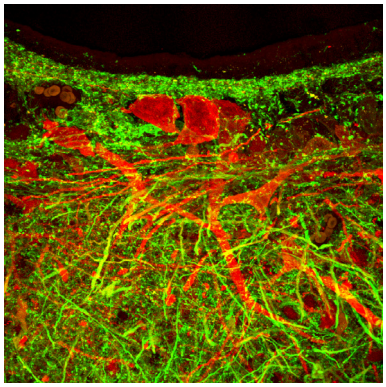
UNIVERSIDAD DE SANTIAGO DE COMPOSTELA
DEPARTAMENTO DE BIOLOGÍA CELULAR Y ECOLOGÍA
ÁREA DE BIOLOGÍA CELULAR

"The glycinergic system of the CNS of the sea lamprey, *Petromyzon marinus*. A developmental study and comparison with GABA"



MEMORIA

Que para optar al Grado de Doctor en Biología presenta

Verona Villar Cerviño

Santiago de Compostela, 2009

MARIA CELINA RODICIO, PROFESORA TITULAR DE BIOLOGÍA CELULAR, Y RAMÓN ANADÓN ÁLVAREZ, CATEDRÁTICO DE BIOLOGÍA CELULAR, DEL DEPARTAMENTO DE BIOLOGÍA CELULAR Y ECOLOGÍA DE LA FACULTAD DE BIOLOGÍA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA,

CERTIFICAN,

Que la presente memoria titulada "The glycinergic system of the CNS of the sea lamprey, *Petromyzon marinus*. A developmental study and comparison with GABA", que para optar al Grado de Doctor en Biología presenta Doña VERONA VILLAR CERVIÑO, ha sido realizada bajo nuestra dirección. Y considerando que constituye trabajo de tesis, autorizamos su presentación al Consejo de Departamento correspondiente.

Y para que así conste, expedimos el presente certificado en Santiago de Compostela, a de de 2009.

El Doctorando | La Directora de la Tesis | El Director de la Tesis

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Esta Tesis Doctoral forma parte de los Proyectos de Investigación “Nuevas aportaciones al estudio neuroquímico del desarrollo y organización del sistema nervioso central de la lamprea de mar” de la Xunta de Galicia (PGIDIT04PXID20003PR; 2004-2007) y “Estudio hodológico inmunohistoquímico de los sistemas aminoacidérgicos durante el desarrollo del sistema nervioso central de la lamprea de mar” del Ministerio de Educación y Ciencia (BFU2007-61056; 2007-2009).

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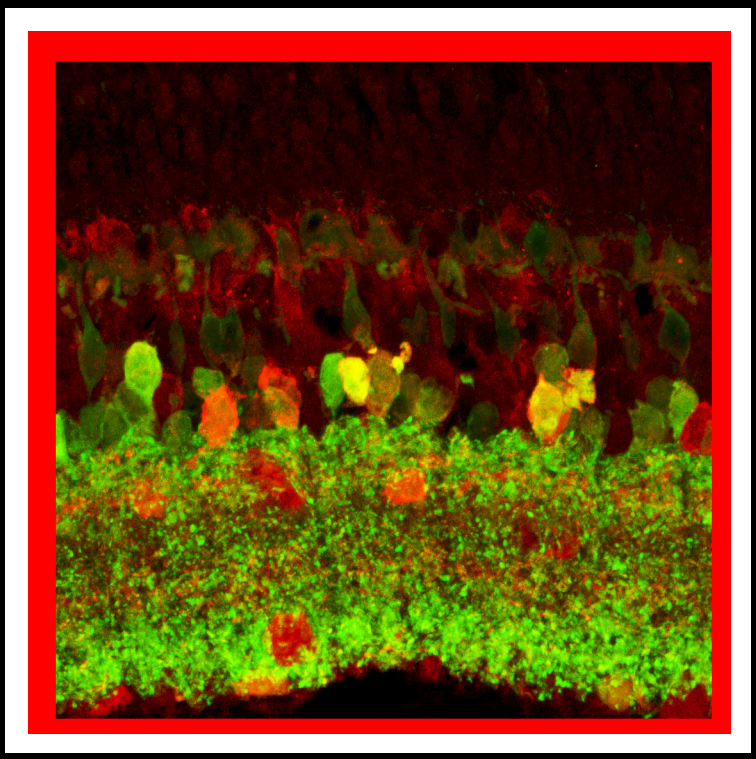
Introduction



INTRODUCTION

1.1. Glycine and GABA as neurotransmitters

Glycine, as well as the γ-aminobutyric acid (GABA), is one of the most important inhibitory neurotransmitters in the central nervous system (CNS) of vertebrates. Glycine is synthesized from serine by the enzyme serine transhydroxymethylase (Sky-Peck et al., 1966; Bridgers, 1968; Shank and Aprison, 1970; Shank et al., 1973; Daly and Aprison, 1974; Daly et al., 1976; Aprison and Daly, 1978) (Fig. 1). The action of glycine released by glycinergic neurons is mediated by the activation of glycine receptors that increases the conductance of chloride in the postsynaptic membrane, hyperpolarizing the postsynaptic neuron (Young and Snyder, 1974; Barker and Ransom, 1978; Betz, 1987; Bormann et al., 1987). This effect ends with the reuptake of glycine by two members of the family of Na^+/Cl^- dependent neurotransmitter transporters (GLYT1 and GLYT2) and by the glycine cleavage system, a mitochondrial and cytosolic enzyme complex (Garrow et al., 1993). GLYT1 is mainly considered a glial transporter, although it is also expressed in neurons, and the GLYT2 is primarily associated with neurons (Cubelos et al., 2005).

In the CNS, glycine appears to be a co-agonist modulator of the N-methyl-D-aspartate (NMDA) receptor (Wood, 2005), and during development it could act, as well as GABA, as an excitatory neurotransmitter (Reichling et al., 1994; Ben-Ari, 2002). This excitatory action is due to the fact that intraneuronal Cl^- concentrations are high during development and glycine or GABA receptor channel opening is followed by Cl^- efflux and membrane depolarization. The relatively high intracellular Cl^- concentration in immature neurons is due to the developmentally early appearance of inwardly directed Cl^- transporters, in particular the Na^+-K^+-Cl^- cotransporter NKCC1 (Clayton et al., 1998). The shift from depolarization to hyperpolarization and thereby the inhibitory action of GABA and glycine depends on the expression of KCC2, a K^+-Cl^- cotransporter that is responsible for the Cl^- extrusion capacity of mature neurons (Rivera et al., 1999).

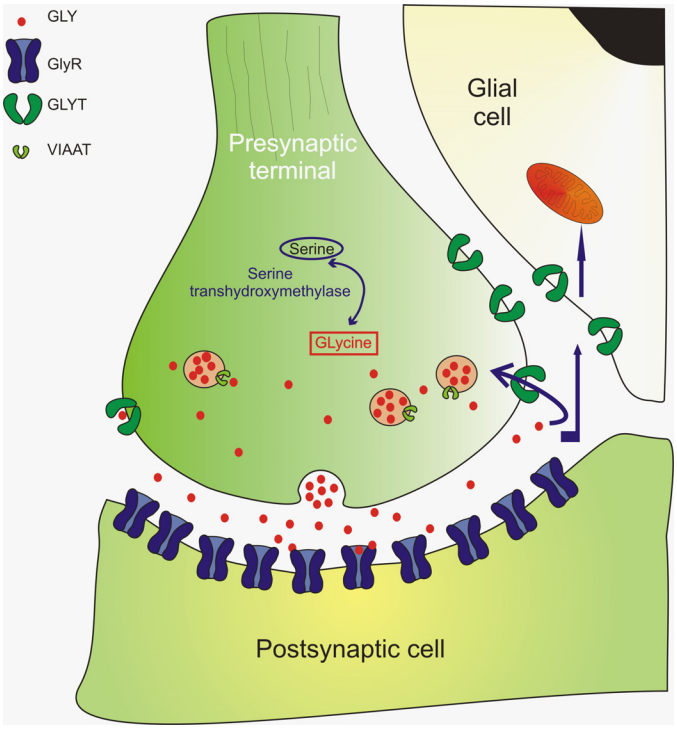


Fig. 1. Schematic drawing of a glycinergic synapse showing the main structures involved in glycine neurotransmission. GLY, glycine; GlyR, glycine receptor; GLYT, glycine transporter; VIAAT, vesicular inhibitory amino acid transporter.

GABA is synthesized preferentially in the CNS from glutamate by glutamate decarboxylase (GAD) enzymes. Early kinetic and inhibition studies showed two GAD isoforms with different affinity for its coenzyme, pyridoxal-50-phosphate, GAD65 and GAD67 (Bayon et al., 1977; Covarrubias and Tapia, 1980; Erlander and Tobin, 1991; Kaufman et al., 1991). These proteins are encoded by different, independently regulated genes. GAD67, located on cell bodies and dendrites, synthesizes a cytoplasmic pool of GABA, and GAD65 synthesizes synaptic GABA in nerve endings (Soghomonian and Martin, 1998). Three types of widely described GABA receptors were found: the ionotropic $GABA_A$ (Olsen, 1982; Alford et al., 1991) and $GABA_C$ receptors (Johnston et al., 1975; Bormann and Feigenspan, 1995), and the metabotropic $GABA_B$ receptor (Bowery et al., 1980; Dolphin and Scott, 1987; Dunlap et al., 1987; Alford and Grillner, 1991). The reuptake of GABA from the synaptic cleft is mediated by different Na^+-Cl^- dependent transporters called GAT1, GAT2, GAT3 and GAT4 (Borden, 1996; Itouji et al., 1996; Johnson et al., 1996; Jursky and Nelson, 1996).

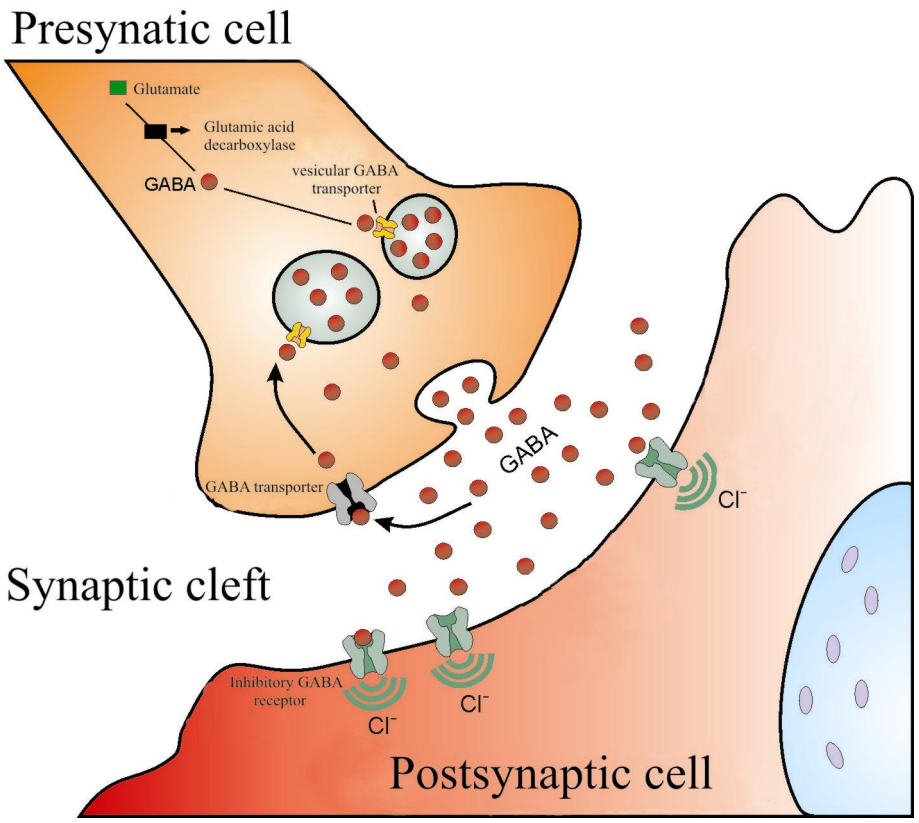


Fig. 2. Schematic drawing of the GABAergic transmission and the main structures involved.

Only a single transporter for the filling of vesicles at both GABAergic and glycinergic synapses has been identified. It is referred to as either vesicular GABA transporter (VGAT) (McIntire et al., 1997) or vesicular inhibitory amino acid transporter (VIAAT) (Sagne et al., 1997). The designation of VIAAT as a GABA/glycine transporter is based on evidences from morphological studies showing the presence of VIAAT in both GABAergic and glycinergic synaptic terminals (Chaudhry et al., 1998), from biochemical studies on neurotransmitter uptake characteristics of synaptic vesicle preparations (Burger et al., 1991; Christensen and Fonnum, 1991), and from electrophysiological studies demonstrating corelease of GABA and glycine, most likely from single vesicles in spinal cord neurons (Jonas et al., 1998).

1.2. Glycine and GABA in the CNS of vertebrates

Although studies on the distribution of glycine in the brain have focused mainly in mammals (Hökfelt and Ljungdahl, 1971; Iversen and Bloom, 1972; Ljungdahl and Hökfelt, 1973; Campistron et al., 1986; Wenthold, 1987; Aoki et al.,

1988; Helfert et al., 1989; Fort et al., 1990, 1993; Walberg et al., 1990; Kolston et al., 1992; Pourcho et al., 1992; Popratiloff et al., 1996; Rampon et al., 1996; Bäurle and Grüsser-Cornehls, 1997; Lue et al., 1997; Spirou and Berrebi, 1997; Merchán et al., 2005; Zeilhofer et al., 2005), some studies have been performed in specific regions of the CNS of other vertebrates (Sheridan et al., 1984; Dale et al., 1986; Reichenberger et al., 1993; Uematsu et al., 1993; Shupliakov et al., 1996; Vesselkin et al., 2000). Glycine is primarily located in neurons of the rhombencephalon and spinal cord (Wenthold, 1987; Aoki et al., 1988; Helfert et al., 1989; Saint Marie et al., 1989, 1991; Walberg et al., 1990; Kolston et al., 1992; Pourcho et al., 1992; Spirou and Berrebi, 1997), but it was also found in neurons of other regions of the CNS (Pourcho et al., 1992; Rampon et al., 1996; Zeilhofer et al., 2005). Glycine plays an important role in the regulation of locomotor behavior and in processing sensory information.

A number of functional and anatomical studies have focused on the neurotransmitters present in the spinal cord of adult lampreys. Electrophysiological and pharmacological studies in the spinal cord and brainstem of adult lampreys have revealed that glycinergic inhibitory interneurons control motor rhythm generation underlying locomotor behavior (Homma and Rovainen, 1978; Matthews and Wickelgren, 1979; Gold and Martin, 1983; Buchanan and Grillner, 1988; Alford and Williams, 1989; Alford et al., 1990a,b; Dubuc et al., 1993a,b). However, there are no studies of distribution of glycine in the brain of lampreys, and only a few studies were performed on the presence of glycine in the adult lamprey spinal cord (Vesselkin et al., 1995, 2000; Shupliakov et al., 1996; Birinyi et al., 2001).

Scant studies have focused on the glycinergic system during development of mammalian or non-mammalian vertebrates. A few studies with glycine immunocytochemistry reported the early development of the glycinergic neurons of the chick and mouse spinal cord, as well as its comparison with development of GABA immunoreactivity (Berki et al., 1995; Allain et al., 2006). Earlier immunocytochemical studies also reported the development glycinergic cells in the spinal cord and medulla oblongata of early embryos of *Xenopus* (Dale et al., 1986; Roberts et al., 1988). In zebrafish, a developmental study with *in situ* hybridization of GLYT2 has provided some data on the early organization of putative glycinergic populations in the hindbrain and spinal cord (Higashijima et al., 2004; Cui et al.,

2005). Similarly, the expression patterns of the glycine transporters xGLYT1 and xGLYT2 in the brain and its coexpression with the mRNA of its synthesizing enzyme glutamic acid decarboxylase (xGAD67) have been recently described by *in situ* hybridization in early stages of *Xenopus* (Wester et al., 2008). The distributions of glycine-immunoreactive (ir) populations in the cerebellum and vestibular nuclear complex of the frog (Reichenberger et al., 1993, 1997) and in the medulla oblongata of plethodontid salamanders (Landwehr and Dicke, 2005) have also been reported.

Glycine is contained or uptaken by some cells of the adult vertebrate retina, mainly amacrine cells (Pourcho and Goebel, 1985, 1987; Fletcher and Kalloniatis, 1996; MacNeil and Masland, 1998; Menger et al., 1998). The distribution of glycine in larval retinas has been investigated only in the tiger salamander (Yang and Yazulla, 1988; Li et al., 1990). In this system, glycine immunoreactivity was found primarily in amacrine cells, in cells of the ganglion cell layer that may be displaced amacrine cells, and rarely in bipolar cells (Yang and Yazulla, 1988). In the rabbit retina, glycine appears to provide an excitatory drive during early retinal development, playing a developmentally regulated role in the initiation and propagation of spontaneous retinal waves (Zhou, 2001).

With regard to the other major inhibitory neurotransmitter, GABA, its distribution in lampreys has been studied previously in the brain of both developing stages (Meléndez-Ferro et al., 2002b, 2003) and adults (Meléndez-Ferro, 2001; Meléndez-Ferro et al, 2000; Meléndez-Ferro et al, 2001; Rodicio et al., 2005; Robertson et al., 2007). There are also reports of the GABA-ir neuronal populations in the spinal cord of developing lamprey (Meléndez-Ferro et al., 2003; Ruiz et al., 2004). Developmental studies in sea lampreys reported that the differentiation of GABA-ir neurons in the diencephalon, in the basal plate of the isthmus, caudal rhombencephalon, and rostral spinal cord begins in late embryos, whereas differentiation in the telencephalon and midbrain was delayed to posthatching stages (Meléndez-Ferro et al., 2002b, 2003). In adult lampreys, GABA-ir cells and fibers are widely distributed from the olfactory bulbs to the spinal cord (Meléndez-Ferro, 2001; Meléndez-Ferro et al, 2000; Meléndez-Ferro et al., 2001; Robertson et al., 2007).

The development of the GABAergic system was also investigated in the brain, spinal cord and retina of various jawed vertebrates by using GABA

immunocytochemistry (teleosts: Östholm et al., 1988; Ekström and Ohlin, 1995; Mueller et al, 2006; Mueller et al, 2008; amphibians: Roberts et al., 1987; Dale et al., 1987a,b) and/or *in situ* hybridization with probes to mRNAs of glutamate decarboxylase (GAD65 and GAD67; mouse: Katarova et al., 2000; frog: Brox et al., 2002; zebrafish: Higashijima et al., 2004; Mueller et al., 2008). Some studies have revealed colocalization of GABA and glycine immunoreactivities in some cells and nerve boutons of the lamprey spinal cord (Vesselkin et al., 1995, 2000; Shupliakov et al., 1996; Birinyi et al., 2001), indicating that inhibitory effects on postsynaptic cells can be mediated by corelease of these neurotransmitters. Colocalization of glycinergic and GABAergic markers in the same neuron (GLYT2 and GAD67, respectively) was also revealed by in situ hybridization in the rat brain (Tanaka et al., 2003; Tanaka and Ezure, 2004). Some glycine receptors of zebrafish, unlike those of mammals, can be activated by GABA and taurine, suggesting possible cooperative functions of these transmitters in some synapses (David-Watine et al., 1999; Imboden et al., 2001). The existence of inhibitory receptors activated by both glycine and GABA has also been suggested for lamprey (Baev et al., 1992).

1.3. The sea lamprey

Biology

Lampreys are extant representatives of the most ancient lineage of vertebrates, the Agnathans. Accordingly, their study is key to understand the phylogeny of the vertebrate brain. Sea lampreys have a complex life cycle that begins in the river with the embryonic period (about 12 days), followed by a short prolarval stage that was subdivided into hatchling (P0–P1), pigmentation (P2–P3), gill cleft (P4–P7), and burrowing (P8–P23) stages, according to Piavis (1971). The larval period is very long and can last for more than eight years. During this stage, the animals are blind filter-feeders that live burrowing in the river bed. Through a complex metamorphosis, larvae transform into sighted young adult lampreys that migrate to the sea and feed parasitically on fish as they grow. After several years they return to rivers to breed and then die (Hardisty and Potter, 1971) (Fig. 3).

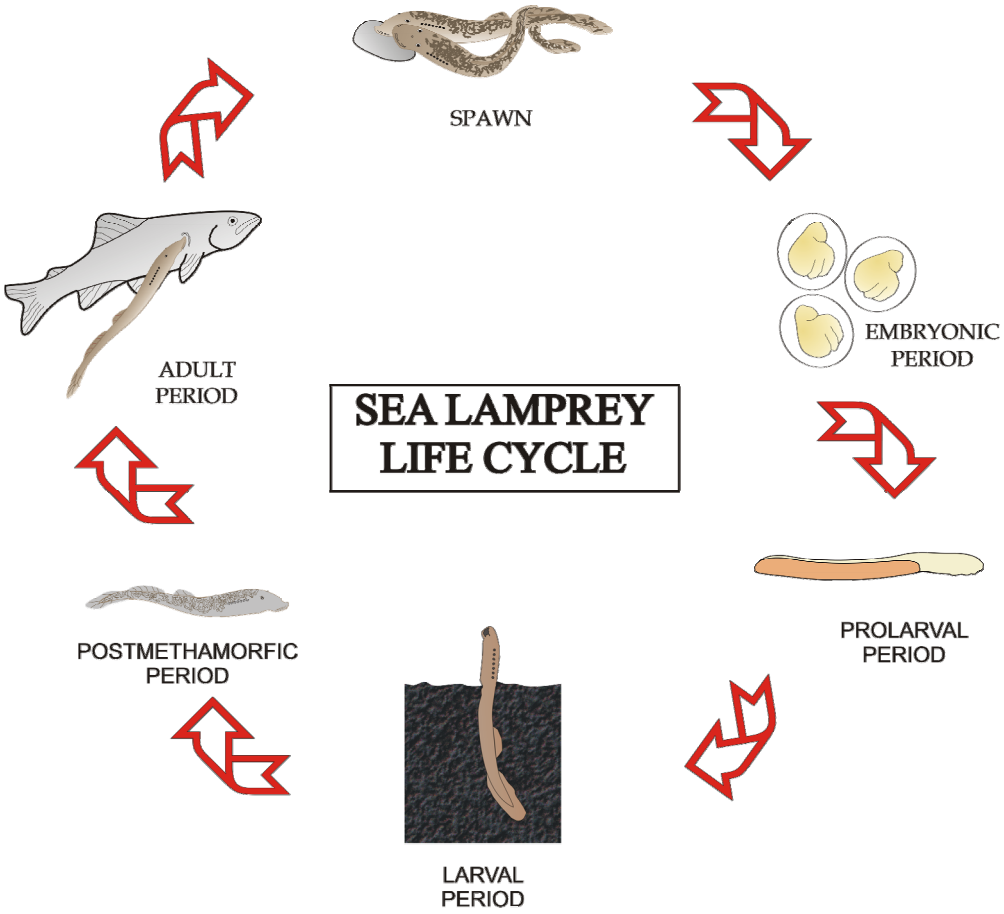


Fig. 3. Life cycle of the sea lamprey showing the different stages: embryos, prolarvae, larvae and adults.

Organization of the lampreys central nervous system

The brain of lampreys is slender and very small, but the main parts of the brain neuraxis present in jawed vertebrates (telencephalon, diencephalon, mesencephalon and rhombencephalon) can be readily identified (Fig. 4).

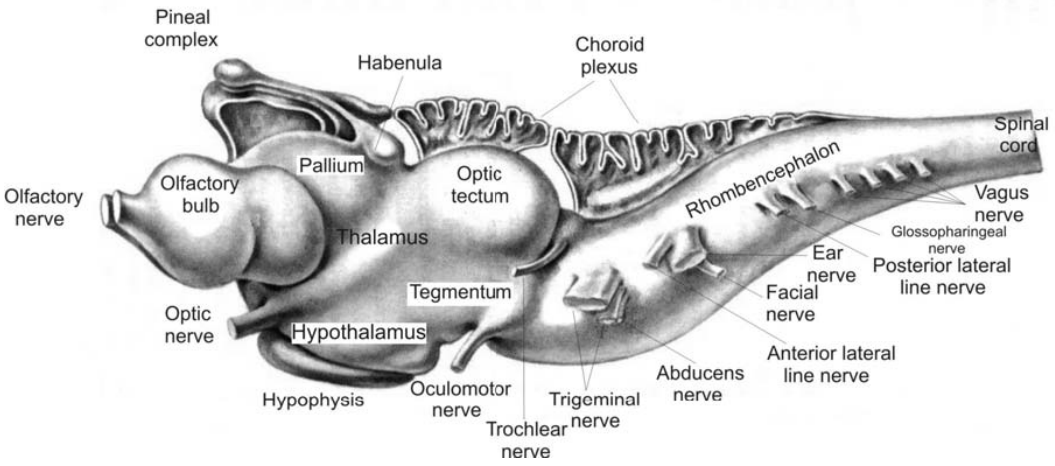


Fig. 4. Schematic drawing of a lateral view of the lamprey brain slightly modified from Nieuwenhuys and Nicholson, 1998.

In the CNS of lampreys, most of the neuronal perikarya are small and located in a continuous grey zone that extends close to the ventricular surface (Butler and Hodos, 1996), although some prominent giant neurons are also present and a number of neurons have migrated away from the periventricular region remaining at lateral positions (Nieuwenhuys and Nicholson, 1998). Another characteristic feature is that the glial system consists only of astrocytes and ependymal cells (Merrick et al., 1995), since the brain and the spinal cord lack oligodendrocytes (Fig. 5). It is also remarkable that all nerve fibers are devoid of myelin sheaths in both the central and peripheral nervous system. Other unusual characteristic of the lamprey CNS is the lack of blood vessels of the larval CNS and adult spinal cord, and the poor vascularization of the adult brain.

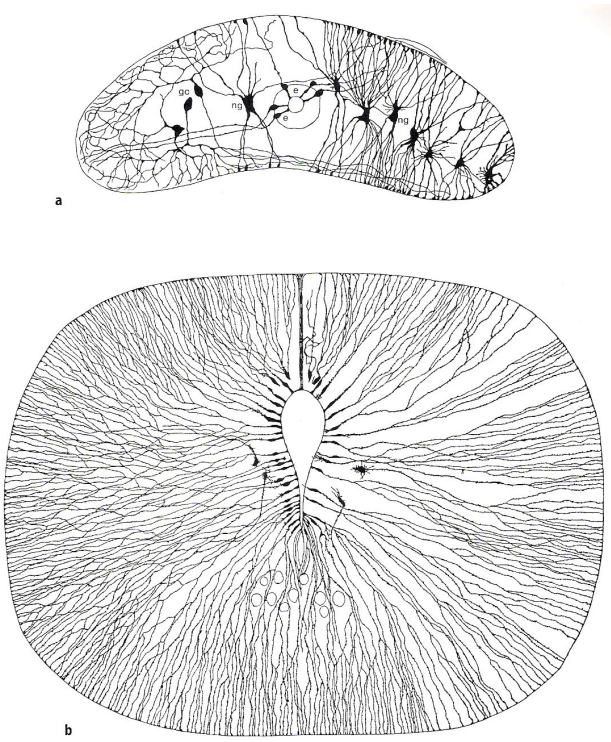


Fig. 5. Drawing of the glial elements in the central nervous system of the lamprey. **a**: Cross-section through the caudal part of the spinal cord. **b**: Cross-section through the caudal part of the rhombencephalon. e, ependymal cells; gc, ganglion cells (neurons); ng, neuroglial cells. From Retzius, 1893.

The pattern of organization of the prosencephalon of lampreys was recently revised by Pombal and Puelles (1999), who proposed a segmental model. In this prosomeric model they indicated the existence of 6 prosomeres (P1-P6). However, recent studies in other vertebrates have shown that only prosomeres P1-P3 could be considered as subdivisions of the caudal diencephalon, while the territory comprised of putative prosomeres P4-P6 (i.e. the telencephalon plus rostral diencephalon or hypothalamus) is nowadays considered as a single region, the secondary prosencephalon (Puelles and Rubenstein, 2003). This modified prosomeric model is supported by developmental studies in lampreys (Meléndez-Ferro et al., 2002b; Osorio et al., 2005; Villar-Cheda et al., 2006).

The following description of the organization of central nervous system of the lamprey is mainly based on the revision by Nieuwenhuys and Nicholson (1998) (Fig. 6), taken also in consideration the results of a number of experimental studies in lampreys.

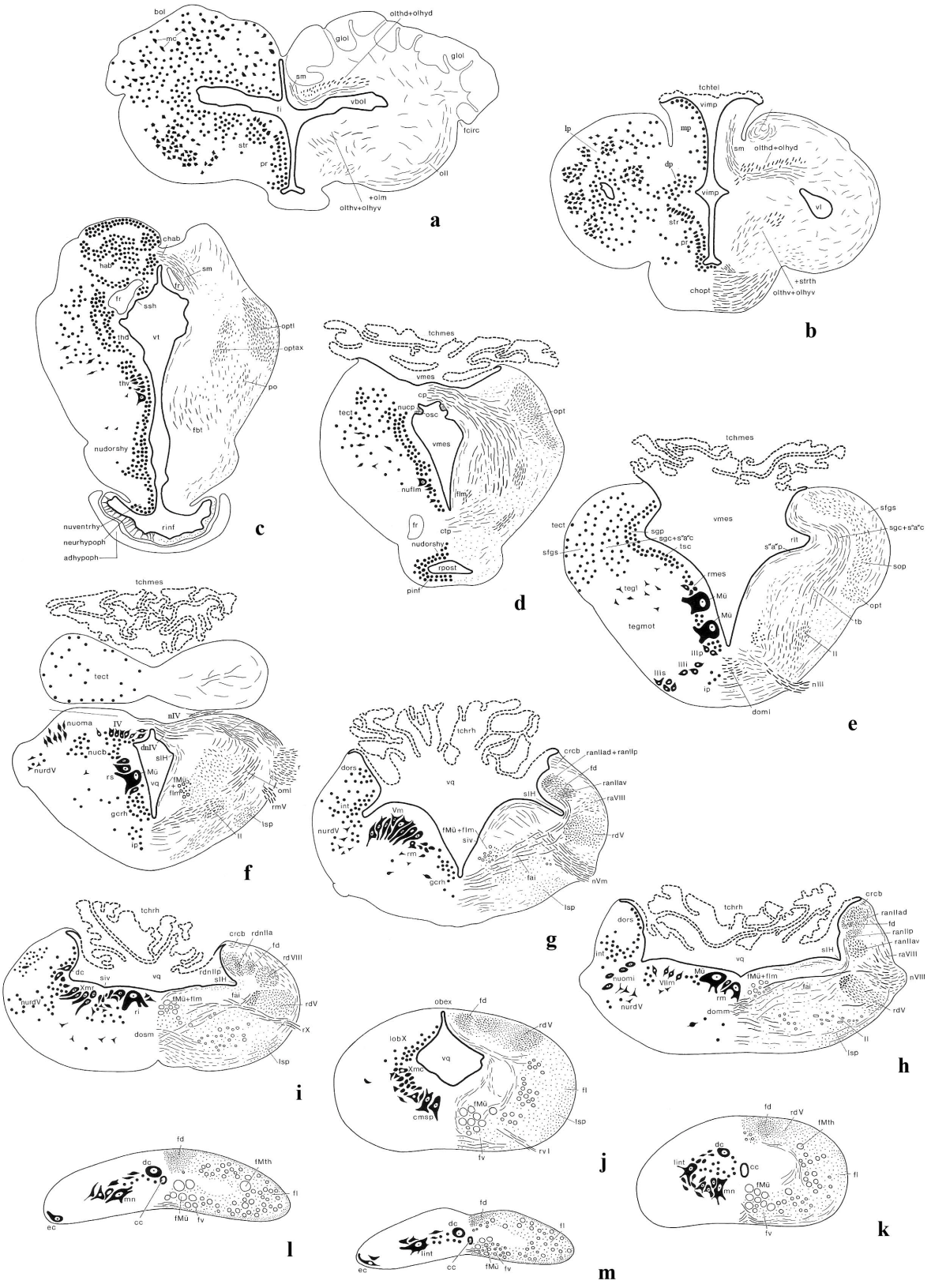


Fig. 6. A series of transverse sections through the brain (a-j) and spinal cord (k-m) of *Lampetra fluviatilis*. The left half shows the cell picture and the right half shows the fiber systems. **a, b**: telencephalon. **c**: diencephalon. **d**: section through the posterior commissure. **e**: mesencephalon. **f**: isthmus. **g, h, i**: rhombencephalon. **j**: obex. **k-m**: spinal cord. Slightly modified from Nieuwenhuys and Nicholson, 1998. For abbreviations see list.

Abbreviations

adhypoph, adenohypophysis

bol, olfactory bulb

cc, central canal

chab, habenular commissure

chopt, optic chiasm

cmsp, spinal motor column

cp, posterior commissure

crcb, cerebellar crest

ctp, posterior tubercle commissure

dc, dorsal cells

dnIV, decussating fibers of the trochlear nerve

doml, decussation of the octavomesencephalic lateral tract

domm, decussation of the medial octavomesencephalic tractus

dors, dorsal nucleus of the octavolateral area

dosm, decussation of the medial octavospinal tract

dp, dorsal pallium

ec, edge cell

fai, arcuate internal fibers

fbt, basal telencephalic fascicle

fcirc, circular fissure

fd, funiculus dorsalis

fl, funiculus lateralis

flm, medial longitudinal fascicle

fMth, fiber of Mauthner

fMü, fibers of Müller

fr, fasciculus retroflexus

fv, funiculus ventralis

gcrh, rhombencephalic central grey

glol, olfactory glomeruli

hab, habenula

IIIi, intermediate oculomotor nucleus

IIIp, periventricular oculomotor nucleus

IIIs, superficial oculomotor nucleus

int, intermediate nucleus of the octavolateral area

ip, interpeduncular nucleus

IV, nucleus of the trochlear nerve

lint, lateral interneurons

ll, lateral lemniscus

lm, lateral pallium

lobX, vagal lobus

lsp, spinal lemniscus

mc, mitral cells

mn, motoneuron

mp, medial pallium

Mü, Müller cells

neurhypophh, neurohypophysis

nIII, oculomotor nerve

nIV, trochlear nerve

nucb, cerebellar nucleus

nucp, nucleus of the posterior commissure

nudorshy, dorsal hypothalamic nucleus

nuflm, medial longitudinal fascicle nucleus

nuoma, anterior octavomotor nucleus

nuomi, intermediate octavomotor nucleus

nurdV, trigeminal dorsal root nucleus

nuventrhy, ventral hypothalamic nucleus

nVIII, octaval nucleus

nVm, motor root of the trigeminal nerve

olhyd, dorsal olfacto-hypothalamic tract

olhyv, ventral olfacto-hypothalamic tract

oll, lateral olfactory tract

olm, medial olfactory tract

olthd, dorsal olfacto-thalamic tract

olthv, ventral olfacto-thalamic tract

opt, optic tract

optax, axial optic tract

optl, lateral optic tract

osc, subcommissural organ

pinf, postinfundibular nucleus

po, postoptic tract

pr, preoptic nucleus

ranllad, dorsal ascending root of the anterior lateral line nerve

ranllav, ventral ascending root of the anterior lateral line nerve

ranllp, ascending root of the posterior lateral line nerve

raVIII, ascending root of the octaval nerve

rdnlla, descendent root of the anterior lateral line nerve

rdnllp, descending root of the posterior lateral line nerve

rdV, descending root of the trigeminal nerve

rdVIII, descending root of the octaval nerve

ri, inferior reticular nucleus

rinf, infundibular recess

rlt, lateral tectal recess

rm, medial reticular nucleus

rpost, posterior recess

rs, superior reticular nucleus

rsV, sensory root of the trigeminal nerve

rv1, ventral root of the spinal nerve

rX, root of the vagal nerve

s "a"p, stratum album periventriculare

s"a"c, stratum album centrale

sfgs, stratum fibrosum et griseum superficiale

sgp, stratum griseum periventriculare

siv, sulcus intermedius ventralis

slH, sulcus limitans of His

sm, stria medullaris

sop, stratum opticum

ssh, subhabenular sulcus

str, striatum

strth, striothalamic tract

tb, tectobulbar tract

tchmes, mesencephalic tela chorioidea

tchrh, rhombencephalic tela chorioidea

tchtel, telencephalic tela chorioidea

tect, mesencephalic tectum

tegl, lateral tegmental nucleus

tegmot, motor tegmentum

thd, dorsal thalamus

thv, ventral thalamus

VIIm, motor nucleus of the facial nerve

vimp, impar telencephalic ventricle

vl, lateral ventricle

Vm, motor nucleus of the trigeminal nerve

vmes, mesencephalic ventricle

vq, fourth ventricle

Xmc, caudal motor nucleus of the vagal nerve

Xmr, rostral motor nucleus of the vagal nerve

Telencephalon

The lamprey telencephalon comprises the olfactory bulbs, the cerebral hemispheres (lateral pallium) and the telencephalon medium (medial pallium, subhippocampal lobe, subpallium and preoptic nucleus). The olfactory bulbs and the lateral pallium are widely continuous and result from paired evagination of the developing forebrain, while the telencephalon medium represents the non-evaginated portion. The lamprey pallium mainly consists of the medial and lateral pallium, the latter often subdivided into dorsal and ventral parts ("primordial dorsal and pyriform pallia", respectively). The connections of the lamprey telencephalon have received considerable attention, and the intra- and extratelencephalic connections of the olfactory bulbs, pallium and striatum have been studied experimentally with modern tract-tracing methods in lampreys (Northcutt and Puzdrowski, 1988; Polenova and Vesselkin, 1993; Northcutt and Wicht, 1997; Pombal et al., 1997a,b). The olfactory bulb projects to the medial and lateral pallia, the subhippocampal lobe, the septum, striatum, preoptic area and the contralateral olfactory bulb, and also to the ventral thalamus, hypothalamus and posterior tuberculum (Polenova and Vesselkin, 1993). Bulbopetal projections originate in the subhippocampal lobe, pallium and probably in the dorsal thalamus. The lateral and medial pallia receive projections from the olfactory bulbs, the other pallial regions, septum, preoptic area, habenular nuclei and thalamus, and project to most of these regions (Northcutt and Wicht, 1997). Additionally, the medial pallium receives inputs from the hypothalamus, the dorsal isthmal grey and midbrain tegmentum and projects to the pretectum and optic tectum (Northcutt and Wicht, 1997).

The striatum receives fibers from several telencephalic and diencephalic regions, including the olfactory bulbs, pallia thalamus and hypothalamus, as well as from some populations of the isthmus and rostral rhombencephalic reticular formation (Pombal et al., 1997a,b).

Diencephalon

The diencephalon of lampreys comprises the prethalamus (P3, ventral thalamus), thalamus (P2, dorsal thalamus), epithalamus (pineal complex and habenula; dorsalmost part of P2) and pretectum (P1) as alar plate derivatives, and the hypothalamus, posterior tubercle and nucleus of the medial longitudinal fascicle as

basal plate derivatives. Most of the diencephalic cells are sited in the periventricular zone with diffuse anatomical limits between the diverse nuclei.

The pineal complex consists of the pineal and parapineal organs that are both neuroendocrine photoreceptive organs that project differentially to the brain (Meiniel and Collin, 1971; Pu and Dowling, 1981; Pombal et al., 1999a; Yáñez et al., 1993, 1999). The habenula is a highly asymmetric structure with right and left parts. Both habenulae mainly receive telencephalic afferents, and send efferents via the fasciculus retroflexus towards the interpeduncular nucleus that caudally continues to the trigeminal region (Yáñez and Anadón, 1994).

The prethalamus receives fibers from the telencephalon, the habenula, the dorsal thalamus, the hypothalamus and from the optic tectum, and its efferent fibers join the medial longitudinal fascicle or course toward the hypothalamus. It is a motor coordinating center that mostly receives inputs from higher centers and conveys outputs to brainstem coordinating centers (El Manira et al., 1997).

The thalamus is comprised of a group of periventricular neurons and scattered cells located laterally. This region receives inputs from the eyes, the pineal organ, and the sensory mesencephalic and rhombencephalic nuclei, process the information and lead information toward the telencephalon (pallium) and coordination centers of the brain (Heier, 1948; Polenova and Vesselkin, 1993).

The pretectum receives inputs from the optic tracts (De Miguel et al., 1990), and pretectal efferent fibers pass to the optic tectum, torus semicircularis, the parapineal ganglion, prethalamus, thalamus, and hypothalamus (Yáñez et al., 1999; Robertson et al., 2006; De Arriba and Pombal, 2007).

The hypothalamus forms the ventral (basal) part of the secondary diencephalon. It can be divided in a dorsal zone, that includes the postoptic commissure and the dorsal hypothalamic commissure nuclei, and a ventral zone, formed by the preinfundibular, ventral hypothalamic (tuberal region) and postinfundibular nuclei (mammillary region) (Heier, 1948). The pattern of the lamprey hypothalamus has been more recently revised by Pombal and Puelles (1999) in the light of the prosomeric model. The ventral part of the hypothalamus contains four commissures: postoptic, preinfundibular, postinfundibular and posterior tubercle commissures. According to Heier, the hypothalamus receives afferents from all parts

of the telencephalon, the dorsal thalamus, the tectum and the midbrain tegmentum. The afferents from the thalamus and mesencephalon decussate partially in the postoptic commissure. Hypothalamic efferent systems reach the mesencephalic tegmentum and the basal plate of the rhombencephalon (hypothalamo-tegmental tract), the tectum, the dorsal and ventral parts of the thalamus, the pallium, and the olfactory bulb (Heier, 1948; El Manira et al., 1997; Northcutt and Wicht, 1997; Pombal et al., 1997b; González et al., 1999; De Arriba and Pombal, 2007). Neurosecretory, cholinergic projections from the preoptic nucleus and the paraventricular nucleus to the hypophysis are present in lampreys (Oztan and Gorbman, 1960; Goossens et al., 1977; Pombal et al., 1999b). A hypothalamo-spinal projection arising from CSF-c cells of the walls of the infundibular and mammillary recesses was also described recently (Barreiro-Iglesias et al., 2008). The posterior tubercle (located in the basal plate of P2; Pombal and Puelles, 1999) is adjacent to the dorsal hypothalamus. Efferent fibers from the posterior tubercle project to various CNS regions including among others the spinal cord (Barreiro-Iglesias et al., 2008), torus semicircularis (González et al., 1999), optic tectum (De Arriba et al., 2007) and the striatum (Pombal et al., 1997a,b).

The nucleus of the medial longitudinal fascicle (considered classically as a mesencephalic derivative) has also been included in the diencephalic basal plate by Pombal and Puelles (1999). It consists of big neurons (Müller cells M1 and M2) and large cells. This nucleus projects mainly ipsilaterally to the spinal cord (Ronan, 1989).

Mesencephalon

The lamprey midbrain consists of the optic tectum, torus semicircularis and tegmentum. The optic tectum exhibited a layered appearance in adults, with a periventricular layer with two or three rows of cells, a deep fiber layer, intermediate cell and fibrous layer and superficial optic fiber layer (Vesselkin et al., 1980; De Miguel and Anadón, 1987). However, during most the larval life the optic tectum is poorly differentiated, as well as the retina and the optic pathways (De Miguel and Anadón, 1987; De Miguel et al., 1990). The main afferents to the optic tectum come from the retina, medial pallium, striatum, ventral and dorsal thalamus, pretectal nuclei, pineal organ, the torus semicircularis, the mesencephalic M5 nucleus, the

mesencephalic reticular area, isthmic nuclei, the octavolateral nuclei, nucleus of the descending trigeminal tract, the dorsal column nucleus and the reticular formation (Kennedy and Rubinson, 1977; Vesselkin et al., 1980; Robertson et al., 2006; De Arriba and Pombal, 2007). The torus semicircularis is continuous anatomically with the tectum. It receives a large number of fibers from vestibular and lateral line centers (González et al., 1999) and is comparable with the inferior colliculus of mammals, whereas the optic tectum is the main visual center, and is homologous to the superior colliculus (Kennedy and Rubinson, 1977; De Arriba Pérez, 2007; De Arriba and Pombal, 2007).

The midbrain tegmentum comprises a dorsal part with the M5 nucleus of Schober and the reticular mesencephalic area, which sent both projections to the retina (Vesselkin et al., 1980; De Miguel et al., 1990; Rodicio et al., 1995), and the oculomotor nucleus in its ventral part. The M5 nucleus of Schober is located ventral to the torus semicircularis and dorsorostral to the M3 giant Müller cell. The oculomotor nucleus of lamprey consists of three subnuclei that each innervate an extraocular muscle; rostral and dorsal rectus (lateral and intermediate subnuclei, respectively), and rostral oblique (dorsomedial subnucleus) (Fritzsch et al., 1990; Pombal et al., 1994, 1995).

Rhombencephalon

The rhombencephalon of adult lampreys includes a horizontal basal plate and a vertical oriented alar plate, which surround ventrally and laterally the fourth ventricle. The most rostral part of the rhombencephalon is known as the isthmus and the region of the most caudal part of the fourth ventricle as the obex.

The alar plate comprises three longitudinal sensory zones that from dorsal to ventral are:

- The special somatosensory zone or octavolateral area occupies almost the rostral part of the rhombencephalic alar plate organized in three different nuclei, dorsal, medial and ventral (Ronan and Northcutt, 1987; González and Anadón, 1992). The first two nuclei represent the end station of the electroreceptive and mechanoreceptive lateral line nerve fibers, respectively, whereas the ventral nucleus receives fibers from the octaval nerve (Fritzsch et al., 1984; Ronan, 1988; Koyama et al., 1989, 1990; González and Anadón, 1992, 1994). The latter nucleus contains three

local cell aggregations that are known as the octavomotor nuclei or as the vestibular nuclei (Stefanelli and Caravita, 1968; González and Anadón, 1994). These three vestibular nuclei give rise to a specific pattern of spinal, reticular, and mesdiencephalic projections (González and Anadón, 1994; Pombal et al., 1995, 1996; González et al., 1997; Bussières et al., 1999). The presence of labyrinth efferents have also been demonstrated in lampreys (Fritzsch et al., 1989; Koyama et al., 1989).

- The general somatosensory zone, which can be subdivided into a trigeminal sensory region that is chiefly related to the sensory fibers of the trigeminal nerve arising either in the trigeminal ganglia or in medullary and spinal dorsal cells (Finger and Rovainen, 1982; Koyama et al., 1987; Anadón et al., 1989), and in a dorsal column nucleus that receives ascending sensory projections of the spinal dorsal column (Dubuc et al., 1993a; Rodicio et al., 2005). The projections of the dorsal column nucleus to the reticulospinal system have been well-characterized (Dubuc et al., 1993a,b)

- The viscerosensory zone, a poorly characterized region that receives fibers from the sensory roots of the facial, glossopharyngeal and vagal nerves (Koyama, 2005).

The rhombencephalic basal plate is clearly divisible into a medial somatomotor region and a lateral visceromotor region. The largest part of the somatomotor region is occupied by the reticular formation centers. The classical rhombencephalic reticular formation is divided into isthmic, trigeminal, middle and posterior regions following the scheme of Stefanelli (1934). A large number of cells of this formation project to the spinal cord (Ronan, 1989; Swain et al., 1993). Descending projections to the reticular formation have been studied experimentally by Zompa and Dubuc (1998).

The somatomotor centers include the rostral part of the spinal motor column (spino-occipital motoneurons), as well as the abducens nucleus and the trochlear nucleus, which exceptionally and characteristically occupies a dorsal position in the lamprey isthmus (Finger and Rovainen, 1978; Fritzsch et al., 1990; Pombal et al., 1994, 2001). Some of the interoculomotor pathways have also been studied experimentally (González et al., 1998). The visceromotor zone mainly consists of a long column of primary motor neurons (cholinergic) that form the motor nuclei of the trigeminal, facial, glossopharyngeal and vagal nerves (Heier, 1948; Koyama et

al., 1987; Koyama, 2005), which are arranged in a segmental way (Pombal et al., 2001). Only the brainstem afferents of the trigeminal motor nucleus have been studied experimentally (Huard et al., 1999).

Spinal cord

The lamprey spinal cord is a ribbon-like structure that receives paired dorsal and ventral spinal roots, but these do not emerge at the same transverse levels and do not unit to form mixed spinal nerves. The gray matter forms bilateral wing-like expansions that contain different cell types like somatomotor neurons, dorsal cells and several types of intrinsic elements. This gray is surrounded by a mantle of longitudinal fibers, the "white matter" (Rovainen, 1979). Large axons running in the ventromedial column of the spinal cord originate in reticular cells and giant interneurons of the brain (Rovainen, 1967a,b, 1979; Ronan, 1989). Axons in the dorsal column are somatosensory, but the majority of the spinal axons probably arise from interneurons (Ronan and Northcutt, 1990).

The spinal motoneurons are located in a lateral column situated on either side of the cord, consisting of different types of neuron that innervate myotome and fin muscles (Rovainen and Birnberger, 1971; Teräväinen and Rovainen, 1971; Wallén et al., 1985; Shupliakov et al., 1992). In the dorsomedial part of the spinal gray the large first order sensory dorsal cells are found (Finger and Rovainen, 1982; Christenson et al., 1988; Anadón et al., 1989). These cells respond to touch and pressure. The intrinsic neurons are mainly small cells that make up most of the spinal gray, but in addition to the small elements, the lamprey spinal cord contains several types of larger intercalated neurons (Rovainen, 1967b, 1979, 1983; Buchanan and Grillner, 1987, 1988; Grillner et al., 1990; Grillner and Matsushima, 1991; Ohta et al., 1991). The lamprey edge cells represent a new type of intraspinal mechanoreceptor neurons (Grillner et al., 1984).

The reticular formation of the brain stem sends a number of projections to the spinal cord, which constitute the most important descending system from the brain to the spinal system (Johnston, 1902; Tretjakoff, 1909a,b; Ronan, 1989; Swain et al., 1993). Vestibulospinal, mesencephalospinal and diencephalospinal projections have been also demonstrated in lampreys (Ronan, 1989; Swain et al., 1993; Bussières et al., 1999; Barreiro-Iglesias et al., 2008).

Two large fiber systems, the dorsal column pathway and the spinal lemniscus, ascend from the spinal cord to the brain stem. The dorsal column fibers ascend in the dorsal funiculus, reach the obex and continue ipsilaterally in the lateral rhombencephalic alar plate (Ronan and Northcutt, 1990; Dubuc et al., 1993a). Spinal lemniscus fibers ascend in the lateral funiculus carrying touch, pain and temperature stimuli to the brain. Spinal lemniscus fibers innervate the octavolateral area, the reticular region and isthmus, and a few fibers reach the mesencephalic tegmentum; a very small population of cells in the far rostral cord of lampreys may project to the optic tectum and diencephalon (Ronan and Northcutt, 1990; De Arriba and Pombal, 2007). The cells of origin of the spinobulbar projections have been studied with tracing methods, which showed several classes of spinal neurons that project to the brainstem (Vinay et al., 1998).

Retina

Adult lampreys have well-developed eyes. Although the general organization of the adult lamprey retina is similar to that of other vertebrates, there are some characteristic differences.

Like in other vertebrates, the adult lamprey retina contains photoreceptors, bipolar, horizontal, amacrine, ganglion and Müller cells (Dickson and Graves, 1981; Rubinson and Cain, 1989). There are two types of photoreceptor in the lamprey retina, short and long, that were identified as cones and rods respectively by using ultrastructural features (Ishikawa et al., 1987) and specific markers (Negishi et al., 1986). They have also two different bipolar cell types, short and long, although again this distinction is not observed in the larval period. The horizontal cells are distributed in two different horizontal rows, an external one or escleral that contacts only with long photoreceptors, and an internal one or vitreal that contacts with both types of photoreceptors (Teranishi et al., 1982). Most of the ganglion cells are located between the inner nuclear layer (INL) and the inner plexiform layer (IPL), whereas some are immersed in the inner plexiform layer, where the bundles of optic nerve fibers are also situated close to the IPL without forming the classical optic fiber layer (Dalil et al., 1990; Dalil-Thiney et al., 1994).

The adult lamprey retina is formed by the following layers:

1. The **pigment epithelium**, with cells with high amounts of melanosomes.

2. The **photoreceptor layer**, with the outer and inner segments of both short and long photoreceptors.
3. The **outer limiting membrane**, which is formed of the adherent junctions between Müller cell processes and photoreceptor cell inner segments below the photoreceptor layer.
4. The **outer nuclear layer** is composed of the cell bodies of short and long photoreceptors.
5. The **outer plexiform layer**, which separates the outer and inner nuclear layers, is the place where the connections between photoreceptors, and vertically running bipolar cells and horizontally oriented horizontal cells occur.
6. The **inner nuclear layer** is composed of the cell bodies of horizontal, bipolar, and Müller cells, and also of a part of the ganglion and amacrine cells.
7. The **inner plexiform layer** of lampreys is a thick neuropil where the optic fibers course externally and some displaced ganglion and amacrine cells are located. No ganglion cell layer is formed below this plexiform layer.

During the larval period, the lamprey eyes are located deep under the skin and show still a very immature appearance, lacking a differentiated lens (Kleerekoper, 1972). The late development of the lamprey retina is exceptional among vertebrates. The differentiation of cells in the neural retina begins early during the postembryonic period, when ganglion cells and short photoreceptors become distinguishable, but it stops during the larval stage and it stills incomplete until the metamorphic period (Kleerekoper, 1972; Dickson and Collar, 1979; de Miguel and Anadón, 1987; Meléndez-Ferro et al., 2002a). Until the first half of the larval period the retina grows slowly, but from this moment the margins became a proliferative epithelium that expands laterally giving rise to a lack photoreceptor undifferentiated retina. During the second half of the larval life an early differentiated central retina and an undifferentiated lateral retina could be observed. The differentiation of the lateral retina and the appearance of the two types of photoreceptors occur during metamorphosis (De Miguel and Anadón, 1987; Villar-Cheda et al., 2008).

AIMS OF THIS THESIS

The sea lamprey, *Petromyzon marinus*, is a representative of the most primitive vertebrates, the Agnathans, which have the same main central nervous system regions as other vertebrates. Because of its key phylogenetic position the lampreys constitute an important model to explore the basic organization and evolution of many neurochemical systems of vertebrates.

In the central nervous system, many neurons use generally glycine as an inhibitory neurotransmitter. In spite of this fact, investigations about this neurochemical system are very scarce in non-mammalian vertebrates. Previous studies of our group have studied the distribution of GABA immunoreactivity cells and fibers in the lamprey brain and spinal cord during development (Meléndez-Ferro et al., 2002b, 2003) and in adults (Meléndez-Ferro 2001; Meléndez-Ferro et al., 2001), so we wished to know more about the presence in the lamprey CNS of the other main inhibitory system, the glycinergic system.

The main aim of the present study was to describe for the first time the different glycine-ir cell groups and fibers in the brain of the adult sea lamprey, and to analyze the changes that have occurred in glycinergic populations from primitive vertebrates to mammals. We also wished to compare the distribution of glycine and GABA immunoreactivities and to study the possible colocalization of both neurotransmitters in the adult lamprey brain. The results of this study are presented in the **chapter 1** entitled: **Distribution of glycine immunoreactivity in the brain of adult sea lamprey (*Petromyzon marinus*). Comparison with gamma-aminobutyric acid.**

We also wanted to investigate the appearance of the glycinergic system in the lamprey brain, the changes occurring in this system in larvae after prolarval stages and whether or not the glycinergic populations observed in larval stages are retained in the adult. A further aim was to compare the developmental pattern of glycinergic and GABAergic populations by using double immunofluorescence methods and confocal microscopy. The results of this study are presented in the **chapter 2** entitled: **Development of glycine immunoreactivity in the brain of the sea lamprey: comparison with gamma-aminobutyric acid immunoreactivity.**

The third aim of this Thesis was to study the early development of the glycinergic populations of the sea lamprey spinal cord and the changes occurring between embryos and adults by using immunohistochemical techniques. A further aim was to compare the development of the glycinergic cell groups with those containing GABA. The results of this study are presented in the **chapter 3** entitled: **Glycine-immunoreactive neurons in the developing spinal cord of the sea lamprey: comparison with the gamma-aminobutyric acidergic system.**

We also wished to study the neurochemical differentiation of neural circuitry in the lamprey retina during the larval period using a set of antibodies directed against several classical neurotransmitters, including glycine, and to analyze the distribution of these neurotransmitters in the retina of recently transformed young lampreys. This work corresponds to the **chapter 4** entitled: **Presence of glutamate, glycine, and gamma-aminobutyric acid in the retina of the larval sea lamprey: comparative immunohistochemical study of classical neurotransmitters in larval and postmetamorphic retinas.**

MATERIAL AND METHODS

Embryos, prolarvae, larvae, postmetamorphic and adult sea lampreys (*Petromyzon marinus* L) were used in the present study. Embryos and prolarvae were obtained from *in vitro* fertilized eggs reared in our laboratory. Larvae, postmetamorphic and adult animals were collected from the River Ulla with permission from the Xunta of Galicia. Adults were also purchased from a commercial supplier. Larvae were maintained in aerated aquaria with a bed of river sediment, whereas postmetamorphic and adult lampreys were used immediately. All experimental procedures were performed under deep anesthesia with benzocaine.

For the characterization of glycine-ir cells and fibers of the developing and adult lamprey central nervous system, and for the study of the differentiation of the retina we performed single immunoperoxidase or single immunofluorescence protocols using polyclonal or monoclonal primary antibodies. For the comparison of several neurotransmitters with GABA immunoreactivity we made double immunofluorescence experiments using a cocktail of a GABA monoclonal antibody with one polyclonal antibody against glycine or other classical neurotransmitters in the case of the retina study (for more details see Material and methods sections of chapters 1, 2, 3 and 4).

In order to facilitate the analysis of some neuronal populations and to assess their locations, we also performed some tract-tracing experiments with neurobiotin (NB) in larvae in combination with glycine immunofluorescence, under deep anesthesia with benzocaine. For this, we performed some experiments in which deeply anesthetized larvae were injected with NB in the eye orbit, which resulted in labeling of the optic tract and the retinopetal system, the ocular motor complex, the trigeminal sensory root and motor nucleus, and the anterior lateral line nerve. Other deeply anesthetized larvae were injected with NB in the otic capsule, which resulted in labeling of the octaval and facial nerves. Some other deeply anesthetized larvae were injected with NB in the rostral spinal cord, which resulted in labeling of the descending brain-spinal neuronal system. Larvae were then maintained in cold water for 2 or 3 days. After this survival period, larvae were re-anesthetized and fixed with the glutaraldehyde-based fixative for the subsequent immunofluorescence procedure. For details of the methods, see the Material and Methods section of chapter 2.

Sections were analyzed and photographed with a spectral confocal microscope TCS-SP2 (Leica, Wetzlar, Germany), that allowed us to best analyzed the colocalization of two neurotransmitters or the presence of glycine in NB retrogradely labeled neurons.

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

REFERENCES

Alford S, Williams TL. 1989. Endogenous activation of glycine and NMDA receptors in lamprey spinal cord during fictive locomotion. J Neurosci 9:2792-2800.

Alford S, Sigvardt KA, Williams TL. 1990a. GABAergic control of rhythmic activity in the presence of strychnine in the lamprey spinal cord. Brain Res 506:303-306.

Alford S, Williams TL, Sigvardt KA. 1990b. Effects of bicuculline and strychnine on synaptic inputs to edge cells during fictive locomotion. Brain Res 509:137-140.

Alford S, Christenson J, Grillner S. 1991. Presynaptic GABAA and GABAB receptor-mediated phasic modulation in axons of spinal motor interneurons. Eur J Neurosci 3:107-117.

Alford S, Grillner S. 1991. The involvement of GABAB receptors and coupled G-proteins in spinal GABAergic presynaptic inhibition. J Neurosci 11:3718-3728.

Allain AE, Baïri A, Meyrand P, Branchereau P. 2006. Expression of the glycinergic system during the course of embryonic development in the mouse spinal cord and its co-localization with GABA immunoreactivity. J Comp Neurol 496:832-846.

Anadón R, De Miguel E, González-Fuentes MJ, Rodicio C. 1989. HRP study of the central components of the trigeminal nerve in the larval sea lamprey: organization and homology of the primary medullary and spinal nucleus of the trigeminus. J Comp Neurol 283:602-610.

Aoki E, Semba R, Keino H, Kato K, Kashiwamata S. 1988. Glycine-like immunoreactivity in the rat auditory pathway. Brain Res 442:63-71.

Aprison MH, Daly EC. 1978. Biochemical aspects of transmission in inhibitory synapses: The role of glycine. Adv Neurochem 3:203-294.

Baev KV, Rusin KI, Safronov BV. 1992. Primary receptor for inhibitory transmitters in lamprey spinal cord neurons. Neuroscience 46:931-941.

Barker JL, Ransom BR. 1978. Amino acid pharmacology of mammalian central neurones grown in tissue culture. J Physiol 280:331-354.

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

Bäurle J, Grüsser-Cornehls U. 1997. Differential number of glycine- and GABA-immunopositive neurons and terminals in the deep cerebellar nuclei of normal and Purkinje cell degeneration mutant mice. J Comp Neurol 382:443-458.

Bayon A, Possani LD, Tapia M, Tapia R. 1977. Kinetics of brain glutamate decarboxylase. Interactions with glutamate, pyridoxal 5'-phosphate and glutamate-pyridoxal 5'-phosphate Schiff base. J Neurochem 29:519–525.

Ben-Ari Y. 2002. Excitatory actions of GABA during development: the nature of the nurture. Nat Rev Neurosci 3:728–739.

Berki ACS, O´Donovan MJ, Antal M. 1995. Developmental expression of glycine immunoreactivity and its colocalization with GABA in the embryonic chick lumbosacral spinal cord. J Comp Neurol 302:583-596.

Betz H. 1987. Biology and structure of the mammalian glycine receptor. Trends Neurosci 10:113-117.

Birinyi A, Parker D, Antal M, Shupliakov O. 2001. Zinc co-localizes with GABA and glycine in synapses in the lamprey spinal cord. J Comp Neurol 433:208-221.

Borden LA. 1996. GABA transporter heterogeneity: pharmacology and cellular localization. Neurochem Int 29:335-356.

Bormann J, Hamill OP, Sakmann B. 1987. Mechanism of anion permeation through channels gated by glycine and gamma-aminobutyric acid in mouse cultured spinal neurones. J Physiol 385:243-286.

Bormann J, Feigenspan A. 1995. $GABA_C$ receptors. Trends Neurosci 12:515-519.

Bowery NG, Hill DR, Hudson AL, Doble A, Middlemiss DN, Shaw J, Turnbull M. 1980. (-) Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. Nature Lond 283:92-94.

Bridgers WF. 1968. Serine transhydroxymethylase in developing mouse brain. J Neurochem 15:1325-1328.

Brox A, Ferreiro B, Puelles L, Medina L. 2002. The telencephalon of the frog *Xenopus* based on calretinin immunostaining and gene expression patterns. Brain Res Bull 57:381-384.

Buchanan JT, Grillner S. 1987. Newly identified 'glutamate interneurons' and their role in locomotion in the lamprey spinal cord. Science 236:312-314.

Buchanan JT, Grillner S. 1988. A new class of small inhibitory interneurones in the lamprey spinal cord. Brain Res 438:404-407.

Burger PM, Hell J, Mehl E, Krasel C, Lottspeich F and Jahn R. 1991. GABA and glycine in synaptic vesicles: storage and transport characteristics. Neuron 7:287-293.

Bussières N, Pflieger JF, Dubuc R. 1999. Anatomical study of vestibulospinal neurons in lampreys. J Comp Neurol 407:512-526.

Butler AB, Hodos W. 1996. Comparative vertebrate neuroanatomy. Evolution and adaptation. Wiley-Liss, New York.

Campistron G, Buijs RM, Geffard M. 1986. Glycine neurons in the brain and spinal cord. Antibody production and immunocytochemical localization. Brain Res 376:400-405.

Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH and Storm-Mathisen J. 1998. The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci 18:9733-9750.

Christensen H, Fonnum F. 1991. Uptake of glycine, GABA and glutamate by synaptic vesicles isolated from different regions of rat CNS. Neurosci Lett 129:217-220.

Christenson J, Boman A, Lagerbäck PA, Grillner S. 1988. The dorsal cell, one class of primary sensory neuron in the lamprey spinal cord. I. Touch, pressure but no nociception--a physiological study. Brain Res 440:1-8.

Clayton GH, Owens GC, Wolff JS and Smith RL. 1998. Ontogeny of cation-Cl^- cotransporter expression in rat neocortex. Dev Brain Res 109:281-292.

Covarrubias M, Tapia R. 1980. Brain glutamate decarboxylase: properties of its calcium-dependent binding to liposomes and kinetics of the bound and the free enzyme. J Neurochem 34:1682–1688.

Cubelos B, Giménez C, Zafra F. 2005. Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. Cereb Cortex 15:448-459.

Cui WW, Low SE, Hirata H, Saint-Amant L, Geisler R, Hume RI, Kuwada JY. 2005. The zebrafish shocked gene encodes a glycine transporter and is essential for the function of early neural circuits in the CNS. J Neurosci 25:6610-6620.

Dale N, Ottersen OP, Roberts A, Storm-Mathisen J. 1986. Inhibitory neurones of a motor pattern generator in *Xenopus* revealed by antibodies to glycine. Nature 324:255-257.

Dale N, Roberts A, Ottersen OP, Storm-Mathisen J. 1987a. The development of a population of spinal cord neurons and their axonal projections revealed by GABA immunocytochemistry in frog embryos. Proc R Soc Lond B 232:205-215.

Dale N, Roberts A, Ottersen OP, Storm-Mathisen J. 1987b. The morphology and distribution of "Kolmer-Agduhr cells", a class of cerebrospinal-fluid-contacting neurons revealed in the frog embryo spinal cord by GABA immunocytochemistry. Proc R Soc Lond B 232:193-203.

Dalil N, Repérant J, Kenigfest N, Vesselkin N, Versaux-Botteri C, Rio JP. 1990. Typology and distribution of ganglion cells in the retina of lamprey (*Lampetra fluviatilis*). C R Acad Sci III 11:403-410.

Dalil-Thiney N, Pochet R, Versaux-Botteri C, Vesselkin N, Repérant J, Nguyen-Legros J. 1994. Immunohistochemical localization of calbindin-D28K and calretinin in the lamprey retina. J Comp Neurol 1:140-147.

Daly EC, Aprison MH. 1974. Distribution of serine hydroxymethyltransferase and glycine transaminase in several areas of the central nervous system of the rat. J Neurochem 22:877-885.

Daly EC, Nadi NS, Aprison MH. 1976. Regional distribution and properties of the glycine cleavage system within the central nervous system of the rat: evidence for an endogenous inhibitor during in vitro assay. J Neurochem 26:179-185.

David-Watine B, Goblet C, de Saint Jan D, Fucile S, Devignot V, Bregestovski P, Korn H. 1999. Cloning, expression and electrophysiological characterization of glycine receptor alpha subunit from zebrafish. Neuroscience 90:303-317.

De Arriba Pérez, MC. 2007. Conexiones del techo óptico. Organización y desarrollo en lamprea y expresión de genes implicados en la vía retinotectal en pez cebra. Tesis Doctoral, Universidad de Vigo.

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

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

De Miguel E, Rodicio MC, Anadón R. 1990. Organization of the visual system in larval lampreys: an HRP study. J Comp Neurol 302:529-542.

Dickson DH, Collard TR. 1979. Retinal development in the lamprey (*Petromyzon marinus* L.): premetamorphic ammocoete eye. Am J Anat 154:321-336.

Dickson HD, Graves DA. 1981. The ultrastructure and development of the eye. In: Potter IC, Hardisty MW, editors. The biology of lampreys, vol 3. London. Academic Press. pp:43–94.

Dolphin AC, Scott RH. 1987. Calcium channels currents and their inhibition by (-)-baclofen in rat sensory neurons: modulation by guanine nucleotides. J Physiol Lond 386:1-17.

Dubuc R, Bongianni F, Ohta Y, Grillner S. 1993a. Anatomical and physiological study of brainstem nuclei relaying dorsal column inputs in lampreys. J Comp Neurol 327:260-270.

Dubuc R, Bongianni F, Ohta Y, Grillner S. 1993b. Dorsal root and dorsal column mediated synaptic inputs to reticulospinal neurons in lampreys: involvement of glutamatergic, glycinergic, and GABAergic transmission. J Comp Neurol 327:251-259.

Dunlap K, Holz GG, Rane SGG. 1987. G proteins as regulators of ion channel function. Trends Neurosci 10:241-244.

Ekström P, Ohlin LM. 1995. Ontogeny of GABA-immunoreactive neurons in the central nervous system in a teleost, *Gasterosteus aculeatus* L. J Chem Neuroanat 9:271-288.

El Manira A, Pombal MA, Grillner S. 1997. Diencephalic projection to reticulospinal neurons involved in the initiation of locomotion in adult lampreys *Lampetra fluviatilis*. J Comp Neurol 389:603-616.

Erlander MG, Tobin AJ. 1991. The structural and functional heterogeneity of glutamic acid decarboxylase: a review. Neurochem Res 16:215–226.

Finger TE, Rovainen CM. 1978. Retrograde HRP labeling of the oculomotoneurons in adult lampreys. Brain Res 154:123-127.

Finger TE, Rovainen CM. 1982. Spinal and medullary dorsal cell axons in the trigeminal nerve in lampreys. Brain Res 240:331-333.

Fletcher EL, Kalloniatis M. 1996. Neurochemical architecture of the normal and degenerating rat retina. J Comp Neurol 376:343-360.

Fort P, Luppi PH, Wenthold R, Jouvet M. 1990. Glycine immunoreactive neurons in the medulla oblongata in cats. C R Acad Sci 311:205-212.

Fort P, Luppi PH, Jouvet M. 1993. Glycine-immunoreactive neurones in the cat brain stem reticular formation. Neuroreport 4:1123-1126.

Fritzsch B, Crapon de Caprona MD, Wächtler K, Körtje KH. 1984. Neuroanatomical evidence for electroreception in lampreys. Z Naturforsch 39:856-858.

Fritzsch B, Dubuc R, Ohta Y, Grillner S. 1989. Efferents to the labyrinth of the river lamprey (*Lampetra fluviatilis*) as revealed with retrograde tracing techniques. Neurosci Lett 96:241-246.

Fritzsch B, Sonntag R, Dubuc R, Ohta Y, Grillner S. 1990. Organization of the six motor nuclei innervating the ocular muscles in lamprey. J Comp Neurol 294:491-506.

Garrow TA, Brenner AA, Whitehead VM, Chen XN, Duncan RG, Korenberg JR, Shane B. 1993. Cloning of human cDNAs encoding mitochondrial and cytosolic serine hydroxymethyltransferases and chromosomal localization. J Biol Chem 268:11910-11916.

Gold MR, Martin AR. 1983. Inhibitory conductance changes at synapses in the lamprey brainstem. Science 221:85-87.

González MJ, Anadón R. 1992. Primary projections of the lateral line nerves in larval sea lamprey, *Petromyzon marinus* L. An HRP study. J Hirnforsch 33:185-194.

González MJ, Anadón R. 1994. Central projections of the octaval nerve in larval lamprey: an HRP study. J Hirnforsch 35:181-189.

González MJ, Manso MJ, Anadón R. 1997. Octavolateral neurons projecting to the middle and posterior rhombencephalic reticular nuclei of larval lamprey: a retrograde horseradish peroxidase labeling study. J Comp Neurol 384:396-408.

González MJ, Pombal MA, Rodicio MC, Anadón R. 1998. The internuclear neurons of the ocular motor system of the larval sea lamprey. J Comp Neurol 401:1-15.

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

Goossens N, Dierickx K, Vandesande F. 1977. Immunocytochemical demonstration of the hypothalamo-hypophysial vasotocinergic system of *Lampetra fluviatilis*. Cell Tissue Res 177:317-323.

Grillner S, Williams T, Lagerbäck PA. 1984. The edge cell, a possible intraspinal mechanoreceptor. Science 223:500-503.

Grillner S, Wallén P, Viana di Prisco G. 1990. Cellular network underlying locomotion as revealed in a lower vertebrate model: transmitters, membrane

properties, circuitry, and simulation. Cold Spring Harb Symp Quant Biol 55:779-789.

