neurons of the nucleus paratubercularis. TH-ir fibers did not enter the conspicuous interpeduncular nucleus neuropil.

In the medulla oblongata, most TH-ir fibers coursed through the dorsolateral portion of the basal plate forming a diffuse catecholaminergic tract. Some fibers formed a periventricular plexus at the level of the motor nuclei of the visceral column (trigeminal to vagal nerves). Dorsally in the isthmic region, numerous TH-ir fibers crossed the midline at the level of the cerebellar commissural plate. The area octavolateralis also received a rather abundant TH-ir innervation. At the transition between the medulla and the spinal cord, the fibers running in the basal plate shifted gradually towards more dorsal locations, and most TH-ir fibers acquired a location dorsal to the lateral horn. Other fibers, probably originated from TH-ir cells of the central canal, coursed close to the ventromedial surface of the spinal cord.

TH immunoreactivity during development

The location of TH-ir cell groups in prolarvae, larvae, and adults is schematic illustrated in sagittal projections of the brain in Figure 1, and the sequence of appearance of the TH-ir populations in the sea lamprey CNS is summarized in Table 1. The study of the larval brain revealed several TH-ir cell groups in the preoptic region and diencephalon, in the most caudal part of the medulla oblongata and in the spinal cord. No TH-ir neuron was observed in other regions of the telencephalon (with the exception of the largest larvae), in the mesencephalon or rostral regions of the

rhombencephalon. No neuronal groups transiently expressing TH during development were found in the sea lamprey.

Only in very large larvae (presumably premetamorphic) a few pale TH-ir cells were observed in the olfactory bulbs, and a few very pale small TH-ir cells were also observed in the striatum.

One or two very faint TH-ir cells appear for the first time close to the optic recess in the preoptic region of late burrowing (P16) prolarvae. In 20-mm larvae, a scarce number of faintly stained TH-ir cells were observed at the end of the lateral optic recesses just at the level of the optic nerve entrance (Fig. 3A). In medium-sized larvae (40-60 mm) the number of TH-ir cells increased and they were also observed in the periventricular layer of the lateral walls of the recess (Fig. 3B) and also over the region of the postoptic commissure, corresponding to a posterior commissure nucleus. Accordingly, the location of this TH-ir nucleus was caudal to the anterior intraencephalic sulcus of von Kupffer and, hence, caudal to the magnocellular preoptic nucleus (see Meléndez-Ferro et al., 2002). This organization was also observed in larger larvae. Differences between larvae were mainly quantitative: the number of cells increased from small larvae to premetamorphic larvae, and the staining intensity of preoptic TH-ir cells was clearly higher in premetamorphic larvae. In these larvae, the morphology of preoptic TH-ir cells is more easily appreciated: most cells, both in the bottom and the lateral walls of the ventricle were CSF-c, those in the lateral walls often showing long and rather thick ventricular dendrites and their axons are clearly visible running to the neurohypophysis.

In the paratubercular region, the first TH-ir cells appeared in early burrowing prolarvae. In late burrowing prolarvae they form a small group of very small cells that exhibit a thin perinuclear TH-ir region. In larvae less than 20 mm in length the number

of cells increased slightly, and TH-ir fibers could be observed only near to the positive cells (Fig. 3C). In larvae of intermediate length (20-60 mm) the paratubercular TH-ir nucleus was conspicuous, and their cells exhibited long processes directed dorsolaterally (Fig. 3D). They formed a rather compact band extending from dorsorostral to ventrocaudal in the dorsolateral wall of the posterior infundibular recess, and some TH-ir cells being found in the midline. In these larvae, the dorsorostral cells had also migrated away from the periventricular cell layer, while the smaller cells were found within this periventricular layer (Fig. 3D). In large larvae, most of these cells were pear-shaped and their dendritic processes extended dorsally or rostrodorsally into the thalamus for long distances, most in a wide region centered on the thalamic course of the fasciculus retroflexus. In longitudinal sections of these larvae, many axons from these cells were seen coursing caudally in the brainstem. The number of paratubercular cells increased throughout the larval period: cell counts indicate that the population increased from about 40 cells in 20 mm larvae (Fig. 3C) to about 260 cells observed in a 112 mm larva.

The CSF-c TH-ir cells of the postinfundibular commissure nucleus were observed for the first time in larvae 60-80 mm in length. These positive cells were small and located around the infundibular recess. In the paraventricular organ, TH-ir cells were first observed in larvae of 40 mm, in which these neurons occupied a medial part of the nucleus in the dorsal region of the hypothalamus, and the population increased in larger larvae (Fig. 3E). The small TH-ir cells were faintly stained even in premetamorphic larvae. Only in very large larvae appeared occasional CSF-c TH-ir cells in the infundibular recess ventral to the paraventricular organ. In the neurohypophysis, TH-ir fibers can be observed from small larvae (Fig. 3F) until the

adult stage. By the end of the larval phase, the caudal rhombencephalon-spinal cord CSF-c TH-ir cells appeared as pale stained small cells.

DA distribution in the sea lamprey

The distribution of DA-ir cell groups in the adult *P. marinus* brain is schematically illustrated in a sagittal projection of the brain and rostral spinal cord in Figure 1. Although there were a few differences in distribution of TH-ir and DA-ir perikarya, the distribution of TH-ir and DA-ir fibers in adult sea lampreys was very similar. However the timing of appearance of DA immunoreactivity in the different cell groups notably differs from that of TH immunoreactivity.

Adult

Cell Bodies

In the brain of adult sea lamprey, groups of DA-ir neurons were found in the caudal preoptic nucleus, the postoptic commissure nucleus, the dorsal hypothalamic nucleus (the putative paraventricular organ), the ventral hypothalamic nucleus, the paratubercular nucleus, the postinfundibular commissure nucleus (mammillary region), the caudal rhombencephalon and the spinal cord. Occasional DA-ir cells were also observed in the striatum, the ventral thalamus and the ventral region of the isthmus. Two regions with TH-ir cells, the olfactory bulbs and the lamina terminalis, did not show DA immunoreactive cells.

Prosencephalon

Occasional DA-ir cells were observed in the striatum. In the caudal wall of the preoptic recess some DA-ir cells were also observed. These DA-ir cells were faint to moderate stained and almost all were in periventricular location over the optic chiasm

(Fig. 4A), but some cells occupied a more lateral position. The DA-ir cells of the postoptic commissure nucleus were scarce, intensely stained and located in the dorsal and ventral periventricular parts of this nucleus.

In the dorsal hypothalamus there is a group of DA-ir cells in the paraventricular organ. It is a population with strongly DA-ir bipolar, CSF-c cells that were distributed in one or two cell rows in the periventricular layer (Fig. 4B). Darkly-stained perikarya, bulbous protrusions in the ventricle and lateral processes could be observed. In the ventral hypothalamus a DA-ir cell group was observed in the ventral hypothalamic nucleus as a dorsal extension of the paraventricular nucleus (Fig. C). It is formed of one or two rows of positive, weakly stained CSF-c cells located periventricularly, which contact with the ventricle and that extend between the postoptic and the infundibular recesses. This DA-ir cell group only was observed in adults.

The paratubercular nucleus has a group of faintly stained DA-ir cells in medial position (Fig. D) that gives rise to processes dorsally directed. These neurons are rather large rounded or pear-shaped cells, . The postinfundibular commissure nucleus consists of strongly stained small rounded DA-ir cells (smaller than paratubercular nucleus cells), and CSF-c, which are in the periventricular layer of cells and contact with the posterior recess (Fig. D). The paraventricular organ, the paratubercular nucleus and the postinfundibular commissure nucleus become confluent in the caudal hypothalamus, but limits between them can be noted because of the clear differences in cell size, staining intensity, population density, and morphology of processes.

Rhombencephalon and rostral spinal cord

In some samples a variable number of DA-ir cells in the isthmus region, mainly in the ventral part, were found. A continuous group of DA-ir cells was observed from the caudal rhombencephalon to the spinal cord. These cells were pear-shaped CSF-c

neurons with a process to the ventricle (Fig. E), which were located in groups in the ventral wall of the central canal. There were also a few non-CSF-c DA-ir bipolar cells that located more laterally (Fig. E).

Fibers

In adults, DA-ir fibers were widely distributed throughout the brain. Regions with dense dopaminergic innervation are the neurohypophysis, striatum, preoptic area, dorsal thalamus, pretectum, midbrain tegmentum, the lateral tegmental region of the rhombencephalon, from small larval stage. DA-ir innervation was scarce in the olfactory bulbs, subhippocampal lobe, epithalamus, optic tectum, and the interpeduncular nucleus.

In the spinal cord, the DA-ir cells formed a ventromedial fiber plexus along the whole spinal cord, which can be observed from larval stages onwards. DA-ir fibers were also observed in the dorsal funiculus, but are lacking in the lateral funiculus.

DA immunoreactivity during development

The location of DA-ir cell groups in prolarvae, larvae, and adults is schematically illustrated in Figure 1 as sagittal projections of the brain. The sequence of appearance of the DA-ir populations in the sea lamprey CNS is summarized in Table 1. Most DA-ir cell groups observed in adult sea lamprey appeared in prolarvae and larvae, with the notable exception of the DA-ir cells of the ventral hypothalamic nucleus which were not present in developing stages. The brain of large larvae was less densely innervated by DA-ir fibers than the adult brain, but the innervation followed the same pattern.

Prosencephalon

Occasional DA-ir cells were observed in the striatum of large larvae (more than 80 mm). The DA-ir cell groups located in the caudal wall of the preoptic recess and in

the postoptic commissure nucleus were observed from early larvae (less than 30 mm in length) onwards (Fig. 5A). The DA-ir cells of the preoptic nucleus were faintly stained and occupied the caudal wall of the preoptic recess; most cells were located periventricularly over the optic chiasm, and some cells occupied a more lateral position close to the optic nerves (Fig. 5B). The DA-ir cells of the postoptic commissure nucleus were more intensely stained than the preoptic cells and located only in the dorsal periventricular part of this nucleus (Fig. 5C). In the paratubercular nucleus, the first DA-ir cells were observed in P8 prolarvae, these cells being located in the outer part of the marginal zone (Fig. 5D). In larvae, these cells increased progressively in number and occupied more medial positions, giving rise to dorsally directed processes (Fig. 5E). The DA-ir cells of the paraventricular organ first appeared in P16 prolarvae (about 8-9 mm in length). Since its appearance, this organ consists of a population of CSF-c, strongly DA-ir bipolar cells that progressively extended ventrocaudally (Fig. 5F). The postinfundibular commissure nucleus first appeared around the posterior infundibular recess (mammillary region) of larvae of about 30 mm. It consists of a population of strongly stained CSF-c cells that increased considerably in number throughout the larval period (Fig. 5E). In large larvae (more than 80 mm), occasional DA-ir cells were observed in the ventral thalamus and the telencephalic lobes. In premetamorphic larvae (more than 120 mm) a few cells (1-5) were observed in the isthmic region.

After the appearance of the paratubercular cells (P8), DA-ir fibers were observed coursing in the lateral diencephalon and reaching the caudal telencephalon. In late prolarvae and larvae, DA-ir fibers formed a lateroventral plexus in the diencephalon, mesencephalon and rhombencephalon. In small larvae, DA-ir fibers were observed in the neurohypophysis (Fig. 5A), striatum, preoptic area, dorsal thalamus, pretectum, midbrain tegmentum, and these fibers increased progressively in number

throughout development. In larvae more than 100 mm in length, DA-ir fibers were widely distributed throughout the brain, though they were scarce in the olfactory bulbs, subhippocampal lobe, epithalamus, optic tectum and interpeduncular nucleus.

Caudal rhombencephalon and rostral spinal cord

The earliest DA-ir cells appeared in the spinal cord of prolarvae 3 days post-hatching (P3). There were rather large, yolk-filled CSF-c cells located under the central canal (Fig. 5G). In early larvae (less than 30 mm in length) some CSF-c DA-ir cells appeared in the caudal rhombencephalon as a rostral prolongation of the CSF-c spinal population, and some non-CSF-c ventral DA-ir cells were also observed in the spinal cord. DA-ir fibers coursing in lateral spinal regions were already present in prolarvae. DA-ir fibers originating from spinal cord cells formed a ventromedial plexus along the whole spinal cord of larvae and adults. In large larvae, DA-ir fibers were numerous in the rhombencephalon and the spinal cord, although being scarce in its lateral funiculus.

DBH distribution in the sea lamprey

The distribution of DBH-like immunoreactive (DBH-ir) cell groups in the adult *P. marinus* is schematically illustrated in a sagittal projection of the brain and rostral spinal cord in Figure 1. Cell bodies generally showed weak immunoreactivity, but the DBH-ir fibers, appeared intensely stained.

Adult

Cell bodies

In the brain of adult sea lamprey, DBH-ir neurons were observed in the paraventricular organ, the synencephalic-mesencephalic tegmentum and the caudal rhombencephalon.

In the paraventricular organ, a group of DBH-ir cells formed a band from an intermediate tuberal levels (the caudal part of paraventricular organ) to the ventral part of the posterior infundibular recess (Fig. 6A). Most of these DBH-ir cells were small CSF-c neurons that located in the inner region of the periventricular layer, but some larger DBH-ir cells were present in the outer region of this layer.

In the synencephalic-mesencephalic tegmentum, in the so-called M2-M5 nucleus of Schöber (1964), a few DBH-ir cells were observed in medial positions, around the M1 and M2 Müller cells (Fig. 6B). In the caudal rhombencephalon, at glossopharyngeal-vagal levels, scattered DBH-ir cells were also observed in the basal region. These cells send a long process to the lateral area (Fig. 6C).

Fibers

Thin, varicose DBH-ir fibers were observed throughout the adult brain, with different densities depending on the brain region. In the telencephalon, DBH-ir fibers were scarce, coursing mainly in the medial region of the olfactory bulbs, the interbulbar commissure, the lamina terminalis, the striatum and the caudoventral region of the preoptic nucleus. Some DBH-ir fibers were also observed in the diencephalon, mainly in the periventricular layer of the postoptic and infundibular recesses and in the superficial region of the lateral area of the thalamus and hypothalamus, and some fibers were also observed among the optic fibers in the optic chiasm and coursing in the ventral part of the habenula. Many DBH-ir fibers coursed to the neurohypophysis. The density of DBH-ir fibers was higher in the mesencephalon, rhombencephalon and spinal cord, being especially numerous is their basal parts. In the optic tectum, immunoreactivity was only observed in the inner layers. In the rhombencephalon, rather numerous DBH-ir fibers were observed on both sides of the ventral glial raphe at

octaval levels (Fig. 6D). In the alar region, DBH-ir fibers were more abundant in the octavolateral area.

DBH immunoreactivity during development

The location of DBH-ir cell groups in the brain of prolarvae and larvae is schematically illustrated in sagittal projections in Figure 1. The sequence of appearance of the DA-ir populations in the sea lamprey CNS is summarized in Table 1.

In prolarvae the presence of DBH-ir cells was occasional. Only one or two very faint cells were observed in the infundibular region (paraventricular organ) of a P16 prolarvae and a few cells more were present in larger prolarvae (Fig. 7A).

In 20 mm larvae, DBH-ir cells formed a fairly conspicuous group in the caudal walls of the third ventricle extending as an oblique band from an intermediate tuberal level rostrally to the rostral part of the posterior infundibular recess caudally. These DBH-ir cells were CSF-c neurons. The number of DBH-ir cells in the infundibular walls increased considerably in medium-sized larvae (Fig. 7B). Most these neurons were small CSF-c cells, but a few larger cells were also observed in the outer region of the cell mantle. The paraventricular nucleus DBH-ir cells increased in number in larger larvae (Figs. 7C, D). Study of parallel series of sections immunostained for TH and DBH, respectively, indicated that DBH-ir and TH-ir cells were co-distributed with in the paraventricular nucleus, but whether DBH and TH are co-localized in the same CSF-c cell could not be assessed. In the mesencephalic tegmentum of large larvae, a few weakly DBH-ir neurons were observed rostral to the M3 Müller cell, at the level of the third nerve entrance. These were small bipolar cells.

In the caudal rhombencephalon of 20 mm larvae, one or two very pale DBH-ir neurons were observed at glossopharyngeal-vagal levels near the sulcus limitans. In

larger larvae their number increased and the group was formed of a few DBH-ir cells distributed among cells of the periventricular layer, near the sulcus limitans. In 60-130 mm larvae, DBH-ir fusiform cells were also observed in more ventral regions (Figs. 7E, F). In premetamorphic larvae these cells are bipolar or multipolar neurons that located in the reticular area of the basal region.

In small larvae, scarce DBH-ir thin fibers were observed in the neuropil of the infundibular region. The number of fibers increased very considerably in medium-sized larvae (30-80 mm). In the telencephalon of these larvae, scarce DBH-ir fibers were distributed in caudoventral regions (e.g. striatum and preoptic region). They were more abundant in the preoptic region, where some of them crossed the midline in the optic chiasm region. DBH-ir fibers were rather abundant in the caudolateral region of the hypothalamus and thalamus. A few DBH-ir fibers were observed to course to the neurohypophysis in 40-60 mm larvae. In the rhombencephalon of larvae from 40 mm onwards, the ventromedial neuropil at the level of the octaval nerve entrance showed a large number of very thin varicose fibers.

In large larvae (80-130 mm), the number of DBH-ir fibers increased by comparison with that observed in medium-sized larvae. The neurohypophysis was rather intensely stained due to the presence of numerous DBH-ir very thin fibers. A few DBH-ir fibers ran to the ventral region of the habenula. In the mesencephalon, DBH-ir fibers were fairly abundant in the lateral tegmentum, and some fibers also coursed in the periventricular region of the optic tectum. In the medulla oblongata, fibers were more abundant in the basal plate than in the alar plate, although DBH-ir fibers were fairly abundant in the octavolateral area. Only a few DBH-ir fibers crossed the midline at the cerebellar plate. A small DBH-ir tract was observed associated with the trigeminal descending root between the entrance levels of the trigeminal and octaval nerves. In the

caudal medulla and spinal cord, most DBH-ir fibers coursed in ventral regions, some of them in close association with thick axons of reticular and Müller neurons, although there were also some DBH-ir fibers in more lateral regions.

DISCUSSION

Comparison of the relative time of development of catecholaminergic cell populations in sea lamprey and gnathostomes reveals striking differences and some similarities. The absence of catecholaminergic cell groups during the entire embryonic period in the sea lamprey is in contrast with its presence in embryos of teleosts (Ekström et al., 1992; Manso et al., 1993; Foster, 1994), amphibians (González et al., 1994a, b), lizard (Medina et al., 1994a, b), chicken (Puelles and Medina, 1994) and mammals (Foster, 1994). This fact can be attributed to the comparatively short embryonic period and lack of brain maturation at hatching in lamprey, so that the prolarval period might be considered as an extension of the embryonic period.

In the sea lamprey, new DA-ir cell groups progressively appear during the larval period and metamorphosis, which is similar to that reported in amphibians (González et al., 1994a, b), though very different from that observed in vertebrates with a direct development, in which by the end of the embryonic period the DA-ir system is similar to that observed in adults (Ekström et al., 1992, 1994; Foster, 1994; Medina et al., 1994a, b; Puelles and Medina, 1994). Results of the present study also show that the dopaminergic cell groups of the sea lamprey change considerably between prolarvae and adults, although the pattern of distribution of catecholaminergic perikarya observed in premetamorphic larvae correspond rather well to that observed in the adult with some exceptions. Interestingly, no DBH-ir cell groups appear during the metamorphic period. No neuronal groups showing transient expression of DA, TH or DBH immunoreactivity were observed in sea lamprey, unlike that reported for TH immunoreactivity in teleosts, (Ekström et al., 1992; Manso et al., 1993), reptiles (Medina et al., 1994a, b) and mammals (Specht et al., 1981b).

Our results also show that the first catecholaminergic neurons appear in early prolarvae, i.e. a few days later than the appearance of first serotoninergic (Abalo et al., 2003; chapter 1) and GABAergic neurons (Meléndez-Ferro et al., 2002, 2003). The delayed appearance of catecholamine system as regards that of serotonin and GABA systems is similar to that reported in a teleost (Ekström et al., 1985, 1992; Ekström and Ohlin, 1995).

Phenotypes of catecholaminergic cells

Based on the pathway of catecholamine biosynthesis, DA-ir neurons should be TH-ir, and immunonegative to DBH and secondary catecholamines (NA, A), whilst noradrenergic neurons should be DBH-ir. In the sea lamprey CNS, four types of populations of catecholaminergic perikarya can be distinguished with the antibodies used here: the first group is formed by cells that only are TH-ir. The second type are TH-ir populations in which some of their cells also present immunoreactivity to DA. The third type is formed by DA-ir perikarya that are either TH-negative or weakly TH-positive and the fourth type consists of DBH-ir perykarya. Discrepancy between distribution of catecholamines (DA, NA, and A) and catecholamine synthesizing enzymes (TH, DBH, and PNMT) has been previously reported in several vertebrates (elasmobranchs: Molist et al., 1993; teleosts: Meek et al., 1989; Sas et al., 1990; amphibians: González and Smeets, 1991; reptiles: Smeets and Steinbusch, 1990).

TH +/DA - /DBH- populations

The olfactory bulb, the lamina terminalis of adult lampreys and the large accompanying cells of the paraventricular were of this type. TH-ir/DA-negative cells have been found in other vertebrates (Sas et al., 1990; Smeets and Steinbusch, 1990; Vincent and Hope, 1990; González and Smeets, 1991) where these neurons seem to be

also immunonegative to secondary catecholamines (NA/A) suggesting that they contain L-dihydroxyphenylalanine (L-DOPA) as their end catecholamine.

TH +/DA ±/DBH- populations

The most conspicuous population of this type was the paratubercular nucleus. Probably all its catecholaminergic cells were TH-ir, but only a part of them exhibit DA immunoreactivity in different grades. Similar results were obtained in the preoptic nucleus and postoptic commissure nucleus. However, while in the paratubercular nucleus TH and DA immunoreactivity appear at the same stage, in the preoptic nucleus TH immunoreactivity appears before than DA immunoreactivity, while in the postoptic commissure nucleus DA immunoreactivity precedes TH immunoreactivity.

A possible explanation for the TH +/DA ± /DBH - phenotype is that they are dopaminergic cells with DA levels too low to be detected in most cells. It is conceivable that the absence of DA immunoreactivity in the large TH-ir cells of the posterior tubercle of lamprey may be due to that dopamine mainly accumulates in fibers as a consequence of a high rate of axonal transport and/or the location of AADC (the enzyme catalyzing the final step in dopamine biosynthesis) mainly in axons, whilst the first enzyme of the synthesizing pathway (TH) is distributed in all cell compartments. Discrepancy would mainly concern to the perikaryon and dendrites, not to axons, but further studies on this problem are clearly necessary.

DA+/TH±/DBH- populations

Most small catecholaminergic cells of the paraventricular organ and the CSF-c cells of the caudal rhombencephalon-spinal cord were of this type. Neuronal groups with similar phenotype were also reported in the hypothalamus of fishes (Meek et al., 1989; Ekström et al., 1990; Sas et al., 1990; Molist et al., 1993), amphibians

(González and Smeets, 1991) and reptiles (Smeets and Steinbusch, 1990) in a similar periventricular location (paraventricular organ). Different hypotheses might explain the presence of DA-containing cells lacking TH or with low TH levels:

- 1) The absence or its very low levels of TH might be secondarily caused either by negative feed-back produced by excess of DA or by gene inactivation during the differentiation (Molist et al., 1993). Results of developmental studies in trout (Manso et al., 1993) seem to rule out this explanation. Thus, CSF-c neurons of these ventricular organs do not express TH in early trout stages (embryos or early larvae), but some neurons can express it in late larval stages and adults. Our results in lamprey also disagree with this possibility, as judged by the early appearance of DA in this organ and the expression of weak TH immunoreactivity in some CSF-c neurons of large larvae, and the increasing amount of CSF-c TH-ir neurons found in adults.
- 2) The existence of two different TH enzymes has recently been reported in teleosts (Candy and Collet, 2005), and it might also occur in lampreys. Three tryptophan-hydroxylase genes, tphD1, tphD2 and tphR (the rate-limiting enzyme of serotonin biosynthesis) have been also found in zebrafish; interestingly, the three genes are expressed in different brain regions (Bellipanni et al., 2002; Teraoka et al., 2004). Therefore, it is possible that lampreys have two different TH enzymes and that they show differential expression in the brain, but the antiserum used only recognizes one of these enzymes. The fact that only in late stages some CSF-c neurons were faint TH-ir (see above) might be explained by the very low affinity of the anti-TH antibody used here for the TH enzyme expressed in these neurons, which only produces detectable cross-reaction when very high levels are present.

- 3) There may be a novel catecholaminergic substance similar to DA in these cells (Sas et al., 1990). However, microspectrofluorimetric studies in CSF-c cells of the dogfish appear to rule out this possibility (Rodríguez-Moldes et al., 1993).
- 4) DA present in these cells is picked up from the ventricular space (Sas et al., 1990; Smeets and Steinbusch 1990; Molist et al., 1993), which has also been hypothesized for the serotonin present in CSF-c cells (Marc et al., 1988). Based in the selective absorption of ^{3}H-DA by CSF-c cells in *Rana* (Nakai et al., 1977), and in results of inhibition experiments in reptiles that agree with hypothesis of the selective uptake of DA (Smeets et al., 1991), the most accepted opinion for TH-, dopaminergic CSF-c cells is the option 4, although it does not account for all our results in lamprey and those of Manso et al. (1993) in trout. Elucidation of the number of TH genes in lampreys and other vertebrates, and of the expression pattern of the genes in the CNS appears clearly necessary for explain some discrepancies between DA and TH immunostaining of cell populations.

Studies in the lamprey spinal cord have evidenced that CSF-c DA-ir neurons are part of synaptic circuits of the cord (Schotland et al., 1996). In addition, some occasional DA-ir and TH-negative cells were found in the ventral thalamus, isthmus and also some non-CSF-c DA-ir cells were observed in the spinal cord.

DBH-ir neurons

In this study, CSF-c, DBH-ir periventricular neurons were observed in the infundibular region, the M2-M5 nucleus of Schöber and in the caudal rhombencephalon of sea lamprey, despite that Pierre et al. (1997) were unable to detected DBH-ir cells in these regions in the river lamprey. The demonstration of NA immunoreactivity in the same two regions of adult *Lampetra fluviatilis* (Steinbusch et al., 1981) supports the

present finding of DBH-like immunoreactivity in sea lamprey, and suggests that both NA and DBH may occur in the same cell. Together with the Steinbusch et al. (1981) results, our results indicate that these infundibular and rhombencephalic cells may synthesize NA. DBH-ir cells have not been described in the diencephalon of other vertebrates (teleosts: Hornby and Piekut, 1990; Sas et al., 1990; Ekström et al., 1990, 1992; reptiles: Smeets and Steinbusch, 1989; mammals: Hökfelt et al., 1984), with the notable exception of the sturgeon (Adrio, 2002). Although CSF-c NA-ir neurons have been observed in the periventricular organ of reptiles, these cells were DBH-negative (Smeets and Steinbusch, 1989, 1990).

The discrepancy reported in reptiles between distribution of NA and its synthesizing enzyme (DBH) suggests dissociation in these infundibular cells between expression of noradrenaline synthesizing enzymes and NA transporters, which was also suggested for the river lamprey (Steinbusch et al., 1981), but it might not apply to sea lamprey. It is interesting to note that in sea lamprey DBH-immunoreactivity appears in infundibular cells from late prolarvae onwards. Our results with TH and DBH immunocytochemistry also suggest that catecholamine synthesizing enzymes and their transporters do occur in dopaminergic and noradrenergic CSF-c cells of primitive vertebrates, and that the absence or late expression of the synthesizing enzymes in these cells indicates a possibly specialization for store of catecholamines. Since the CSF-c DBH-ir neurons are co-distributed with the DA-ir population in the lamprey hypothalamus (present results), there exists the possibility that TH and DBH immunoreactivities were co-localized in the same cell.

DBH-ir cells were observed in the caudal rhombencephalon of the sea lamprey, which is in agreement with the presence of NA immunoreactive cells in the caudal rhombencephalon of all vertebrate groups (review in Smeets and González, 2000). Our

results in sea lamprey failed to reveal consistently a DBH-ir and/or TH-ir population in the rostral rhombencephalon (isthmus) that might correspond to the locus coeruleus of jawed vertebrates. Whether this is a primitive or derived feature of sea lamprey is not known.

Comparison with adult river lamprey

DA-ir neurons

The distribution of DA-ir groups observed in the adult sea lamprey is largely similar to that reported for the river lamprey (Pierre et al., 1997; Pombal et al., 1997), although there are some interspecies differences. Specifically, in the sea lamprey we did not find the DA-ir cells reported in the olfactory bulbs (Pierre et al., 1997; Pombal et al., 1997), and the optic tectum (Pombal et al., 1997) of the river lamprey. Accordingly, the numerous DA-ir fibers observed in the sea lamprey telencephalic lobes (subpallium, pallium, and caudal olfactory bulbs) appear to originate from preoptic/diencephalic DA-ir populations. Occasional DA-ir cells were observed in the ventral thalamus of the sea lamprey, whereas no DA-ir cells were observed in the ventral thalamus of river lamprey (Pierre et al., 1997). In this lamprey, DA-negative TH-ir cells were located in the dorsal thalamus and pretectum (Pierre et al., 1997). The DA-ir CSF-c cells observed in the ventral posterior reticular nucleus of the river lamprey (Pierre et al., 1997) appear to correspond with the rostral extension of the central canal DA-ir population of the sea lamprey that was found in large larvae and adults. These between-species differences in the DA-ir system suggest some divergence during the long evolutionary history of lampreys.

DBH-ir neurons

Three DBH-ir populations were observed in the brain of adult sea lamprey, which were located in the paraventricular organ, the mesencephalic M2-M5 nucleus of Schöber and the caudal rhombencephalon. As indicated above, this pattern of DBH-ir nuclei is very different of that reported in the river lamprey, in which DBH-ir cells only were observed in the nucleus isthmi rhombencephali dorsalis posterior (Pierre et al., 1997). In only one of our samples a DBH-ir cell was observed in this location. However, two of the DBH-ir populations of the sea lamprey, the paraventricular organ and the caudal rhombencephalon (present results), were also described as NA-ir in the river lamprey using an antibody to NA (Steinbusch et al., 1981). This strongly suggests that these two NA-ir brain populations are shared by sea and river lampreys.

The DBH-ir cell population in the M2-M5 nucleus of Schöber was not previously described. However, the presence of DBH-ir fibers passing from the head of the optic nerve in the adult retina had been already reported (Yáñez and Anadón, 1994).The origin of retinopetal fibers in lamprey was traced experimentally to the M2-M5 nucleus of Schöber in both the river and sea lamprey (Vesselkin et al., 1984; Rodicio et al., 1995). Present results, together with those of Yáñez and Anadón (1994) in the retina, strongly suggest the presence of NA-ir retinopetal cells in the M2-M5 nucleus of the sea lamprey.

Development of Catecholaminergic Systems

Comparison with previous reports in lampreys

Present results extend our knowledge on the development of the catecholaminergic system of lampreys to embryonic, prolarvae and early larvae periods. In sea lamprey larvae, two DA-ir groups have previously been reported (Yáñez et al.,

1992). The first group, composed of the ventral hypothalamic nucleus and the dorsal hypothalamic nucleus (paraventricular organ), was found in larvae ranging from 14 mm to 135 mm in length. Our results indicate that the paraventricular organ DA-ir group appears in late prolarvae. A second group of CSF-c DA-ir cells around the posterior infundibular recess (postinfundibular commissure nucleus) was only reported in large larvae. Our results indicate that this group appears in larvae of about 30 mm in length. The DA-ir groups in the paratubercular nucleus, the caudal preoptic and the postoptic commissure nuclei, the caudal rhombencephalon and spinal cord cells were not previously reported in larvae with DA immunocytochemistry. With TH immunocytochemistry, TH-ir cells were also observed in most of these nuclei, although in some of them they appear in larvae larger than those showing DA-ir cells (Yáñez et al, 1992; present results).

The development of the TH-immunoreactive systems was previously reported in the brain of the river lamprey (Pierre-Simons et al., 2002). This study has only included larvae from 29 mm long onwards (the larval stage begins with larvae about 9 mm long), which were subdivided into three groups, group I (29-54 mm), group II (55-80 mm) and group III (81-120 mm). In groups I and II, only the preoptic and paratubercular TH-ir populations were present, which is roughly similar to that observed in sea lamprey (present results), but the origin of these populations was not followed to early developmental stages. Similar to river lamprey adults, the group III larvae showed TH-ir cells in the olfactory bulbs, which was not the case for sea lamprey.

The time of appearance of the TH-ir preoptic and hypothalamic populations appears roughly similar in the two species of lamprey, although we have noted more gradation in appearance than that reported in river lamprey, were the remainder TH-ir brain populations do not appear until group III larvae. A striking difference between

river and sea lampreys is the appearance of TH-ir cells in the spinal cord of the river lamprey group II, while in the sea lamprey they do not appear until very large larvae. Since DA-ir cell appear in the spinal cord of sea lamprey prolarvae, between-species differences appear to be due to differential phenotype expression during development. Moreover, some TH-ir populations reported in the dorsal thalamus and in the rhombencephalon of the adult river lamprey were not observed at any developmental or adult stage of the sea lamprey.

Finally, our results showed that DBH-ir cells appear in the mesencephalic tegmentum of large larvae. By their location, these cells pertain to the M2-M5 nucleus of Schöber, which contains retinopetal neurons and develops very early in larval lampreys (Rodicio et al., 1995). The presence of these putative NA-ir cells in large larvae suggests that their fibers reach the retina during the larval period. Which role(s) may play NA in the larval and adult lamprey retina are not known.

Comparison with gnathostomes

This study shows that the DA-ir CSF-c cells of the spinal cord were the first catecholaminergic group to appear in sea lamprey (although TH immunoreactivity is not detected in these cells till premetamorphic larvae), being already present in early prolarvae. This result is similar to that reported using TH antibodies in *Xenopus* (González et al., 1994a, b) and an elasmobranch (Sueiro et al., 2003; Carrera et al., 2005). However, no DA-ir cells have been found in the spinal cord of both developing and adult teleosts investigated (Ekström et al., 1992, 1994; Manso et al., 1993) and in reptiles, spinal catecholaminergic cells appear at intermediate stages of the catecholaminergic system development (Medina et al., 1994a, b).

The second catecholaminergic nucleus by order of appearance in lamprey is the DA-ir posterior tubercle nucleus (paratubercular). The earliest catecholaminergic brain

nucleus of lamprey corresponds to the posterior tuberal nucleus of elasmobranchs (Carrera et al., 2005) and the posterior tuberal nucleus (Manso et al., 1993) or paraventricular organ accompanying cells (Ekström et al., 1992) of teleosts, which are the earliest catecholaminergic brain populations of these fishes (Ekström et al., 1992; Manso et al., 1993; Carrera et al., 2005), and may correspond to the common primordium of the dorsal infundibular nucleus and tegmental neurons of amphibians (Smeets et al., 1993; González et al., 1994a, b, 1995), that also develop very early (Ekström et al., 1992, 1994; Foster, 1994; González et al., 1994a, b; Medina et al., 1994a, b). It is also noteworthy that in the sheep the earliest TH-ir neurons appear in the region of the mesencephalic flexure (Tillet and Thibault, 1987), which suggests a highly conserved pattern of development in vertebrates.

Combined tract-tracing and immunohistochemical studies in river lamprey have shown that this nucleus gives rise to DA-ir projections to the striatum (Pombal et al., 1997), and this projection may be comparable to the meso-striatal DA-ir projections characteristic of amphibians (González et al., 1994c; Marín et al., 1995) and amniotes (see Medina and Reiner, 1995). However, unlike in amniotes, this projection is not massive and, as observed in sagittal and horizontal sections, the same nucleus gives rise to extra-striatal ascending projections and to descending projections to the brainstem. The mesostriatal DA-ir projection develops very early in reptiles (Medina et al., 1994a, b) and mammals (Specht et al., 1981a), which does not occur with the paratubercular nucleus-striatal projections in lampreys.

TH-ir cells appear in the preoptic nucleus by the same time than in the paratubercular nucleus, although DA immunoreactivity appears latter. On the contrary, in teleosts, amphibians, reptiles and birds TH-ir cells of the preoptic area appear later than in the posterior tubercle (Ekström et al., 1992, 1994; Manso et al., 1993; González

et al., 1994a, b, 1995; Medina et al., 1994a, b). Whereas in the suprachiasmatic nucleus of other vertebrates TH immunoreactivity appears by the same time than in the preoptic nucleus, in lampreys it appears latter. The CSF-c DA-ir cells of the paraventricular organ (dorsal hypothalamic nucleus) appear comparatively early in sea lamprey, as do TH-ir cells in the paraventricular organ other vertebrates (amphibians: González et al., 1994b; Medina et al., 1994a, b; elasmobranchs: Carrera et al., 2005).

DBH-ir cells appear in the paraventricular organ of lampreys by the same time as DA-ir cells do. As far as we are aware, no developmental studies of the hypothalamic DBH-ir cells were reported in other vertebrates, which preclude comparative analysis. At intermediate larval stages (30-40 mm), there appear DA-ir cells in the postinfundibular commissure nucleus (TH-ir, DA-ir, DBH-negative), which latter become continuous with the paraventricular organ population. This is similar to development of the paraventricular organ of trout, which arises by confluence of separated lateral recess nucleus, posterior recess nucleus, and hypothalamic vascular organ primordia (Manso et al., 1993). The last hypothalamic catecholaminergic group to appear is the ventral hypothalamic nucleus. The different time of appearance of hypothalamic groups in lampreys and the different cell morphologies of these groups, together, suggest that they actually form distinct neuronal populations.

In the caudal rhombencephalon, DBH-ir (noradrenergic) cells were observed from 20 mm larvae onwards. At early larval stages they were located near the sulcus limitans, but by the end of the larval period DBH-ir cells also appear in the reticular region of the basal plate, but always in periventricular position. Two NA-ir cell groups are present in the caudal brainstem of all gnathostomes studied, a ventrolateral tegmental group (A1) and a dorsomedial group located in the solitary tract nucleus/ area

postrema (A2). However, whether one or both of these caudal NA-ir groups correspond to lamprey caudal DBH-ir cells is not clear.

Occasional DA-ir cells were found in a position ventral in the isthmus of adult and premetamorphic sea lamprey, though not in earlier stages. In river lamprey, TH-ir cells but not DA-ir cells were observed in the dorsal isthmus (Pierre et al., 1997). NA-ir cells have been found in the isthmus region (locus coeruleus) of most vertebrates (elasmobranchs: Stuesse et al., 1991; teleosts: Ekström et al., 1986, 1992; Hornby and Piekut, 1988, 1990; Roberts et al., 1989; Batten et al., 1993; Manso et al., 1993; amphibians: González and Smeets, 1991; González et al., 1994, 1995; reptiles: Wolters et al., 1984; Smeets, 1988; birds: Guglielmone and Panzica, 1984; mammals: Specht et al., 1981b; Tillet and Thibault, 1987) and these cells appear very early in development (Ekström et al., 1992, 1994; Manso et al., 1993; González et al., 1994a, b; Medina et al., 1994a, b). The present results with TH and DBH immunocytochemistry in the sea lamprey isthmus cast doubt about the presence of a possible homologous of the locus coeruleus in this species.

In the sea lamprey, among the latest catecholaminergic cells to appear are those of the olfactory bulbs, though they were present in river lamprey group III larvae (Pierre-Simons et al., 2002). These neurons were also among the last to appear in elasmobranchs (Carrera et al., 2005), teleosts (Ekström et al., 1992; Manso et al., 1993), amphibians (González and Smeets, 1992; González et al., 1994, 1995), reptiles (Medina et al., 1994a, b), birds (Puelles and Medina, 1994) and rat (Specht et al., 1981a, b). Lampreys undergo complex morpho-physiological and behavioral changes at metamorphosis (Hardisty and Potter, 1971). The late expression of TH in neurons of the olfactory bulbs could be related with functional changes of the olfactory system in the transformation from larvae (sedentary, burrowing filter-feeders) to adults (free living

and parasitic). In trout, TH-ir neurons of the olfactory bulb first appear at the time when the viteline reserve of fry was exhausted and they have to search food actively (Manso et al., 1993).

In lampreys and teleosts, the mesencephalic tegmentum lacks DA-ir neurons, but mesencephalic DA-ir neurons are present in amphibians and amniotes, although they appear to have different origins from rostral and caudal regions (Smeets et al., 1993; González et al., 1994a, b, 1995). Elasmobranchs have also catecholaminergic cells in the midbrain, which are rather late-appearing (Carrera et al., 2005). Another difference between sea lamprey (present results) from that reported in sauropsids (Medina et al., 1994a, b; Puelles and Medina, 1994) is the lack of complex migratory movements of TH-ir cells during differentiation, suggesting that the lamprey catecholaminergic system has a simpler differentiation pattern.