Grillner S, Matsushima T. 1991. The neural network underlying locomotion in lamprey--synaptic and cellular mechanisms. Neuron 7:1-15.

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

Heier P. 1948. Fundamental principles in the structure of the brain. A study of the brain of *Petromyzon fluviatilis*. Acta Anat 6:3-213.

Helfert RH, Bonneau JM, Wenthold RJ, Altschuler RA. 1989. GABA and glycine immunoreactivity in the guinea pig superior olivary complex. Brain Res 501:269-286.

Higashijima S, Mandel G, Fetcho JR. 2004. Distribution of prospective glutamatergic, glycinergic, and GABAergic neurons in embryonic and larval zebrafish. J Comp Neurol 480:1-18.

Hökfelt T, Ljungdahl A. 1971. Light and electron microscopic autoradiography on spinal cord slices after incubation with labeled glycine. Brain Res 32:189-194.

Homma S, Rovainen CM. 1978. Conductance increases produced by glycine and gamma-aminobutyric acid in lamprey interneurones. J Physiol 279:231-252.

Huard H, Lund JP, Veilleux D, Dubuc R. 1999. An anatomical study of brainstem projections to the trigeminal motor nucleus of lampreys. Neuroscience 91:363-378.

Imboden M, De Saint Jan D, Leulier F, Korn H, Goblet C, Bregestovski P. 2001. Isolation and characterization of an alpha 2-type zebrafish glycine receptor subunit. Neuroscience 103:799-810.

Ishikawa M, Takao M, Washioka H, Tokunaga F, Watanabe H, Tonosaki A. 1987. Demonstration of rod and cone photoreceptors in the lamprey retina by freeze-replication and immunofluorescence. Cell Tissue Res 249:241-246.

Itouji A, Sakai N, Tanaka C, Saito N. 1996. Neuronal and glial localization of two GABA transporters (GAT1 and GAT3) in the rat cerebellum. Mol Brain Res 37:309-316.

Iversen LL, Bloom FE. 1972. Studies of the uptake of ^{3}H-GABA and [^{3}H]glycine in slices and homogenates of rat brain and spinal cord by electron microscopic autoradiography. Brain Res 41:131-143.

Johnson J, Chen TK, Rickman DW, Evans C, Brecha NC. 1996. Multiple γ-aminobutyric acid plasma membrane transporters (GAT-1, GAT-2, GAT-3) in the rat retina. J Comp Neurol 375:212-224.

Johnston JB. 1902. The brain of Petromyzon. J Comp Neurol 12:2-86.

Johnston GAR, Curtis DR, Beart PM, Game CJA, McCulloch RM, Twitchin B. 1975. Cis-and trans-4-amicrotonic acid as GABA analogues of restricted conformation. J Neurochem 24:157-160.

Jonas P, Bischofberger J, Sandkuhler J. 1998. Corelease of two fast neurotransmitters at a central synapse. Science 281:419-424.

Jursky F, Nelson N. 1996. Developmental expression of GABA transporters GAT1 and GAT4 suggests involvement in brain maturation. J Neurochem 67:857-867.

Katarova Z, Sekerková G, Prodan S, Mugnaini E, Szabó G. 2000. Domain-restricted expression of two glutamic acid decarboxylase genes in midgestation mouse embryos. J Comp Neurol 424:607-627.

Kaufman DL, Houser CR, Tobin AJ. 1991. Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. J Neurochem 56:720–723.

Kennedy MC, Rubinson K. 1977. Retinal projections in larval, transforming and adult sea lamprey, *Petromyzon marinus*. J Comp Neurol 171:465-479.

Kleerekoper H. 1972. The sense organs. In: MW Hardisty and IC Potter, editors. The Biology of Lampreys, vol. 2. London. Academic Press. pp. 373-404.

Kolston J, Osen KK, Hackney CM, Ottersen OP, Storm-Mathisen J. 1992. An atlas of glycine- and GABA-like immunoreactivity and colocalization in the cochlear nuclear complex of the guinea pig. Anat Embryol 186:443-465.

Koyama H. 2005. Organization of the sensory and motor nuclei of the glossopharyngeal and vagal nerves in lampreys. Zoolog Sci 22:469-476.

Koyama H, Kishida R, Goris RC, Kusunoki T. 1987. Organization of sensory and motor nuclei of the trigeminal nerve in lampreys. J Comp Neurol 264:437-448.

Koyama H, Kishida R, Goris RC, Kusunoki T. 1989. Afferent and efferent projections of the VIIIth cranial nerve in the lamprey *Lampetra japonica*. J Comp Neurol 280:663-671.

Koyama H, Kishida R, Goris RC, Kusunoki T. 1990. Organization of the primary projections of the lateral line nerves in the lamprey *Lampetra japonica*. J Comp Neurol 295:277-289.

Landwehr S, Dicke U. 2005. Distribution of GABA, glycine, and glutamate in neurons of the medulla oblongata and their projections to the midbrain tectum in plethodontid salamanders. J Comp Neurol 490:145-162.

Li T, Wu SM, Lam DM, Watt CB. 1990. Localization of classical neurotransmitters in interneurons of the larval tiger salamander. Invest Ophthalmol Vis Sci 31:262-271.

Ljungdahl A, Hökfelt T. 1973. Accumulation of ^{3}H-glycine in interneurons of the cat spinal cord. Histochemie 33:277-280.

Lue JH, Shieh WF, Chen SH, Shieh JY, Wen CY. 1997. Morphometric study of glycine-immunoreactive neurons and terminals in the rat cuneate nucleus. J. Anat 191:375-385.

MacNeil MA, Masland RH. 1998. Extreme diversity among amacrine cells: implications for function. Neuron 20:971-982.

Matthews G, Wickelgren WO. 1979. Glycine, GABA and synaptic inhibition of reticulospinal neurones of lamprey. J Physiol 293:393-415.

McIntire SL, Reimer RJ, Schuske K, Edwards RH and Jorgensen EM. 1997. Identification and characterization of the vesicular GABA transporter. Nature 389:870-876.

Meiniel A, Collin JP (1971) Le complexe pinéal de l'ammocète (*Lampetra planeri*, Bl.). Z Zellforsch 117:345–380.

Meléndez-Ferro M. 2001. Distribución de la inmunorreactividad al ácido gamma-aminobutírico (GABA) en el sistema nervioso central de la lamprea de mar durante el desarrollo y la etapa adulta. Tesis Doctoral, Universidad de Santiago de Compostela.

Meléndez-Ferro M, Pérez-Costas E, González MJ, Pombal MA, Anadón R, Rodicio MC. 2000. GABA-immunoreactive internuclear neurons in the ocular motor system of lampreys. Brain Res 855:150-157.

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

Meléndez-Ferro M, Villar-Cheda B, Abalo XM, Pérez-Costas E, Rodríguez-Muñoz R, Degrip WJ, Yáñez J, Rodicio MC, Anadón R. 2002a. Early development of the retina and pineal complex in the sea lamprey: comparative immunocytochemical study. J Comp Neurol 442:250-265.

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

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

Menger N, Pow DV, Wässle H. 1998. Glycinergic amacrine cells of the rat retina. J Comp Neurol 401:34-46.

Merchán M, Aguilar LA, Lopez-Poveda EA, Malmierca MS. 2005. The inferior colliculus of the rat: quantitative immunocytochemical study of GABA and glycine. Neuroscience 136:907-925.

Merrick SE, Pleasure SJ, Lurie DI, Pijak DS, Selzer ME, Lee VM. 1995. Glial cells of the lamprey nervous system contain keratin-like proteins. J Comp Neurol 355:199-210.

Mueller T, Vernier P, Wullimann MF. 2006. A phylotypic stage in vertebrate brain development: GABA cell patterns in zebrafish compared with mouse. J Comp Neurol 494:620-634.

Mueller T, Wullimann MF, Guo S. 2008. Early teleostean basal ganglia development visualized by zebrafish *Dlx2a, Lhx6, Lhx7, Tbr2 (eomesa)*, and *GAD67* gene expression. J Comp Neurol 507:1245-1257.

Negishi K, Kiyama H, Kato S, Teranishi T, Hatakenaka S, Katayama Y, Miki N, Tohyama M. 1986. An immunohistochemical study on the river lamprey retina. Brain Res 362:389-393.

Nieuwenhuys R, Nicholson C. 1998. Lampreys, Petromyzontoidea. In: "The central nervous system of vertebrates". Nieuwenhuys R, ten Donkelaar HJ, Nicholson C (Eds.). Springer-Verlag (Berlin) Vol. 1:397-495.

Northcutt RG, Puzdrowski RL. 1988. Projections of the olfactory bulb and nervus terminalis in the silver lamprey. Brain Behav Evol 32:96-107.

Northcutt RG, Wicht H. 1997. Afferent and efferent connections of the lateral and medial pallia of the silver lamprey. Brain Behav Evol 49:1-19.

Ohta Y, Dubuc R, Grillner S. 1991. A new population of neurons with crossed axons in the lamprey spinal cord. Brain Res 564:143-148.

Olsen RW. 1982. Drug interactions at the GABA receptor ionophore complex. Annu Rev Pharmacol 22:245-277.

Osorio J, Mazan S, Rétaux S. 2005. Organisation of the lamprey (*Lampetra fluviatilis*) embryonic brain: insights from LIM-homeodomain, Pax and hedgehog genes. Dev Biol 288:100-112.

Östholm T, Ekström P, Bruun A, van Veen T. 1988. Temporal disparity in pineal and retinal ontogeny. Brain Res 470:1-13.

Oztan N, Gorbman A. 1960. The hypophysis and hypothalamo-hypophyseal neurosecretory system of larval lampreys and their responses to light. J Morphol 106:243-261.

Piavis GW. 1971. Embryology. In: Hardisty MW, Potter IC , editors. The biology of lampreys, vol 1. London: Academic Press. p 361-400.

Polenova OA, Vesselkin NP. 1993. Olfactory and nonolfactory projections in the river lamprey (*Lampetra fluviatilis*) telencephalon. J Hirnforsch 34:261-279.

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

Pombal MA, Rodicio MC, Anadón R. 1994. Development and organization of the ocular motor nuclei in the larval sea lamprey, *Petromyzon marinus* L.: an HRP study. J Comp Neurol 341:393-406.

Pombal MA, Rodicio MC, Anadón R. 1995. The anatomy of the vestibulo-ocular system in lampreys. In: Delgado-García JM, Godaux E, Vidal P-P, eds. Information Processing Underlying Gaze Control, London, Pergamon Press, p 1-11.

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

Pombal MA, El Manira A, Grillner S. 1997a. Organization of the lamprey striatum - transmitters and projections. Brain Res 766:249-254.

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

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

Pombal MA, Marín O, González A. 1999b. Choline acetyltransferase immunoreactivity in the hypothalamoneurohypophysial system of the lamprey. Eur J Morphol 37:103-106.

Pombal MA, Marín O, González A. 2001. Distribution of choline acetyltransferase-immunoreactive structures in the lamprey brain. J Comp Neurol 431:105-126.Popratiloff A, Valtschanoff JG, Rustioni A, Weinberg RJ. 1996. Colocalization of GABA and glycine in the rat dorsal column nuclei. Brain Res. 706:308-312.

Pourcho RG, Goebel DJ. 1985. Immunocytochemical demonstration of glycine in retina. Brain Res 348:339-342.

Pourcho RG, Goebel DJ. 1987. Visualization of endogenous glycine in cat retina: an immunocytochemical study with Fab fragments. J Neurosci 7:1189-1197.

Pourcho RG, Goebel DJ, Jojich L, Hazlett JC. 1992. Immunocytochemical evidence for the involvement of glycine in sensory centers of the rat brain. Neuroscience 46:643-656.

Pu GA, Dowling JE. 1981. Anatomical and physiological characteristics of pineal photoreceptor cell in the larval lamprey, *Petromyzon marinus*. J Neurophysiol 46:1018-1038.

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

Rampon C, Luppi PH, Fort P, Peyron C, Jouvet M. 1996. Distribution of glycine-immunoreactive cell bodies and fibers in the rat brain. Neuroscience 75:737-755.

Reichenberger I, Streit P, Ottersen OP, Dieringer N. 1993. GABA- and glycine-like immunoreactivities in the cerebellum of the frog. Neurosci Lett 154:89-92.

Reichenberger I, Straka H, Ottersen OP, Streit P, Gerrits NM, Dieringer N. 1997. Distribution of GABA, glycine, and glutamate immunoreactivities in the vestibular nuclear complex of the frog. J Comp Neurol 377:149-164.

Reichling DB, Kyrozis A, Wang J, MacDermott AB. 1994. Mechanisms of GABA and glycine depolarization-induced calcium transients in rat dorsal horn neurons. J Physiol 476:411-421.

Retzius G. 1983. Ependym and neuroglia bei den Cyclostomen. Biol Untersuch 5:15-18.

Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K. 1999. The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397:251-255.

Roberts A, Dale N, Ottersen OP, Storm-Mathisen J. 1987. The early development of neurons with GABA immunoreactivity in the CNS of *Xenopus laevis* embryos. J Comp Neurol 261:435-449.

Roberts A, Dale N, Ottersen OP, Storm-Mathisen J. 1988. Development and characterization of commissural interneurones in the spinal cord of *Xenopus* laevis embryos revealed by antibodies to glycine. Development 103:447-461.

Robertson B, Saitoh K, Ménard A, Grillner S. 2006. Afferents of the lamprey optic tectum with special reference to the GABA input: combined tracing and immunohistochemical study. J Comp Neurol 499:106-119.

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

Rodicio MC, Pombal MA, Anadón R. 1995. Early development and organization of the retinopetal system in the larval sea lamprey, Petromyzon marinus L. An HRP study. Anat Embryol 192:517-526.

Rodicio MC, Villar-Cerviño V, Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R. 2005. Chemoarchitecture of the dorsal column nucleus of the larval sea lamprey. Brain Res Bull 66:536-540.

Ronan M. 1988. Anatomical and physiological evidence for electroreception in larval lampreys. Brain Res 448:173-177.

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

Ronan M, Northcutt RG. 1987. Primary projections of the lateral line nerves in adult lampreys. Brain Behav Evol 30:62-81.

Ronan M, Northcutt RG. 1990. Projections ascending from the spinal cord to the brain in petromyzontid and myxinoid agnathans. J Comp Neurol 291:491-508.

Rovainen CM. 1967a. Physiological and anatomical studies on large neurons of central nervous system of the sea lamprey (*Petromyzon marinus*). I. Müller and Mauthner cells. J Neurophysiol 30:1000-1023.

Rovainen CM. 1967b. Physiological and anatomical studies on large neurons of central nervous system of the sea lamprey (*Petromyzon marinus*). II. Dorsal cells and giant interneurons. J Neurophysiol 30:1024-1042.

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

Rovainen CM. 1983. Identified neurons in the lamprey spinal cord and their roles in fictive swimming. Symp Soc Exp Biol 37:305-330.

Rovainen CM, Birnberger KL. 1971. Identification and properties of motoneurons to fin muscle of the sea lamprey. J Neurophysiol 34:974-982.

Rubinson K, Cain H. 1989. Neural differentiation in the retina of the larval sea lamprey (*Petromyzon marinus*). Vis Neurosci 3:241-248.

Ruiz Y, Pombal MA, Megías M. 2004. Development of GABA-immunoreactive cells in the spinal cord of the sea lamprey, *P. marinus*. J Comp Neurol 470:151-163.

Sagne C, El Mestikawy S, Isambert MF, Hamon M., Henry JP, Giros B, Gasnier B. 1997. Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases. FEBS Lett 417:177-183.

Saint Marie RL, Ostapoff EM, Morest DK, Wenthold RJ. 1989. Glycine-immunoreactive projection of the cat lateral superior olive: possible role in midbrain ear dominance. J Comp Neurol 279:382-396.

Saint Marie RL, Benson CG, Ostapoff EM, Morest DK. 1991. Glycine immunoreactive projections from the dorsal to the anteroventral cochlear nucleus. Hear Res 51:11-28.

Shank RP, Aprison MH. 1970. The metabolism in vivo of glycine and serine in eight areas of the rat central nervous system. J Neurochem 17:1461-1475.

Shank RP, Aprison MH, Baxter CF. 1973. Precursors of glycine in the nervous system: comparison of specific activities in glycine and other amino acids after administration of [U-^{14}C]glucose, [3,4-^{14}C]glucose, [1-^{14}C]glucose, [U-^{14}C]serine or [1,5-^{14}C]citrate to the rat. Brain Res 52:301-308.

Sheridan PH, Youngs LJ, Krieger NR, Selzer ME. 1984. Glycine uptake by lamprey spinal neurons demonstrated by light microscopic autoradiography. J Comp Neurol 223:252-258.

Shupliakov O, Wallén P, Grillner S. 1992. Two types of motoneurons supplying dorsal fin muscles in lamprey and their activity during fictive locomotion. J Comp Neurol 321:112-123.

Shupliakov O, Fagersted P, Ottersen OP, Storm-Mathiesen J, Grillner S, Brodin L. 1996. Immunocytochemical localization of glycine in the lamprey spinal cord with reference to GABAergic and glutamatergic synapses: a light and electron microscopic study. Acta Biol Hung 47:393-410.

Sky-Peck HH, Rosenbloom C, Winzler RJ. 1966. Incorporation of glucose into the protein-bound amino acids of one-day-old mouse brain in vitro. J Neurochem 13:223-228.

Soghomonian JJ, Martin DL. 1998. Two isoforms of glutamate decarboxylase: why? Trends Pharmacol Sci 19:500–505.

Spirou GA, Berrebi AS. 1997. Glycine immunoreactivity in the lateral nucleus of the trapezoid body of the cat. J Comp Neurol 383:473-488.

Stefanelli A. 1934. I centri tegmentali dell'encefalo dei Petromizonti. Arch Zool Ital 20:117-202.

Stefanelli A, Caravita S. 1968. Sui dispositivi sinaptici delle cellule dei nuclei ottavo-motori (tangenziali) di lampreda (*Lampetra planeri*). R C Accad Naz Lincei Ser VIII, 44:455-462.

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

Tanaka I, Ezure K. 2004. Overall distribution of GLYT2 mRNA-containing versus GAD67 mRNA-containing neurons and colocalization of both mRNAs in midbrain, pons, and cerebellum in rats. Neurosci Res 49:165-178.

Tanaka I, Ezure K, Kondo M. 2003. Distribution of glycine transporter 2 mRNA-containing neurons in relation to glutamic acid decarboxylase mRNA-containing neurons in rat medulla. Neurosci Res 47:139-151.

Teranishi T, Negishi K, Kato S. 1982. Two types of light-induced response recorded from horizontal cells in the river lamprey retina. Neurosci Lett 33:41-46.

Teräväinen H, Rovainen CM. 1971. Fast and slow motoneurons to body muscle of the sea lamprey. J Neurophysiol 34:990-998.

Tretjakoff D. 1909a. Das Nervensystem von Ammocoetes. I. Das Rückenmark. Arch Mikrosk Anat 73:607-680.

Tretjakoff D. 1909b. Das Nervensystem von Ammocoetes. II. Gehirn. Arch Mikrosk Anat 74:636-779.

Uematsu K, Shirasaki M, Storm-Mathisen J. 1993. GABA- and glycine-immunoreactive neurons in the spinal cord of the carp, *Cyprinus carpio*. J Comp Neurol 332:59-68.

Vesselkin NP, Ermakova TV, Repérant J, Kosareva AA, Kenigfest NB. 1980. The retinofugal and retinopetal systems in *Lampetra fluviatilis*. An experimental study using radioautographic and HRP methods. Brain Res 195:453-460.

Vesselkin NP, Rio JP, Adanina VO, Kenigfest NB, Repérant J. 1995. Colocalization of glutamate and glycine in giant fiber synapses of the lamprey spinal cord. J Hirnforsch 36:229-237.

Vesselkin NP, Rio JP, Adanina VO, Repérant J. 2000. GABA- and glycine-immunoreactive terminals contacting motoneurons in lamprey spinal cord. J Chem Neuroanat 19:69-80.

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

Villar-Cheda B, Abalo XM, Villar-Cerviño V, Barreiro-Iglesias A, Anadón R, Rodicio MC. 2008. Late proliferation and photoreceptor differentiation in the transforming lamprey retina. Brain Res. 1201:60-67.

Vinay L, Bussières N, Shupliakov O, Dubuc R, Grillner S. 1998. Anatomical study of spinobulbar neurons in lampreys. J Comp Neurol 397:475-492.

Walberg F, Ottersen OP, Rinvik E. 1990. GABA, glycine, aspartate, glutamate and taurine in the vestibular nuclei: an immunocytochemical investigation in the cat. Exp Brain Res 79:547-563.

Wallén P, Grillner S, Feldman JL, Bergelt S. 1985. Dorsal and ventral myotome motoneurons and their input during fictive locomotion in lamprey. J Neurosci 5:654-661.

Wenthold RJ. 1987. Evidence for a glycinergic pathway connecting the two cochlear nuclei: an immunocytochemical and retrograde transport study. Brain Res 415:183-187.

Wester MR, Teasley DC, Byers SL, Saha MS. 2008. Expression patterns of glycine transporters *(xGlyT1, xGlyT2*, and *xVIAAT*) in *Xenopus laevis* during early development. Gene Expr Patterns 8:261-270.

Wood PL. 2005. The NMDA receptor complex: a long and winding road to therapeutics. IDrugs 8:229-235.

Yang CY, Yazulla S. 1988. Light microscopic localization of putative glycinergic neurons in the larval tiger salamander retina by immunocytochemical and autoradiographical methods. J Comp Neurol 272:343-357.

Yáñez J, Anadón R, Holmqvist BI, Ekström P. 1993. Neural projections of the pineal organ in the larval sea lamprey (*Petromyzon marinus* L.) revealed by indocarbocyanine dye tracing. Neurosci Lett 164:213-216.

Yáñez J, Anadón R. 1994. Afferent and efferent connections of the habenula in the larval sea lamprey (*Petromyzon marinus* L.). An experimental study. J Comp Neurol 345:148-160.

Yáñez J, Pombal MA, Anadón R. 1999. Afferent and efferent connections of the parapineal organ in lampreys: a tract tracing and immunocytochemical study. J Comp Neurol 403:171-189.

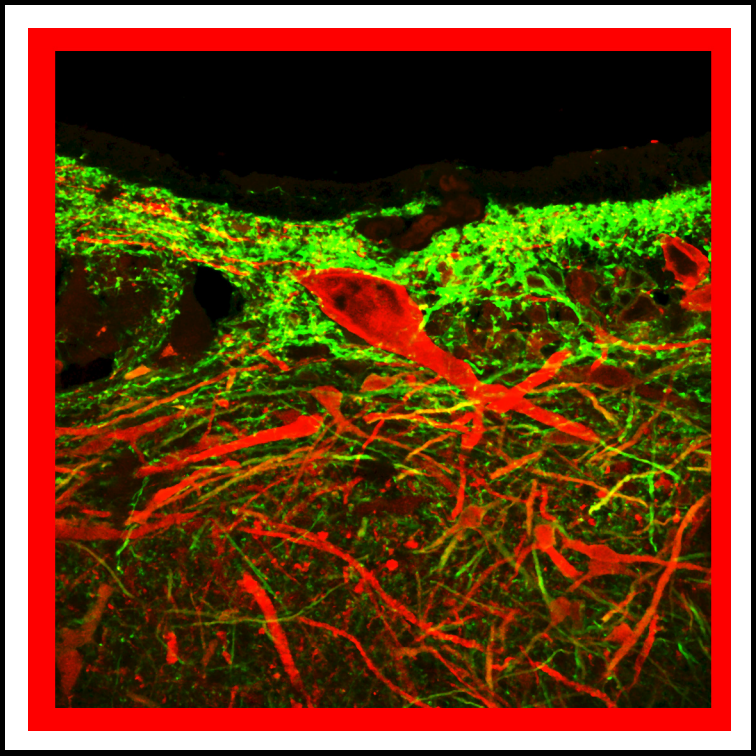
Young AB, Snyder SH. 1974. The glycine synaptic receptor: evidence that strychnine binding is associated with the ionic conductance mechanism. Proc Natl Acad Sci U S A 71:4002-4005.

Zeilhofer HU, Studler B, Arabadzisz D, Schweizer C, Ahmadi S, Layh B, Bosl MR, Fritschy JM. 2005. Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. J Comp Neurol 482:123-141.

Zhou ZJ. 2001. A critical role of the strychnine-sensitive glycinergic system in spontaneous retinal waves of the developing rabbit. J Neurosci 21:5158-5168.

Zompa IC, Dubuc R. 1998. Diencephalic and mesencephalic projections to rhombencephalic reticular nuclei in lampreys. Brain Res 802:27-54.

Distribution of Glycine Immunoreactivity in the Brain of Adult Sea Lamprey (*Petromyzon marinus*). Comparison with GABA



Chapter 1

INTRODUCTION

The amino acid glycine is one of the main inhibitory neurotransmitters in the vertebrate brain. The inhibitory action of glycine results from increased conductance of chloride in the postsynaptic membrane when glycine receptors are activated (Young and Snyder, 1974; Barker and Ransom, 1978; Betz, 1987; Bormann et al., 1987). This action is terminated through glycine reuptake by two members of the family of Na^+/Cl^- dependent neurotransmitter transporters and by the glycine cleavage system, a mitochondrial and cytosolic enzyme complex (Garrow et al., 1993). Whereas the glycine transporter GLYT1 is considered a glial transporter, the GLYT2 transporter is primarily associated with neurons, although recent studies have shown that GLYT1 is also expressed in neurons (Cubelos et al., 2005). Glycine is also a modulator co-agonist of the N-methyl-D-aspartate (NMDA) glutamate receptor, which is a complex ion channel with multiple protein subunits that act as binding sites for glutamate and as allosteric regulatory binding sites that bind glycine and/or D-serine as co-agonists (for revision see Wood, 2005).

Studies on the brain distribution of glycinergic neurons and/or fibers have mainly focused on mammals. Early studies of the glycinergic system examined *in vivo* accumulation of labeled glycine followed by autoradiography to localize putative glycinergic cells (Hökfelt and Ljungdahl, 1971; Iversen and Bloom, 1972; Ljungdahl and Hökfelt, 1973; Sheridan et al., 1984). The introduction of antibodies against glycine coupled to protein carriers allowed studies of distribution of glycine-containing cells and fibers in the brain (Campistron et al., 1986; Dale et al., 1986; Aoki et al., 1988; Helfert et al., 1989; Fort et al., 1990, 1993; Walberg et al., 1990; Kolston et al., 1992; Pourcho et al., 1992; Reichenberger et al., 1993; Uematsu et al., 1993; Popratiloff et al.,1996; Rampon et al.,1996; Shupliakov et al., 1996; Bäurle and Grüsser-Cornehls, 1997; Lue et al., 1997; Spirou and Berrebi, 1997; Vesselkin et al., 2000; Merchán et al., 2005). The results of early studies suggested that putative glycinergic synapses were mainly distributed in the brainstem and spinal cord, but now it is known that glycinergic cells and fibers are more widely distributed throughout the central nervous system (Rampon et al., 1996; Zeilhofer et al., 2005). Even so, most studies were performed in caudal regions of the brain (Wenthold, 1987; Aoki et al., 1988; Helfert et al., 1989; Saint Marie et al., 1989, 1991; Walberg et al., 1990; Kolston et al., 1992; Pourcho et al., 1992; Spirou and Berrebi, 1997),

where putative glycinergic populations are more numerous. In non-mammalian vertebrates, a developmental study in zebrafish, with *in situ* hybridization of the transporter GLYT2, has provided some data on the early organization in columns of putative glycinergic populations in the hindbrain (Higashijima et al., 2004), although the adult populations to which these columns give rise have not been explored. The distribution of glycine-immunoreactive (ir) populations in the cerebellum and vestibular nuclear complex of the frog (Reichenberger et al., 1993, 1997) and in the medulla oblongata of plethodontid salamanders (Landwehr and Dicke, 2005) have also been reported. As far as we are aware, there are no studies about the evolution of glycinergic populations in vertebrates.

With regard to the other major inhibitory neurotransmitter, γ-aminobutyric acid (GABA), its distribution has been studied previously in the brain of both developing (Meléndez-Ferro et al., 2002, 2003) and adult lampreys (Meléndez-Ferro, 2001; Rodicio et al., 2005; Robertson et al., 2007). These studies revealed a conserved pattern of GABA distribution. In the spinal cord of lampreys, some studies have revealed colocalization of GABA and glycine immunoreactivities in some cells and nerve boutons (Shupliakov et al., 1996; Vesselkin et al., 1995, 2000; Birinyi et al., 2001; Villar-Cerviño et al., 2008), indicating that inhibitory effects on postsynaptic cells can be mediated by co-release of these neurotransmitters. Some glycine receptors of zebrafish, unlike those of mammals, can be activated by GABA and taurine, suggesting the possibility of cooperative functions of these transmitters in some synapses (David-Watine et al., 1999; Imboden et al., 2001). The existence of inhibitory receptors activated by both glycine and GABA has also been suggested in lamprey (Baev et al., 1992).

Lampreys are living representatives of the most primitive group of vertebrates, the Agnathans (Nieuwenhuys and Nicholson, 1998). Accordingly, they are essential subjects for deciphering the early history of the vertebrate nervous system. Although there are a number of functional studies on glycinergic transmission in the spinal cord and brain stem reticular formation of lamprey, mainly involving infusion of glycine and/or the glycine channel specific antagonist strychnine (Homma and Rovainen, 1978; Matthews and Wickelgren, 1979; Gold and Martin, 1983; Rovainen, 1983; Buchanan and Grillner, 1988; Alford and Williams, 1989; Alford et al., 1990a,b; McPherson et al., 1994; Bongianni et al., 2006), studies

on the distribution of putative glycinergic populations in the lamprey nervous system have been restricted to the spinal cord (Vesselkin et al., 1995, 2000; Shupliakov et al., 1996) and retina (Villar-Cerviño et al., 2006). The main aim of the present study was to describe for the first time the different glycine-ir cell groups and fibers in the brain of the sea lamprey, and to analyse the changes that have occurred in glycinergic populations from primitive vertebrates to mammals. A further aim was to investigate possible colocalization of GABA in glycine-immunoreactive brain populations using double immunofluorescence methods.

MATERIAL AND METHODS

Subjects

Adult (N = 8) sea lampreys (*Petromyzon marinus* L) were used in the present study. Animals were collected from the River Ulla (Galicia, northwest Spain) and used immediately. All experiments were approved by the Ethics committee of the University of Santiago de Compostela and conformed to the European Community guidelines on animal care and experimentation.

Tissue Collection and Processing

Animals were deeply anaesthetized with benzocaine (Sigma, St. Louis, MO; 0.05%) and killed by decapitation. Brains and spinal cords of adult lampreys were dissected out prior to fixation. All samples were fixed by immersion in 5% glutaraldehyde and 1% sodium metabisulfite in 0.05M Tris buffered saline (TBS; pH 7.4) for 17 h. The fixed samples were embedded in Tissue Tek (Sakura, Torrance, CA), frozen in liquid nitrogen-cooled isopentane, sectioned on a cryostat in the transverse or sagittal plane (16 μm thick) and mounted on Superfrost® Plus glass slides (Menzel, Braunschweig, Germany).

Immunofluorescence

For immunofluorescence, sections were pretreated with 0.2% $NaBH_4$ in deionized water for 45 minutes at room temperature to quench autofluorescence. Sections were incubated for 3 days at 4ºC with rabbit polyclonal anti-glycine antibody (Immunosolution, Jesmond, Australia; code IG1003, batch 1953; dilution 1:3,000; or Chemicon, Temecula, CA, code AB139, lots 25050133 and 0508007382; dilution 1:100) in 0.05M TBS with 1% sodium metabisulfite and 0.2% Triton X-100. The samples were rinsed in TBS with 1% sodium metabisulfite, then incubated for 1 hour with Cy3 conjugated goat anti-rabbit immunoglobulin (Chemicon; 1:200) and mounted with fluorescence anti-fade mounting medium (Vectashield; Vector, Burlingame, CA). All antibodies were diluted in TBS (pH 7.4) containing 0.2% Triton X-100 and 3% normal goat serum.

To compare the distributions of glycine and γ-aminobutyric acid (GABA) immunoreactivities, some series were treated as above and stained with a cocktail of polyclonal rabbit anti-glycine (Immunosolution, dilution 1:3,000) and monoclonal

mouse anti-GABA (Sigma; clone GB-69, No A 0310, dilution 1:1,200) antibodies, then incubated for 1 hour with a cocktail of Cy3-conjugated goat anti-rabbit immunoglobulin (Chemicon; 1:200) and fluorescein conjugated goat anti-mouse IgG immunoglobulin (Chemicon; 1:50), and mounted in Vectashield.

Antibodies

One of the glycine antibodies (Immunosolution) was raised against a glycine-porcine thyroglobin conjugate and tested by the supplier in sections of retina and cerebellum from various mammals and other vertebrates, as well as in dot blot immunoassays with a variety of amino acid conjugates including the standard 20 amino acids found in proteins, the non-protein amino acids D-serine, D-alanine and D-aspartate, GABA and the glycine-containing tripeptide glutathione (GSH), which did not yield significant reactivity. This antibody has been developed by Dr. David V. Pow (University of Newcastle, New South Wales, Australia), and used in a number of studies on glycinergic neurons of the retina, brain and spinal cord. The other glycine antiserum (Chemicon) was raised against a glycine-BSA conjugate. The specificity of this glycine antiserum was previously tested in lamprey spinal cord tissue homogenates reacted with fixative in the presence of GABA, L-glutamate, glycine or L-aspartate, and showed high specificity for glycine-protein conjugates (Vesselkin et al., 2000). Moreover, preadsorption of this glycine antibody with BSA did not block immunostaining in lamprey. The immunohistochemical results obtained with both anti-glycine antibodies revealed the same pattern of glycine-ir cell populations in both the brain and spinal cord. For tissue processing controls, primary antisera were omitted from some tissue sections. No staining was observed in these controls. In addition, the antibodies were tested by Western blotting with lamprey brain protein extracts (Villar-Cerviño et al., 2006; unpublished results). No protein band was stained in these blots.

The monoclonal anti-GABA antibody (Sigma) was raised against GABA conjugated to BSA with glutaraldehyde and it was evaluated for activity and specificity by dot blot immunoassay by the supplier. No cross-reaction is observed with BSA, L-α-aminobutyric acid, L-glutamic acid, L-aspartic acid, glycine, δ-aminovaleric acid, L-threonine, L-glutamine, taurine, putrecine, L-alanine, and carnosine. The antibody showed weak cross-reaction with β-alanine. Furthermore,

the sections of the brain and retina of sea lamprey incubated with this antibody revealed the same pattern of immunostaining revealed in studies with other anti-GABA antibodies (Meléndez-Ferro, 2001; Meléndez-Ferro et al., 2002, 2003; Villar-Cerviño et al., 2006; Robertson et al., 2007). No immunoreactivity was detected when the primary antibody was omitted from the immunohistochemical processing. In addition, the antibody was tested by Western blotting with lamprey brain protein extracts (unpublished results). No protein band was stained in these blots.

Image Acquisition

Sections were analysed and photographed with a fluorescence microscope fitted with an Olympus DP 70 colour digital camera and/or with a spectral confocal microscope TCS-SP2 (Leica, Wetzlar, Germany). Confocal stacks were acquired and processed with LITE software (Leica). For presentation of most of confocal figures, stack projections were converted to greyscale, inverted and then adjusted for brightness and contrast in Corel Photo-Paint (Corel, Ottawa, Canada). Similarly, fluorescence colour photomicrographs were converted to greyscale, inverted and adjusted for brightness and contrast in Corel Photo-Paint.

Measurements

The cell diameters were measured on confocal photomicrographs of transverse sections with either LITE software (Leica) or ImageJ software (NIH). In each population 10-20 cells were measured. Values are expressed as the mean lesser diameter ± standard deviation.

RESULTS

Both anti-glycine antibodies yielded similar results in the adult brain, but the cells and fibers showed better morphology with the Immunosolution (Pow's) antibody. We describe the distribution of glycine-ir cells and/or fibers in the different brain regions, as revealed by these antisera, in transverse and sagittal sections of upstream migrating adult brain. Schematic drawings of transverse sections showing the location of glycine-ir cell populations and fibers are presented in Figure 1, and the organization of these populations is schematically represented in Figure 2. In the present study, we mostly followed the nomenclature of Pombal and Puelles (1999) for the forebrain, and that of Pombal et al. (1997a, 2001, 2006) for the brainstem.

Telencephalon

The telencephalon of sea lamprey showed a scattered population of glycine-ir cells distributed in inner regions of the olfactory bulb (Fig. 1A, 2A). The pallium and subpallium lacked glycine-ir cells but were innervated by abundant glycine-ir fibers (Fig. 1B,C).

Olfactory bulbs. In the olfactory bulb, spindle-shaped or tripolar intensely glycine-ir cells (9.6 ± 0.9 μm in diameter) were observed in the inner granular layer (Fig. 1A, 3A), far from the glomeruli, extending caudally to the limit with the telencephalic lobes. These cells exhibited long, non-branched dendrites. Very faint glycine immunoreactivity was observed in other neurons of the olfactory bulb, such as the mitral cells (Fig. 3B).

Intensely glycine-ir beaded fibers were observed in both inner (inner granular layer) and outer regions of the olfactory bulb, but were more numerous in inner regions and rather scarce in the olfactory glomeruli (Figs. 1A, 3A,B). Very scarce glycine-ir fibers were also observed in the olfactory fiber layer.

Pallium. The lamprey pallium mainly consists of an evaginated lateral pallium with a thick pallial wall, and a medial pallium that faces the mid telencephalic ventricle.

A number of strongly glycine-ir beaded fibers innervated the lateral pallium, the inner half of the wall showing the densest innervation and the outer layer showing thinner and paler fibers, mostly in the inner layer (Figs. 1B,C). In sagittal

sections that pass superficially through the junction of the telencephalic lobe and the diencephalon, numerous intensely glycine-ir, and rather thick, straight, fibers were observed coursing in parallel between the lateral pallium and the diencephalon (not shown), but whether they arose from telencephalic neurons or from more caudal glycine-ir populations was not established.

The medial pallium showed a rather rich innervation by strong glycine-ir beaded fibers (Figs. 1C, 3C), but no glycine-ir neurons were observed. Fibers showing thick beaded swellings were only observed in a periventricular location, contacting perikarya of periventricular neurons. Similar coarsely beaded glycine-ir fibers were also observed in the subhippocampal lobe (Figs. 1C, 3D), a pallial region located ventrally to the medial pallium.

Subpallium. The lamprey subpallium consists of two main regions, the septum-terminal lamina region and the striatum. The terminal lamina is a thin glial sheet poor in fibers and neurons located ventrally between the two telencephalic lobes. Some strongly glycine-ir fibers crossed the terminal lamina giving rise to large spherical dilatations (Fig. 1B, arrow; see also Fig. 3E). Smaller beads are also common in the adjacent septal region. The entire septal region was innervated by fairly numerous beaded glycine-ir fibers. The striatum is characterized morphologically by the presence of a rather compact row of cells that becomes separated from the ventricular surface in a dorsolateral direction by a rich neuropil region. The striatum had no glycine-ir neurons but received moderate innervation by intensely glycine-ir fibers (Figs. 1B,C, 3F).

Preoptic region, hypothalamus and posterior tubercle

Some cells located in the thick cell band of the magnocellular preoptic nucleus parallel to the preoptic recess exhibited faint glycine immunoreactivity (Figs. 1C, 4A). The preoptic region was scarcely to moderately innervated by strongly glycine-ir beaded fibers that were more abundant in the lateral neuropil areas (Fig. 4A). Strongly glycine-ir fibers decussated in the postoptic commissural region. No glycine-ir neurons were observed in the parvocellular preoptic nucleus. In the proximal stump of the optic nerve, a few thin, intensely glycine-ir fibers were observed. Thick optic nerve fibers only exhibited faint glycine immunoreactivity.

In the tuberal and mammillary regions of the hypothalamus, no glycine-ir neurons were observed. Moderate innervation by glycine-ir beaded fibers was observed in hypothalamic areas lateral to the periventricular cell bands, but innervation was scant in subventricular areas (Fig. 1D). Some thin glycine-ir fibers were observed in the neurohypophysis (Fig. 1D).

Prethalamus, thalamus, epithalamus and pretectum

In these diencephalic regions derived from embryonic prosomeres 1-3 (see Pombal and Puelles, 1999; Meléndez-Ferro et al., 2002), only an intensely glycine-ir neuronal population was observed in the caudal region of the thalamus (P2; "dorsal thalamus") near the fasciculus retroflexus (Figs. 1D, 2A). The thalamic glycine-ir population consisted of intensely stained scattered cells with perikarya (7.9 ± 0.5 μm in diameter) located externally to the periventricular cell layer, which characteristically exhibited rather thick long dendritic processes bifurcating with sharp angles and extending laterally through about one half of the thickness of the thalamus (Figs. 1D, 4B). These tripolar, spindle-shaped or more complex neurons located in the area external to this cell layer also send dendrites parallel to the ventricle or in other directions. In this thalamic region, faint glycine-ir perikarya were located in the periventricular cell layer (Figs. 1D, 4C). They showed dendritic processes extending laterally.

The prethalamus (P3; ventral thalamus) and thalamus showed a rather rich innervation by glycine-ir beaded fibers, both in the lateral and periventricular neuropil regions (Figs. 1D, 4C). In lampreys, the habenula (derived from the dorsal region of prosomere 2) is highly asymmetric; the right habenula is much larger than the left. Thin glycine-ir beaded fibers coursed in the habenular commissure and innervated cell-rich regions of the both habenulae, giving rise to small boutons of a density similar to that observed in the adjacent thalamus (Figs. 1D, 4D). In the right habenula, there was also an intermediate neuropil that showed glycine-ir fibers with rather large beads (Fig. 4D). The glycine-ir innervation of the pretectal region was scarcer than in the thalamus, especially in lateral regions through which the optic tract courses.

Midbrain

The midbrain showed glycine-ir populations located in the caudal region of the optic tectum, in the torus semicircularis and in the caudal tegmentum (Figs. 1E,F, 2A). Most regions of the midbrain were richly innervated by glycine-ir beaded fibers (Fig. 1E).

Optic tectum. The optic tectum of the adult lamprey is a layered structure in which up to eight layers have been distinguished. From inner to outer, these are the ependymal layer, the stratum cellulare periventriculare, the inner fiber layer, the inner cell and fiber layer, the central fiber layer, the external cell and fiber layer, the optic layer and the superficial fibrous layer. In the lateral region of the optic tectum, there was a small population of small glycine-ir cells located in the central fiber layer and the adjacent inner cell and fiber layer (Figs. 1E, 5A). The fiber layers are rather richly innervated by glycine-ir beaded fibers (Fig. 5A), and innervation is denser in the periventricular and in the superficial layers than in other layers (Fig.1E).

Torus semicircularis. The torus semicircularis (TS) is continuous with the tectum. It consists of a cellular layer, two or three cells thick, that ran parallel to the ependymal layer, from which it was separated by a thick fiber layer, and a wide lateral region that is continuous with the mesencephalic reticular area. A conspicuous population of intensely glycine-ir cells (10.2 ± 1.5 μm in diameter) was observed in the TS (Fig. 1E,F, 2A, 5B). Their perikarya were located in the cell-rich layer or just lateral to it and gave rise to rather thick long dendrites to the adjacent lateral area, where they often bifurcated and formed a wide dendritic network. This population of glycine-ir cells extended toward the midline in the caudal pole of the midbrain tectum (Figs. 1F, 5C). Careful observation of sagittal sections of the midbrain tectum indicated that its caudal pole lacked the cell and fiber layer organization characteristic of the optic tectum (see above), suggesting that the torus semicircularis of adult lampreys formed actually the caudal part of the midbrain tectum (see Discussion). Smaller and paler glycine-ir cells were also observed in more ventral regions of the periventricular cell layer of the torus semicircularis (Fig. 1E). Glycine-ir fibers were abundant in the lateral area and periventricular fiber layer, which were more densely innervated than the adjacent tectal regions (Fig. 1E,F, 5B).

M5 nucleus. Ventrally, the cell layers of the TS are continuous with the M5 nucleus of Schober (1964), which extends from the subpretectal tegmentum, embracing the M1 and M2 Müller cells, to the level of the midbrain Müller cell (M3). Small, intensely glycine-ir cells were observed in the caudal tegmentum dorsally to the M3 cell (Figs. 1E, 2A, 5D), in the region that contains internuclear neurons of the oculomotor nucleus (González et al., 1998). The midbrain tegmentum was rather richly innervated by glycine-ir beaded fibers and abundant fibers surrounded the M3 cell perikaryon (Figs. 1E, 5D). As seen in sagittal sections, rather thick glycine-ir fibers coursed longitudinally in the midbrain and isthmus (not shown).

Oculomotor nucleus. The oculomotor nucleus of lamprey consists of three subnuclei (Fig. 1E) that each innervate an extraocular muscle; rostral and dorsal rectus (lateral and intermediate subnuclei, respectively), and rostral oblique (dorsomedial subnucleus; see Fritzsch et al., 1990; González et al., 1998). The rostral rectus subnucleus consists of large cells clustered in a compact lateral group just dorsal to the oculomotor nerve exit. The large, rounded neurons of this subnucleus were surrounded by some glycine-ir fibers and small boutons. This nucleus received very thick glycine-negative axons that surrounded motoneuron perikarya. The intermediate population of motoneurons (dorsal rectus subnucleus) received some glycine-ir fibers (Fig. 1E). These medium-sized neurons send long dendrites dorsolaterally (Fritzsch et al., 1990) towards a fiber region through which a number of glycine-ir fibers coursed longitudinally. The dorsomedial population of motoneurons (rostral oblique subnucleus) was likewise moderately innervated by glycine-ir fibers (Figs. 1E, 5D).

Rhombencephalon

Alar plate regions

Isthmus. The lamprey isthmus is separated from the midbrain dorsally by the dorsal isthmic commissure, and ventrally by the decussation of the rostral octavomotor fibers and the appearance of the interpeduncular nucleus. Caudally, it extends approximately till the rostral pole of the trigeminal motor nucleus, which in adults forms a large protrusion toward the fourth ventricle and consists of large motoneurons. Intensely glycine-ir populations were observed in the isthmus: two

dorsal (alar plate) isthmic populations (dorsomedial and dorsolateral) and a conspicuous ventral (basal plate) reticular population (see below) (Figs. 1F,G, 2A,B). A further glycine-ir population was observed ventrally in the rostral region of the interpeduncular nucleus (Figs. 1F, 2A,B).

Just dorsal to the sulcus limitans, there was a thick, longitudinal band of small glycine-ir cells (6.7 ± 0.6 μm in diameter) located close and parallel to the ventricle (Figs. 1G, 6A). A conspicuous dorsolateral isthmus population of very intensely glycine-ir cells (11.5 ± 1.5 μm in diameter) was located just lateral to this dorsomedial periventricular population, forming a rather compact band of perikarya and thick dendrites extending in the middle of the dorsal isthmic region (Figs. 1G, 6B,C). These cells were bipolar, tripolar or multipolar and exhibited straight, thick dendrites forming a conspicuous sheet laterally to the perikarya. The position of this cell sheet closely corresponds to that of the "dorsal isthmal gray" reported in sea lamprey with immunohistochemistry against neuropeptide FF (Pombal et al., 2006). Most cells were spindle-shaped, with long, thick dendrites extending laterally and, in some cells, an axon coursing to the periventricular neuropil, whereas cells further lateral in the sheet were more variable in shape (Fig. 6B,C). The periventricular neuropil exhibited a dense mat of glycine immunoreactivity. In lateral regions of the isthmus, glycine-ir processes were scarcer than in periventricular regions.

Octavolateral area. The octavolateral area of adult lamprey extends in the alar plate from levels of the rostral isthmus to the region rostral to the obex and consists of three main longitudinal columns with unclear topographical limits: the dorsal nucleus (receiving anterior lateral line nerve electroreceptive fibers), the medial nucleus (receiving lateral line nerves mechanoreceptive fibers), and the ventral nucleus or octaval region that exhibits groups of large neurons, the superior, intermediate and inferior octavomotor nuclei. The dorsomedial region of the alar plate (dorsal nucleus) did not exhibit any glycine-ir neurons (Figs. 1H, 2B). The dorsal nucleus was innervated by thin, glycine-ir, beaded fibers. In the medial nucleus, scattered, small neurons (8.7 ± 1.6 μm in diameter) that showed glycine immunoreactivity were located in periventricular regions (Figs. 1H,I, 6D). Abundant glycine-ir thin fibers were observed in the rostral region (at trigeminal levels) of this nucleus. Caudally to the octaval nerve entrance, the number of glycine-ir cells in this nucleus diminished sharply, and glycine-ir cells were barely appreciated. The ventral

nucleus proper, which includes the conspicuous superior, intermediate and posterior octavomotor nuclei (see Stefanelli, 1937; Stefanelli and Caravita, 1970; González et al., 1997), did not show any glycine-ir cells, although it was innervated by some glycine-ir fibers (Fig. 1H-J).

Dorsal column nucleus. The dorsal column nucleus is a long nucleus extending rostrally in the alar plate of the caudal rhombencephalon from obex levels. The nucleus consists of a periventricular region rich in cell perikarya, and a dorsolateral region that consists mainly of fibers coursing longitudinally and neuropil with some scattered neurons. Sections through the dorsal column nucleus of adult lamprey revealed the presence of numerous glycine-ir neurons in the periventricular cell layer (Fig. 1K, 2B, 6E) and of fibers and dendritic processes in the dorsolateral region. Glycine-ir neurons were pear-shaped or tripolar and sent straight dendrites to the fiber layer, and most were located in the periventricular cell layer or adjacent to it in the fiber layer (Fig. 6E).

Basal plate regions

Large numbers of glycine-ir cells were observed in rhombencephalic basal plate regions. For purposes of description, they were referred to as either the medial (magnocellular) or lateral (parvocellular) zones of the rhombencephalic reticular formation. We subdivided the reticular glycine-ir populations of the classical rhombencephalic reticular formation into isthmic, trigeminal, middle and posterior regions, following the scheme of Stefanelli (1934). These large-celled regions correspond to the medial zone of the reticular formation (Fig. 2B). In addition, some glycine-ir cells were located more laterally, more or less closely associated with the visceromotor column. Most of these cells can be included in an ill-defined broad, lateral, parvocellular reticular zone (Fig. 2B). The lateral glycine-ir reticular populations will be described in relation to the somatomotor (abducens, spinooccipital) and visceromotor (trigeminal, facial, glossopharyngeal, and vagal) nuclei.

Medial zone of the rhombencephalic reticular formation

Isthmic region. In the intermedioventral reticular region, there was a loose band of intensely glycine-ir cells (10.2 ± 1.5 μm in cell diameter) extending ventrolaterally in intermediate reticular levels (Figs. 1F,G, 2A,B). This band was

formed of numerous spindle-shaped or tripolar cells (Fig. 7A). Larger cells exhibited long, rather thick dendrites extending in a network of processes in the lateral fiber and neuropil region.

There was a small population of moderately to strongly glycine-ir, small cells (7.1 ± 0.8 μm in diameter) with round or triangular perikarya and generally rather thin processes, in the rostral region of the interpeduncular nucleus (Fig. 1F,G), just caudal to the decussation of anterior octavomotor axons. The interpeduncular neuropil exhibited a much paler appearance than neighbouring regions with glycine immunofluorescence, owing to the scarcity of glycine-ir fibers. A few thin, glycine-ir, beaded fibers crossed the interpeduncular nucleus.

Trigeminal levels. Some intensely glycine-ir cells were observed caudally to the isthmus, in the medial zone of the basal plate (Fig. 1H, 2B, 7B), which corresponds to the medial part of the trigeminal reticular formation. At levels medial to the trigeminal motor nucleus, the number of glycine-ir cells was low, with a few cells per section, distributed mainly in the rostral trigeminal region. The perikarya (11.1 ± 1.2 μm in diameter) were bipolar or tripolar in appearance and were located either in a lateral position in the periventricular cell mantle or scattered in the adjacent ventral reticular area, to which they extended long straight dendrites.

Middle rhombencephalic reticular region. In the middle reticular formation, located at facial motor nucleus/octaval nerve levels and caudal to the trigeminal motor nucleus, the number of glycine-ir cells in the medial zone increased considerably (Figs. 1I, 7C,D). They were small to large, spindle-shaped or tripolar cells (19.4 ± 7.9 μm in diameter) with thick dendrites that branched abundantly in the ventral region, forming a conspicuous mat of straight and rather coarse glycine-ir dendritic processes in the inner half of the reticular area, as observed in transverse sections. In sagittal sections, however, these dendrites mostly coursed perpendicular to the longitudinal brain axis (not shown). The largest perikarya in the strongly glycine-ir cells of this region (28.5 ± 4.6 μm in diameter) were close to the ependyma close to the Mauthner cell (Figs. 1I, 2B, 7C,D), whereas smaller cells generally occupied more ventral positions. These intensely glycine-ir cells were associated with medium-sized to large reticular neurons of this region, which were glycine negative or faintly positive and whose perikarya were surrounded by some highly glycine-ir fibers and boutons.

Posterior rhombencephalic reticular region. In the posterior rhombencephalic reticular formation, there were numerous small to medium-sized spindle-shaped or tripolar cells (11.7 ± 2.0 μm in diameter) with thick dendrites that branched abundantly in the ventral region, forming a conspicuous mat of straight and rather coarse glycine-ir dendritic processes in the inner half of the reticular area (Fig. 1J). These intensely glycine-ir cells were associated with the large reticular neurons characteristic of this region, which showed faint or very faint glycine immunoreactivity and whose perikarya were surrounded by some highly glycine-ir fibers and boutons.

Glycine-ir reticular populations associated with somatomotor nuclei

Region of the abducens nucleus. The lamprey abducens nucleus innervates two extraocular muscles, the posterior and ventral rectus muscles (see Fritzsch et al., 1990; González et al., 1998). Abducens motoneurons were located either in the reticular region ventral to the facial and glossopharyngeal motor nuclei at intermediate dorsoventral levels (abducens ventral subgroup or caudal rectus subnucleus) or in periventricular regions medial to the facial and glossopharyngeal motor nuclei (abducens dorsal subgroup or ventral rectus subnucleus) (Fig. 1I). Large numbers of strong glycine-ir cells with long thick dendrites were observed at these reticular levels (Figs. 1I, 8A). The region through which the abducens neurons extended their dendrites was richly innervated by glycine-ir fibers, but whether these motoneurons are contacted by glycine-ir fibers was not investigated.

Spinooccipital motoneurons. This column of large, cholinergic motoneurons located in the caudal rhombencephalon is continuous with the spinal motoneuron column (Pombal et al., 2001). The region just ventral and lateral to these large perikarya contained small glycine-ir cells (9.3 ± 1.9 μm in diameter) with long dendrites, and a number of glycine-ir axons coursed in this fiber region (Figs. 1K, 2B, 8B). However, glycine-ir processes were rather scarce in the region containing the motoneuron perikarya.

Glycine-ir reticular populations associated with visceromotor nuclei

Trigeminal motor nucleus. The trigeminal motor nucleus of the adult lamprey consists of large neurons with perikarya that form a large prominence towards the fourth ventricle. Just lateral to the motor nucleus, there was a cell population

containing small, strongly glycine-ir cells (9.1 ± 0.5 μm in diameter) intermediate between the motor nucleus and the rostral octavolateral area (Figs. 1H, 2B, 8C). These cells gave rise to thin dendrites extending laterally towards the trigeminal descending root, as observed in sagittal sections.

Motoneuron perikarya are separated from the ependymal layer by a thick, subventricular fiber region, to which motoneurons send thin proximal dendrites (see Homma, 1978; Koyama et al., 1987; Pombal et al., 2001). Thick, distal dendrites of trigeminal motoneurons project ventrolaterally to a wide region. The region of the motor nucleus exhibited a number of glycine-ir fibers along with glycine-ir boutons surrounding motoneuron perikarya and, at a higher density, the proximal trunk of dendrites (Fig. 8C). Abundant glycine-ir boutons were also observed in the periventricular fiber region (Fig. 8C). The intricate glycine-ir network of the periventricular region was also appreciated in sagittal sections of the nucleus (not shown).

Facial motor nucleus. Medium-sized (12.0 ± 1.9 μm in diameter) glycine-ir, spindle-shaped or tripolar neurons were distributed in the region just ventrolateral to the large perikarya of the facial motor nucleus (Fig. 8D). Dendrites of these glycine-ir cells were intermingled with the proximal portion of motoneuron dendrites, which were also surrounded by some glycine-ir fibers.

Glossopharyngeal-vagal motor column. There was a group of glycine-ir cells (10.1 ± 1.7 μm in diameter) lateral and ventral to the large neurons of the glossopharyngeal-vagal motor column (see Pombal et al., 2001; Koyama, 2005) (Figs. 1J, 2B, 8E). Because most glycine-ir cells were intermediate between the visceromotoneuron column and solitary tract nucleus or the descending trigeminal nucleus, unequivocal ascription of these cells to one or other of these regions was not possible. Similar small glycine-ir cells were also observed just medial to motoneurons and scattered in the neuropil ventral to the motor column. Glycine-ir processes were abundant throughout the lateral and periependymal area, and the motoneuron perikarya also appeared partially covered by glycine-ir boutons.

Commissural glycine-ir systems in the lamprey brain

Because contralateral inhibition mediated by glycine appears important for motor control, we investigated the distribution of glycine-ir commissural fibers in the lamprey brain.

In the telencephalon, glycine-ir fibers exhibiting large swellings crossed the ventral midline in the terminal lamina and in the dorsal interbulbar commissural region (Fig. 1B). In the preoptic region, large numbers of thin, beaded, glycine-ir commissural fibers were observed in the postoptic commissural plate and, to a lesser extent, accompanying the decussating optic fibers. The posterior tubercle showed scarce glycine-ir commissural fibers. The habenular commissure was crossed by fairly abundant glycine-ir, thin fibers. A number of glycine-ir fibers also crossed in the posterior commissure, which is located over the subcommissural organ and rostral to an extensive choroid plexus covering the rostral roof of the midbrain ventricle.

In the midbrain tectum, the midline raphe caudal to the choroid roof is crossed by fairly abundant glycine-ir processes, especially in its caudal region (Fig. 1F). Very scarce glycine-ir commissural fibers were observed in the midbrain tegmentum.

Glycine-ir commissural fibers in the region of the interpeduncular nucleus (isthmus) and the trigeminal region are scarce. Instead, numerous glycine-ir fibers, some of them rather thick, cross the midline at middle and posterior rhombencephalic reticular levels (Fig. 8F). At the midline level, many of these fibers changed their direction, as was usual in arcuate fiber systems. In the caudal medulla, the number of glycine-ir commissural fibers was low again, and they were generally thin.

Colocalization of GABA immunoreactivity in glycine-ir neurons

To assess whether the glycine-rich neurons of the lamprey brain also exhibit GABA immunoreactivity, we performed double-labeling immunofluorescence experiments. The general distribution of GABA-ir perikarya and fibers observed in these double-labeling experiments was similar to that reported in other studies of the sea lamprey brain (Meléndez-Ferro, 2001; Robertson et al., 2007) and will not be considered here. In the following, we investigate possible colocalization of GABA in

nuclei and regions containing glycine-ir cells. Colocalization of GABA immunoreactivity in glycine-ir perikarya varied widely among nuclei and regions.

In the olfactory bulb, numerous glycine-ir cells are located in the inner granular layer. Although this layer contains a number of GABA-ir cells, double immunofluorescence did not show colocalization with GABA immunoreactivity in these intensely glycine-ir cells (Fig. 9A). Although abundant GABA-ir cells were observed in the lateral pallium, these cells did not show any colocalization of glycine.

In the diencephalon, the only intensely glycine-ir neuronal population was observed in the thalamus. Double immunofluorescence revealed that these cells were not GABA-ir (Fig. 9B). Some cells located in the thick cell band of the magnocellular preoptic nucleus parallel to the preoptic recess exhibited faint glycine immunoreactivity, but again they did not show any GABA immunoreactivity. Small GABA-ir/glycine-negative neurons were located in the parvocellular preoptic nucleus just ventrocaudal to the magnocellular preoptic nucleus.

In the optic tectum, there was a large number of faintly GABA-ir cells as well as a population of more intensely stained cells. Although there was a small population of glycine-ir cells located in the optic tectum, mainly at lateral regions, no colocalization with GABA was observed in double immunofluorescence experiments. In the torus semicircularis, small GABA-ir cells were observed in the cell layer and in the adjacent neuropil region, where a conspicuous population of medium-sized, intensely glycine-ir cells was observed extending in the caudal region of the midbrain tectum. Double immunofluorescence did not reveal colocalization of GABA and glycine in torus semicircularis cells (Fig. 9C). Small GABA-ir cells were also observed in the region of the M5 nucleus in the midbrain tegmentum, which contained small, intensely glycine-ir cells. Double immunofluorescence revealed colocalization of GABA and glycine in a few of these M5 cells, but most cells were either GABA-ir or glycine-ir.

In the isthmus, most of the small glycine-ir cells observed in the rostral region of the interpeduncular nucleus also showed GABA immunoreactivity, although this population also contained a few only GABA-ir and only glycine-ir neurons. In the isthmic reticular region, many of the intensely glycine-ir cells showed colocalization

with GABA, although glycine-ir/GABA-negative and GABA-ir/glycine-negative cells were also observed (Fig. 9D). The intensely glycine-ir cells of the dorsal isthmus (the "dorsal isthmal grey") showed no colocalization with GABA, although smaller GABA-ir/glycine-negative cells were intermingled with cells of this glycine-ir population. In addition, the cell band located parallel to the ventricle containing small glycine-ir cells showed a few small, GABA-ir cells, but double immunofluorescence did not reveal colocalization of GABA and glycine in these cells.

The dorsal nucleus of the octavolateral region showed GABA-ir cells but did not exhibit glycine-ir neurons. In the medial nucleus of this region, some glycine-ir neurons also showed GABA immunoreactivity, although other cells were either GABA-ir or glycine-ir (Fig. 9E). In the caudal rhombencephalon, the dorsal column nucleus contained both GABA-ir and glycine-ir neurons. Double immunofluorescence showed colocalization of GABA and glycine immunoreactivities in some cells of the periventricular layer (Fig. 9F), although most juxtaependymal cells and the smallest cells of the periependymal cell layers were only GABA-ir, and the largest cells with thick dendrites were mostly only glycine-ir.

At rhombencephalic reticular levels, a large number of strong glycine-ir cells was observed in the medial zone (see above), where some GABA-ir cells were also present. Double immunofluorescence indicated that GABA and glycine immunoreactivities were mainly localized in different neurons (Fig. 9G,H). In the lateral reticular zone that was associated with the visceromotor column, there were also small, GABA-ir neurons, mainly lateral to the trigeminal motor nucleus. In this trigeminal region, double immunofluorescence indicated the colocalization of glycine and GABA immunoreactivities in a few cells (Fig. 9I). The glycine-ir neurons distributed throughout the region just ventrolateral to the facial motor nucleus were not GABA-ir. Lateral to the glossopharyngeal-vagal motor column was a group of small glycine-ir cells. Double labeling indicated that glycine and GABA immunoreactivities were colocalized in a few of these cells (Fig. 9J). With regard to the small glycine-ir cells observed among cells of the spinooccipital motor column, they did not show colocalization with GABA.

DISCUSSION

This study represents the first analysis of the glycine-ir cell groups and fibers in the whole brain of a nonmammalian vertebrate. Results with the anti-glycine antiserum in the spinal cord (Villar-Cerviño et al., 2008) are consistent with those of previous spinal cord studies (Shupliakov et al., 1996; Vesselkin et al., 2000). The different distribution of GABA and glycine observed with double immunofluorescence in the adult retina (Villar-Cerviño et al., 2006), brain (present results) and spinal cord (Villar-Cerviño et al., 2008), together with the controls performed by us in the lamprey and by the supplier of the antiserum, indicate that we are demonstrating the presence of glycine.

With regard to the characteristics of the cells revealed by glycine immunohistochemistry in lamprey, all brain regions show neuronal morphology and exhibit specific distributions, which identify them as neurons. The glia of the lamprey brain consists mostly of ependymocytes primitive glial cells containing keratin-like intermediate filaments (Merrick et al., 1995), whereas oligodendrocytes are lacking (Bullock et al., 1984). Throughout the brain ventricles, the ependyma was glycine-negative or very faintly glycine-ir, again indicating that glycine-rich cells observed in the lamprey brain were neurons.

Comparison of the distribution of glycine in the lamprey with that reported in the brain of several mammals (rat, mice, cat) reveal a number of differences but also interesting resemblances.

Unlike the case in mammals, the adult lamprey brain exhibits putative glycinergic cell populations in the forebrain

For the forebrain of the adult sea lamprey, we describe for the first time the presence of glycine-ir cell populations in three different regions: the olfactory bulbs, the preoptic nucleus and the thalamus. These results notably contrast with those obtained in the forebrain of mammals (Rampon et al., 1996; Zeilhofer et al., 2005), in which only a few cells expressing the glycine transporter GlyT2 were observed in the posterior hypothalamus with use of a bacterial artificial chromosome in transgenic mice (Zeilhofer et al., 2005), whereas immunohistochemical studies in the rat showed a few glycine-ir cells in two forebrain structures: the subfornical organ and the lateral habenular nucleus (Rampon et al., 1996).

In the olfactory bulbs, the presence of some glycine-ir cells in the granular layer and the abundance of glycine-ir fibers suggest that this neurotransmitter is involved in the processing of olfactory information in the lamprey. In mice, only very scarce glycine-ir fibers reached the olfactory bulb (Zeilhofer et al., 2005), suggesting a reduction in glycinergic circuitry with evolution. In the olfactory bulbs of vertebrates, including lampreys (Meléndez-Ferro et al., 2001; Robertson et al., 2007), large numbers of cells, mainly granule cells, were observed to be GABAergic. In mammals, GABA is present in both periglomerular cells and granular cells (Mugnaini et al., 1984). Although the absence of glycine immunoreactivity in cells in the mammalian olfactory bulbs precludes its colocalization with GABA, GABA-ir cells have been reported in the inner granular layer in lampreys (Meléndez-Ferro et al., 2001; Robertson et al., 2007). However, our double-labeling experiments indicate that, in the bulb, the GABA-ir and glycine-ir cells represent separate populations. Whether these glycine-ir cells are granule cells or another type of neuron is not known. The greater abundance GABA-ir cells and processes than of glycine-ir cells suggests that GABA is the main inhibitory transmitter, although it is possible that glycine is important in lamprey olfactory circuits.

Direct comparison of telencephalic structures of lamprey and mammals is not possible, but experimental studies have revealed that the lateral pallium of lamprey is an olfactory recipient region (Northcutt and Puzdrowski, 1988; Northcutt and Wicht, 1997) and thus appears to be homologous to the mammalian olfactory cortex, which among other regions comprises the olfactory tubercle, the anterior olfactory nucleus, and the piriform and entorhinal cortices. Our results reveal that glycine-ir cells are lacking in the lamprey pallium. No glycinergic cell was found in the pallium of rodents (Rampon et al., 1996; Zeilhofer et al., 2005), which is similar to that observed in lamprey. Instead, GABAergic inhibitory cells are abundant in mammalian pallium, as well as in the lateral pallium of lampreys (Meléndez-Ferro, 2001; Meléndez-Ferro et al., 2002; Robertson et al., 2007), suggesting that they are the origin of pallial local inhibitory circuits.

With regard to the glycine-ir innervation of the lamprey telencephalon, abundant immunoreactive fibers were observed in the lateral pallium but also in subpallial regions such as the striatum (for characterization see Pombal et al., 1997a,b) and the region around the terminal lamina (putative septum). These fibers

are widely distributed, but it is unknown whether they originate from the olfactory bulb and/or from caudal glycine-ir populations. The medial pallium contains comparatively scarce glycine-ir fibers. In rodents, only the basal forebrain contained a relatively high density of glycinergic axons, notably the medial septum, the nucleus of the diagonal band of Broca, and the substantia innominata, whereas, in the remaining telencephalon, the innervation was either sparse or almost absent (Rampon et al. 1996; Zeilhofer et al., 2005). In the absence of data on other vertebrates, these results suggest a reduction of glycinergic inhibitory mechanisms in rostral brain regions during evolution.

With regard to the diencephalon, we only observed some glycine-ir cells only in the thalamus of the lamprey, among longitudinal fiber systems. In mammals, scarce glycine-ir cells have only been found in the lateral habenular nucleus of rats (Rampon et al., 1996), which indicates that diencephalic glycine-ir populations of lamprey (thalamic) and rat (habenular) are unrelated. However, a sparse putative glycinergic population has recently been demonstrated in the posterior hypothalamus of mice, among fiber bundles coursing between the dorsal hypothalamus and the central grey (Zeilhofer et al., 2005), which judged by its position might correspond to the thalamic glycine-ir cells of lamprey. Whether similar neurons are present in the thalamus of other vertebrate groups is not known. GABA-ir cells are widely distributed in the lamprey diencephalon (Meléndez-Ferro et al., 2002; Robertson et al., 2007), but GABA is absent from glycine-ir cells of the dorsal thalamus.

Most regions of the lamprey diencephalon exhibit rich glycine-ir innervation, including the habenula, dorsal thalamus, prethalamus, and hypothalamus, suggesting involvement of glycine in a number of diencephalic circuits. Judging from the near absence of diencephalic glycine-ir cells, most of these fibers are diencephalic afferents originating from other brain regions, as in mammals. The diencephalic regions that receive the most widespread glycinergic innervation in rodents are the preoptic area and the hypothalamus (Rampon et al., 1996; Zeilhofer et al., 2005). In these regions and in the thalamus, the distribution was highly specific and differed across different nuclei, which is in contrast to the distribution observed in the lamprey. Marked right-left asymmetry, with regard to glycine-ir innervation, was also observed in the habenula, in addition to other asymmetries such as that of the

small size of the left habenula and its preferential afferent connection from the parapineal organ (Yáñez et al., 1999).

Although studies on other vertebrate groups are lacking, these results suggest that the putative forebrain glycinergic system of early vertebrates was rather extensive and became severely reduced through evolution. These data also suggest that glycinergic circuits in the forebrain have been specialized in mammals, although the absence of studies in other vertebrate groups precludes further speculation.

The midbrain glycine-ir cell populations of the lamprey are associated with octavolateral and visual regions of the mesencephalic tectum

We observed three glycine-ir cell populations in the midbrain of the lamprey: in the caudal region of the optic tectum, in the torus semicircularis, and in the caudal tegmentum. The torus semicircularis of the lamprey receives a large number of fibers from vestibular and lateral line centers (González et al., 1999) and is comparable to the inferior colliculus of mammals, whereas the optic tectum is the main visual center and is homologous to the superior colliculus (De Arriba Pérez, 2007; De Arriba and Pombal, 2007). However, *in situ* hybridization did not reveal GlyT-2 transporter mRNA in cells in the inferior colliculus of either rat or mice (Tanaka and Ezure, 2004; Zeilhofer et al., 2005), whereas occasional glycinergic cells appeared in the superior colliculus (Zeilhofer et al., 2005). Again, putative glycinergic cells are far more abundant in the lamprey midbrain than in that of mammals. For mammals, glycine-ir neuron populations have been described in the ventrolateral part of the periaqueductal grey and in the deep mesencephalic nucleus (rat: Campistron et al., 1986; Pourcho et al., 1992; Rampon et al., 1996; cats: Fort et al., 1990). Because the central grey and deep mesencephalic nuclei have not been formally described in the midbrain of lampreys, we only can speculate on the possibility that some of these mammalian populations would correspond to some of the glycine-ir cells of the lamprey midbrain tegmentum.