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TABLES

Table 1

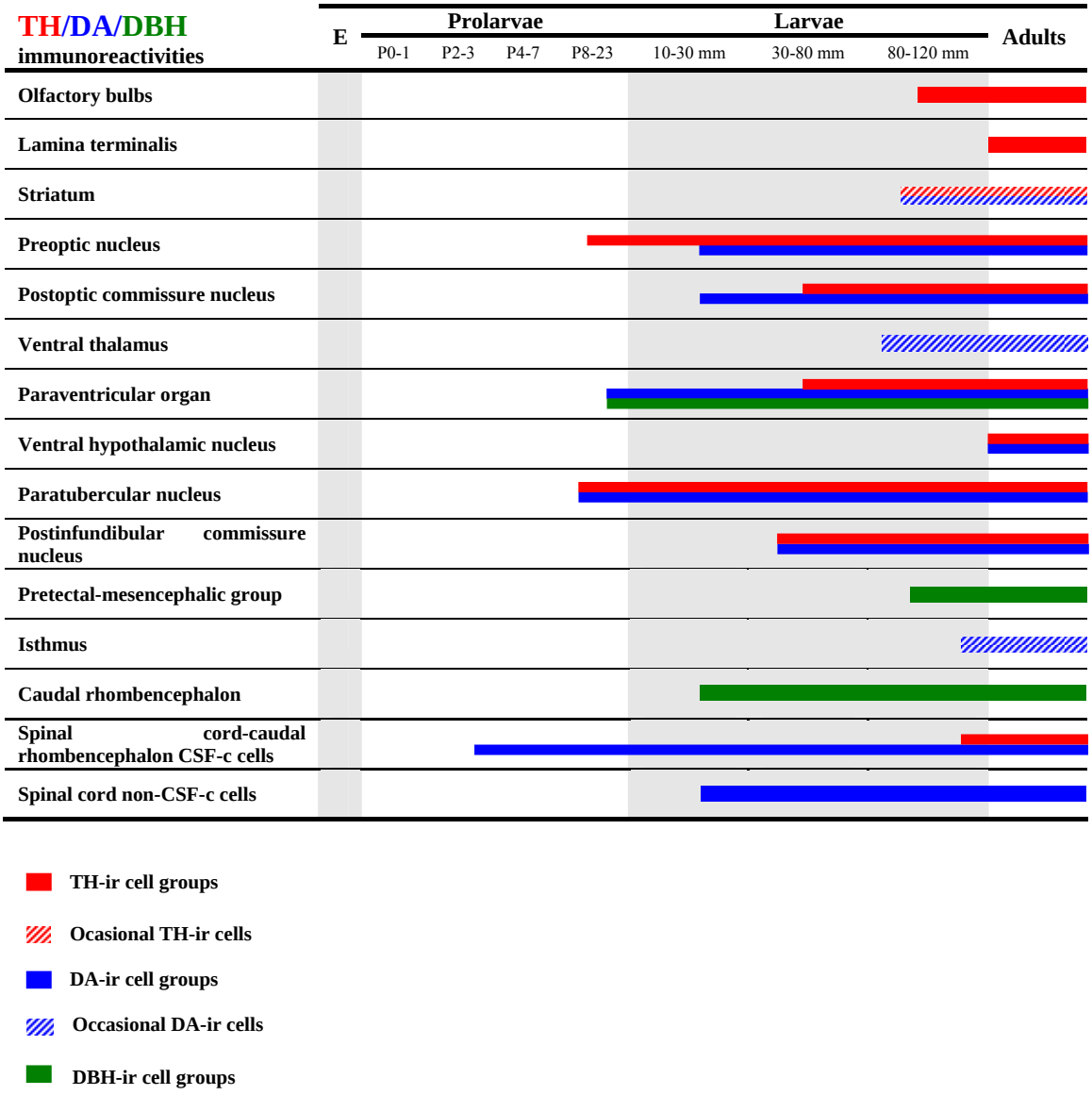


FIGURE LEGENDS

Table 1: Time of appearance of TH-ir, DA-ir, and DBH-ir cell groups in the central nervous system of the sea lamprey. Red stripes show TH-ir groups, blue stripes show DA-ir groups, dotted blue stripes show occasional DA-ir cells, and green stripes show DBH-ir cells.

Figure 1: Sagittal drawings of adult, larval, and prolarval brains of sea lamprey showing the TH-ir (red dots), DA-ir (blue dots), and DBH-ir (green dots) cell groups, and some putative limits into the brain. For abbreviations, see list. Scale bars: adult = 1 mm; larva = 500 μm; prolarva = 250 μm.

Figure 2: Photomicrographs of brain sections of adult sea lamprey showing TH-ir cell groups and fibers. **A**: Transverse section of the olfactory bulbs showing few faintly stained TH-ir cells (arrows). **B**: Transverse section of the lamina terminalis showing TH-ir bipolar cells (arrows). **C**: Transverse section of the suprachiasmatic region showing TH-ir cells of the caudal preoptic nucleus (arrow), some of them contacting with the ventricle (outlined arrow), and their distal processes (asterisks). **D**: Transverse section of the caudal hypothalamus showing TH-ir cells of the paratubercular nucleus (arrow), some of them contacting with the ventricle (outlined arrow), and their dendritic processes running dorsalward (asterisks). **E**: Transverse section of the caudal hypothalamus showing TH-ir cells of the postinfundibular commissure nucleus (arrows). **F**: Transverse section of the caudal rhombencephalon showing TH-ir cells (arrow). For abbreviations, see list. Scale bars = 100 μm.

Figure 3: Photomicrographs of brain sections of sea lamprey larvae showing TH-ir cell groups and fibers. **A, B**: Transverse sections of the preoptic region of 20 mm (A) and 60 mm (B) larvae showing few faintly stained TH-ir cells (arrows). **C, D**: Transverse sections of the caudal hypothalamus of 20 mm (A) and 60 mm (B) larvae showing TH-ir cells (arrows), and lateral fibers (asterisk). **E**: Transverse section of the dorsal hypothalamus of a 115 mm larva showing TH-ir cells of the paraventricular organ (arrow) and dendrites of these cells contacting with the ventricle (outlined arrow). **F**: Transverse section of the caudal hypothalamus showing TH-ir fibers in the hypophysis (asterisks). For abbreviations, see list. Scale bars: A, B, C, E = 50 μm; D = 100 μm; F = 25 μm.

Figure 4: Photomicrographs of brain sections of adult sea lamprey showing DA-ir cell groups and fibers. **A**: Transverse section of the telencephalon showing the DA-ir cells caudal preoptic nucleus located over the optic chiasm (arrow). **B**: Transverse section of dorsal hypothalamus showing DA-ir bipolar cells (arrow) and the processes to the ventricle (outlines arrow) of the paraventricular organ. **C**: Transverse section of the hypothalamus showing DA-ir cells (arrow) and their ventricular dendrites (outlined arrow) in the ventral hypothalamic nucleus around a lateral infundibular recess. **D**: Transverse section of the caudal hypothalamus showing DA-ir CSF-c cells of the postinfundibular commissure nucleus (arrow), and few pale DA-ir cells of the paratubercular region (arrowhead). **E**: Transverse section of the transition between the rhombencephalon and the spinal cord showing DA-ir CSF-c cells close to the ventricle (arrow) and non-CSF-c bipolar cells located more laterally (arrowhead). The asterisk points to the plexus of strongly DA-ir fibers that runs in the ventromedial zone, and the

star indicates the giant Müller axons. For abbreviations, see list. Scale bars: A, D, E = 100 μm; B, C = 50 μm.

Figure 5: Photomicrographs of sections of the sea lamprey CNS showing DA-ir cell groups. **A**: Sagittal section of the hypothalamus of a 31 mm larva showing the DA-ir cells in the paratubercular nucleus (outlined arrow), the paraventricular organ (arrow) and the postoptic commissure nucleus (outlined arrowhead), and DA-ir fibers in the neurohypophysis (double arrowhead). **B**: Transverse section of the preoptic region of a 119 mm larva showing DA-ir cells in the caudal preoptic nucleus (arrow). **C**: Transverse section through the postoptic commissure nucleus of a 119 mm larva showing DA-ir cells (arrows). **D**: Transverse section of the caudal hypothalamus of a P8 prolarva showing DA-ir cells in the paratubercular nucleus (arrows). **E**: Transverse section of the paratubercular region of a 119 mm larva showing DA-ir cells in the paratubercular nucleus (outlined arrows) and in the postinfundibular commissure nucleus (arrows). **F**: Transverse section of the paraventricular organ of a P16 prolarva showing DA-ir cells (black arrow) and fibers (white arrows). **G**: Transverse section of the spinal cord of a P8 prolarva showing a DA-ir CSF-c cell in the floor plate (arrow). For abbreviations, see list. Scale bars: G = 25 μm; D, F, B = 50 μm; A, E, C = 100 μm.

Figure 6: Photomicrographs of brain sections of adult sea lamprey showing DBH-ir cell groups and fibers. **A**: Transverse section of the diencephalon showing the small (black arrow) and large (outlined arrow) DBH-ir cells of the caudal paraventricular organ. **B**: Transverse section of the mesencephalon showing some DBH-ir cells (arrow). The asterisk indicates a Müller cell. **C**: Transverse section of the caudal rhombencephalon showing DBH-ir cells (arrows). **D**: Transverse section of the ventromedial

rhombencephalon showing DBH-ir fibers (asterisks). For abbreviations, see list. Scale bars = 50 μm.

Figure 7: Photomicrographs of sections of the larval lamprey brain showing DBH-ir cells (arrows). **A**-**D**: Transverse sections of a P23 prolarva (**A**), and 40 mm (**B**), 73 mm (**C**) and 92 mm (**D**) larvae showing DBH-ir cells in the paraventricular organ. **E, F**: Transverse sections of 90 mm (**E**) and 115 mm (**F**) larvae showing DBH-ir cells in the caudal rhombencephalon. For abbreviations, see list. Scale bars: A, B = 50 μm; C-F = 100 μm.

ABBREVIATIONS

CC; central canal

Ch; optic chiasm

DTh; dorsal thalamus

DV; diencephalic ventricle

Hyp; hypophysis

I; isthmus

IR; infundibular recess

LIR; "lateral" infundibular recess

LP; lateral pallium

LT; lamina terminalis

M; mesencephalon

M1-3; Müller 1-3 cells

MI; Müller isthmic cell

MP; medial pallium

OB; olfactory bulbs

ON; optic nerve

OT; optic tectum

P: pineal complex

PCNd; postoptic commissure nucleus, pars dorsalis

PCNv; postoptic commissure nucleus, pars ventralis

PCN; postoptic commissure nucleus

PiCN; postinfundibular commissure nucleus

PON; preoptic nucleus

PoC; postoptic commissure

PoR; preoptic recess

PopR; postoptic recess

PR; posterior recess

Pt; pretectum

PTl; primordium telencephali

PTN; paratubercular nucleus

PVO; paraventricular organ

Rh; rhombencephalon

RV; rhombencephalic ventricle

SC; spinal cord

Str; striatum

T; telencephalon

TL; telencephalic lobe

TrN; trigeminus motor nucleus

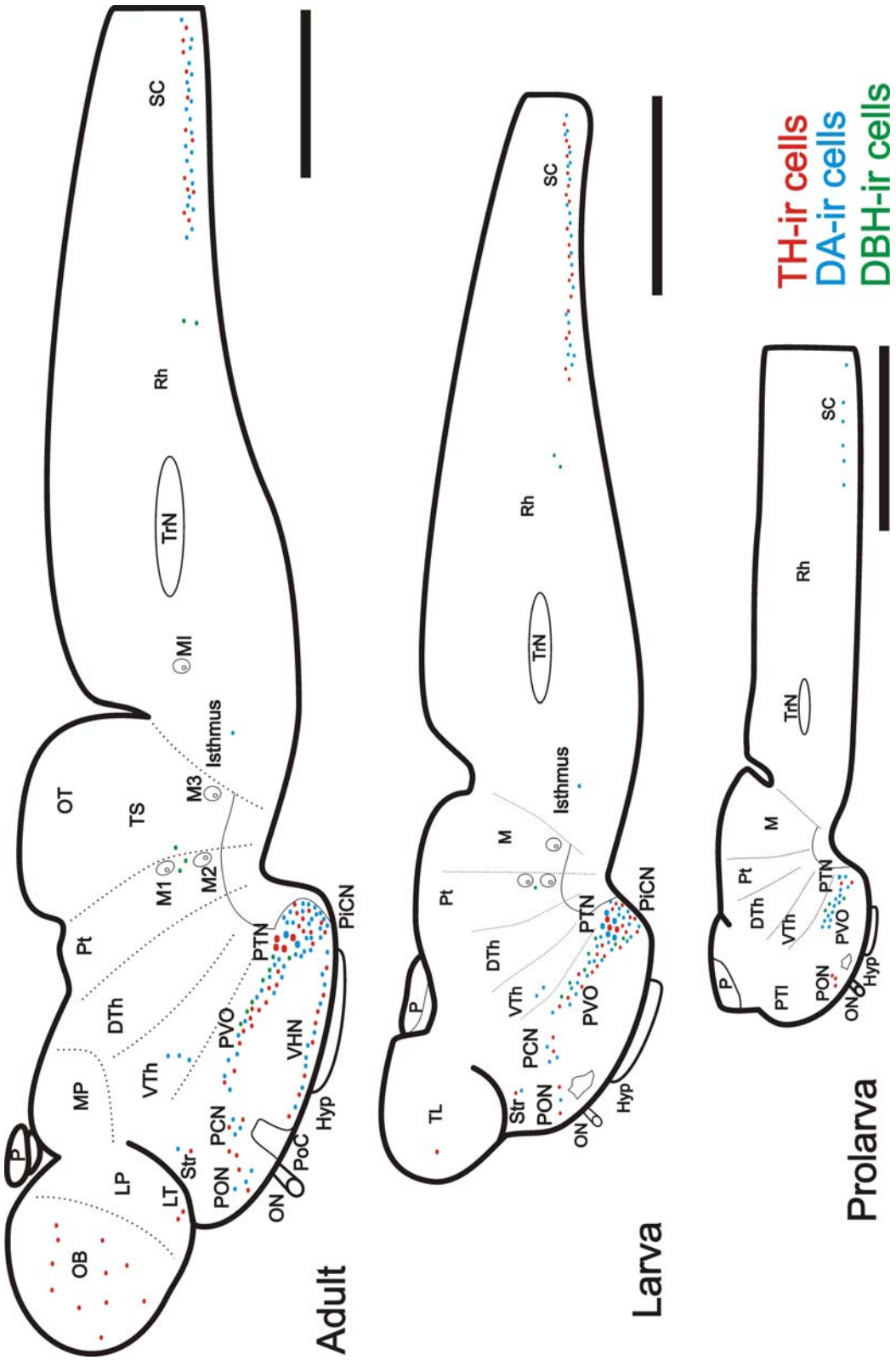
TS; torus semicircularis

VHN; ventral hypothalamic nucleus

VTh; ventral thalamus

FIGURES

Figure 1



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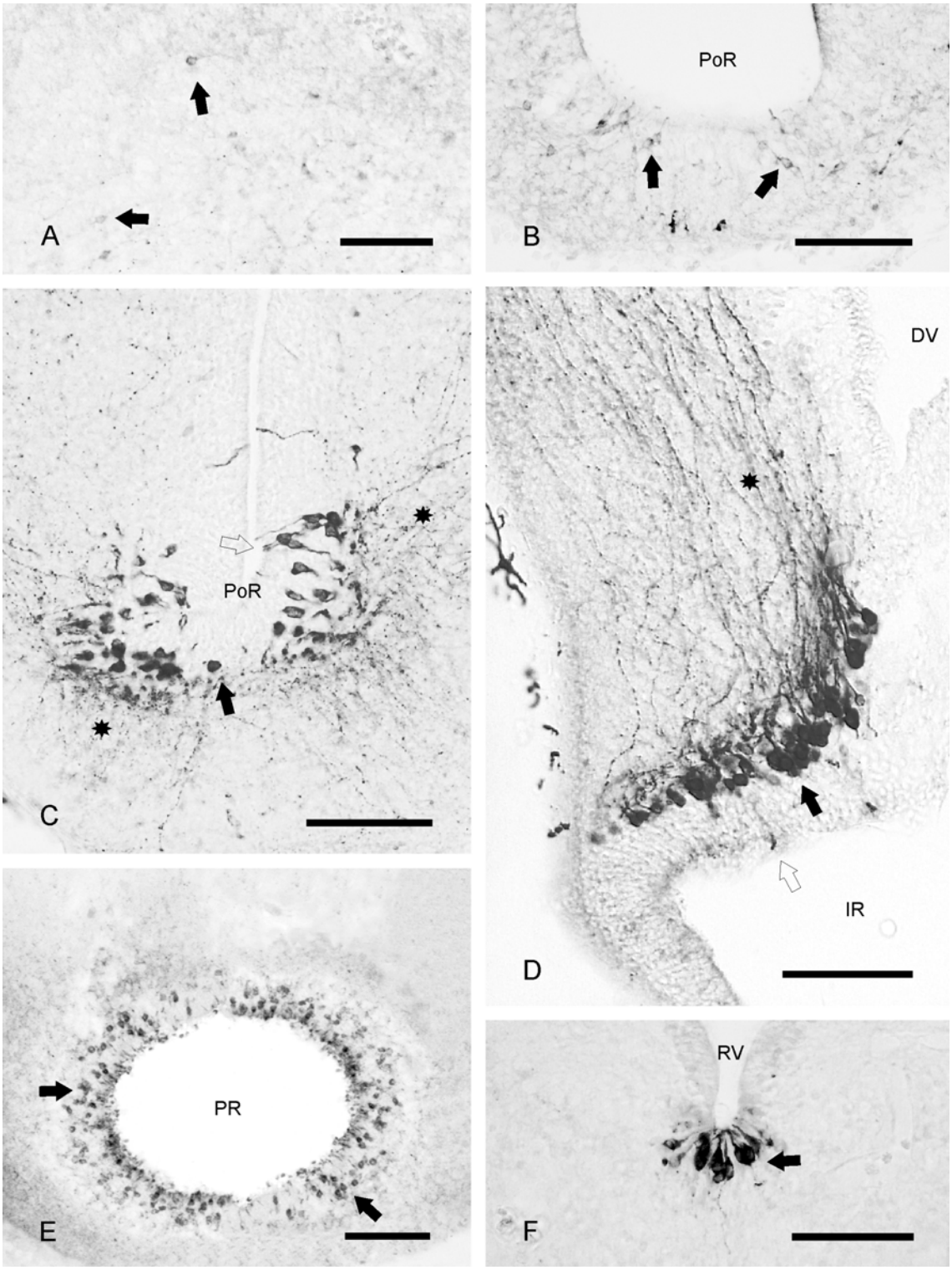

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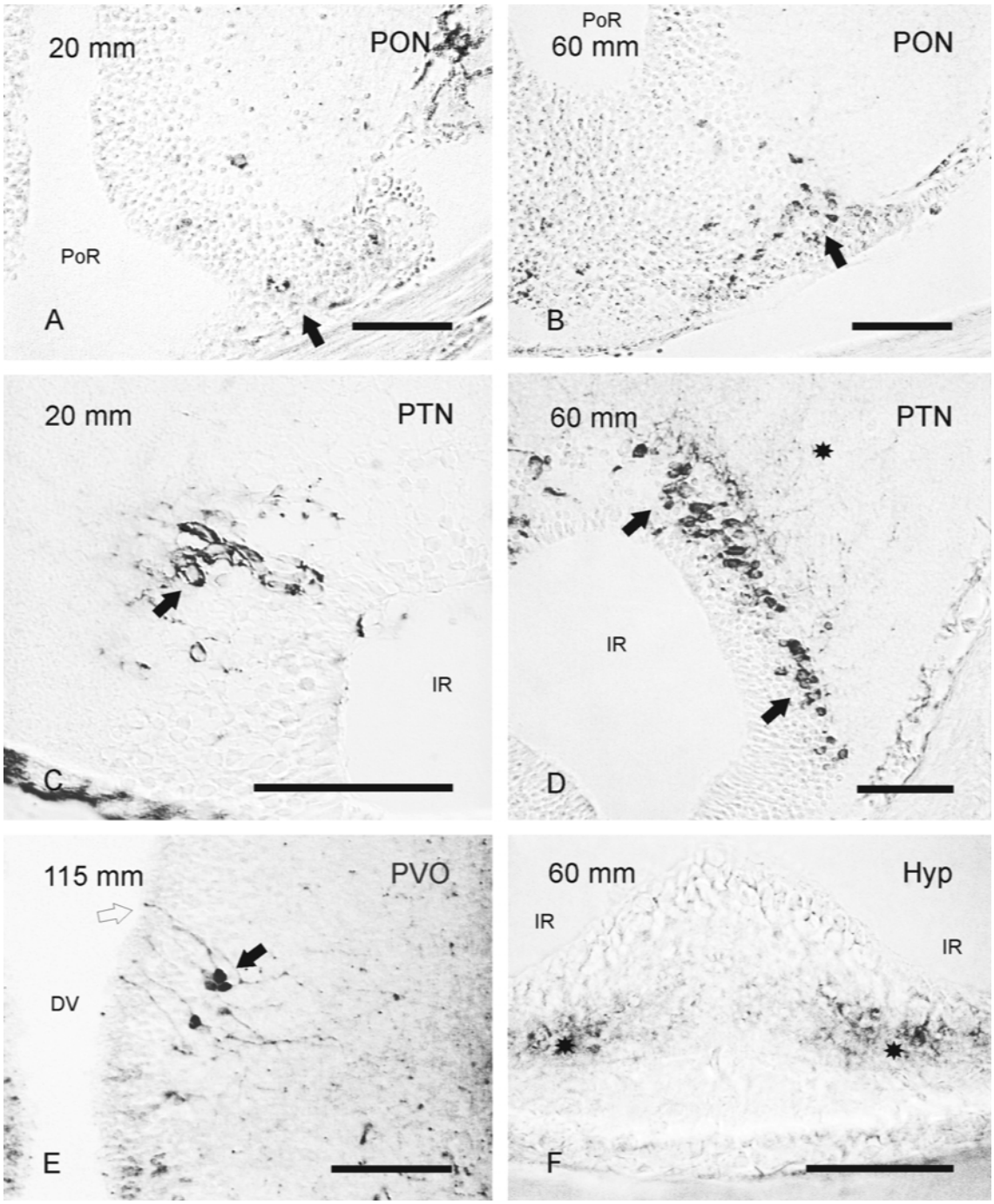
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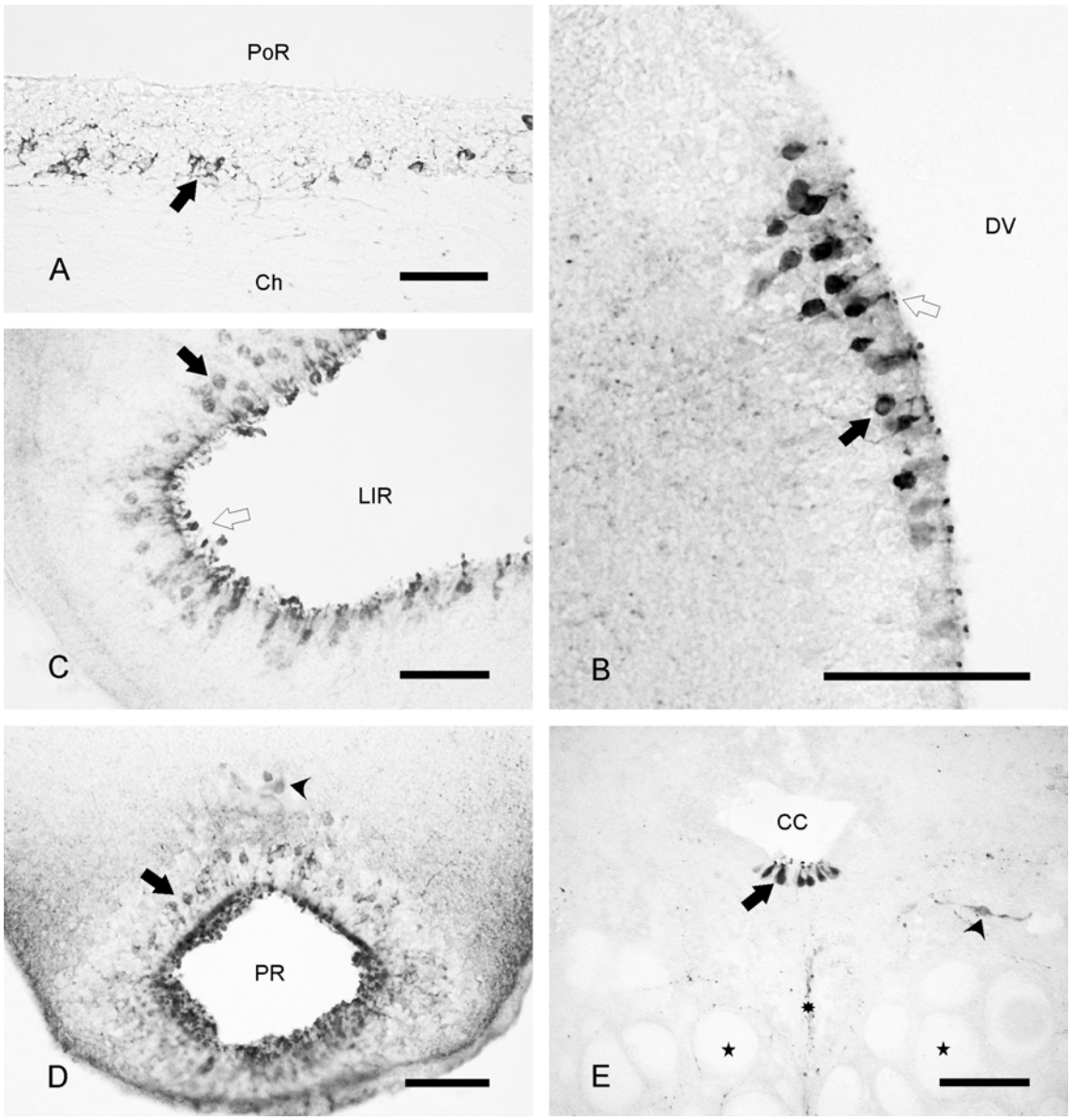
Figure 4



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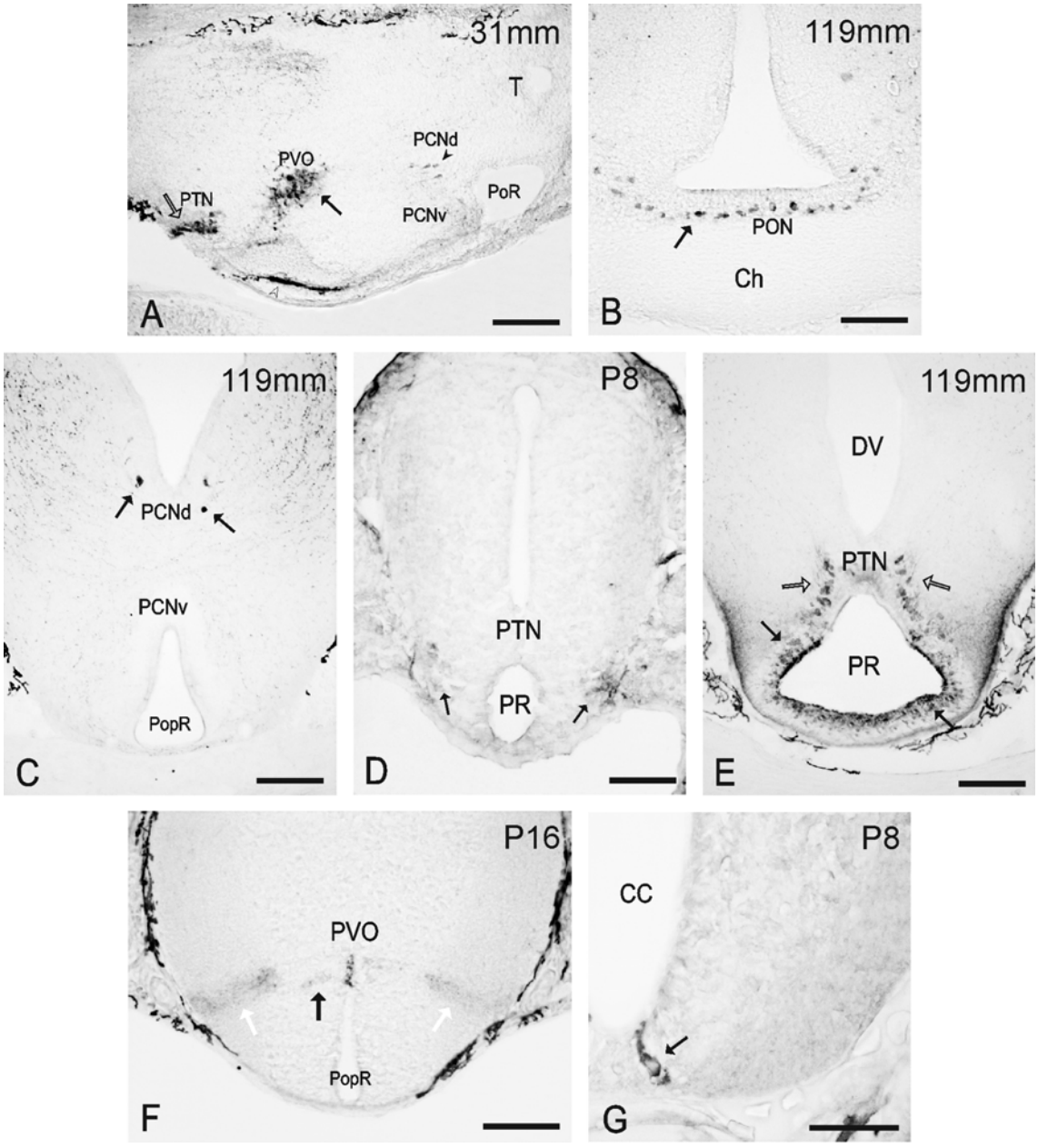
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Figure 6

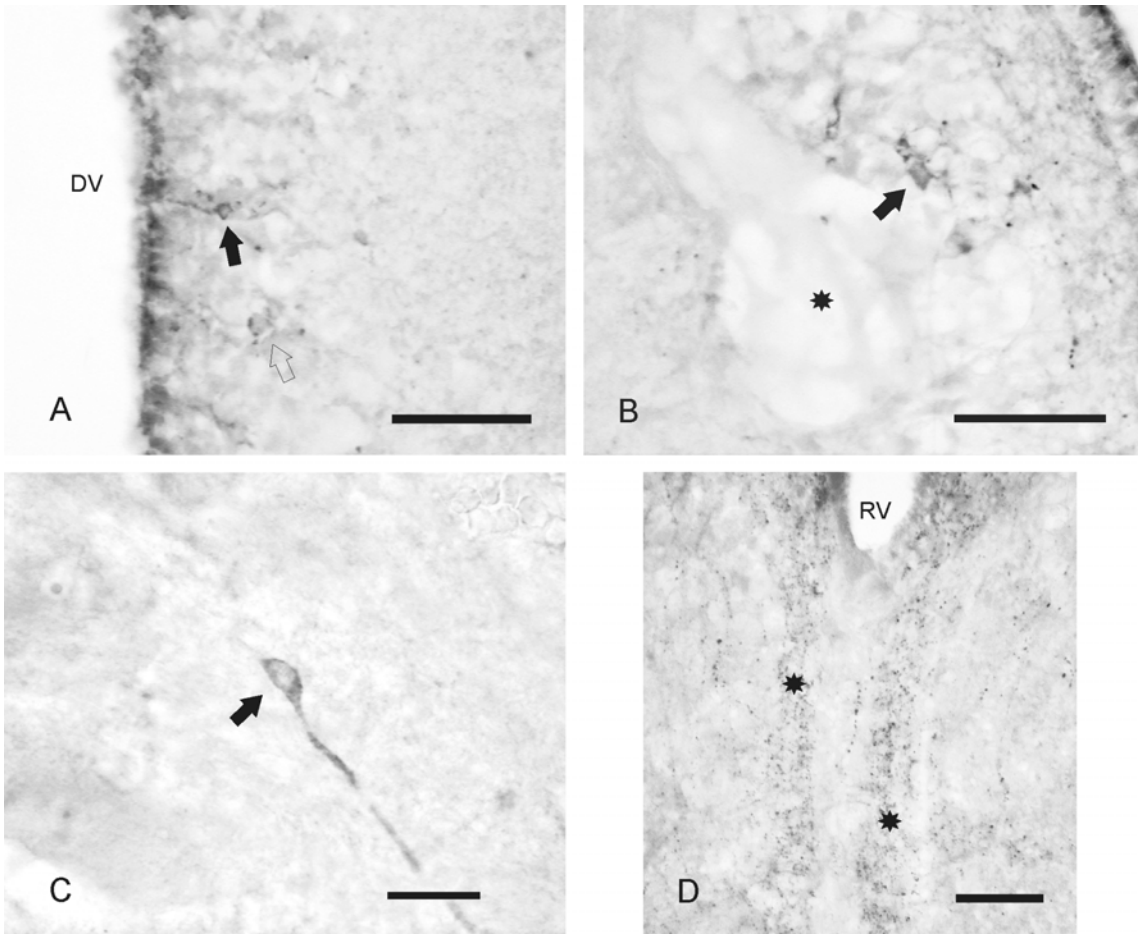
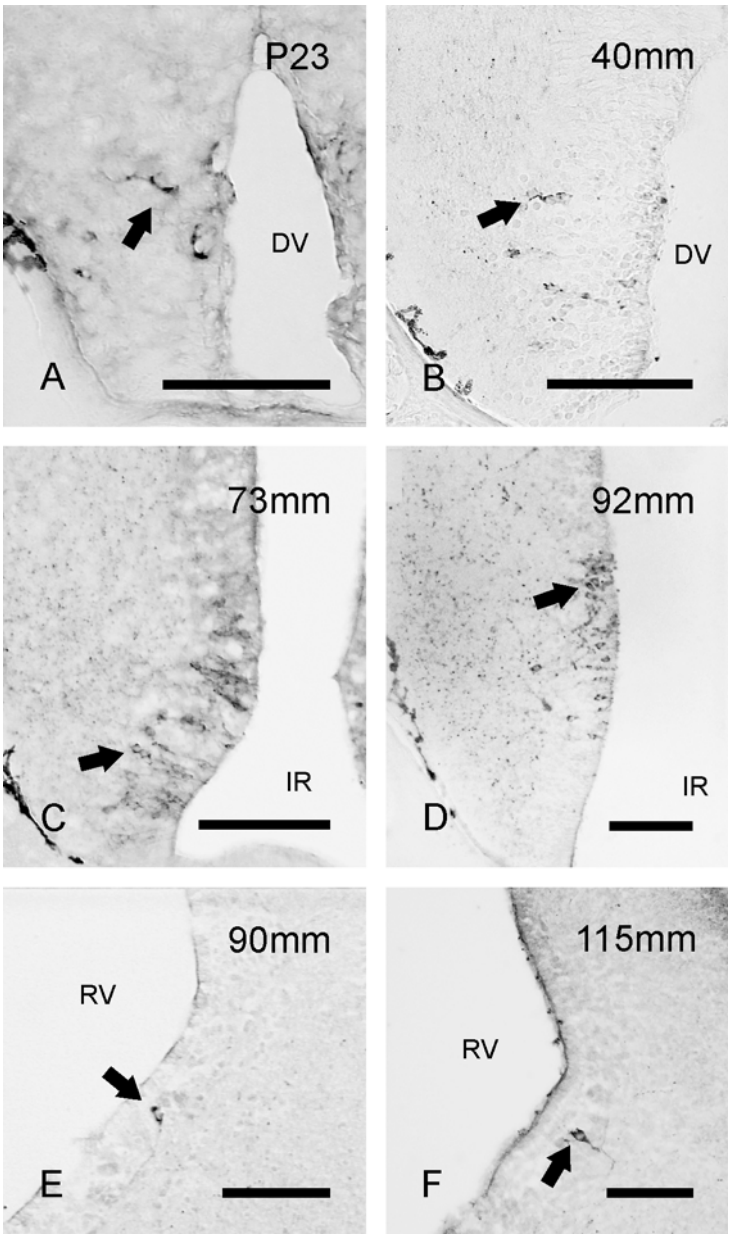
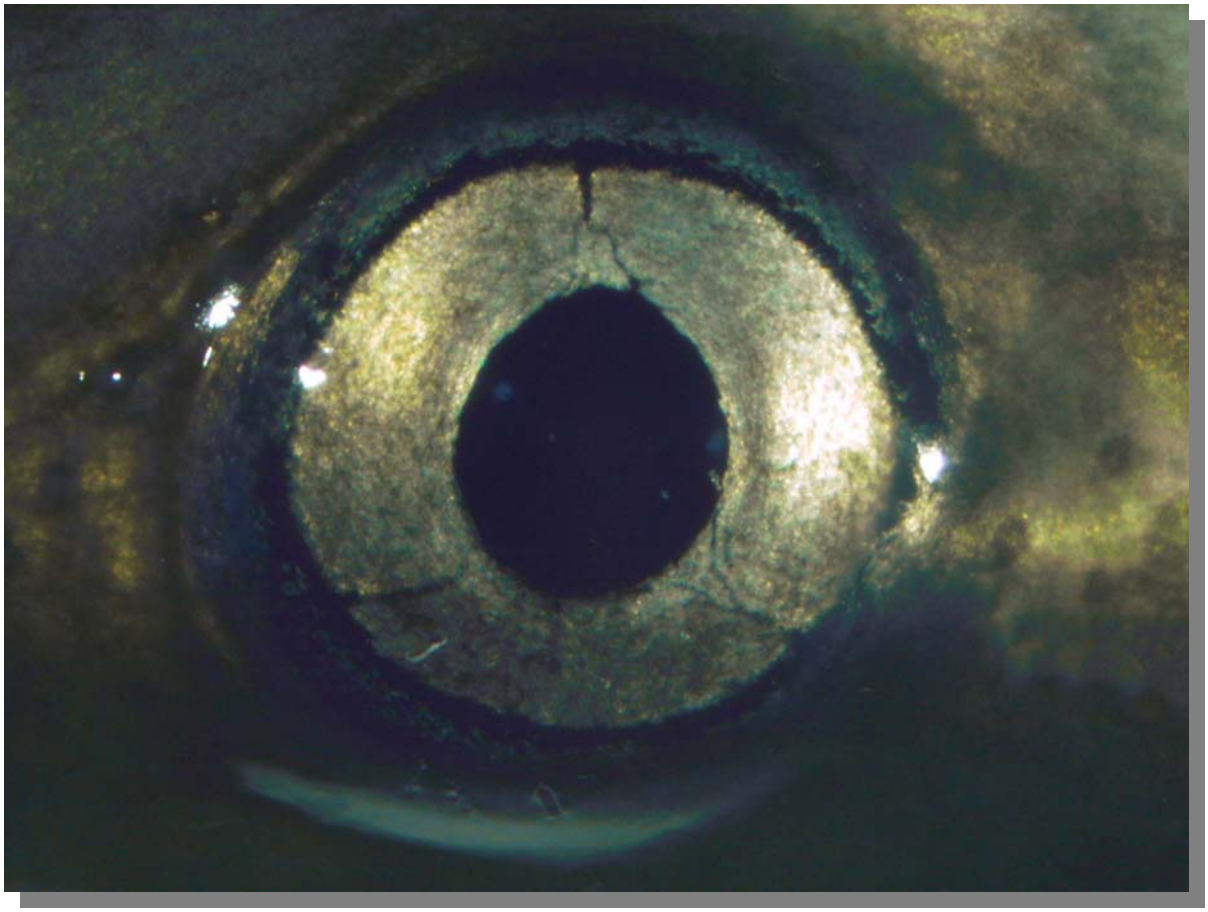


Figure 7





Chapter 3

Chapter 3

Neurochemical Changes in the Retina of the Sea Lamprey (*Petromyzon marinus*) during Metamorphosis: A Serotonin, Tyrosine Hydroxylase, Dopamine, and Choline Acetyltransferase Immunocytochemical Study

ABSTRACT

Lampreys belong to the most ancient group of extant vertebrates, the Agnathans. Characteristically, lampreys have a long larval stage very different from the adult, passing through a complex metamorphosis to achieve the adult appearance. The larval retina is an interesting model for developmental studies because of its biphasic development. The first phase begins in prolarvae and gives rise to a differentiated central retina, while in the second phase growth around the central retina during the middle-late larval period produces a wide neuroblastic lateral retina that differentiates during metamorphosis. Here, we study the neurochemical maturation of the sea lamprey retina during metamorphosis by using antibodies against serotonin, tyrosine hydroxylase, dopamine, and choline acetyltransferase. In the adult sea lamprey retina amacrine cells immunoreactive to all these substances were present. However, no structure immunoreactive to any of these substances was observed in the retina of larvae, either in the central or the lateral region of the retina. The sea lamprey metamorphosis was subdivided by Youson and Potter (1979) from stages M1 to M7. Serotonergic and cholinergic structures were first observed at M1 stage, while the appearance of catecholaminergic structures is delayed until mid-metamorphosis (M3). The immunopositive cells to all these substances were described as amacrine cells, since

no immunoreactive fibers were observed in the outer plexiform layer or in the outermost inner nuclear layer. The order of appearance of serotonergic, cholinergic and catecholaminergic cells in the metamorphic retina recalls the order of appearance of these systems observed in the brain of sea lamprey prolarvae and larvae. The delayed maturation of amacrine cells in the lamprey retina is unique in vertebrates and can be related to the peculiar cycle of life of this specie.

INTRODUCTION

Lampreys are considered the sister group of jawed vertebrates. Because of the phylogenetic position of lamprey, their study is a key to understanding the development and phylogeny of vertebrates. The development of the lamprey eye and retina is exceptional among vertebrates and it is conditioned by the special life cycle with very different larval and adult stages. In the anadromous sea lamprey (*Petromyzon marinus* L.), the short embryonic (11-12 days after fertilization) and prolarval (22-24 days after hatching) stages, are followed by a long filter-feeding larval stage or ammocoetes (4-7 years long) that lives buried in the bottom of the rivers were they were born. Larvae undergo a complex metamorphosis (about 4 months) to give adult predatory individuals (Hardisty and Potter, 1971) that descend to the sea to grow before to regain the river to reproduce and dye. Lamprey larvae are considered blind, their inconspicuous eyes are located under thick skin layers and probably work only as ocellus-like photoreceptor organs, while adults have well-developed eyes with a fully functional retina (de Miguel and Anadón, 1987). Therefore, maturation of the larval visual system appears to occur during metamorphosis, as indicated by the complete rearrangement of the retina.