Our results showing the presence of both glycine-ir cells and fibers in the optic tectum indicate that glycine is involved in visual circuits of lampreys. Previous results with GABA also indicated the presence of abundant GABAergic cells and fibers in the optic tectum of adult lampreys (Meléndez-Ferro, 2001; Robertson et al., 2007), although they do not appear in larval stages (Meléndez-Ferro, 2001;

Meléndez-Ferro et al., 2002). Together, these results indicate that inhibition by glycine and GABA coexist in the lamprey optic tectum, although colocalization of these neurotransmitters was not observed at the cellular level. Glycine-ir innervation of the optic tectum appears to have mixed origins. The presence of glycine-ir cells in the optic tectum suggests that a part of this tectal innervation originates from these local neurons. However, glycine-ir fibers passing from the isthmus toward the optic tectum were also observed in sagittal sections (results not shown), indicating that the tectum also receives extrinsic glycine-ir innervation. The presence of extrinsic inhibitory GABAergic innervation of the optic tectum originating from a few forebrain and midbrain nuclei has been reported recently for lampreys (Robertson et al., 2006). These forebrain nuclei do not contain glycine-ir cells (present results). On the other hand, Robertson et al. (2006) showed GABAergic projections from the torus semicircularis and the M5 to the tectum, with both nuclei also containing glycine-ir cells. Colocalization of GABA and glycine was only observed in some M5 cells, which suggests that most forebrain and midbrain fibers afferent to the tectum might use either GABA or glycine, pertaining largely to different systems.

The presence of glycine-ir neurons projecting to the optic tectum in the lateral (vestibular) and medial (reticular) zones of the medulla oblongata has been demonstrated experimentally in plethodontid salamanders (Landwehr and Dicke, 2005). Several nuclei of the isthmic region project to the optic tectum (Robertson et al., 2006; De Arriba and Pombal, 2007), and this region also has a number of glycine-ir populations, some of which may project to the tectum. Although only occasional putative glycinergic cells have been observed in the superior colliculus of mammals (Tanaka and Ezure, 2004), glycinergic fibers appear rather abundant (Rampon et al., 1996). Judged by the quite complete absence of glycinergic cells in the forebrain and midbrain of mammals (see above), the afferent glycinergic innervation of the superior colliculus probably originates from rhombencephalic populations, as seen in the lamprey.

In rats, inhibitory neurons within the inferior colliculus are primarily GABAergic, and no glycine-ir cells have been found (Merchán et al., 2005). Rather, the torus semicircularis of lamprey appears to contain an abundant glycine-ir population, mainly in caudal regions, where they extend to the caudal pole of the optic tectum. A recent experimental study has shown that in adult lampreys this optic

tectum caudal pole receives mainly octavolateral fibers, but no optic fibers (De Arriba Pérez, 2007). Together with the present results with glycine, this suggests that the caudal pole of the so-called lamprey optic tectum is actually the caudal part of the torus semicircularis, which would be reminiscent of the presence of visual and acoustic centers (superior and inferior colliculi) in the rostral and caudal parts of the midbrain tectum of mammals, respectively.

Major glycine-ir populations were observed in the isthmus and the rhombencephalic reticular formation

In mammalian brains, most putative glycinergic cells are located in the hindbrain (see Rampon et al., 1996; Tanaka and Ezure, 2004). The distribution is nuclei specific, and a number of sensory and reticular regions showed the presence of glycinergic neurons in variable proportions. The presence of glycine-ir cells has been reported in the four longitudinal zones (dorsal, lateral, medial and median) of the medulla oblongata of plethodontid salamanders (Landwehr and Dicke, 2005). In the developing brain of zebrafish, GlyT-2 expressing cells (putative glycinergic neurons) were found only caudal to the midbrain-hindbrain boundary and were observed in longitudinal cell bands (Higashijima et al., 2004). However, the location of glycinergic cells in the adult teleost brain is not known. Present results in adult lamprey reveal that the numerically most important glycine-ir populations in the brain are distributed caudal to the midbrain-hindbrain boundary in various nuclei and columns. As indicated above, and unlike the case in developing zebrafish, several putative glycinergic populations are observed in the lamprey midbrain and forebrain.

Three main intensely glycine-ir populations were observed in the lamprey isthmus: one conspicuous reticular population (see below) and two dorsal isthmic populations, dorsomedial and dorsolateral. By comparison, the isthmus proper of mammals exhibits scarce glycinergic populations (Rampon et al., 1996; Tanaka and Ezura, 2004), all located in basal plate derived regions. These differences are intriguing and may be related to the possible lack of a cerebellum in lampreys (Lannoo and Hawkes, 1997). In mammals, the cerebellar nuclei (Bäurle and Grüsser-Cornehls, 1997) and the cerebellar cortex (see Zeilhofer et al., 2005) exhibit conspicuous glycinergic neuronal populations, and some glycine-ir cells have also been observed in the frog cerebellum (Reichenberger et al., 1993). The true

cerebellum appeared in jawed vertebrates from specialization of the most dorsolateral region of the alar plate, the rhombic lip, and, in the most primitive extant jawed vertebrates, the elasmobranchs, the cerebellar cortex is accompanied by a conspicuous cerebellar nucleus (Álvarez-Otero et al., 1996). From a parsimonious point of view, it is plausible that the rhombic lip of the isthmic region was homologous as a field of the region giving rise to the cerebellum in gnathostomes and that dorsal glycine-ir populations similar to those observed in the lamprey isthmus may have evolved into cerebellar populations such as those observed in mammals.

In the rhombencephalon of mammals, glycine-ir cells are localized in a number of nuclei involved in processing auditory and vestibular information, such as the dorsal and ventral cochlear nuclei, the superior olive, nucleus of the trapezoid body, nuclei of the lateral lemniscus, and vestibular nuclei (Wenthold, 1987; Aoki et al., 1988; Helfert et al., 1989; Saint Marie et al., 1989, 1991; Walberg et al., 1990; Kolston et al., 1992; Pourcho et al., 1992; Rampon et al., 1996; Spirou and Berrebi, 1997; Zeilhofer et al., 2005). The cochlear and vestibular nuclei of the rhombencephalon also exhibit some glycine-ir cells in amphibians (Reichenberger et al., 1997; Landwehr et al., 2005). The octavolateral region of lampreys, as in other fishes, receives fibers from the octaval and the lateral line nerves (Northcutt, 1979; Ronan and Northcutt, 1987; González and Anadón, 1992, 1994). This region contains three main nuclei: the dorsal, medial, and ventral octavolateral nuclei, vestibular fibers entering in the latter nucleus (Northcutt, 1979; Koyama et al., 1989; González and Anadón, 1994). For this vestibular nucleus, we observed abundant glycine-ir fibers, but, unlike the case in vestibular nuclei of mammals and amphibians, very scarce glycine-ir cells were observed. Likewise, this nucleus contains few GABA-ir cells (Meléndez-Ferro et al., 2003; Robertson et al., 2007). However, abundant glycine-ir cells can be observed in reticular areas just medial to this nucleus (see below), suggesting that polysynaptic inhibitory effects on the ipsilateral rhombencephalic reticular formation observed after vestibular nerve stimulation (Matthews and Wickelgren, 1979) might be mediated by glycinergic cells. Application of either glycine or GABA to large reticular neurons leads to hyperpolarisation of the cell membrane of these cells (Matthews and Wickelgren, 1979).

With regard to the primary nuclei of the lateral line nerves, the dorsal octavolateral nucleus receives projections from electroreceptive organs (via the dorsal root of the anterior lateral line nerve) and the medial nucleus from mechanoreceptive organs, via the ventral and intermediate roots of the anterior lateral line nerve and the posterior lateral line nerve (Ronan and Northcutt, 1987; González and Anadón, 1992). Differences between the primary projections of these nerves (González and Anadón, 1992) and the cellular organization of the nuclei (González et al., 1997) were also noted. The present results reveal major differences between the medial and the dorsal nucleus with respect to the abundance of putative glycinergic cells in the former and the lack of such cells in the latter. These neurochemical differences must be correlated with functional differences between the nuclei, but as far as we know, no functional studies have been carried out to distinguish them. No major differences between the dorsal and the medial nucleus of the octavolateral area have been observed with GABA immunocytochemistry (Meléndez-Ferro, 2001; Robertson et al., 2007), although the embryonic columns originating the GABAergic populations of the dorsal and medial nucleus were different (Meléndez-Ferro et al., 2003).

The dorsal column nucleus is a relay center for tactile and proprioceptive somatosensory information. In this region of adult lampreys, most of the glycine-ir cells were located in the periventricular layer, as previously reported in larval sea lampreys (Rodicio et al., 2005). Thus, the lamprey dorsal column nucleus appears to be similar to that in mammals in that it has putative glycinergic neurons (Pourcho et al., 1992; Popratiloff et al., 1996; Rampon et al., 1996; Lue et al., 1997). Stimulation of the dorsal column of lampreys mainly produces inhibition of identified reticulospinal neurons, probably via glycinergic synapses (Dubuc et al., 1993a,b). However, the finding of colocalization of GABA and glycine in numerous neurons of this nucleus suggests that both neurotransmitters are used simultaneously in the dorsal column nucleus circuits.

Most studies on the reticular formation of lampreys have emphasized the distribution of large reticular cells, but other studies have also shown some small neurons, some of which are GABA-ir (Meléndez-Ferro, 2001; Meléndez-Ferro et al., 2003; Robertson et al., 2007), located in both medial and lateral reticular regions. The large reticulospinal neurons of the lamprey rhombencephalon exhibit a

segmental pattern from embryonic stages (Murakami et al., 2004), and a segmental pattern can be also observed in the distribution of glycine-ir cells of adults. To compare the reticular glycine-ir cells of adult lamprey with other species, we found the classification of the medial reticular formation into four regions-isthmic, trigeminal, octaval and posterior (Stefanelli, 1934)-useful. This appears better suited to study the organization of glycine-ir populations than the generally used classification into three rhombencephalic reticular nuclei, anterior (ARRN), middle (MRRN) and posterior (PRRN) (Nieuwenhuys, 1972). The segmental correspondence of Stefanelli's regions is: isthmic (rhombomere 1; ARRN), trigeminal (rhombomeres 2 and 3; includes I3 and I4 Müller cells; includes part of the MRRN), middle or octaval (rhombomeres 4 and 5; rhombomere 4 includes the largest cells of the MRRN and the anterior and posterior Mauthner cells), and posterior (rhombomeres 6 and 7; coincides with the PRRN).

With respect to the distribution of glycine-ir reticular cells, the isthmic region was clearly different from the trigeminal region in that it exhibits a large glycine-ir reticular population with conspicuous cells with dendrites perpendicular to main longitudinal tracts. As observed in sagittal sections, these cells may give rise to axons coursing both rostrally and caudally, which suggests that this population is a source of a number of putative glycinergic fibers in various brain regions. The glycine-ir population of the lamprey isthmic reticular formation may correspond to the glycinergic population of the oral part of the pontine reticular nucleus described in rats (Rampon et al., 1996; Tanaka and Ezure, 2004). Because this reticular nucleus is close to the midbrain, it is not surprising that it was referred to as mesencephalic in studies in which the isthmic Müller cell has often been used as a marker of the region (Antri et al., 2006). However, careful morphological studies in adult and developing lamprey have revealed that the midbrain-hindbrain boundary becomes an oblique plane and that, in "transverse brain sections", the caudal midbrain covers the wedge-shaped rostral portion of the rhombencephalon (Pombal and Puelles, 1999; Meléndez-Ferro et al., 2003; Abalo et al., 2007). It is important to mention this because the region referred to in recent studies as the "mesencephalic locomotor region" (Ménard et al., 2007), in fact corresponds to this rostral region of the lamprey hindbrain. It is this hindbrain region that contains large populations of serotonin-ir (Abalo et al., 2007), GABA-ir (Meléndez-Ferro, 2001; Meléndez-Ferro et al., 2003)

and glycine-ir (present results) neurons, not the mesencephalic tegmentum. Many of the glycine-ir cells of this region also colocalize GABA. In addition to its own GABA-ir and/or glycine-ir neurons, this region, which is important in terms of generating locomotion, also receives descending GABA-ir projections from some telencephalic and diencephalic regions (Ménard et al., 2007). Injection of the GABA antagonist Gabazine in this region elicits body movements, indicating that it is under tonic inhibition (Ménard et al., 2007). Whether glycine accomplishes a similar role in the rostral hindbrain is not known.

The number of putative glycinergic reticular cells in the trigeminal region is low, and they are located near the midline in a parvocellular region that also shows some serotoninergic cells (Abalo et al., 2007). The number of glycine-ir cells is much higher in the middle rhombencephalic reticular region than in the trigeminal reticular region, and the cells, some of them rather large, are characteristically distributed in both periventricular and more ventral regions. The largest glycine-ir cells are associated with the Mauthner neuron. Notably, this reticular region shows numerous glycine-ir commissural fibers, including rather thick fibers, which appears to be specific of this rhombencephalic region. The pattern of distribution of glycine-ir cells in the posterior reticular formation is different, and most are located in a periventricular location among larger glycine-negative reticular cells. Putative glycinergic neurons have also been reported in several reticular nuclei of plethodontid salamanders (Landwehr and Dicke, 2005) and the rat (Rampon et al., 1996; Tanaka and Ezure, 2004), which indicates that they are conserved through evolution. As in lamprey, colocalization of GABA and glycine was observed in some cells of this region in salamanders (Landwehr and Dicke, 2005).

Several experimental studies have reported the important roles of the reticular regions in the generation of locomotor patterns in lampreys, with symmetrical or asymmetrical alternative contraction of the two sides of the body (see Grillner and Wallén, 2002). Although most known lamprey reticulospinal neurons have excitatory effects on spinal motoneurons and interneurons (Ohta and Grillner, 1989; Brodin et al., 1989), some reticulospinal neurons act monosynaptically on spinal neurons via glycinergic transmission (Wannier et al., 1995). The presence of glycine-ir cells in all reticular nuclei, together with the abundance of small reticulospinal neurons (McClellan et al., 2006), provide a possible morphological substrate for these results,

but experimental studies with tracers are required to reveal the location of cells that project to the spinal cord. Many glycine-ir reticular cells may be involved in the generation of locomotor patterns via ipsilateral and/or contralateral connections with other reticular cells, as suggested by the numerous glycine-ir fibers observed (including commissural fibers), and may be a substrate for coordination of the different reticular nuclei. Dorsal column nucleus and trigeminal inhibitory inputs to reticulospinal neurons were mediated by glycine (Dubuc et al., 1993a; Viana di Prisco et al., 1995). The presence of a variety of GABA-ir (Meléndez-Ferro, 2001; Meléndez-Ferro et al., 2003; Robertson et al., 2007) and glycine-ir (present results) populations in the lamprey reticular formation, together with the presence of glycine-ir and/or GABA-ir cells in several brain centers that project to it, including the dorsal column nucleus, octavolateral nuclei, trigeminal descending nucleus, torus semicircularis, and optic tectum (González et al., 1997; Zompa and Dubuc, 1998; Pflieger and Dubuc, 2004; Viana di Prisco et al., 2005), suggests that inhibitory circuits in this region are complex. The relative importance and differential functions of GABA and glycine in the generation of patterns of body movements in different aspects such as its initiation and termination, vigour of locomotion, and steering and equilibrium control, must be investigated.

Adult sea lampreys attach to other fish and feed by rhythmical coordinated movements of a sucker and a movable tongue-like structure called the apicalis. These specialized feeding mechanisms utilize a complex and unique group of muscles that control the sucker, the apicalis, pharynx, and velum (see Hardisty and Rovainen, 1982). All these muscles are innervated by branches of the trigeminal nerve. Cobalt-lysine application to the trigeminal motor nucleus has revealed a densely packed column of labeled neurons medial to this nucleus on the ipsilateral side, extending further rostrally in the isthmic region. More interestingly for the present discussion, continuous columns of labeled cells were observed in the lateral reticular formation on each side ventral to rhombencephalic cranial motor nuclei (Huard et al., 1999). The shape (spindle-shaped, tripolar) and location of these retrogradely labeled cells are notably similar to that of glycine-ir cells observed in the same regions, suggesting that some of these glycine-ir cells may be involved in coordination of feeding movements.

The facial, glossopharyngeal and vagal motor nuclei innervate the gills in a segmental manner, and characteristically the vagal motoneurons were larger that those of the facial and glossopharyngeal nerves (Guimond et al., 2003). Respiratory rhythm generation is complex, and the region of the trigeminal motor nucleus appears involved in its regulation (Rovainen, 1983). Recent physiological studies suggest that rhythmic respiratory changes occur during locomotion and that some change is programmed to adjust ventilation prior to motor activity (Gravel et al., 2007). Pharmacological studies suggest that GABA- and glycine-mediated inhibition is not essential for respiratory rhythm generation in the adult lamprey, although it appears to exert potent influences on respiratory activity and to have a role in maintaining a stable and regular breathing pattern (Rovainen, 1983; Bongianni et al., 2006). The close association of many glycine-ir cells in the trigeminal-pretrigeminal regions involved in burst activity prior to respiratory generation (Rovainen, 1983) might also be involved in the regulation of respiratory activity. Together, these results suggest that rhombencephalic glycinergic cells are involved in coordination of a number of basic visceromotor circuits.

Significance of colocalization of glycine and GABA

From present double immunofluorescence experiments, glycine-ir and GABA-ir neurons of lamprey brain appear to represent largely separate populations, except in a few locations. In this regard, the present results are similar to those reported in the hindbrain of zebrafish embryos and 4-5 days postfertilization larvae using *in situ* hybridization with RNA probes complementary to the neuronal glycine transporter GLYT2 and glutamate decarboxylase (GAD) (Higashijima et al., 2004). However, in nuclei such as the reticular isthmic nucleus, a proportion of glycine-ir cells was also GABA-ir, suggesting the possibility that these neurons release both glycine and GABA in synapses. The release of GABA at these glycinergic synapses would increase the glycine receptor channel activation, as experiments with zebrafish glycine receptors suggest (David-Watine et al., 1999; Imboden et al., 2001), but whether these synapses behave differently than those releasing glycine alone is not known. In addition, glycine is a modulator coagonist of the NMDA glutamate receptor (Wood, 2005). In lamprey, NMDA has been widely used to elicit fictive locomotion in isolated spinal cord preparations (Brodin et al., 1985). Moreover, reticulospinal neurons of the lamprey reticular formation are directly responsive to

bath NMDA application, which produces a long-lasting depolarizing plateaus accompanied by Ca^{2+} entry into the cell (Viana Di Prisco et al., 1997). However, the possible interaction of glycine with lamprey NMDA glutamate receptors is not known.

Final considerations

The present results in the lamprey reveal that the distribution of putative glycinergic cells in the brain has varied significantly from the first vertebrates to mammals. Evolutionary changes affected brain populations differently, because of severe reduction of putative glycinergic cell groups in rostral brain regions of mammals and a notable specialization of cell groups in the hindbrain. Likewise, important changes involving specialization of the glycinergic innervation pattern have occurred between lampreys and mammals. In most populations containing both GABA-ir and glycine-ir neurons, glycinergic neurons represent a separate population, although colocalization has been observed in some populations. Despite the advance that this study represents in knowledge of the anatomical organization of the putative glycinergic system in the earliest vertebrates, there is a wide gap regarding its evolution and organization in other vertebrate groups.

Abbreviations

DCN	dorsal column nucleus
DIG	dorsal isthmic grey
DIsC	dorsal isthmic commissure
dl	dorsolateral DIG population
dm	dorsomedial DIG population
DN	dorsal nucleus of the octavolateral area
dV	descending root of the trigeminal nerve
fr	fasciculus retroflexus
GL	glomeruli
Ha	habenula
HY	hypothalamus
IGL	inner granular layer
IIIi	intermediate (dorsal rectus) oculomotor subnucleus
IIIl	lateral (rostral rectus) oculomotor subnucleus
IIId	dorsomedial (rostral oblique) oculomotor subnucleus
IP	interpeduncular nucleus
Is	isthmus
IsRF	isthmic reticular formation
IV	trochlear nucleus
LP	lateral pallium
M	mesencephalon
M1-4	Müller cell 1-4
M5	Schober's M5 nucleus
MN	medial nucleus of the octavolateral area
MO	medulla
MP	medial pallium
MRF	middle rhombencephalic reticular formation
Mth	Mauthner cell
NH	neurohypophysis
OB	olfactory bulb
OLA	octavolateral area
ON	optic nerve
OT	optic tectum
P	pineal complex
PO	preoptic nucleus
PoC	postoptic commissure
PRF	posterior rhombencephalic reticular formation
PT	pretectal region
PTh	prethalamus (ventral thalamus)
PTN	paratubercular nucleus
SC	spinal cord
ShL	subhippocampal lobe
sl	sulcus limitans
SOC	spino-occipital motor column
SP	septum
ST	striatum
STN	solitary tract nucleus
Th	thalamus (dorsal thalamus)
TRF	trigeminal reticular formation
TS	torus semicircularis
VId	dorsal (ventral rectus) abducens subnucleus
VIIm	facial motor nucleus
VIv	ventral (caudal rectus) abducens subnucleus
Vm	trigeminal motor nucleus
VN	ventral nucleus of the octavolateral area
Xm	vagal motor nucleus

F**ig. 1.** Schematic drawing of transverse sections of the adult sea lamprey forebrain and midbrain showing the distribution of glycine immunoreactive cells and fibers (at right), and main brain structures (left). The level of sections is indicated in the upper right figure. The size of glycinergic cells was doubled for better visualization. Arrow in B points to a field with very coarsely beaded fibers. Correspondence with photomicrographs in other figures is indicated by squared areas. For abbreviations, see list. Scale bar = 50 μm.

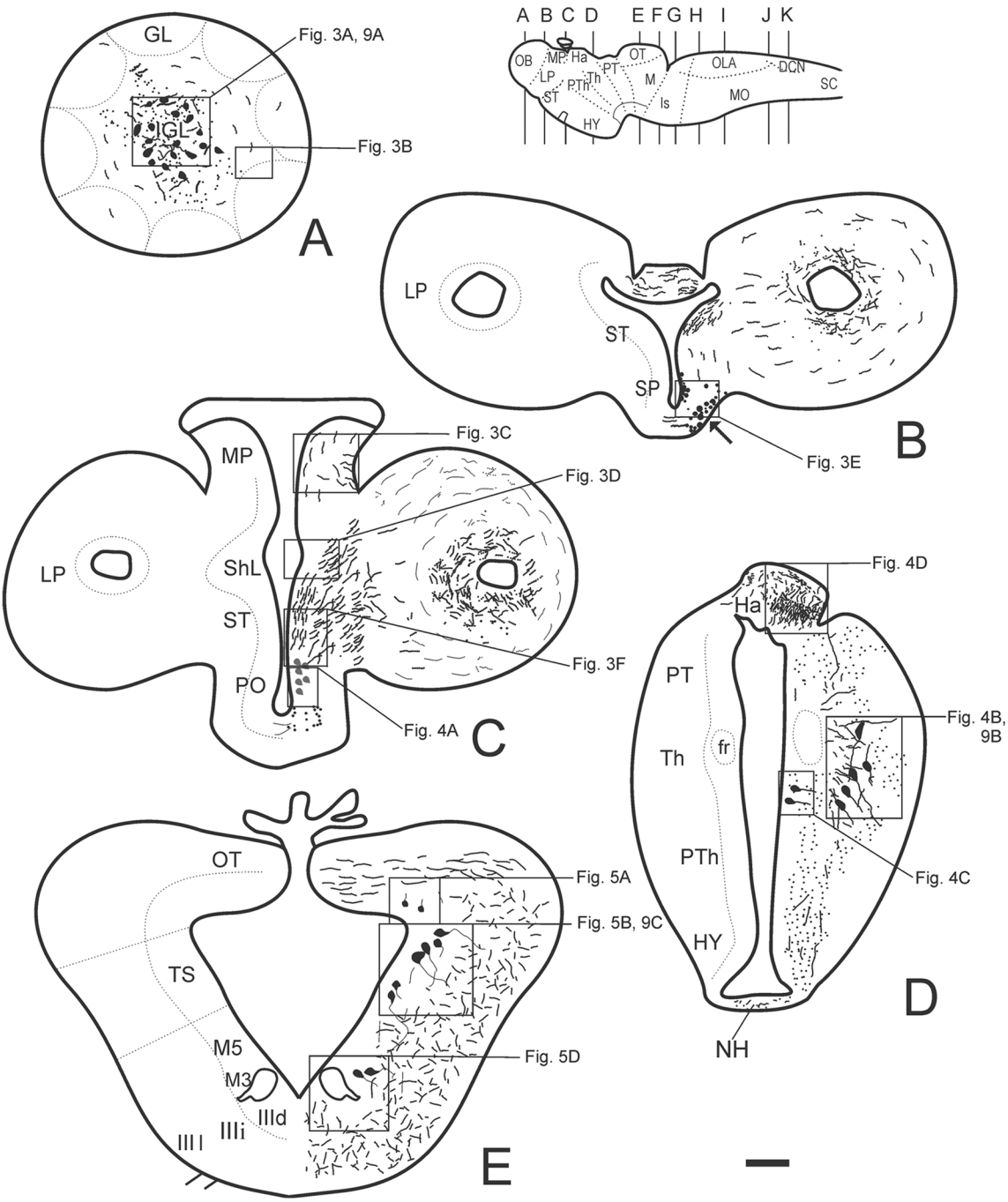

A B C D E F G H I J K
OB MP Ha OT
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Fig. 3A, 9A
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Fig. 3E
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Fig. 3C
Fig. 3D
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Fig. 3F
Fig. 4A
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Fig. 4D
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Fig. 4B, 9B
Fig. 4C
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Fig. 5A
Fig. 5B, 9C
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M3
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IIIi
III l
Fig. 5D
E

Fig.1.

Fig. 1. (continuation). Schematic drawing of transverse sections of the adult sea lamprey hindbrain showing the distribution of glycine immunoreactive cells and fibers (at right), and main brain structures (left). The level of sections is indicated in the upper right figure. The size of glycinergic cells was doubled for better visualization. Correspondence with photomicrographs in other figures is indicated by squared areas. For abbreviations, see list. Scale bar = 50 μm.

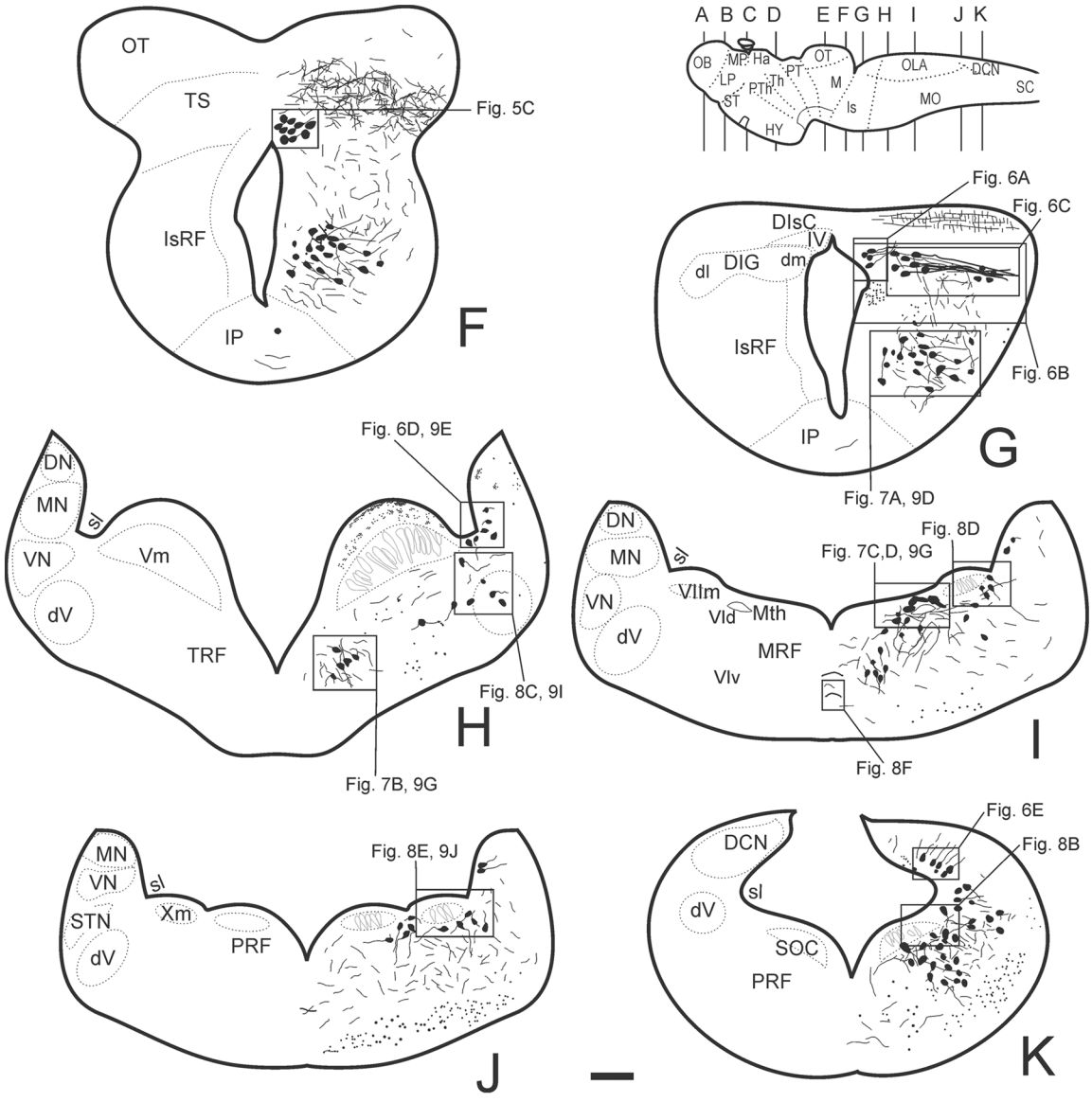
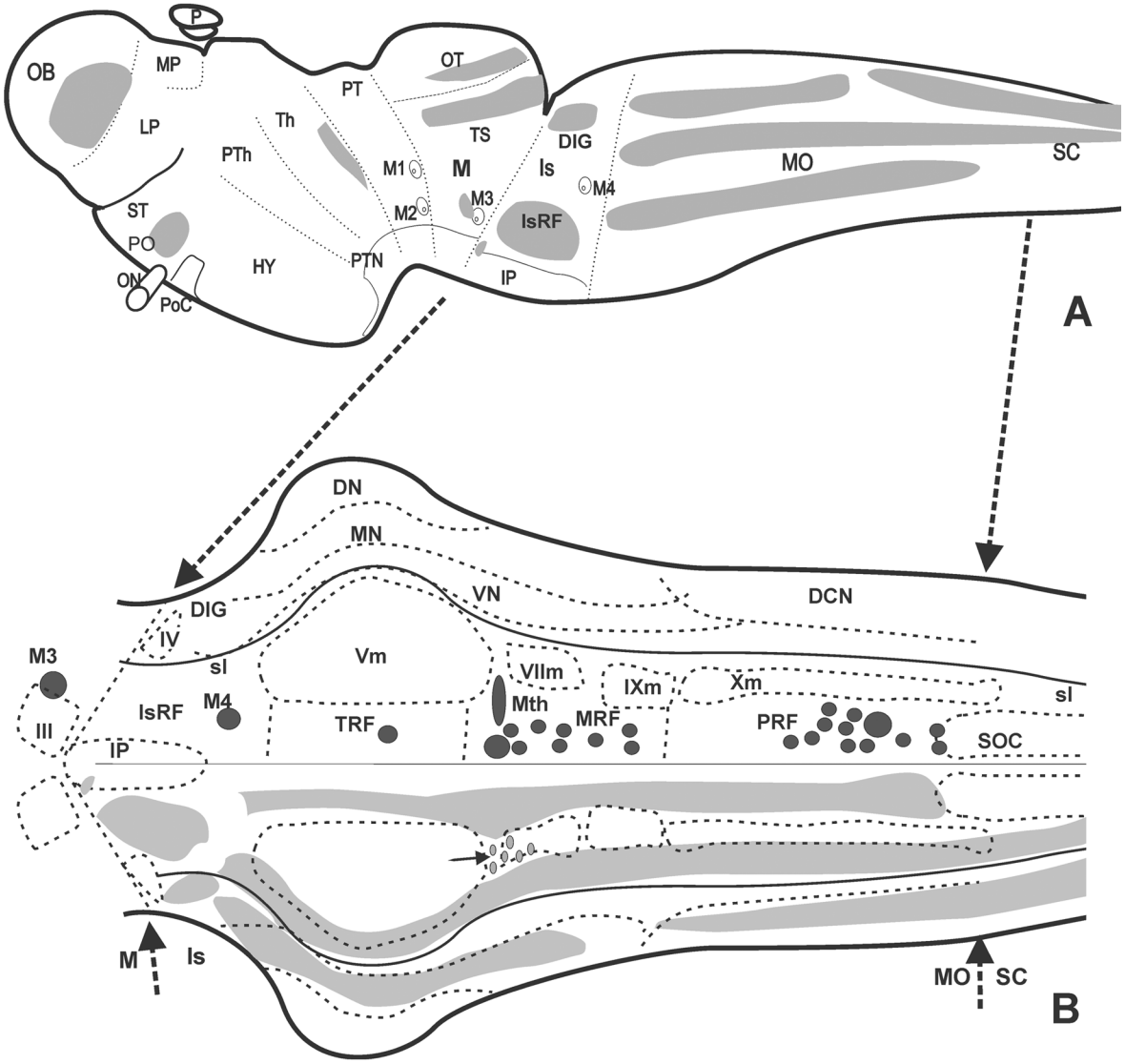


Fig.1. (continuation)

Fig. 2. A, Schematic lateral view of the brain showing the distribution of glycine-immunoreactive neuronal populations in the forebrain, midbrain, and hindbrain. **B,** Schematic drawing of a dorsal view of a projection of the rhombencephalon showing the distribution of the main glycine-immunoreactive populations (lower half). Solid arrow points to large glycinergic cells associated with the Mauthner neuron. Main nuclei and regions, as well as some large reticulospinal cells (in black) are represented in the upper half of the figure. Dashed arrows indicate the midbrain–hindbrain and hindbrain–spinal cord boundaries. The dorsal projection was adapted from Nieuwenhuys (1972).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IXm
Xm
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M4
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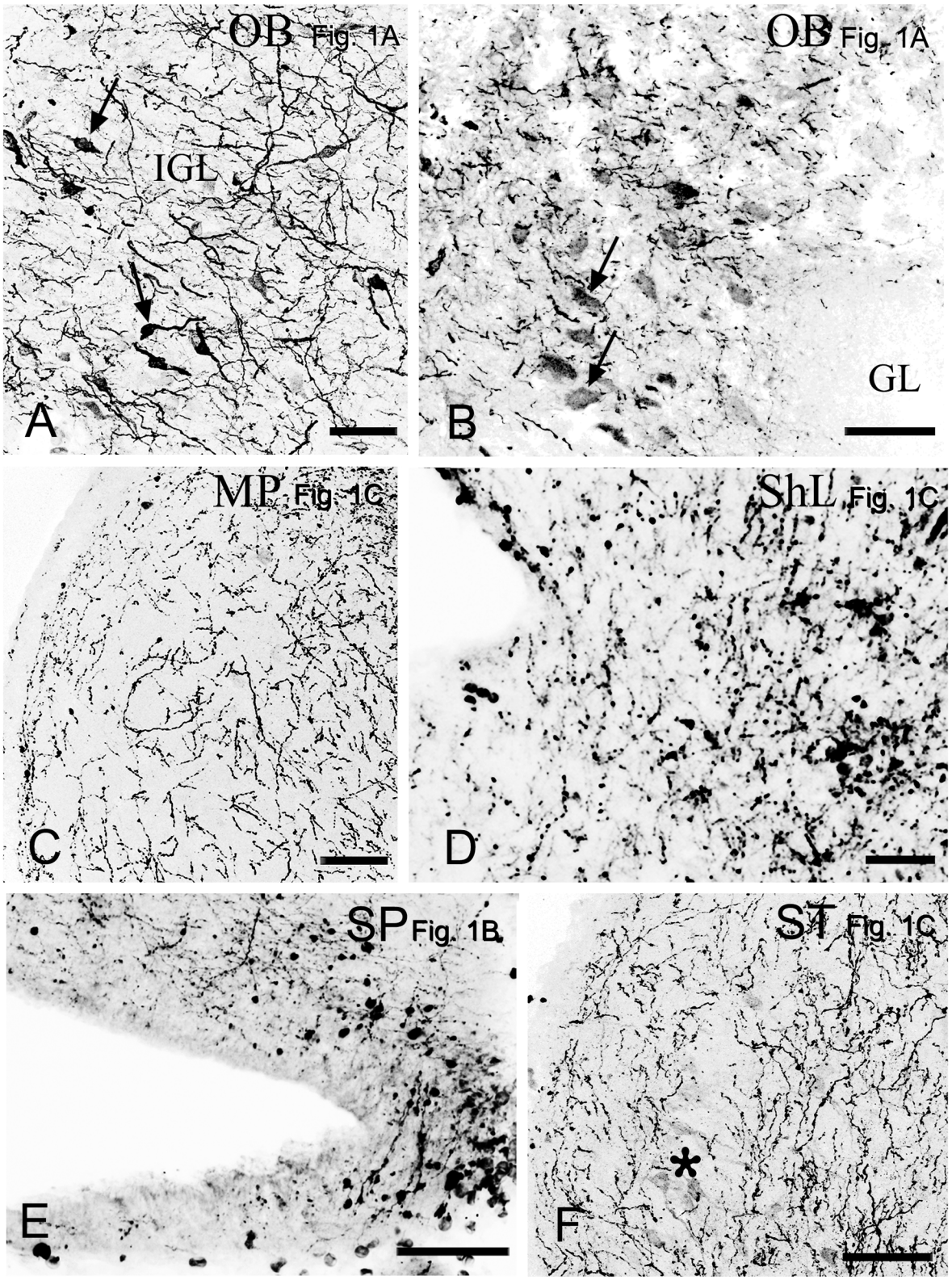
Fig.2.

Fig. 3. Inverted greyscale photomicrographs of transverse sections of the lamprey forebrain showing glycine-ir structures. **A**, Section of the olfactory bulb showing glycine-ir cells (arrows) and processes in the granular layer. **B**, Section of the olfactory bulb showing faint glycine-ir mitral cells (arrows). Asterisk, olfactory glomerulus. **C**, Section showing glycine-ir fibers in the medial pallium. **D**, Section of the subhippocampal lobe showing strongly glycine-ir fibers. **E**, Section of the septum/terminal lamina showing strongly glycine-ir, coarse beaded fibers. **F**, Section of the striatum showing abundant glycine-ir fibers. A part of the neighbour magnocellular preoptic nucleus is also shown (asterisk). For abbreviations, see list. Correspondence with schemes of figure 1 is indicated in the upper right corner. A-C and F are confocal micrographs; D and E are fluorescence photomicrographs. Scale bars = 50 μm in A-C,E and F; 25 μm in D.


OB Fig. 1A
IGL
A
OB Fig. 1A
GL
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MP Fig. 1C
C
ShL Fig. 1C
D
SP Fig. 1B
E
ST Fig. 1C
*
F

Fig.3.

Fig. 4. Inverted greyscale confocal photomicrographs of transverse sections of the preoptic nucleus (A), thalamus (B, C) and habenula (D) showing glycine-ir structures. **A**, Section showing faint glycine-ir neurons in the cell layer of the magnocellular preoptic nucleus (arrows) and numerous glycine-ir fibers in subependymal and lateral regions. **B**, Section showing strong glycine-ir cells of the lateral region of the thalamus (arrows). Note also thin positive fibers. **C**, Section showing glycine-ir neurons (arrows) in the periventricular region of the thalamus. Note the dense subventricular mat of glycine-ir processes and the abundance of fibers in regions lateral to the cell rows. **D**, Section of the right habenula showing the dense glycine-ir innervation. Note coarse beaded positive fibers in intermediate regions (arrows). For abbreviations, see list. Correspondence with schemes of figure 1 is indicated in the upper right corner. Scale bars = 50 μm.

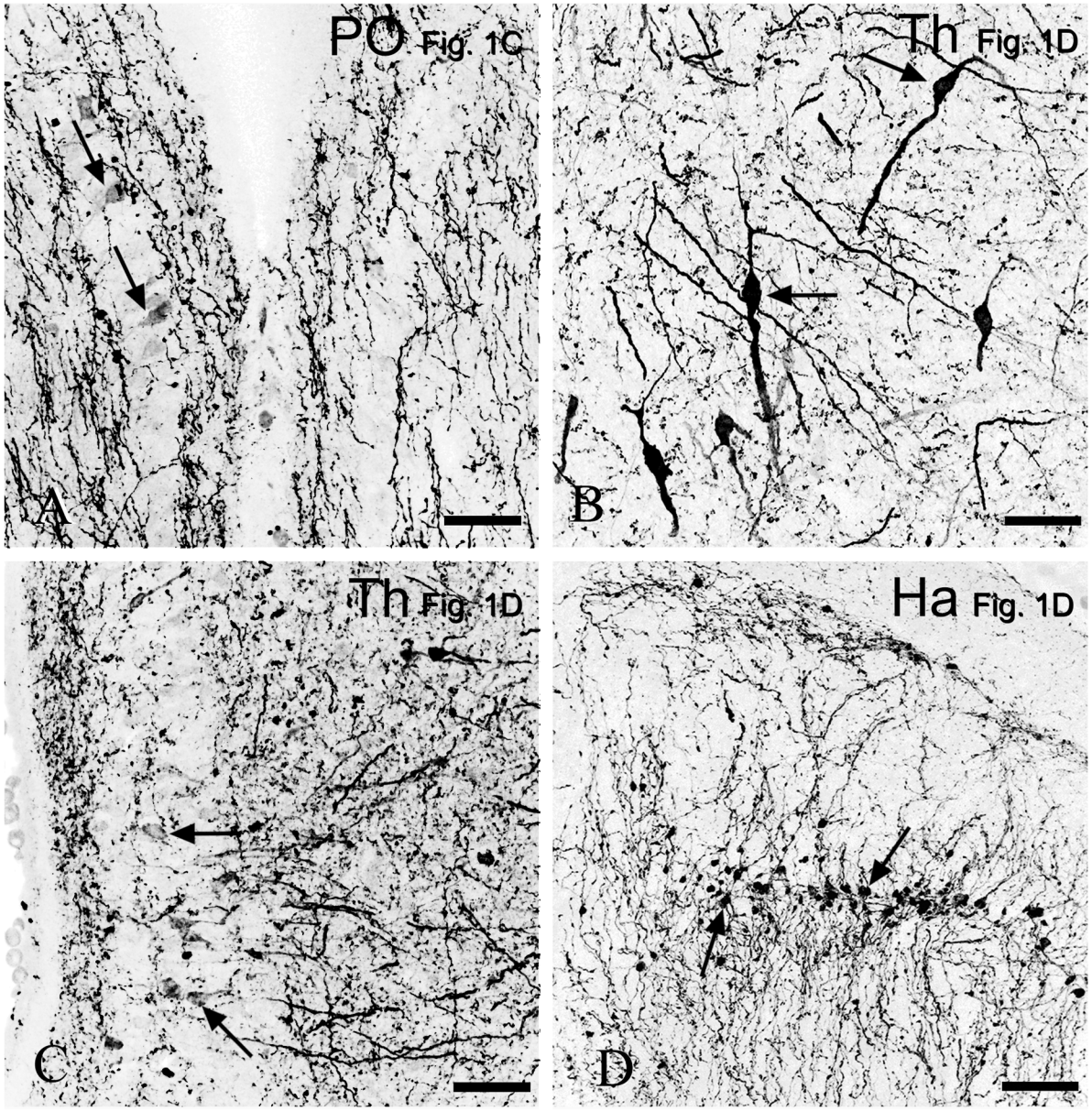


Fig.4.

Fig. 5. Inverted greyscale confocal photomicrographs of transverse sections through the mesencephalon showing glycine-ir structures. **A**, Section through the lateral region of the optic tectum showing small glycine-ir cells (arrow) in the stratum cellulare periventriculare. Note also the abundance of glycine-ir fibers. **B**, Section through the torus semicircularis showing strong glycine-ir neurons (arrows) and processes. **C,** Section through the most caudal region of the tectum mesencephali showing the caudal part of the glycine-ir toral population. **D**, Section at the level of the M3 Müller cell (asterisk) showing glycine-ir cells in the M5 nucleus (arrows). Star, dorsomedial oculomotor subnucleus. For abbreviations, see list. Correspondence with schemes of figure 1 is indicated in the upper right corner. Scale bars = 50 μm.

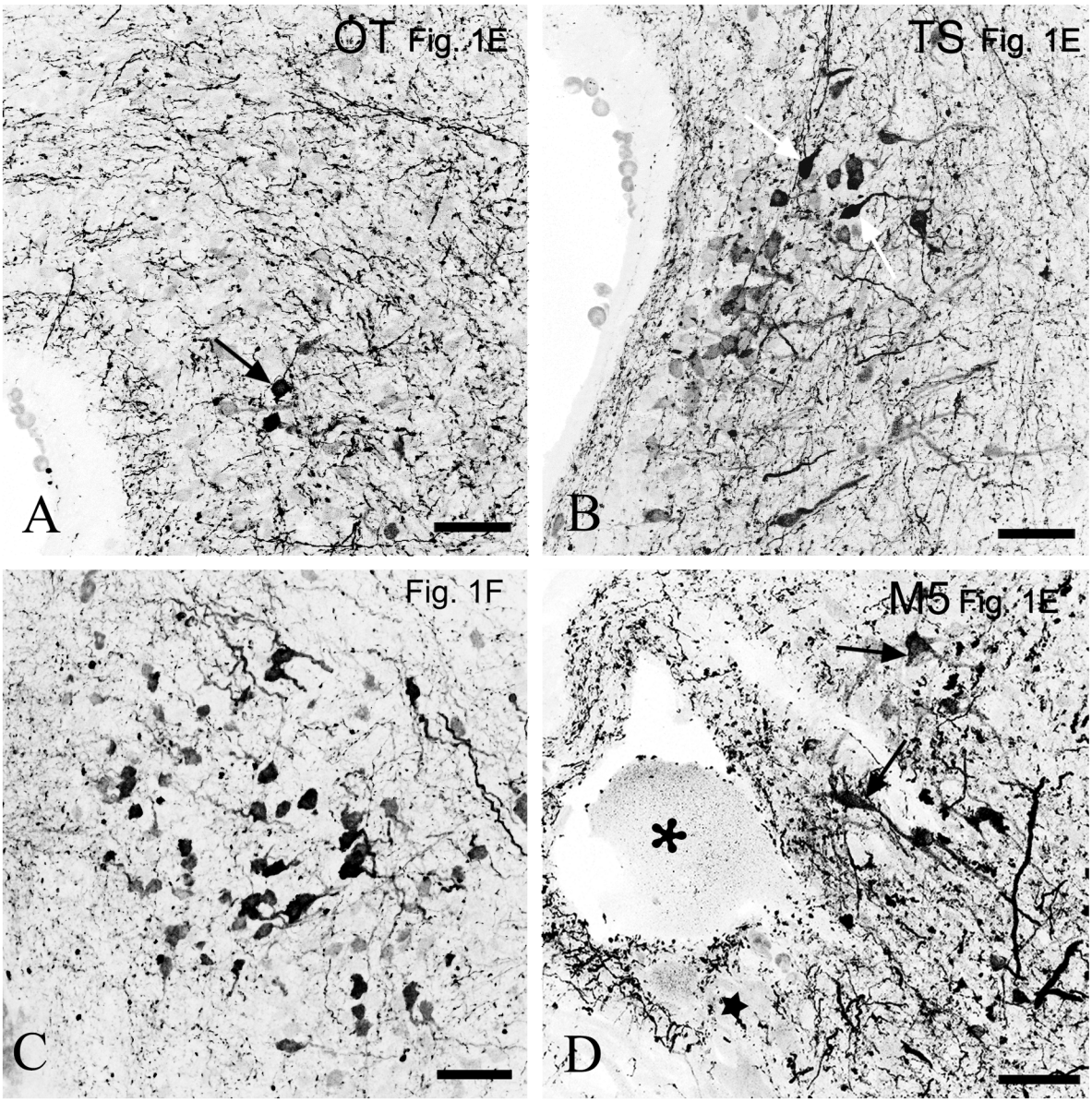


Fig.5.

Fig. 6. Inverted greyscale photomicrographs of transverse sections through the alar plate of the rhombencephalon showing strongly glycine-ir cells. **A**, Section through the medial region of the dorsal isthmus showing a dorsomedial population of small glycine-ir cells. Note the abundance of processes in the cerebellar plate region (top). **B**, Section showing the conspicuous dorsolateral population of strong glycine-ir cells of the dorsal isthmic grey with long and thick lateral dendrites. Asterisk, large glycine-negative reticular cells. Black arrow, negative fibers of the anterior octavomotor tract. **C**, Detail of the dorsolateral glycine-ir population of the dorsal isthmic grey. **D**, Section showing strong glycine-ir neurons (arrows) in the medial nucleus of the octavolateral area. **E**, Detail of the strong glycine-ir neurons of the dorsal column nucleus. For abbreviations, see list. Correspondence with schemes of figure 1 is indicated in the upper right corner. A and B are fluorescence photomicrographs, C-E are confocal photomicrographs. Scale bars = 50 μm in A and C, 100 μm in B, 25 μm in D and E.

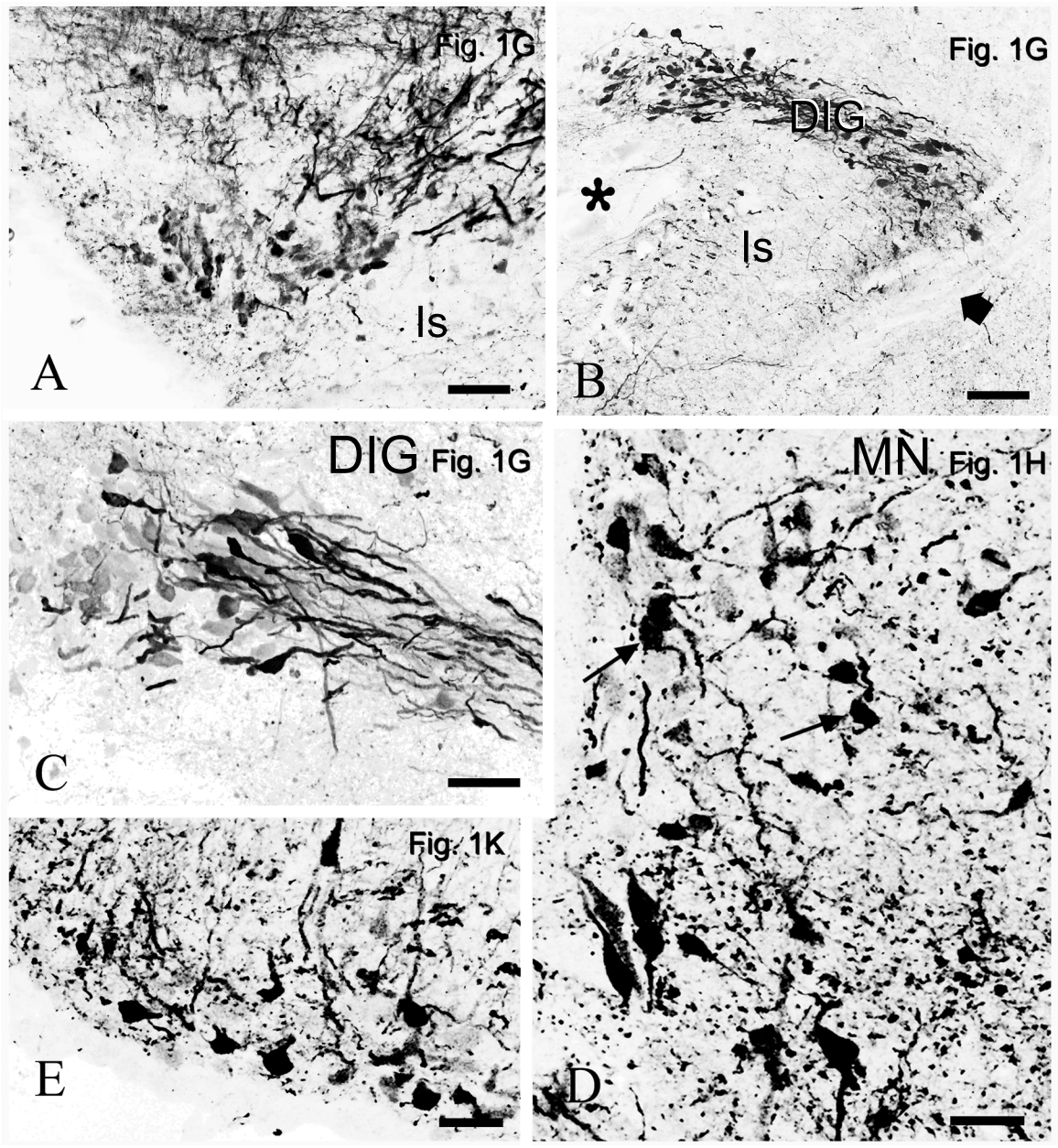


Fig.6.

Fig. 7. Inverted greyscale confocal photomicrographs of transverse sections through the medial zone of the reticular formation showing glycine-ir reticular cells. **A**, Section showing glycine-ir cells of the isthmic reticular formation. **B**, Section showing glycine-ir cells of the trigeminal levels of the reticular formation. **C,D**, Section showing strong glycine-ir large cells of the middle rhombencephalic reticular formation associated with the Mauthner cell (asterisk in C). Note their thick branched dendrites. Arrow in D, small glycine-ir neuron. For abbreviations, see list. Correspondence with schemes of figure 1 is indicated in the upper right corner. Scale bars = 50 μm.

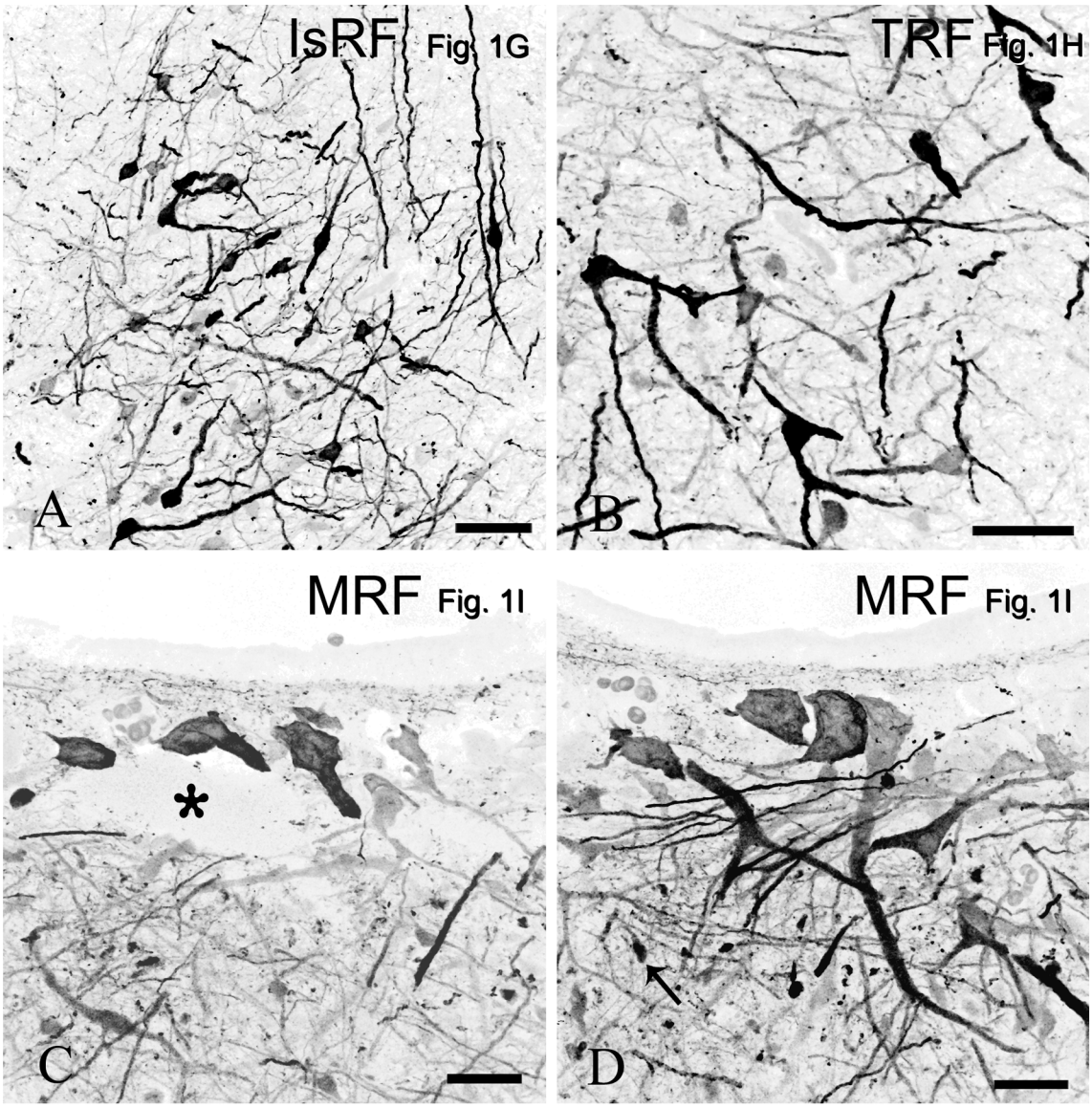


Fig.7.

Fig. 8. Inverted greyscale confocal photomicrographs of transverse sections through basal regions of the rhombencephalon showing glycine-ir cells and fibers. **A**, Section showing ventrally migrated glycine-ir cells in the region of the abducens nucleus. **B**, Section showing glycine-ir neurons (arrows) lateral to the spino-occipital somatomotor column (asterisk). Note the absence of glycine-ir cells in medial regions. **C**, Section showing a group of glycine-ir cells located just lateral to the trigeminal motor nucleus (asterisk). Note also the abundance of glycine-ir fibers in this region. **D**, Sections showing abundant glycine-ir reticular cells associated with the facial motor nucleus (asterisk). **E**, Section through the vagal motor nucleus (asterisk) showing numerous glycine-ir reticular cells. **F**, Section through the midline raphe at the level of the middle rhombencephalic reticular formation showing thick strongly glycine-ir commissural fibers (arrow). For abbreviations, see list. Correspondence with schemes of figure 1 is indicated in the upper right corner except in A, which corresponds to a level intermediate between schemes 1I and 1J. Scale bars = 50 μm.

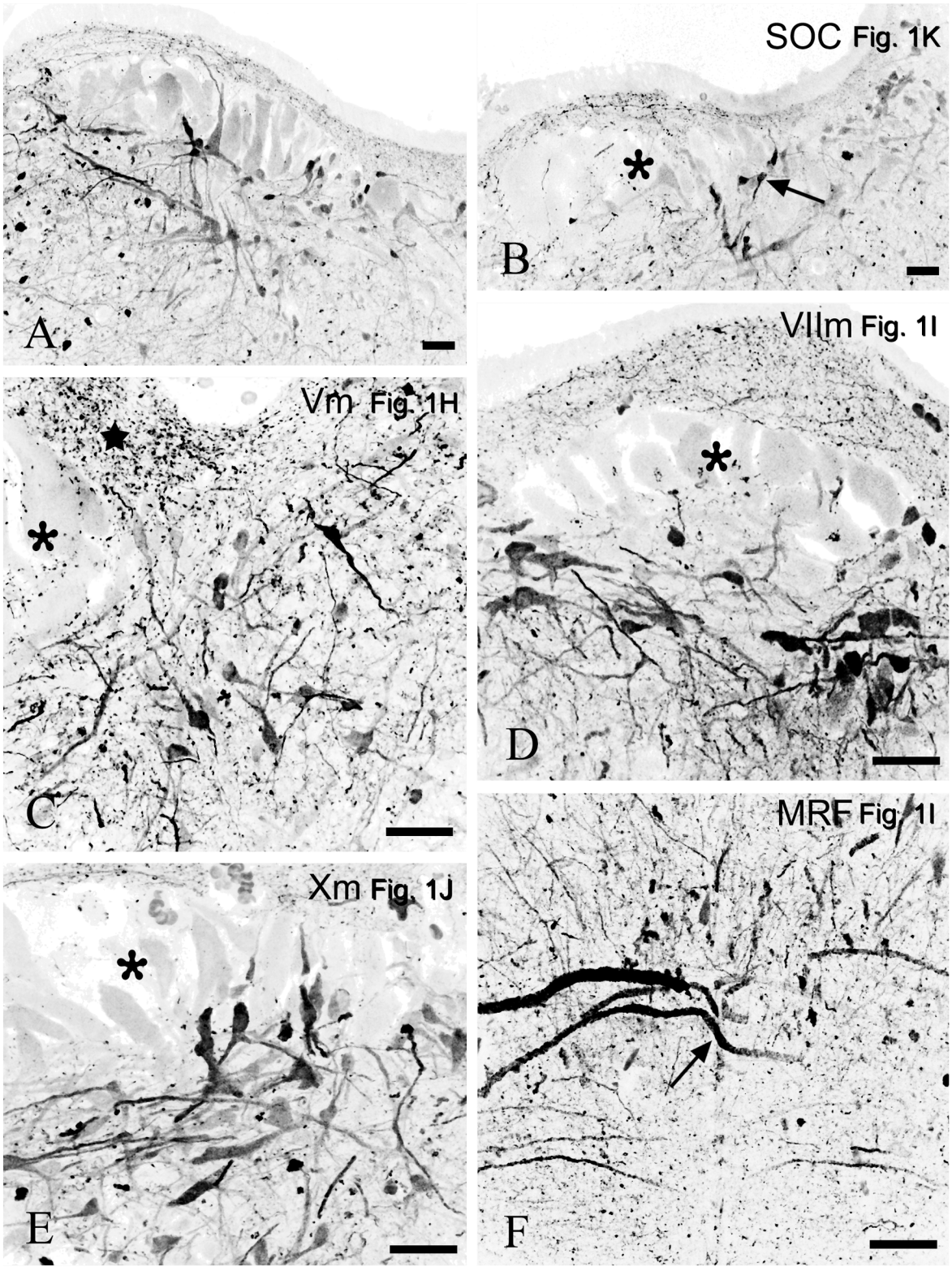
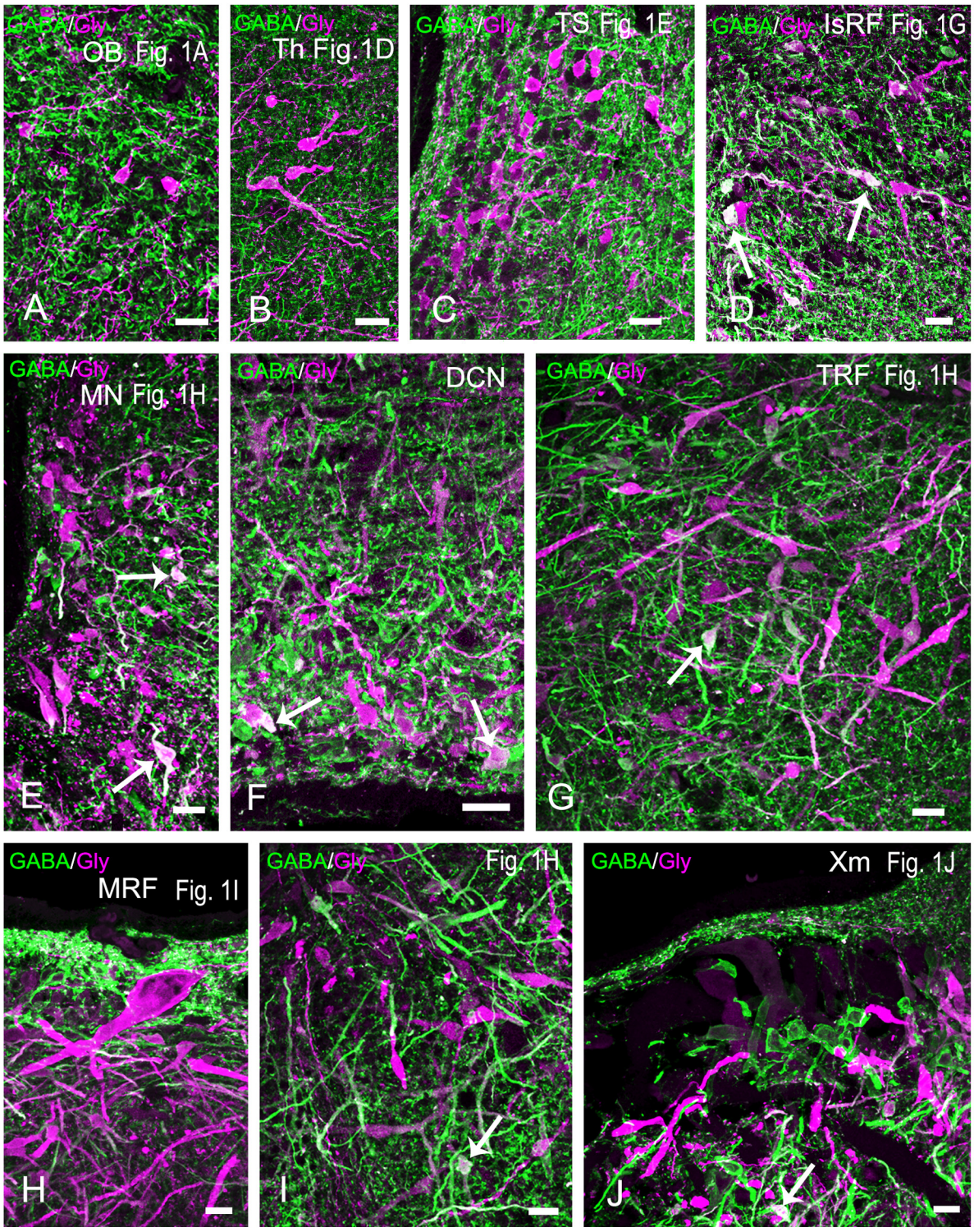


Fig.8.

Fig. 9. Confocal photomicrographs of double immunolabelled sections showing glycine (Gly)-ir (magenta) and GABA-ir (green) structures in several brain regions. Note that in most regions colocalization of GABA and glycine (white structures) is either absent (A-C, H) or present in a few cells (arrows in E-G, I-J), whereas only the isthmic reticular population shows a high proportion of colocalization (D). **A**, Olfactory bulb. **B**, Thalamus. **C**, Torus semicircularis. **D**, Isthmic reticular formation **E**, Medial nucleus of the octavolateral area. **F**, Dorsal column nucleus. **G**, Trigeminal reticular formation. **H**, Middle rhombencephalic reticular formation. Note the large glycine-ir cell. **I**, Reticular cells associated with the trigeminal motor nucleus. **J,** Reticular cells associated with the vagal motor nucleus. Correspondence with other figures is indicated in the upper right corner. Scale bars = 25 μm.


GABA/Gly
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GABA/Gly
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IsRF Fig. 1G
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MN Fig. 1H
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G
GABA/Gly
MRF Fig. 1I
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Fig. 1H
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GABA/Gly
Xm Fig. 1J
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Fig.9.

REFERENCES

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

Alford S, Williams TL. 1989. Endogenous activation of glycine and NMDA receptors in lamprey spinal cord during fictive locomotion. J Neurosci 9:2792-2800.

Alford S, Sigvardt KA, Williams TL. 1990a. GABAergic control of rhythmic activity in the presence of strychnine in the lamprey spinal cord. Brain Res 506:303-306.

Alford S, Williams TL, Sigvardt KA. 1990b. Effects of bicuculline and strychnine on synaptic inputs to edge cells during fictive locomotion. Brain Res 509:137-140.

Álvarez-Otero R, Pérez SE, Rodríguez MA, Anadón R. 1996. Organization of the cerebellar nucleus of the dogfish, *Scyliorhinus canicula* L.: A light microscopic, immunocytochemical and ultrastructural study. J Comp Neurol 368 :487-502.

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

Aoki E, Semba R, Keino H, Kato K, Kashiwamata S. 1988. Glycine-like immunoreactivity in the rat auditory pathway. Brain Res 442:63-71.

Baev KV, Rusin KI, Safronov BV. 1992. Primary receptor for inhibitory transmitters in lamprey spinal cord neurons. Neuroscience 46:931-941.

Barker JL, Ransom BR. 1978. Amino acid pharmacology of mammalian central neurones grown in tissue culture. J Physiol 280:331-354.

Bäurle J, Grüsser-Cornehls U. 1997. Differential number of glycine- and GABA-immunopositive neurons and terminals in the deep cerebellar nuclei of normal and Purkinje cell degeneration mutant mice. J Comp Neurol 382:443-458.

Betz H. 1987. Biology and structure of the mammalian glycine receptor. Trends Neurosci 10:113-117.

Birinyi A, Parker D, Antal M, Shupliakov O. 2001. Zinc co-localizes with GABA and glycine in synapses in the lamprey spinal cord. J Comp Neurol 433:208-221.

Bongianni F, Mutolo D, Nardone F, Pantaleo T. 2006. GABAergic and glycinergic inhibitory mechanisms in the lamprey respiratory control. Brain Res 1090:134-145.

Bormann J, Hamill OP, Sakmann B. 1987. Mechanism of anion permeation through channels gated by glycine and gamma-aminobutyric acid in mouse cultured spinal neurones. J Physiol 385:243-286.

Brodin L, Grillner S, Rovainen CM. 1985. N-Methyl-D-aspartate (NMDA), kainate and quisqualate receptors and the generation of fictive locomotion in the lamprey spinal cord. Brain Res 325:302-306.

Brodin L, Ohta Y, Hökfelt T, Grillner S. 1989. Further evidence for excitatory amino acid transmission in lamprey reticulospinal neurons: selective retrograde labeling with (^{3}H)D-aspartate. J Comp Neurol 281:225-233.

Buchanan JT, Grillner S. 1988. A new class of small inhibitory interneurones in the lamprey spinal cord. Brain Res 438:404-407.

Bullock TH, Moore JK, Fields RD. 1984. Evolution of myelin sheaths: both lamprey and hagfish lack myelin. Neurosci Lett 48:145-148.

Campistron G, Buijs RM, Geffard M. 1986. Glycine neurons in the brain and spinal cord. Antibody production and immunocytochemical localization. Brain Res 376:400-405.

Cubelos B, Giménez C, Zafra F. 2005. Localization of the glycineT1 glycine transporter at glutamatergic synapses in the rat brain. Cereb Cortex 15:448-459.

Dale N, Ottersen OP, Roberts A, Storm-Mathisen J. 1986. Inhibitory neurones of a motor pattern generator in Xenopus revealed by antibodies to glycine. Nature 324:255-257.

David-Watine B, Goblet C, de Saint Jan D, Fucile S, Devignot V, Bregestovski P, Korn H. 1999. Cloning, expression and electrophysiological characterization of glycine receptor alpha subunit from zebrafish. Neuroscience 90:303-317.

De Arriba Pérez, MC. 2007. Conexiones del techo óptico. Organización y desarrollo en lamprea y expresión de genes implicados en la vía retinotectal en pez cebra. PhD Dissertation, University of Vigo.

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

Dubuc R, Bongianni F, Ohta Y, Grillner S. 1993a. Dorsal root and dorsal column mediated synaptic inputs to reticulospinal neurons in lampreys: involvement of glutamatergic, glycinergic, and GABAergic transmission. J Comp Neurol 327:251-259.

Dubuc R, Bongianni F, Ohta Y, Grillner S. 1993b. Anatomical and physiological study of brainstem nuclei relaying dorsal column inputs in lampreys. J Comp Neurol 327:260-270.

Fort P, Luppi PH, Wenthold R, Jouvet M. 1990. Glycine immunoreactive neurons in the medulla oblongata in cats. C R Acad Sci 311:205-212.

Fort P, Luppi PH, Jouvet M. 1993. Glycine-immunoreactive neurones in the cat brain stem reticular formation. Neuroreport 4:1123-1126.

Fritzsch B, Sonntag R, Dubuc R, Ohta Y, Grillner S. 1990. Organization of the six motor nuclei innervating the ocular muscles in lamprey. J Comp Neurol 294:491-506.

Garrow TA, Brenner AA, Whitehead VM, Chen XN, Duncan RG, Korenberg JR, Shane B. 1993. Cloning of human cDNAs encoding mitochondrial and cytosolic serine hydroxymethyltransferases and chromosomal localization. J Biol Chem 268:11910-11916.

Gold MR, Martin AR. 1983. Inhibitory conductance changes at synapses in the lamprey brainstem. Science 221:85-87.

González MJ, Anadón R. 1992. Primary projections of the lateral line nerves in larval sea lamprey, *Petromyzon marinus* L. An HRP study. J Hirnforsch 33:185-194.

González MJ, Anadón R. 1994. Central projections of the octaval nerve in larval lamprey: an HRP study. J Hirnforsch 35:181-189.