The early development (Meléndez-Ferro et al., 2002) and the proliferation and differentiation of the lamprey retina were recently studied (Villar-Cheda, 2005). The retinal development begins in embryos with the evagination of the prosencephalic floor giving the optic vesicles, and their posterior development into rudimentary lateral eyes in prolarval stages. Cell differentiation already begins in the prolarval retina, as shown by the appearance of proliferating cell nuclear antigen (PCNA)-negative cells and opsin-positive photoreceptors (Meléndez-Ferro et al., 2002).

In larval stages, the eyes are covered by a thick non-transparent skin and have an immature lens, indicating that it is not an image-forming eye (Kleerekoper, 1972),

which is comparable to the pineal organ (Meléndez-Ferro et al., 2002). The larval retina consists of an early differentiated small central zone, surrounding the optic nerve head, which remains almost unchanged through larval life, and of a lateral zone that grows since 60-mm larvae and mainly consists of a thick neuroblastic layer (de Miguel and Anadón, 1987). The larval differentiated zone has a single type of photoreceptor (de Miguel and Anadón, 1987; Rubinson and Cain, 1989; Villar-Cheda, 2005), and bipolar and ganglion cells, while in the lateral retina only ganglion cells could be observed (de Miguel et al., 1989; Meléndez-Ferro et al., 2002; Villar-Cheda et al., 2006; Villar-Cerviño et al., accepted). The larval eye appears to be a simple photoreceptor organ: selective illumination of one lateral larval eye consistently evoked a negative phototactic reaction, i.e. a turning movement away from the light source followed by locomotion (Ullén et al., 1993).

From 60 mm larvae onwards, the retina grows by lateral extension of the undifferentiated retina, that in large larvae becomes subdivided into a marginal germinal zone and a more thickened intermediate differentiating zone, where ganglion cells are early differentiated and give rise to retinofugal projections (de Miguel and Anadón, 1987; de Miguel et al., 1990b). By the end of the larval stage, three different zones can be distinguished in the retina.

The differentiation of the larval lamprey retina does not occur until the metamorphosis. Youson and Potter (1979) divided the lamprey metamorphosis in 7 stages (M1-M7) attending to the eye transformation, and other external features. The layering and differentiation of the lateral retina and the appearance of the two types of adult photoreceptors occur during metamorphosis (de Miguel and Anadón, 1987). Therefore, the whole retina develops into an efficient visual organ which possesses capacity to form images and send visual information to the brain; the eyes differentiate

photoreceptors, retinal layers, and neuronal connections and acquire vision (Rubinson and Cain, 1989; Rubinson, 1990).

The use of immunohistochemical procedures is useful to demonstrate the classical neurotransmitters or their synthesis/degradation enzymes in retinal cells. These techniques have clearly demonstrated the existence of various neuroactive substances in distinct morphological types of amacrine cells (Karten and Brecha, 1983). Specific antibodies have been used to detect serotonin (5-HT), tyrosine hydroxylase (TH), dopamine (DA), or choline acetyltransferase (ChAT) in retinas of vertebrates. Previous studies in lampreys have analyzed the presence of several classical neurotransmitters in larval (Anadón et al., 1998; Pombal et al., 2003; Villar-Cerviño et al., accepted) and adult (Negishi et al., 1983, 1986; de Miguel et al., 1990a; Versaux-Botteri et al., 1991; Rio et al., 1993; Yáñez and Anadón, 1994; Pombal et al., 2003; Rio et al., 2003) retinas. In larvae, the presence of GABA immunoreactive retinopetal fibers (Rodicio et al., 1995; Anadón et al., 1998; Meléndez-Ferro et al., 2002), and of some glutamate and glycine immunoreactive cells and fibers were observed in the central retina (Villar-Cerviño et al., accepted), while no immunoreactivity to choline acetyltransferase (Pombal et al., 2003), serotonin or dopamine (Villar-Cerviño et al., accepted; present results) was found. Otherwise, all of these substances were observed in retinal cells of adult lampreys (Negishi et al., 1983, 1986; de Miguel et al., 1990a; Versaux-Botteri et al., 1991; Yáñez and Anadón, 1994; Pombal et al., 2003). Whereas these studies suggest that neurochemical maturation of the retina occurs during metamorphosis, the sequence of appearance of immunoreactivity to these classical neurotransmitter systems in the lamprey retina was not known. The aim of this study is to shed light on the neurochemical differentiation of sea lamprey retina during metamorphosis, reporting the

appearance of the immunoreactivity to serotonergic, dopaminergic and cholinergic markers.

MATERIALS AND METHODS

Animals

Larvae, metamorphic individuals (M1, M2, M3, M5, M6, M7), and postmetamorphic and upstream migrating adults of sea lamprey (*Petromyzon marinus*) were used in the experiments. Larvae were staged by total body length, whereas metamorphic individuals were staged according to Youson and Potter (1979). Larvae were obtained by digging the banks of the River Ulla (Galicia, Northwest Spain) and kept in an aerated aquarium until processing. Postmetamorphic individuals are down-stream migrating young adult lampreys that go to the sea, and were provided for the "Estación Biolóxica de Ximonde" that belongs to the "Xunta de Galicia". The up-stream migrating adults are individuals that come back to the river to reproduce, and were purchased from a local supplier. Before experiments, all specimens were deeply anesthetized with 0.05% benzocaine (Sigma, St. Louis, MO) and killed by decapitation. Larvae were processed without to remove the eyes, but the eyes of metamorphic and adult specimens were dissected out, carefully opened to remove the lens, and fixed either in cold 4% paraformaldehyde in 0.1 M Tris-buffered saline pH 7.4 (TBS) for 4-6 h (for serotonin, TH or ChAT detection), or in cold 5% glutaraldehyde in TBS containing 1% sodium metabisulfite overnight (for DA detection). Samples were cryoprotected by immersion in a 30% sucrose solution in TBS overnight before to embedding them in Tissue Tek (Sakura, Torrance, CA), freezing with liquid nitrogen-cooled isopentane, and cutting sections on cryostats (Leica 1800 and Leica CM 1850) at 14-18 µm thick. The sections were stuck on gelatinized slides and dried. After the

immunocytochemical procedure, the sections were dehydrated and mounted with Eukitt (Panreac). All experiments were conducted in accordance with European Community guidelines on animal care and experimentation to minimize pain and discomfort.

Immunocytochemistry

After washing in TBS, the sections were developed by the peroxidase-antiperoxidase (PAP) method or by immunofluorescence. For the PAP method, the samples were pretreated with 10% H_2O_2 in Tris-buffered saline (TBS) for 30 min to eliminate endogenous peroxidase activity. The PAP method involves a sequential incubation in: (1) one of the polyclonal primary antibodies raised in rabbit [anti-serotonin (Incstar, Stillwater, MN; 1:5000), anti-DA (Steinbusch, The Netherlands; 1:900), anti-TH (Chemicon, Temecula, CA; 1:1000)] overnight at room temperature, or in goat anti-ChAT (Chemicon, Temecula, CA; 1:100) for two days at 4ºC. (2) Secondary goat anti-rabbit antibody (Sigma; diluted 1:100) for serotonin, TH and DA detection, or rabbit anti-goat antibody for ChAT detection (Chemicon; diluted 1:50), for 1 h at RT. (3) Rabbit PAP complex (Sigma; diluted 1:500), or goat PAP complex (Chemicon; diluted 1:600), for 1 h at RT. (4) To reveal the immune complex the samples were finally incubated with 3-3´diaminobenzidine (Sigma; 0.5 mg/ml) and 0.01% H_2O_2 in 0.05 M TBS for 1-20 min at RT. For immunofluorescence detection of serotonin, TH, and DA, the sections were incubated in primary rabbit antibody as in PAP method step 1 (see above), and then incubated with FICT-conjugated or rhodamine-conjugated goat anti-rabbit-antibodies (Dako; 1:30) as secondary antibodies.

The sections were rinsed three times in TBS (10 min each) before each step. All antibodies and the PAP complex were diluted with 0.2% Triton X-100 and 3% normal goat serum (Vector Laboratories, Burlingame, CA) in TBS. The specificity of primary

antibodies was thoroughly tested by the suppliers. As a negative control, the primary antiserum was omitted from some sections of each specimen in each experiment. This resulted in no immunostaining at all.

In addition, some sections of different retina stages were stained with haematoxylin-eosin for topographical purposes.

Photographs were taken in an Olympus AX-70 photomicroscope equipped with a color digital camera (Olympus DP-12). Images were adjusted for contrast and brightness with Corel Photo-Paint 12 (Corel, Ottawa, Canada), and photomontage and lettering were done using Corel Draw 12.

RESULTS

Here, we will follow the nomenclature of Fritzsch and Collin (1990) for the adult retina, though somewhat simplified.

General organization of the adult lamprey retina (Fig. 1A)

The adult lamprey retina contains two photoreceptor types, short and long (Ishikawa et al., 1987; Negishi et al., 1987; Collin et al., 2003). In addition, as in other vertebrates, it shows bipolar cells, horizontal cells, amacrine cells, and ganglion cells, as well as retinopetal fibers and Müller cells (Rubinson and Cain, 1989; Fritzsch and Collin, 1990; Rio et al., 1993, 2003). However, layering of lamprey retina is somewhat different to that observed in jawed vertebrates (Öhman, 1976; Fritzsch and Collin, 1990). The most striking feature of lamprey retina is that there is not a defined ganglion cell layer. Lampreys have a presumably primitive ganglion cell organization compared to that of gnathostomes, with ganglion cells in two separate sublayers into the inner nuclear layer (INL) and the inner plexiform layer (IPL) (Dalil et al., 1990; Fritzsch and

Collin, 1990; Rio et al., 1998). The IPL can be subdivided into outer (oIPL) and inner (iIPL) sublayers. Other distinctive features are that the optic fiber bundles course in the IPL (Dalil et al., 1990; Fritzsch and Collin, 1990), immediately adjacent to the INL (Holmberg, 1978), and the INL is thick and complex, consisting of two outer horizontal cell layers, and an inner layer with the perikarya of bipolar cells, amacrine cells, and most ganglion cells (Fig. 1A).

Immunoreactivities to 5HT, DA, TH and ChAT in the adult retina of ***P. marinus***

Serotonin (5HT)

In adult lampreys, perikarya of serotonin-immunoreactive (5HT-ir) amacrine cells were observed in the inner part of the INL (Fig. 1B) and, occasionally, close to the inner limiting membrane. The morphology of 5HT-ir cells was not homogeneous (Fig. 1B). In vertical sections, were either pear-shaped perikarya included in the INLi, or bipolar or flattened and located in the limit between the INL and the IPL. In tangential sections, bipolar or triangular morphologies were appreciable. The 5HT-ir perikarya sent processes that could be easily followed to the IPL and that, as observed in vertical sections, were stratified in two subplexuses into the iIPL and oIPL (Fig. 1B). 5HT-ir processes extended far laterally from the perikaryon, so that only in favorable instances the process could be followed for a considerable way. In a number of instances, branches of the same process could be followed to both fiber subplexuses, indicating that at least some cells are bistratified processes. However, the possibility that some 5HT-ir cells contact only one or other subplexus cannot be ruled out. No process from 5HT-ir cells was observed coursing to the outer plexiform layer.

Dopamine (DA) and tyrosine hydroxylase (TH)

Since no obvious differences between the pattern of distribution of TH and dopamine immunoreactivity was found in the adult lamprey retina, it is assumed that all the TH-ir cells are dopaminergic. Accordingly, the observations with both dopaminergic markers are presented together. In adult retina, TH-ir and DA-ir cell perikarya were observed scattered in the innermost part of the INL (Fig. 1C). They were fairly less abundant than serotonergic cells, and different of TH or DA immunopositive cells between distinct regions of the adult retina were not observed. They showed mostly bipolar and polygonal perikarya (Fig. 1C). Thin immunoreactive processes could be observed coursing tangentially in the innermost INL and the outer part of the IPL, and occasionally in the inner IPL sublayer. No immunoreactivity to TH or DA was observed in outer retinal layers (OPL, ONL, and photoreceptor layer) or in processes ascending in the INL toward these layers.

Choline acetyltransferase (ChAT)

ChAT immunocytochemistry revealed that ChAT-immunoreactive (ChAT-ir) cells are mostly located in the inner sublayer of the IPL (Fig. 1D-F). Vertical sections of the retina of adult *P. marinus* showed bipolar or multipolar ChAT-ir perikarya. Some cells located in the outer region of the IPL are often extremely flattened. In tangential sections and in whole mounts, bipolar, triangular or stellate morphologies were also appreciable. ChAT-ir processes were rather thin, sometimes branching. In radial sections most ChAT-ir processes occupied a central region of the IPL, but a thin ChAT-ir sublamina was sometimes distinguishable external to the main band of ChAT-ir processes.

Morphological changes during metamorphosis of the sea lamprey retina

In early stages of metamorphosis (M1 and M2), the neural retina consists of four cell layers; a primordial inner plexiform layer band, and two compact neuroblastic layers (outer and inner) separated by a thin layer of primordial horizontal cells (Fig. 2A). The presence of photoreceptors in these stages allows to distinguishing the small central region (with differentiated larval photoreceptors) from the lateral retina (without photoreceptors), whereas the appearance of the other retinal layers was rather homogeneous throughout most parts of the retina (de Miguel and Anadón, 1987). In intermediate stages of metamorphosis (M3-M5), the IPL and the horizontal cell layer of the INL have thickened, and the horizontal cells were enlarged and flattened in the tangential plane, forming a distinct cell band between the outer nuclear layer and the inner part of the INL (Fig. 3A). The differentiation of opsin-expressing photoreceptors in the lateral retina starts in M3 (Villar-Cheda, 2005) and extends progressively to all the extension of the retina, which in M5 shows morphologically identifiable photoreceptors throughout (de Miguel and Anadón, 1987; Villar-Cheda, 2005).

At late stages of metamorphosis (M6 and M7), the retina has acquired an adult-like organization. The neural retina exhibited a well-layered pattern, with photoreceptor, outer nuclear, outer plexiform, horizontal cell, inner part of the INL, and IPL layers (Fig. 4A). Photoreceptors were present over the entire neural retina, though they were longer in M7 than in M6.

Between the late transforming stages and the upstream-migrating adults, the eye diameter and retinal area increase very considerably, but the increase in retinal area is paralleled by a thinning of most retinal layers. Therefore, in the M7 retina the inner retinal layers were thicker than in upstream-migrating adults, facilitating topographical

analysis of structures. Whole metamorphic changes in retina and optic tectum complete the functional development of the visual system and provide for the adult lamprey's predatory and reproductive behavior (Rubinson, 1990).

There are dividing cells in the retina during metamorphosis, as revealed by PCNA immunocytochemistry, while no PCNA-ir cells were observed in the retina of young lampreys and upstream-migrating adults. This indicates the absence of significant proliferation in postmetamorphic stages, the neurogenesis in the lamprey retina being apparently exhausted after transformation (Villar-Cheda, 2005).

Immunoreactivities to 5HT, DA, TH and ChAT during transformation

The immunocytochemical study of neurotransmitters and neurotransmitter-synthesizing enzymes revealed the sequence of appearance of characteristic neuronal populations, which in recently metamorphosed sea lamprey showed a layered distribution.

All cells immunopositive to the substances investigated were amacrine cells; no positivity to them was observed in photoreceptors, bipolar, horizontal or ganglion cells. The expression of these neurotransmitters/neurotransmitter-synthesizing enzymes starts early in metamorphosis, the serotonergic and cholinergic systems become appreciable in the retina earlier than the catecholaminergic system.

Development of serotonin immunoreactivity

No 5HT-ir structures were observed in larval retinae (Fig. 2B). The immunostaining of serotonergic structures starts in early stages of metamorphosis (M1-M2). In M1-M2 there are a few scattered pear-shaped, intensely 5HT-ir cells in the INL throughout the retina (Fig. 2C). In later metamorphic stages, the number of serotonergic

cells increases slightly, and occasional 5HT-ir cells can be also observed in the innermost IPL. Already in M3, the 5HT-ir amacrine cells exhibited thin branched processes that exhibited a clear stratification, forming two well-defined plexuses in the oIPL and iIPL (Fig. 3B). Radial processes related these two serotonergic plexuses. Processes of the inner plexus appeared to contact perikarya of cells of the "putative ganglion cell layer", whereas those of the outer plexus were located in the neuropil. No serotonergic fibers were observed in the optic nerve or in the OPL, indicating that these cells were not ganglion cells or interplexiform neurons.

From M5 to M7, rather abundant pear-shaped 5HT-ir neurons were observed in the INL (Fig. 4B). The number of 5HT-ir cells is similar in M7 and postmetamorphic adults. These cells exhibited a main process directed to the IPL, branching in the iIPL and oIPL sublayers, where they form similarly dense plexuses (Fig. 4B). Tangential sections showed that the horizontal branches can be rather long. Moreover, the main process or an horizontal branch give rise to a vertical or oblique process relating the inner and outer 5HT-ir plexuses. Since this process often is not a straight prolongation of the main process, but arises at a certain distance from it, it is not always possible to observe it. However, comparison of the number (density) of cell perikarya and the number of radial processes crossing the central region of the IPL suggests that most, if not all, the 5HT-ir neurons contribute to the two plexuses, inner and outer. The similar size of 5HT-ir neurons, together with process distribution in both plexuses, suggests they are part of a single 5HT-ir cell population. No 5HT-ir fibers were observed in the optic nerve, or in the INL, ONL and OPL. The density of 5HT-ir cells is higher than those of TH-ir cells, but much less than those GABA or glycine immunoreactive cells (data not shown).

Development of catecholaminergic cells

No TH/DA immunoreactive structures were observed in the retina of larvae or early metamorphic stages (M1 and M2). In M3, some small TH-ir or DA-ir cells were scattered in the innermost INL (Fig. 3C). These cells appeared faintly or moderately stained, and occasionally very thin processes with small beads were observed coursing among negative cells (ganglion cell and/or amacrine perikarya) to the IPL.

In M6 and M7 stage retinas, TH-ir or DA-ir cells were observed scattered in the inner INL (Fig. 4C). They were flattened or polygonal cells with a few thin processes coursing horizontally in the outer IPL, and occasional processes were also observed coursing to inner sublayers of the IPL. No TH-ir process was observed ascending from these cells to the OPL or the ONL in these stages. The staining of processes was very clear, and no immunoreactivity was observed in the photoreceptor layer, outer limiting membrane, ONL or OPL, or ascending in the INL toward these layers.

In the retina of recently metamorphosed lampreys scarce DA-ir amacrine cells were observed in the innermost INL, with their processes coursing in the IPL. The appearance of these cells and distribution of their processes closely correspond with the immunoreactive cells observed in the late metamorphic stages, which send processes only to the IPL (DA-ir amacrine cells). No evidence of the presence of catecholaminergic ganglion cells was obtained.

Development of ChAT immunoreactivity

No ChAT-ir structures were observed in the retina of larvae. ChAT-ir cells were first observed in early transforming stages (M1 and M2), appearing as very faint immunoreactive cells in the primordial IPL (Fig. 2D).

In middle metamorphic stages (M3) faint ChAT-ir perikarya with thin processes were distributed through the inner sublayer of the by now rather thick IPL (Fig. 3D), although stratification of ChAT-ir processes in the IPL was not observed. No differences in ChAT immunoreactivity were observed between the photoreceptor-bearing central retina and the lateral retina of M1-M3 transforming larvae. In M6-M7 retinas, the IPL showed ChAT-ir cells mainly close to the inner limiting membrane, and in a loose cell sublayer at about 60% of the depth of the IPL, which was better defined in M6 stage (the ‘‘inner ganglion cell layer’’ of Fritzsch and Collin, 1990).

In late transforming stages (M6-M7), the ChAT-ir elements displayed a clear laminar organization (Fig. 4D), which is unlike the retinas of upstream-migrating adults. In these stages, the inner retinal layers were also thicker than in adults, facilitating analysis of the distribution of ChAT-ir structures. Most ChAT-ir perikarya (about 76%) were observed in the IPL near the inner limiting membrane. Some ChAT-ir perikarya (about 12%) were observed in close association with the inner ChAT-ir IPL sublamina, some of them among ChAT-ir processes; other ChAT-ir perikarya (again about 12%) were observed external to the outer ChAT-ir IPL sublamina, either in the IPL or in the inner INL. There is a clearly asymmetrical stratification of ChAT-ir processes in the IPL, forming two sublaminae that extended throughout the entire retina (Fig. 4D). In M7 retinas, the inner ChAT-ir IPL sublamina occupied between about 65% and 82% of the depth of the IPL, whereas the outer sublamina was located vitreal to the bands of optic nerve fibers between about 26% and 32% of the depth of the IPL (i.e. considerably thinner than the inner sublamina). The ChAT-ir pear-shaped neurons found in the INL or outer sublayer of the IPL sent processes toward the outer ChAT-ir sublamina. These cells resemble the occasional flattened cells observed in the outer sublayer of the IPL of adult retinas.

DISCUSSION

General considerations

The aim of this study was to investigate the development of the serotonergic, catecholaminergic, and cholinergic systems in the retina of sea lamprey using immunocytochemical methods. The antibodies used here have been used previously to investigate serotonergic (Pierre et al., 1992; Abalo et al., 2003), dopaminergic (Pierre et al., 1997; Abalo et al., 2005), and cholinergic (Pombal et al., 2001) systems in the lamprey brain.

A striking result of the present study was the absence of immunoreactivity to the studied substances throughout all larval stages, both in the early-developing central retina and the late-developing lateral retina. The presence of a simple neuronal circuitry with glutamate-immunoreactive photoreceptors, bipolar, and ganglion cells, as well as and some calretinin- and glycine-immunoreactive cells (Villar-Cheda et al., 2006; Villar-Cerviño et al., accepted), of opsin-expressing photoreceptors (Meléndez-Ferro et al., 2002) had been reported in the larval central retina. This is contrast to the absence of immunoreactivity to typical amacrine cell markers such as GABA (Anadón et al., 1998), DA (Yáñez and Anadón, 1994), and ChAT (present results). In contrast to larval lamprey, the larvae of jawed anamniotes possess amacrine cells that are immunoreactive to acetylcholine, GABA, dopamine/TH and serotonin (van Veen et al., 1984; Östholm et al., 1988; Zhu and Straznicky, 1992; González et al., 1995; Huang and Moody, 1998; Dunker, 1999; López et al., 2002). This, together with the lack of structures necessary for image formation in larval lamprey, support the hypothesis that the larval eye is an "ocellus" that only becomes an image-forming eye, due to the development of the cornea and lens, the chemical maturation of horizontal and amacrine cells in the retina and the formation of functional image-analysis circuits during the metamorphosis.

Accordingly, differences found between retinas of larval and adult lampreys appear to be related to the different functionality of both eyes.

The early appearance of serotonin immunoreactivity in the metamorphic lamprey retina is consistent with the early appearance of serotonin in brains of lampreys (Pierre et al., 1992; chapter 1) and other vertebrates (Bolliet and Ali, 1992; Ekström et al., 1985; Bolliet et al., 1994; Wallace and Lauder, 1982). Moreover, catecholamine immunoreactivity appears later than serotonergic immunoreactivity in lamprey retina, which is consistent with the order of appearance of serotonergic and catecholaminergic structures in the brain (Pierre et al., 1997; Villar-Cerviño, et al., accepted; chapter 2).

Whereas in most teleosts the retina steadily grows by years without any sharp discontinuity (Johns, 1977; Raymond and Rivlin, 1987; Reh and Levine, 1998; Candal, 2002), in lampreys there is a clear distinction between retinal growth before and after metamorphosis (Villar-Cheda, 2005; Villar-Cerviño et al., accepted). Therefore, after metamorphosis, the eye diameter and retinal area increase considerably due to cellular reorganizations, a considerable enlargement of the cells and their processes, and a slimming of most retinal layers, since in the lamprey retina there is not proliferation from late metamorphosis (Villar-Cheda, 2005), contrary to that pointed in most of teleosts (Johns, 1977; Raymond and Rivlin, 1987; Reh and Levine, 1998; Candal, 2002), amphibians (Reh and Levine, 1998), and chick (Fischer and Reh, 2000). The retina of these vertebrates has a marginal proliferating zone throughout life. The comparison of the distribution of serotonin and catecholamine immunoreactive cells between the late metamorphosis and the upstream-migrating adult retinas corroborates that cell density of immunoreactive cells declines dramatically with age. A great decrease of the serotonergic cell density with the increase of the retinal area was also observed in amphibians (Zhu and Straznicky, 1992). Since the ‘‘inner ganglion cell

layer'' in the retina of lampreys is poorly defined in the vitreal region (Dalil et al., 1990; Fritzsch and Collin, 1990; Rio et al., 1998), unlike most vertebrates, ganglion and amacrine cells cannot be reliably distinguished solely on the basis of their distribution.

Serotonin immunoreactivity in lamprey retina

Serotonin immunoreactivity was observed in the retina of all studied animals, which have positive cells and fibers. However, there is not unanimity referring to the kind of cell that possesses serotonin, since amacrine, bipolar and even ganglion cells are able to store end even synthesize serotonin, depending on the species.

Serotonergic amacrine cells have been described in the retina of most vertebrates, excepting mouse (Brunken et al., 1993; Lima and Urbina, 1998; Pérez-León et al., 2004; present results). These amacrine cells were distributed non-uniformly across the INL and their processes ramify in the IPL in lampreys (Negishi et al., 1986; Tamotsu et al., 1990; Versaux-Botteri et al., 1991; present results), teleosts (Hayashi et al., 1986; Jaffe et al., 1987; Marc et al., 1988), elasmobranches, (Ritchie and Leonard, 1983; Bruun et al., 1984), amphibians (Schutte and Witkovsky, 1990; Zhu and Straznicky, 1990; Zhu and Straznicky, 1992; Liu and Debski, 1993; Dunker, 1999), reptiles (Witkovsky et al., 1984; Engbretson and Battelle, 1987), chicken (Rios et al., 1997; Fosser et al., 2005), and mammals (Ehinger, 1983; Yew et al., 1991; Chanut et al., 2002).

Serotonergic cells were also observed in the putative "ganglion cell layer" of the sea lamprey (present results), some amphibian species (Zhu and Straznicky, 1990; Liu and Debski, 1993; Dunker, 1999), the chameleon (Bennis and Versaux-Bottery, 1995), and the cat (Wassle et al., 1987), these neurons corresponding to displaced amacrine cells.

In lampreys and in most fishes, putative amacrine cells were the only serotonergic retinal cell type (Ritchie and Leonard, 1983; Bruun et al., 1984; Hayashi et al., 1986; Jaffe et al., 1987; Marc et al., 1988; Versaux-Botteri et al., 1991; present results). In addition, 5HT-ir bipolar cells were observed in skates (Bruun et al., 1984), some amphibians (Schutte and Witkovsky, 1990; Zhu and Straznicky, 1992; Liu and Debski, 1993; Dunker, 1999), the turtle and chick retinas (Witkovsky et al., 1984; Wilhelm et al., 1993), and even 5HT-ir ganglion cells were observed the ganglion cell layer of the turtle retina (Weiler and Ammermuller, 1986). In most of 5HT-ir bipolar cells, ganglion cells and some of the amacrine cells, the synthesizing enzyme for serotonin could not be detected, which suggests that these serotonin-immunoreactive cells possibly only accumulate serotonin (Wilhelm et al., 1993).

In lampreys the uneven distribution of serotonergic cells is not accompanied of a different cell density, as in amphibians, which has a higher concentration in the central than in the peripheral region of the retina (Zhu and Straznicky, 1990; Liu and Debski, 1993).

Serotonin-immunoreactive amacrine cells and fibers were observed in the adult retina of all investigated lampreys: *Lampetra japonica* (Negishi et al., 1986; Tamotsu et al., 1990), *Lampetra fluviatilis* (Versaux-Botteri et al., 1991), and *Petromyzon marinus* (present results). Thus, while in *L. japonica* a single type of 5HT-ir amacrine cells was described, in *L. fluviatilis* 5HT-ir amacrine cells were subdivided in four cell types attending to cell size, the location of perikarya, and the arrangement of their processes that branch in outer and/or inner sublaminae into the IPL (Versaux-Botteri et al., 1991). As it is better observed in late transforming stages, our study in *P. marinus* indicates that most 5HT-ir amacrine cells send processes into both iIPL and oIPL plexuses, which reveals that they were similar to the type 3 described in *L. fluviatilis*. If the sea lamprey

retina also contains 5HT-ir cells with processes only to one plexus could not be determined in this study.

The presence of inner and outer serotonergic plexuses in the IPL of lampreys is consistent with the results obtained in most vertebrates (Witkovsky et al., 1984; Hayashi et al., 1986; Engbretson and Battelle, 1987; Jaffe et al., 1987; Marc et al., 1988; Rios et al., 1997; Fosser et al., 2005), although in most amphibians serotonergic fibers branched diffusely and densely throughout all levels of the IPL (Schutte and Witkovsky, 1990; Zhu and Straznicky, 1990; Zhu and Straznicky, 1992; Liu and Debski, 1993; Dunker, 1999). No 5HT-ir fibers were observed in the optic nerve of lampreys, unlike that reported in amphibians, rodents, frog, and stingray (Ritchie and Leonard, 1983; Liu and Debski, 1993; Lima and Urbina, 1998; Dunker, 1999). This immunoreactivity would be related to the presence of 5HT-ir ganglion cells in these vertebrates.

In other vertebrates the synaptic contacts formed in the outer serotoninergic plexus are involved in transmission of OFF signals while synapses in the inner serotoninergic plexus are implicated in the ON pathway (Brunken et al., 1993). The organization of the processes of the serotoninergic amacrine cells in two plexuses in the IPL strongly suggest that serotonergic amacrine cells could be involved in the modulation of the activity of OFF-center and ON-center ganglion cells.

Development of serotonin immunoreactivity in the retina of P. marinus

No immunoreactivity to serotonin was observed in the larval lamprey retina (Meléndez-Ferro et al., 2002; Villar-Cerviño et al., accepted; present results), which precludes any role in the early eye development. However, serotonin is involved in *Xenopus* retinal histogenesis and eye morphogenesis by supporting cell proliferation and survival (de Lucchini et al., 2005). In the lamprey retina, first serotonin

immunoreactive cells appear at early metamorphosis (M1-M2, present results), and then a progressive increment of serotonergic elements was observed, similar to that pointed in the amphibian retina (Liu and Debski, 1993). In lamprey a sequence of development outer to inner (as in humans; Yew et al., 1991), inner to outer, or even from the central region to lateral ones (as in amphibians; Liu and Debski, 1993) were not observed. At late sea lamprey metamorphic stages (M6-M7), the adult pattern of serotonergic structures is achieved. It is also achieved in chicken at hatching, which is temporally correlated with the establishment of synapses in the retina and with the emergence of the typical adult electroretinogram (Rios et al., 1997; Fosser et al., 2005).

Catecholaminergic cells in the lamprey retina

Catecholamines are neuroregulatory substances widely distributed in the CNS of vertebrates. The presence of catecholaminergic cells in the retina of lampreys and other vertebrates has been established (Ehinger, 1983; Yáñez and Anadón, 1994; Smeets and González, 2000).

Dopamine (DA) is the major catecholamine in vertebrate retinas. It is localized in a subset of amacrine and/or interplexiform cells, depending on the species. These two types of DA-ir neurons were observed in most of the studied animals (Ehinger, 1983). The dopaminergic amacrine cells contact only other amacrine cells. They receive synapses from other amacrine cells which are likely to operate with, e.g. GABA or glycine as neurotransmitter. DA-ir interplexiform neurons are well characterized in teleosts (Osborne et al., 1984; Yazulla and Zucker, 1988; Kallionatis and Marc, 1990; Wagner and Behrens, 1993; Fröhlich et al., 1995), amphibians (Zhu and Straznicky, 1991), avian (Dkhissi et al., 1993), and mammals (Lugo-García and Blanco, 1993; Guimaraes and Hokoc, 1997). They have approximately the same contacts in the IPL as

the amacrine cells, but, in addition, they send processes to the OPL and there contact both horizontal and bipolar cells (Ehinger, 1983). In this study, both DA and TH immunoreactive cells do not send positive processes in the OPL of the transforming or adult retina, which supports that they are amacrine cells as reported by Yáñez and Anadón (1994) and not interplexiform cells as described by other authors (de Miguel and Wagner, 1990a; Dalil-Thiney et al., 1996).

Dopamine has multiple trophic roles in retinal function related to circadian rhythmicity, cell survival, and eye growth (Witkovsky, 2004). It controls several aspects of retinal physiology, with special emphasis in the role of DA as a mediator of the inhibitory effect of light on melatonin synthesis in retinas of lower vertebrates (Zawilska and Nowak, 1991). In mammals there is evidence to suggest that DA-ir amacrine cells brings the surround response into the rod system through synapses with the rod amacrine cell, and that an indoleamine, probably serotonin, increases the signal in the ON pathway through a feedback synapse onto the rod bipolar terminal (Daw et al., 1990). Dopaminergic neurons are also involved in the light/dark adaptation process in mammalian retina (Djamgoz and Wagner, 1992). At the present, possible roles of the lamprey retina DA-ir amacrine cells only can be speculative.

Development of dopaminergic cells

In the larval lamprey no immunoreactivity to TH or DA was observed either in the central or the lateral retina (Villar-Cerviño et al., accepted; present results). TH/DA immunoreactivity is first observed at middle stages of metamorphosis (M3). When compared with expression of serotonin and ChAT, dopamine is expressed later in development, similarly to that reported in other vertebrates (Nguyen-Legros et al., 1983; Kato et al., 1984; Mitrofanis et al., 1989; Mitrofanis and Finlay, 1990; Wang et al.,

1990). The temporal precedence of the serotonergic system over the catecholaminergic system in retina is consistent with data obtained in the lamprey brain (Abalo et al., 2005). At late stages of metamorphosis (M6-M7) the adult pattern of catecholaminergic structures is achieved. In amphibians, dopaminergic amacrine cells are generated continuously throughout life (Zhu and Straznicky, 1991), but this does not appear to occur in lampreys, which lack retinal cell proliferation in adults (Villar-Cheda, 2005).

Cholinergic cells in the lamprey retina

ChAT-ir perikarya were observed in the IPL of the retina of adult lamprey. The horizontal extent and layer distribution of processes of these cells is uncertain, since they were faintly stained and mostly embedded in the rich ChAT-ir plexuses. Due to the difficulty to distinguishing between ganglion cells and amacrine cells in basis of their location, these cells might be amacrine and/or ganglion cells. However, the well-characterized ganglion cells of adult *L. fluviatilis* and *I. unicuspis* (Dalil et al., 1990; Fritzsch and Collin, 1990) do not correspond either in size or in IPL distribution with the ChAT-ir cells (Pombal et al., 2003). Moreover, no ChAT-ir fibers were observed in the optic nerve (present results), and the optic tracts in the brain were also ChAT-negative (Pombal et al., 2001), which strongly suggests that the ChAT-ir neurons were amacrine cells. The finding of cholinergic amacrine cells in the lamprey retina is similar to that reported in the retinas of teleosts (Ekström and Korf, 1986), elasmobranchs (Brandon, 1991), amphibians (López et al., 2002, 2003), turtles (Nguyen et al., 2000), chick (Millar et al, 1985), and mammals (Voigt, 1986).

In jawed vertebrates, most ChAT-ir amacrine cells are consistently found arranged symmetrically on both sides of the IPL (i.e. with mirror-like distribution), in the inner INL and in the GCL, respectively (orthotopic and displaced amacrine cells),

and processes of these amacrine cells are precisely stratified into two sublaminae of the IPL (teleosts: Ekström and Korf, 1986; amphibians: López et al., 2002, 2003; turtles: Nguyen et al., 2000; chick: Millar et al, 1985; mammals: Voigt, 1986). In mammals, the characteristic symmetric radial branching pattern of these mirror-like ChAT-ir cells correspond to that of the well known starburst cells identified in rabbit (Flamiglietti, 1983). Similar starburst-like morphologies of cholinergic displaced amacrine cells have been observed in an elasmobranch (Brandon, 1991). In addition, other types of ChAT-ir amacrine cells with non-starburst morphology have been described in other vertebrates (Millar et al., 1985; Conley et al., 1986; Spira et al., 1987; Guiloff and Kolb, 1992; Nguyen et al., 2000; Cuenca et al., 2003). In the adult lamprey retina, the distribution of ChAT-ir perikarya in the inner retinal layers is asymmetrical, and their processes in the IPL are not stratified into two similar sublayers, unlike in the retinas of most gnathostomes.

In lampreys, most ChAT-ir cells are displaced amacrine cells, and appear to be at least of three different types: stellate cells that resemble the ChAT-ir starburst cells described in other vertebrates retinas, bipolar or triangular cells that are clearly non-starburst cells, and flattened cells of the outer region of the IPL.

The rich cholinergic system observed in the lamprey retina may have similar functions to those proposed for the cholinergic systems of jawed vertebrates. The best characterized cholinergic retinal cells of vertebrates are the starburst cells, which appear to be critical for the directional sensitivity of ON-OFF responding ganglion cells (Famiglietti, 2002). The connectivity and properties of the non-starburst cells observed in other vertebrates are not known in detail. The ChAT-ir plexuses observed in lampreys appear adequate for establishing contacts with ganglion cell dendrites, but also with processes of amacrine cells and bipolar terminals, and it is not clear whether

ChAT-ir cells form direct contacts with ganglion cells. Possible roles of acetylcholine and cholinergic cells in retinal processing in lamprey remain to be investigated.

Development of ChAT immunoreactivity

No ChAT-ir structures were observed in the retina of larval lampreys, either in the early-developing central retina or in the lateral retina. This is very unlike to the developmental pattern reported in vertebrates as diverse as amphibians (López et al., 2002, 2003), turtle (Nguyen et al., 2000), chick (Spira et al., 1987), and rat (Kim et al., 2000). Present results with immunocytochemistry support that the appearance of ChAT-ir amacrine cells in the retina during metamorphosis is linked to differentiation of functional image-analysis circuits.

As regards the pattern of development of cholinergic structures, faint ChAT immunoreactivity could be observed in the innermost rows of cells at stages M1-M2, stages in which the IPL is hardly differentiated. An expanded IPL is observed in stage M3, but ChAT immunoreactivity was faint and no accumulation of ChAT-ir processes in IPL sublaminae could be observed until stage M6. By stages M6 and M7, two outstanding ChAT-ir IPL sublaminae were observed throughout the retina, the outer sublamina being thinner than the inner one. The appearance of these ChAT-ir sublaminae appears to be coordinated with differentiation of adult photoreceptors, which by stage M5 are observed in throughout the retina (de Miguel and Anadón, 1987), and hence with the presence of a functional visual circuitry. Comparison of the distribution of ChAT-ir processes in the IPL of stage M7 with that observed in upstream-migrating adults indicates that the outer sublamina becomes very thin in these adults, the outer ChAT-ir perikarya often appearing very flattened and closely associated with this rather inconspicuous band, whereas in transforming stages most

processes are distributed in a wide inner IPL band, apparently reflecting the thickness differences in the inner ChAT-ir sublamina. The reasons for this marked asymmetry in the distribution of ChAT-ir processes are not known, but it might be related to the atypical location of most ganglion cells in the INL (‘‘displaced’’ ganglion cells) and the course of the optic nerve fibers scleral to the INL, which imply that the orientation of the dendritic trees of these ganglion cells is unlike those of typical ganglion cells of jawed vertebrates.

Finally, comparison of the distribution of ChAT-ir cells in the retina of upstream-migrating adults and in the M7 stage also corroborates that the cell density in the inner retina declines dramatically with age, inversely to the increase of the retinal surface.

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TABLES

Table 1

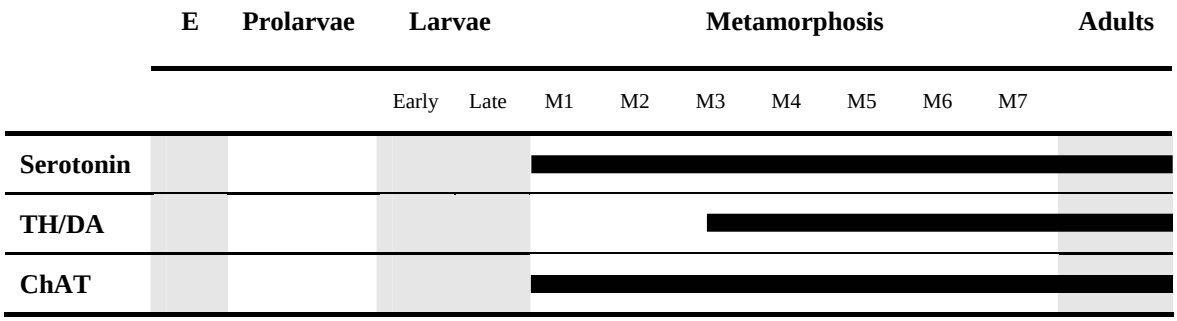


FIGURE LEGENDS

Table 1: Timetable of the appearance of the studied neurotransmitter systems in the retina of sea lamprey.

Fig. 1. Vertical sections of the retina of an upstream-migrating adult. **A**: hematoxylin-eosin staining showing the different layers. **B**: Section showing a 5HT-ir amacrine cell in the INL (arrow) and 5HT-ir processes in the oIPL (outlined arrow) and iIPL (arrowhead). **C**: Section showing a DA-ir amacrine cell in the INL (arrow) and a plexus of DA-ir fibers in the outer IPL (outlined arrow). Note the total absence of dopamine immunoreactivity in the outer INL and the photoreceptor layer. **D**-**F**: Sections showing ChAT-ir cells in the inner (arrowheads) and middle (arrows) regions of the IPL. In D, the thin outer IPL sublamina of ChAT-ir processes (outlined arrow) is hardly distinguishable. Scale bars: A, C= 50 µm; B = 25 µm; D-F= 12.5 µm.