González MJ, Manso MJ, Anadón R. 1997. Octavolateral neurons projecting to the middle and posterior rhombencephalic reticular nuclei of larval lamprey: A retrograde horseradish peroxidase labelling study. J Comp Neurol 384:396-408.

González MJ, Pombal MA, Rodicio MC, Anadón R. 1998. The internuclear neurons of the ocular motor system of the larval sea lamprey. J Comp Neurol 401:1-15.

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

Gravel J, Brocard F, Gariépy JF, Lund JP, Dubuc R. 2007. Modulation of respiratory activity by locomotion in lampreys. Neuroscience 144:1120-1132.

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

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

Hardisty MW, Rovainen CM. 1982. Morphological and functional aspects of the muscular system. In: Hardisty MW, Potter IC, editors. The Biology of Lampreys, vol 4. London: Academic Press. p 137-231.

Helfert RH, Bonneau JM, Wenthold RJ, Altschuler RA. 1989. GABA and glycine immunoreactivity in the guinea pig superior olivary complex. Brain Res 501:269-286.

Higashijima S, Mandel G, Fetcho JR. 2004. Distribution of prospective glutamatergic, glycinergic, and GABAergic neurons in embryonic and larval zebrafish. J Comp Neurol 480:1-18.

Hökfelt T, Ljungdahl A. 1971. Light and electron microscopic autoradiography on spinal cord slices after incubation with labeled glycine. Brain Res 32:189-194.

Homma S. 1978. Organization of the trigeminal motor nucleus before and after metamorphosis in lampreys. Brain Res 140:33-42.

Homma S, Rovainen CM. 1978. Conductance increases produced by glycine and gamma-aminobutyric acid in lamprey interneurones. J Physiol 279:231-252.

Huard H, Lund JP, Veilleux D, Dubuc R. 1999. An anatomical study of brainstem projections to the trigeminal motor nucleus of lampreys. Neuroscience 91:363-378.

Imboden M, De Saint Jan D, Leulier F, Korn H, Goblet C, Bregestovski P. 2001. Isolation and characterization of an alpha 2-type zebrafish glycine receptor subunit.Neuroscience 103:799-810.

Iversen LL, Bloom FE. 1972. Studies of the uptake of 3 H-gaba and (3 H) glycine in slices and homogenates of rat brain and spinal cord by electron microscopic autoradiography. Brain Res 41:131-143.

Kolston J, Osen KK, Hackney CM, Ottersen OP, Storm-Mathisen J. 1992. An atlas of glycine- and GABA-like immunoreactivity and colocalization in the cochlear nuclear complex of the guinea pig. Anat Embryol 186:443-465.

Koyama H. 2005. Organization of the sensory and motor nuclei of the glossopharyngeal and vagal nerves in lampreys. Zool Sci 22:469-476.

Koyama H, Kishida R, Goris RC, Kusunoki T. 1987. Organization of sensory and motor nuclei of the trigeminal nerve in lampreys. J Comp Neurol 264:437-448.

Koyama H, Kishida R, Goris RC, Kusunoki T. 1989. Afferent and efferent projections of the VIIIth cranial nerve in the lamprey *Lampetra japonica*. J Comp Neurol 280:663-671.

Landwehr S, Dicke U. 2005. Distribution of GABA, glycine, and glutamate in neurons of the medulla oblongata and their projections to the midbrain tectum in plethodontid salamanders. J Comp Neurol 490:145-162.

Lannoo MJ, Hawkes R. 1997. A search for primitive Purkinje cells: zebrin II expression in sea lampreys (*Petromyzon marinus*). Neurosci Lett 237:53-55.

Ljungdahl A, Hökfelt T. 1973. Accumulation of 3 H-glycine in interneurons of the cat spinal cord. Histochemie 33:277-280.

Lue JH, Shieh WF, Chen SH, Shieh JY, Wen CY. 1997. Morphometric study of glycine-immunoreactive neurons and terminals in the rat cuneate nucleus. J. Anat 191:375-385.

Matthews G, Wickelgren WO. 1979. Glycine, GABA and synaptic inhibition of reticulospinal neurones of lamprey. J Physiol 293:393-415.

McClellan AD, Zhang L, Palmer R. 2006. Fluorogold labeling of descending brain neurons in larval lamprey does not cause cell death. Neurosci Lett 401:119-124.

McPherson DR, Buchanan JT, Kasicki S. 1994. Effects of strychnine on fictive swimming in the lamprey: evidence for glycinergic inhibition, discrepancies with model predictions, and novel modulatory rhythms. J Comp Physiol A 175:311-321.

Meléndez-Ferro M. 2001. Distribución de la inmunorreactividad al ácido gamma-aminobutírico (GABA) en el sistema nervioso central de la lamprea de mar durante el desarrollo y la etapa adulta. Ph.D. Dissertation, University of Santiago de Compostela.

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

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

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B, Rodríguez-Muñoz R, Anadón R, Rodicio MC. 2003. Ontogeny of γ-aminobutyric acid-immunoreactive

neurons in the rhombencephalon and spinal cord of the sea lamprey. J Comp Neurol 464:17-35.

Ménard A, Auclair F, Bourcier-Lucas C, Grillner S, Dubuc R. 2007. Descending GABAergic projections to the mesencephalic locomotor region in the lamprey *Petromyzon marinus*. J Comp Neurol 501:260-273.

Merchán M, Aguilar LA, Lopez-Poveda EA, Malmierca MS. 2005. The inferior colliculus of the rat: quantitative immunocytochemical study of GABA and glycine. Neuroscience 136:907-925.

Merrick SE, Pleasure SJ, Lurie DI, Pijak DS, Selzer ME, Lee VM. 1995. Glial cells of the lamprey nervous system contain keratin-like proteins. J Comp Neurol 355:199-210.

Mugnaini E, Oertel WH, Wouterlood FF. 1984. Immunocytochemical localization of GABA neurons and dopamine neurons in the rat main and accessory olfactory bulbs. Neurosci Lett. 47:221-226.

Murakami Y, Pasqualetti M, Takio Y, Hirano S, Rijli FM, Kuratani S. 2004. Segmental development of reticulospinal and branchiomotor neurons in lamprey: insights into the evolution of the vertebrate hindbrain. Development 131:983-995.

Nieuwenhuys R. 1972. Topological analysis of the brain stem of the lamprey *Lampetra fluviatilis*. J Comp Neurol 145:165-178.

Nieuwenhuys R, Nicholson C. 1998. Lampreys, Petromyzontoidea. In: Nieuwenhuys R, ten Donkelaar HJ, Nicholson C, editors. The central nervous system of vertebrates, vol 1. Berlín: Springer Verlag. pp:397-486.

Northcutt RG. 1979. Central projections of the eighth cranial nerve in lampreys. Brain Res 167:163-167.

Northcutt RG, Puzdrowski RL. 1988. Projections of the olfactory bulb and nervus terminalis in the silver lamprey. Brain Behav Evol 32:96-107.

Northcutt RG, Wicht H. 1997. Afferent and efferent connections of the lateral and medial pallia of the silver lamprey. Brain Behav Evol 49:1-19.

Ohta Y, Grillner S. 1989. Monosynaptic excitatory amino acid transmission from the posterior rhombencephalic reticular nucleus to spinal neurons involved in the control of locomotion in lamprey. J Neurophysiol 62:1079-1089.

Pflieger JF, Dubuc R. 2004. Vestibulo-reticular projections in adult lamprey: their role in locomotion. Neuroscience 129:817-829.

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

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

Pombal MA, El Manira A, Grillner S. 1997b. Organization of the lamprey striatum - transmitters and projections. Brain Res 766:249-254.

Pombal MA, Marín O, González A. 2001. Distribution of choline acetyltransferase-immunoreactive structures in the lamprey brain. J Comp Neurol 431:105-126.

Pombal MA, López JM, de Arriba MC, Megías M, González A. 2006. Distribution of neuropeptide FF-like immunoreactive structures in the lamprey central nervous system and its relation to catecholaminergic neuronal structures. Peptides 27:1054-1072.

Pourcho RG, Goebel DJ, Jojich L, Hazlett JC. 1992. Immunocytochemical evidence for the involvement of glycine in sensory centers of the rat brain. Neuroscience 46:643-656.

Popratiloff A, Valtschanoff JG, Rustioni A, Weinberg RJ. 1996. Colocalization of GABA and glycine in the rat dorsal column nuclei. Brain Res. 706:308-312.

Rampon C, Luppi PH, Fort P, Peyron C, Jouvet M. 1996. Distribution of glycine-immunoreactive cell bodies and fibers in the rat brain. Neuroscience 75:737-755.

Reichenberger I, Streit P, Ottersen OP, Dieringer N. 1993. GABA- and glycine-like immunoreactivities in the cerebellum of the frog. Neurosci Lett 154:89-92.

Reichenberger I, Straka H, Ottersen OP, Streit P, Gerrits NM, Dieringer N. 1997. Distribution of GABA, glycine, and glutamate immunoreactivities in the vestibular nuclear complex of the frog. J Comp Neurol 377:149-164.

Robertson B, Saitoh K, Menard A, Grillner S. 2006. Afferents of the lamprey optic tectum with special reference to the GABA input: combined tracing and immunohistochemical study. J Comp Neurol 499:106-119.

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

Rodicio MC, Villar-Cervino V, Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R. 2005. Chemoarchitecture of the dorsal column nucleus of the larval sea lamprey. Brain Res Bull 66:536-540.

Ronan M, Northcutt RG. 1987. Primary projections of the lateral line nerves in adult lampreys. Brain Behav Evol 30:62-81.

Rovainen CM. 1983. Generation of respiratory activity by the lamprey brain exposed to picrotoxin and strychnine, and weak synaptic inhibition in motoneurons. Neuroscience 10:875-882.

Saint Marie RL, Ostapoff EM, Morest DK, Wenthold RJ. 1989. Glycine-immunoreactive projection of the cat lateral superior olive: possible role in midbrain ear dominance. J Comp Neurol 279:382-396.

Saint Marie RL, Benson CG, Ostapoff EM, Morest DK. 1991. Glycine immunoreactive projections from the dorsal to the anteroventral cochlear nucleus. Hear Res 51:11-28.

Schober W. 1964. Vergleichend-anatomische Untersuchungen am Gehirn der Larven und adulten Tiere von *Lampetra fluviatilis* (Linné, 1758) und *Lampetra planeri* (Bloch, 1784). J Hirnforsch 7:107-209.

Sheridan PH, Youngs LJ, Krieger NR, Selzer ME. 1984. Glycine uptake by lamprey spinal neurons demonstrated by light microscopic autoradiography. J Comp Neurol 223:252-258.

Shupliakov O, Fagersted P, Ottersen OP, Storm-Mathiesen J, Grillner S, Brodin L. 1996. Immunocytochemical localization of glycine in the lamprey spinal cord

with reference to GABAergic and glutamatergic synapses: a light and electron microscopic study. Acta Biol Hung 47:393-410.

Spirou GA, Berrebi AS. 1997. Glycine immunoreactivity in the lateral nucleus of the trapezoid body of the cat. J Comp Neurol 383:473-488.

Stefanelli A. 1934. I centri tegmentali dell'encefalo dei Petromizonti. Arch Zool Ital 20:117-202.

Stefanelli A. 1937. Il sistema statico dei Petromizonti (sistema laterale, sistema vestibolare, cervelletto). I: Centri nervosi e vie centrali. Arch Zool Ital 24:209-273.

Stefanelli A, Caravita S. 1970. Ultrastructural features of the synaptic complex of the vestibular nuclei of *Lampetra planeri* (Bloch). Z Zellforsch 108:282-296.

Shupliakov O, Fagersted P, Ottersen OP, Storm-Mathiesen J, Grillner S, Brodin L. 1996. Immunocytochemical localization of glycine in the lamprey spinal cord with reference to GABAergic and glutamatergic synapses: a light and electron microscopic study. Acta Biol Hung 47:393-410.

Tanaka I, Ezure K. 2004. Overall distribution of GLYT2 mRNA-containing versus GAD67 mRNA-containing neurons and colocalization of both mRNAs in midbrain, pons, and cerebellum in rats. Neurosci Res 49:165-178.

Uematsu K, Shirasaki M, Storm-Mathisen J. 1993. GABA- and glycine-immunoreactive neurons in the spinal cord of the carp, *Cyprinus carpio*. J Comp Neurol 332:59-68.

Vesselkin NP, Rio JP, Adanina VO, Kenigfest NB, Repérant J. 1995. Colocalization of glutamate and glycine in giant fiber synapses of the lamprey spinal cord. J Hirnforsch 36:229-237.

Vesselkin NP, Rio JP, Adanina VO, Repérant J. 2000. GABA- and glycine-immunoreactive terminals contacting motoneurons in lamprey spinal cord. J Chem Neuroanat 19:69-80.

Viana Di Prisco G, Ohta Y, Bongianni F, Grillner S, Dubuc R. 1995. Trigeminal inputs to reticulospinal neurones in lampreys are mediated by excitatory and inhibitory amino acids. Brain Res 695:76-80.

Viana Di Prisco G, Pearlstein E, Robitaille R, Dubuc R. 1997. Role of sensory-evoked NMDA plateau potentials in the initiation of locomotion. Science 278:1122-1125.

Viana Di Prisco G, Boutin T, Petropoulos D, Brocard F, Dubuc R. 2005. The trigeminal sensory relay to reticulospinal neurones in lampreys. Neuroscience 131:535-546.

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

Villar-Cerviño V, Holstein GR, Martinelli GP, Anadón R, Rodicio MC. 2008. Glycine-immunoreactive neurons in the developing spinal cord of the sea lamprey. A comparison with the GABAergic system. J Comp Neurol 508:112-130.

Walberg F, Ottersen OP, Rinvik E. 1990. GABA, glycine, aspartate, glutamate and taurine in the vestibular nuclei: an immunocytochemical investigation in the cat. Exp Brain Res 79:547-563.

Wannier T, Orlovsky G, Grillner S. 1995. Reticulospinal neurones provide monosynaptic glycinergic inhibition of spinal neurones in lamprey. Neuroreport 6:1597-1600.

Wenthold RJ. 1987. Evidence for a glycinergic pathway connecting the two cochlear nuclei: an immunocytochemical and retrograde transport study. Brain Res 415:183-187.

Wood PL. 2005. The NMDA receptor complex: a long and winding road to therapeutics. IDrugs 8:229-235.

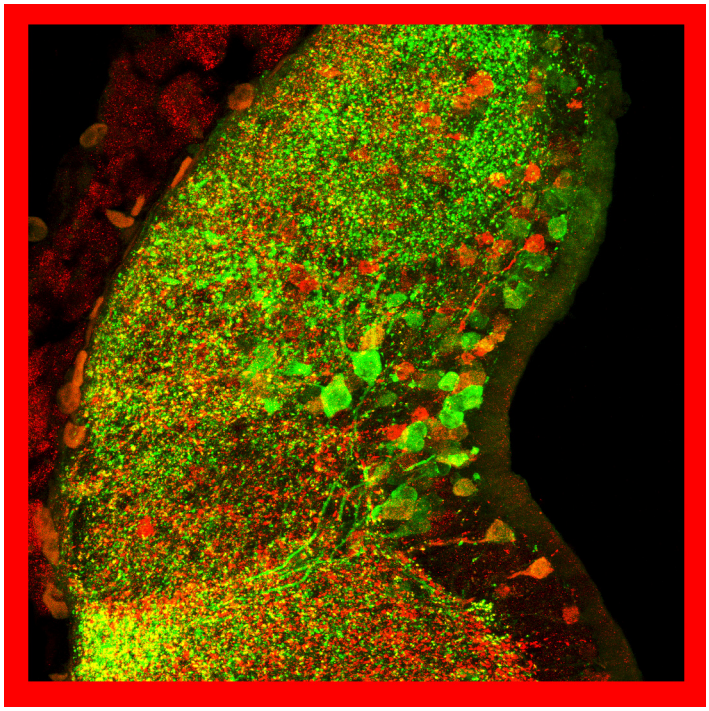
Yáñez J, Pombal MA, Anadón R. 1999. Afferent and efferent connections of the parapineal organ in lamprey: A tract tracing and immunocytochemical study. J Comp Neurol 403:171-189.

Young AB, Snyder SH. 1974. The glycine synaptic receptor: evidence that strychnine binding is associated with the ionic conductance mechanism. Proc Natl Acad Sci U S A 71:4002-4005.

Zeilhofer HU, Studler B, Arabadzisz D, Schweizer C, Ahmadi S, Layh B, Bosl MR, Fritschy JM. 2005. Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. J Comp Neurol 482:123-141.

Zompa IC, Dubuc R. 1998. Diencephalic and mesencephalic projections to rhombencephalic reticular nuclei in lampreys. Brain Res 802:27-54.

Development of Glycine Immunoreactivity in the Brain of the Sea Lamprey (*Petromyzon marinus*). Comparison with GABA



Chapter 2

INTRODUCTION

Glycinergic and GABAergic neurons represent the main rapid inhibitory systems in the brain and spinal cord of vertebrates. They appear early on in embryonic development, when they may be transiently involved in excitatory transmission, as suggested by the different ionic composition of the intra- and extracellular milieu of embryonic neurons with respect to those in the adult brain (Spitzer, 2006). Despite their functional importance, few comprehensive studies of the distribution of glycinergic cells in the adult brain of mammals have been carried out, and almost all refer to rodents (Zafra et al., 1995a; Tanaka et al., 2003; Tanaka and Ezure, 2004). Other studies on mammalian glycinergic cells have focused on the auditory nuclei (Aoki et al., 1988; Osen et al., 1990; Saint Marie et al., 1991; Kolston et al., 1992; Henkel and Brunso-Bechtold, 1995; Winer et al., 1995; Kemmer and Vater, 1997) and medullary respiratory centers (Schmid and Böhmer, 1989; Schreihofer et al., 1999; Ezure et al., 2003). Unlike GABAergic cells, which are rather widespread in many brain regions, most glycinergic neurons of the rat central nervous system are restricted to the rhombencephalon and spinal cord. In a previous immunofluorescence study with rabbit polyclonal antibodies that specifically recognized glycine in glutaraldehyde-fixed tissues, we described for the first time the distribution of glycinergic cells in the adult brain of a non-mammalian vertebrate, the adult sea lamprey *Petromyzon marinus* (Villar-Cerviño et al., 2008a). Glycine-immunoreactive (ir) neurons were found at midbrain and hindbrain levels but, unlike in the rat, also in the olfactory bulbs, the preoptic nucleus and the thalamus. Some of these glycine-ir cells showed colocalization with γ-aminobutyric acid (GABA) immunoreactivity (Villar-Cerviño et al., 2008a), which is similar to the colocalization of glycinergic and GABAergic markers (glycine transporter GLYT2 and glutamic acid decarboxylase GAD67, respectively) revealed by in situ hybridization in the rat (Tanaka et al., 2003; Tanaka and Ezure, 2004). Rich innervation by glycine-ir fibers was also observed in most regions of the adult lamprey brain (Villar-Cerviño et al., 2008a).

The development of the GABAergic system has been investigated in the brain, spinal cord and retina of various vertebrates by use of immunocytochemistry of GABA (lampreys: Meléndez-Ferro et al., 2002, 2003; Villar-Cerviño et al., 2006; teleosts: Östholm et al., 1988; Ekström and Ohlin, 1995; Mueller et al, 2006;

amphibians: Roberts et al., 1987; Dale et al., 1987a,b) and/or *in situ* hybridization with probes of the enzymes that synthesize GABA, i.e., glutamate decarboxylase (GAD65 and GAD67; mouse: Katarova et al., 2000; frog: Brox et al., 2002; zebrafish: Higashijima et al., 2004; Mueller et al., 2008). Both types of approaches have revealed that the early developmental pattern of the GABA system in vertebrates is conserved, and passes through a similar phylotypic stage (Katarova et al., 2000; Meléndez-Ferro et al., 2002, 2003; Mueller et al., 2006).

As regards the development of the glycinergic system in vertebrates, the available data are both scarce and fragmentary. Early studies with glycine immunocytochemistry reported the early development of the glycinergic neurons of the spinal cord and medulla oblongata of embryos of a frog (Dale et al., 1986; Roberts et al., 1988). The development of glycine immunoreactivity has recently been described in the spinal cord of mouse (Allain et al., 2006) and lamprey (Villar-Cerviño et al., 2008b). The development of glycinergic neurons in the rat auditory system has also been studied with immunocytochemistry against the neuronal glycine transporter GLYT2 and/or by *in situ* hybridization with probes to this transporter (Friauf et al., 1999). GLYT2 in situ hybridization also revealed the early organization of prospective glycinergic populations in the spinal cord and medulla oblongata of zebrafish (Higashijima et al., 2004; Cui et al., 2005). Similarly, the expression patterns of the glycine transporters xGlyT1 and xGlyT2 in the brain and its coexpression with xGAD67 have been described by *in situ* hybridization in early stages of *Xenopus* (Wester et al., 2008). However, the glycinergic cells of the brain in zebrafish and *Xenopus* embryos have not been identified at the level of populations and the distribution of glycinergic neurons in adults of these species has not been reported. Moreover, as far as we are aware there has been no comprehensive study of the development of the glycinergic brain populations from embryos to adults in any vertebrate.

Lampreys are extant representatives of the most ancient lineage of vertebrates, the Agnathans (the sister group of jawed vertebrates), and their study is key to understanding the phylogeny of the vertebrate brain. The main aims of this study were to describe the early appearance of the glycinergic system in the lamprey brain, the changes occurring after prolarval stages in the larvae and whether or not the glycinergic populations observed in larval stages are retained in the adult. We

also aimed to compare the developmental pattern of the glycine populations with that of the GABAergic system in the lamprey brain by use of double immunofluorescence methods and confocal microscopy.

MATERIAL AND METHODS

Subjects

Embryos, prolarvae and larvae of sea lampreys (*Petromyzon marinus* L) were used in the present study. Embryos (9 and 12 days postfertilization: E9 and E12; three embryos of each stage) and prolarvae (hatchlings, and 1, 2, 4, 8, 15 and 22 days posthatching: P0, P1, P2, P4, P8, P15 and P22; three prolarvae of each stage) were obtained from *in vitro* fertilized eggs reared in the laboratory. Larvae (total body length comprised between 30 mm and 100 mm; N = 12) were collected from the River Ulla (Galicia, northwest Spain) with permission from the Xunta of Galicia, and maintained in aerated aquaria with a bed of river sediment. All experiments were approved by the Ethics committee of the University of Santiago de Compostela and conformed to the European Community guidelines on animal care and experimentation.

Tissue Collection and Processing

Animals were deeply anaesthetized with benzocaine (Sigma, St. Louis, MO; 0.05%) and, in the case of larvae, killed by decapitation. All samples were fixed by immersion in 5% glutaraldehyde and 1% sodium metabisulphite in 0.05M Tris buffered saline (TBS; pH 7.4) for 17 h. The fixed samples were embedded in Tissue Tek (Sakura, Torrance, CA), frozen in liquid nitrogen, sectioned on a cryostat in the transverse plane (embryos and prolarvae, 10µm thick; larvae, 16 µm thick) and mounted on Superfrost® Plus glass slides (Menzel, Braunschweig, Germany).

Immunofluorescence

For single immunofluorescence, sections were pretreated with 0.2% $NaBH_4$ in deionized water for 45 minutes at room temperature to quench glutaraldehyde-induced autofluorescence. Sections were incubated for 3 days at 4ºC with rabbit polyclonal anti-glycine antibody (Immunosolution, Jesmond, Australia; code IG1003, batch 1953; dilution 1:3,000; or Chemicon, Temecula, CA, code AB139, lots 25050133 and 0508007382; dilution 1:100) in 0.05M TBS with 1% sodium metabisulphite and 0.2% Triton X-100. The samples were rinsed in TBS with 1% sodium metabisulphite, then incubated for 1 hour with Cy3 conjugated goat anti-rabbit immunoglobulin (Chemicon; 1:200) and mounted with fluorescence anti-fade

mounting medium (Vectashield; Vector, Burlingame, CA). All antibodies were diluted in TBS (pH 7.4) containing 0.2% Triton X-100 and 3% normal goat serum.

In order to compare the distributions of glycine and GABA immunoreactivities, some series were treated as above and stained with a cocktail of polyclonal rabbit anti-glycine (Immunosolution, dilution 1:3,000 or Chemicon 1:100) and monoclonal mouse anti-GABA (Sigma; clone GB-69, No A 0310, dilution 1:1,200) antibodies, then incubated for 1 hour with a cocktail of Cy3-conjugated goat anti-rabbit immunoglobulin (Chemicon; 1:200) and fluorescein conjugated goat anti-mouse IgG immunoglobulin (Chemicon; 1:50), and mounted in Vectashield.

Antibodies

We used two different rabbit polyclonal glycine antibodies, as previously reported (Villar-Cerviño et al 2008a,b). One of the glycine antibodies (Immunosolution) was raised against a glycine-porcine thyroglobin conjugate, purified against thyroglobulin and tested by the supplier in sections of retina and cerebellum from various mammals and other vertebrates, as well as in dot blot immunoassays with a variety of amino acid conjugates, including the standard 20 amino acids found in proteins, the non-protein amino acids D-serine, D-alanine and D-aspartate, GABA and the glycine-containing tripeptide glutathione (GSH), which did not yield significant reactivity. The antibody was developed by Dr. David V. Pow (University of Newcastle, New South Wales, Australia), and has been used in a number of studies on glycinergic neurons of the retina, brain and spinal cord. The other glycine antiserum (Chemicon) was raised against a glycine-BSA conjugate. The specificity of this glycine antiserum was previously tested in lamprey spinal cord tissue homogenates reacted with fixative in the presence of GABA, L-glutamate, glycine or L-aspartate, and showed high specificity for glycine-protein conjugates (Vesselkin et al., 2000). Moreover, preadsorption of this glycine antibody with BSA did not block immunostaining in lamprey. The immunohistochemical results obtained with both anti-glycine antibodies revealed the same pattern of glycine-ir cell populations in the brain and spinal cord. For tissue processing controls, primary antisera were omitted from some tissue sections. No staining was observed in these controls. In addition, these two antibodies were tested by Western blotting with lamprey brain protein extracts (Villar-Cerviño et al., 2006; unpublished results). No

protein band was stained in these blots, which indicated that the antibodies did not react with lamprey brain native proteins.

The monoclonal mouse anti-GABA antibody (Sigma) has previously been used in our laboratory (Villar-Cerviño et al., 2008a). The antibody was raised against GABA conjugated to BSA with glutaraldehyde and was evaluated by the supplier for activity and specificity, by use of dot blot immunoassay. No cross-reaction was observed with BSA, L-α-aminobutyric acid, L-glutamic acid, L-aspartic acid, glycine, δ-aminovaleric acid, L-threonine, L-glutamine, taurine, putrescine, L-alanine or carnosine. The antibody showed weak cross-reaction with β-alanine. Furthermore, the sections of the brain and retina of sea lamprey incubated with this antibody revealed the same pattern of immunostaining revealed in studies with other anti-GABA antibodies (Meléndez-Ferro, 2001; Meléndez-Ferro et al., 2002, 2003; Villar-Cerviño et al., 2006; Robertson et al., 2007; Villar-Cerviño et al 2008b). Moreover, preadsorption of this GABA antibody with BSA did not block immunostaining in lamprey. No immunoreactivity was detected when the primary antibody was omitted from the immunohistochemical procedure. In addition, the antibody was tested by Western blotting with lamprey brain protein extracts (unpublished results). No protein band was stained in these blots.

Tract-tracing experiments

The brain of lamprey larvae shows scarce size differences and morphological differentiation among neuronal populations, which sometimes makes it difficult to distinguish cell populations. In order to facilitate analysis and to assess the location of some cell populations, we also performed some tract-tracing experiments with neurobiotin (NB) -in 50-80 mm long larvae- in combination with glycine immunofluorescence, under deep anaesthesia with benzocaine. For this, small amounts of NB were injected with the aid of a minute pin, in three types of experiments. Two larvae were injected with NB in the eye orbit, which resulted in labeling of the optic tract and retinopetal system (see de Miguel et al., 1990), the ocular motor complex (see Pombal et al., 1994), the trigeminal sensory root and motor nucleus (see Anadón et al., 1989) and the anterior lateral line nerve (see González and Anadón, 1992). Two further larvae were injected with NB in the otic capsule, which resulted in labeling of the octaval (see González and Anadón, 1994)

and facial nerves. Two other larvae were injected with NB in the rostral spinal cord, which resulted in labeling of the descending brain-spinal neuronal system. Larvae were allowed to survive for two-three days after injection, and then fixed as above. Neurobiotin was revealed with fluorescein isothiocyanate (FITC)-labeled avidin D (Vector Laboratories, Burlingame, CA; 1:1000), and then single immunofluorescence was performed as above with Cy3-coupled goat anti-rabbit polyclonal antibody (Chemicon; dilution 1:200).

Image Acquisition and Analysis

Sections were analyzed and photographed with a spectral confocal microscope TCS-SP2 (Leica, Wetzlar, Germany), with a combination of red and green lasers. Confocal stacks were acquired and processed with LITE software (Leica). For presentation of most of the confocal figures, stack projections were converted to grey scale, inverted and then adjusted for brightness and contrast in Corel Photo-Paint (Corel, Ottawa, Canada).

For qualify colocalization of GABA and glycine in neurons, we considered only clearly stained glycine-ir and GABA-ir neurons unambiguously detected on projections of confocal stacks of alternate sections, and we did not take into account other neurons that were very weakly stained and possibly contained minimally detectable levels of glycine, as in a previous study (Villar-Cerviño et al., 2008b). For this goal, we used the "maximum projection" command of the LITE software filtered by adjusting the threshold to 80 with rescaling to 255 (eight-bit scale, 0-255 range).

Measurements

The cell diameters were measured on confocal photomicrographs of transverse sections, with LITE software (Leica). Ten cells were measured in each population. For this goal, only cells showing nuclei and with the maximum diameter within the stack of optical sections (i.e. showing decreasing values both above and below this maximum optical section) were considered. Values are expressed as the means of the smallest diameter ± standard deviation.

Terminology

Whenever possible we used the same terminology for the different glycinergic populations of developing lamprey as we employed in a previous study

of adult sea lamprey brain (Villar-Cerviño et al., 2008a). For identification of very early populations, we also used the terminology that we employed in other developmental studies on GABAergic populations, in which colocalization or codistribution with this transmitter was observed (Meléndez-Ferro et al., 2002, 2003). Identification of nuclei and regions of the larval brain was also carried out following the criteria used in previous tract-tracing (Anadón et al., 1989; de Miguel et al., 1990; González and Anadón, 1992, 1994; Pombal et al., 1994, 1996; Yáñez and Anadón, 1994; Rodicio et al., 1995; González et al., 1997, 1998, 1999; Yáñez et al., 1999) and immunohistochemical studies carried out in our lab (Rodicio et al., 2005; Abalo et al., 2005, 2007; Villar-Cheda et al., 2005, 2006; Barreiro-Iglesias et al., 2008), and following their neuromeric distribution as far as possible (Pombal and Puelles, 1999; Meléndez-Ferro et al., 2001, 2002, 2003; Villar-Cheda et al., 2005, 2006).

RESULTS

Glycine-ir populations during the embryonic and prolarval period

In the brain of late embryos (E12), only a few glycine-ir cells that send ascending fibers to isthmic levels were observed in the caudal rhombencephalon (Fig. 1A). No GABA-ir cells were observed in the brain of these stages.

The prolarval period was subdivided into hatchlings (P0-P1), pigmentation (P2-P3), gill cleft (P4-P7), and burrowing (P8-P23) stages, in accordance with Piavis (1971). In the brain of hatchlings, glycine-ir cell populations were found only in the caudal rhombencephalon, located at intermedioventral positions (Fig. 1B). Glycine-ir fibers coursed mainly caudally from this group. At isthmic levels only a few glycine-ir fibers were observed and no glycine immunoreactivity was shown at more rostral brain regions (Fig. 1C). In the pigmentation stage, the first glycine-ir cell groups became apparent in the prosencephalon. These new glycine-ir cell populations appeared in the postoptic region of the hypothalamus and in the nucleus of the medial longitudinal fascicle (nmlf) (Fig. 1D). The hypothalamic population consisted of some cerebrospinal fluid-contacting (CSF-c) glycine-ir cells showing a thin ventricular dendrite (Fig. 1D). At this stage, new glycine-ir cell groups were also observed in the rhombencephalon, both in the isthmus and at the level of the otic vesicle (Figs. 1E-F). The caudal rhombencephalic glycine-ir neuronal population and the glycine-ir fibers coursing in the marginal region were greater in number than in hatchlings (Fig. 1G).

At the gill cleft stage the postoptic hypothalamus and nucleus of the nmlf showed a few glycine-ir cells (Fig. 1H). At isthmic levels, two glycine-ir cell populations were now distinguished, dorsally and ventrally (Fig. 1I). The dorsal population, which consisted of some pear-shaped glycine-ir cells, may correspond to the primordium of the conspicuous dorsal isthmic grey population previously described in the adult lamprey brain (Villar-Cerviño et al., 2008a). The ventral cell group was formed by round glycine-ir neurons located near the lateral neuropil. This population represents the primordium of the isthmic reticular population. At trigeminal and octaval levels (rhombomeres 2 to 5), the number of glycine-ir cells was very low at this stage, although strong glycine-ir fibers coursed in the mid-ventral marginal layer (Figs. 1J-K). In the caudal rhombencephalon numerous

glycine-ir cells appeared in the periventricular region at ventral and intermediate levels (basal plate), and some dorsally located (alar plate) glycine-ir cells were also observed (Fig.1L).

At the beginning of the burrowing stage (P8), a new glycine-ir cell population consisting of some faintly stained CSF-c cells appeared in the septum (Fig. 2A) and some glycine-ir cells were also present at thalamic levels (Fig. 2C). As regards the hypothalamic glycine-ir population, some of these cells exhibited a long dendrite that contacted the ventricle (Figs. 2B-C). As in the gill cleft stage, a group of glycine-ir cells was present in the nmlf (Fig. 2B). At isthmic levels, the number of glycine-ir neurons increased, mainly in the ventral population (Fig. 2D). At trigeminal and octaval levels, a few glycine-ir populations were distributed in a dorsal and a ventral group (Figs. 2E-F), but from levels of the otic capsule to the caudal rhombencephalon, numerous glycine-ir cells were observed and the separation of the two cell groups became less clear (Figs. 2G-H).

At the middle of the burrowing stage (P15), no glycine immunoreactivity was observed in the telencephalon, except for some glycine-ir fibers that coursed mainly in the lateral marginal region (Fig. 3A). Some faint to moderate glycine-ir populations were observed in the diencephalon. There was a faint glycine-ir group of small CSF-c cells in the preoptic nucleus (Fig. 3B) and glycine immunoreactivity was also found in CSF-c cells in the postoptic-tuberal cell group (Fig. 3C). Numerous glycine-ir fibers crossed the midline at the postoptic commissural region (Fig. 3B). Some glycine-ir cells were found in the ventral thalamus, dorsal thalamus and pretectum (Fig. 3C). Intense glycine-ir cells were observed at the nmlf, (Fig. 3D). Some glycine-ir cells appeared in the region of the M5 nucleus of Schober in the mesencephalic tegmentum. At isthmic, trigeminal and octaval levels, the ventral and dorsal glycine-ir populations increased in number in the cells and fibers of the ventrolateral neuropil (Figs. 3E-G). The glycine-ir ventral cell population was strongly stained and abundant in the caudal rhombencephalon (vagal region) (Fig. 3H), and scarce glycine-ir cells were observed at dorsal locations. No significant changes were observed in the brain of late prolarvae with respect to that of middle burrowing stage.

Colocalization of GABA and glycine immunoreactivities during prolarval stages

Colocalization of GABA and glycine was observed in all glycine-ir cell groups of the prolarval prosencephalon (Fig. 4A-E). In the septum of early burrowing stage prolarvae (P8), most GABA-ir cells were also glycine-ir, while many only glycine-ir neurons were observed (Fig. 4A). In the preoptic nucleus, hypothalamus and in the nmlf most neurons that exhibited glycine immunoreactivity also showed GABA immunoreactivity (Figs. 4B-D). GABA-ir cells were more numerous than glycine-ir neurons in the ventral thalamus, and colocalization of GABA and glycine immunoreactivity was observed in only a few cells (Fig. 4E). There were three types of cells in the dorsal thalamus: glycine-ir only, GABA-ir only and glycine-ir/GABA-ir cells (Fig. 4E). In contrast, most GABA-ir neurons observed in the pretectum were also glycine-ir (Fig. 4E).

In the ventral population of the isthmus (primordium of the isthmic reticular population) all GABA-ir cells were also glycine-ir, but some glycine-ir/GABA-negative neurons were observed (Fig. 4F). The ventral and dorsal populations of trigeminal levels were formed by glycine-ir-only cells, GABA-ir-only cells and also glycine-ir/GABA-ir neurons (Fig. 4G-H). In the octaval region GABA-ir neurons were very faint and scarce, but all of them also contained glycine. In the caudal rhombencephalon, glycine-ir cells were more numerous and larger than GABA-ir cells, so that the glycine-ir-only cells were predominant in this area (Fig. 4I).

Glycine-ir populations in the larval brain

The distribution of glycine was mainly studied in the brain of larvae of lengths of between 30 mm and 90 mm, and the following description corresponds to that observed in these larvae.

Telencephalon

Olfactory bulbs. Scarce spindle-shaped glycine-ir cells (5.5 ± 0.9 μm in diameter) were observed in the inner cell layer of the olfactory bulbs, at caudal positions close to the limit with the telencephalic lobes, (Figs. 5A, 6A). Intense glycine-ir beaded fibers were found in most regions of olfactory bulbs, and were more numerous in inner regions and very scarce in, or absent from, glomerular regions.

Pallium. The larval lamprey pallium consists of three main regions, the lateral, medial and dorsal pallium. Of these regions, the medial pallium is very poorly developed in these larvae (Villar-Cheda et al., 2006). No glycine-ir cells were found at any of these levels. However, the lateral pallium was richly innervated by strongly glycine-ir beaded fibers, while the innervation of both the medial and dorsal pallium was scarcer (Fig. 5B).

Subpallium. The lamprey subpallium comprises two main regions, the septum-terminal lamina region and the striatum, which did not show glycine-ir neurons in larvae. Some strongly glycine-ir fibers crossed in the terminal lamina giving rise to spherical fiber dilatations (Fig. 5B). The septal region was innervated by numerous glycine-ir beaded fibers mainly coursing lateral to the periventricular cell layer. The striatum received moderate innervation by intensely glycine-ir fibers.

Preoptic region and diencephalon

Preoptic region, hypothalamus and posterior tubercle. Only a scarce and faint glycine-ir cell population was observed in the preoptic nucleus located parallel to the preoptic recess (Fig. 5C). The lateral preoptic region was innervated by strongly glycine-ir beaded fibers. No glycine-ir neurons were observed in the larval hypothalamus, although some glycine-ir beaded fibers innervated both tuberal and mammillary regions, except the subventricular areas.

Prethalamus, thalamus, epithalamus and pretectum. The thalamus was the only region of these diencephalic regions that exhibited glycine-ir cells in larvae (Figs. 5D, 6B). This population was present in the prolarval stage, but no glycine-ir neurons were observed in this cell group in the first half of the larval period (from 10 mm to 60 mm). Thalamic glycine-ir cells reappeared in the second half of the larval period (between 60 mm and transformation, which in sea lamprey occurs in larvae of about 120-150 mm in length), located just dorsal to the fasciculus retroflexus and ventral to the GABAergic pretectal population (Fig. 8B). It was formed by some pear-shaped strongly glycine-ir cells (5.3 ± 0.5 μm in diameter). Numerous glycine-ir fibers were present at the level of the prethalamus (ventral thalamus), thalamus (dorsal thalamus) and pretectum, except at periventricular regions. In the habenula, scarce thin glycine-ir fibers gave rise to small beads preferentially located near the

dorsal edge. GABA-ir cells were found in the prethalamus, thalamus and pretectal nucleus.

Midbrain

The midbrain showed glycine-ir populations located in two different nuclei, one in the M5 nucleus of Schober (Schober, 1964) and the other one in the dorsomedial region of the oculomotor nucleus.

M5 nucleus. The M5 nucleus is continuous with the cell layers of the torus semicircularis and extends between the subpretectal tegmentum, embracing the M1 and M2 Müller cells, and the level of the midbrain Müller cell (M3). Some pear-shaped glycine-ir cells (6.8 ± 0.8 μm in diameter) with thick dendrites extending laterally were found here (Figs. 5E, 6C). These cells were located with the retinopetal periventricular cells demonstrated by Rodicio et al. (1995), which was confirmed by double labeling experiments after application of NB to the eye orbit (Fig. 7A).

Oculomotor nucleus. The oculomotor nucleus of lampreys consists of three subnuclei innervating each extraocular muscle; rostral and dorsal rectus (lateral and intermediate subnuclei, respectively), and rostral oblique (dorsomedial subnucleus) (Fritzsch et al., 1990; Pombal et al., 1994; present results). A few oval-shaped glycine-ir cells (5.8 ± 0.7 μm in diameter) were found in the region of the dorsomedial oculomotor subnucleus at the transverse levels showing the entrance of the oculomotor nerve, thus, this region also contained internuclear interneurons (Figs. 5E, 6D). The location of these glycine-ir cells was assessed in double labeling experiments after application of NB to the eye orbit (Fig. 7B). Both lateral and intermediate oculomotor subnuclei were innervated by scarce glycine-ir fibers, while the dorsomedial subnucleus exhibited more abundant glycine-ir processes.

Rhombencephalon

Alar plate regions

Isthmus. Two different glycine-ir populations were observed in the alar plate of the isthmic region. Just dorsal to the sulcus limitans and close to the ventricle, there was a group of round glycine-ir cells with dendrites extending laterally (Figs. 5F, 6E), and just lateral to this periventricular population, the "dorsal isthmal grey"

exhibited a large number of very strongly glycine-ir cells (4.6 ± 0.4 μm in diameter). These cells extended dendrites laterally spreading as a sheet (Fig. 6E). The position of these glycine-ir populations was caudal to the trochlear nucleus, as assessed in double labeling experiments after application of NB to the eye orbit (not shown). Also, these glycine-ir populations were dorsal to the population of medium-sized reticulospinal cells that lay dorsal to the large I1 Müller cell, as revealed in double labeling experiments after application of NB to the rostral spinal cord (Fig. 7C).

Octavolateral area. The octavolateral area of the larval lamprey forms the dorsolateral region of the medulla oblongata, from caudal isthmic levels to the region of the vagal nerve entrance. It is composed by three main longitudinal columns, the dorsal (lateral line electroreceptive), medial (lateral line mechanoreceptive) and ventral (octaval) octavolateral nuclei. Intensely stained round glycine-ir cells (5.4 ± 0.4 μm in diameter) were located at periventricular regions in the medial nucleus, mainly in rostral (trigeminal) regions of the column (Figs. 5G, 6F). There were also a few glycine-ir cells located more laterally (Fig. 6F). The dorsal and ventral nuclei of the larva lacked glycine-ir neurons. The octavolateral area was richly innervated by strongly glycine-ir fibers.

Dorsal column nucleus. The dorsal column nucleus extends rostrally from the obex in the alar plate of the caudal rhombencephalon. The nucleus consists of a periventricular region rich in cell perikarya, and a dorsolateral region with fibers and some scattered neurons. The nucleus lies medial to the descending trigeminal root, as observed in double labeling experiments after application of NB to the eye orbit (Fig. 7D). In this nucleus, glycine-ir oval-shaped perikarya (5.3 ± 0.9 μm in diameter) that sent thin dendrites to the fiber layer were found in both the periventricular layer and in the lateral neuropil, where numerous intense glycine-ir fibers were observed (Figs. 5I-J, 6G). This lateral neuropil receives numerous ascending fibers that course longitudinally in the dorsal column, as revealed in double labeling experiments after application of NB to the rostral spinal cord (Fig. 7E).

Trigeminal spinal nucleus. An alar plate region containing abundant glycine-ir and GABA-ir cells was identified in the caudal rhombencephalon on the basis of its association with the descending root of the trigeminal nerve, which became labeled after application of NB to the eye orbit (Fig. 7D). The organization of the glycine-ir population of this nucleus is similar to that reported in the dorsal column

nucleus, although these glycine-ir cells did not receive ascending fibers from the dorsal column that became anterogradely labeled after application of NB to the spinal cord (Figs 5J, 7E).

Basal plate regions

Medial zone of the rhombencephalic reticular formation

Isthmic region. A group of numerous pear-shaped glycine-ir cells (5.9 ± 0.9 μm in diameter) was observed in the ventral isthmic reticular formation (Figs. 5F, 6H). Some of these cells exhibited a laterally extending dendrite. Fairly abundant glycine-ir fibers were observed through reticular regions. However, the interpeduncular nucleus showed only scarce glycine-ir fibers.

Trigeminal levels. There were some glycine-ir cells (6.6 ± 0.6 μm in diameter) ventral to the trigeminal motor nucleus in the periventricular cell layer, with small laterally extending dendrites (Figs. 5G, 6I). These were located in the same region of medium-sized and large reticulospinal neurons demonstrated by application of NB (Fig. 7F). A few scattered glycine-ir cells were observed in the ventrolateral neuropil.

Middle rhombencephalic reticular region. Rather numerous glycine-ir cells were observed in the middle reticular region (Figs. 5H, 6J). These pear-shaped cells (6.1 ± 0.7 μm in diameter) were located in the periventricular layer, and most of them exhibited a dendrite extending laterally towards the adjacent neuropil (Fig. 6J). They were located in the same region containing large and medium-sized reticulospinal neurons, as demonstrated by triple labeling, although some glycine-ir perikarya were observed away from the ventricle in neuropil regions. A number of intense glycine-ir fibers innervated the middle rhombencephalic reticular region and some boutons were observed surrounding large glycine-negative reticular neurons (Fig. 6J).

Posterior rhombencephalic reticular region. A compact group of round to oval-shaped intense glycine-ir cells (5.8 ± 0.7 μm in diameter) were located just ventromedial to the glossopharyngeal-vagal motor column, in the periventricular cell layer (Figs. 5I, 6K). As observed in the middle reticular region, the medial zone of the posterior reticular region was richly innervated by intensely stained glycine-ir fibers.

Glycine-ir reticular populations associated with somatomotor nuclei

Region of the abducens nucleus. The abducens motoneurons are either scattered in the reticular region ventral to the facial and glossopharyngeal motor nuclei (abducens ventral subgroup), or in periventricular regions medial to the facial and glossopharyngeal motor nuclei (abducens dorsal subgroup) (Pombal et al., 1994). In this region, glycine-ir cells were observed in both periventricular and migrated regions (Figs. 5H, 6L). As shown by NB experiments, only a few oval glycine-ir cells were observed in the reticular region containing migrated cells of the abducens nucleus, where a number of intense glycine-ir processes were observed (Fig. 7G).

Spino-occipital motoneurons. This spino-occipital nucleus extends from just caudal to the vagal motor nucleus to the rostral spinal cord, and their neurons are located near the midline (Pombal et al., 2001). At this level a compact group of intensely stained oval glycine-ir cells (6.2 ± 0.7 μm in diameter) was observed, with dendrites coursing to the adjacent neuropil, where there were also many glycine-ir fibers (Figs. 5J, 6M).

Glycine-ir reticular populations associated with visceromotor nuclei

Trigeminal motor nucleus. In larval lampreys, the trigeminal motor nucleus is a continuous column of medium-sized cells located ventral to the sulcus limitans, which extends between the entrance levels of the trigeminal and octaval nerves. As shown by tract-tracing experiments, larval trigeminal motoneurons were grouped closely near the ventricle (Anadón et al., 1989; present results). Scarce faint to moderate glycine-ir cells (6.2 ± 0.6 μm in diameter) were observed just dorsolateral to the trigeminal motor nucleus in the periventricular cell layer, and sent dendrites extending towards the trigeminal descending root (Figs. 5G, 6N).

Facial motor nucleus. In lamprey larvae, facial motoneurons showed medium-sized perikarya located near the ventricle and caudal to the trigeminal motor nucleus. Some oval-shaped glycine-ir cells were intermingled with facial motoneurons and gave rise to thin dendrites extending towards the adjacent neuropil (Figs. 5H, 6O). A group of scarce round to oval glycine-ir cells could be observed dorsolateral to this motor nucleus (Fig. 6O). The facial nucleus was labeled from the otic capsule region in a triple labeling experiment (data not shown). The neuropil of

this region was richly innervated by glycine-ir fibers, but no glycine-ir fibers were found among motoneuron perikarya.

Glossopharyngeal-vagal motor column. In the glossopharyngeal-vagal motor column, which extends caudally to that of the facial nerve, some oval-shaped glycine-ir cells were located both lateral to and intermingled with motoneurons (Figs. 5I, 6P).

Colocalization of GABA immunoreactivity in larval glycine-ir neurons

Double immunofluorescence methods in the larval brain revealed the distribution of GABA-ir and glycine-ir structures in the same sections. Glycine-ir/GABA-ir cells, glycine-ir only cells and/or GABA-ir only cells were observed in different locations. In the following we describe these populations as regards colocalization with GABA.

Both glycine and GABA-ir cells were observed in the olfactory bulbs (Fig. 8A), thalamus (Fig. 8B), and in the oculomotor nucleus region (Fig. 8C), but double immunofluorescence did not reveal colocalization of GABA and glycine in the same cells. In the M5 nucleus of Schober of larvae most of the glycine-ir cells observed also showed GABA immunoreactivity, although this population also contained a few GABA-ir only and glycine-ir only neurons (Fig. 8D).

Double immunofluorescence revealed three different cell populations in the isthmic reticular formation as regards glycine and GABA immunoreactivity. The cells located near the ependymal layer were GABA-ir only, cells at adjacent positions showed both GABA and glycine immunoreactivity, and at the most lateral positions the cells were glycine-ir only (Fig. 8E). GABA-ir cells were more abundant than glycine-ir cells in the octavolateral region, and most medial nucleus cells showed one or other type of immunoreactivity (Fig. 8F). Only a few cells were doubly labeled in this region (Fig. 8F). In the dorsal column nucleus, some glycine-ir cells of the periventricular layer also exhibited GABA immunoreactivity (Fig. 8G). At rhombencephalic reticular levels, there was a low degree of colocalization of glycine and GABA in the medial zone at the level of the trigeminal and glossopharyngeal-vagal nuclei, while in the medial zone of the facial nucleus many of the glycine-ir cells were also GABA-ir (Fig. 8H). In the lateral reticular zone associated with the trigeminal (Fig 8F) and facial motor nuclei (Fig. 8I), most of the

glycine-ir cells were also GABA-ir, but the glycine-ir population associated with the glossopharyngeal-vagal motor column did not exhibit GABA immunoreactivity. Finally, double immunofluorescence did not reveal colocalization of GABA and glycine in cells associated with the spino-occipital motor column.

DISCUSSION

This is the first study of the development of glycine immunoreactivity in the sea lamprey brain. Together with previous results in the adult sea lamprey brain (Villar-Cerviño et al., 2008a), it represents the first comprehensive study of development of glycinergic system in a vertebrate brain.

The development of glycine immunoreactive neurons in the sea lamprey

The complex life cycle of the sea lamprey comprises five different stages: embryos, prolarvae, larvae, metamorphic stages and adults. The first body movements can be observed in late embryonic stages, prolarvae progressively acquire the brain organization and locomotor functionality observed in larvae, which are blind filter-feeders that burrow in the bottom of rivers. At the end of the pigmentation stage, the prolarvae can make full swimming movements and they become adept swimmers at the gill-cleft stage (Piavis, 1971). After a long larval period that can last for up to eight years, the sea lamprey becomes a sighted parasitic feeder passing through a complex metamorphosis in which retina differentiates neurochemically (Abalo et al., 2008) and visual structures of the brain acquire the characteristic organization of adults (De Miguel and Anadón, 1987). The present results suggest that the appearance of glycine immunoreactivity in some neuronal populations is related to different phases of development.

In the lamprey, the first glycine-ir cells appear in the caudal rhombencephalon and spinal cord of late embryos (Villar-Cerviño et al., 2008b; present results), roughly at the time when the first body movements are observed. The caudal hindbrain and rostral spinal cord of the *Xenopus* embryo contains glycinergic commissural interneurons, which play an important role in the generation of the swimming motor pattern (Dale et al., 1986; Roberts et al., 1988). The early appearance in lamprey of cells in a similar location suggests that they are involved in similar reciprocally crossed inhibitory circuits in antagonistic motor systems (Soffe et al., 2001). Moreover, during locomotion, muscle contractions must be coordinated longitudinally to produce the pattern of movements observed in swimming (Soffe et al., 2001). As in *Xenopus* embryos, longitudinally coursing glycine-ir axons were observed in the hindbrain and spinal cord shortly after the first appearance of the first glycinergic neurons.

In the prolarval prosencephalon, the first glycinergic cells became apparent at the prolarval pigmentation stage, i.e. several days after the appearance of the caudal populations. The two glycine-ir cell groups that appear in the prolarval diencephalon are closely related to GABA-ir cells in the same location (Meléndez-Ferro et al., 2002), the axons of which form part of the early axonal longitudinal scaffold (Barreiro-Iglesias et al., 2008). A group of glycine-ir cells was also observed in the telencephalon of prolarvae at the beginning of the burrowing stage. This suggests that glycine is expressed by cells involved in the regulation of the earliest premotor circuits. Cells of the ventral thalamus and posterior tubercle project to the rhombencephalic reticular formation in adult lampreys (El Manira et al., 1997), and it is possible that some GABA- and/or glycine-ir cells observed in the prolarval diencephalon associated with early longitudinal tracts may also project to the brainstem premotor centers. In larvae and adult lamprey, only three glycine-ir cell groups were distinguished in the prosencephalon at levels of the olfactory bulbs, the thalamus and the preoptic nucleus (Villar-Cerviño et al., 2008a; present results). A group of glycine-ir cells appeared in the mesencephalic tegmentum at the end of the prolarval period, when burrowing occurs by lateral head movements and whip-like contractions of the tail. These cells probably correspond to those of the M5 nucleus observed at later stages.

During the long larval period, the lampreys are burrowing filter feeders. Their eyes are still very immature, are located deep under the skin and lack a differentiated lens, and appear more like an ocellus. The retina is poorly differentiated but exhibits glutamate-ir cells in the vertical visual system (Villar-Cerviño et al., 2006) and projects to the diencephalon and - in larvae longer than 70-80 mm - to the immature optic tectum (De Miguel et al., 1990). At metamorphosis, lampreys undergo dramatic changes related to adaptation to the adult sighted parasitic feeding stage; these changes include chemical differentiation of most retinal cells, including the glycine-ir interplexiform neurons (Villar-Cerviño et al., 2006; Abalo et al., 2008). In larval and adult stages, immunoreactivity to glycine was present in cells throughout the brain, predominantly in the isthmus, rhombencephalon and spinal cord (Villar-Cerviño et al., 2008a, b; present results). The prosencephalic, isthmic and rhombencephalic glycine-ir neurons present in larvae were also observed in adults. However, important changes in the midbrain glycine-ir populations were observed in

adults. In the larval midbrain, glycine-ir cells were only found in the M5 nucleus of Schober and the dorsomedial oculomotor region, while in adults two new glycine populations appeared in the alar midbrain, one in the torus semicircularis and other in the optic tectum (Villar-Cerviño et al., 2008a; present results). Since these structures lack glycine-ir cells (the optic tectum also mostly lacked GABA-ir cells) in larval lampreys, this suggests that appearance of these cells is related to the maturation of the optic tectum that accompanies acquisition of fully functional eyes. Although a torus semicircularis is present in larvae (González et al., 1999), the absence of glycine-ir cells in this structure also suggests the existence of important differences between larval and adult lampreys in the organization and circuitry of octavolateral centers.

Comparison with other vertebrates

As indicated above, the first glycine-ir cells appear in the caudal rhombencephalon and spinal cord of late embryos (Villar-Cerviño et al., 2008b; present results). In *Xenopus* embryos, the first glycine-ir neurons were found in the caudal hindbrain region (stage 22), the population increased and extended caudally to the spinal cord (Dale et al., 1986; Roberts et al., 1988), which is similar to that observed in lamprey. In this frog, the onset of expression of the neuronal glycine transporter GLYT2 was observed earlier in embryonic brain development than in the rostral spinal cord (Wester et al., 2008). In zebrafish embryos 36 hours postfertilization (hpf), numerous cells express GLYT2 mRNA in the hindbrain (Cui et al., 2005). Zebrafish are hatched and free-swimming by 4-5 days postfertilization (dpf), which may correspond to either late prolarvae or early larvae in lamprey. At this time, glycinergic cells were numerous in the zebrafish hindbrain and adopted a columnar pattern reminiscent of that observed in prolarvae (Higashijima et al., 2004). In the rat brain, the expression of GLYT2 mRNA and protein has only been studied by Northern blot and Western blot in spinal cord and cerebellar extracts (Zafra et al., 1995b), and data are difficult to compare with those obtained in *Xenopus*, zebrafish and lamprey. However, expression of this transporter begins in late foetal life in the rat spinal cord. However, glycine immunoreactivity was observed in earlier stages in the mouse spinal cord (Allain et al., 2006).

Two glycine-ir cell groups were observed in the diencephalon of the pigmentation stage of prolarval lamprey, and a cell group expressing transient glycine immunoreactivity was also observed in the telencephalon at the beginning of the burrowing stage. In *Xenopus laevis,* GLYT2 mRNA was expressed in an undetermined region of the lateral prosencephalon from hatching stages (Wester et al., 2008), while in zebrafish no GLYT2 expression was found in the prosencephalon of embryos or early larvae (Higashijima et al., 2004; Cui et al., 2005).

In burrowing lamprey prolarvae, a group of glycine-ir cells appeared in the mesencephalic tegmentum. In *Xenopus,* mesencephalic GLYT2 expressing populations were observed at the hatchling stage (Wester et al., 2008), which is earlier than expression of glycine immunoreactivity in the lamprey mesencephalon. In zebrafish 4-5 dpf larvae only a very small number of cells expressing GLYT2 were found in the ventral region of the midbrain (Higashijima et al., 2004), although they were not characterized as an identifiable group. In late lamprey prolarvae, glycine-ir neurons were distributed in the prosencephalon, mesencephalon, rhombencephalon and spinal cord (Villar-Cerviño et al., 2008b; present results). Further comparison of the glycinergic populations of larval lamprey with other vertebrates is precluded by the lack of comparable studies in other species.

Transient glycine immunoreactivity in prolarval lamprey

In some prosencephalic populations glycine immunoreactivity was observed in prolarval stages, but disappeared in larvae. In the telencephalon, transient glycine-ir neurons were found in the septum at the burrowing stage. In the diencephalon, the nmlf contained glycine-ir cells from the pigmentation stage to the end of the prolarval period, but not in larval and adult lampreys, and the same occurs with the glycine-ir cells observed in the hypothalamus, ventral thalamus and pretectum (Villar-Cerviño et al., 2008a; present results). During the prolarval stage, glycine immunoreactivity was found colocalizing with GABA in cells of the hypothalamus, nmlf and the pretectum, but in larvae only GABA-ir cells were observed. In the ventral thalamus of prolarvae, GABA-ir cells were more numerous than glycine-ir cells, and in larvae only GABA-ir neurons remained. In adult lampreys, the nmlf projects to the spinal cord (Ronan, 1989; Swain et al., 1993) and the ventral thalamus, and the pretectal nucleus project to the rhombencephalic reticular

formation, where they are involved in the initiation of rhythmic locomotion (El Manira et al., 1997). It is possible that glycine is involved in these circuits in early stages by acting as a neurotransmitter and/or a trophic signal involved in development. During development, the activation of chloride conducting ion channels gated by GABA or glycine results in depolarization, a phenomenon thought to influence many aspects of embryonic neurodevelopment (Kriegstein and Owens, 2001; Tapia et al., 2001; Ben-Ari, 2002; Owens and Kriegstein, 2002; Banks et al., 2005; Fiumelli and Woodin, 2007). It is also known that chloride channels provide much of the depolarizing activity at times when glutamatergic transmission is primarily silent (Liao and Malinow, 1996). The trophic depolarizing actions of GABA and glycine are thought to be involved in proliferation, migration, differentiation, axon pathfinding, dendritic arborization, and synaptogenesis in both immature (Owens and Kriegstein, 2002; Spitzer, 2006) and adult (Ge et al., 2007) nervous systems.

In the dorsal thalamus, glycine-ir cells were observed in the prolarval period, but they disappeared in the first half of the larval life. In the second half of the larval period, some glycine-ir cells were again observed in the dorsal thalamus and these populations continued in the adult brain (Villar-Cerviño et al., 2008a, present results). Whether the glycine-ir cells observed in the thalamus of prolarvae are the same population as that observed during late development and in adults is not known. The transient expression of a glycinergic marker was previously described in cerebellar interneurons of mouse after study of the expression of GlyT2 gene promoter-driven enhanced green fluorescent protein in BAC transgenic mice (Simat et al., 2007).

Comparison of the developmental pattern of glycine populations with that of GABA

Both glycine-ir and GABA-ir cells were distinguished in late embryos, but GABAergic neurons were more extended in the brain than glycine-ir cells. At this stage, GABA immunoreactivity was found in the diencephalon, in the basal plate of the isthmus and in the caudal rhombencephalon (Meléndez-Ferro et al., 2002, 2003), whereas glycine-ir neurons were only present in the caudal rhombencephalon. Moreover, no glycine-ir cells were observed in the diencephalon until the

pigmentation stage (present results). In the diencephalon of prolarvae, glycine and GABA immunoreactivities appear codistributed in several cell groups, and in most they were located in the same cells, except in the ventral thalamus where only scarce cells showed colocalization of both neurotransmitters. Most of these doubly labeled populations lose glycine immunoreactivity in larvae (see above), although in the larval and adult stages these populations are GABAergic (Meléndez-Ferro et al., 2002; Robertson et al., 2007; Villar-Cerviño et al., 2008a; present results), which indicates a shift in the neurotransmitter phenotype. The same occurs in the telencephalon, where the septum contained GABA-ir cells that also showed transient glycine immunoreactivity in prolarvae. This shift appears to follow a direction opposite to that reported in mammals for some neuronal systems. A profound transition from GABAergic to glycinergic transmission was observed in the lateral superior olive of young rodents (Kotak et al., 1998; Nabekura et al., 2004). It was also described that GABAergic transmission predominates over that of glycine in the spinal cord of early stages rats and that either the number of glycinergic synapses or the probability of vesicular glycine release increased during the period studied (Gao et al., 2001). This may reflect disparity in the time of appearance of glycine with respect to GABA (Allain et al., 2006).

Glycine and GABA populations were not distinguished in the mesencephalic tegmentum until late prolarval or early larval stages (Meléndez-Ferro et al., 2002; present results). Most cells of the M5 nucleus showed colocalization of both immunoreactivities in larvae. In the isthmus, the pattern of codistribution of glycine-ir and GABA-ir cells appears to be similar from prolarval to adult stages. The dorsal isthmal grey population showed only glycine-ir cells throughout development, whereas colocalization of glycine and GABA and also glycine-ir/GABA-negative and GABA-ir/glycine-negative cells was observed in the same cells in the isthmic reticular region (Villar-Cerviño et al., 2008a; present results). This suggests that neurotransmitter phenotype is maintained in these populations.

In the trigeminal region of the lamprey brain glycine-ir only, GABA-ir only and double labeled neurons were observed from prolarvae to adults. In contrast, in the octaval region of prolarvae GABA-ir cells were scarce and all of them also displayed glycine immunoreactivity, but in larvae and adult lampreys glycine-ir/GABA-ir, glycine-ir/GABA-negative and GABA-ir/glycine-negative cells were

observed (Villar-Cerviño et al., 2008a; present results). In the caudal rhombencephalon, the degree of colocalization of glycine and GABA was lower than in rostral levels during development and in adult lampreys. Higashijima et al. (2004) observed that in 4-5 dpf zebrafish larvae GAD-positive cells tend to lie relatively dorsal (periventricular) in the hindbrain, while GLYT2-positive cells tend to be located more ventrally (away from the ventricle). In the larval lamprey hindbrain differences in location of glycine-ir and GABA-ir perikarya with respect the ventricular surface were not appreciable in most regions. In general, lamprey neurons are mainly located in periventricular positions in larvae, which suggests that the initial organization of populations reported by Higashijima et al. (2004) in zebrafish reflects the more advanced and complex organization of the teleost brain.

Final considerations

In lamprey, the first glycine-ir cells appeared in the caudal rhombencephalon and spinal cord of late embryos, which suggests that they play a role in generation of the swimming motor pattern. Several glycine-ir cell groups were observed in the prosencephalon during the prolarval period, but in larvae and adult lamprey, only three glycine-ir cell groups were distinguished at the level of the olfactory bulbs, the thalamus and the preoptic nucleus. The presence of transient glycine-ir populations in the brain of prolarvae suggests that they may play different roles in development. From late prolarvae to adults, glycine-ir cells were present in the prosencephalon, mesencephalon, rhombencephalon and spinal cord. Although the first mesencephalic glycine-ir neurons appeared at the end of the prolarval period, major glycine-ir populations of the adult midbrain such as those of the optic tectum and torus semicircularis were not detected in the larval period studied, which suggests that the appearance of these populations is delayed until metamorphosis.

GABA immunoreactivity was more widely distributed than glycine in the brain of lampreys from late embryonic stages onwards. In the prosencephalon of the sea lamprey a shift in neurotransmitter phenotype was observed during development from glycine-ir/GABA-ir to only GABA-ir, which is the opposite of the trend reported in some nuclei of mammals. In contrast, the isthmic and trigeminal populations maintained their neurotransmitter phenotype from prolarvae to adults. In

all developmental stages, the degree of colocalization of glycine and GABA in the caudal rhombencephalon was lower than at rostral levels.

Abbreviations

DC	dorsal column
DCN	dorsal column nucleus
DIG	dorsal isthmic grey
DN	dorsal nucleus of the octavolateral area
DTh	dorsal thalamus
dV	descending root of the trigeminal nerve
FR	fasciculus retroflexus
GL	glomeruli
Ha	habenula
Hy	hypothalamus
I1	I1 Müller cell
IGL	inner granular layer
IIID	dorsomedial (rostral oblique) oculomotor subnucleus
IIIL	lateral (rostral rectus) oculomotor subnucleus
Ip	interpeduncular nucleus
Is	isthmus
IsRF	isthmic reticular formation
IX	glossopharyngeal motor nucleus
LP	lateral pallium
M	mesencephalon
M3	Müller cell 3
M5	Schober's M5 nucleus
MN	medial nucleus of the octavolateral area
MO	medulla
MRF	middle rhombencephalic reticular formation
N	notochord
nmlf	nucleus of the medial longitudinal fascicle
OB	olfactory bulb
OLA	octavolateral area
OT	optic tectum
OV	otic vesicle
PoC	postoptic commissure
PoR	postoptic recess
Po-Tu	postoptic-tuberal region
PRF	posterior rhombencephalic reticular formation
Pro	preoptic nucleus
Pt	pretectal region
SC	spinal cord
SOC	spino-occipital motor column
Sp	septum
Th	thalamic region
TL	terminal lamina
TRF	trigeminal reticular formation
TS	torus semicircularis
TSN	trigeminal spinal nucleus
vDTh	ventral part of the dorsal thalamus
Vg	trigeminal ganglion
VId	dorsal (ventral rectus) abducens subnucleus
VIIm	facial motor nucleus
VIv	ventral (caudal rectus) abducens subnucleus
Vm	trigeminal motor nucleus
VN	ventral nucleus of the octavolateral area
VTh	ventral thalamus (prethalamus)
Xm	vagal motor nucleus
ZL	zona limitans intrathalamica

Fig. 1. Inverted grey scale confocal micrographs of transverse sections through the brain of embryonic (A), hatchling (B-C), pigmentation (D-G) and gill cleft (H-L) sea lampreys, showing glycine-ir structures. **A**, Section showing glycine-ir cells (arrow) in the caudal rhombencephalon of an E12 embryo. **B**, Section showing glycine-ir cells (arrow) in the caudal rhombencephalon of a P0 prolarvae. **C**, Section through isthmic levels of a P0 prolarvae showing glycine-ir fibers. **D**, Section showing glycine-ir cells in the nmlf and CSF-c glycine-ir cells in the postoptic region of the hypothalamus (double arrow) of a P2 prolarvae. **E**, Section through the isthmus of a P2 prolarvae showing glycine-ir cells (arrows). **F**, Section of the rhombencephalon of a P2 prolarvae at the level of the otic vesicle showing ventral glycine-ir cells. **G**, Section of a P2 caudal rhombencephalon showing glycine-ir neurons (arrows) and glycine-ir fibers coursing in the marginal region. **H**, Section through the postoptic hypothalamus and the nmlf of a P4 prolarvae showing glycine-ir cells. **I**, Section through the isthmic level of a P4 prolarvae showing a dorsal (thin arrow) and a ventral (thick arrow) glycine-ir cell group. **J**, Section through the trigeminal level of the rhombencephalon of a P4 prolarvae with some glycine-ir cells (arrow). **K**, Section through the octaval level of the rhombencephalon of a P4 prolarvae showing scarce glycine-ir cells (arrow). **L**, Section through the caudal rhombencephalon of a P4 prolarvae showing numerous glycine-ir cells (arrows). For abbreviations, see list. Scale bars = 25 μm.

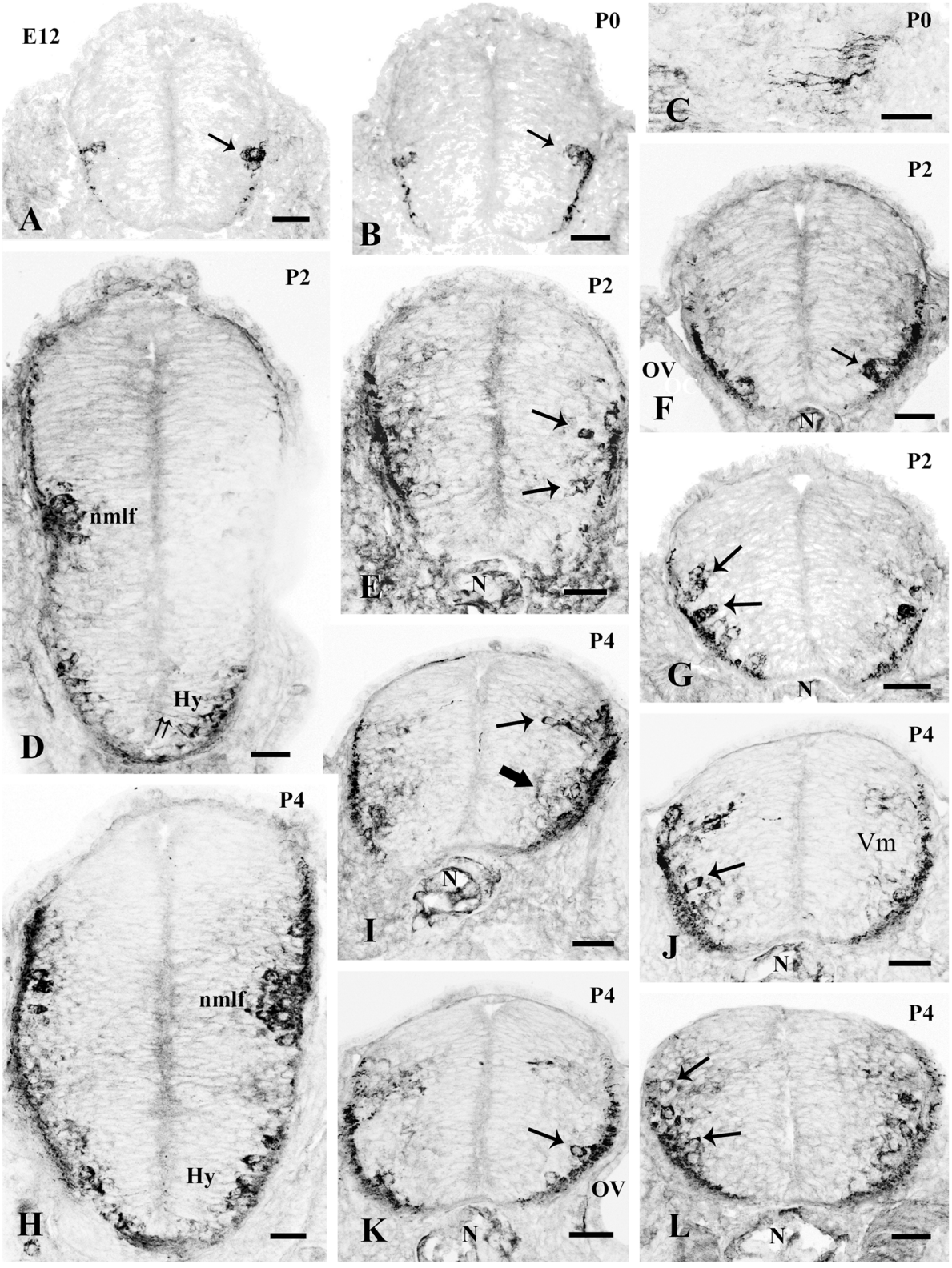

E12
A
P0
B
P0
C
P2
nmlf
Hy
D
P2
E
N
P2
OV
F
N
P2
G
N
P4
I
N
P4
Vm
J
N
P4
nmlf
Hy
H
P4
K
N
OV
P4
L
N

Fig.1.