Fig. 2. Vertical sections of the retina of M2 stage transforming lampreys (A, C, D) and of a 53 mm larva (B). **A**: hematoxylin-eosin stained retina showing the different layers. **B**: transverse section showing the absence of 5-HT immunoreactivity in the retina (arrow), in spite of the presence of strongly stained 5-HTir fibers of the peripheral nervous system (outlined arrows). **C, D**: Sections immunostained for serotonin (C) and ChAT (D) showing the faint immunoreactivity of the primordial IPL-"ganglion cell layer". The arrow shows positive serotonergic cells. The retinal pigment epithelium is detached in A. Scale bars: A, C, D = 25 µm; B = 12.5.

Fig. 3. Vertical sections of the retina of M3 stage transforming lampreys. **A**: hematoxylin-eosin stained retina showing the different layers. **B-D**: sections immunostained for serotonin (B), TH (C), and ChAT (D) showing immunoreactive perikarya (arrows). Note in B the two 5HT-ir plexuses into the IPL (outlined arrows). Scale bars: A, B, D = 25 µm; C = 10 µm.

Fig. 4. Vertical sections of the retina at late metamorphosis (M6-M7). **A**: Hematoxylin-eosin stained retina of a M6 stage showing the different layers. **B**: Section of a M7 retina immunostained for serotonin showing positive cells (arrow) and processes (outlined arrow). **C**: Section of a M6 retina immunostained for TH showing positive cells (arrows). **D**: Section of a M6 retina immunostained for ChAT showing most ChAT-ir perikarya close to the inner limiting membrane (arrowhead). Note the inner (thick solid arrow) and outer (outlined arrow) ChAT-ir sublaminae of the IPL. Scale bars: A, D = 25 µm; B, C= 50 µm.

FIGURES

Figure 1

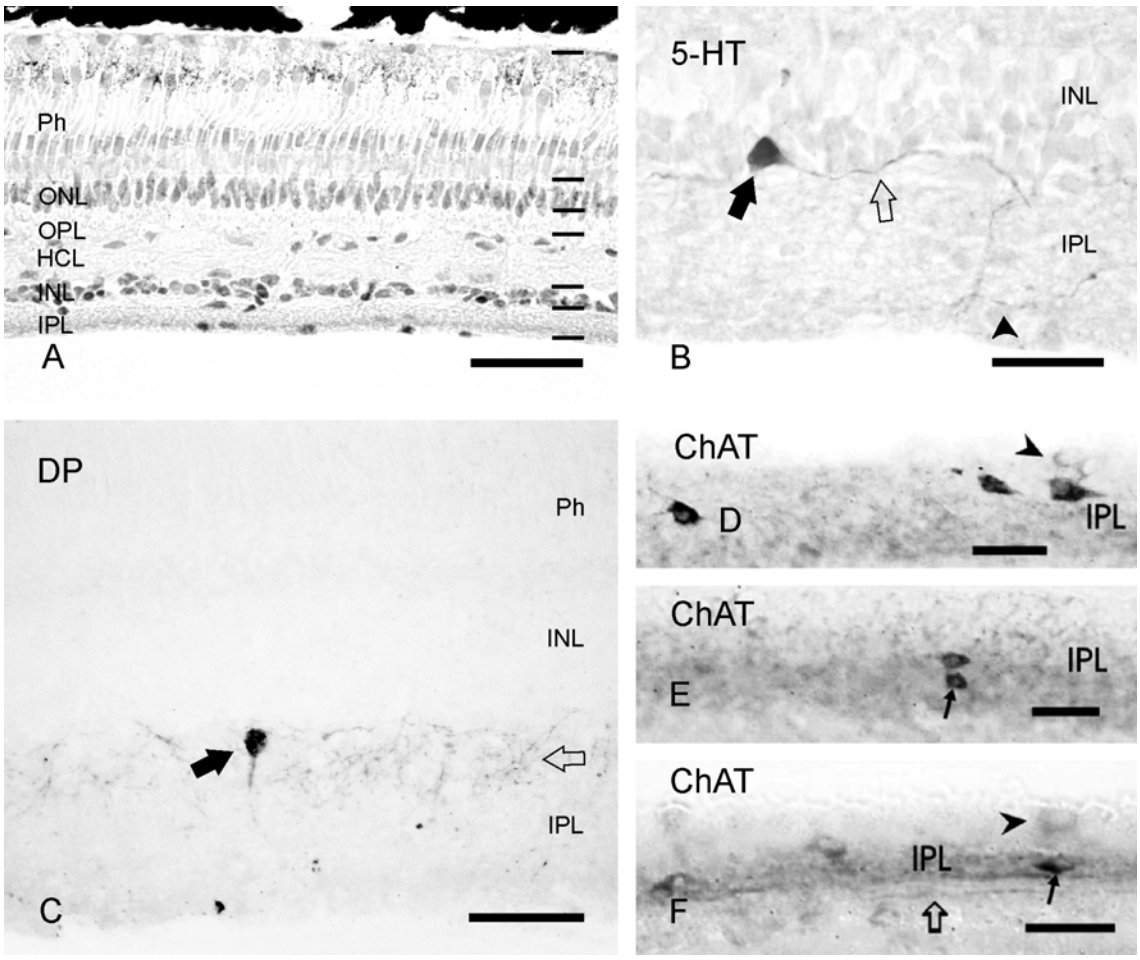
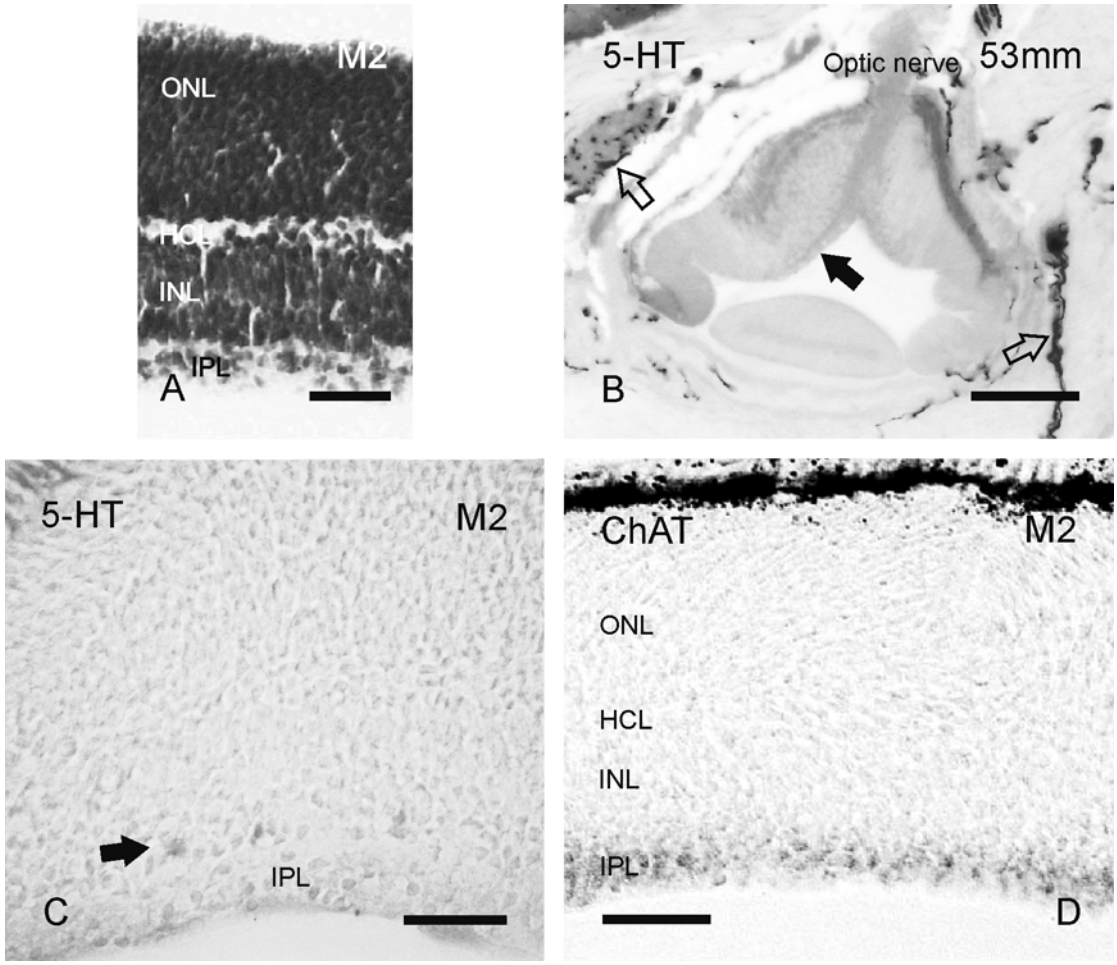


Figure 2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D

Figure 3

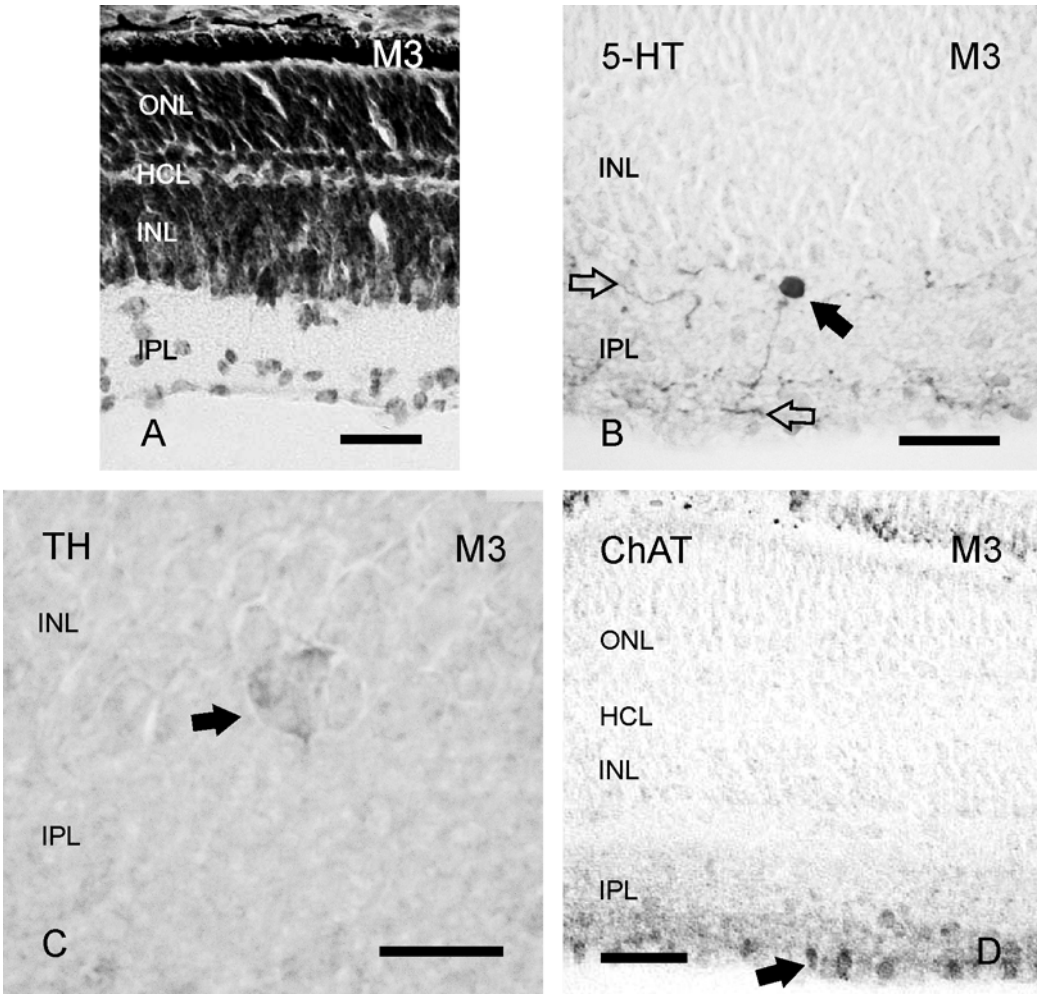
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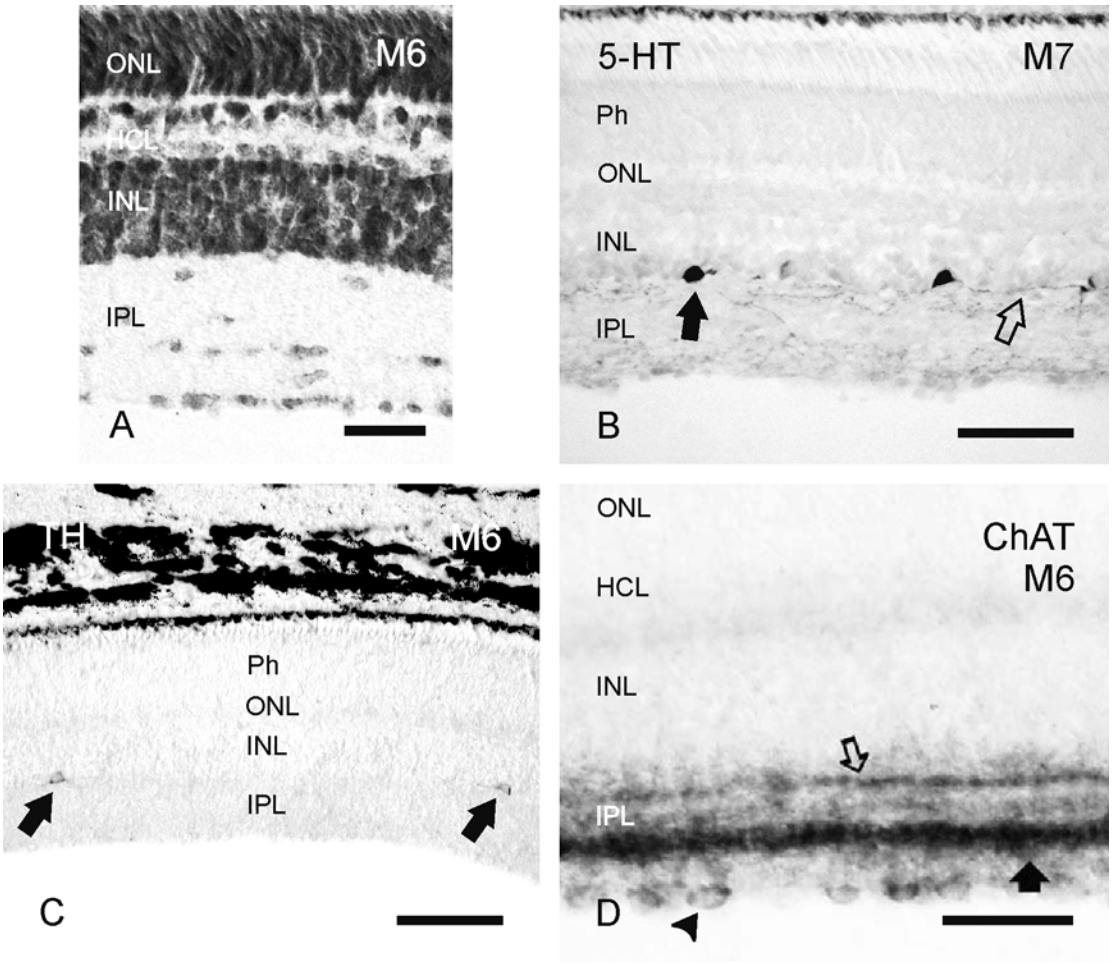
Figure 4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Conclusions

Conclusions

We have studied with immunocytochemical methods the adult organization and development of the serotonergic and catecholaminergic systems in the central nervous system of the sea lamprey. From the analysis of the results, we obtained the following conclusions:

1. In the CNS of sea lamprey, serotonin-immunoreactive (5HT-ir) cell groups were observed in the diencephalon, rhombencephalon and spinal cord, and occasional 5HT-ir cells were also observed in the telencephalon and in the caudal mesencephalon. This distribution is roughly similar to that reported in the adult river lamprey (Pierre et al., 1992).

2. In the adult lamprey CNS, tyrosine hydroxylase-immunoreactive (TH-ir) cell groups were observed in the prosencephalon, the most caudal part of the rhombencephalon and the spinal cord. The TH-ir cell groups found in the adult sea lamprey brain were also dopamine-immunoreactive (DA-ir), except those of the olfactory bulbs and lamina terminalis. However, the distribution of TH-ir and DBH-ir structures in the lamprey brain is very different. DBH-ir neurons were observed in the paraventricular organ, the synencephalic-mesencephalic tegmentum and the caudal rhombencephalon.

3. In the sea lamprey CNS, the various serotonergic cell groups appear at different times from the embryonic period to metamorphosis, the earliest population being observed in the isthmus of late embryos. The earliest catecholaminergic (dopaminergic) cells appear in spinal cord of prolarvae. The adult pattern of serotonin, TH and DA immunoreactivity is settled during the metamorphosis, while that of DBH immunoreactivity is settled by the end of the larval period. Our results indicate that

neuronal groups with the same monoaminergic phenotype begin to express it at different developmental periods and this probably involves differences in the mechanisms of cell specification.

4. Comparison of serotonergic and catecholaminergic systems showed that the serotonergic system appears earlier than the catecholaminergic system. This result is similar to that found in the other vertebrates, which suggests it is a shared feature of vertebrates.

5. No neuronal groups showing transient expression of serotonin, DA, TH or DBH immunoreactivity were observed in sea lamprey, which is unlike that reported in some other vertebrates.

6. The first appearance of catecholaminergic cell groups in the sea lamprey is delayed to the prolarval period, which is in contrast with its presence in embryos of other vertebrates. This can be attributed to a lack of brain maturation at hatching in lamprey. In addition, the progressive appearance of catecholaminergic cell groups during the larval period and metamorphosis in the sea lamprey is similar to that found in amphibians.

7. The study of the development of the serotonergic, catecholaminergic and cholinergic systems in the lamprey retina showed that serotonergic and cholinergic structures were first observed at the beginning of the metamorphosis, while the appearance of catecholaminergic structures is delayed until mid-metamorphosis. The immunopositive cells to all these substances were described as amacrine cells, since no immunoreactive fibers were observed in the outer plexiform layer or coursing in the optic nerve.

8. The order of appearance of serotonergic, cholinergic and catecholaminergic cells in the sea lamprey metamorphic retina parallels the order of appearance of these systems observed in the developing brain .

9. The absence of immunoreactivity to ChAT, 5HT, DA and TH in the retina of all larval stages, and the delayed maturation of amacrine cells in the lamprey retina is unique to vertebrates and can be related to the peculiar cycle of life of this species. This, together with the lack of differentiation of structures necessary for image formation in larval eye, support the hypothesis that it is an “ocellus” that only becomes an image-forming eye during the metamorphosis.

Summary

Resumen

La lamprea es un representante del grupo de los vertebrados vivientes más antiguos, los Agnatos. Dentro de la superclase de los agnatos existen varios géneros que viven hoy en día, y dentro del género *Petromyzon* se encuentra el animal objeto de nuestro estudio; *Petromyzon marinus* (la lamprea de mar). La lamprea de mar presenta un ciclo de vida complejo dado que es un animal anádromo, es decir, que comienza su ciclo vital en el río, migra al mar, en donde pasa la etapa adulta, y finalmente vuelve al río para desovar y posteriormente morir.

Su ciclo vital comienza con una breve etapa embrionaria de entre 9 y 12 días de duración que termina con la eclosión de los huevos, le sigue una etapa prolarvaria de entre 22 y 25 días de duración que termina con la completa reabsorción del vitelo, y una etapa larvaria, anormalmente larga y variable, entre 4 y 7 años en la que las larvas son prácticamente ciegas. Al término de esta etapa larvaria ocurre la metamorfosis que provoca cambios drásticos en el animal, tanto a nivel morfológico como fisiológico. Con la metamorfosis se desarrolla el sistema visual y las larvas cambian incluso su modo de alimentación, y así, de una etapa en la que se alimenta por filtración de la materia en suspensión, pasa a un modo de vida parasitario alimentándose de los fluidos internos de otros peces. Este cambio tan drástico de alimentación conlleva la transformación de la boca larvaria en un eficiente aparato suctor. Una vez que la metamorfosis ha terminado los individuos jóvenes descienden el río hasta alcanzar el mar, donde pasan alrededor de dos años de etapa adulta, para regresar al río.

El desarrollo del sistema nervoso central es un proceso complejo y secuencial. Comienza en la etapa embrionaria con la formación de la placa neural que se desarrolla en el surco neural y posteriormente en el tubo neural. Este tubo hueco deriva en su zona

rostral en el cerebro y el resto en la médula espinal. Dentro de la formación del cerebro se distinguen varios estadíos según el número de vesículas que se van diferenciando. De esta manera se puede apreciar inicialmente un estadío de tres vesículas; prosencéfalo, mesencéfalo y rombencéfalo. En la mayoría de los vertebrados el prosencéfalo posteriormente se diferencia en telencéfalo y diencéfalo, el mesencéfalo permanece igual y el rombencéfalo se subdivide en metencéfalo y mielencéfalo. Sin embargo, en la lamprea la subdivisión del rombencéfalo no se produce.

El sistema nervioso central de la lamprea de mar presenta el patrón general de los vertebrados. Se considera un cerebro laminar en referencia a la lámina periventricular en la que permanecen la mayoría de los cuerpos neuronales. Aunque la lamprea presenta una historia filogenética independiente, probablemente retiene muchas características del supuesto antecesor común de los vertebrados, siendo de esta manera un excelente punto de partida para estudios del desarrollo, estructura y función del sistema nervioso central.

El cerebro de la lamprea de mar puede subdividirse en las partes anteriormente citadas; telencéfalo, diencéfalo, mesencéfalo y rombencéfalo. Cada uno puede, a su vez, ser subdividido en distintas regiones: en el telencéfalo podemos distinguir los bulbos olfatorios, los palios dorsal, medial y ventral, el septo, el estriado y la región preóptica. El diencéfalo fue clásicamente dividido en epitálamo, tálamo dorsal, tálamo ventral e hipotálamo, pero estudios recientes (basados en la teoría neuromérica) parecen confirmar la hipótesis de una subdivisión en prosómeros. De esta manera todo el prosencéfalo quedaría subdividido en los prosómeros 1, 2 y 3 y dos zonas sin división en prosómeros que serían el telencéfalo y el hipotálamo. Dentro del hipotálamo, a su vez, se pueden observar distintas regiones; postóptica, infundibular, tuberal o mamilar. El rombencéfalo se puede subdividir en 4 zonas en el eje dorso-ventral atendiendo a su

localización respecto a los distintos surcos y con una diferente funcionalidad. Rostrocaudalmente en el rombencéfalo se pueden apreciar 7 u 8 subdivisiones, los rombómeros. El rombencéfalo se continúa caudalmente con la médula espinal que se puede a su vez subdividir en una zona dorsal sensitiva y otra ventral motora.

La retina de la lamprea es similar a la retina de todos los vertebrados estudiados con algunas características específicas, estructurales y de desarrollo. La retina del adulto de lamprea es una estructura laminada que de fuera adentro esta formada por: el epitelio pigmentario, que no pertenece a la retina neural propiamente dicha. Por debajo de ésta se encuentran los fotorreceptores, que son dos tipos; largos y cortos, cuyo soma se localiza en la capa nuclear externa. La capa plexiforme externa es fina, mientras que la capa nuclear interna es compleja y consta de una región externa que contiene dos subcapas de células horizontales y las células bipolares grandes y una región interna con las células bipolares pequeñas, las células amacrinas y la mayoría de las células ganglionares. No hay una verdadera capa de células ganglionares ni capa de fibras del nervio óptico; la capa plexiforme interna es compleja y en ella se encuentran además algunas células ganglionares. Las células gliales de Müller están situadas en el capa nuclear interna y sus procesos forman las membranas limitantes externa e interna.

El desarrollo y la diferenciación de la retina de la lamprea son únicos en vertebrados. Comienza con la formación de las vesículas ópticas en la etapa embrionaria y el desarrollo posterior de un ojo rudimentario ya en la etapa prolarvaria. En la retina larvaria se distingue una pequeña zona central diferenciada que aparece muy pronto y posee un tipo de fotorreceptor, células ganglionares, bipolares y horizontales y una zona lateral que empieza a crecer en larvas de mediano tamaño, aumenta considerablemente en larvas grandes, en las que solo se diferencian células ganglionares y termina su

desarrollo durante la metamorfosis, transformándose en una estructura laminada en la que los fotorreceptores son los últimos en diferenciarse.

La serotonina es una monoamina biógena, sintetizada a partir del aminoácido esencial triptófano, presente en el torrente sanguíneo. Forma parte del grupo de las monoaminas junto con las catecolaminas. Presenta una amplia distribución en el Reino Animal, incluyendo especies tanto de vertebrados como de invertebrados. Se descubrió a mediados del siglo XX. Casi desde el principio se determinó su presencia en el sistema nervioso central, aunque su papel como neurotransmisor no se estableció hasta los años 80. Para determinar su distribución en el SNC se usaron inicialmente técnicas de fluorescencia inducida por la exposición a vapores de formaldehído (FIF) y de autorradiográfia. Actualmente se utilizan técnicas inmunohistoquímicas y de hibridación *in situ*, que son más específicas.

La síntesis de serotonina comienza con la hidroxilación inicial de L-triptófano a 5-hidroxitriptófano por la enzima triptófano-5-hidroxilasa, enzima limitante de la síntesis de serotonina. El 5-hidroxitriptófano es rápidamente descarboxilado por la aminoácido descarboxilasa (AADC, también presente en síntesis de las catecolaminas) que da como producto la 5-hidroxitriptamina, o serotonina. La serotonina se almacena en vesículas y se libera a través de un proceso de exocitosis calcio-dependiente. Una vez en la hendidura sináptica, ésta se une a receptores tanto pre- como postsinápticos. En la célula postsináptica la serotonina transmite su señal química, mientras que en los autorreceptores de la célula presináptica da lugar a una señal de "feedback" positivo o negativo. En la membrana de la célula presináptica hay transportadores específicos para la recaptación de la serotonina de la hendidura sináptica. Una vez en la célula presináptica, la serotonina se puede almacenar de nuevo en vesículas o bien ser

degradada por la enzima mitocondrial monoamino oxidasa (MAO, también presentan en la ruta de la degradación de los catecholamines).

Existen al menos siete tipos de receptores de serotonina, y cada uno tiene un patrón de distribución y respuesta biológica específica. La mayoría de las sinapsis serotoninérgicas son inhibidoras sobre la neurona postsináptica, aunque algunas son excitatorias. La serotonina puede trabajar como un neurotransmisor o como modulador de la neurotransmisión, y participa en la regulación del desarrollo neuronal. La cantidad de serotonina es más alta durante el desarrollo que en la etapa del adulto, lo que supondría un importante papel de esta monoamina en el desarrollo temprano del sistema nervioso central.

Las acciones de las neuronas serotoninérgicas son complejas, por ello generalizar sobre sus acciones resulta difícil. Sin embargo, hay un patrón de desarrollo y neuroquímico similar en el sistema serotoninérgico de todas las especies estudiadas. El sistema serotoninérgico se asocia con la regulación de la temperatura corporal, la presión arterial, el movimiento rápido del ojo durante el sueño, los ritmos circadianos y la percepción del dolor.

Las catecolaminas son una familia de neurotransmisores sintetizados en el tejido nervioso a partir de un sustrato común: la tirosina, que se obtiene del torrente sanguíneo. La tirosina hidroxilasa, enzima soluble inicial y limitante en la síntesis de la catecolaminas, se concentra en los terminales axónicos de todas las neuronas que contienen catecolaminas. Transforma la tirosina en L-DOPA, que a su vez rápidamente descarboxilada por la enzima citosólica soluble L-DOPA-descarboxilasa para dar dopamina. La dopamina se almacena en vesículas en todas las neuronas catecolaminérgicas. Dado que la L-DOPA-descarboxilasa no es específica de la L-

DOPA y descarboxila otros aminoácidos aromáticos, el nombre preferido para esta enzima es de aminoácido aromático descarboxilasa. La dopamina-β-hydroxylase (DBH) es una enzima soluble intravesicular que hidroliza la dopamina para conseguir noradrenalina en neuronas noradrénergicas y adrenérgicas. La noradrenalina es convertida en adrenalina por la enzima feniletanolamina N-metiltransferasa solamente en neuronas adrénergicas.

Una vez en la hendidura sináptica, el neurotransmisor se une a su receptor, que abarca también los autorreceptores de la membrana presináptica y que regulan la síntesis de las catecolaminas. Aproximadamente el 80% de las catecolaminas son eliminadas de la hendidura sináptica e introducidas de nuevo en la célula presináptica por un transportador de recaptación. Una vez en la célula presináptica, las catecolaminas bien se realmacenan en vesículas o bien son degradadas por enzimas como la monoamino oxidasa o la catecol-o-metiltransferasa. Las catecolaminas se asocian clásicamente a alteraciones del humor y al control de la actividad locomotora, y su ausencia produce varios síntomas relacionados con la esquizofrenia o la enfermedad de Parkinson. Las catecolaminas desarrollan también un importante papel durante el desarrollo además de regular la actividad de varias hormonas hipofisarias en teleósteos.

La acetilcolina fue el primer neurotransmisor descubierto. Es la única amina de bajo peso molecular aceptada como sustancia neurotransmisora que no es ni aminoácido ni derivada directamente de ningún aminoácido. Se sintetiza en los terminales axónicos a partir de los sustratos colina y acetil-coenzima-A. La enzima encargada de este proceso es la colina acetiltransferasa, aunque sintetizada en el soma, es transportada al terminal nervioso para ejercer su función. Las neuronas no producen colina, y puesto que ésta no puede cruzar la barrera hematoencefálica, su aportación al cerebro deriva de

la ruptura de la fosfatidilcolina. Las moléculas de acetilcolina se almacenan en vesículas (2000-10000 moléculas por vesícula) y se liberan al terminal sináptico después de la despolarización. Una vez en la hendidura sináptica, la acetilcolina se une a receptores pre y postsinápticos y es degradada por la acetilcolinesterasa.

Hay dos familias de receptores de acetilcolina: los receptores nicotínicos y los muscarínicos. Existen, además, dos tipos de acetilcolinesterasa: una soluble y otra transmembrana presente en los terminales pre y postsinápticos. Una vez degradada la acetilcolina, la colina puede ser transportar a la neurona presináptica para ser reutilizada. Las sinapsis colinérgicas son conservadoras y reciclan alrededor el 50% de la colina. La acetilcolina es el neurotransmisor usado por las motoneuronas espinales y por lo tanto se usa en todas las uniones neuro-musculares de vertebrados. En el sistema nervioso autónomo la acetilcolina es el neurotransmisor utilizado por todas las neuronas preganglionares y postganglionares. La acetilcolina se usa además como neurotransmisor en varias áreas del cerebro y de la retina.

CAPÍTULO 1

Desarrollo del sistema serotoninérgico en el sistema nervioso central de la lamprea de mar

La caracterización de los grupos neuronales y su desarrollo puede aportar información decisiva para un completo entendimiento de la evolución temprana del sistema nervioso de los vertebrados. Es este capítulo se intentó dilucidar el desarrollo del sistema serotoninérgico en el sistema nervioso central de la lamprea de mar mediante técnicas inmunohistoquímicas desde el período embrionario a la etapa adulta. La aparición de las diferentes poblaciones neuronales serotoninérgicas presentes en individuos adultos ocurre entre la fase embrionaria y la metamorfosis. Las primeras

neuronas aparecen al final del período embrionario en la placa basal de la región ístmica. En la etapa prolarvaria aparecen progresivamente nuevos grupos celulares serotoninérgicos: primero en la médula espinal, luego en el órgano pineal, la región tuberal y la zona limitans intratalámica, y por último en la parte caudal del rombencéfalo. Ya en la etapa larvaria temprana aparece un nuevo grupo celular serotoninérgico en la región mamilar. Posteriormente, en larvas de tamaño medio aparece un grupo celular en la región pretectal, y en la etapa larvaria tardía las células serotoninérgicas de la glándula parapineal. Las primeras fibras del sistema serotoninérgico aparecen en el período prolarvario temprano, mostrando fibras del grupo celular ístmico que cursan tanto rostralmente como caudalmente. El número de fibras inmunorreactivas aumenta progresivamente hasta el período adulto. Con estos resultados

La comparación de la aparición espacio-temporal de células serotoninérgicas en agnatos y gnatóstomos demuestra semejanzas en la localización y el momento de aparición de los grupos serotoninérgicos del cerebro medio. Esta comparación también apoya la tendencia evolutiva de una desaparición progresiva de los grupos serotoninérgicos rostrales y su aumento en el cerebro medio. Nuestros resultados también revelan que la secuencia de desarrollo y el tiempo de aparición de las vías serotoninérgicas ascendentes y descendentes tempranas son similares entre vertebrados. Las diferencias más llamativas del desarrollo del sistema serotoninérgico de la lamprea con respecto al resto de los vertebrados se basan en la diferenciación tardía en lamprea de las poblaciones pretectales y tectales, que parece estar relacionada con el peculiar desarrollo del sistema visual de esta especie.

CAPÍTULO 2

Desarrollo del sistema catecolaminérgico en el sistema nervioso central de la lamprea de mar

Es este capítulo se describe el desarrollo del sistema catecolaminérgico en el cerebro y la médula espinal de la lamprea de mar desde el período embrionario hasta la etapa adulta utilizando anticuerpos contra tirosina hidroxilasa (TH), dopamina (DA) y dopamina β-hidroxilasa (DBH). Pretendemos dar una visión integrativa sobre el desarrollo de este sistema en un representante del grupo más primitivo de los vertebrados.

El desarrollo de la inmunorreactividad a estas tres sustancias comienza la etapa prolarvaria tardía. Los primeros grupos celulares TH-inmunorreactivos (TH-ir) aparecen en los núcleos paratubercular y preóptico de prolarvas. En larvas aparecen progresivamente grupos TH-ir en el núcleo de la comisura postinfundibular, el núcleo de la comisura postóptica, el órgano paraventricular, el estriado, bulbos olfatorios y en médula espinal. En el adulto aparecen células TH-ir en la lámina terminal y en el núcleo hipotalámico ventral.

Los primeros grupos celulares DA-ir aparecen en la médula espinal, el núcleo del tubérculo posterior y el núcleo hipotalámico dorsal de prolarvas. En larvas se observa la aparición de nuevos grupos celulares DA-ir en el núcleo preóptico caudal, el núcleo de la comisura postóptica, el núcleo de la comisura postinfundibular y en el rombencéfalo caudal. Todos estos grupos celulares DA-ir fueron también observados en adultos, en los que aparece además un grupo DA-ir adicional en el núcleo hipotalámico ventral. La distribución de la inmunorreactividad a TH y DA se asemeja bastante, aunque en los bulbos olfatorios y la lámina terminal hay células TH-ir que no son DA-

inmunorreactivos (DA-ir), y en algunas poblaciones el momento de aparición de la inmunorreactividad es diferente.

La distribución y el desarrollo de estructuras DBH-inmunorreactivas (DBH-ir) en el cerebro de la lamprea varían sensiblemente de las de TH. Las primeras células DBH-ir aparecen en el órgano paraventricular de prolarvas, y ya en larvas en el tegmento sinencéfalo-mesencéfalo y en el rombencéfalo caudal. Hay diferencias llamativas entre la lamprea de mar y el resto de vertebrados con respeto al desarrollo relativo de la inmunorreactividad a DA, lo cual se relaciona probablemente con el complejo ciclo vital de la lamprea de mar. Como en otros vertebrados el desarrollo de los sistemas serotoninérgico y GABAérgico precede al del sistema catecolaminérgico.

CAPÍTULO 3

Cambios neuroquímicos en la retina de la lamprea de mar (Petromyzon marinus) durante la metamorfosis: estudio inmunocitoquímico de serotonina, tirosina hidroxilasa, dopamina y colina acetiltransferasa

Las lampreas tienen una larga etapa larvaria bien diferenciada del individuo adulto en la que los individuos son prácticamente ciegos y su retina funciona meramente como fotorreceptor. La retina larvaria es un modelo interesante para estudios de desarrollo debido a su desarrollo bifásico. La primera fase comienza en prolarvas y da lugar a una retina central escasamente diferenciada, mientras que en la segunda fase se produce un crecimiento alrededor de la retina central durante el período larvario medio-tardío que provoca la formación de una retina lateral ancha prácticamente indiferenciada, y que no se diferenciará hasta la metamorfosis.

Con este trabajo pretendemos estudiar la maduración neuroquímica de la retina de la lamprea de mar durante metamorfosis usando anticuerpos contra serotonina,

tirosina hiroxilasa, dopamina y colina acetiltransferasa. Las células amacrinas de la retina del individuo adulto son inmunorreactivas a estas sustancias, mientras que esto no ocurre en cualquiera de las regiones de la retina larvaria. La metamorfosis de la lamprea de mar fue subdividida por Youson y Potter (1979) en 7 etapas, de M1 a M7. La inmunorreactividad en estructuras serotoninérgicas y colinérgicas fue observada por primera vez en la etapa M1, mientras que en las estructuras catecolaminérgicas esta aparición se retrasa hasta M3. Todas las células inmunorreactivas a estas sustancias fueron descritas como células amacrinas, puesto que no se observaron fibras inmunorreactivas en la capa plexiforme externa o en la parte externa de la capa nuclear interna. El orden de aparición de las células serotoninérgicas, colinérgicas y catecolaminérgicas durante la metamorfosis se asemeja al orden de aparición de estos mismos sistemas en el cerebro de la lamprea de mar. La maduración tardía de las células amacrinas en la retina de la lamprea es única en vertebrados y se puede relacionar con el peculiar ciclo de la vida de esta especie.

CONCLUSIONES

Este trabajo es un estudio sobre el desarrollo de los sistemas serotoninérgico y catecolaminérgico en el sistema nervioso central de la lamprea de mar. Del análisis detallado de los resultados extraemos las siguientes conclusiones:

1. En el sistema nervioso central de la lamprea de mar se observaron grupos celulares serotoninérgicos en el diencéfalo, el rombencéfalo y la médula espinal, además de células ocasionales en el telencéfalo y el mesencéfalo caudal. Esta distribución es similar a la encontrada en la lamprea de río.

2. Grupos celulares inmunorreactivos a tirosina hidroxilasa (TH-ir) se observaron en el prosencéfalo, la parte más caudal del rombencéfalo y en la médula espinal del individuo adulto. Estos grupos se demostraron inmunorreactivos también a dopamina, excepto en los bulbos olfatorios y la lámina terminal. Sin embargo, la distribución de estructuras TH-ir y DBH-ir es muy diferente. Las neuronas DBH-ir se encontraron en el órgano paraventricular, el tegmento del sinencéfalo-mesencéfalo y en el rombencéfalo caudal.

3. Los grupos celulares serotoninérgicos del sistema nervioso central de la lamprea de mar aparecen en diferente momento desde la etapa embrionaria y la metamorfosis. Las células catecolaminérgicas aparecen por primera vez en la fase prolarvaria. El patrón de distribución de la inmunorreactividad a TH y DA en adultos no se completa hasta la metamorfosis, mientras que la inmunorreactividad a DBH se completa al final del período larvario. Estos resultados indican que grupos neuronales son el mismo fenotipo comienzan a expresarlo en diferente momento del desarrollo, lo que probablemente implica diferencias en los mecanismos de especificación celular.

4. La comparación del desarrollo de los sistemas serotoninérgico y catecolaminérgico muestra que el serotoninérgico tiene una aparición más temprana. Este resultado es similar a lo encontrado en otros vertebrados, lo que sugiere que debe ser una característica altamente conservada dentro del desarrollo del sistema nervioso de los vertebrados.

5. No se encontró expresión transitoria de serotonina, TH, DA o DBH en ningún grupo celular inmunorreactivo a estas sustancias, a pesar de lo descrito en otros vertebrados.

6. La ausencia de grupos celulares catecolaminérgicos durante el período embrionario y su progresiva aparición durante el período prolarvario de la lamprea de

mar contrasta con su masiva presencia en embriones en otros grupos de vertebrados. Este hecho se puede atribuir al comparativamente corto período embrionario y la falta de maduración del cerebro en el momento de la eclosión. Además, la aparición progresiva de los grupos celulares catecolaminérgicos entre el período prolarvario y la metamorfosis en la lamprea de mar es similar a lo encontrado en anfibios, y contrasta con lo observado en vertebrados sin metamorfosis.

7. El estudio del desarrollo de los sistemas serotoninérgico, catecolaminérgico y colinérgico en la retina de la lamprea de mar mostró que mientras el serotoninérgico y el colinérgico comienzan su expresión al comienzo de la metamorfosis, el catecolaminérgico retrasa su aparición hasta la mitad de la misma. Todas las células inmunorreactivas a estas sustancias fueron descritas como células de tipo amacrino, dado que no se encontraron fibras inmunorreactivas en la capa plexiforme externa ni en la parte externa de la capa nuclear interna.

8. El orden de aparición de estos sistemas en la retina durante la metamorfosis coincide con el orden de aparición en el cerebro en la lamprea de mar durante las etapas prolarvaria y larvaria.

9. La ausencia de inmunorreactividad a estos sistemas en la retina larvaria y el retraso de la maduración de las células amacrinas son características únicas entre los vertebrados y seguramente está relacionado con el peculiar ciclo de vida de esta especie. Esto, junto con la falta de estructuras necesarias para la formación de imágenes en la retina larvaria, apoyan la hipótesis de que el ojo larvario es una estructura parecida a un "ocelo" que se desarrolla en un ojo funcional durante la metamorfosis.

Os *Cartier Breson* colaboradores;

Garci; portada

Chusiña; salmón en Ximonde

Verona; frezadeiro lampreeiro

Pablo; lamprey´s pose

Fruqui; lamprea á Cesureña

Eu tamén fixen algunha

Elixe o que che gustaría facer de balde o resto da túa vida e logo atopa a maneira de que cobrar por iso.

(Anónimo)