Fig. 2. Inverted greyscale confocal photomicrographs of transverse sections through the brain of an early burrowing stage prolarvae (P8). **A**, Section through the telencephalon showing CSF-c glycine-ir cells in the septum (arrow). Note the prolongation of the CSF-c cells to the ventricle (double arrow). **B**, Section through the diencephalon showing glycine-ir cells in the nmlf and the hypothalamus. Note hypothalamic glycine-ir cells (arrow) that exhibit a long dendrite that contacts with the ventricle (double arrow). **C**, Section showing glycine-ir cells at thalamic levels (arrow). **D**, Section showing the dorsal (thin arrow) and the ventral (thick arrow) glycine-ir populations of the isthmus. **E**, Section through the trigeminal rhombencephalic level showing the dorsal (thin arrow) and ventral (thick arrow) glycine-ir cell groups. **F**, Section through the octaval rhombencephalic level showing the dorsal (thin arrow) and ventral (thick arrow) glycine-ir cell groups. **G**, Section through the caudal rhombencephalon with glycine-ir cells. **H**, Section through the most caudal rhombencephalon with glycine-ir cells in a single group. For abbreviations, see list. Scale bars = 50 μm in A, B, C and D, 25 μm in E, F, G and H.

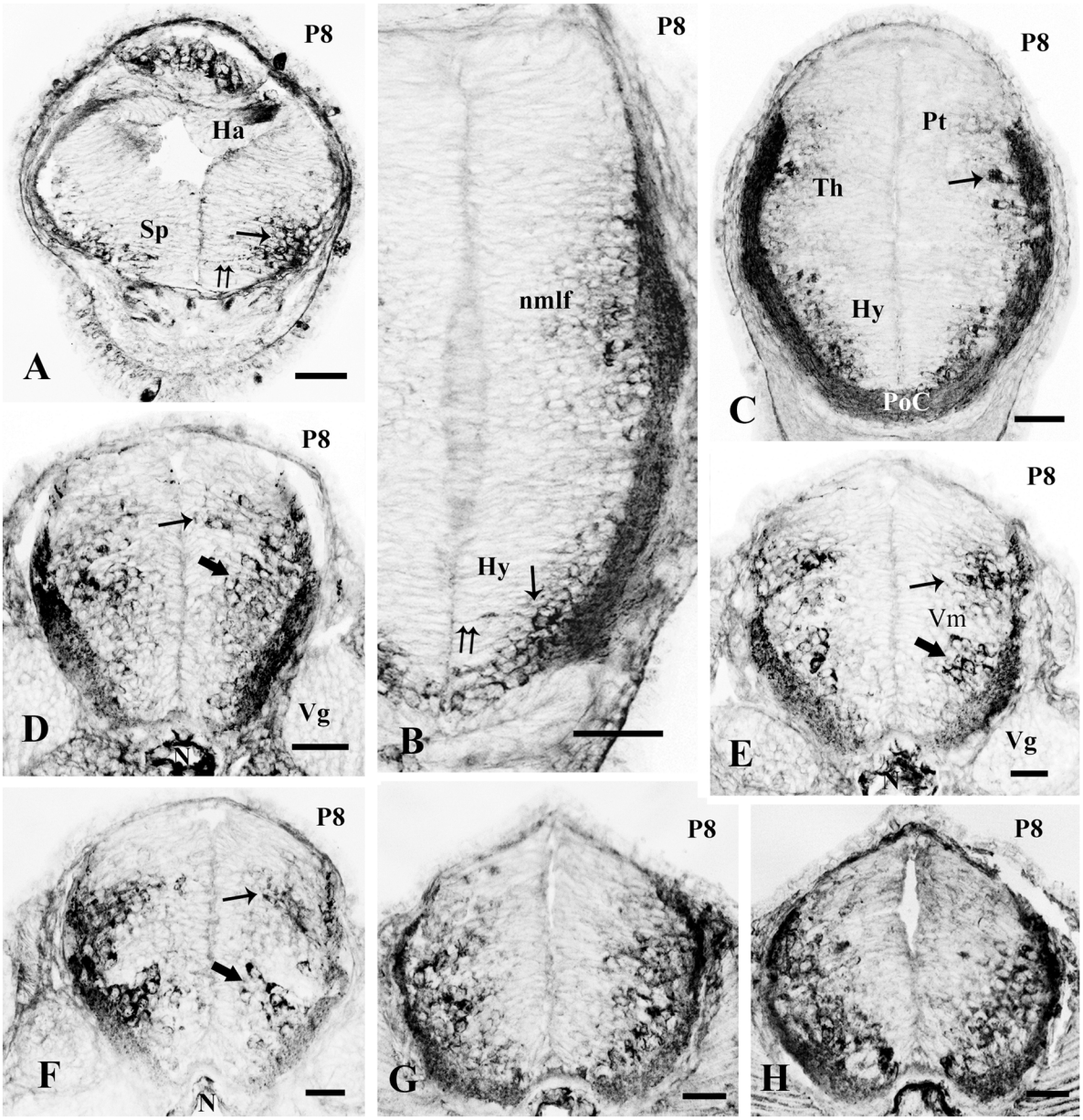

P8
Ha
Sp
A
P8
nmlf
Hy
B
P8
Pt
Th
Hy
PoC
C
P8
Vg
N
D
P8
Vm
Vg
N
E
P8
N
F
P8
G
P8
H

Fig.2.

Fig. 3. Inverted grey scale confocal photomicrographs of transverse sections through the brain of an intermediate burrowing stage prolarvae (P15). **A**, Section through the telencephalon showing glycine-ir fibers coursing mainly in the lateral marginal region (double arrow). **B**, Section through the diencephalon showing glycine-ir cells in both the thalamus and the preoptic nucleus. Note also the numerous glycine-ir fibers that cross the midline at the postoptic commissural region. **C**, Section through the diencephalon showing glycine-ir cell groups in the pretectum, dorsal thalamus, ventral thalamus (arrows) and in the postoptic-tuberal hypothalamus. **D**, Section showing glycine-ir cells in the M5 nucleus and in the nmlf. **E**, Section showing the dorsal (thin arrow) and ventral (thick arrow) glycine-ir populations of the isthmus. **F**, Section through the trigeminal rhombencephalic level showing the dorsal (thin arrow) and ventral (thick arrow) glycine-ir cell groups. **G**, Section through the octaval rhombencephalic level showing the dorsal (thin arrow) and ventral (thick arrow) glycine-ir cell groups. **H**, Section through the caudal rhombencephalon (vagal region) showing scarce dorsal glycine-ir cells (thin arrow) and numerous ventral glycine-ir cells (thick arrow). For abbreviations, see list. Scale bars = 50 μm.

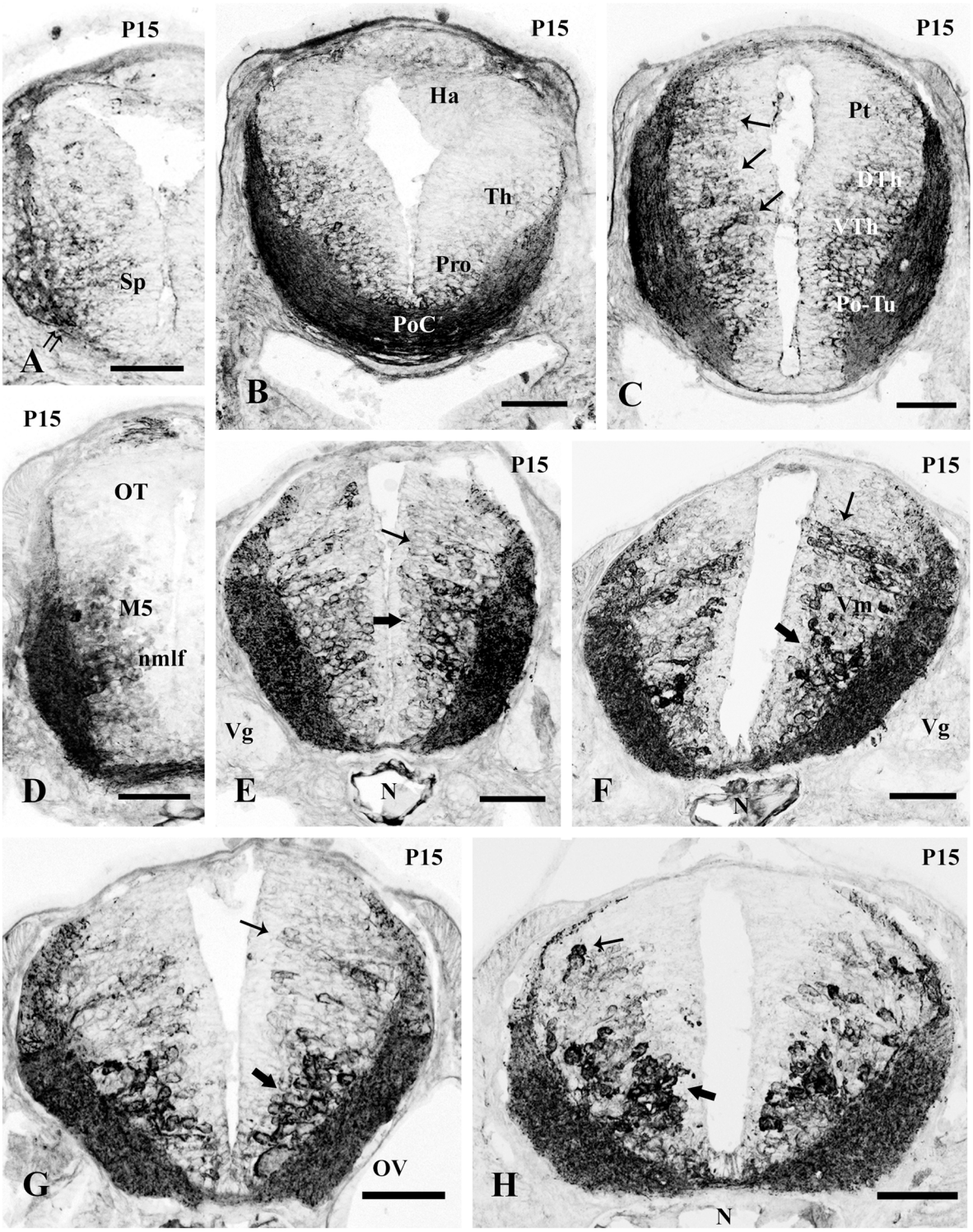

P15
Sp
A
P15
Ha
Th
Pro
PoC
B
P15
Pt
DTh
VTh
Po-Tu
C
P15
OT
M5
nmlf
D
P15
Vg
N
E
P15
Vm
Vg
N
F
P15
OV
G
P15
N
H

Fig.3.

Fig. 4. Confocal photomicrographs of double immunolabeled sections showing glycine-ir (magenta) and GABA-ir (green) structures in several brain prolarvae regions. **A-A''**, Section of a P4 prolarvae through the septum of early burrowing stage prolarvae where colocalization of glycine and GABA can be observed (arrow). **B-B''**, Detail of the preoptic nucleus of a P15 prolarvae with many double labeled cells (arrow). **C-C''**, Section through the hypothalamus of a P8 prolarvae where most cells exhibit double labeled immunoreactivity. Note dendrites of the CSF-c cells (double arrow). **D-D''**, Detail of the double labeled cells (arrow) in the nflm of a P4 prolarvae. **E-E''**, Section through the diencephalon of a P15 prolarvae showing colocalization of glycine and GABA in the pretectum, ventral part of the dorsal thalamus (just adjacent to the zona limitans) and ventral thalamus (arrows). **F-F''**, Detail of the ventral isthmus (primordium of the isthmic reticular population) of a P8 prolarvae showing the colocalization of glycine and GABA in this cell group (arrow). **G-G''**, Detail of the ventral trigeminal population of a P8 prolarvae with some glycine-ir cells are also GABA-ir (arrow). **H-H''**, Detail of the dorsal trigeminal population of a P8 prolarvae showing colocalization of glycine and GABA (arrow). **I-I''**, Section through the caudal rhombencephalon of a P15 prolarvae in which glycine-ir cells are more numerous and larger than GABA-ir cells. The green cells located in the meninges are autofluorescent red blood cells. Scale bar = 25 μm.

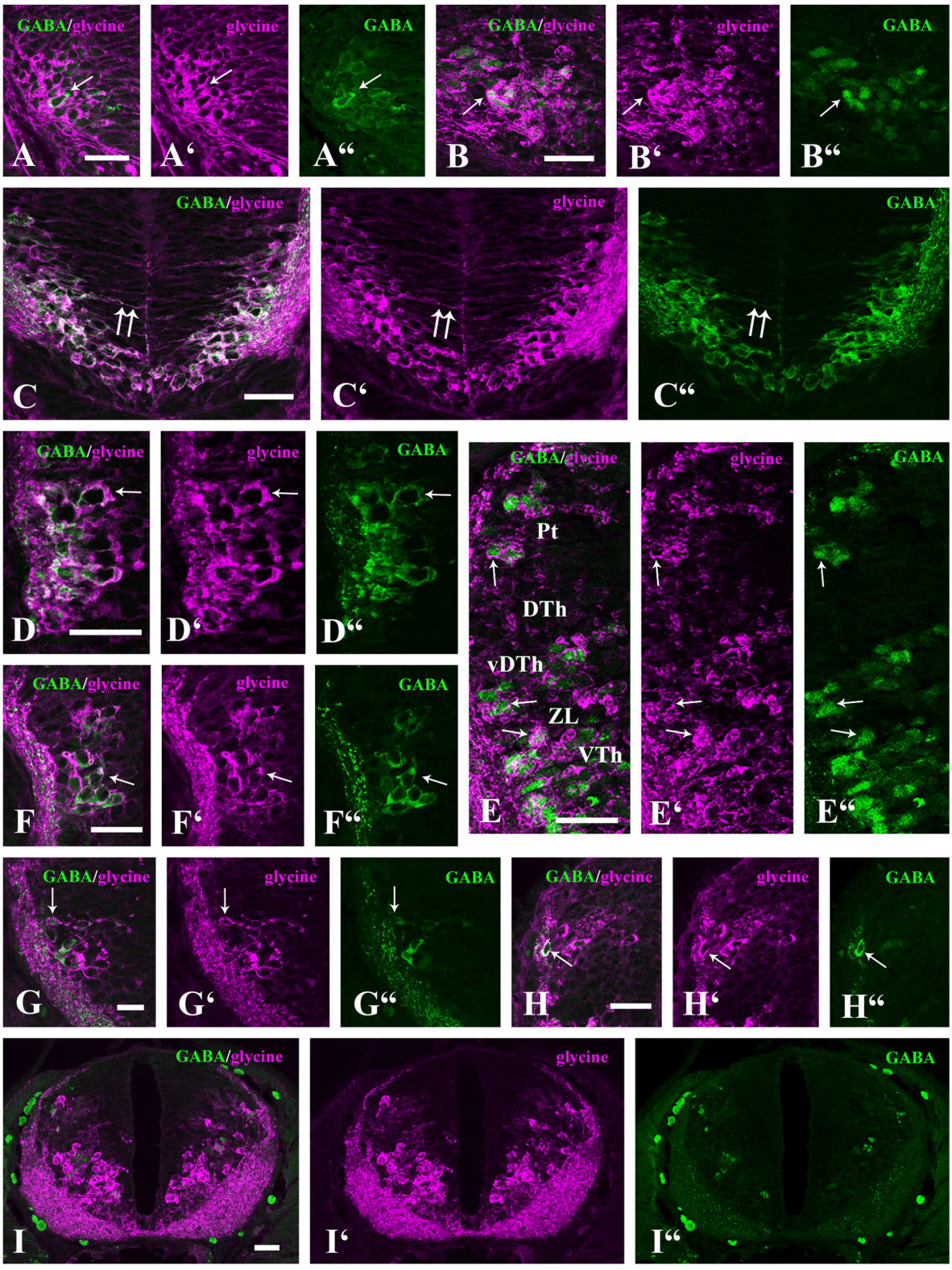

GABA/glycine
glycine
GABA
A
A‘
A“
B
B‘
B“
C
C‘
C“
D
D‘
D“
Pt
DTh
vDTh
ZL
VTh
E
E‘
E“
F
F‘
F“
G
G‘
G“
H
H‘
H“
I
I‘
I“

Fig.4.

Fig. 5. Schematic drawing of transverse sections of the larvae sea lamprey brain showing the distribution of glycine immunoreactive cells and fibers (on the right), and main brain structures (on the left). **A**, Olfactory bulb. **B**, Telencephalon. **C-D**, Diencephalon. **E**, Mesencephalon. **F**, Isthmus. **G-J**, Rhombencephalon. The level of sections is indicated in the upper right-hand-side figure. The glycinergic cells were magnified by two times for better visualization. Correspondence with photomicrographs in other figures is indicated by squared areas. For abbreviations, see list. Scale bar = 50 μm.

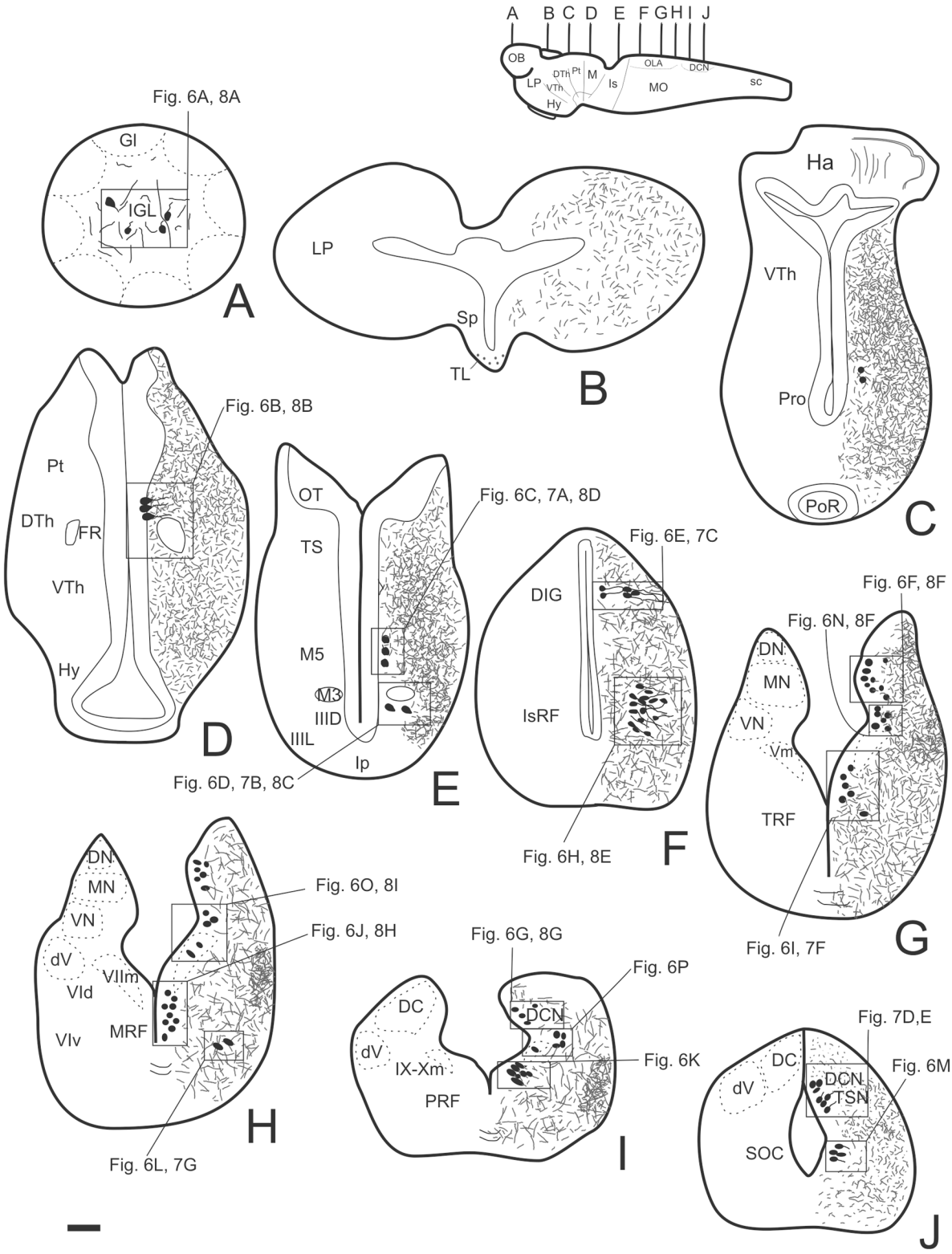

A B C D E F G H I J
OB
LP
DTh
VTh
Pt
M
Hy
Is
OLA
MO
DCN
SC
Fig. 6A, 8A
Gl
IGL
A
LP
Sp
TL
B
Ha
VTh
Pro
PoR
C
Fig. 6B, 8B
Pt
DTh
FR
VTh
Hy
D
OT
TS
M5
M3
IIID
IIIL
Ip
Fig. 6C, 7A, 8D
Fig. 6D, 7B, 8C
E
DIG
IsRF
Fig. 6E, 7C
Fig. 6H, 8E
F
Fig. 6F, 8F
Fig. 6N, 8F
DN
MN
VN
Vm
TRF
Fig. 6I, 7F
G
DN
MN
VN
dV
VIId
VIIm
VIv
MRF
Fig. 6O, 8I
Fig. 6J, 8H
Fig. 6L, 7G
H
Fig. 6G, 8G
Fig. 6P
DC
DCN
dV
IX-Xm
PRF
Fig. 6K
I
Fig. 7D,E
DC
dV
DCN
TSN
Fig. 6M
SOC
J

Fig.5.

Fig. 6. Inverted grey scale confocal photomicrographs of transverse sections through several brain levels of a 50 mm larvae. **A**, Section showing glycine-ir cells in the olfactory bulbs. **B**, Detail of the glycine-ir cells of the dorsal thalamus. **C**, Section through the M5 nucleus of Schober showing glycine-ir cells with thick dendrites extending laterally (arrow). **D**, Detail of the glycine-ir cells (arrow) at the level of the oculomotor nucleus. **E**, Section through the dorsal isthmus showing a glycine-ir population close to the ventricle (arrowheads) and glycine-ir cells in the dorsal isthmal grey (arrows). **F**, Section through the octavolateral area showing glycine-ir cells both in periventricular regions (arrowheads) and more lateral positions (arrow). **G**, Detail of the dorsal column nucleus showing glycine-ir cells in the periventricular layer (arrowheads) and in the lateral neuropil (arrows). **H**, Section showing glycine-ir cells in the ventral isthmic reticular formation. **I**, Section showing glycine-ir cells located ventral to the trigeminal motor nucleus. **J**, Section through the middle rhombencephalic reticular region with glycine-ir cells located in the periventricular layer. Note the dendrite extending laterally from some of these cells (arrows). **K**, Section through the posterior rhombencephalic reticular region showing a compact group of glycine-ir cells located ventromedial to the glossopharyngeal-vagal motor column. **L**, Section through the abducens nucleus showing glycine-ir cells both in periventricular (arrowheads) and lateral regions (arrow). **M**, Section through the spino-occipital nucleus showing intense glycine-ir cells with dendrites coursing to the adjacent neuropil (arrow). **N**, Detail of the region dorsal to the trigeminal motor nucleus where glycine-ir cells are located in the periventricular cell layer (arrowheads). Note the dendrites extending towards the trigeminal descending root (arrow). **O**, Section through the facial motor nucleus showing glycine-ir cells intermingled with facial motoneurons. **P**, Detail of the glossopharyngeal-vagal motor column with glycine-ir cells located both lateral to and intermingled with motoneurons. Correspondence with photomicrographs in other figures is indicated by squared areas. For abbreviations, see list. Scale bar = 25 μm.

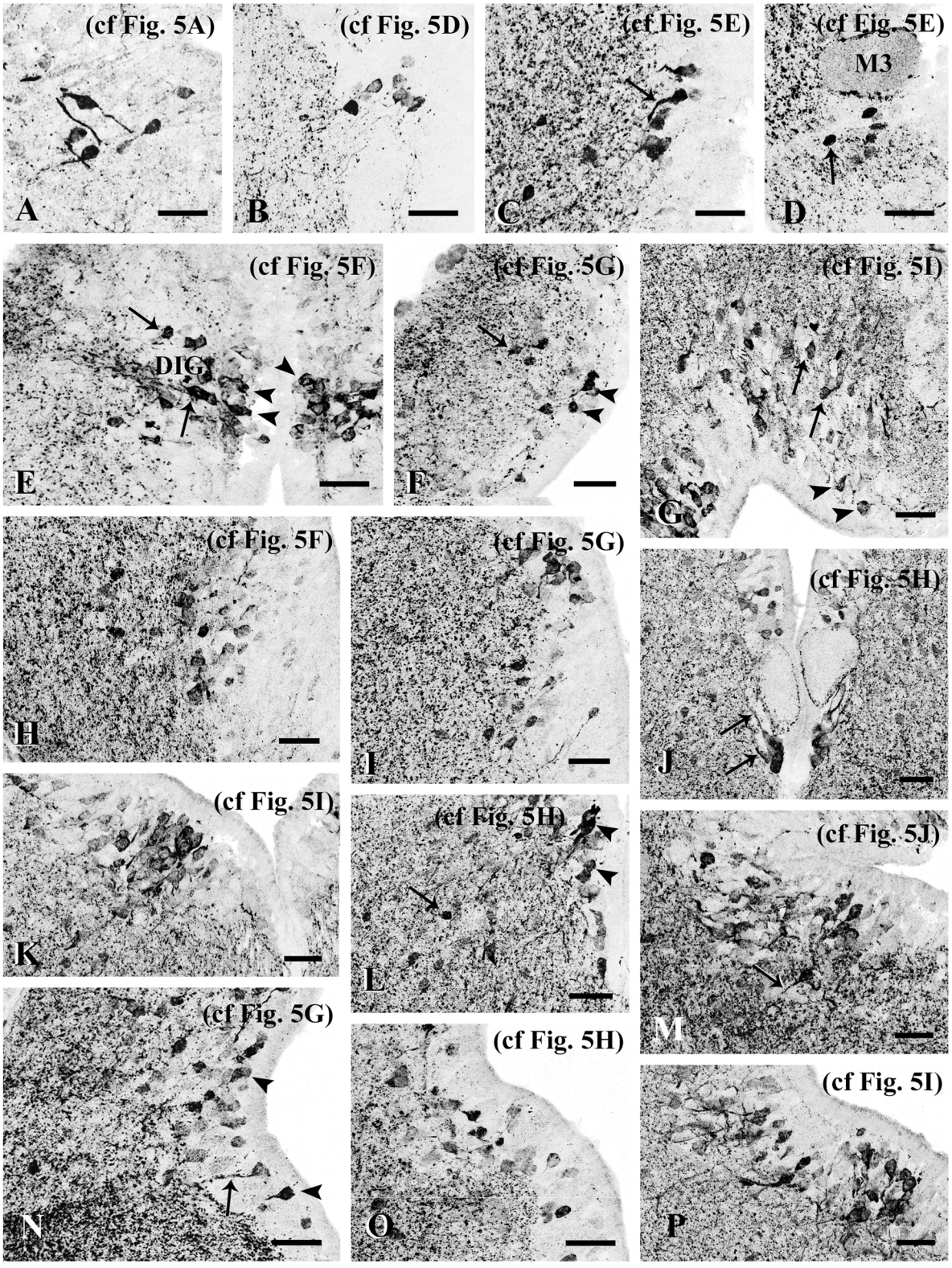

(cf Fig. 5A)
A
(cf Fig. 5D)
B
(cf Fig. 5E)
C
(cf Fig. 5E)
M3
D
(cf Fig. 5F)
DIG
E
(cf Fig. 5G)
F
(cf Fig. 5I)
G
(cf Fig. 5F)
H
(cf Fig. 5G)
I
(cf Fig. 5H)
J
(cf Fig. 5I)
K
(cf Fig. 5H)
L
(cf Fig. 5J)
M
(cf Fig. 5G)
N
(cf Fig. 5H)
O
(cf Fig. 5I)
P

Fig.6.

Fig. 7. Confocal photomicrographs of double immunolabeled sections showing glycine-ir (magenta) and NB (green) structures in several larval brain regions. **A**, Detail of the glycine-ir cells at the level of the M5 nucleus of Schober labeled after the application of NB to the eye orbit. **B**, Detail of the glycine-ir cells at the level of the oculomotor nucleus labeled after application of NB to the eye the orbit. **C**, Section through the isthmus showing the position of the glycine-ir cells of the dorsal isthmal grey with respect to the big I1 Müller cell labeled after application of NB to the rostral spinal cord. **D**, Detail of the glycine-ir cell groups of the DCN and TSN labeled after application of NB to the eye orbit. **E**, Detail of the location of glycine-ir cell groups of the DCN and TSN labeled after application of NB to the rostral spinal cord. **F**, Section showing glycine-ir cells located ventral to the trigeminal motor nucleus labeled after the application of NB to the eye orbit. **G**, Detail of glycine-ir cells in the reticular region containing migrated cells of the abducens nucleus labeled after the application of NB to the eye orbit. For abbreviations, see list. Scale bar = 50 μm in C, 25 μm in A, B, D, E, F and G.

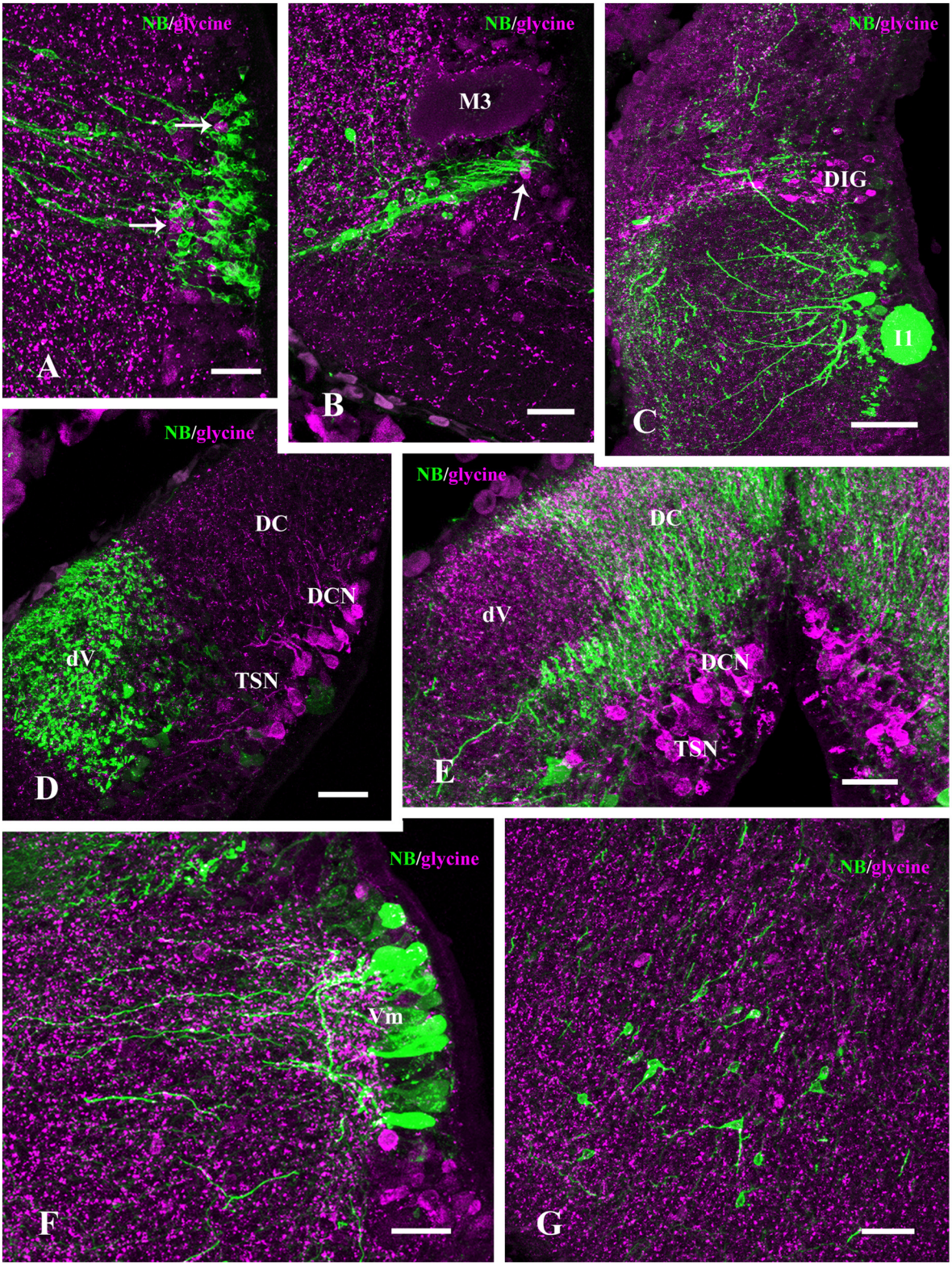

NB/glycine
A
NB/glycine
M3
B
NB/glycine
DIG
I1
C
NB/glycine
DC
DCN
dV
TSN
D
NB/glycine
DC
dV
DCN
TSN
E
NB/glycine
Vm
F
NB/glycine
G

Fig.7.

Fig. 8. Confocal photomicrographs of double immunolabeled sections showing glycine-ir (magenta) and GABA-ir (green) structures in several larval brain regions. **A**, Detail of the inner layer of the olfactory bulb showing glycine and GABA immunoreactivity in different cells. **B**, Detail of glycine-ir cells of the dorsal thalamus. **C**, Detail at the level of the oculomotor nucleus where glycine and GABA do not colocalize in the same cells. **D**, Detail at the level of the M5 nucleus of Schober showing double labeled cells (arrow). **E-E''**, Section through the isthmic reticular formation where cells located near the ependymal layer are GABA-ir only, at adjacent positions there are cells that show both GABA and glycine immunoreactivity (arrow), and at most lateral positions cells are glycine-ir only. **F-F''**, Section through the octavolateral area and the lateral region of the trigeminal motor nucleus. Note that most medial nucleus cells show only glycine-ir cells or only GABA-ir cells, whereas in the lateral reticular zone most cells are double labeled (arrows). **G-G''**, Section through the DCN showing some double labeled glycine-ir cells. **H-H''**, Section through the medial zone of the facial nucleus showing many cells that display both glycine and GABA immunoreactivity (arrows). **I**, Section through the lateral reticular zone associated with the facial motor nuclei showing high colocalization of glycine and GABA (arrows). Correspondence with photomicrographs in other figures is indicated by squared areas. For abbreviations, see list. Scale bar = 25 μm.

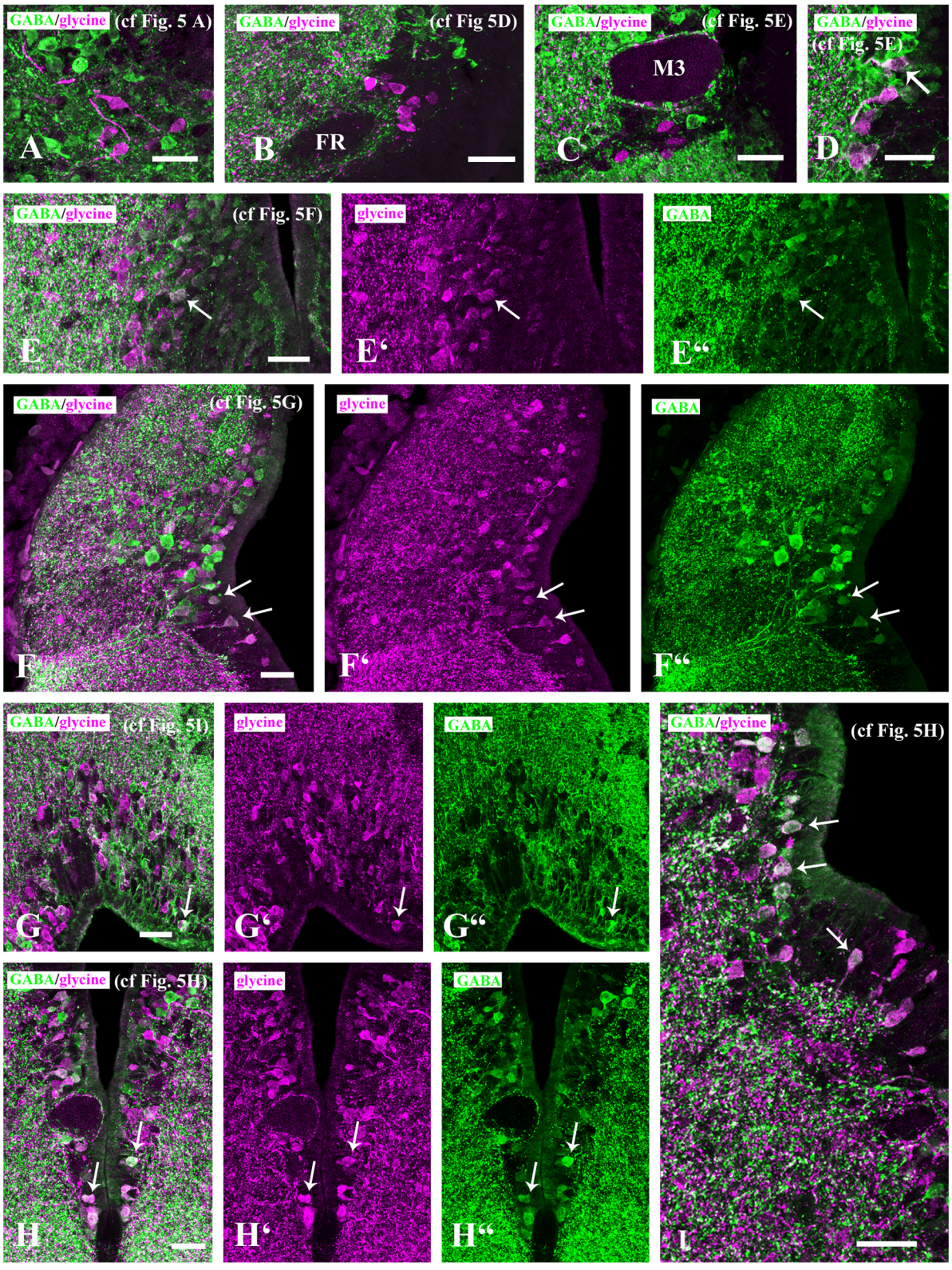

GABA/glycine (cf Fig. 5 A)
A
GABA/glycine (cf Fig 5D)
B
FR
GABA/glycine (cf Fig. 5E)
M3
C
GABA/glycine (cf Fig. 5E)
D
GABA/glycine (cf Fig. 5F)
E
glycine
E‘
GABA
E“
GABA/glycine (cf Fig. 5G)
F
glycine
F‘
GABA
F“
GABA/glycine (cf Fig. 5I)
G
glycine
G‘
GABA
G“
GABA/glycine (cf Fig. 5H)
I
GABA/glycine (cf Fig. 5H)
H
glycine
H‘
GABA
H“

Fig.8.

REFERENCES

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

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

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

Allain AE, Baïri A, Meyrand P, Branchereau P. 2006. Expression of the glycinergic system during the course of embryonic development in the mouse spinal cord and its co-localization with GABA immunoreactivity. J Comp Neurol 496:832-846.

Anadón R, De Miguel E, González-Fuentes MJ, Rodicio C. 1989. HRP study of the central components of the trigeminal nerve in the larval sea lamprey: organization and homology of the primary medullary and spinal nucleus of the trigeminus. J Comp Neurol 283:602-610.

Aoki E, Semba R, Keino H, Kato K, Kashiwamata S. 1988. Glycine-like immunoreactivity in the rat auditory pathway. Brain Res 442:63-71.

Banks GB, Kanjhan R, Wiese S, Kneussel M, Wong LM, O'Sullivan G, Sendtner M, Bellingham MC, Betz H, Noakes PG. 2005. Glycinergic and GABAergic synaptic activity differentially regulate motoneurons survival and skeletal muscle innervation. J Neurosci 25:1249-1259.

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

Ben-Ari Y. 2002. Excitatory actions of GABA during development: the nature of the nurture. Nat Rev Neurosci 3:728-739.

Brox A, Ferreiro B, Puelles L, Medina L. 2002. The telencephalon of the frog *Xenopus* based on calretinin immunostaining and gene expression patterns. Brain Res Bull 57:381-384.

Cui WW, Low SE, Hirata H, Saint-Amant L, Geisler R, Hume RI, Kuwada JY. 2005. The zebrafish shocked gene encodes a glycine transporter and is essential for the function of early neural circuits in the CNS. J Neurosci 25:6610-6620.

Dale N, Ottersen OP, Roberts A, Storm-Mathisen J. 1986. Inhibitory neurones of a motor pattern generator in *Xenopus* revealed by antibodies to glycine. Nature 324:255-257.

Dale N, Roberts A, Ottersen OP, Storm-Mathisen J. 1987a. The development of a population of spinal cord neurons and their axonal projections revealed by GABA immunocytochemistry in frog embryos. Proc R Soc Lond B 232:205-215.

Dale N, Roberts A, Ottersen OP, Storm-Mathisen J. 1987b. The morphology and distribution of "Kolmer-Agduhr cells", a class of cerebrospinal-fluid-contacting neurons revealed in the frog embryo spinal cord by GABA immunocytochemistry. Proc R Soc Lond B 232:193-203.

De Miguel E, Anadón R. 1987. The development of retina and the optic tectum of *Petromyzon marinus*, L. A light microscopic study. J Hirnforsch 28:445-56.

De Miguel E, Rodicio MC, Anadón R. 1990. Organization of the visual system in larval lampreys: an HRP study. J Comp Neurol 302:529-542.

Ekström P, Ohlin LM. 1995. Ontogeny of GABA-immunoreactive neurons in the central nervous system in a teleost, *Gasterosteus aculeatus* L. J Chem Neuroanat 9:271-288.

El Manira A, Pombal MA, Grillner S. 1997. Diencephalic projection to reticulospinal neurons involved in the initiation of locomotion in adult lampreys Lampetra fluviatilis. J Comp Neurol 389:603-616.

Ezure K, Tanaka I, Kondo M. 2003.Glycine is used as a transmitter by decrementing expiratory neurons of the ventrolateral medulla in the rat. J Neurosci 23:8941-8948.

Fiumelli H, Woodin MA. 2007. Role of activity-dependent regulation of neuronal chloride homeostasis in development. Curr Opin Neurobiol 17:81-86.

Friauf E, Aragón C, Löhrke S, Westenfelder B, Zafra F. 1999. Developmental expression of the glycine transporter GLYT2 in the auditory system of rats suggests involvement in synapse maturation. J Comp Neurol 412:17-37.

Fritzsch B, Sonntag R, Dubuc R, Ohta Y, Grillner S. 1990. Organization of the six motor nuclei innervating the ocular muscles in lamprey. J Comp Neurol 294:491-506.

Gao BX, Stricker C, Ziskind-Conhaim L. 2001. Transition from GABAergic to glycinergic synaptic transmission in newly formed spinal networks. J Neurophysiol 86:492-502.

Ge S, Pradhan DA, Ming GL, Song H. 2007. GABA sets the tempo for activity-dependent adult neurogenesis. Trends Neurosci 30:1-8.

González MJ, Anadón R. 1992. Primary projections of the lateral line nerves in larval sea lamprey, *Petromyzon marinus* L. An HRP study. J Hirnforsch 33:185-194.

González MJ, Anadón R. 1994. Central projections of the octaval nerve in larval lamprey: an HRP study. J Hirnforsch 35:181-189.

González MJ, Manso MJ, Anadón R. 1997. Octavolateral neurons projecting to the middle and posterior rhombencephalic reticular nuclei of larval lamprey: a retrograde horseradish peroxidase labeling study. J Comp Neurol 384:396-408.

González MJ, Pombal MA, Rodicio MC, Anadón R. 1998. The internuclear neurons of the ocular motor system of the larval sea lamprey. J Comp Neurol 401:1-15.

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

Henkel CK, Brunso-Bechtold JK. 1995. Development of glycinergic cells and puncta in nuclei of the superior olivary complex of the postnatal ferret. J Comp Neurol 354:470-480.

Higashijima S, Mandel G, Fetcho JR. 2004. Distribution of prospective glutamatergic, glycinergic, and GABAergic neurons in embryonic and larval zebrafish. J Comp Neurol 480:1-18.

Katarova Z, Sekerková G, Prodan S, Mugnaini E, Szabó G. 2000. Domain-restricted expression of two glutamic acid decarboxylase genes in midgestation mouse embryos. J Comp Neurol 424:607-627.

Kemmer M, Vater M. 1997. The distribution of GABA and glycine immunostaining in the cochlear nucleus of the mustached bat (*Pteronotus parnellii*). Cell Tissue Res 287:487-506.

Kolston J, Osen KK, Hackney CM, Ottersen OP, Storm-Mathisen J. 1992. An atlas of glycine-like and GABA-like immunoreactivity and colocalization in the cochlear nuclear complex of the guinea pig. Anat Embryol 186:443-465.

Kotak VC, Korada S, Schwartz IR, Sanes DH. 1998. A developmental shift from GABAergic to glycinergic transmission in the central auditory system. J Neurosci 18:4646-4655.

Kriegstein AR, Owens DF. 2001. GABA may act as a self-limiting trophic factor at developing synapses. Sci STKE 95:PE1.

Liao D, Malinow R. 1996. Deficiency in induction but not expression of LTP in hippocampal slices from young rats. Learn Mem 3:138-149.

Meléndez-Ferro M. 2001. Distribución de la inmunorreactividad al ácido gamma-aminobutírico (GABA) en el sistema nervioso central de la lamprea de mar durante el desarrollo y la etapa adulta. Ph.D. Dissertation, University of Santiago de Compostela.

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

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

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

Mueller T, Vernier P, Wullimann MF. 2006. A phylotypic stage in vertebrate brain development: GABA cell patterns in zebrafish compared with mouse. J Comp Neurol 494:620-634.

Mueller T, Wullimann MF, Guo S. 2008. Early teleostean basal ganglia development visualized by zebrafish Dlx2a, Lhx6, Lhx7, Tbr2 (eomesa), and GAD67 gene expression. J Comp Neurol 507:1245-1257.

Nabekura J, Katsurabayashi S, Kakazu Y, Shibata S, Matsubara A, Jinno S, Mizoguchi Y, Sasaki A, Ishibashi H. 2004. Developmental switch from GABA to glycine release in single central synaptic terminals. Nat Neurosci 7:17-23.

Osen KK, Ottersen OP, Storm-Mathisen J. 1990. Colocalization of glycine-like and GABA-like immunoreactivities: a semiquantitative study of neurons in the dorsal cochlear nucleus of the cat. In: Ottersen OP, Storm-Mathisen J, editors. Glycine neurotransmission. Chichester: Wiley. p 417-451.

Östholm T, Ekström P, Bruun A, van Veen T. 1988. Temporal disparity in pineal and retinal ontogeny. Brain Res 470:1-13.

Owens DF, Kriegstein AR. 2002. Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715-727.

Piavis GW. 1971. Embryology. In: Hardisty MW, Potter IC , editors. The biology of lampreys, vol 1. London: Academic Press. p 361-400.

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

Pombal MA, Rodicio MC, Anadón R. 1994. Development and organization of the ocular motor nuclei in the larval sea lamprey, *Petromyzon marinus* L.: an HRP study. J Comp Neurol 341:393-406.

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

Pombal MA, Marín O, González A. 2001. Distribution of choline acetyltransferase-immunoreactive structures in the lamprey brain. J Comp Neurol 431: 105-126.

Roberts A, Dale N, Ottersen OP, Storm-Mathisen J. 1987. The early development of neurons with GABA immunoreactivity in the CNS of *Xenopus laevis* embryos. J Comp Neurol 261:435-449.

Roberts A, Dale N, Ottersen OP, Storm-Mathisen J. 1988. Development and characterization of commissural interneurones in the spinal cord of *Xenopus laevis* embryos revealed by antibodies to glycine. Development 103:447-461.

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

Rodicio MC, Pombal MA, Anadón R. 1995. Early development and organization of the retinopetal system in the larval sea lamprey, *Petromyzon marinus* L. An HRP study. Anat Embryol 192:517-526.

Rodicio MC, Villar-Cerviño V, Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R. 2005. Chemoarchitecture of the dorsal column nucleus of the larval sea lamprey. Brain Res Bull 66:536-540.

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

Saint Marie RL, Benson CG, Ostapoff E-M, Morest DK. 1991. Glycine immunoreactive projections from the dorsal to the anteroventral cochlear nucleus. Hear Res 51:11-28.

Schmid K, Böhmer G. 1989. Involvement of fast synaptic inhibition in the generation of high-frequency oscillation in central respiratory system. Brain Res 485:193-198.

Schober W. 1964. Vergleichend-anatomische Untersuchungen am Gehirn der Larven und adulten Tiere von *Lampetra fluviatilis* (Linné, 1758) und *Lampetra planeri* (Bloch, 1784). J Hirnforsch 7:107-209.

Schreihofer AM, Stornetta RL, Guyenet PG. 1999. Evidence for glycinergic respiratory neurons: Bötzinger neurons express mRNA for glycinergic transporter 2. J Comp Neurol 407:583-597.

Simat M, Ambrosetti L, Lardi-Studler B, Fritschy JM. 2007. GABAergic synaptogenesis marks the onset of differentiation of basket and stellate cells in mouse cerebellum. Eur J Neurosci 26:2239-2256.

Soffe SR, Zhao FY, Roberts A. 2001. Functional projection distances of spinal interneurons mediating reciprocal inhibition during swimming in *Xenopus* tadpoles. Eur J Neurosci 13:617-627.

Spitzer NC. 2006. Electrical activity in early neuronal development. Nature 444:707-712.

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

Tanaka I, Ezure K. 2004. Overall distribution of GLYT2 mRNA-containing versus GAD67 mRNA-containing neurons and colocalization of both mRNAs in midbrain, pons, and cerebellum in rats. Neurosci Res 49:165-178.

Tanaka I, Ezure K, Kondo M. 2003. Distribution of glycine transporter 2 mRNA-containing neurons in relation to glutamic acid decarboxylase mRNA-containing neurons in rat medulla. Neurosci Res 47:139-151.

Tapia JC, Mentis GZ, Navarrete R, Nualart F, Figueroa E, Sánchez A, Aguayo LG. 2001. Early expression of glycine and GABA(A) receptors in developing spinal cord neurons. Effects on neurite outgrowth. Neuroscience 108:493-506.

Vesselkin NP, Rio JP, Adanina VO, Repérant J. 2000. GABA- and glycine-immunoreactive terminals contacting motoneurons in lamprey spinal cord. J Chem Neuroanat 19:69-80.

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

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

Villar-Cerviño V, Holstein GR, Martinelli GP, Anadón R, Rodicio MC. 2008b. Glycine-immunoreactive neurons in the developing spinal cord of the sea lamprey: Comparison with the gamma-aminobutyric acidergic system. J Comp Neurol 508:112-130.

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

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

Wester MR, Teasley DC, Byers SL, Saha MS. 2008. Expression patterns of glycine transporters (xGlyT1, xGlyT2, and xVIAAT) in *Xenopus laevis* during early development. Gene Expr Patterns 8:261-270.

Winer JA, Larue DT, Pollak GD. 1995. GABA and glycine in the central auditory system of the mustache bat: structural substrates for inhibitory neuronal organization. J Comp Neurol 355:317-353.

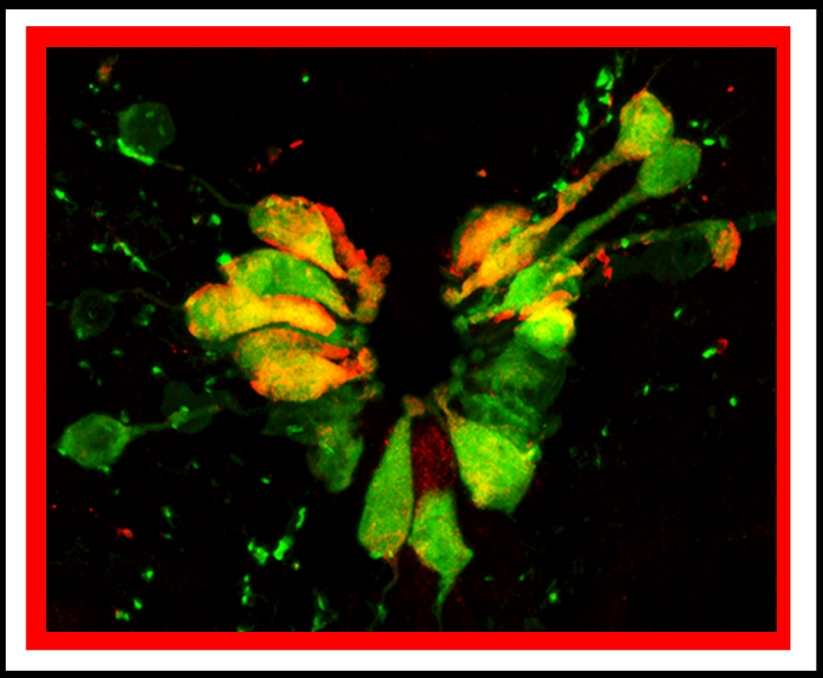
Yáñez J, Anadón R. 1994. Afferent and efferent connections of the habenula in the larval sea lamprey (*Petromyzon marinus* L.): an experimental study. J Comp Neurol 345:148-160.

Yáñez J, Pombal MA, Anadón R. 1999. Afferent and efferent connections of the parapineal organ in lampreys: a tract tracing and immunocytochemical study. J Comp Neurol 403:171-189.

Zafra F, Aragón C, Olivares L, Danbolt NC, Giménez C, Storm-Mathisen J. 1995a. Glycine transporters are differentially expressed among CNS cells. J Neurosci 15:3952-3969.

Zafra F, Gomeza J, Olivares L, Aragón C, Giménez C. 1995b. Regional distribution and developmental variation of the glycine transporters GLYT1 and GLYT2 in the rat CNS. Eur J Neurosci 7:1342-1352.

Glycine-Immunoreactive Neurons in the Developing Spinal Cord of the Sea Lamprey: A Comparison with the GABAergic System



Chapter 3

INTRODUCTION

Glycine is a major inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates, including lampreys (Aprison and Werman, 1965; Aprison and Daly, 1978; Homma and Rovainen, 1978; Matthews and Wickelgren, 1979; Gold and Martin, 1983; Buchanan and Grillner, 1988; Alford and Williams, 1989; Alford et al., 1990a,b; O'Donovan et al., 1992; Dubuc et al., 1993; Uematsu et al., 1993; Berki et al., 1995). The inhibitory action of glycine results from an increase chloride conductance of the postsynaptic membrane upon ligand binding to glycine receptors (Young and Snyder, 1974; Barker and Ransom, 1978; Betz, 1987; Bormann et al., 1987). Although glycine is a major inhibitory neurotransmitter in the adult CNS, it is excitatory during embryonic development and the perinatal period (Reichling et al., 1994). This is attributable to a positive chloride equilibrium potential in the postsynaptic neurons, resulting in chloride efflux upon receptor activation and in depolarization. This glycine receptor excitatory activity is important for synaptogenesis, because the glycine-mediated increase in intracellular [Ca^{2+}] is crucial for the correct formation of postsynaptic glycinergic membrane specializations (Kirsch and Betz, 1998). The action of glycine is terminated through reuptake by two members of the family of Na^+/Cl^--dependent neurotransmitter transporters and by the glycine cleavage system, a mitochondrial and cytosolic enzyme complex (Garrow et al., 1993). The glycine transporter GLYT1 is considered a glial transporter, whereas the GLYT2 transporter is primarily associated with neurons. Recent studies have shown that GLYT1 is also expressed in neurons and that it is associated with a subpopulation of glutamatergic synapses (Cubelos et al., 2005). Although glycinergic synapses are localized mainly in the spinal cord and brainstem of mammals, it is now well established that the glycinergic system is more widely distributed throughout the CNS (Malosio et al., 1991; Rampon et al., 1996). On the other hand, developmental studies of glycinergic neurons in the spinal cord of vertebrates are scant (*Xenopus*: Dale et al., 1986; Roberts et al., 1988; zebrafish: Higashijima et al., 2004a,b; chick: O'Donovan et al., 1992; Berki et al., 1995; mouse: Allain et al., 2006).

Lampreys are living representatives of the most primitive group of vertebrates, the Agnathans (Nieuwenhuys and Nicholson, 1998). Lampreys have a complex life cycle that begins in a river with the embryonic period (about 12 days),

followed by a short prolarval stage and a very long larval stage when the animals lives by burrowing in the riverbed and filter-feeding. Through a complex metamorphosis, larvae transform into young adult lampreys that feed parasitically on fish as they grow, breed, and then die (Hardisty and Potter, 1971). The complex life cycle and early appearance in vertebrate phylogeny make lampreys critical subjects for deciphering the early history of the nervous system of vertebrates and for studying adaptive changes to markedly different larval and adult life styles.

A number of functional and anatomical studies have focused on the neurotransmitters present in the spinal cord of adult lamprey. Electrophysiological and pharmacological studies in the spinal cord and brainstem of adult lampreys have revealed that glycinergic inhibitory interneurons control the motor rhythm generation underlying locomotor behavior (Homma and Rovainen, 1978; Matthews and Wickelgren, 1979; Gold and Martin, 1983; Buchanan and Grillner, 1988; Alford and Williams, 1989; Alford et al., 1990a,b; Dubuc et al., 1993). These inhibitory glycinergic synapses, together with those utilizing γ-aminobutyric acid (GABA), play a major role in spinal circuits, although the effects of glycine and GABA on the locomotor pattern generation may be different. For instance, in neonatal mouse spinal cord, GABAergic and glycinergic synapses play different roles in regulating spontaneous activity and bilaterally alternating rhythms (Hinckley et al., 2005). Several ultrastructural studies have dealt with the features and possible colocalization of GABA and glycine immunoreactivities in synapses in the lamprey spinal cord (Shupliakov et al., 1996; Vesselkin et al., 1995, 2000) as well as the colocalization of these neurotransmitters with zinc (Birinyi et al., 2001; Gustafsson et al., 2002). Whereas GABA-immunoreactive (GABA-ir) neurons in the spinal cord of adult lampreys have been described by several studies (Batueva et al., 1990; Brodin et al., 1990; Christenson et al., 1991; Shupliakov et al., 1996; Ruiz et al., 2004), the characterization of glycine-immunoreactive (glycine-ir) cells has received little attention (Shupliakov et al., 1996; Gustafsson et al., 2002). There are also reports of the GABA-ir neuronal populations in the spinal cord of developing lamprey (Meléndez-Ferro et al., 2003; Ruiz et al., 2004), but as far as we are aware there are no comparable studies on the development of the glycinergic system.

The aim of the present developmental study was to characterize for the first time the appearance of and changes in the glycinergic populations in the spinal cord

of a primitive vertebrate, the sea lamprey (*Petromyzon marinus*), from embryo to adult, by using immunohistochemical techniques. A further aim was to compare the development of the glycinergic cell groups with those containing GABA. Results of this study reveal that the appearance of glycine-ir neurons precedes that of GABA-ir neurons in lampreys. Also, the number of glycine-ir cells is higher than that of GABA-ir cells in the lateral and dorsomedial spinal cord gray matter throughout development. Several glycine-ir neuron subtypes are distinguishable in the gray matter populations by their morphological features. Only a portion of the neurons studied show double immunoreactivity for GABA and glycine, revealing a wide separation between these populations.

MATERIALS AND METHODS

Subjects

Embryos (stages E9-E12; N = 8), prolarvae (stages P0-P22; N = 18), larvae (8-130 mm; N = 55), postmetamorphic (N = 7), and adult (N = 5) sea lampreys (*Petromyzon marinus* L.) were used in the present study. Embryos and prolarvae were obtained from in-vitro-fertilized eggs reared in our laboratory. Embryonic and prolarval stages are defined by the number of days from fertilization and hatching, respectively. Larvae, postmetamorphic, and adult animals were collected from the River Ulla (Galicia, Northwest Spain). Larvae were maintained in aerated aquaria with a bed of river sediment, whereas postmetamorphic and adult lampreys were used immediately.

Tissue collection and processing

Animals were deeply anaesthetized with benzocaine (Sigma, St. Louis, MO; 0.05%), and larvae and adult lampreys were killed by decapitation. Brains and spinal cords of postmetamorphic and adult lampreys were dissected out prior to fixation. All experiments were approved by the Ethics Committee of the University of Santiago de Compostela and conformed to the European Community guidelines on animal care and experimentation. All samples were fixed by immersion in 5% glutaraldehyde and 1% sodium metabisulfite in Tris-buffered saline (TBS; pH 7.4) for 17 hours. The fixed samples were embedded in Tissue Tek (Sakura, Torrance, CA); frozen in liquid nitrogen-cooled isopentane; sectioned on a cryostat in the transverse, horizontal, or sagittal plane (embryos and prolarvae: 10 μm thick; larvae, postmetamorphic, and adults: 16 μm thick); and mounted on Superfrost Plus glass slides (Menzel, Braunschweig, Germany).

Immunohistochemistry for brightfield microscopy

For brightfield microscopy, sections were processed by the avidin-biotin (ABC) technique. Sections were treated with 10% H2O2 to abolish endogenous peroxidase, preincubated with 10% normal goat serum and incubated with a rabbit polyclonal anti-glycine antiserum (Chemicon, Temecula, CA; 1:200). The tissue was then sequentially incubated with goat anti-rabbit biotinylated immunoglobulin (Sigma; 1:100) and rabbit ABC complex (Vector, Burlingame, CA). All dilutions

were done in TBS containing 1% sodium metabisulfite. The immunocomplexes were developed by immersion in a fresh solution of 3,3'-diaminobenzidine (Sigma; 0.6 mg/ml) and 0.003% H2O2. Photomicrographs were obtained with a photomicroscope with an Olympus DP 70 color digital camera. Images were converted to gray scale and adjusted for brightness and contrast in Corel Photo-Paint (Corel, Ottawa, Ontario, Canada).

Immunofluorescence

For immunofluorescence, sections were pretreated with 0.2% NaBH4 in deionized water for 45 minutes at room temperature to quench autofluorescence. Sections were subsequently incubated for 3 days at 4°C with a mixture of rabbit polyclonal anti-glycine antibody (IG1003; Immunosolution, Jesmond, Australia; dilution 1:3000; or Chemicon; dilution 1:100) and mouse monoclonal anti-GABA antibody (GABA93; Prof. G.P. Martinelli; 1:50) in TBS with 1% sodium metabisulfite. After rinsing in TBS with 1% sodium metabisulfite, samples were incubated for 1 hour with Cy3-conjugated goat anti-rabbit IgG (Chemicon; 1:200) and fluorescein-conjugated goat anti-mouse IgG (Chemicon; 1:50) and mounted with fluorescence antifade mounting medium (Vectashield; Vector). All reagents were diluted in TBS (pH 7.4) containing 0.2% Triton X-100 and 3% normal goat serum. For tissue processing controls, primary antisera were omitted from some tissue sections. No staining was observed in these controls. Moreover, preadsorption of GABA and glycine antibodies with BSA did not block immunostaining in lamprey. In addition, these antibodies were tested by Western blotting, with lamprey brain protein extracts (Villar-Cerviño et al., 2006; unpublished results). No protein band was stained in the blots with either glycine antisera or the GABA antibody, which strongly suggests that these antisera do not cross-react with native proteins of the lamprey CNS.

Antibody characterization

The specificity of primary antibodies has been well characterized by the suppliers. According to the supplier, the cross-reactivity of one of the glycine antisera (Immunosolution; code IG1003) was raised against a glycine-glutaraldehyde-porcine thyroglobin conjugate and tested in sections of retina and cerebellum from various mammals and other vertebrates as well as in dot blot

immunoassays against a variety of amino acid-carrier protein conjugates, including the standard 20 amino acids found in proteins; the nonprotein amino acids D-serine, D-alanine, and D-aspartate; GABA; and the glycine-containing tripeptide glutathione, which did not yield significant reactivity. The other glycine antiserum (Chemicon; code AB139) was tested by ELISA or RIA assays, and the results indicated that it only weakly or very weakly cross-reacts with alanine (1/100)-, GABA (1/500)-, taurine (1/1,000)-, aspartate (1/20,000)-, or glutamate (1/20,000)-BSA conjugates, in comparison with the glycine-BSA conjugate. The mab93 monoclonal GABA antibody (provided by Prof. G.P. Martinelli) was raised against GABA-glutaraldehyde-BSA conjugates and tested by ELISA against BSA conjugates of GABA, 16 protein amino acids, histamine, serotonin, adrenaline, noradrenaline, and histamine; it showed high specificity for GABA conjugate and negligible levels of cross-reactivity with the other conjugates (Holstein et al., 2004).

Image acquisition

Sections were analyzed and photographed with a fluorescence microscope with an Olympus DP 70 color digital camera and/or with a spectral confocal microscope TCSSP2 (Leica, Wetzlar, Germany). Confocal stacks were acquired and processed in LITE software (Leica). Photomicrographs were converted to gray scale, inverted, and adjusted for brightness and contrast in Adobe Photoshop (Adobe, San Jose, CA) or Corel Photo-Paint.

Quantitative analysis of cell proportions

For quantitative analysis of proportions of cell phenotypes, we counted only clearly stained glycine-ir and/or GABA-ir neurons unambiguously detected on projections of confocal stacks of alternate sections, and we did not take into account other neurons that were very weakly stained and possibly contained minimally detectable levels of glycine. For this goal, we used the “maximum projection” command of the LITE software filtered by adjusting the threshold to 80 with rescaling to 255 (eight-bit scale, 0-255 range). This threshold value was chosen because it filtered out all very faintly fluorescent cells that were judged as nonpositive above background by two different observers and left only the cells judged unequivocally as positive (Fig. 1). Only cells visible in these projections were taken into account. With this procedure, we did not take into account nonglycinergic

neurons that were very weakly stained but possibly contained detectable levels of glycine (Fig. 1A,B). For each stage considered (prolarvae, larvae, and adults), three individuals and seven transverse sections of the rostral one-third of the spinal cord from each one (representing 70 μm of spinal cord per prolarva and 112 μm of spinal cord per larval or adult animal) were counted. The total number of cells analyzed in each stage was 314 cells (prolarvae), 691 cells (larvae), and 960 cells (adults).

Because our purpose was to estimate possible shifting during development in relative proportion of glycine-ir *vs*. GABA-ir cells in the lamprey spinal cord, and, because larger cells have a higher probability of being encountered than smaller ones in sections, a correction factor (Abercrombie's factor; T/T + H, where T is the section thickness and H the mean height of the cells; Abercrombie, 1946) was considered for calculations (Table 1). Owing to the high polarization of most lamprey spinal cells in the transverse plane of the spinal cord, H was directly estimated in sagittal or horizontal sections from 12 cells of each phenotype showing nuclei (prolarvae, larvae, and adults). For descriptive purposes, neurons with a short diameter of less than 10 μm were considered small, and cells with diameters of between 10 and 20 μm were considered as medium-sized. A roughly similar H was obtained for the GABA-ir and glycine-ir cell phenotypes in each cell population [lateral, dorsomedial, and cerebrospinal fluid-contacting (CSF-c)] within each stage, so this procedure together with the number of cells analyzed ensured enough precision in cell proportions for our goals. Cell measures were expressed as mean ± SD (Table 1). In addition, the corrected numbers of cells observed in prolarvae, larvae, and adults corresponded to both a section and a spinal cord segment (Table 2). Because the length of segments vary largely from embryos to adults, to calculate the segment length we used the linear function that correlates the length of spinal segments, obtained by Ruiz et al. (2004) in the sea lamprey (Galician breed). By using this function ($y = 0.0066x + 0.058$, expressed in mm), we determined the mean segment length of the prolarvae (0.11 mm), larvae (0.47 mm), and adults (4.67 mm) used for calculation.

Measurement of axons

For measurement of axonal diameters, confocal micrographs of a transverse section of the rostral spinal cord of an adult lamprey were used. The white matter

was divided into four regions: dorsomedial (DM), ventromedial (VM), dorsolateral (DL), and ventrolateral (VL). DM and VM correspond to the dorsal and ventral columns of Van Dongen et al. (1985), respectively, whereas the lateral column of these authors was subdivided into dorsal and ventral parts. In total, 451 cross-sectioned glycine-ir axons of more than 1 μm in diameter were measured with LITE software (DM, 29 axons; DL, 127 axons; VL, 169 axons; VM, 126 axons). These values are representative of the number of axons of more than 1 μm in diameter coursing longitudinally in these quadrants. For comparison, the mean diameter of GABA-ir axons coursing longitudinally was obtained from measures of 40 axons of diameters higher than 1 μm.

RESULTS

Glycine immunoreactivity precedes the appearance of GABA immunoreactivity in the spinal cord of embryonic and prolarval lampreys

The first glycine-ir cells of the sea lamprey spinal cord were observed in late embryos, in which they formed a discontinuous longitudinal band (zero to two neurons per 10 μm-thick section on each side of the cord) in the primordial mantle zone (Fig. 2A). These cells were oval and did not contact the central canal, which in these embryos was a long vertical slit. These cells often appeared filled with yolk platelets. A few immunoreactive fibers were also observed in the ventrolateral marginal region at these embryonic stages. In hatchlings (P0– P1), new faintly glycine-ir cells appeared dorsal to the earliest neurons, and the number of glycine-ir fibers increased (Fig. 2B). At the pigmentation (P2-P3) and gill cleft (P4-P7) stages, the number of small glycine-ir cells (5.3 ± 0.7 μm) increased, but their organization as loose intermediate and ventral longitudinal bands was conserved (Fig. 2C). In late prolarvae (P8-P23 or burrowing stage), glycine-ir cells appeared more dorsally in the spinal cord, resulting in the appearance of dorsal, intermediate, and ventral longitudinal bands of glycine-ir somata (Fig. 2D,E). The ventral band, which contained much more glycine-ir neurons than the intermediate and dorsal bands, was assigned to the basal plate, whereas the two other bands were tentatively considered of alar plate origin (Fig. 2D,E). Bands were better observed in parasagittal sections (Fig. 2F), and the ventral band contained the largest number of glycine-ir cells. At this stage, a few glycine-ir CSF-c cells were faintly stained in the most ventral region of the slit-shaped central canal (Fig. 2E). The number of glycine-ir fibers increased markedly in the ventrolateral marginal region (Fig. 2D,E), and some glycine-ir fibers also extended into the thin marginal zone of the dorsal regions (Fig. 2E). The presence of faintly glycine-ir fibers in the ventral commissure was outlined since P1 prolarvae (not shown), but commissural fibers become outstanding in later prolarvae (Fig. 2G,H).

The appearance of GABA immunoreactivity followed a developmental pattern rather different from that observed with glycine. The only GABA-ir neurons appearing in embryos were occasional CSF-c neurons (Kolmer-Agduhr cells), and these cells were the only GABA-ir spinal cells detectable until P3 prolarvae. GABA

immunoreactivity in perikarya of intermediate and dorsal bands appeared gradually after this stage (Meléndez-Ferro et al., 2003; present results). Colocalization studies in the P10-P15 prolarvae indicated that almost all glycine-ir cells were GABA negative (98.2% in the ventral column and 94.3% in the dorsal plus intermediate columns; see Table 2).

The pattern of glycine- and GABA-ir populations is acquired in larval lampreys

The lamprey spinal cord becomes flattened dorsoventrally in early larvae (Fig. 2I), and the gray matter extends laterally from the central canal, which arises from the ventral part of the prolarval vertical ependymal slit (see also Meléndez-Ferro et al., 2003). During this spinal transformation, ventral glycine-ir neuronal populations became displaced laterally, whereas most dorsal glycine-ir populations remained putatively in the dorsomedial region of the gray matter (Fig. 2I). Only CSF-c neurons maintain the original position close to the central canal observed in prolarvae and progressively surround the larval central canal. It was derived from the ventral portion of the slit-shaped prolarval canal.

The distribution of glycine-ir and GABA-ir populations of the spinal cord in larval stages was similar to that observed in young postmetamorphic and adult lampreys (see below), although the cell bodies were smaller in the larvae. Most glycine-ir perikarya were distributed along the dorsomedial and lateral margins of the gray matter (Figs. 3, 4A-F, 5A,B), and faintly glycine-stained CSF-c cells were also observed in the central canal walls (Figs. 3A,B,D, 4A, 5C,C'). In horizontal sections, most glycine-ir cells in the dorsomedial and lateral regions of the gray matter were small to medium-sized (8.5 ± 1.9 μm) and showed processes coursing in the transverse plane (Fig. 4A-F). In the dorsomedial region, most glycine-ir cells were pear-shaped (Fig. 4B), but a few glycine-ir cells exhibited a spindle-shaped appearance coursing obliquely to the longitudinal axis (Fig. 4C). Some tripolar or bipolar neurons with one or two processes coursing longitudinally were also observed (Fig. 4D). Processes of some glycine-ir cells of the dorsomedial region crossed the midline dorsally to the central canal (Fig. 4A,C). In the lateral population, most glycine-ir cells were pear-shaped or spindle-shaped, showing processes coursing in the transverse plane (Fig. 4E,F). Some cells in this region showed a bipolar or tripolar appearance, with a thick process coursing rostrally (Fig.

4F). Small bipolar or monopolar cells showing longitudinal orientation were also observed.

The small glycine-ir CSF-c cells (6.5 ± 0.65 μm) were concentrated in the dorsolateral walls of the central canal, with about three or four cells per transverse section (Fig. 5C). Glycine-ir cells were also scarce outside the gray matter: some edge cells (Figs. 3A,B, 4G, 5D,D'), a few small interstitial neurons (conic neurons) located in the ventromedial white matter near the giant reticulospinal axons (Figs. 4H, 6A), and occasional interstitial cells located in the dorsal column showed glycine immunoreactivity (Fig. 6B). Glycine-ir edge cells of the larval spinal cord showed a small (7.9 ± 1.3 μm) perikaryon and a short, thick dendrite. These cells were located laterally in the cord near the intensely GABA-ir marginal neuropil (Fig. 5D).

Processes of glycine-ir cells coursed to the adjacent white matter and only occasionally traversed the gray matter. Dorsomedial cells also sent dendritic processes to the dorsal column (Fig. 6C). Some small interstitial neurons were observed in the ventromedial column. These cells showed a short plump process that appeared to contact giant axons (Fig. 6A). In horizontal sections, these small neurons appeared as elongated cells arranged transversely to the longitudinal axis (Fig. 4H). These cells correspond to those already reported as "conic neurons" in the adult river lamprey (Gustafsson et al., 2002).

In the larval spinal cord, GABA-ir cells were observed in most regions also containing glycine-ir cells: dorsomedial and lateral regions of the gray matter and in the walls of the central canal (CSF-c cells) (Fig. 3A,C,D). The morphology of these dorsomedial and lateral cells in transverse sections was roughly similar to those of glycine-ir of the same region. Occasional GABA-ir edge cells were also observed. At the transition between the spinal cord and rhombencephalon, the number of glycine-ir and GABA-ir cells per section increased considerably in dorsomedial regions, reflecting the transition to the dorsal column nucleus. The distribution of glycine, GABA, and glutamate in the dorsal column nucleus of larval lamprey has been reported previously (Rodicio et al., 2005).

Colocalization with GABA was observed in some glycine-ir spinal populations (Figs. 3A-C, 5A-C), although the proportion of double-labeled cells varied greatly among populations. It was scarcer in the gray matter regions but high

in the CSF-c population. In the dorsomedial population, only about 11% of total immunoreactive neurons showed colocalization of glycine and GABA. The lowest proportion (2%) of double-stained cells with respect to all immunoreactive somata was observed in the lateral population, whereas the highest proportion (23%) was observed in the CSF-c cells (Table 2). Most of these CSF-c cells exhibited strong GABA-ir (94.4%). In addition, a few of the glycine-ir edge cells showed also GABA-ir. In the larval spinal cord, the most abundant glycine-ir fibers coursed in the dorsolateral, ventrolateral, and ventral white matter columns, whereas the dorsal column contained only scarce glycine-ir fibers (Fig. 3A,B). Numerous glycine-ir fibers coursing in the ventral column crossed the midline ventral to the central canal (Figs. 4I, 6D). Likewise, GABA-ir fibers in the spinal cord were fairly numerous in the white matter, with the notable exception of the dorsal column. The highest GABA-ir fiber density was observed adjacent to the dorsal column, itself showing very few GABA-labeled processes (Fig. 3A,C). GABA-ir fibers crossing in the ventral commissural region are very scarce.

Colocalization of glycine and GABA immunoreactivities in the spinal cord populations of postmetamorphic and adult sea lamprey is only partial

The spinal cord of postmetamorphic and adult sea lamprey contained numerous glycine-ir cells, most located in the gray matter (Fig. 7). As in larvae, these gray matter glycine-ir perikarya were classified as dorsomedial cells, lateral cells, and CSF-c cells. Glycine-ir edge cells were also observed in the white matter near the lateral border of the cord.

The intensely glycine-ir dorsomedial cells showed small to medium-sized pear- or spindle-shaped perikarya (Fig. 7A,A',D,D') and long, slender, poorly branched dendrites that coursed laterally or dorsally in the dorsal column or crossed the midline just dorsal to the central canal. In horizontal or parasagittal sections, tripolar or more complex morphologies were also observed (Fig. 7B,C). These perikarya (16.4 ± 3.2 μm in diameter in adults) were located most commonly in the border between the gray and the white matters, and rarely within the dorsal column. Some spindle-shaped and tripolar neurons showed long processes coursing in a longitudinal or oblique direction (Fig. 7C). The same region contained a population of GABA-ir neurons with similar morphologies (Fig. 7A,A'',C), although, as a

mean, GABA-ir perikarya were smaller (12.8 ± 2.8 μm) than those of the glycine-ir neurons. Double immunofluorescence in upstream-migrating adults revealed colocalization of GABA and glycine in 23.7% of these dorsomedial neurons, whereas 34.8% of all immunoreactive cells were GABA-ir/glycine-negative and 41.4% were glycine-ir/GABA-negative (see Table 2).

At the junction of the spinal cord and rhombencephalon, the dorsomedial populations of GABA- and/or glycine-ir cells were replaced by those of the dorsal column nucleus, which could be distinguished by the large number and periventricular location of small GABA-ir and glycine-ir cells (not shown). In addition, some GABA-ir and glycine-ir cells were located within the dorsal column nucleus, among the fibers of the dorsal column.

The lateral region of the adult lamprey contained two main types of intensely glycine-ir cells: those that were spindle-shaped and those that were triangular or multipolar (Fig. 7E,F). These cells (14.7 ± 2.3 μm) were situated in the margin of the gray matter or interspersed with large motoneuron or interneurons perikarya, which were glycine-negative or only very faintly stained. Dendrites of the intensely glycine-ir cells extended into the dorsolateral, lateral, and ventrolateral regions of the white matter. Immunofluorescence revealed the presence in this lateral region of GABA-ir neurons (15.4 ± 3.5 μm) with morphologies similar to those of the glycine-ir cells (Fig. 7E,F). Colocalization of GABA and glycine was observed in 18.8% of immunostained cells, whereas about 14.7% of all immunoreactive perikarya were solely GABA-ir and 66.3% of the cells were exclusively glycine-ir.

Some faint or moderately labeled glycine-ir small CSF-c cells (7.4 ± 1.0 μm) were observed in the central canal walls. In general, these cells were situated mostly dorsolateral to the central canal and showed a short ventricular dendrite that ended as a small club protruding into the canal (Fig. 8A,A'). A large proportion of CSF-c cells (91.3% of the GABA-ir plus glycine-ir populations) was strongly GABA-ir (Fig. 8A-A''), and about 40.7% of these cells were also glycine-ir (Table 2). The perikarya of some CSF-c GABA-ir/glycine-ir cells were located outside the ependymal layer, and their dendrites were longer than those of cells located in the ependymal layer (Fig. 8A-A''). The glycine-ir and/or GABA-ir CSF-c cells formed a dense cell column along the spinal cord that extended rostrally in the caudal fourth ventricle, forming two separated ventromedial bands of CSF-c cells.

In addition, the adult spinal cord contained a few glycine-ir edge cells located in the lateral marginal region. Some of these immunostained somata were also surrounded by glycine-ir boutons. These edge cell bodies were observed in close proximity to the intensely GABA-ir longitudinal marginal neuropil that was adjacent to the lateral margin of the cord and to which these cells appeared to send dendritic branches. This neuropil showed only faint glycine-ir processes, and occasional GABA-ir neurons were observed near the marginal neuropil. A few of these cells showed colocalization with glycine.

The glycine- and GABA-ir fibers differ substantially in diameter in the adult lamprey spinal cord

In the rostral spinal cord of upstream-migrating adult lamprey, glycine-ir fibers coursing in the four white matter regions were thicker than the GABA-ir fibers, and most traveled longitudinally in the white matter (Fig. 8B-F). The most abundant glycine-ir longitudinal fibers were found in the dorsolateral, ventrolateral, and ventral columns, whereas the dorsal column and the adjacent region of the spinal trigeminal tract neuropil (located just laterally to the dorsal column) had far fewer glycine-ir longitudinal fibers (Fig. 8B-F). Similarly, glycine-ir fibers coursing through the gray matter were rather scarce in comparison with the number of GABA-ir fibers traversing the same region. In the white matter columns, glycine-ir longitudinal fibers were rather thick (dorsolateral: 6.5 ± 2.9 μm, n = 127; ventrolateral: 7.1 ± 3.6 μm, n = 169; ventromedial: 5.4 ± 2.3 μm, n = 126) and showed a continuous spectrum of diameters (Fig. 9). A few fibers exceeding 20 μm in diameter were observed in the ventral and ventrolateral columns (Figs. 8D,F, 9). In the dorsomedial column, glycine-ir fibers were scarce (Fig. 8B) and rather thin (mean diameter, 3.4 ± 1.1 μm, n = 29; maximum diameter, 5.8 μm). GABA-ir fibers were rather abundant in white matter regions (Fig. 8B), especially in the dorsal region adjacent to the dorsal column (spinal tract of the trigeminal nerve), whereas they were rather scarce in the dorsal column caudal to the dorsal column nucleus. By comparison, GABA-ir fibers were rather thin and generally did not exceed 2-3 μm in diameter, most being about 1 μm thick. Only two GABA-ir fibers of the ventromedial column were comparatively rather thick, about 10 μm in diameter (Fig. 8D). Double immunofluorescence showed that most of these coarse longitudinal axons were either glycine-ir or GABA-ir (Fig. 8B,C), and colocalization of these

transmitters was not observed in those of the ventromedial column (Fig. 8D). In the gray matter, GABAand glycine-ir was observed in some thin fibers and boutons, but GABA-ir boutons were far more numerous (Fig. 8E). Colocalization of these transmitters was observed in some thin fibers and boutons in the gray matter, but no attempt to quantify the proportion of boutons/thin fibers showing colocalization of glycine and GABA was made. Moreover, GABA-ir boutons/thin fibers were abundant among the giant reticulospinal fibers. These giant fibers showed no or very faint glycine immunofluorescence (Fig. 8D).

DISCUSSION

This is the first study on the development of glycine-ir neurons in the spinal cord of a lamprey. Glycine-ir cells appear in the spinal cord (late embryos and prolarvae) earlier than GABA-ir cells, and the glycine-ir populations maintain glycine-ir throughout development. Accordingly, glycine-ir neurons are the earliest inhibitory neurons to appear in the lamprey spinal cord, which suggests the importance of glycine in nascent inhibitory spinal circuits. A specific function for glycine in the developing spinal cord was suggested on the basis of studies in early zebrafish embryos, where bursts of glycinergic synaptic activity were the first synaptic activity recorded from motoneurons (Saint-Amant and Drapeau, 2000).

A rapid change of glycinergic populations occurs in prolarvae, whereas the pattern of the glycine-ir populations is settled in early larvae. Double immunofluorescence allowed us to compare easily the distributions of glycine and GABA-ir in the same cells. With the exception of the CSF-c neurons located around the central canal, which are predominantly GABA-ir, development of glycine-ir neurons in gray matter regions precedes that of those showing GABA immunoreactivity. Among the total number of GABA- and/or glycine-immunolabeled cells counted in the gray matter of the adult spinal cord (dorsomedial plus lateral populations), 52.6% of the neurons were glycine-ir only, 25.7% were GABA-ir only, and colocalization was observed in 21% of these cells. In larvae, 61.8% of these neurons were glycine-ir only, 29.4% were GABA-ir only, and 8.6% were double-labeled cells. How these different proportions are related to different life styles of larval and adult lamprey is not known.

Results on the development of GABA and glycine populations in the lamprey spinal cord reveal important differences from the mouse and chick

The spinal glycinergic cells form numerically important populations at early developmental stages. Early in ontogeny, these cells can be classified into ventral, intermediate, and dorsal populations that exhibit a roughly longitudinal columnar organization, albeit less clearly than that of the GABA-ir populations (Meléndez-Ferro et al., 2003), a fact attributable to the large number of glycine-ir cells. The first of these cell groups to appear is that located at intermediate-ventral levels, followed by the intermediate cells, and finally by the dorsal population. Glycine expression in

the developing mouse also showed a variable time course of development of the glycine-ir cell groups, with those of the ventral horn appearing at least 1 day before those of the dorsal horn (alar plate; Allain et al., 2006). At these mouse embryo stages, the spinal cord exhibits a vertical, slit-shaped central canal similar to that found in early lamprey prolarvae, although the alar plate occupies a wider extension than is seen in lamprey. In chick embryos, however, glycine-ir cells appear rather late in the same day (E8) in both the dorsal and the ventral horn (Berki et al., 1995). Our results suggest that the ventral glycine-ir populations correspond to those of the mouse and chick ventral horn, whereas the late appearing dorsal glycine-ir cells correspond to those of the dorsal horn. Whether the intermediate glycine-ir prolarval population observed in lamprey prolarvae is alar or basal could not be assessed. In lamprey, the lateral migration of the basal plate cells extends the ventral populations (including the motoneurons) laterally, whereas those located dorsally in embryos do not change their position appreciably, as reported for GABA-ir populations (Meléndez-Ferro et al., 2003). However, it was not possible to trace individually the three glycine-ir neuronal columns observed in prolarvae to the different glycine-ir populations observed in larvae and adults.

The present results in lamprey reveal important differences from the distributions of GABA and glycine in the developing spinal cord of the chick and mouse (Berki et al., 1995; Allain et al., 2004, 2006). Studies in chick and mouse embryos indicate that GABA-ir cells mature earlier (4 days and 1 day, respectively) than glycine-ir cells (Berki et al., 1995; Allain et al., 2006), whereas in the lamprey spinal cord most glycine-ir cells are detectable several days earlier than GABA-ir cells, except those of the CSF-c cells, which exhibit little glycine-ir overall. Moreover, studies utilizing glutamate decarboxylase (GAD; the GABA-synthesizing enzyme) and GABA in developing chick and rodent spinal cord have revealed a progressive loss of GAD/GABA-ir in early developing GABAergic ventral cells (Berki et al., 1995; Phelps et al., 1999; Allain et al., 2004), leading to the hypothesis that some spinal neurons are only transiently GABAergic. Similarly, for the rabbit outer retina, it has been suggested that GABA is replaced by glycine during the ontogeny of some cells (Messersmith and Redburn, 1992, 1993). However, some studies in developing rat suggest that the observed decrease in perikaryal GAD/GABA-ir may be due to selective transport of GAD to axons in mature neurons

rather than to the loss of the GABAergic phenotype (Tran et al., 2003). Our results also indicate that the proportion of double-labeled GABA-ir/ glycine-ir neurons in the dorsomedial and lateral spinal populations increases between larvae and adults and that the proportion of the dorsomedial and lateral spinal cells that exhibit glycine immunoreactivity only diminishes between larvae and adults. These results do not support the notion that the GABAergic phenotype is progressively replaced by the glycinergic phenotype during development, as suggested in chick and mammalian studies.

Cell counts in sea lamprey revealed that the number of GABA-ir perikarya per spinal segment increases markedly between prolarvae and adults owing to the continuous increase in length of segments, although the numbers of cells in 100 μm of spinal cord vary moderately (Ruiz et al., 2004). From the results shown in Table 2, it is clear that the absolute number of glycine-ir cells per segment increases considerably from prolarvae to adults in all spinal populations and that this increase is more marked for CSF-c cells. This is rather similar to that reported for GABA-ir cells by Ruiz et al. (2004). These authors suggested that the progressive increase in number of GABA-ir cells in the lamprey spinal cord serves to adapt the inhibitory control of locomotion to new locomotor requirements during development. The present results with glycinergic cells point in the same direction.

As regards the order of appearance of glycine-ir populations in spinal longitudinal zones of lamprey, first in the ventral column (putative basal plate) and then in the alar plate, it appears similar to that in the mouse, where basal plate populations are the first to appear (Allain et al., 2006). However, dorsal and ventral populations appear at the same day in chick (Berki et al., 1995). Moreover, in mouse, the dorsal horn population shows a notable increase in number, whereas the ventral horn cells are reduced in number at birth (Allain et al., 2006), which is unlike the case in lamprey. These differences are probably related to the much larger development of primary sensory circuits in mammals than in lampreys, including a profusion of spinal nerve fibers and terminals. Moreover, developing glycine-ir cells become scattered throughout the dorsal and ventral horns in chick and mouse (Berki et al., 1995; Allain et al., 2006), whereas in lampreys they are arranged roughly as a cell sheet on the dorsal side of the characteristic wing-like expansions of the gray matter (as seen in transverse sections), although the lateral glycine-ir neurons

become interspersed among other types of neurons, as is observed in the mouse. This differential developmental pattern probably reflects the fact that many lamprey spinal cells extend long dendrites to the "white matter" (Vesselkin et al., 2000), whereas in birds and mammals the dendrites of spinal neurons are primarily restricted to the gray matter.

On the other hand, the early presence of glycine-ir cells during development in lamprey spinal cord is in agreement with observations in early zebrafish revealing that the glycinergic cells are clearly more abundant than the GABAergic cells (Higashijima et al., 2004a,b). These zebrafish studies were performed by in situ hybridization with probes for GAD and the neuronal glycine transporter (GLYT2) as markers of GABAergic and glycinergic cells, respectively (Higashijima et al., 2004a,b). Similarly to the present results in the lamprey, these authors have observed GAD expression in Kolmer-Agduhr (CSF-c) cells of developing zebrafish, but these cells do not hybridize in situ with the GLYT2 probe. Immunocytochemical studies of GAD-ir in a dogfish revealed the early appearance of the GABAergic phenotype in Kolmer-Agduhr cells and continued expression of this marker in adult, which supports the results obtained with GABA antibodies in sea lamprey (Meléndez-Ferro et al., 2003; Ruiz et al., 2004; present results).

Double immunofluorescence in larval and adult lamprey allows determination of the morphology and transmitter phenotype of inhibitory neurons

The approach employed in the present study allows us to distinguish the shapes of the cells and some part of the extent of their dendrites, in addition to transmitter phenotype. We found that most glycine-ir spinal cells were small neurons, and a portion of them may correspond to the small inhibitory ipsilateral neurons described by Buchanan and Grillner (1988). Some glycine-ir cells of the dorsomedial group send long dendritic processes contralaterally through the dorsal gray commissure and may also send processes to the dorsal funiculus, suggesting that they may receive bilateral inputs. Dendritic processes of neurons of the lateral group appear to ramify scarcely within the dorsolateral and ventral funiculi, and some course longitudinally. These results suggest that these cell populations are roughly comparable to dorsal and ventral horn cell groups of the mammalian spinal cord, respectively, which is supported by the developmental results (see above). Moreover,

our results indicate that less than 24% of the immunoreactive cells in these gray matter populations exhibit colocalization of GABA and glycine, the majority of cells being either glycine-ir/GABA-negative or GABA-ir/glycine-negative. These results are not in good agreement with those reported for adult *Lampetra fluviatilis* and *Ichthyomyzon unicuspis* by Shupliakov et al. (1996), who reported that more than half of the glycine-ir cells (19 cells of 32; 59%) contained GABA immunoreactivity. Instead, in the adult sea lamprey, these values are 23.7% for glycine-ir cells of the dorsomedial population and a mere 18.8% for those of the lateral population. These differences probably are due to the small number of cells counted by these authors, although the existence of between-species differences cannot be ruled out. An ultrastructural immunocytochemical study of the spinal cord in river lamprey reported colocalization of GABA and glycine in about one-third of the synaptic boutons contacting motoneuron dendrites exhibiting glycine and/or GABA (Vesselkin et al., 2000). This value is also higher than that observed by us in cell perikarya, but percentages observed in perikarya and in synapses can be compared only if glycine-ir and GABA-ir cells bear the same number of terminals per cell. Moreover, the presence of descending reticulospinal axons could disturb any conclusion.

As regards the glycine-ir cells of the gray matter, they appear somewhat heterogeneous with respect to their size, morphology, and orientation of their processes. This heterogeneity is clearly observed in the spinal cord of larvae but is more evident in that of upstream-migrating adults. The presence of small inhibitory interneurons, probably glycinergic, has been characterized physiologically by Buchanan and Grillner (1988). They also injected identified cells with horseradish peroxidase, allowing them to reconstruct their morphology. The inhibitory interneurons revealed by these authors have a small body with relative few dendrites extending transversely toward the lateral margin and the midline, although their appearance (size, branching pattern) is not homogeneous. By their location, size, and appearance, these cells appear to correspond to some of the glycine-ir cell subtypes of the lateral population of the gray matter reported here.

Consistently, the circumventricular CSF-c cells exhibit strong GABA-ir from early stages of development (late larvae) through adulthood. Our GABA immunofluorescence results are in agreement with other studies of adult (Batueva et

al., 1990; Christenson et al., 1991; Shupliakov et al., 1996) and developing (Meléndez-Ferro et al., 2003; Ruiz et al., 2004) lamprey. A subset of the CSF-c cells shows glycine-ir, and these generally occupy dorsal or dorsolateral regions around the central canal. The GABAergic CSF-c cells are considered to be the source of the rich GABA-ir plexus along the lateral margin of the spinal cord (Christenson et al., 1991), which shows very intense GABA-ir but faint glycine-ir in axonal processes (Shupliakov et al., 1996; present results). This suggests that GABA is the main inhibitory neurotransmitter released by axons in this neuropil, which is in agreement with the results of physiological studies on edge cells in lamprey (Christenson et al., 1991). Although glycine has been found to be a source of tonic inhibition of lamprey edge cells during fictive swimming (Alford et al., 1990b; Vinay et al., 1996), it is unclear whether glycine is released by axon terminals in this special neuropil or is released by synaptic boutons contacting other parts of edge cells such as the perikarya or primary dendrites.

Some edge cells (intraspinal stretch receptors) of the sea lamprey are glycine-ir (Shupliakov et al., 1996; present results), whereas other edge cells appear to be immunoreactive to glutamate and/or aspartate (unpublished results). These results are in agreement with physiological results on this intraspinal mechanoreceptor system, showing the existence of both excitatory and inhibitory edge cells (see Rovainen, 1974; Grillner and Wallén, 2002). Studies on edge cells indicate that glycinergic spinal interneurons provide phasic inhibition on these cells, whereas the GABA-ir CSF-c system exerts a tonic inhibition (Vinay et al., 1996). The presence of GABA-ir in some edge cells has also been reported (Batueva et al., 1990; Ruiz et al., 2004). Present results indicate that some of the glycine-ir edge cells were also GABA-ir, suggesting that actions of these inhibitory edge cells may be mediated by both neurotransmitters.

Differences in diameters of GABA and glycine fiber systems suggest different roles in spinal cord inhibitory circuits

Comparison of the strongly glycine-ir fibers and the GABAergic fibers coursing longitudinally in the white matter of the rostral spinal cord of upstream-migrating adults reveals large differences in diameter between these two types of fiber; most glycine-ir fibers are several times thicker than the GABA-ir fibers of the

same funicular region. Differences are less marked in larvae, but glycine-ir axons are also thicker than GABA-ir fibers at this stage. The large diameter of many axons in adult lamprey appears closely related to the absence of myelin in its nervous system. Data available indicate modest conduction velocities (5 m/second) in the largest axons of Müller cells (about 50 μm in diameter) and a slope of 0.613 in the power relation of diameter to conduction velocity (Rovainen, 1982). Together, the present results suggest that glycine-ir axons as a mean may have conduction velocities two to three times higher than those of GABA-ir axons, revealing a notable specialization of spinal inhibitory fiber systems into fast and slow-conducting systems. This fact appears to have been overlooked in other studies. Moreover, some thick glycine-ir axons might originate from the group of reticulospinal neurons that inhibits their target neurons via glycine receptors (Wannier et al., 1995). The presence of rather large glycine-ir reticular cells in the rhombencephalic reticular formation has been recently reported (Villar-Cerviño et al., 2008). Because recruitment of successively larger and hence more rapidly conducting reticulospinal neurons for successively more rapid swimming has been reported in lamprey (Wannier and Senn, 1998), the presence of a continuum of axon diameters might also be important for modulation of spinal circuits to different swimming cycles.

Glycine immunocytochemistry also reveals the presence of a number of commissural fibers crossing below the central canal. However, only occasional commissural GABA-ir fibers have been observed in developing and adult lampreys (Meléndez-Ferro et al., 2003; present results). In embryonic and larval zebrafish, studies of expression of the glycine transporter GLYT2, GABAergic markers, and neuronal tracers indicate that early glycinergic cells are of various commissural and circumferential types, whereas GABAergic neurons are cells with longitudinal axons and Kolmer-Agduhr cells (Higashijima et al., 2004b). Commissural glycine-ir cells have also been reported in the spinal cord of amphibian embryos (Dale et al., 1986; Roberts et al., 1988). Unlike the case in lampreys and zebrafish, conspicuous commissural GABAergic fiber systems have been reported in the developing spinal cord of the rat (Phelps et al., 1999) and an elasmobranch (*Scyliorhinus canicula*; Sueiro et al., 2004), which suggests the existence of important neurochemical differences in the crossed inhibitory spinal circuits among vertebrates. Comparison of hemicord preparations of lamprey spinal cord with the intact cord have provided

strong evidence that the reciprocal inhibition between opposite hemisegments ensures left-right alternation in the locomotor pattern and promotes multiple action potentials per cycle in network neurons (Cangiano and Grillner, 2005). Glycine-ir fibers represent the majority of the commissural fibers with inhibitory neurotransmitters in the lamprey spinal cord, providing further morphological evidence for these crossed interactions.

Finally, the giant reticulospinal (Müller) axons exhibited very faint or no glycine-ir in adult sea lamprey with the Immunosolution antiglycine antibody. This result is not in good agreement with results of immunoelectron microscopy by Vesselkin et al. (1995), who reported colocalization of glycine and glutamate in these axons. These authors also speculated on the possibility that coreleased glycine and glutamate at giant synapses of these axons modulate the N-methyl-D-aspartate (NMDA)-type glutamate receptors at the membrane of postsynaptic cells. Alternatively, the very faint glycine-ir observed sometimes in these axons might represent metabolic glycine at concentrations high enough to be demonstrated by the present approach.

Final considerations

Glycine-ir neurons appear in late lamprey embryos, before the appearance of GABA-ir neurons in prolarvae. They are the first inhibitory neurons to appear, which is unlike the case reported in mammals. The precedence of glycine-ir over GABA-ir spinal cells during lamprey ontogeny suggests a fundamental, primitive feature of vertebrates. Glycinergic cells arise from both alar and basal regions and, at the transition to larval stages, become distributed in the dorsomedial and lateral regions of the wing-shaped gray matter characteristic of larval and adult lamprey spinal cord. These lamprey populations are roughly similar to the dorsal and ventral horn populations of mammals, respectively. The changes in glycine-ir populations observed in lampreys between larvae and adults involve an increase in the number and size of immunoreactive cells, but the same glycine-ir cell types are observed in larvae and adults, despite the major alterations in head anatomy and life style that occur during transformation. GABA and glycine are colocalized in a low proportion of immunoreactive cells in larvae, and this proportion increases in adults. A number of rather thick glycine-ir axons course longitudinally in the rostral spinal cord, most

of which were not GABA-ir. Finally, since early stages, commissural glycine-ir fibers are abundant in the spinal cord. These results indicate fundamental roles for glycinergic neurons in spinal circuits throughout development in the earliest extant vertebrates.

Table 1. Minor diameters of glycine-ir and GABA-ir neurons in the major populations of the spinal cord, and Abercrombie's factor (CF) used for correction of cell numbers *

Population	Phenotype	Prolarvae (n = 12)	Larvae (n = 12)	Adults (n = 12)
Dorsomedial gray (prolarval alar region)	Glycine +	5.3 ± 0.7 µm CF = 0.65	8.5 ± 1.9 µm CF = 0.65	16.4 ± 3.2 µm CF = 0.49
	GABA +	5.3 ± 0.7 µm CF = 0.65	8.1 ± 1.1 µm CF = 0.66	12.8 ± 2.8 µm CF = 0.55
Lateral gray (prolarval basal region)	Glycine +	5.3 ± 0.7 µm CF = 0.65	8.5 ± 1.9 µm CF = 0.65	14.7 ± 2.3 µm CF = 0.52
	GABA +	5.3 ± 0.7 µm CF = 0.65	8.1 ± 1.1 CF = 0.66	15.4 ± 3.5 µm CF = 0.50
CSF-c cells	Glycine and/or GABA	5.3 ± 0.7 µm CF = 0.65	6.5 ± 0.6 µm CF = 0.71	7.4 ± 1.0 µm CF = 0.68

* For doubly labeled cells we used the mean of the CFs of GABA-ir and glycine-ir cells of that population. Thickness of sections: 10 µm (prolarvae), 16 µm (larvae and adults).

Table 2. Proportions of glycine-ir only, GABA-ir only, and doubly immunolabeled neurons in the major populations of the spinal cord in prolarval, larval and adult lamprey

Population	Phenotype	Prolarvae (n=3)	Larvae (n=3)	Adults (n=3)
Dorsomedial gray (prolarval alar region)	Glycine +/ GABA -	94.3% (1.55; 17.0) *	56.6% (4.88; 143.4)	41.4% (3.21; 936.7)
	Glycine +/ GABA +	0% (0; 0)	11.1% (1.05; 30.8)	23.7% (1.84; 536.8)
	GABA +/ Glycine -	5.7% (0.09; 1.0)	32.3% (2.78; 81.7)	34.8% (2.69; 787.3)
Lateral gray (prolarval basal region)	Glycine +/ GABA -	98.2% (6.90; 75.9)	73.3% (3.24; 95.4)	66.3% (4.21; 1,230.7)
	Glycine +/ GABA +	0% (0; 0)	2.0% (0.09; 2.6)	18.8% (1.20; 350.1)
	GABA +/ Glycine -	1.8% (0.12; 1.3)	24.7% (1.09; 32.1)	14.7% (0.93; 273.8)
CSF-c cells	Glycine +/ GABA -	26.5% (0.27; 3.0)	5.6% (0.50; 14.7)	8.7% (1.08; 317.4)
	Glycine +/ GABA +	20.5% (0.21; 2.4)	23.0% (2.04; 60.1)	40.7% (5.08; 1,484.4)
	GABA+/ Glycine -	53.0% (0.55; 6.1)	71.4% (6.38; 187.4)	50.6% (6.33; 1,848.5)

*The numbers of cells (parenthesis) were pooled from three individuals, corrected by Abercrombie's factor (see Table 1) and corresponded to a single spinal section and to the mean length of a spinal cord segment (prolarvae: 110 µm; larvae: 470 µm; adult: 4,670 µm), respectively.

Fig. 1. Confocal projections of a section of the adult spinal cord showing glycine (**A,B**; red channel) and GABA (**C,D**; green channel) immunostaining, without (A,C) and after (B,D) threshold filtering (see Material and Methods). Arrows indicate faintly fluorescent cells considered as negative. Arrowheads point to clearly immunopositive cells. Asterisks, blood vessel with autofluorescent red blood cells. F, filtered; NF, nonfiltered. Scale bar = 50 μm.

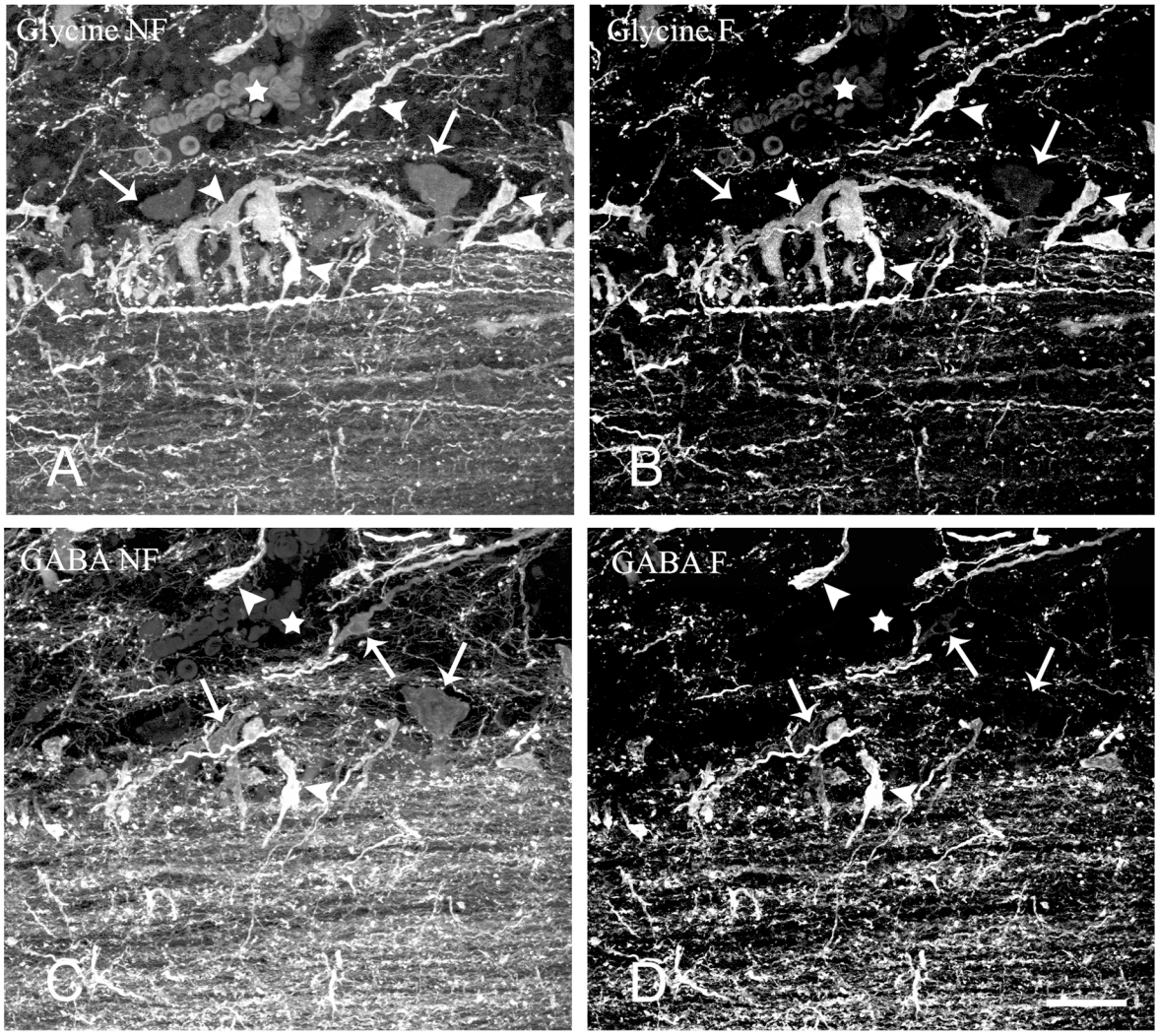


Fig.1.

Fig. 2. Photomicrographs of sections of the spinal cord of a E12 embryo (A), prolarvae (B-H), and an early (8 mm) larva (I) of sea lamprey showing the distribution of glycine-ir cells. **A-E:** Transverse sections showing changes in glycine-ir populations from embryos to prolarvae. Note the vertical, slit-shaped ependymal canal (star) in transverse sections of embryos and prolarvae. Dotted lines indicate the presumptive alar/basal boundary. In prolarvae, thin arrows indicate ventral (basal) cells, open arrows intermediate cells, curved arrow (in E) CSF-c cells, and arrowheads dorsal glycine-ir cells. The prolarval stage is indicated in photographs. **F:** The columnar arrangement of glycine-ir cells at ventral, intermediate, and dorsal levels is better appreciated in a parasagittal section (the left side is closer to the midline than the right side). **G,H:** Details of the ventral commissure of prolarvae showing crossed glycine-ir fibers (arrows). **I:** Transverse section showing the flattened shape of the larval spinal cord produced by lateral extension of the gray matter and partial closure of the central canal. Dotted lines separate putative dorsomedial (Dm) and lateral (L) populations of glycine-ir cells. Double arrows (A-C) and asterisks (D-I), glycine-ir fibers in the marginal layer/white matter. B,C,E,G-I are confocal micrographs. Scale bars = 10 μm in A-E,G-I; 50 μm in F.

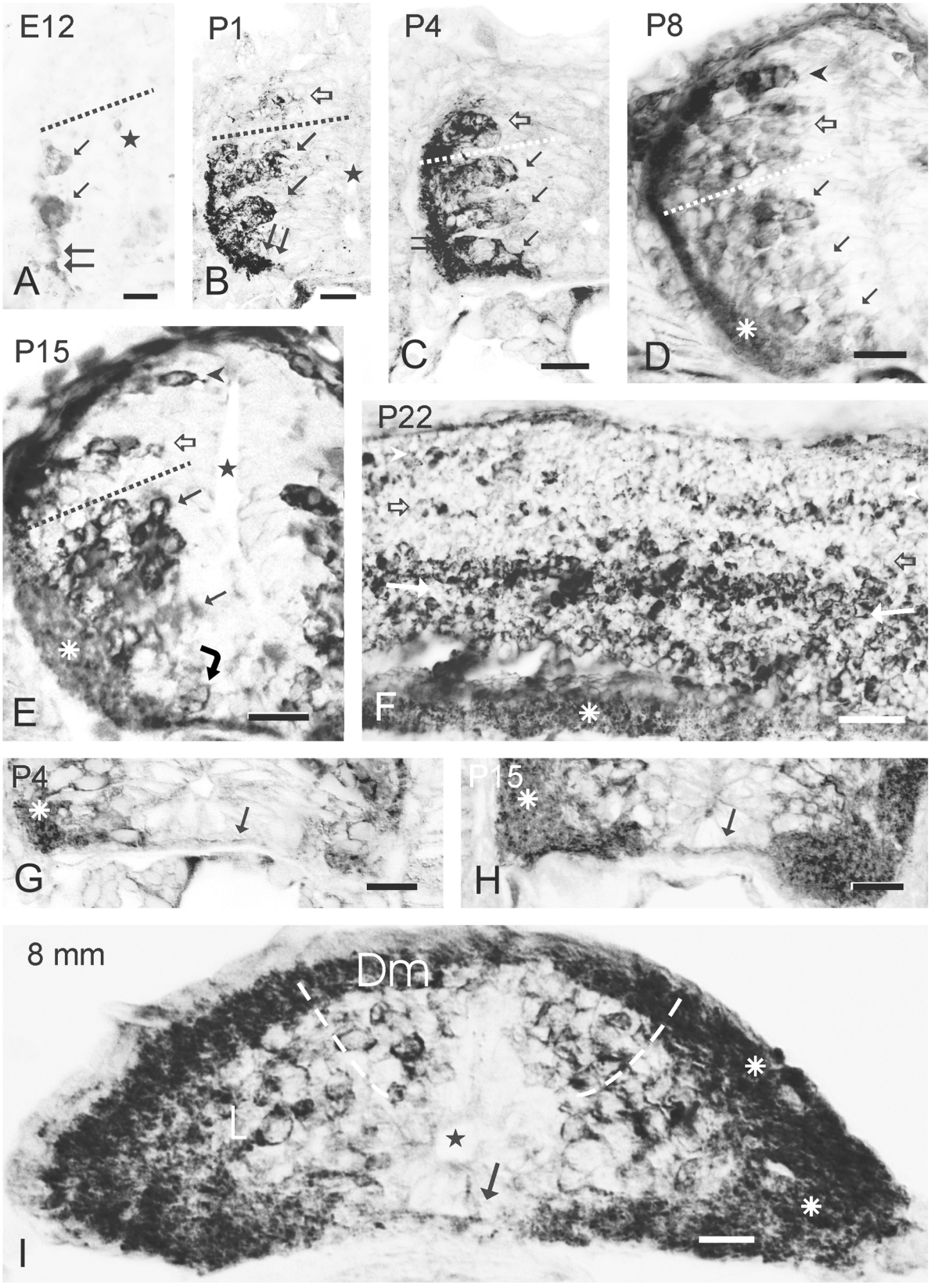


Fig.2.

Fig. 3. A-C: Confocal photomicrographs of a transverse section of the spinal cord of a larva (50 mm in length) showing glycine and GABA immunofluorescence. A: Overlay; B: glycine (magenta); C: GABA (green). Note the abundance of GABA-ir fibers close to the dorsal column (DC; black asterisks) and the marginal neuropil (thick arrows in A and C). Arrowheads point to double-labeled cells (whitish); thin arrows point to single labeled cells. White stars (A-C) indicate CSF-c neurons. Curved arrow (A,B), glycine-ir edge cell. **D:** Schematic drawing showing the distribution of the major glycine-ir cell populations (color code below) in the larval spinal cord. The red star and the black asterisk indicate occasional cells displaced in the white matter dorsal and ventromedial columns. Scale bars = 50 μm.

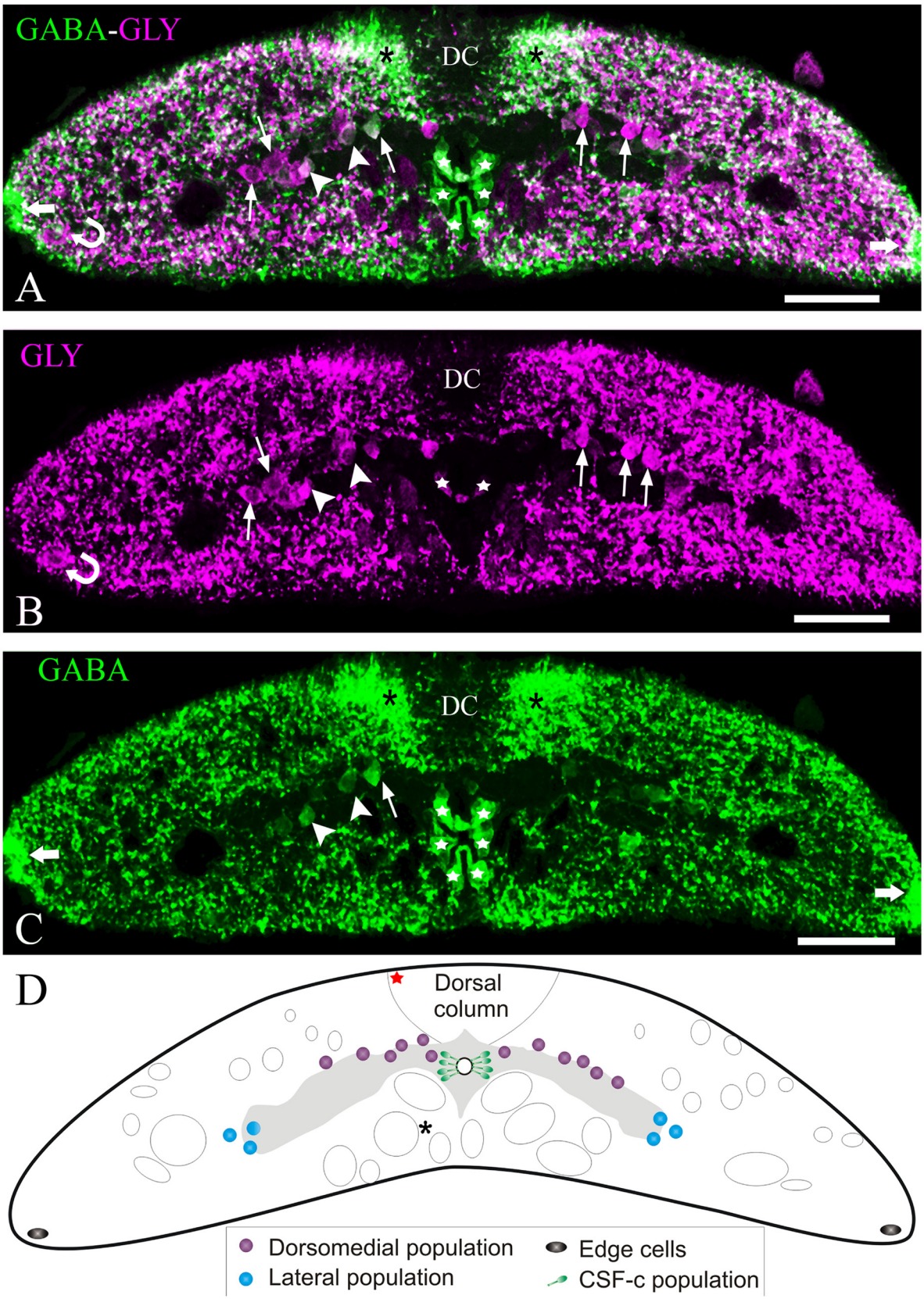

GABA-GLY
DC
A
GLY
DC
B
GABA
DC
C
D
Dorsal column
Dorsomedial population
Lateral population
Edge cells
CSF-c population

Fig.3.

Fig. 4. Confocal photomicrographs of horizontal sections through the spinal cord of a large larva (130 mm) showing glycine-ir cells and processes. **A:** Section passing through the dorsomedial (Dm), lateral (L), and CSF-c (double arrow) glycine-ir populations. Note glycine-ir processes crossing the midline dorsally to the central canal (arrowheads). The thin arrow points to a bipolar cell with a process coursing rostrally, the large-headed arrow a spindle-shaped neuron of L, and the open arrow the dorsal midline. **B:** Section showing dorsomedial (Dm) and lateral (L) glycine-ir populations. The arrows point to small bipolar cells of Dm with longitudinal or oblique processes. Note also processes of lateral cells coursing in the transverse plane. **C:** Detail of an oblique spindle-shaped glycine-ir neuron of the dorsomedial population showing a thick dendrite giving rise to several collaterals (arrowheads). **D:** Detail of a small tripolar glycine-ir cell of the dorsomedial population (arrow). **E:** Section passing through the lateral glycine-ir population showing the pear-shaped morphology of most neurons and their processes coursing in the transversal plane. **F:** Detail of a spindle-shaped neuron of the lateral glycine-ir population showing the rostral orientation of its main process (arrow). **G:** Glycine-ir edge cell (arrow) near the lateral margin of the cord. **H:** Small glycine-ir conic neuron (arrow) closely associated with large ventromedial axons (asterisks). **I:** Section passing through the ventral midline commissure (indicated by open arrows) showing numerous glycine-ir commissural fibers. Asterisk, giant Müller axon. Scale bars = 25 μm.

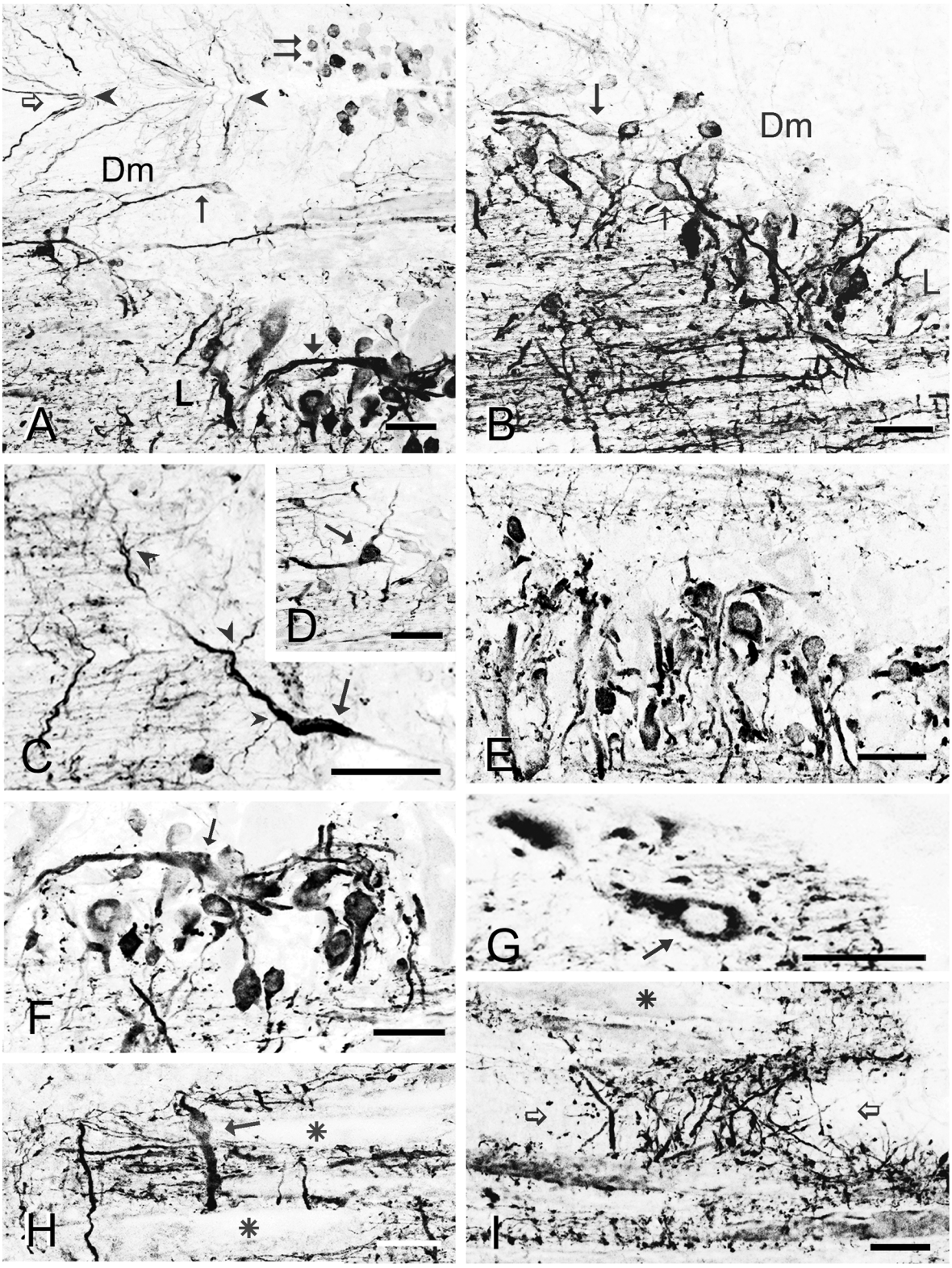


Fig.4.

Fig. 5. Confocal photomicrographs of details of transverse sections of the larval spinal cord gray matter showing cells with glycine and/or GABA immunofluorescence. Note cells with single (arrows) and double immunolabeling (arrowheads). Left column, overlay (double channel); central column, glycine (magenta); right column, GABA (green). **A-A’’:** Dorsomedial populations. **B-B’’:** Lateral populations. **C-C’’:** CSF-c neurons. White stars, central canal. **D-D’’:** Glycinergic edge cell (arrow; asterisk, cell nucleus) and GABA-ir marginal neuropil. In A-A’’, B-B’’, and D-D’’, medial is to the right. Scale bars = 25 μm in A; 10 μm in B; 5 μm in C,D.

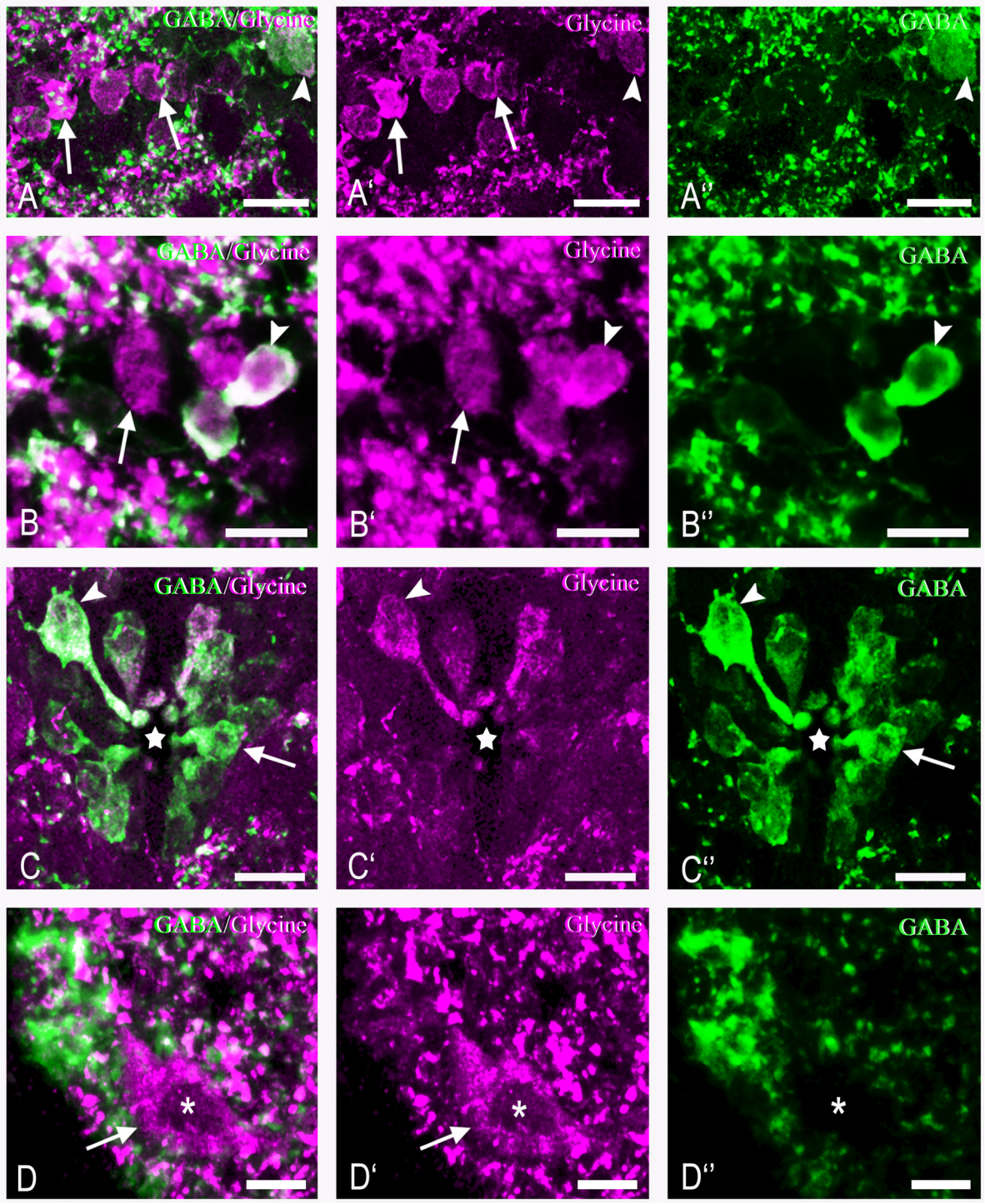


Fig.5.

Fig. 6. Photomicrographs of transverse sections of the larval spinal cord showing glycine-ir cells (A-C) and fibers (D). **A:** Glycine-ir conic neuron (arrowhead) in the ventromedial white matter close to a giant reticulospinal axon (RF). Note the short ventral dendrite directed to the giant axon. **B:** Interstitial glycine-ir cell (arrowhead) in the dorsal column. Note the scarcity of positive processes. **C:** Glycine-ir cell in dorsomedial location sending a process (arrow) to the dorsal funiculus. **D:** Ventral funiculus showing glycine-ir fibers crossing to the contralateral side below the central canal (arrow) and fibers surrounding and possibly contacting giant reticulospinal axons (arrowheads). In A and D, thick arrows point to the ventral midline. A, C, and D are inverted and gray-scale-converted fluorescent micrographs; B shows brightfield microscopy. Larval length: A and D, 130 mm; B and C, 105 mm. Scale bars = 20 μm.

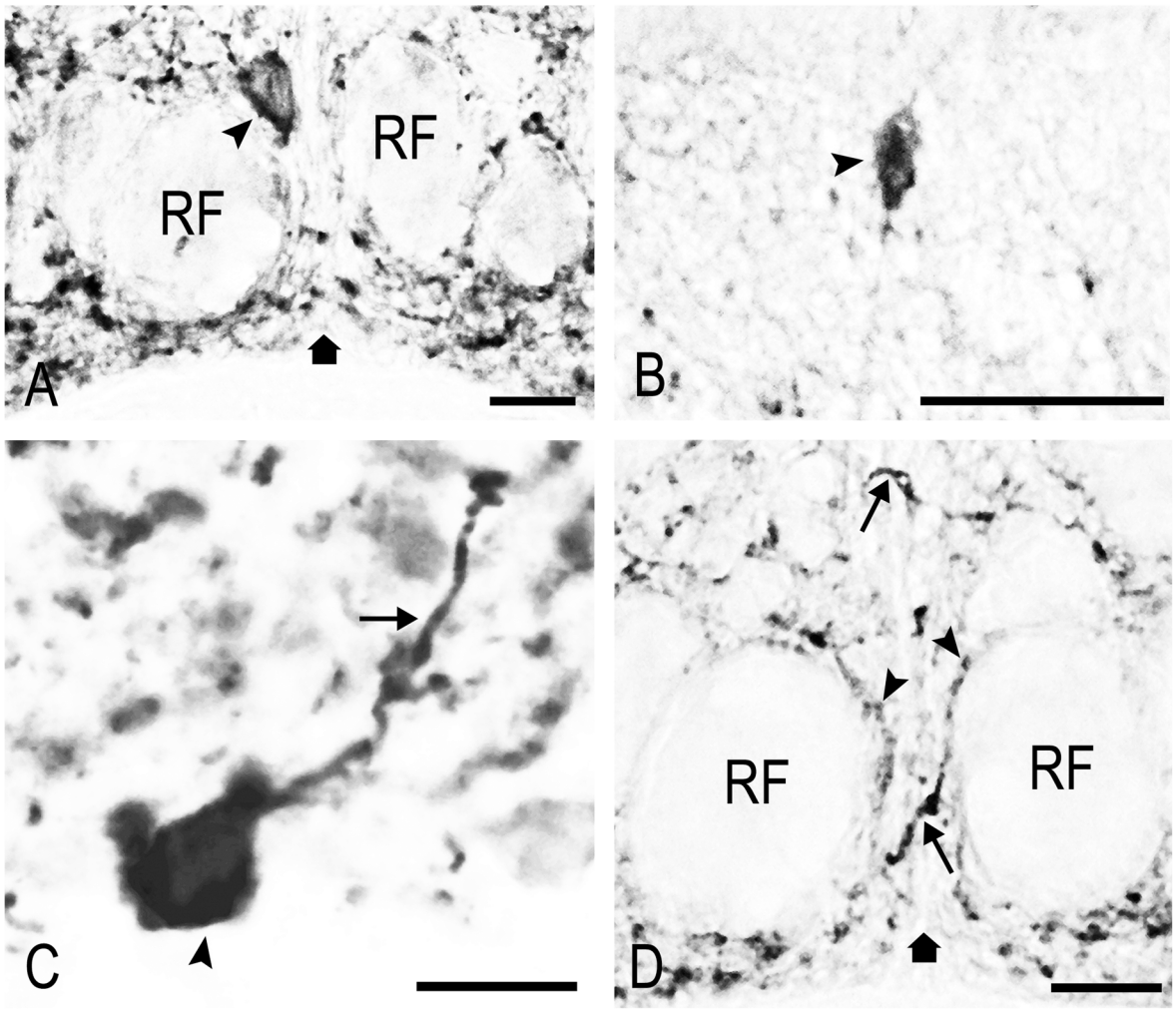


Fig.6.

Fig. 7. Confocal photomicrographs of the spinal cord of postmetamorphic (A,D) and upstream-migrating adult lampreys (B,C,E,F) showing the distribution of glycine (magenta) and GABA (green) immunofluorescence. Cells showing double immunolabeling appear whitish to pinkish (A-F). **A:** Detail of dorsomedial cells in a transverse section. **B,C:** Dorsomedial cells of the adult in parasagittal sections. Note the presence of tripolar and spindle-shaped cells. **D:** Transverse section of the spinal cord at the transition with the rhombencephalon showing dorsomedial cells. **E:** Lateral glycine-ir cell population of the adult in a parasagittal section. Note synaptic boutons (mostly GABA-ir) around immunonegative profiles of motoneurons and interneurons (asterisks). **F:** Parasagittal section through the lateral population showing a large glycine-ir cell with thick dendrites and the axon exit. Arrows point to singly labeled cells; arrowheads point to doubly labeled cells. A-F: Overlay; A'-D': glycine; A''-D'': GABA. Scale bars = 25 μm.

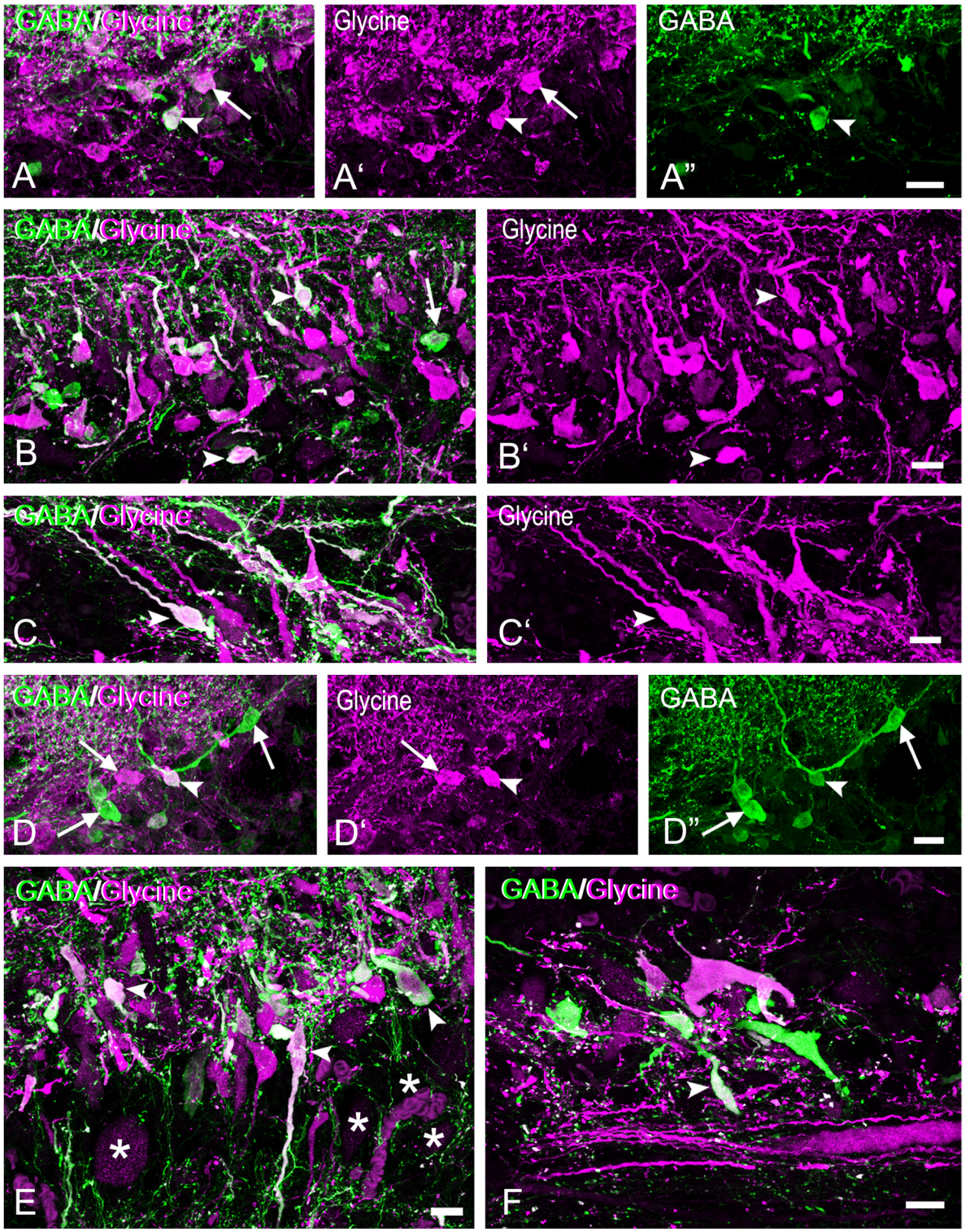

GABA/Glycine
A
Glycine
A‘
GABA
A”
GABA/Glycine
B
Glycine
B‘
GABA/Glycine
C
Glycine
C‘
GABA/Glycine
D
Glycine
D‘
GABA
D”
GABA/Glycine
E
GABA/Glycine
F

Fig.7.

Fig. 8. Photomicrographs of sections of adult spinal cord showing glycine (magenta) and GABA (green) immunoreactivities. **A:** Transverse section through the central canal of the adult spinal cord showing CSF-c neurons with intraependymal (arrowhead) and periependymal (arrow) perikarya. Stars, central canal. A: Merged; A': glycine; A'': GABA. **B-D:** Transverse section of the adult spinal cord showing GABA-ir and glycine-ir fibers in the dorsal (B), dorsolateral (C), and ventromedial (D) white matter columns. In B, the dorsomedial region (left half) shows mostly thin GABA-ir fibers. Note a few doubly labeled fibers in the dorsal and dorsolateral columns (in white, arrowheads in B,C). The thick arrow in C points to the GABA-ir marginal neuropil. In D, note the coarse glycine-ir axons (asterisks), thick Müller axons (stars), and a rather thick GABA-ir fiber (thick arrow). In B-D, medial is to the left. **E:** Transverse section through the lateral border of the gray matter showing several GABA-ir lateral neurons and numerous thin bouton-like structures, mostly GABA-ir. Note thick glycine-ir axons coursing in the neighbor white matter. **F:** Parasagittal section through the lateral white matter of the rostral spinal cord of an adult lamprey showing that most glycine-ir and GABA-ir axons course longitudinally. Note thick (asterisks) and thin (thin arrow) glycine-ir axons and a rather thick (thick arrow) and very thin (arrowheads) GABA-ir fibers. A-E are confocal photomicrographs; F is a fluorescence photomicrograph. Scale bars = 10 μm in A; 25 μm in B-F.

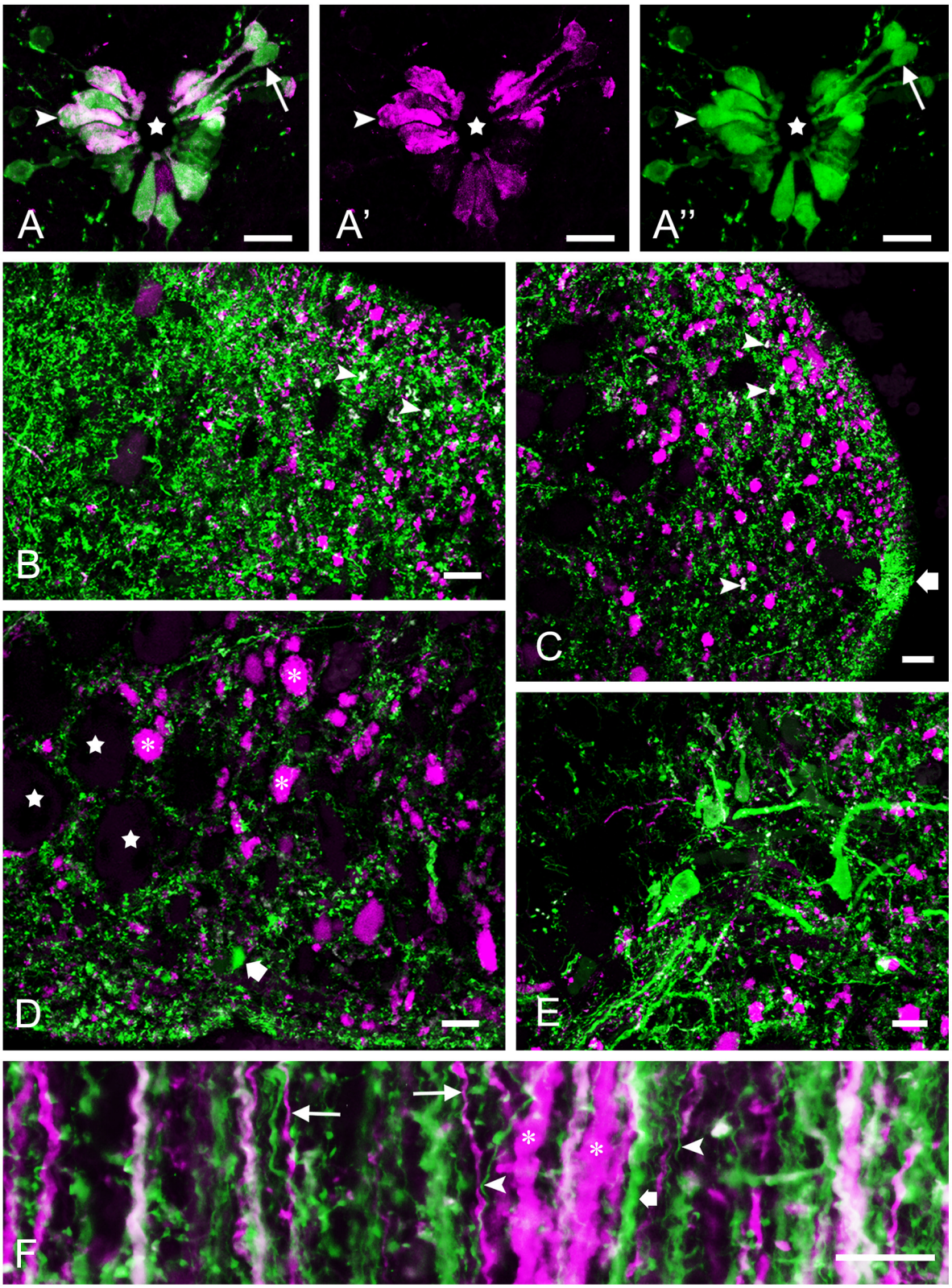


Fig.8.

Fig. 9. Graphic representation of the diameter distribution of glycine-ir fibers coursing longitudinally in the dorsomedial (DM), dorsolateral (DL), ventromedial (VM), and ventrolateral (VL) white matter columns of the adult rostral spinal cord. Fibers less than 2 μm thick were not measured. Values in the y axis correspond to the frequency of fibers, those in the x axis to the axon diameter (micrometers).

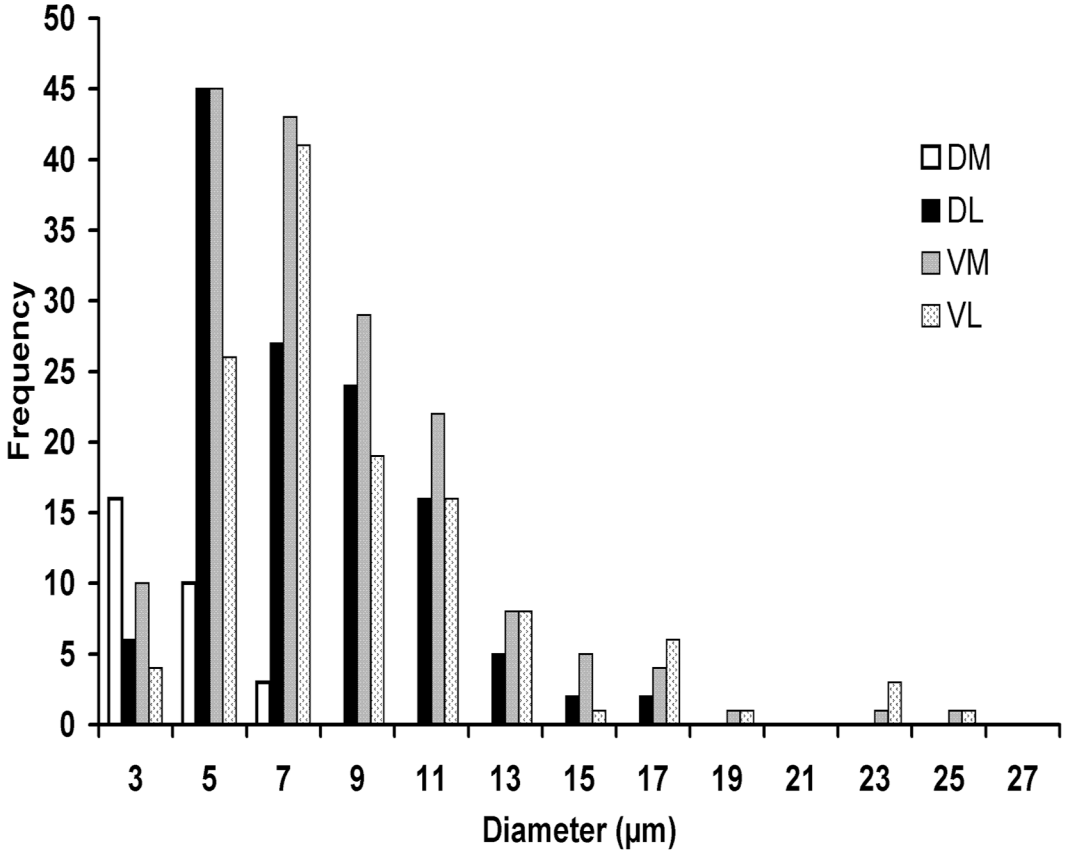

50
45
40
35
30
25
20
15
10
5
0
Frequency
DM
DL
VM
VL
3
5
7
9
11
13
15
17
19
21
23
25
27
Diameter (µm)

Fig.9.

REFERENCES

Abercrombie M. 1946. Estimation of nuclear population from microtome sections. Anat Rec 94:239-247.

Alford S, Williams TL. 1989. Endogenous activation of glycine and NMDA receptors in lamprey spinal cord during fictive locomotion. J Neurosci 9:2792-2800.

Alford S, Sigvardt KA, Williams TL. 1990a. GABAergic control of rhythmic activity in the presence of strychnine in the lamprey spinal cord. Brain Res 506:303-306.

Alford S, Williams TL, Sigvardt KA. 1990b. Effects of bicuculline and strychnine on synaptic inputs to edge cells during fictive locomotion. Brain Res 509:137-140.

Allain AE, Baïri A, Meyrand P, Branchereau P. 2004. Ontogenic changes of the GABAergic system in the embryonic mouse spinal cord. Brain Res 1000:134-147.

Allain AE, Baïri A, Meyrand P, Branchereau P. 2006. Expression of the glycinergic system during the course of embryonic development in the mouse spinal cord and its co-localization with GABA immunoreactivity. J Comp Neurol 496:832-846.

Aprison MH, Daly EC. 1978. Biochemical aspects of transmission in inhibitory synapses: the role of glycine. Adv Neurochem 3:203-294.

Aprison MH, Werman R. 1965. The distribution of glycine in cat spinal cord and roots. Life Sci 4:2075-2083.

Barker JL, Ransom BR. 1978. Amino acid pharmacology of mammalian central neurones grown in tissue culture. J Physiol 280:331-354.

Batueva V, Suderevskaya EI, Vesselkin NP, Pierre J, Repérant J. 1990. Localisation of GABA-immunopositive cells in the river lamprey spinal cord. J Hirnforsch 31:739-745.

Berki ACS, O'Donovan MJ, Antal M. 1995. Developmental expression of glycine immunoreactivity and its colocalization with GABA in the embryonic chick lumbosacral spinal cord. J Comp Neurol 302:583-596.

Betz H. 1987. Biology and structure of the mammalian glycine receptor. Trends Neurosci 10:113-117.

Birinyi A, Parker D, Antal M, Shupliakov O. 2001. Zinc co-localizes with GABA and glycine in synapses in the lamprey spinal cord. J Comp Neurol 433:208-221.

Bormann J, Hamill OP, Sakmann B. 1987. Mechanism of anion permeation through channels gated by glycine and gamma-aminobutyric acid in mouse cultured spinal neurones. J Physiol 385:243-286.

Brodin L, Dale N, Christenson J, Storm-Mathisen J, Hökfelt T, Grillner S. 1990. Three types of GABA-immunoreactive cells in the lamprey spinal cord. Brain Res 508:172-175.

Buchanan JT, Grillner S. 1988. A new class of small inhibitory interneurones in the lamprey spinal cord. Brain Res 438:404-407.

Cangiano L, Grillner S. 2005. Mechanisms of rhythm generation in a spinal locomotor network deprived of crossed connections: the lamprey hemicord. J Neurosci 25:923-935.

Christenson J, Alford S, Grillner S, Hökfelt T. 1991. Co-localized GABA and somatostatin use different ionic mechanisms to hyperpolarize target neurons in the lamprey spinal cord. Neurosci Lett 134:93-97.

Cubelos B, Giménez C, Zafra F. 2005. Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. Cereb Cortex 15:448-459.

Dale N, Ottersen OP, Roberts A, Storm-Mathisen J. 1986. Inhibitory neurones of a motor pattern generator in *Xenopus* revealed by antibodies to glycine. Nature 324:255-257.

Dubuc R, Bongianni F, Ohta Y, Grillner S. 1993. Dorsal root and dorsal column mediated synaptic inputs to reticulospinal neurons in lampreys: involvement

of glutamatergic, glycinergic, and GABAergic transmission. J Comp Neurol 327:251-259.

Garrow TA, Brenner AA, Whitehead VM, Chen XN, Duncan RG, Korenberg JR, Shane B. 1993. Cloning of human cDNAs encoding mitochondrial and cytosolic serine hydroxymethyltransferases and chromosomal localization. J Biol Chem 268:11910-11916.

Gold MR, Martin AR. 1983. Inhibitory conductance changes at synapses in the lamprey brainstem. Science 221:85-87.

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

Gustafsson JS, Birinyi A, Crum J, Ellisman M, Brodin L, Shupliakov O. 2002. Ultrastructural organization of lamprey reticulospinal synapses in three dimensions. J Comp Neurol 450:167-182.

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

Higashijima S, Mandel G, Fetcho JR. 2004a. Distribution of prospective glutamatergic, glycinergic, and GABAergic neurons in embryonic and larval zebrafish. J Comp Neurol 480:1-18.

Higashijima S, Schaefer M, Fetcho JR. 2004b. Neurotransmitter properties of spinal interneurons in embryonic and larval zebrafish. J Comp Neurol 480:19-37.

Hinckley C, Seebach B, Ziskind-Conhaim L. 2005. Distinct roles of glycinergic and GABAergic inhibition in coordinating locomotor-like rhythms in the neonatal mouse spinal cord. Neuroscience 131:745-758.

Holstein GR, Martinelli GP, Henderson SC, Friedrich VL Jr, Rabbitt RD, Highstein SM. 2004. γ-Aminobutyric acid is present in a spatially discrete subpopulation of hair cells in the crista ampullaris of the toadfish *Opsanus tau*. J Comp Neurol 471:1-10.

Homma S, Rovainen CM. 1978. Conductance increases produced by glycine and gamma-aminobutyric acid in lamprey interneurones. J Physiol 279:231-252.

Kirsch J, Betz H. 1998. Glycine-receptor activation is required for receptor clustering in spinal neurons. Nature 392:717-720.

Malosio ML, Marqueze-Pouey B, Kuhse J, Betz H. 1991. Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. EMBO J 10:2401-2409.

Matthews G, Wickelgren WO. 1979. Glycine, GABA and synaptic inhibition of reticulospinal neurones of lamprey. J Physiol 293:393-415.

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

Messersmith EK, Redburn DA. 1992. γ-Aminobutyric acid immunoreactivity in multiple cell types of the developing rabbit retina. Vis Neurosci 8:201-211.

Messersmith EK, Redburn DA. 1993. The role of GABA during development of the outer retina in the rabbit. Neurochem Res 18:463-470.

Nieuwenhuys R, Nicholson C. 1998. Lampreys, Petromyzontoidea. In: Nieuwenhuys R, ten Donkelaar HJ, Nicholson C, editors. The central nervous system of vertebrates, vol 1. Berlin: Springer Verlag. p 397-486.

O'Donovan M, Sernagor E, Sholomenko G, Ho S, Antal M, Yee W. 1992. Development of spinal motor networks in the chick embryo. J Exp Zool 261:261-273.

Phelps PE, Alijani A, Tran TS. 1999. Ventrally located commissural neurons express the GABAergic phenotype in developing rat spinal cord. J Comp Neurol 409:285-298.

Rampon C, Luppi PH, Fort P, Peyron C, Jouvet M. 1996. Distribution of glycine-immunoreactive cell bodies and fibers in the rat brain. Neuroscience 75:737-755.

Reichling DB, Kyrozis A, Wang J, MacDermott AB. 1994. Mechanisms of GABA and glycine depolarization-induced calcium transients in rat dorsal horn neurons. J Physiol 476:411-421.

Roberts A, Dale N, Ottersen OP, Storm-Mathisen J. 1988. Development and characterization of commissural interneurones in the spinal cord of *Xenopus laevis* embryos revealed by antibodies to glycine. Development 103:447-461.

Rodicio MC, Villar-Cervino V, Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R. 2005. Chemoarchitecture of the dorsal column nucleus of the larval sea lamprey. Brain Res Bull 66:536-540.

Rovainen CM. 1974. Synaptic interactions of identified nerve cells in the spinal cord of the sea lamprey. J Comp Neurol 154:189-206.

Rovainen CM. 1982. Neurophysiology. In: Hardisty MW, Potter IC, editors. The biology of lampreys, vol. 4A. London: Academic Press. p 2-136.

Ruiz Y, Pombal MA, Megías M. 2004. Development of GABA-immunoreactive cells in the spinal cord of the sea lamprey, *P. marinus*. J Comp Neurol 470:151-163.

Saint-Amant L, Drapeau P. 2000. Motoneuron activity patterns related to the earliest behavior of the zebrafish embryo. J Neurosci 20:3964-3972.

Shupliakov O, Fagersted P, Ottersen OP, Storm-Mathiesen J, Grillner S, Brodin L. 1996. Immunocytochemical localization of glycine in the lamprey spinal cord with reference to GABAergic and glutamatergic synapses: a light and electron microscopic study. Acta Biol Hung 47: 393-410.

Sueiro C, Carrera I, Molist P, Rodrı´guez-Moldes I, Anadón R. 2004. Distribution and development of glutamic acid decarboxylase immunoreactivity in the spinal cord of the dogfish *Scyliorhinus canicula* (elasmobranchs). J Comp Neurol 478:189-206.

Tran TS, Alijani A, Phelps PE. 2003. Unique developmental patterns of GABAergic neurons in rat spinal cord. J Comp Neurol 456:112-126.

Uematsu K, Shirasaki M, Storm-Mathisen J. 1993. GABA- and glycine-immunoreactive neurons in the spinal cord of the carp, *Cyprinus carpio*. J Comp Neurol 332:59-68.

Van Dongen PAM, Hökfelt T, Grillner S, Verhofstad AAJ, Steinbusch HWM, Cuello AC, Terenius L. 1985. Immunohistochemical demonstration of some putative neurotransmitters in the lamprey spinal cord and spinal ganglia: 5-

hydroxytryptamine-, tachykinin-, and neuropeptide-Y-immunoreactive neurones and fibers. J Comp Neurol 234:501-522.

Vesselkin NP, Rio JP, Adanina VO, Kenigfest NB, Repérant J. 1995. Colocalization of glutamate and glycine in giant fiber synapses of the lamprey spinal cord. J Hirnforsch 36:229-237.

Vesselkin NP, Rio JP, Adanina VO, Repérant J. 2000. GABA- and glycine-immunoreactive terminals contacting motoneurons in lamprey spinal cord. J Chem Neuroanat 19:69-80.

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

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

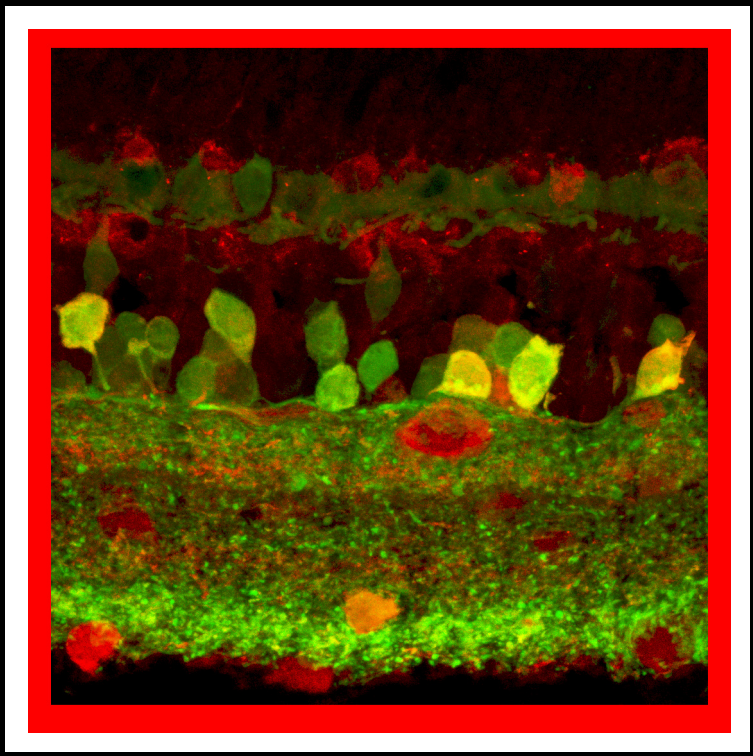
Vinay L, Barthe JY, Grillner S. 1996. Central modulation of stretch receptor neurons during fictive locomotion in lamprey. J Neurophysiol 76: 1224-1235.

Wannier T, Senn W. 1998. Recruitment of reticulospinal neurones and steady locomotion in lamprey. Neural Netw 11:1005-1015.

Wannier T, Orlovsky G, Grillner S. 1995. Reticulospinal neurones provide monosynaptic glycinergic inhibition of spinal neurones in lamprey. Neuroreport 6:1597-1600.

Young AB, Snyder SH. 1974. The glycine synaptic receptor: evidence that strychnine binding is associated with the ionic conductance mechanism. Proc Natl Acad Sci U S A 71:4002-4005.

Presence of Glutamate, Glycine and GABA in the Retina of the Larval Sea Lamprey: A Comparative Immunohistochemical Study of Classical Neurotransmitters in Larval and Postmetamorphic Retinas



Chapter 4

INTRODUCTION

The development of the lamprey retina and eye is exceptional among vertebrates. After an initial embryonic period when a small retina with ganglion cells and a single type of photoreceptor appears (Kleerekoper, 1972; Dickson and Collar, 1979; De Miguel and Anadón, 1987; Meléndez-Ferro et al., 2002a), retinal growth slows until the mid-larval stage (starting in sea lamprey larvae of about 60 mm in body length) when the margins of the retina are transformed into a highly proliferating neuroepithelium that produces by progressive lateral expansion a large undifferentiated retina lacking photoreceptors. During the second half of the larval period, between 60 mm and transformation at 120 mm length in sea lamprey, the retina is comprised of a small early-differentiated central region and an extensive lateral undifferentiated zone. The layering and differentiation of the lateral retina and the appearance of the two types of adult photoreceptors occur during transformation (De Miguel and Anadón, 1987). Previous studies have shown that the expression of opsin in the central retina occurs early in prolarvae, before differentiation of outer photoreceptor segments (Meléndez-Ferro et al., 2002a). Tract-tracing studies and GABA immunocytochemistry have revealed the early appearance of ganglion cells and retinofugal projections (De Miguel et al., 1989, 1990), as well as retinopetal GABA-immunoreactive fibers (Rodicio et al., 1995; Anadón et al., 1998; Meléndez-Ferro et al., 2002a). Both ganglion cells and GABAergic retinopetal fibers are present in the lateral retina during the second larval period (Anadón et al., 1998). However, despite Golgi impregnation studies indicating that the major classes of retinal neurons appear during the late larval period (Rubinson and Cain, 1989), to the best of our knowledge the presence of classical neurotransmitters has not been reported in any of these cells, either in the central or the lateral larval retina. In fact, we recently reported that cholinergic amacrine cells do not appear in the lamprey retina until early metamorphosis (Pombal et al., 2003). Previous studies thus suggest that, aside from the GABAergic fibers of central origin, all neurotransmitters are absent from cells of the larval retina. Accordingly, it is not known whether the early differentiated (opsin-expressing) central retina has functional neural circuitry.

In the present study, the putative neurochemical differentiation of neural circuitry in the lamprey retina was analyzed during the larval period using a set of antibodies directed against several classical neurotransmitters (glutamate, GABA,

glycine, aspartate, serotonin and dopamine) and a neurotransmitter-synthesizing enzyme (tyrosine hydroxylase). For comparison, the retina of recently transformed young lampreys was also analyzed. The results provide the first demonstration of glutamate immunoreactivity in neurons of the central and lateral retina of larvae, and reveal further details of the centrifugal innervation by GABA – immunoreactive (ir) fibers. The significance of these findings is discussed in the context of lamprey biology and its remarkable retinal development.

MATERIAL AND METHODS

Animals

Larval sea lampreys, *Petromyzon marinus* L, ranging from 30 to 156 mm in length, were collected from the River Ulla (Galicia, Northwest Spain) and maintained in aerated aquaria before processing. Four recently metamorphosed young sea lamprey, provided by the Estación Biolóxica de Ximonde, were also used for comparison. All procedures conformed to European Community guidelines on animal care and experimentation. Animals were deeply anaesthetized with benzocaine (0.05%; Sigma, St. Louis, MO) prior to fixation. For glycine (GLY; n=12), γ-aminobutyric acid (GABA; n=12), glutamate (GLU, n=8), aspartate (n=5) and dopamine (DA; n=10) immunohistochemistry, heads of 40 larvae and eyes of 4 young postmetamorphic lampreys were fixed by immersion in 5% glutaraldehyde/1% sodium metabisulfite in 0.05M Tris buffer (pH 7.4). For serotonin (5HT; n=10), calretinin (CR; n=5) and tyrosine-hydroxylase (TH; n=6) immunohistochemistry, larval heads and two recently metamorphosed lampreys were fixed by immersion in cold 4% paraformaldehyde in 0.1M phosphate buffer (PB) at pH 7.4. To visualize these antisera, samples were embedded in Tissue Tek (Sakura, Torrance, CA) and cut on a cryostat (16 µm thick). For opsin immunocytochemistry (n=3), larval heads were fixed in Bouin's fluid, embedded in paraffin and sectioned on a rotary microtome (10 µm thick).

Two additional larvae were fixed with 2% glutaraldehyde and 2% paraformaldehyde in Tris buffer containing 1% metabisulfite and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA). Semithin sections were obtained with glass knives on an ultramicrotome and mounted on glass slides.

Single label immunohistochemistry

For bright-field light microscopy, specimens were processed according to the peroxidase-antiperoxidase (PAP) technique. Sections were incubated in one of the following primary antisera raised in rabbits: 5HT (Incstar, Stillwater, MN; dilution 1:5000); DA (H.W.M. Steinbusch, Maastricht, The Netherlands; 1:900); TH (Chemicon, Temecula, CA; 1:1000); GABA (Affiniti, Mamhead, U.K.; 1:1000); GLU (Sigma; 1:2000); GLY (Chemicon; 1:200); aspartate (Chemicon; 1:200); opsin

(CERN-922 anti-bovine rod opsin provided by Prof. W. DeGrip; 1:1000) and calretinin (SWant, Bellinzona, Switzerland; 1:1500) (see Table 1). The tissue was subsequently incubated with goat anti-rabbit immunoglobulin (Sigma; 1:100) and rabbit PAP complex (Sigma; 1:400). Other sections were sequentially incubated in mouse anti-GABA (GABA93, Martinelli; 1:50), rabbit anti-mouse IgG (Dako, Glostrup, Denmark; 1:600) and mouse PAP complex (Sigma; 1:600). The immunocomplexes were developed by immersion in 3,3´-diaminobenzidine (Sigma; 0.6 mg/ml) with 0.003% H_2O_2. Photomicrographs were obtained using an Olympus DP 12 color digital camera (Tokyo, Japan). Images were converted to gray scale and adjusted for brightness and contrast using Corel Photo-Paint (Corel, Ottawa, Canada).

Prior to GLU, GABA and GLY immunostaining, semithin plastic sections were deplasticized with ethanol-NaOH, rinsed four times with PB and then immunostained following a similar protocol to that used for cryostat sections.

Double immunofluorescence and confocal laser scanning microscopy

For double immunofluorescence, sections were pretreated with 0.2% $NaBH_4$ in water for 45 minutes at room temperature. Alternate series' of sections were sequentially incubated for 3 days at 4ºC with a mixture of either 1) rabbit polyclonal anti-GLY antiserum (Chemicon; 1:100) and mouse monoclonal anti-GABA (GABA93, Holstein et al.; 1:50), or 2) rabbit polyclonal anti-GLU antiserum (Chemicon; 1:1000) and mouse monoclonal anti-GABA (GABA93; 1:50), or rabbit polyclonal anti-aspartate (Chemicon; 1:100) and mouse monoclonal anti-GABA (GABA93; 1:50). All sections were subsequently incubated with Cy3-conjugated goat anti-rabbit immunoglobulin (Chemicon; 1:200) and fluorescein-conjugated goat anti-mouse IgG (Chemicon; 1:50). Sections were observed and photographed using a spectral confocal microscope (Leica TCS-SP2). For presentation of some figures, single channel stack projections were inverted and then adjusted for brightness and contrast using Adobe Photoshop (Adobe, San Jose, CA).

All antibodies for both single and double immunolabeling were diluted in Tris-buffered saline containing 0.2% Triton X-100 and 3% normal goat serum.

Specificity controls

Control sections were processed as above, except for the omission of primary antisera. No staining was observed in these controls. Moreover, staining of antisera against neurotransmitter-BSA (glycine, aspartate, GABA, serotonin, dopamine) or neurotransmitter-KLH (glutamate) conjugates was not modified by preabsorption of the primary antiserum with BSA (Sigma) or KLH (Sigma), respectively. As positive controls for cases of negative immunostaining of the larval retina, we used the brain tissue adjacent in the same head sections and the brain and the retina of metamorphosed lampreys.

The specificity of all primary antibodies has been well-characterized by the suppliers (Table 1). According to the supplier, the anti-glutamate antiserum recognizes L-glutamic acid immobilized on an affinity membrane and no cross-reaction is observed with L-aspartic acid, L-glutamine, L-asparagine, and L-alanine. Weak cross-reactivity is observed with Gly-Asp, GABA, β-alanine, glycine and 5-aminovaleric acid (amino acid concentration 5-10 mM). The anti-aspartate antiserum recognizes L-aspartic acid immobilized on affinity membrane. No cross-reaction of this antiserum is observed with L-glutamic acid, L-glutamine and L-alanine. Weak cross-reactivity is observed with L-asparagine, GABA, β-alanine, glycine and 5-aminovaleric acid (amino acid concentration 5-10 mM). The cross-reactivity of the glycine antiserum determined by ELISA or RIA assays indicates that it only weakly or very weakly cross-reacts with alanine- (1/100), GABA-(1/500), taurine- (1/1000), aspartate- (1/20000) or glutamate-BSA (<1/20000) conjugates as regards the glycine-BSA conjugate. The aspartate antiserum is highly specific of the L-aspartate-BSA conjugate, the cross-reactivities determined using an ELISA test by competition experiments with GLU-BSA and GABA-BSA conjugates are much lower (1/30000 and >1/100000, respectively) than with L-aspartate-BSA conjugate. The mab93 monoclonal GABA antibody was tested by ELISA against BSA conjugates of GABA, 16 amino acids, histamine, serotonin, adrenaline, noradrenaline and histamine: it showed high specificity for GABA conjugate and negligible levels of cross-reactivity with the other conjugates (Holstein et al., 2004). The specificity of the rabbit anti-GABA antiserum has been extensively characterized by ELISA and immunohistochemical techniques, and was adsorbed against BSA-glutaraldehyde. The dopamine antiserum does cross-react with noradrenaline for less that 10% and

with other monoamines for less than 1%. The anti-serotonin antibody does not cross-react with 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid and dopamine. The calretinin antiserum does not cross-react with calbindin D-28k or other known calcium binding proteins, as determined by its distribution in the brain, as well as by immunoblots. This antiserum was already tested by Western blotting in lamprey brain extracts, showing a single stained band of the appropriate molecular weight (Villar-Cheda et al., 2006). The anti-opsin antiserum was shown to stain specifically photoreceptors in lampreys (García-Fernández et al., 1997; Meléndez-Ferro et al., 2002a) and teleosts (Candal et al., 2005). Since this immunoreaction was only intended for demonstrating photoreceptors, characterization of opsin type specificity was not done.

As a further specificity control, antisera to glutamate, GABA, glycine, aspartate and serotonin were analyzed by Western blotting using protein extracts of adult sea lamprey brain, as already detailed (Villar-Cheda et al., 2006). Briefly, brains (including meninges) were homogenized at 4 °C in 6 volumes of modified RIPA buffer (50 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 0.1% sodium dodecylsulfate, 5 μg/ml aprotinin, pH 7.4). The homogenate was centrifuged at 20,000 × g and the supernatant was precipitated with 6 volumes of 100% methanol. The precipitate was resuspended in modified RIPA buffer and 25 μg of total protein per lane loaded onto 12% acrylamide gels, resolved by SDS-PAGE and then electroblotted in a Mini-Protean 3 cell (Biorad, Hercules, CA) onto 0.2 μm polyvinylidene difluoride membranes (Biorad). Nonspecific binding sites on the membrane were blocked by incubating in 5% powdered non-fat milk dissolved in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 h. After blocking, lanes in membranes were cut off, rinsed in TBST and each incubated overnight with one of the neurotransmitter primary antibodies. The membranes were then rinsed in TBST, incubated with either goat anti-rabbit or goat anti-mouse HRP-conjugated antibodies (Biorad, diluted 1:15000), rinsed again in TBST, and incubated with enhanced chemiluminescent reagent (Biorad Immun-Star HRP kit). Precision Plus protein standards (Biorad) were used as molecular weight (MW) markers.

In western blots, no protein band was stained with the glycine, GABA, serotonin and aspartate antibodies (Fig. 1A), strongly suggesting that these antisera

do not cross-react with native proteins of the lamprey CNS. The anti-glutamate antibody stained a protein band of about 100 kDa in blots (Fig. 1A). In order to investigate distribution of this glutamate-like immunoreactivity, cryostat sections of lamprey brain fixed in buffered 4% paraformaldehyde were submitted to the immunofluorescence procedure detailed above. In these sections, strong staining was observed in large ovoid meningeal cells, whereas neurons and nerve fibers of the adjacent nervous tissue were not stained (Fig. 1B). Likewise, in sections fixed in buffered 5% glutaraldehyde these large meningocytes were also stained. Since neurons and fibers were not stained in these paraformaldehyde-fixed controls, together these results support that the native protein that cross-reacts in western blots with the anti-glutamate antibody is located in meningocytes and, accordingly, does not interfere with immunocytochemical analysis of glutaraldehyde-coupled glutamate in the nervous tissue. A protein band of about 135 kDa was stained in blots with the dopamine antiserum (Fig. 1A). However, demonstration of DA immunoreactivity in the brain and retina was strictly dependent of fixation with glutaraldehyde and addition of metabisulfite to incubation solutions. Since distribution of dopamine immunoreactivity revealed with this antiserum in brain and retina matched with that of tyrosine hydroxylase (Pombal et al., 1997; Abalo et al., 2005; present results), this strongly suggests that the substance demonstrated in lamprey is dopamine.

Additional material

Series of larval lamprey heads from our collection stained with hematoxylin-eosin were used for topographical landmarks.

RESULTS

General organization of the larval eye and retina

With hematoxylin-eosin staining, the eyes of larval lampreys between 60 and 156 mm in length showed the two retinal regions characteristic of the second half of larval life (De Miguel and Anadón, 1987): a central retina with differentiated photoreceptor, outer nuclear and inner nuclear/ganglion cell layers (Fig. 2A), and a lateral region consisting primarily of a thick neuroepithelium in which only a thin optic fiber/inner plexiform layer (IPL) is distinguishable in the innermost region. This latter layer enlarges considerably near the optic nerve head. The central retina is mostly located dorsal to the optic nerve, which has an asymmetrical location in the eye.

Opsin and calretinin immunoreactivities

The CERN 922 anti-opsin antibody revealed the presence of opsin-ir photoreceptor cells in the central retina of the larvae. The antibody stained both segments of the photoreceptors as well as their perikarya, thereby allowing the unequivocal localization of the cells to the central retina. Photoreceptor perikarya formed two rows of cells below the outer limiting membrane in larvae ranging from 38 - 156 mm in length. The cell bodies were oval-shaped and lacked any conspicuous inner processes (Fig. 2B), consistent with previous electron microscopic observations (De Miguel et al., 1989).

In the retina of adult sea lamprey, the calretinin (CR) antibody intensely stained two types of bipolar cell as well as some other cells in the inner nuclear layer (INL) (inner horizontal cells, a few amacrine cells and some ganglion cells; Villar-Cheda et al., 2006), which is similar to that reported in the retina of river lamprey (Dalil-Thiney et al., 1994). In the larval retina, intense CR immunoreactivity was observed in bipolar cells of the central retina (Fig. 2C), and faint to moderate staining was obtained in the putative ganglion cells of both the central and lateral retinal areas (Fig. 2C-D). (The location of ganglion cells in larval sea lamprey has been demonstrated in previous tract-tracing experiments; De Miguel et al., 1989; Anadón et al., 1998). The CR-ir bipolar cells exhibit outer and inner radial processes (Fig. 2C); the inner processes branch in the region of the inner plexiform layer. The

perikarya of CR-ir bipolar cells are more numerous caudal to the optic nerve exit, and are located in a central band that is well-separated from the rows of photoreceptors (Fig. 2C).

Neurotransmitter immunoreactivity in the larval retina and brain

Of the six classical neurotransmitters (glutamate, GABA, glycine, aspartate, serotonin and dopamine) and the catecholamine-synthesizing enzyme (TH) investigated in the larval retina, we observed immunoreactivity for glutamate, glycine and GABA only. The negative results for aspartate, serotonin, dopamine and TH were not due to false negative results, as indicated by the positive staining of cells and fibers in the brain present in the same head sections of the larvae. Each of the antibodies yielded intense immunostaining of specific neuronal populations and of numerous fibers throughout the brain. The distribution of these GABA-, serotonin-, dopamine-, and TH-ir systems is consistent with that reported in previous studies in sea lamprey larvae (Meléndez-Ferro et al., 2002b, 2003; Abalo et al., 2005; Rodicio et al., 2005) and/or with the distributions of serotonin and dopamine in adult river lamprey (Pierre et al., 1992, 1997). A comprehensive study of glutamate and glycine systems in the larval lamprey brain is currently in progress, but further description is beyond the scope of the present report. As a positive control in the retina, we used retinas of recently metamorphosed lampreys (see below).

Glutamate immunoreactivity in the larval retina

Central retina. Glutamate immunoreactivity was observed in both the central and lateral regions of the larval retina, although with distinctly different staining patterns (Figs. 3A-B, 4A-D). Several types of GLU-ir cells were observed in the larval central retina, including photoreceptors, ganglion cells and putative bipolar cells (Figs. 3A-B, 4A-C). These neurons did not show immunoreactivity to GABA (Fig. 3A-B), precluding the possibility that the GLU staining reflected its role as an intermediary metabolite in the synthesis of GABA. Faint GLU immunoreactivity was observed in the inner segments of the photoreceptors, whereas the apical and basal poles of the oval-shaped photoreceptor perikarya showed intense GLU-ir and the cell nuclei were immunonegative (Figs. 3A-B, 4A,C).

The position of horizontal cells in the INL of the central retina of the larval sea lamprey is known from Golgi impregnation studies (Rubinson and Cain, 1989).

These cells can be appreciated as a loose cell region just below the outer nuclear layer in hematoxylin-eosin-stained retinae (Fig. 2A). These horizontal cells are not GLU-immunostained in our material. In the middle of the INL there are small, loosely scattered, faintly GLU-ir cells with elongated perikarya (Figs. 3A-B, 4A,C). On the basis of their shape and position in the outer part of the INL, correlated with cells of similar position and appearance in Golgi stains (Rubinson and Cain, 1989), the cells were identified as bipolar cells. They were located in the same position as the bipolar cells revealed by calretinin immunocytochemistry.

Intensely GLU-ir neurons with rather large spherical perikarya were observed in the inner part of the INL, the IPL and the ganglion cell layer (Figs. 3A-C, 4A-C). Based on their morphology and position in comparison with our previous tract-tracing results (De Miguel et al., 1989; Anadón et al., 1998), these cells were interpreted as ganglion cells. Some of these cells exhibited characteristic ascending processes that reached the outer plexiform layer (OPL) (Fig. 4A) and occasionally branched. Ganglion cells with processes ascending to the OPL correspond to the biplexiform ganglion cells described by De Miguel et al. (1989). It has yet to be determined whether all GLU-ir ganglion cells of the central retina possess such ascending processes. GLU-ir axons of ganglion cells of the central and lateral retina course in the IPL/optic fiber layer to the optic nerve.

Lateral retina. In the lateral retina, GLU immunolabeling was observed only in cells located in the innermost retinal layer. Immunostaining was most prominent toward the central retina and very scarce, if at all present, laterally (Fig. 4C-D). GLU-ir cells were smaller than the ganglion cells of the central retina, but by their size and distribution appeared to correspond to the ganglion cells demonstrated in previous tract-tracing studies (De Miguel et al., 1989; Anadón et al., 1998). Optic fibers were also GLU-immunopositive, providing additional support for the interpretation of the GLU-ir cells as ganglion cells. Near the central retina, some ganglion cells issued long radial processes directed toward the outer regions of the retina (Fig. 4C), similar to the processes of some ganglion cells of the central region. The cells of the very thick neuroblastic layer of the lateral retina did not show GLU immunoreactivity above background. In the lateral retina, occasional GLU-ir processes were observed to ascend through the neuroblastic layer, contacting the outer limiting membrane (Fig. 4D). These processes appeared at the transition

between the lateral region which contained GLU-ir structures, and the marginal region which is exclusively neuroblastic.

Colocalization of glutamate and GABA

Labeling experiments using our monoclonal anti-GABA antibody resulted in specific immunostaining of fibers in the optic nerve and retina, in close agreement with previous results from our laboratory (Anadón et al., 1998). Given the absence of any GABA-immunolabeled perikarya in the larval retina, these processes are interpreted as retinopetal fibers. Additional details of the retinopetal GABA-ir system in relation to retinal ganglion cells were obtained through double label studies using polyclonal anti-glutamate and monoclonal anti-GABA antibodies and spectral laser confocal microscopy.

The immunolabeling with the green-fluorescent secondary antibody used for GABA demonstration may be confused with yellowish autofluorescent bodies, mainly located in the central retina near the outer limiting membrane but also occasionally scattered through the INL, as seen in control sections. Spectral confocal microscopy allowed us to differentiate these autofluorescent bodies from the GABA-ir fibers and boutons by means of image capture using two closely spaced wavelength ranges, one specific for the green fluorophore emission spectrum (490-524 nm) and the other covering a slightly wider range of emission (482-539 nm). Using this approach, the retinopetal fibers were seen to be distributed throughout the lateral and central retina, although the density of such fibers in the central part far exceeded that of the lateral region (Fig. 3A-C). In the central retina, the GABA-ir fibers are found in both the IPL and the adjacent INL (Fig. 3B-C). The highly specific labeling of the monoclonal GABA antibody together with the high spatial resolution of the confocal microscopy revealed that GABA-ir boutons are associated with ganglion cells (Fig. 3B-C). This association is a distinctive feature of retinal ganglion cells. Analysis of 0.5 μm thick confocal sections did not reveal clear labeling of glutamate in GABA-ir boutons, suggesting that these boutons do not accumulate glutamate above basal levels.

Glycine immunoreactivity

Glycine immunoreactivity was observed in differentiated cells of both the central and lateral regions of the retina (Fig. 3D), whereas the thick neuroblastic layer of the lateral retina did not show GLY immunoreactivity above background. In the central retina, the perikarya of photoreceptors were moderately or strongly GLY-ir, whereas the inner photoreceptor segments showed fainter immunoreactivity (Fig. 3D). In addition, moderate to high intensity GLY-immunoreactivity was observed in scattered cells of the outer and inner sides of the IPL characterized by its GABA-ir fiber plexus, i.e. a region also containing putative ganglion cells (Fig. 3D). However, the number of GLY-ir cells was clearly minor in comparison to that of GLU-ir cells. In addition, comparison of photographs of double immunostaining GABA/GLY (Fig. 3D) with those of GABA/GLU (Fig. 3A-C) clearly indicates that the GLY-ir cells did not form a cap over the IPL as was observed with glutamate immunostaining, the large ganglion cells of this region being only very faintly GLY-ir (Fig. 3D). Moreover, no ascending GLY-ir processes were observed, in contrast to the results obtained with glutamate immunocytochemistry. In the lateral retina, some cells close to the primordial IPL showed faint to moderate GLY immunoreactivity (Fig. 3D). As in the central retina, these cells appeared in the region containing ganglion cells. Whereas double GABA/GLY immunostaining (Fig. 3D) suggests that GABA-ir retinopetal fibers might contact GLY-ir cells processes, no specific relationship of these fibers with glycinergic perikarya was observed.

GABA, glycine and glutamate immunostaining in control semithin plastic sections

To confirm the results obtained by confocal microscopy, some semithin plastic sections were stained with antisera against GLU, GABA and GLY. To facilitate identification of structures of these sections, a similar hematoxylin-eosin-stained section is shown (Fig. 5A). In plastic sections, GLU, GABA and GLY immunoreactivities were observed in similar locations of the larval retina to those observed with confocal microscopy (Fig. 5B-D). GLU-ir cells were present in the regions of the photoreceptor cells, bipolar cells and ganglion cells (Fig. 5B). In semithin sections, the monoclonal GABA antibody only stained fibers in the IPL (Fig. 5C), which were clearly identifiable as centrifugal fibers by the absence of any stained retinal perikaryon. Photoreceptors showed GLY immunoreactivity in

semithin plastic sections and a few GLY-ir cells were also observed in the innermost central retina (Fig. 5D).

Neurotransmitter immunoreactivity in the postmetamorphic retina

For comparison with the larvae and to serve as positive controls, we examined retinas from recently metamorphosed lampreys. In these retinas, each of the antibodies against neurotransmitters revealed the presence of characteristic populations of neurons, as shown in Figures 6A-D and 7A-K. Strongly GABA-ir amacrine cells were numerous in the inner part of the inner nuclear layer, and processes of these cells extended to the inner plexiform layer where they formed a dense GABA-ir plexus (Figs. 6A'-D', 7A). Faint to moderate GABA immunoreactivity was observed in the outer row of horizontal cells (HC1), and in small bipolar cells (Figs. 6A'-D', 7A-D). GABA-ir horizontal cells exhibited short, thick dendritic trunks that gave rise to numerous delicate appendages in the outer plexiform layer (Fig. 7A-D). Thick axons of these cells coursed horizontally below the layer of perikarya before ascending to branch in the OPL (Fig. 7A, D). GABA-ir bipolar cell perikarya were located either among horizontal cells or in a position intermediate between amacrine cells and horizontal cells. Some of these GABA-ir cells exhibited ascending processes that coursed to the outer limiting membrane, i.e. Landolt's clubs (Fig. 7A-B). Other retinal cells were GABA-negative. Some GABA-ir fibers were also observed coursing in the bundles of optic fibers that, characteristically in lamprey, are located adjacent to the INL (Fig. 7E).

Numerous glutamate-ir cells were observed in the postmetamorphic retina. Photoreceptors were faintly or very faintly GLU-ir, whereas inner horizontal cells (HC2), large bipolar cells with perikarya at the level of HC1 cells, and ganglion cells were moderately or strongly GLU-ir (Figs. 6A, 7F). Small bipolar cells and some putative amacrine cells also exhibited faint to moderate glutamate immunoreactivity (Fig. 7F). Numerous GLY-ir cells were observed both in the INL (orthotopic amacrine cells) and in the inner plexiform layer, both in its innermost part or interspersed in this layer (Figs. 6B, 7G). Double immunofluorescence confocal microscopy revealed that most GLY-ir cells in the INL do not correspond to the GABA-ir amacrine cell population, although colocalization was observed in some cells (Fig. 6B). Most GLY-ir cells in the IPL and close to the inner limiting membrane probably correspond to displaced amacrine cells. Some GLY-ir small

cells of the INL showed processes ascending to the outer plexiform layer (Figs. 6B, inset in 7G), suggesting they are glycinergic interplexiform cells. In addition, some GLY-ir boutons are found in the OPL just above of HC1 cells. Faint to moderately aspartate-ir cells were observed in the postmetamorphic retina in the outer and inner parts of the INL and in the IPL (Figs. 6C-D, 7H). Some large cells of the INL can clearly be recognized as giant ganglion cells (Figs. 6C, 7H). Other aspartate-ir cells of this layer may be displaced ganglion cells, bipolar cells and/or amacrine cells, whereas immunopositive cells of the IPL may correspond to orthotopic ganglion cells and/or displaced amacrine cells.

Scarce dopamine-ir amacrine cells were observed in the inner part of the INL, their processes coursing in the IPL (Fig. 7 I). No DA-ir processes were observed in the OPL or the ONL, indicating that the dopaminergic cells of the sea lamprey are amacrine cells. The appearance of these cells and distribution of their processes closely correspond to the TH-immunoreactivity observed in the postmetamorphic retina (not shown). Serotonin (5HT)-ir amacrine cells were observed in the inner part of the INL (Fig. 7J) and, occasionally, close to the inner limiting membrane (Fig. 7K). They are more abundant than dopaminergic cells, and have processes that ramify on both sides of the IPL (Fig. 7J-K).

DISCUSSION

The neurochemistry of the larval lamprey retina differs notably from the developing retinae of other vertebrates

One surprising result from the present study is that no GABA-ir, serotonin-ir, aspartate-ir or dopamine-ir perikarya were observed in the larval lamprey retina, either centrally or laterally, despite the reported presence of serotonin-ir, GABA-ir, dopamine-ir and TH-ir cells in adult lamprey (Negishi et al., 1986; De Miguel and Wagner, 1990; Versaux-Botteri et al., 1991; Rio et al., 1993; Yáñez and Anadón, 1994; present results). In addition, a recent study examining the localization of choline acetyltransferase noted the absence of putative cholinergic cells in the central and lateral parts of the larval retina (Pombal et al., 2003). In contrast to larval lamprey, the larvae of jawed anamniotes possess amacrine cells that are immunoreactive to ChAT, 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). Unlike the developing retinae of mammals (Redburn et al., 1992; Fletcher and Kallionatis, 1997), we have never observed GABA immunoreactivity in perikarya of the larval lamprey retina (Anadón et al., 1998; present results). In other vertebrates, these substances (ChAT, GABA, dopamine/TH and serotonin) are first expressed around or shortly after the time of photoreceptor differentiation, which does not occur in the central retina of the larval lamprey. Since the central retina contains photoreceptors that express opsin immunoreactivity throughout the entire larval period (5-7 years long; Meléndez-Ferro et al., 2002a; present results), the absence of cells expressing ChAT, GABA, dopamine/TH or serotonin represents a fundamental difference between the retinae of larval lamprey and other anamniote vertebrates. This difference appears genuine: immunocytochemistry with antisera against GABA, aspartate, serotonin, dopamine, TH and ChAT produced reliable staining of numerous cells and fibers in the larval brains but not the retinae within the same head sections. Moreover, the distributions of GABA-, serotonin-, dopamine- and TH-immunoreactive neurons observed in the brain were coextensive with previous reports in larval (Meléndez-Ferro et al., 2002b, 2003; Abalo et al., 2005) and/or adult lamprey (Pierre et al., 1992, 1997; Pombal et al., 1997). Likewise, the positive results obtained with the same protocols in the postmetamorphic retina rule out the possibility of false negative results in larvae.

Glutamate and glycine immunocytochemistry reveals neurons in the retina of larval lamprey

A common point with other developing vertebrates is that the larval lamprey retina contains glutamate-ir cells and glycine-ir cells. Immunocytochemistry using antisera against glutamate and glycine produced staining of numerous cells and fibers in both the brain and retina of lamprey larvae. Unpublished observations in the brain using double immunofluorescence to visualize glutamate or glycine together with GABA indicate that many GABA-ir populations are GLU-negative and/or GLY-negative, and conversely that many glutamate or glycine immunolabeled structures are GABA-immunonegative. These findings support the antibody specificity studies demonstrating that the monoclonal GABA antibody does not recognize either of the other two amino acids (Holstein et al., 2004). In line with this evidence in the brain, no correspondence was observed between the glutamate- and GABA-immunoreactive, and glycine- and GABA-immunoreactive structures in double-labeling experiments in the larval retina.

Glutamate-immunoreactive cells of the larval lamprey retina: Comparison with the retinae of other vertebrates

Developmental studies of glutamate-ir cells in the vertebrate retina are very scarce: as far as we are aware, this has been addressed exclusively in mammals (Redburn et al., 1992; Pow et al., 1994; Fletcher and Kallionatis, 1997). In the rabbit retina, GLU immunoreactivity first appears by embryonic day 20 (E20). Ganglion cells and photoreceptors are GLU-ir at birth (Redburn et al., 1992), and bipolar cells and some amacrine cells also become GLU-ir around postnatal day 10 (P10) (Pow et al., 1994). Some horizontal cells are reported to be GLU-ir at birth by Redburn et al., (1992), although not by Pow et al. (1994).

The unique retina of larval lampreys affords the opportunity to explore simultaneously the immunoreactivity to glutamate and GABA during three distinct developmental phases in the same larvae. Whereas the central retina has a layered organization with photoreceptors, horizontal cells, bipolar cells and ganglion cells, the lateral retina mainly consists of a thick neuroblastic layer as well as differentiating ganglion cells and retinopetal fibers in the innermost region, and the marginal retina is a purely neuroblastic region lacking identifiable ganglion cells or

retinopetal fibers (De Miguel and Anadón, 1987; De Miguel et al., 1989; Anadón et al., 1998; Meléndez-Ferro et al., 2002; present results). The distribution of glutamate in the central retina of the larval sea lamprey is reminiscent of that reported before eye-opening in the vertical system of rabbit, immunoreactivity being observed in photoreceptors, some bipolar cells and in ganglion cells. These GLU-ir cells of the larval lamprey retina are not GABA-ir in the postmetamorphic lamprey, with the exception of GABA expression in a bipolar cell subtype (present results), and no GLU immunoreactivity was observed in the region of future GABAergic amacrine cells. These findings are consistent with our interpretation that glutamate is the neurotransmitter utilized by neurons of the vertical pathways in the larval retina.

In the lateral and marginal retina, our results demonstrate the absence of GLU immunoreactivity above background in cells of the neuroblastic layers. In fact, the presence of GLU-ir cells in the innermost layers provides a biological marker to distinguish the lateral from the marginal regions. One interesting observation concerns the long GLU-ir processes of ganglion cells ascending through the neuroblastic layer. These processes may be viewed as either transient trailing processes of recently migrated cells, or processes of biplexiform cells awaiting the differentiation of photoreceptors and the OPL. On the other hand, the absence of GLU-ir and GABA-ir fibers and cells in the marginal region reinforces the exclusively proliferative nature of this zone.

With regard to the retinae of adult vertebrates, most studies indicate the presence of glutamate in most photoreceptors, bipolar cells and ganglion cells (*teleosts*: Kageyama and Meyer, 1989; Van Haesendonck and Missotten, 1990; Marc et al., 1990, 1995; Connaughton et al., 1999; *amphibians*: Yang and Yazulla, 1994; *reptiles*: Schutte, 1995; *birds*: Kalloniatis and Fletcher, 1993; *mammals*: Davanger et al., 1991; Crooks and Kolb, 1992; Jojich and Pourcho, 1996; Kalloniatis et al., 1996). Glutamate has also been reported in a GABA-negative horizontal cell type in goldfish (Marc et al., 1995), and in some horizontal cells of the tiger salamander (Yang and Yazulla, 1994). The colocalization of glutamate with GABA or glycine has been observed in amacrine cells of tiger salamander (Yang and Yazulla, 1994), amacrine and horizontal cells of chicken (Kalloniatis and Fletcher, 1993), and amacrine cells of some mammals (Jojich and Pourcho, 1996). Present results indicate the presence of immunoreactivity to glutamate in some cell types of the retina of

postmetamorphic sea lamprey, including some horizontal and amacrine cells. As reported in goldfish, the GLU-ir horizontal cells of lamprey (inner row of horizontal cells; HC2 cells) do not correspond to the faint GABA-ir horizontal cells (outer row; HC1 cells). In postmetamorphic lamprey retina, photoreceptors and small bipolar cells exhibit only faint GLU immunoreactivity.

Biplexiform ganglion cells of larval lamprey are demonstrated with glutamate immunocytochemistry

The presence of biplexiform ganglion cells has been reported using retrograde tract-tracing in larval (De Miguel et al., 1989; Anadón et al., 1998) and adult (Fritzsch and Collin, 1990; Rio et al., 1998) lamprey retinae. Ultrastructural observations indicate that these ganglion cells, first described in the primate (Mariani, 1982), are postsynaptic to photoreceptors in larval (De Miguel et al., 1989) and adult (Rio et al., 1998) lamprey. Our immunocytochemical results reveal for the first time that the processes of larval ganglion cells ascending toward the OPL (in the central retina) or through the neuroblastic layer (in the lateral retina) are GLU-ir. These processes can be distinguished clearly from retinopetal fibers, which are GABA-ir and do not ascend to the OPL/neuroblastic layer (Anadón et al., 1998; present results), further suggesting that the parent cells are glutamatergic. Although biplexiform cells have been reported in the retinae of other vertebrates (*anurans*: Toth and Straznicky, 1989; *bony fishes*: Cook and Becker, 1991; Cook et al., 1992; Collin and Northcutt, 1993; *mice*: Doi et al., 1995), neither the contacts with photoreceptors nor the neurotransmitter(s) used by these cells have been investigated.

An intriguing observation is the presence of conspicuous GLU-ir processes ascending to the outer limiting membrane in the limit between the marginal pure neuroblastic region and the larval lateral retina. Similar processes, and occasional bipolar ganglion cell perikarya resulted labeled in the same transition region after application of horseradish peroxidase to the optic nerve of larval lampreys (De Miguel et al., 1989). Since present observations preclude that these processes were retinopetal fibers, the most plausible explanation is that they represent processes of recently differentiated ganglion cells migrated to the ganglion cell layer that maintain transiently a process contacting the outer limiting membrane, in a manner roughly similar to the Landolt's club of some bipolar cells of the adult lamprey retina (Dalil-Thiney et al., 1994; Villar-Cheda et al., 2006).

Glycine immunoreactivity in the larval retina

In the larval lamprey, glycine immunoreactivity was observed in photoreceptors and in some cells in inner retinal regions containing putative ganglion cells. Presumably the photoreceptors are the same cells that express glutamate immunoreactivity, although this was not directly assessed. The distribution of GLY-ir cells of the inner retina is rather similar to that of glutamate, at least in the lateral retina. In the central retina, we did not observe GLY-ir bipolar cells similar to those immunolabeled for glutamate, or ascending processes of putative ganglion cells. Moreover, the large, strongly GLU-ir ganglion cells detected by immunocytochemistry (present results) and HRP transport (De Miguel et al., 1989) near the optic nerve exit appear to be very faintly GLY-ir. Whether this finding indicates that ganglion cells are neurochemically heterogeneous, that some cells labeled near the inner plexiform/optic fiber layer are in fact not ganglion cells but immature amacrine cells, or that glutamate/glycine differences are simply due to the relatively low levels of glycine present in these cells/processes is not yet known.

The distribution of glycine in other larval retinas has only been investigated in tiger salamander (Yang and Yazulla, 1988; Li et al., 1990). In that system, glycine immunoreactivity is found primarily in amacrine cells, in cells of the ganglion cell layer that may be displaced amacrine cells, and rarely in bipolar cells (Yang and Yazulla, 1988). This distribution is different from that observed in larval lamprey, perhaps due to several unique features of the larval lamprey eye (see below) since glycine immunoreactivity in the postmetamorphic lamprey retina is primarily restricted to amacrine and interplexiform cells (present observations). In rabbit, glycine appears to provide an excitatory drive during early retinal development, playing a developmentally regulated role in the initiation and propagation of spontaneous retinal waves (Zhou, 2001). The existence of glycine in photoreceptors and some cells (possibly amacrine cells) of the innermost layer in the larval retina may also serve a developmental function.

The GABA-ir retinopetal fibers of larval lamprey are glutamate- and glycine-negative

The retinopetal system of lampreys has been studied extensively (Vesselkin et al., 1980, 1984, 1989, 1996; De Miguel et al., 1989; Rio et al., 1993, 2003; Rodicio et al., 1995; Anadón et al., 1998; Meléndez-Ferro et al., 2002a). Several immunocytochemical studies have revealed the presence of GABA in retinopetal cells of the midbrain and in fibers of the IPL, both in adult and larval lampreys (Rio et al., 1993; Vesselkin et al., 1996; Anadón et al., 1998; Meléndez-Ferro et al., 2002a). An experimental electron microscopic study in adult lamprey found that GABA-ir fibers comprise 45% of all tracer-labeled retinopetal fibers, but that glutamate immunoreactivity is present in all retinopetal fibers (Rio et al., 2003). The present results obtained using double immunofluorescence and spectral confocal microscopy in the larval retina confirm the presence of boutons of GABA-ir retinopetal fibers in the larval IPL/optic fiber layer, where they appear to contact ganglion cell perikarya. Moreover, these GABA-ir fibers and boutons do not show glutamate or glycine immunolabeling above background level. Lastly, our results regarding glutamate-positive/GABA-negative retinopetal fibers in larval retinae are inconclusive, since the numerous GLU-ir processes of ganglion cells in the IPL/optic nerve layer preclude the distinction of these retinopetal fibers using the present methods.

Neurotransmitters of the postmetamorphic lamprey retina

The retinae of recently metamorphosed sea lamprey show a layered distribution of immunoreactivities to the neurotransmitters investigated. Our results reveal numerous GABA-ir amacrine cells in the inner part of the INL, in agreement with results in other vertebrates (Mosinger et al., 1986; Osborne et al., 1986; Connaughton et al., 1999). A small portion of these cells were also glycine immunoreactive, as also observed in a lizard (Sherry et al., 1993). Faint to moderate GABA immunoreactivity was observed in the outer row of horizontal cells, in line with results in teleost retinas (Osborne et al., 1986; Connaughton et al., 1999). Although GABA immunoreactivity was noted in some small bipolar cells of sea lamprey, similar to that reported in amphibians (Mosinger et al., 1986; Osborne et al., 1986; Yang and Yazulla, 1994; Yang et al., 2003), the significance of these cells is not well understood. It has been proposed that these GABA-ir bipolar cells co-

release glutamate and GABA, thereby contributing to modulation of ganglion cell responses (Yang and Yazulla, 1994).

Our results reveal for the first time the presence of glycine immunoreactivity in numerous amacrine cells of the lamprey retina. GLY-ir orthotopic amacrine cells are generally located more externally in the INL than the GABA-ir amacrine cells, and displaced GLY-ir cells are rather abundant in the innermost region of the IPL. Some small GLY-ir cells of the INL are interplexiform glycinergic cells, as indicated by the presence of processes coursing to the OPL. The presence of GLY-ir interplexiform cells has also been observed in teleosts (Marc and Lam, 1981; Kallionatis and Marc, 1990; Yazulla and Studholme, 1990; Connaughton et al., 1999), amphibians (Yang and Yazulla, 1988; Vitanova et al., 2004), reptiles (Eldred and Cheung, 1989; Sherry et al., 1993) and chick (Kallionatis and Fletcher, 1993). This glycine distribution is roughly similar to that reported in teleosts (Yazulla and Studholme, 1990; Connaughton et al., 1999). Our results also indicate that glutamate immunoreactivity is abundant in cells and processes of the INL and IPL, with high levels in inner horizontal cells and in other INL cells (ganglion cells, large bipolar cells, putative amacrine cells). Colocalization of GABA and glutamate was observed in some amacrine cells. The presence of glutamate immunoreactivity in some GABA- or glycine-ir amacrine cells has been also reported in the retina of tiger salamander (Yang, 1996). Aspartate immunoreactivity is abundant in putative ganglion cells, including giant ganglion cells, as well as in some amacrine cells, and moderate levels were also observed in inner horizontal cells, suggesting colocalization of aspartate and glutamate in some cell populations.

Our results using antibodies against both dopamine and tyrosine hydroxylase (TH) do not reveal immunoreactive processes in the OPL of the postmetamorphic sea lamprey retina, confirming the identify of the dopaminergic cells as amacrine cells (Yáñez and Anadón, 1994). Although the presence of dopaminergic interplexiform cells in the sea lamprey retina has been reported using an antibody against TH (De Miguel and Wagner, 1990), this staining might be due to cross-reaction with other substances. Unlike sea lamprey, many teleost retinas show a well-developed dopaminergic interplexiform cell system (Osborne et al., 1984; Yazulla and Zucker, 1988; Kallionatis and Marc, 1990; Wagner and Behrens, 1993; Frohlich et al., 1995), although in pure-rod teleost retinas dopaminergic cells are amacrine cells (Frohlich et

al., 1995). The serotonin-ir amacrine cells of the postmetamorphic sea lamprey retina are mostly orthotopic, and their processes branch in outer and/or inner sublaminae of the IPL. These amacrine cells are similar to those described in river lamprey (Versaux-Botteri et al., 1991).

Functional considerations

The eye of the larval lamprey is covered by a thick non-transparent skin and has an immature lens, indicating that it is not an image-forming eye (Kleerekoper, 1972). Thus, larval lampreys must wait several years until metamorphosis in order to acquire a truly functional camera-type eye. However, the larval central retina shows numerous traits suggesting functional maturation beginning in early posthatching stages, including the presence of opsin-expressing photoreceptors with well-developed outer segments (Meléndez-Ferro et al., 2003), the presence of brain-projecting ganglion cells and of centrifugal fibers (De Miguel et al., 1989; Anadón et al., 1998), and the expression of glutamate in cells of the vertical pathway (present results). Together, these traits strongly suggest that at least some parts of the larval retina are functional, but perhaps as a non-directional or broadly directional photoreceptive organ like a simple ocellus. In this regard, the absence of neurotransmitter-immunoreactive amacrine cells throughout the entire larval period may be interpreted in terms of immaturity of the image-forming circuitry, which needs opposing influences to extract the different qualities of the image (movement versus static background, light/dark contrast, etc). In some way, the larval lamprey eye is comparable to the lamprey pineal organ, which also lacks image-forming circuitry.

It is likely that the functional roles of glutamate and GABA in the retina of larval lamprey depend on the specific neuronal types and retinal regions. Although glutamate and GABA are viewed as the major excitatory and inhibitory neurotransmitters in the vertebrate brain, their various actions depend upon developmental stage, and on the nature and subcellular distribution of pre- and post-synaptic ionotropic and metabotropic receptor subtypes(s) (Ben-Ari et al., 1990; Cherubini et al., 1991, 1998). It is well-documented that GABA acts as an excitatory neurotransmitter during retinal development, due to the high membrane potential of developing neurons and/or action through elevation of Ca^{++} levels (Redburn-Johnson, 1998).

In addition to its functions in the adult vertebrate retina, glutamate appears to participate in several processes that occur during development. In the developing cat retina, stratification of ON and OFF ganglion cell dendrites depends on activity mediated by metabotropic glutamate receptors (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995). In addition, responses of early fetal ganglion cells to ionotropic glutamate receptor agonists before synaptogenesis in the INL appear remarkably similar to those of postnatal cells: glutamate and AMPA produce fast desensitizing currents, kainate yields large steady-state currents, and the application of NMDA results in multiple channel openings (Liets and Chalupa, 2001). Ganglion cells in the developing chick retina (E5-E6 stages) begin to express ionotropic glutamate receptor subunits before any distinction of an IPL has occurred (Silveira dos Santos Bredariol and Hamassaki-Britto, 2001). Since the appearance of the chick embryo retina at these stages is remarkably similar to that observed in the lateral retina of larval lamprey, it is conceivable that the GLU-ir ganglion cells observed there are also glutamate-receptive. Although it may be assumed that larval lamprey photoreceptors, like those of other vertebrates, are hyperpolarized by light and release glutamate in the dark, the actual functions of these neurotransmitters in the retina cannot be deduced from their distributions. Moreover, the precise actions of glutamate and GABA may be different in the central and in the lateral regions of the larval lamprey retina. In the lateral retina, GABA released by retinopetal fibers is probably the most influential neurotransmitter: perhaps GABA exerts some excitation on differentiating GLU-ir ganglion cells that are long-term deprived of sensory vertical input. In addition, a role for glutamate released by the lateral retinal ganglion cells, or by glutamatergic retinopetal fibers if they are present, on the maintenance or differentiation of this part of the retina cannot be ruled out. In the central retina, however, the glutamatergic vertical neuronal system appears functional, and it is more probable that GABA acts as an inhibitory neurotransmitter.

Abbreviations

ASP Aspartate

DA Dopamine

GLU Glutamate

GLY Glycine

HCL Horizontal cell sublayer

HC1 Outer horizontal cells

HC2 Inner horizontal cells

INL Inner nuclear layer

IPL Inner plexiform layer

IS Inner segment of larval photoreceptors

NB Neuroblastic layer

ONL Outer nuclear layer

OPL Outer plexiform layer

PE Pigment epithelium

Ph Photoreceptor layer

5HT Serotonin

Table 1. Antisera Used

Antisera	Host	Source[1] and Code	Dilution	Lot	Immunogen
Glycine	rabbit	Chemicon Code AB139	1:200 1:100*	24080975	GLY-glutaraldehyde-BSA conjugate
Aspartate	rabbit	Chemicon Code AB132	1:200 1:100*	24030893	L-aspartate-glutaraldehyde-BSA conjugate
Glutamate	rabbit	Sigma Code G6642	1:2000 1:1000*	113K4824	GLU-key limpet haemocyanin conjugate
GABA	mouse	Holstein et al., 2004 GABA93 MAb	1:50*		GABA-glutaraldehyde-BSA conjugate
GABA	rabbit	Affiniti Code GA1159	1:1000	200493	GABA-glutaraldehyde-BSA conjugate
Serotonin	rabbit	Incstar Code 20080	1:5000	051007	Serotonin-formaldehyde-BSA conjugate
Dopamine	rabbit	HWM Steinbusch, U. Maastricht, Netherland	1:900		Dopamine-glutaraldehyde-BSA conjugate
TH	rabbit	Chemicon Code AB152	1:1000	0509011790	Denatured TH from cat pheochromocytoma
Opsin	rabbit	Gift of Prof. W. De Grip Code CERN 922	1:1000		Bovine rod-opsin
Calretinin	rabbit	Swant Code 7699/4	1:1500	18299	Recombinant human calretinin

[1] Sources include Chemicon, Temecula, CA; Affiniti, Mamhead, U.K.; Sigma; St. Louis, MO; SWant, Bellinzona, Switzerland. BSA, bovine serum albumin.

*, dilution used for immunofluorescence

Fig. 1. A, Western blotting of adult brain protein extracts immunostained with antisera to the different neurotransmitters used here. Note the native protein bands stained in the glutamate and dopamine lanes. **B**, Fluorescence micrograph of a section of a paraformaldehyde-fixed adult brain stained with the anti-glutamate antiserum (the photograph was inverted and converted to gray scale). Note that the only stained structures were large meningocytes (open arrows), which suggests that the native protein revealed by this antibody in blots is located in these cells. Star, optic tectum; asterisk, telencephalon. Bar, 50 μm.

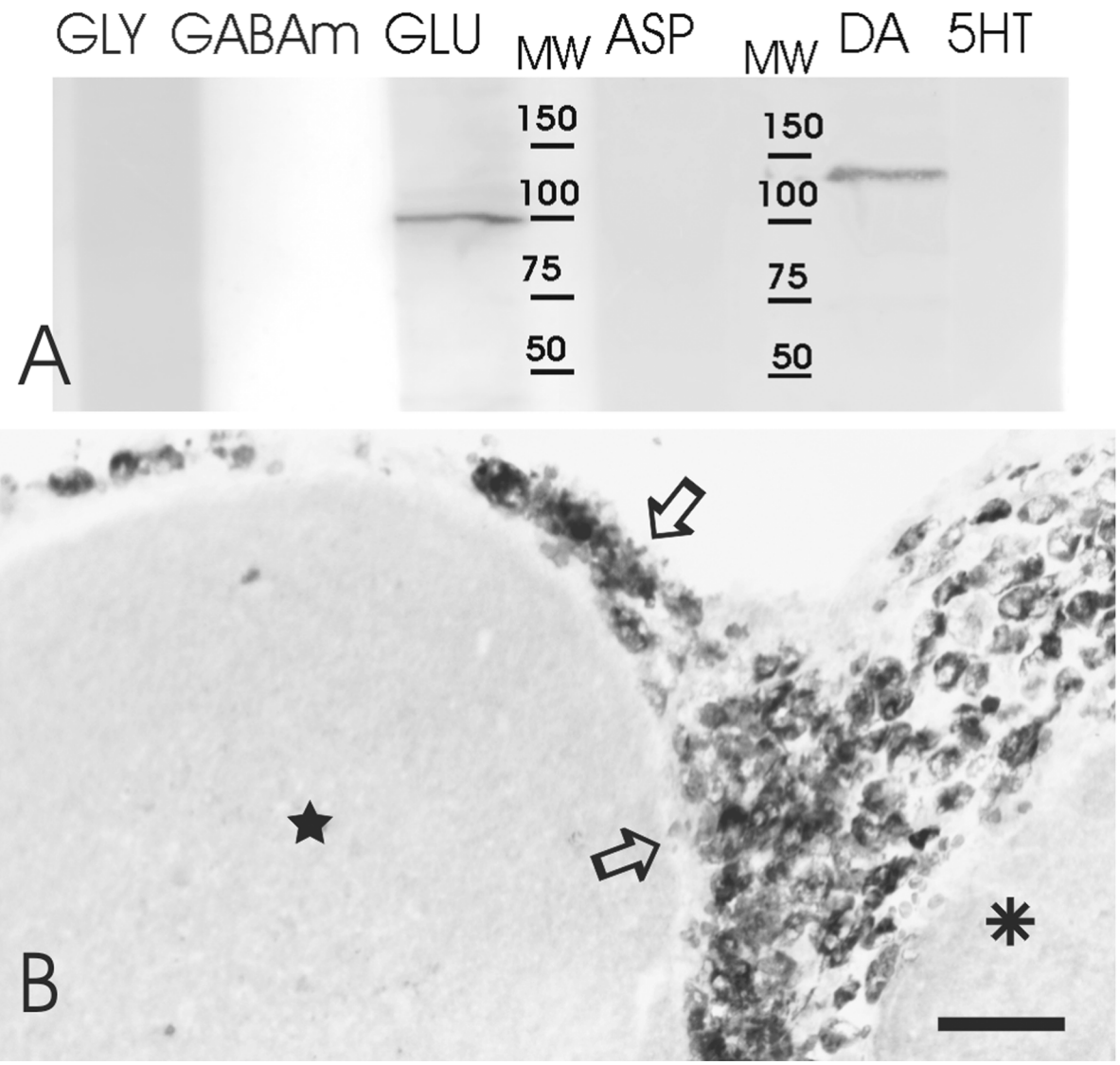


Fig.1.

Fig. 2. Photomicrographs of vertical sections through the central retina (A-C) and the dorsal part of the lateral retina (D) of larval sea lamprey. **A**, Central retina, showing the photoreceptors (Ph), the outer nuclear (ONL) and inner nuclear (INL) layers, and the inner plexiform and optic fiber layer (asterisk). The arrows indicate the outer limiting membrane. Arrowheads point to the ganglion cell bodies located in the INL. NB, neuroblastic layer of the lateral retina. Hematoxylin-eosin staining. **B**, Photoreceptors immunostained with the CERN 922 anti-opsin antibody in the central retina. The arrows point to the outer limiting membrane. **C**, Photomicrograph of the caudal pole of the central retina showing intense calretinin immunoreactivity in bipolar cells (open arrows). Note that these cells partially encircle the ganglion cells. Arrowheads point to Landolt's clubs of bipolar cells. Arrows indicate the outer limiting membrane. Asterisk, inner plexiform layer receiving bipolar cell CR-ir inner fibers and terminals. **D**, Photomicrograph showing that putative ganglion cells of the dorsal region of the lateral retina are moderately CR-ir (arrows). In all photographs, the vitreum is to the right, dorsal is above and ventral is at the bottom. Larval lengths: A, 87 mm; B, 156 mm; C-D, 114 mm. Scale bars, 12.5 μm.

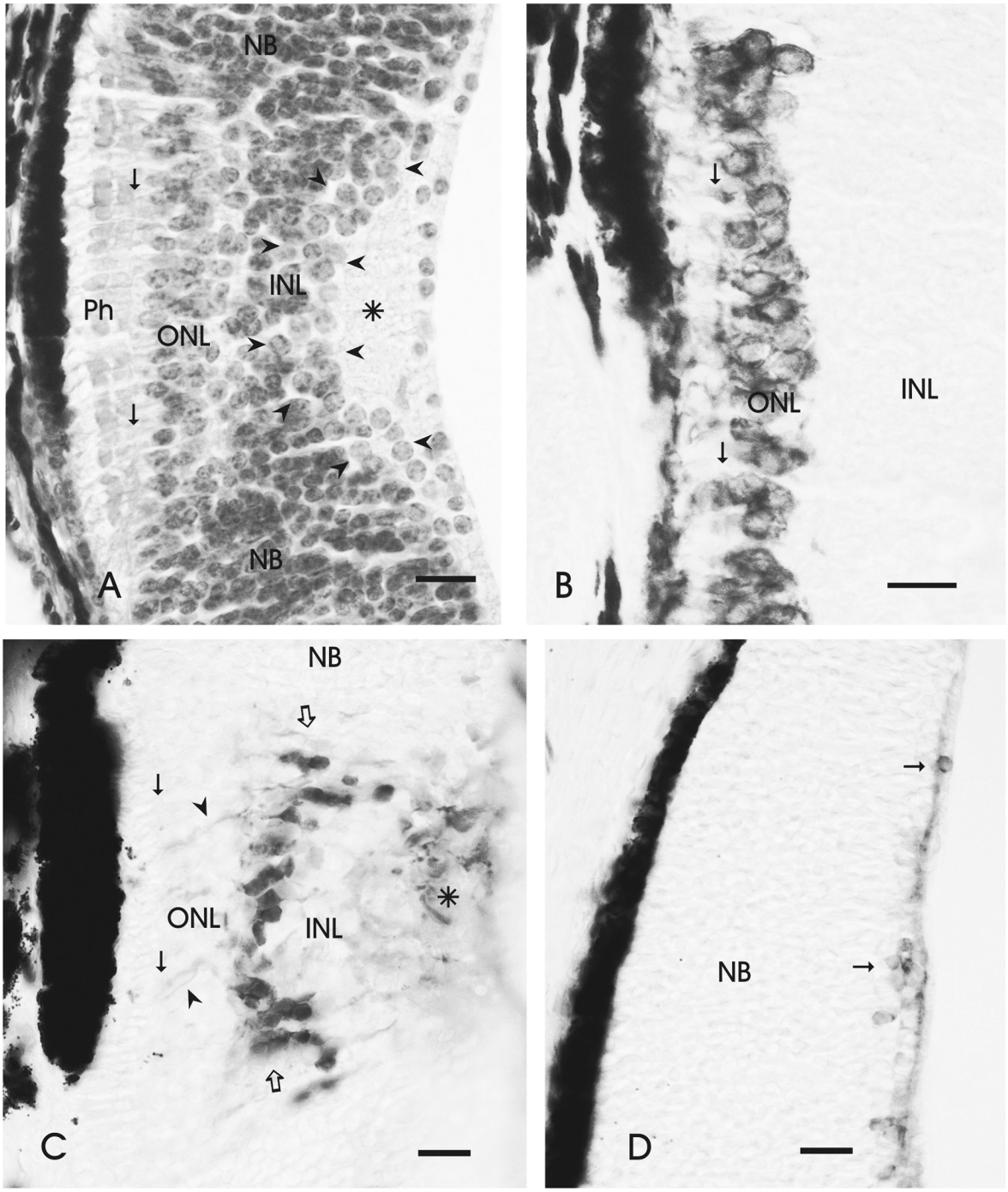


Fig.2.

Fig. 3. Projections of stacks of 0.5 μm thick confocal microscope optical sections of double-labeled retinae of larval (A-D). **A**, Vertical section at the level of the optic nerve entrance (asterisk). Most GLU-ir structures are in the central retina, whereas the adjacent neuroblastic layer of the lateral retina lacks significant immunoreactivity. The thick solid arrow indicates the photoreceptor layer, the thin arrow the photoreceptor perikarya, the curved arrow the layer of bipolar cells, and the open arrow points to ganglion cells. **B**, Detail of the GLU-ir ganglion cells at the level of the optic nerve entrance. Note the rich GABA-ir innervation of the IPL (star) and numerous GABA-ir boutons outlining ganglion cell perikarya. All puncta are uniquely stained in single confocal image planes, the yellow color in some boutons is due to superposition of other structures in the image stacks. **C,** Detail of a single confocal 0.5 μm thick section of the region containing ganglion cells showing the distinct glutamate (in red in the left panel), GABA (in green channel in the central panel) and double GLU/GABA immunofluorescence (in the right panel). **D**, Section just caudal to the optic nerve entrance showing GLY-ir photoreceptors (thick arrows) and perikarya in the ONL (thin arrows) and some GLY-ir cells (arrowheads) in the inner part of the IPL that extend in the lateral retina. Note the position of GLY-ir cells as regards the plexus of GABA-ir retinopetal fibers. Open arrows indicate the region containing ganglion cells in the differentiated retina. Curved arrow points to the pigmented retinal epithelium. In all panels, the vitreum is to the right (asterisk in D), dorsal is above and ventral is at the bottom. Scale bars: 20 μm (A-B, D), 10 μm (C).

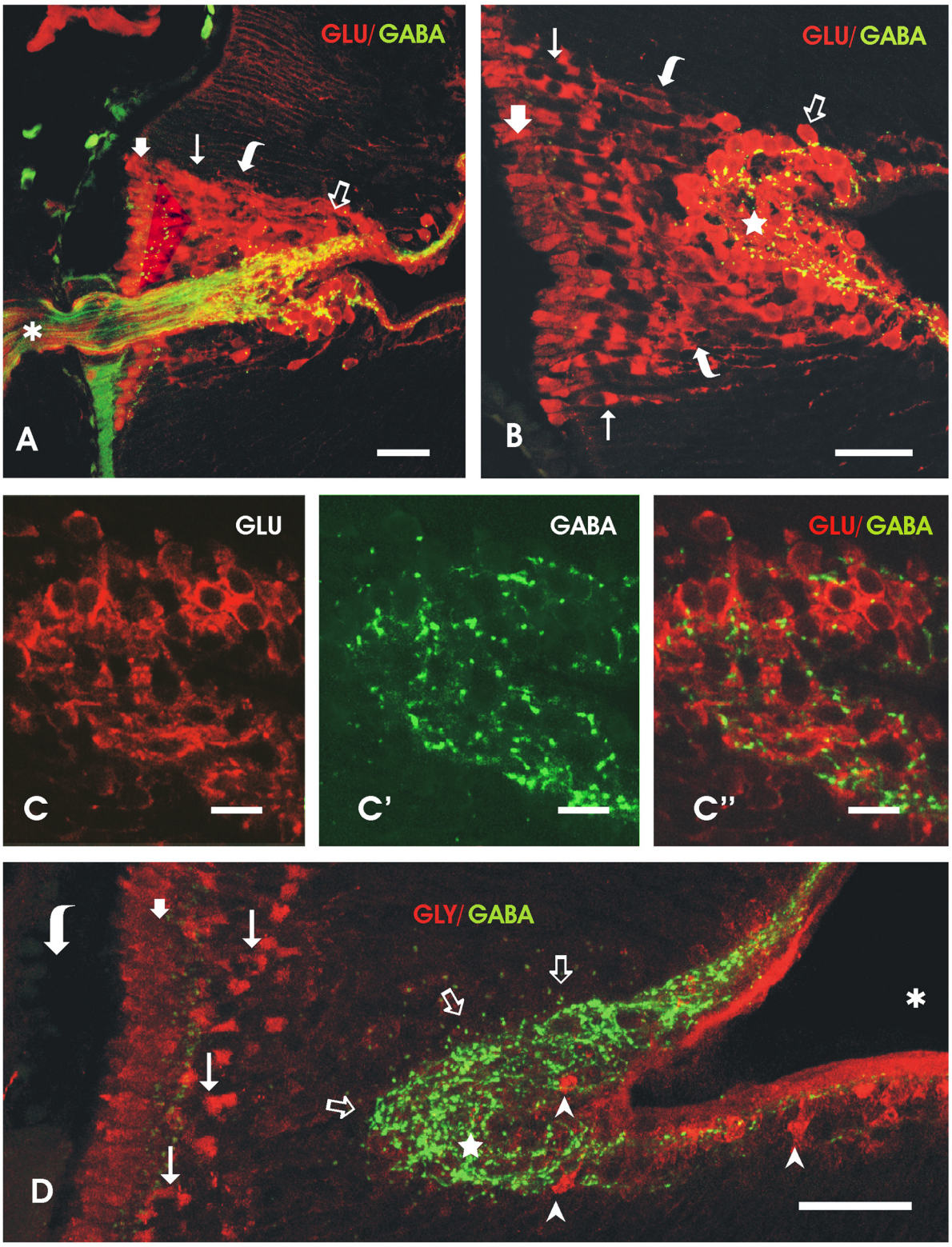


Fig.3.

Fig. 4. Photomicrographs showing the morphology of glutamate-ir cells and processes of the larval retina. All figures are single channel projections of a few confocal 0.5 µm sections (inverted and converted to gray scale). **A,** Section of the central retina showing glutamate immunoreactivity in the inner segment (IS) and perikarya of photoreceptors (filled arrows), bipolar cells (curved arrows) and ganglion cells (open arrows). Note the differences in immunoreactivity between the three neuronal types. The arrowheads point to dendritic processes of biplexiform ganglion cells ascending to the OPL. **B,** Detail of a 3 section projection showing ganglion cells (open arrow) and a bipolar cell (curved arrow). **C,** Section of the lateral retina showing glutamate-ir neurons (thin arrows) close to the primordial IPL, the thin layer of cells and processes close to the vitreum (below). Some of the processes of putative biplexiform ganglion cells ascend through the very thick neuroblastic layer (star). Note at the upper left the border of the differentiated central retina showing GLU-ir photoreceptors (thick arrow), bipolar cells (curved arrow) and ganglion cells (open arrow). **D,** Section through the lateral retina of a large larva showing a few conspicuous glutamate-ir processes ascending to the outer limiting membrane between the lateral (star) and the marginal (asterisk) regions. In all figures, the vitreum is to the right, dorsal is above and ventral is at the bottom. Scale bars: 20 µm (A), 30 µm (B-D).

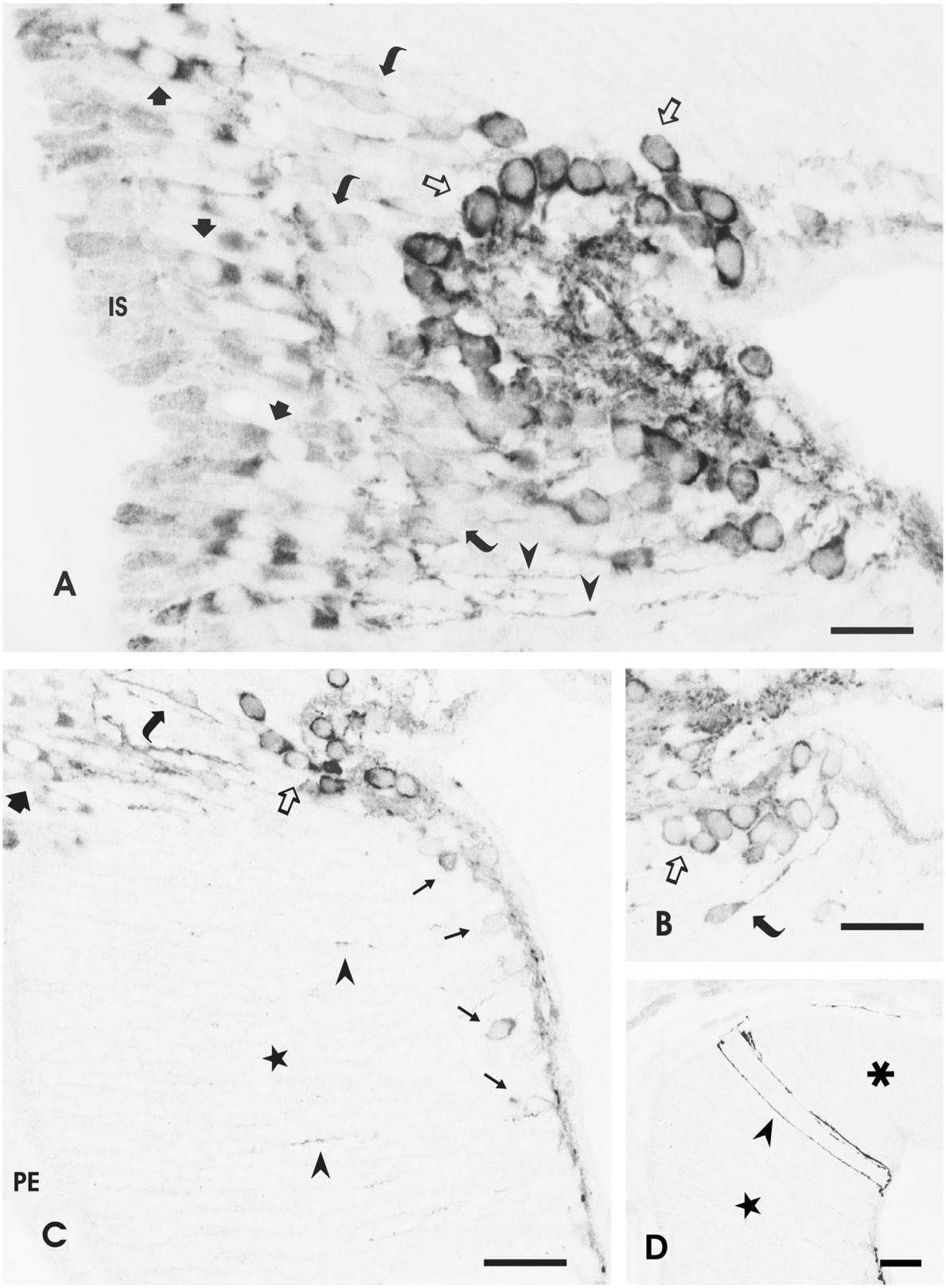


Fig.4.

Fig. 5. Hematoxylin-eosin stained section (A), and post-embedding stained, semithin plastic vertical sections of the larval retina showing immunoreactivity to glutamate (B), GABA (C) and glycine (D). **A,** Photomicrograph showing a section of the retina at a level similar to that in panel C. Star, ventral unpigmented part of pigment epithelium; open arrow, optic nerve entrance; black arrow, layer of photoreceptor perikarya. **B**, Section showing glutamate immunoreactivity in cells of the vertical pathways. The thick arrow points to the GLU-ir photoreceptor perikarya, the curved arrow designates the layer of bipolar cells, and the open arrow indicates ganglion cells. The star marks the unpigmented ventral region of the pigment epithelium. **C,** Section through the level of the optic nerve (asterisk) showing that GABA immunoreactivity is restricted to centrifugal fibers (arrows). **D,** Section showing glycine immunoreactivity in photoreceptors and in scarce cells (open arrow) of the inner part of the retina. In all figures, the vitreum is to the right, dorsal is above and ventral is at the bottom. Bright field microscopy. Scale bars: 50 μm (A), 20 μm (B-D).

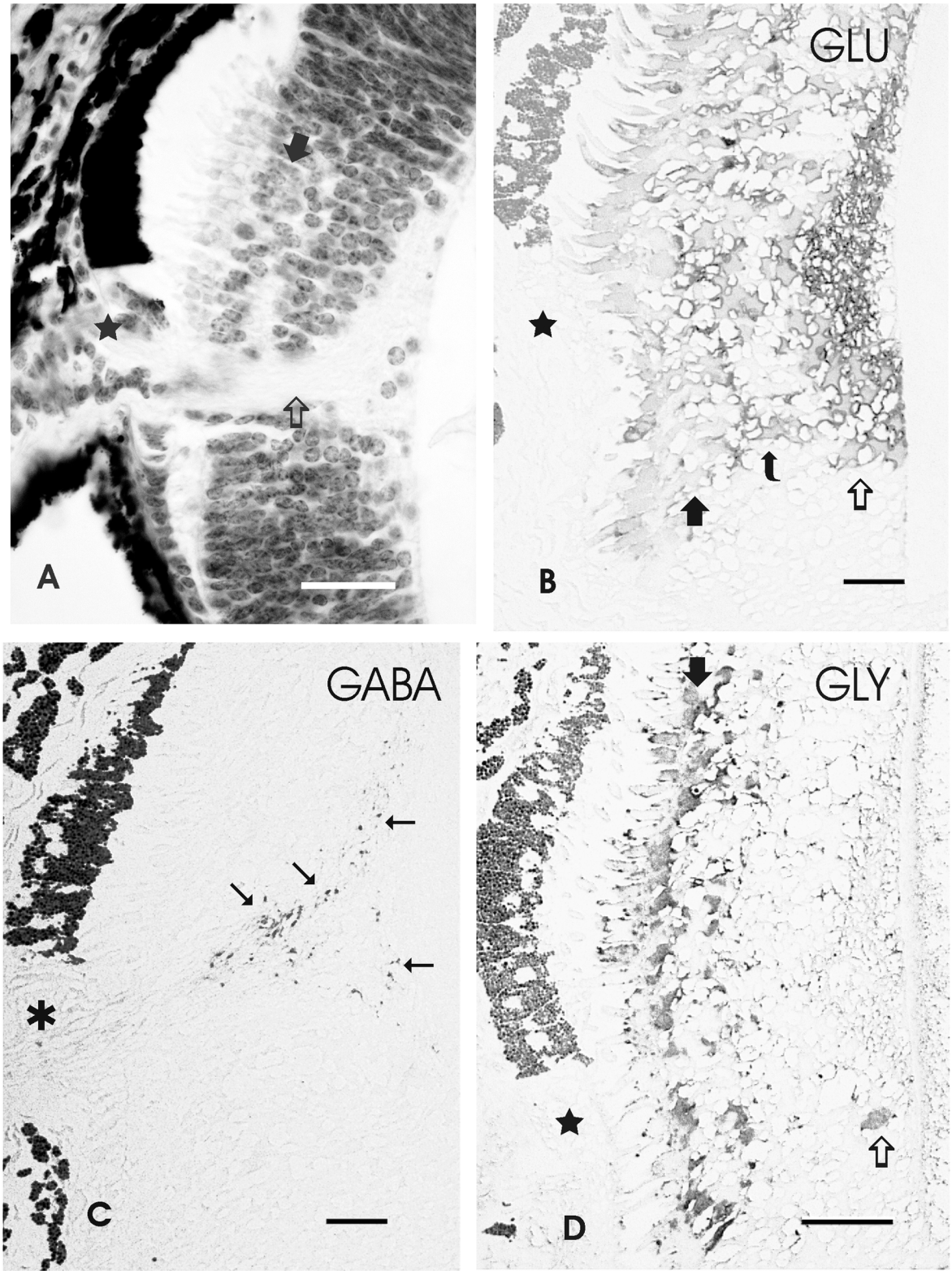


Fig.5.

Fig. 6. Projections of stacks of 0.5 μm thick confocal microscope optical sections of double-labeled retinae of postmetamorphic sea lamprey showing in the left panels glutamate (A), glycine (B), and aspartate (C-D) immunofluorescence (in the red channel), in the central panels immunoreactivity to GABA (in green), and in the right panels, double immunolabeling. **A,** Section showing abundant glutamate immunoreactivity in horizontal cells and putative ganglion cells (arrowhead). Colocalization of GABA and glutamate is occasionally found in cells of the INL and IPL (thin arrows). The curved arrow points to a bundle of optic fibers. Small arrows point to clearly double-labeled cells. Curved arrows, fascicles of optic fibers running close to the INL. **B,** Sections showing glycine immunoreactivity in amacrine cells of the INL and the IPL. Colocalization with GABA is observed in some amacrine cells (thin arrow). The open arrows point to glycinergic interplexiform cells. Arrowheads, interplexiform cell processes in the outer plexiform layer. **C-D,** Sections showing abundant aspartate immunoreactivity in some horizontal cells, ganglion cells (arrowheads) and in some cells of the IPL. Thin arrows point to putative amacrine cells showing colocalization of aspartate and GABA immunoreactivity. In A, thick white arrows point to the outer limiting membrane. In all photographs sclera is toward the upper side, and the vitreum at the bottom. Scale bars: 20 μm.

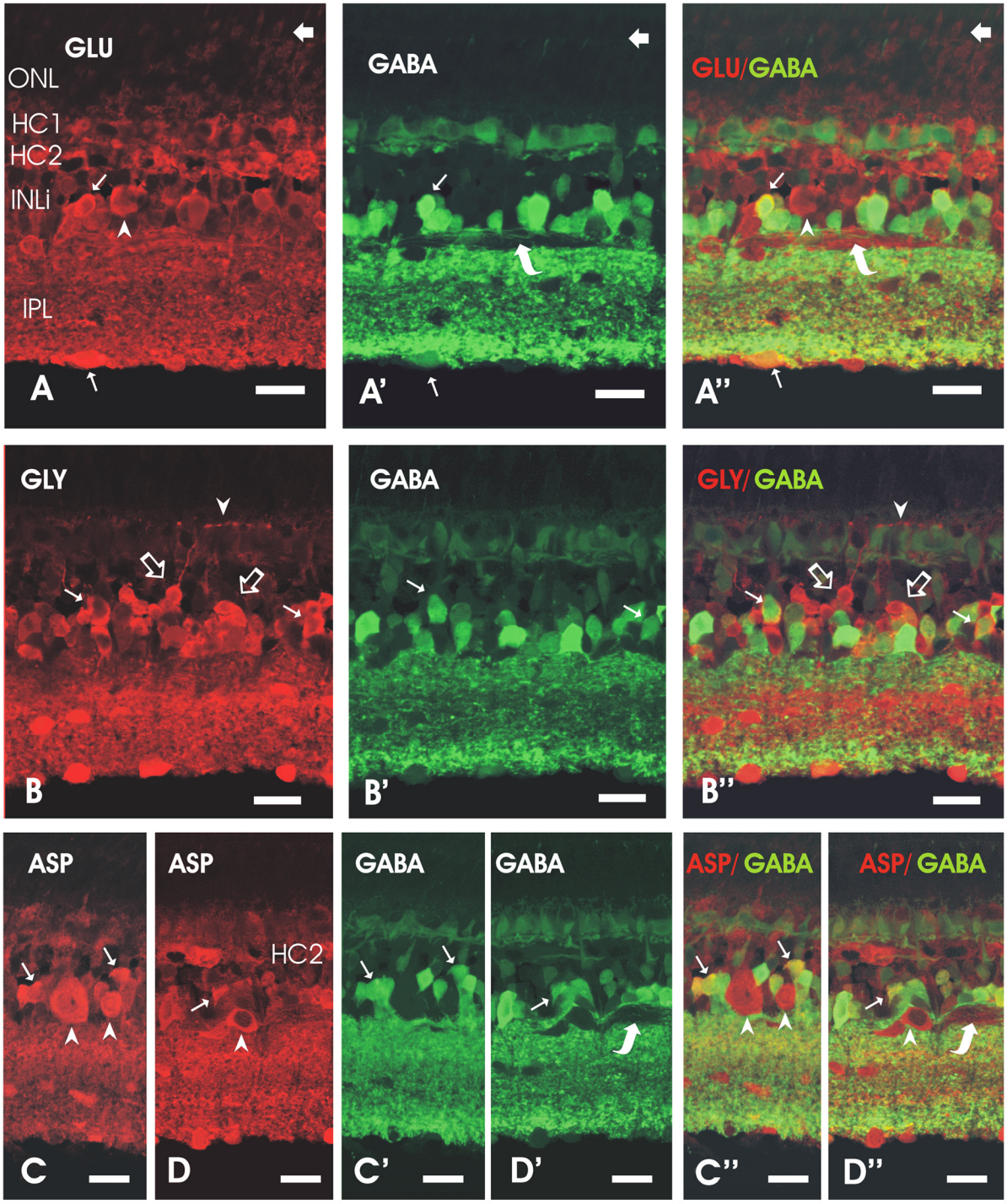

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Fig.6.

Fig. 7. Photomicrographs of vertical sections of the postmetamorphic retina showing the immunoreactivity to GABA (A-E), glutamate (F), glycine (G), aspartate (H), dopamine (I) and serotonin (J-K). **A,** Section showing strong GABA-immunoreactivity in some amacrine cells, and moderate immunoreactivity in type 1 horizontal cells and in some bipolar cells (open arrows). The arrowheads point to appendages of horizontal cells in the OPL. Note Landolt's clubs of bipolar cells coursing to the outer limiting membrane (thin arrows). **B,** Detail of the Landolt's club (arrow) of a GABA-ir bipolar cell with perikaryon located among horizontal cells. **C,** Detail of dendritic appendages of GABA-ir horizontal cells. **D,** Detail of axons of GABA-ir horizontal cells coursing below the perikarya (thick arrows) and ascending to the OPL (thin arrow). **E,** Detail of several putative retinopetal GABA-ir fibers coursing in a fascicle of optic fibers close to amacrine cells of the INL. **F,** Section showing abundant glutamate immunoreactivity in HC2 horizontal cells (open arrow) and in other neurons of the INL and the IPL. Thin arrows, small bipolar cells; arrowheads, large bipolar cells with perikarya just below the outer plexiform layer. **G,** Section showing the distribution of glycine-ir amacrine cells in the INL and IPL. The arrowhead points to the outer plexiform layer showing a few GLY-ir boutons. **Inset,** Detail of a glycinergic interplexiform cell (open arrow) showing an ascending process (thin arrow) coursing to the OPL (arrowhead). **H,** Section showing a large aspartate immunoreactive ganglion cell (double arrow) and other cells showing abundant immunoreactivity in the INL and IPL (single arrows). **I,** Detail of a dopamine-ir amacrine cell of the INL. Note that DA-ir processes course in outer parts of the IPL. **J,** Serotonin-ir amacrine cells located in the INL. Note that 5HT-ir fibers mainly course in outer and inner sublaminae of the IPL. **K,** Section showing a serotoninergic displaced amacrine cell (arrow) that is close to the inner limiting membrane. A-H, single channel projections of confocal stacks (inverted and converted to gray scale); I-K, bright field microscopy. In these photographs of the adult retina, the sclera is above, and the vitreum at the bottom. Scale bars: 20 μm (A-H), 25 μm (I-J). The scale in E applies to B-D.

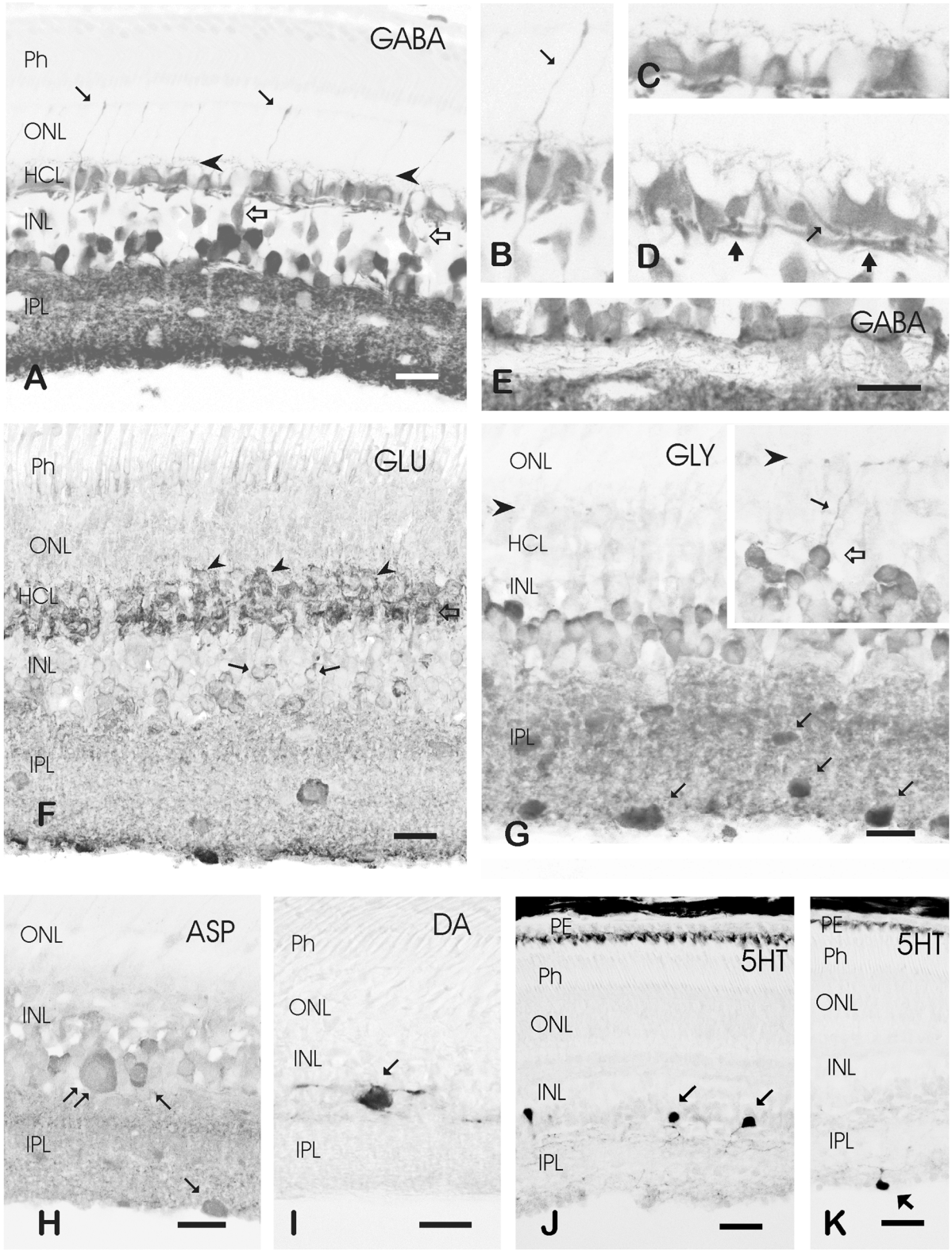
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Fig.7.

REFERENCES

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

Anadón R, Meléndez-Ferro M, Pérez-Costas E, Pombal MA, Rodicio MC. 1998. Centrifugal fibers are the only GABAergic structures of the retina of the larval sea lamprey: an immunocytochemical study. Brain Res 782:297-302.

Ben-Ari Y, Rovira C, Gaiarsa JL, Corradetti R, Robain O, Cherubini E. 1990. GABAergic mechanisms in the CA3 hippocampal region during early postnatal life. Prog Brain Res 83:313-321.

Bodnarenko SR, Chalupa LM. 1993. Stratification of ON and OFF ganglion cell dendrites depends on glutamate-mediated afferent activity in the developing retina. Nature 364:144-146.

Bodnarenko SR, Jeyarasasingam G, Chalupa LM. 1995. Development and regulation of dendritic stratification in retinal ganglion cells by glutamate-mediated afferent activity. J Neurosci 15:7037-7045.

Candal E, Anadón R, DeGrip WJ, Rodríguez-Moldes I. 2005. Patterns of cell proliferation and cell death in the developing retina and optic tectum of the brown trout. Dev Brain Res 154:101-119.

Cherubini E, Gaiarsa JL, Ben-Ari Y. 1991. GABA: an excitatory transmitter in early postnatal life. Trends Neurosci 14:515-519.

Cherubini E, Martina M, Sciancalepore M, Strata F. 1998. GABA excites immature CA3 pyramidal cells through bicuculline-sensitive and -insensitive chloride-dependent receptors. Perspect Dev Neurobiol 5:289-304.

Collin SP, Northcutt RG. 1993. The visual system of the Florida garfish, *Lepisosteus platyrhincus* (Ginglymodi). III. Retinal ganglion cells. Brain Behav Evol 42:295-320.

Connaughton VP, Behar TN, Liu WL, Massey SC. 1999. Immunocytochemical localization of excitatory and inhibitory neurotransmitters in the zebrafish retina. Vis Neurosci 16:483-490.

Cook JE, Becker DL. 1991. Regular mosaics of large displaced and non-displaced ganglion cells in the retina of a cichlid fish. J Comp Neurol 306:668-684.

Cook JE, Becker DL, Kapila R. 1992. Independent mosaics of large inner- and outer-stratified ganglion cells in the goldfish retina. J Comp Neurol 318:355-366.

Crooks J, Kolb H. 1992. Localization of GABA, glycine, glutamate and tyrosine hydroxylase in the human retina. J Comp Neurol 315:287-302.

Dalil-Thiney N, Pochet R, Versaux-Botteri C, Vesselkin N, Repérant J, Nguyen-Legros J. 1994. Immunohistochemical localization of calbindin-D28K and calretinin in the lamprey retina. J Comp Neurol 340:140-147.

Davanger S, Ottersen OP, Storm-Mathisen J. 1991. Glutamate, GABA, and glycine in the human retina: an immunocytochemical investigation. J Comp Neurol 311:483-494.

De Miguel E, Anadón R. 1987. The development of retina and the optic tectum of *Petromyzon marinus*, L. A light microscopic study. J Hirnforsch 28:445-56.

De Miguel E, Wagner HJ. 1990. Tyrosine hydroxylase immunoreactive interplexiform cells in the lamprey retina. Neurosci Lett 113:151-155.

De Miguel E, Rodicio MC, Anadón R. 1989. Ganglion cells and retinopetal fibers of the larval lamprey retina: an HRP ultrastructural study. Neurosci Lett 106:1-6.

De Miguel E, Rodicio MC, Anadón R. 1990. Organization of the visual system in larval lampreys: an HRP study. J Comp Neurol 302:529-542.

Dickson DH, Collard TR. 1979. Retinal development in the lamprey (*Petromyzon marinus* L.): premetamorphic ammocoete eye. Am J Anat 154:321-336.

Doi M, Uji Y, Yamamura H. 1995. Morphological classification of retinal ganglion cells in mice. J Comp Neurol 356:368-386.

Dunker N. 1999. Serotonergic neurons and processes in the adult and developing retina of *Ichthyophis kohtaoensis* (Amphibia; Gymnophiona). Anat Embryol 199:35-43.

Eldred WD, Cheung K. 1989. Immunocytochemical localization of glycine in the retina of the turtle (*Pseudemys scripta*). Vis Neurosci 2:331-338.

Fletcher EL, Kallionatis M. 1997. Localisation of amino acid neurotransmitters during postnatal development of the rat retina. J Comp Neurol 380:449–471.

Fritzsch B, Collin SP. 1990. Dendritic distribution of two populations of ganglion cells and the retinopetal fibers in the retina of the silver lamprey (*Ichthyomyzon unicuspis*). Vis Neurosci 4:533-545.

Frohlich E, Negishi K, Wagner HJ. 1995. The occurrence of dopaminergic interplexiform cells correlates with the presence of cones in the retinae of fish. Vis Neurosci 12:359-369.

García-Fernández JM, Jiménez AJ, González B, Pombal MA, Foster RG. 1997. An immunocytochemical study of encephalic photoreceptors in three species of lamprey. Cell Tissue Res 288:267-278.

González A, Marín O, Smeets WJ. 1995. Development of catecholamine systems in the central nervous system of the newt *Pleurodeles waltlii* as revealed by tyrosine hydroxylase immunohistochemistry. J Comp Neurol 360:33-48.

Holstein GR, Martinelli GP, Henderson SC, Friedrich VL Jr, Rabbitt RD, Highstein SM. 2004. Gamma-aminobutyric acid is present in a spatially discrete subpopulation of hair cells in the crista ampullaris of the toadfish *Opsanus tau*. J Comp Neurol 471:1-10.

Huang S, Moody SA. 1998. Dual expression of GABA or serotonin and dopamine in *Xenopus* amacrine cells is transient and may be regulated by laminar cues. Vis Neurosci 15:969-977.

Jojich L, Pourcho RG. 1996. Glutamate immunoreactivity in the cat retina: a quantitative study. Vis Neurosci 13:117-133.

Kageyama GH, Meyer RL. 1989. Glutamate-immunoreactivity in the retina and optic tectum of goldfish. Brain Res 503:118-127.

Kalloniatis M, Fletcher EL. 1993. Immunocytochemical localization of the amino acid neurotransmitters in the chicken retina. J Comp Neurol 336:174-193.

Kalloniatis M, Marc RE. 1990. Interplexiform cells of the goldfish retina. J Comp Neurol 297:340-358.

Kalloniatis M, Marc RE, Murry RF. 1996. Amino acid signatures in the primate retina. J Neurosci 16:6807-6829.

Kleerekoper H. 1972. The sense organs. In: MW Hardisty and IC Potter, editors. The Biology of Lampreys, vol. 2. London: Academic Press. p 373-404.

Li T, Wu SM, Lam DM, Watt CB. 1990. Localization of classical neurotransmitters in interneurons of the larval tiger salamander. Invest Ophthalmol Vis Sci 31:262-271.

Liets LC, Chalupa LM. 2001. Glutamate-mediated responses in developing retinal ganglion cells. Prog Brain Res 134:1-16.

López JM, Moreno N, González A. 2002. Localization of choline acetyltransferase in the developing and adult retina of *Xenopus laevis*. Neurosci Lett 330:61-64.

Marc RE, Lam DM. 1981. Glycinergic pathways in the goldfish retina. J Neurosci 1:152-165.

Marc RE, Liu WL, Kalloniatis M, Raiguel SF, van Haesendonck E. 1990. Patterns of glutamate immunoreactivity in the goldfish retina. J Neurosci 10:4006-4034.

Marc RE, Murry RF, Basinger SF. 1995. Pattern recognition of amino acid signatures in retinal neurons. J Neurosci 15:5106-5129.

Mariani AP. 1982. Biplexiform cells: ganglion cells of the primate retina that contact photoreceptors. Science 216:1134-1136.

Meléndez-Ferro M, Villar-Cheda B, Abalo XM, Pérez-Costas E, Rodríguez-Muñoz R, DeGrip WJ, Yáñez J, Rodicio MC, Anadón R. 2002a. Early development of the retina and pineal complex in the sea lamprey: comparative immunocytochemical study. J Comp Neurol 442:250-265.

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

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B, Rodríguez-Muñoz R, Anadón R, Rodicio MC. 2003. Ontogeny of gamma-aminobutyric acid-

immunoreactive neurons in the rhombencephalon and spinal cord of the sea lamprey. J Comp Neurol 464:17-35.

Mosinger JL, Yazulla S, Studholme KM. 1986. GABA-like immunoreactivity in the vertebrate retina: a species comparison. Exp Eye Res 42:631-644.

Negishi K, Kiyama H, Kato S, Teranishi T, Hatakenaka S, Katayama Y, Miki N, Tohyama M. 1986. An immunohistochemical study on the river lamprey retina. Brain Res 362:389-393.

Osborne NN, Patel S, Vigny A. 1984. Dopaminergic neurones in various retinas and the postnatal development of tyrosine-hydroxylase immunoreactivity in the rabbit retina. Histochemistry 80:389-393.

Osborne NN, Patel S, Beaton DW, Neuhoff V. 1986. GABA neurones in retinas of different species and their postnatal development in situ and in culture in the rabbit retina. Cell Tissue Res 243:117-123.

Östholm T, Ekström P, Bruun A, van Veen T. 1988. Temporal disparity in pineal and retinal ontogeny. Brain Res 470:1-13.

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

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

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

Pombal MA, Abalo XM, Rodicio MC, Anadón R, González A. 2003. Choline acetyltransferase-immunoreactive neurons in the retina of adult and developing lampreys. Brain Res 993:154-163.

Pow DV, Crook DK, Wong RO. 1994. Early appearance and transient expression of putative amino acid neurotransmitters and related molecules in the developing rabbit retina: an immunocytochemical study. Vis Neurosci 11:1115-1134.

Redburn DA, Agarwal SH, Messersmith EK, Mitchell CK. 1992. Development of the glutamate system in rabbit retina. Neurochem Res 17:61-66.

Redburn-Johnson D. 1998. GABA as a developmental neurotransmitter in the outer plexiform layer of the vertebrate retina. Perspect Dev Neurobiol 5:261-267.

Rio JP, Vesselkin NP, Kirpitchnikova E, Kenigfest NB, Versaux-Botteri C, Repérant J. 1993. Presumptive GABAergic centrifugal input to the lamprey retina: a double-labeling study with axonal tracing and GABA immunocytochemistry. Brain Res 600:9-19.

Rio JP, Vesselkin NP, Repérant J, Kenigfest NB, Versaux-Botteri C. 1998. Lamprey ganglion cells contact photoreceptor cells. Neurosci Lett 250:103-106.

Rio JP, Repérant J, Vesselkin NP, Kenigfest-Rio NB, Miceli D. 2003. Dual innervation of the lamprey retina by GABAergic and glutamatergic retinopetal fibers. A quantitative EM immunogold study. Brain Res 959:336-342.

Rodicio MC, Pombal MA, Anadón R. 1995. Early development and organization of the retinopetal system in the larval sea lamprey, *Petromyzon marinus* L. An HRP study. Anat Embryol 192:517-526.

Rodicio MC, Villar-Cerviño V, Abalo XM, Villar-Cheda B, Meléndez-Ferro M, Pérez-Costas E, Anadón R. 2005. Chemoarchitecture of the dorsal column nucleus of the larval sea lamprey. Brain Res Bull 66:536-540.

Rubinson K, Cain H. 1989. Neural differentiation in the retina of the larval sea lamprey (*Petromyzon marinus*). Vis Neurosci 3:241-248.

Schutte M. 1995. Effects of kainic acid and piperidine dicarboxylic acid on displaced bipolar cells in the turtle retina. J Neurocytol 24:361-369.

Sherry DM, Micich A, Yazulla S. 1993. Glycine in the lizard retina: comparison to the GABA system. Vis Neurosci 10:693-702.

Silveira dos Santos Bredariol A, Hamassaki-Britto DE. 2001. Ionotropic glutamate receptors during the development of the chick retina. J Comp Neurol 441:58-70.

Storm-Mathisen J, Ottersen OP. 1990. Antibodies and fixatives for the immunocytochemical localization of glycine. In: Ottersen OP, Storm-Mathisen J, editors. Glycine neurotransmission. New York: John Wiley & Sons. p 281-302.

Toth P, Straznicky C. 1989. Biplexiform ganglion cells in the retina of *Xenopus laevis*. Brain Res 499:378-382.

Van Haesendonck E, Missotten L. 1990. Glutamate-like immunoreactivity in the retina of a marine teleost, the dragonet. Neurosci Lett 111:281-286.

van Veen T, Ekström P, Nyberg L, Borg B, Vigh-Teichmann I, Vigh B. 1984. Serotonin and opsin immunoreactivities in the developing pineal organ of the three-spined stickleback, *Gasterosteus aculeatus* L. Cell Tissue Res 237:559-564.

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

Vesselkin NP, Ermakova TV, Reperant J, Kosareva AA, Kenigfest NB. 1980. The retinofugal and retinopetal systems in *Lampetra fluviatilis*. An experimental study using radioautographic and HRP methods. Brain Res 195:453-460.

Vesselkin NP, Repérant J, Kenigfest NB, Miceli D, Ermakova TV, Rio JP. 1984. An anatomical and electrophysiological study of the centrifugal visual system in the lamprey (*Lampetra fluviatilis*). Brain Res 292:41-56.

Vesselkin NP, Repérant J, Kenigfest NB, Rio JP, Miceli D, Shupliakov OV. 1989. Centrifugal innervation of the lamprey retina. Light- and electron microscopic and electrophysiological investigations. Brain Res 493:51-65.

Vesselkin NP, Rio JP, Repérant J, Kenigfest NB, Adanina VO. 1996. Retinopetal projections in lampreys. Brain Behav Evol 48:277-286.

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

Vitanova L, Haverkamp S, Wassle H. 2004. Immunocytochemical localization of glycine and glycine receptors in the retina of the frog *Rana ridibunda*. Cell Tissue Res 317:227-235.

Wagner HJ, Behrens UD. 1993. Microanatomy of the dopaminergic system in the rainbow trout retina. Vision Res 33:1345-1358.

Yang CY. 1996. Glutamate immunoreactivity in the tiger salamander retina differentiates between GABA-immunoreactive and glycine-immunoreactive amacrine cells. J Neurocytol 25:391-403.

Yang CY, Yazulla S. 1988. Light microscopic localization of putative glycinergic neurons in the larval tiger salamander retina by immunocytochemical and autoradiographical methods. J Comp Neurol 272:343-357.

Yang CY, Yazulla S. 1994. Glutamate-, GABA-, and GAD-immunoreactivities co-localize in bipolar cells of tiger salamander retina. Vis Neurosci 11:1193-1203.

Yang CY, Zhang J, Yazulla S. 2003. Differential synaptic organization of GABAergic bipolar cells and non-GABAergic (glutamatergic) bipolar cells in the tiger salamander retina. J Comp Neurol 455:187-197.

Yáñez J, Anadón R. 1994. Are the dopaminergic cells of the lamprey retina interplexiform cells? A dopamine, tyrosine hydroxylase and dopamine beta-hydroxylase immunocytochemical study. Neurosci Lett 165:63-66.

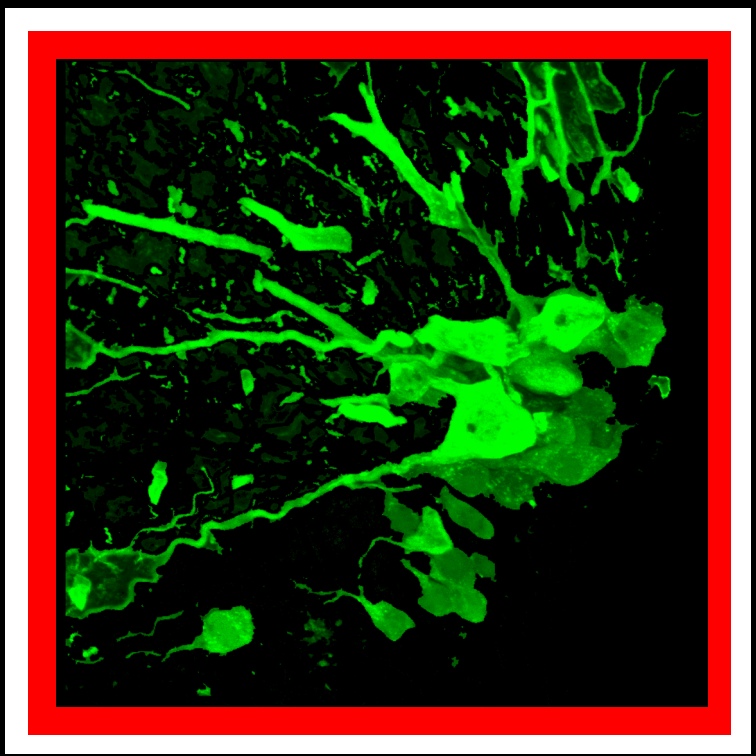
Yazulla S, Studholme KM. 1990. Multiple subtypes of glycine-immunoreactive neurons in the goldfish retina: single- and double-label studies. Vis Neurosci 4:299-309.

Yazulla S, Zucker CL. 1988. Synaptic organization of dopaminergic interplexiform cells in the goldfish retina. Vis Neurosci 1:13-29.

Zhou ZJ. 2001. A critical role of the strychnine-sensitive glycinergic system in spontaneous retinal waves of the developing rabbit. J Neurosci 21:5158-5168.

Zhu B, Straznicky C. 1992. Large serotonin-like immunoreactive amacrine cells in the retina of developing *Xenopus laevis*. Dev Brain Res 69:109-116.

Summary and Conclusions



SUMMARY

Glycine and γ-aminobutyric acid (GABA) are the main inhibitory neurotransmitters of the vertebrate central nervous system. Although both are very important in the inhibitory control of neural circuits, only the GABAergic system was extensively studied in different vertebrate groups, including lampreys. In contrast, the study of the glycinergic system was mainly focused in rhombencephalic or spinal cord populations of mammals. Whereas GABA is widely distributed in neurons throughout the brain, glycine is largely restricted to cells of the brain stem and spinal cord, being involved in sensory circuits and locomotor behavior. Glycine and GABA appeared frequently accumulated in the same cells, although the functional significance of the colocalization and possible corelease of these neurotransmitters is not well known yet.

Lampreys are living representatives of the most ancient vertebrate group, the Agnathans, so they are important subjects for the knowledge of the early evolutionary history of the central nervous system of vertebrates. The complex life cycle of the sea lamprey allowed us to study the various changes occurring in the central nervous system between developmental stages (embryos, prolarvae and larvae) and adults, which appear to be adapted to different life ways and environments. During the long larval period, sea lampreys are blind filter feeders that burrow in the bottom of rivers, but after a complex metamorphosis they became sighted parasitic feeders that migrate to the sea to live there a part of the adult period before to reenter rivers to breed and dye.

Because of the knowledge about the glycinergic system in the brain of adult non-mammalian vertebrates was very scarce, the main aim of the present Thesis was to describe for the first time the glycine-immunoreactive (ir) neuronal populations of the brain of the adult sea lamprey, and to analyze the changes that have occurred in glycinergic populations between jawless vertebrates and mammals (Chapter 1). We also wanted to investigate the early appearance of the glycinergic system in the lamprey brain, the changes occurring in this system in larvae after prolarval stages and whether or not all the glycinergic populations observed in larval stages are retained in the adult lamprey (Chapter 2).

Glycine is an important inhibitory transmitter in the adult lamprey spinal cord, although the spinal neuronal populations have not been thoroughly

investigated. A further aim of this Thesis was to study the early development of the glycinergic populations of the sea lamprey spinal cord and the changes occurring between embryos and adults (Chapter 3).

The retina of larval lampreys exhibits an immature appearance except in a small central region, which is the only part exhibiting photoreceptors and ganglion cells although lacking a layering similar to the adult retina. We also wished to study the possible neurochemical differentiation of neural circuitry in the lamprey retina during this exceptionally long larval phase using a set of antibodies directed against several classical neurotransmitters, including glycine, and to compare the larval pattern with the distribution of these neurotransmitters in the retina of recently transformed young lampreys (Chapter 4).

Possible colocalization of inhibitory neurotransmitters and phenotypic changes of inhibitory populations during development have not been studies in lampreys. Further aims were to compare the similarities and differences of the developmental patterns of glycine and GABA in the lamprey brain and spinal cord from early stages to adults (Chapters 2, 3 and 4) and to study their colocalization in neurons (All chapters).

To achieve these objectives we used immunoperoxidase or immunofluorescence techniques in brains and spinal cords of embryos, prolarvae, larvae, postmetamorphic and adults, and also in retinas of larvae and adults. For the comparison of immunoreactivities to glycine or other neurotransmitters with GABA immunoreactivity we used double immunofluorescence methods. We also used tract tracers in combination with immunofluorescence in order to identify some immunoreactive neuronal populations of the larval brain.

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

Chapter 1

Distribution of glycine immunoreactivity in the brain of adult sea lamprey (Petromyzon marinus). Comparison with gamma-aminobutyric acid

The distribution of glycinergic cells in the brain of non-mammalian vertebrates is still unknown. Lampreys are the most primitive extant vertebrates and they may provide important data on the phylogeny of this system. Here, we studied for the first time the distribution of glycine immunoreactivity in the sea lamprey brain, and compared it with GABAergic populations. Most glycine-ir neurons were found at midbrain and hindbrain levels, and most of these cells did not exhibit GABA immunoreactivity. We describe glycine-ir cell populations in the olfactory bulbs, the preoptic nucleus and the thalamus of the sea lamprey, which is in striking contrast to their lack in the mammalian forebrain. We also observed glycine-ir populations in the optic tectum, the torus semicircularis and the midbrain tegmentum, the isthmus, the octavolateral area, the dorsal column nucleus, the abducens nucleus, the trigeminal motor nucleus, the facial motor nucleus and the rhombencephalic reticular formation. In these populations, colocalization with GABA was only observed in some cells of the tegmental M5 nucleus, ventral isthmus, medial octavolateral nucleus, dorsal column nucleus and lateral reticular region. The present results allow us to conclude that the distribution of glycine-ir cells changed notably from lamprey to mammals, with a decrease in glycinergic populations in the forebrain and a specialization of brain stem cell groups. Although knowledge of the glycinergic populations in lampreys is important for understanding the early evolution of this system, there is a notable gap of information regarding its organization in the brain of other non-mammalian vertebrates.

Chapter 2

Development of glycine immunoreactivity in the brain of the sea lamprey: comparison with gamma-aminobutyric acid immunoreactivity

The development of glycine immunoreactivity in the brain of the sea lamprey was studied by use of immunofluorescence techniques from embryonic to larval stages. Glycine distribution was also compared with that of GABA by use of double immunofluorescence. The first glycine-ir cells appeared in the caudal rhombencephalon of late embryos, diencephalon of early prolarvae, and mesencephalon of late prolarvae, in which glycine-ir cells were observed in several prosencephalic regions (preoptic nucleus, hypothalamus, ventral thalamus, dorsal thalamus, pretectum, and nucleus of the medial longitudinal fascicle), mesencephalon (M5), isthmus, and rhombencephalon. In larvae, glycine-ir populations were observed in the olfactory bulbs, preoptic nucleus and thalamus (prosencephalon), M5 and oculomotor nucleus (mesencephalon), dorsal isthmic gray, isthmic reticular formation, and various alar and basal plate rhombencephalic populations. No glycine-ir cells were observed in the larval optic tectum or torus semicircularis, which contain glycine-ir populations in adults. A wide distribution of glycine-ir fibers was observed, which suggests involvement of glycine in the function of most lamprey brain regions.

Colocalization of GABA and glycine in prolarvae was found mainly in cell groups of the diencephalon, in the ventral isthmic group, and in trigeminal populations. In larvae, colocalization of GABA and glycine was principally observed in the M5 nucleus, the reticular formation, and the dorsal column nucleus. The present results reveal for the first time the complex developmental pattern of the glycinergic system in lamprey, including early glycine-ir populations, populations transiently expressing glycine, and late-appearing populations, in relation to maturation changes that occur during metamorphosis.

Chapter 3

Glycine-immunoreactive neurons in the developing spinal cord of the sea lamprey: comparison with the gamma-aminobutyric acidergic system

The development and cellular distribution of the inhibitory neurotransmitter glycine in the spinal cord of the sea lamprey were studied by immunocytochemistry and double immunofluorescence and compared with the distribution of GABA. Results in lamprey embryos and prolarvae reveal that the appearance of glycine-ir spinal neurons precedes that of GABA-ir neurons. Throughout development, glycine-ir cells in the lateral and dorsomedial gray matter of the spinal cord are more numerous than the GABA-ir cells. Only a subset of these neurons shows colocalization of GABA and glycine, suggesting that they are primarily disparate neuronal populations. In contrast, most cerebrospinal fluid (CSF)-contacting neurons of the central canal walls are strongly GABA-ir, and only a portion of them are faintly glycine-ir. Some edge cells (lamprey intraspinal mechanoreceptors) were glycine-ir in larvae and adults. The glycine-ir and GABA-ir neuronal populations observed in the adult spinal cord were similar to those found in larvae. Comparison of glycine-ir and GABA-ir fibers coursing longitudinally in the spinal cord of adult lamprey revealed large differences in diameter between these two types of fiber. Commissural glycine-ir fibers appear in prolarvae and become numerous at larval stages, whereas crossed GABA-ir fibers are scarce. Taken together, results in this primitive vertebrate indicate that the spinal glycinergic cells do not arise by biochemical shift of preexisting GABAergic cells but instead suggest that glycine is present in the earliest circuitry of the developing lamprey spinal cord, where it might act transiently as an excitatory transmitter.

Chapter 4

Presence of glutamate, glycine, and gamma-aminobutyric acid in the retina of the larval sea lamprey: comparative immunohistochemical study of classical neurotransmitters in larval and postmetamorphic retinas

The neurochemistry of the retina of the larval and postmetamorphic sea lamprey was studied by immunocytochemistry using antibodies directed against the major candidate neurotransmitters (glutamate, glycine, GABA, aspartate, dopamine, serotonin), and the neurotransmitter-synthesizing enzyme tyrosine hydroxylase. Immunoreactivity to rod opsin and calretinin was also used to distinguish some retinal cells. Two retinal regions are present in larvae: the central retina with opsin-immunoreactive photoreceptors, and the lateral retina, which lacks photoreceptors and is mainly neuroblastic. We observed calretinin-immunostained ganglion cells in both retinal regions; immunolabeled bipolar cells were detected in the central retina only. Glutamate immunoreactivity was present in photoreceptors, ganglion cells and bipolar cells. Faint to moderate glycine immunostaining was observed in photoreceptors and some cells of the ganglion cell/inner plexiform layer. No GABA-immunolabeled perikarya were observed. GABA-ir centrifugal fibers were present in the central and lateral retina. These centrifugal fibers contacted glutamate-immunostained ganglion cells. No aspartate, serotonin, dopamine or TH immunoreactivity was observed in larvae, whereas these molecules, as well as GABA, glycine and glutamate, were detected in neurons of the retina of recently transformed lamprey. Immunoreactivity to GABA was observed in outer horizontal cells, some bipolar cells and numerous amacrine cells, whereas immunoreactivity to glycine was found in amacrine cells and interplexiform cells. Dopamine and serotonin immunoreactivity was found in scattered amacrine cells. Since amacrine and horizontal cells did not express classical neurotransmitters (with the possible exception of glycine) during larval life, transmitter-expressing cells of the larval retina appear to participate only in the vertical processing pathway.

CONCLUSIONS

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

1. In the sea lamprey CNS, the first glycine-ir neurons appear early in development in the caudal rhombencephalon and spinal cord, just when the first body movements are observed, which suggests that glycine is involved in the generation of the swimming motor pattern. Unlike in mammals, the first spinal cord glycine-ir neurons appear before GABA-ir neurons.

2. The presence of transient glycine-ir brain populations during the prolarval period indicates that glycine may have different roles during development.

3. The changes observed in midbrain glycine-ir populations from larvae to adult appear related with the maturation of the optic tectum and visual system occurring during metamorphosis. Changes observed in the rhombencephalon also indicate differences in the organization of octaval centers between larval and adult lampreys.

4. The changes of glycine-ir populations observed in the lamprey spinal cord between larvae and adults comprise an increase in the number and size of immunoreactive cells, but the same glycine-ir cell types were observed from larvae to adults.

5. Like in mammals, GABAergic neurons are more widely distributed in the lamprey brain than glycinergic neurons from early developmental stages to adults, and these neuron types appear to represent largely separate neuronal populations except in the prolarval period, when colocalization of both neurotransmitters in neurons is most common.

6. The presence of some glycine-ir cell groups and the rich glycinergic innervation of the sea lamprey forebrain suggest that the glycinergic system of early vertebrates was rather extensive but became highly reduced in higher brain regions throughout evolution.

7. The presence of glycine-ir cells and fibers in the olfactory bulbs of the sea lamprey suggests that this neurotransmitter is involved in processing of olfactory

information, suggesting that glycinergic olfactory circuits were reduced during evolution. However, the greater abundance of GABA-ir cells in the olfactory bulbs suggests that GABA is the main inhibitory neurotransmitter in lamprey olfactory circuits.

8. The observation that the caudal pole of the optic tectum receives mainly octavolateral fibers, but no optic fibers (De Arriba, 2007) together with our results of glycinergic cell distribution, suggests that the caudal pole of the midbrain tectum is actually a caudal part of the torus semicircularis, which is reminiscent of the distribution of visual and acoustic centers in rostral and caudal parts of the midbrain tectum of mammals, respectively.

9. Glycine-ir cells are present in all reticular nuclei where they may be involved in control of locomotor pattern generation centers via their ipsilateral and/or contralateral projections, probably representing a substrate for coordination of the different reticular nuclei. The presence of glycine-ir neurons in several reticular nuclei of other vertebrates indicates that this feature was conserved throughout evolution.

10. The close association of some glycinergic cell groups with hindbrain visceromotor nuclei suggests that they are involved in the coordination of a number of basic visceromotor circuits.

11. Unlike the developing retinas of other vertebrates, serotonin, GABA, dopamine or TH is not expressed in cells of retina of larval sea lampreys, either central or lateral.

12. Unlike in other larval vertebrates, only the central portion of the developing retina of the sea lamprey shows glutamate expression in all cell types of the vertical visual pathways. Glycine immunoreactivity was also observed in photoreceptors and ganglion cells of this region.

13. The lateral retina of larvae shows glutamate expression only in putative ganglion cells, being mostly formed of a neurochemically undifferentiated neuroblastic layer. On the other hand, the absence of any neurotransmitter expression in the marginal retina supports its only proliferative nature.

14. The expression of glutamate in cells of the central retina of larvae, together the expression of opsins in photoreceptors (Meléndez-Ferro et al., 2002a), the presence of ganglion cells that project to the brain and the existence of centrifugal fibers (de Miguel et al., 1989; Anadón et al., 1998), suggest that the rudimentary central retina of larval lampreys may be functional, despite the absence of neurochemically differentiated cells of the horizontal system (amacrine cells, horizontal cells).

15. The absence of neurotransmitters in amacrine and horizontal cells during the larval period indicates immaturity of the neural circuits necessary for analysis of images. In this sense, the larval lamprey eye, which lacks a differentiated lens, could be compared to simple photoreceptor organs such as the pineal organ, which also lacks horizontal neural circuits and ancillary eye structures necessary for formation and analysis of images.

RESUMEN

La glicina y el ácido γ-aminobutírico (GABA) son los principales neurotransmisores inhibidores presentes en el sistema nervioso central de los vertebrados. A pesar de su importancia en los circuitos inhibidores, solamente el GABA ha sido estudiado de forma exhaustiva en diferentes grupos de vertebrados, incluidas las lampreas. Por el contrario, los estudios sobre el sistema glicinérgico se han centrado básicamente en las poblaciones neuronales de la médula espinal y rombencéfalo de algunos mamíferos, aunque también se han encontrado células glicinérgicas en otras regiones del sistema nervioso central. Mientras que el GABA se distribuye en poblaciones neuronales a lo largo de todo el cerebro, la glicina se encuentra generalmente restringida a neuronas del rombencéfalo y de la médula espinal. En el sistema nervioso central, la glicina es un neurotransmisor utilizado en circuitos sensoriales relacionados principalmente con la visión y la audición, así como por interneuronas que intervienen en el control de la generación de ritmos motores durante la locomoción. Aunque algunos estudios han revelado la colocalización de glicina y GABA en algunas neuronas, y se ha visto también que ambos neurotransmisores son almacenados en las vesículas sinápticas por el mismo transportador (transportador vesicular de aminoácidos inhibidores, VIAAT), el significado funcional de la colocalización y coliberación de estos dos neurotransmisores aun no es del todo conocido. De todos modos, parece que en esas sinapsis el GABA puede estar actuando sobre los receptores de glicina, aumentando de este modo el efecto inhibidor de la glicina sobre la célula postsináptica.

Las lampreas y los mixinos son los representantes del grupo mas primitivo y antiguo de los vertebrados actuales, los Agnatos, grupo hermano de los vertebrados con mandíbulas. Por ello, el estudio de su sistema nervioso resulta esencial para el conocimiento de la evolución temprana del sistema nervioso central de los vertebrados. El complejo ciclo de vida de la lamprea de mar, con fases larvaria y adulta muy largas y diferenciadas y una metamorfosis muy compleja, permite el estudio pausado de los diversos cambios que se producen en el sistema nervioso central durante las distintas etapas de desarrollo (embriones, prolarvas, larvas y adultos), pudiéndose relacionar con la adaptación de dichas fases a los distintos modos de vida y a diferentes ambientes. Durante el largo periodo larvario, la lamprea de mar es ciega, se alimenta mediante filtración y vive enterrada bajo el sedimento de

los ríos. Tras una compleja metamorfosis, tanto el órgano olfatorio como los ojos se hacen funcionales, siendo su diferenciación esencial para la localización de las presas durante su vida adulta, en la que actúa como ectoparásito. Parte de su etapa adulta la pasa en el mar, hasta que vuelve a remontar el río para realizar la freza y posteriormente morir.

En el sistema nervioso central de los vertebrados muchas neuronas utilizan la glicina como neurotransmisor inhibidor. A pesar de esto, las investigaciones acerca del sistema glicinérgico han sido muy escasas en los vertebrados no mamíferos. Estudios previos de nuestro grupo habían revelado la organización de las neuronas y fibras inmunorreactivas a GABA en el cerebro y médula espinal de la lamprea durante el desarrollo y en la etapa adulta. De esos estudios se deducía que el patrón de desarrollo inicial del sistema GABAérgico en las lampreas presentaba notables semejanzas con el de ratones y teleósteos, únicos vertebrados estudiados hasta la fecha, y además se confirmaba la utilidad de la aproximación ontogenética para poder comparar ese sistema entre especies evolutivamente muy distantes.

Debido a la ausencia de datos previos sobre la organización de las poblaciones inmunorreactivas a glicina en el sistema nervioso central de vertebrados no mamíferos, nuestros primeros objetivos fueron el estudio de su distribución en el cerebro adulto de la lamprea de mar (*Petromyzon marinus L*), y el análisis comparativo para entender los posibles cambios ocurridos en las poblaciones glicinérgicas entre los vertebrados agnatos y los mamíferos. En este contexto, también hemos querido comparar la distribución de la glicina y el GABA para poder estudiar su grado de colocalización en el cerebro adulto (Capítulo 1).

Otro objetivo de esta Tesis ha sido la investigación de la desarrollo temprano del sistema glicinérgico en el cerebro de la lamprea, así como de los cambios que ocurren en este sistema tras el periodo prolarvario, incluida la comprobación de si todas las poblaciones glicinérgicas observadas durante la fase larvaria se mantienen o no en el adulto. Además, también quisimos comparar el patrón de desarrollo de las poblaciones glicinérgicas y GABAérgicas, usando para ello métodos de doble inmunofluorescencia y microscopía confocal (Capítulo 2).

El tercer objetivo de esta Tesis se centró en el estudio del desarrollo temprano de las poblaciones glicinérgicas de la médula espinal de la lamprea de mar y de los cambios observados entre la fase embrionaria y la adulta usando técnicas inmunohistoquímicas. Además, también quisimos comparar el desarrollo de los grupos celulares glicinérgicos con aquellos que presentaban inmunorreactividad a GABA (Capítulo 3).

Por ultimo hemos querido estudiar el grado de diferenciación neuroquímica de la retina de la lamprea, que durante el periodo larvario se ha considerado como no funcional, usando un conjunto de anticuerpos dirigidos contra varios neurotransmisores clásicos, incluyendo la glicina. Como referencia de comparación y control, también hemos querido analizar la distribución de estos neurotransmisores en la retina de lampreas adultas recientemente metamorfoseadas (Capítulo 4).

Con esta tesis pretendemos llenar parte de la profunda laguna que existe sobre el conocimiento del sistema glicinérgico en los vertebrados no mamíferos.

Capítulo 1

Distribución de la inmunorreactividad a glicina en el cerebro de la lamprea de mar adulta (*Petromyzon marinus*). Comparación con el GABA

Aunque existen algunos estudios sobre el sistema glicinérgico en el sistema nervioso central de mamíferos, su distribución en el cerebro de vertebrados no mamíferos continúa siendo desconocida. En este trabajo hemos estudiado por primera vez la distribución de neuronas y fibras inmunorreactivas a glicina en el cerebro de la lamprea de mar, el vertebrado actual más primitivo, y lo hemos comparado con las poblaciones GABAérgicas. Para ello hemos empleado técnicas de doble inmunofluorescencia y de microscopía confocal que nos permiten analizar la codistribución y colocalización de ambos neurotransmisores.

La mayoría de las neuronas inmunorreactivas a glicina se han encontrado en el mesencéfalo y rombencéfalo, donde la mayor parte de ellas no mostraron inmunorreactividad a GABA. En el prosencéfalo, hemos descrito poblaciones celulares inmunorreactivas a glicina en los bulbos olfatorios, el núcleo preóptico y en el tálamo. Estos resultados difieren notablemente de la ausencia casi general de poblaciones glicinérgicas descrita en el prosencéfalo de mamíferos. También hemos observado poblaciones inmunorreactivas a glicina en el techo óptico, en el torus semicircularis y en el tegmento mesencefálico, así como en el istmo, el área octavolateral, en el núcleo de la columna dorsal, asociadas a los núcleos abducente, motor del trigémino y motor del facial y en la formación reticular rombencefálica. En estas poblaciones, se observó colocalización de glicina y GABA fundamentalmente en algunas células del núcleo M5 del tegmento mesencefálico, del istmo ventral, del núcleo octavolateral medial, del núcleo de la columna dorsal y en la región reticular lateral.

Estos resultados nos permiten concluir que la distribución de células inmunorreactivas a glicina difiere mucho entre lampreas y mamíferos, con un descenso de las poblaciones glicinérgicas en el prosencéfalo. Aunque el conocimiento de la distribución de poblaciones glicinérgicas en el cerebro de la lamprea es importante para comprender la evolución temprana de este sistema, existe una importante falta de información sobre su organización en cerebros de otros vertebrados no mamíferos.

Capítulo 2

Desarrollo de la inmunorreactividad a la glicina en el cerebro de la lamprea de mar: Comparación con el GABA

En este capítulo estudiamos el desarrollo de la inmunorreactividad a glicina utilizando técnicas inmunohistoquímicas tanto en embriones como en prolarvas y larvas de lamprea de mar (*Petromyzon marinus* L). También hemos comparado la distribución de glicina con la de GABA usando técnicas de doble inmunofluorescencia y microscopía confocal.

Las primeras células inmunorreactivas a glicina aparecen sucesivamente en el rombencéfalo caudal de embriones tardíos, en el diencéfalo de prolarvas tempranas y en el mesencéfalo de prolarvas tardías. En este último estado, se encontraron células inmunorreactivas a glicina en varias regiones del prosencéfalo (núcleo preóptico, hipotálamo, tálamo ventral, tálamo dorsal, pretecho y núcleo del fascículo longitudinal medial), en el núcleo mesencefálico M5, en el istmo dorsal y ventral, y en el rombencéfalo. Las larvas mostraron poblaciones inmunorreactivas a glicina en el prosencéfalo (bulbos olfatorios, núcleo preóptico y tálamo dorsal), en el mesencéfalo (núcleo M5 y núcleo oculomotor), en el istmo (gris ístmica dorsal y formación reticular ístmica), así como en poblaciones rombencefálicas distribuidas tanto en la placa alar como en la basal. No encontramos células inmunorreactivas a glicina ni en el techo óptico ni en el torus semicircularis, poblaciones que sí contienen glicina en el adulto. Las fibras inmunorreactivas a glicina se encontraron ampliamente distribuidas por el cerebro de la lamprea, lo que sugiere su participación en las funciones de la mayor parte de las regiones cerebrales.

En cuanto a la colocalización de la glicina y el GABA, en prolarvas se ha encontrado fundamentalmente en grupos celulares diencefálicos, en el grupo ístmico ventral y en poblaciones de la región del trigémino. En larvas, hemos encontrado colocalización de GABA y glicina en el núcleo M5, la formación reticular y en el núcleo de la columna dorsal.

Estos resultados muestran por primera vez el complejo patrón de desarrollo del sistema glicinérgico en la lamprea, incluyendo poblaciones tempranas y poblaciones que expresan glicina de forma transitoria, así como la aparición tardía de poblaciones relacionadas con los cambios que ocurren durante la metamorfosis.

Capítulo 3

Neuronas inmunorreactivas a glicina en la médula espinal en desarrollo de la lamprea de mar: Comparación con el sistema GABAérgico

Mediante el empleo de técnicas inmunohistoquímicas simples y de doble inmunofluorescencia hemos estudiado el desarrollo de las poblaciones que expresan el neurotransmisor inhibidor glicina en la médula espinal de la lamprea de mar, y hemos comparado su distribución con la de las poblaciones que expresan GABA. Los resultados obtenidos en embriones y prolarvas revelan que la aparición de neuronas glicinérgicas en la médula espinal precede a la aparición de las neuronas GABAérgicas. A lo largo del desarrollo, las células inmunorreactivas a glicina de la sustancia gris lateral y dorsomedial de la médula espinal son más numerosas que las células inmunorreactivas a GABA. Solo una parte de estas neuronas mostraron colocalización de glicina y GABA, sugiriendo que son fundamentalmente poblaciones neuronales diferentes. Por el contrario, la mayoría de las células que contactan con el líquido cefalorraquídeo localizadas en las paredes del canal central son fuertemente inmunorreactivas a GABA, y solamente una parte presenta una inmunorreactividad débil a glicina. También encontramos que algunas "células de borde" (mecanorreceptores intraespinales) presentan inmunorreactividad a glicina tanto en larvas como en adultos. Las poblaciones neuronales inmunorreactivas a glicina y a GABA encontradas en la médula espinal de ejemplares adultos son similares a aquellas observadas en larvas. La comparación de las fibras inmunorreactivas a glicina y a GABA que transcurren longitudinalmente en la médula espinal de ejemplares adultos reveló una importante diferencia en sus diámetros. Las fibras comisurales inmunorreactivas a glicina aparecen ya en las prolarvas y se hacen mas numerosas en el estado larvario, mientras que las fibras comisurales inmunorreactivas a GABA son escasas. Los resultados obtenidos en este vertebrado primitivo indican que las células glicinérgicas espinales no surgen a partir de un cambio bioquímico en células GABAérgicas preexistentes, sino que la glicina ya está presente en los circuitos tempranos de la médula espinal en desarrollo, donde podría actuar de forma transitoria como un neurotransmisor excitador.

Capítulo 4

Presencia de glutamato, glicina y GABA en la retina de la larva de lamprea de mar: estudio inmunohistoquímico comparado de neurotransmisores clásicos en retinas de larvas e individuos postmetamórficos

En este capítulo estudiamos la neuroquímica de la retina de larvas de la lamprea de mar, así como de ejemplares postmetamórficos mediante técnicas inmunohistoquímicas usando anticuerpos contra distintos neurotransmisores (glutamato, glicina, GABA, aspartato, dopamina y serotonina), y contra la enzima de síntesis de catecolaminas tirosina hidroxilasa (TH). Para distinguir algunos tipos celulares también hemos empleado anticuerpos contra opsinas y calretinina.

En la retina larvaria avanzada se pueden diferenciar dos regiones: una central con fotorreceptores inmunorreactivos a opsinas, y una lateral que carece de fotorreceptores y es fundamentalmente neuroblástica. En la retina central se pueden distinguir tanto células ganglionares como células bipolares inmunorreactivas a calretinina, mientras que en la retina lateral sólo lo son las células ganglionares. Tanto los fotorreceptores, como las células bipolares y las ganglionares mostraron inmunorreactividad a glutamato. La inmunorreactividad a glicina se encontró en fotorreceptores y en algunas células de la capa de células ganglionares. A diferencia del glutamato, no encontramos pericariones inmunorreactivos a GABA en la retina larvaria, aunque sí fibras centrífugas GABAérgicas que, tanto en la retina central como en la lateral, contactan con células ganglionares inmunorreactivas a glutamato. En la retina larvaria no hallamos ninguna inmunorreactividad a aspartato, serotonina, dopamina o TH, aunque estas moléculas, junto con el GABA, la glicina y el glutamato pueden ser detectadas en neuronas de la retina de lampreas adultas (postmetamórficas). En estos ejemplares adultos encontramos inmunorreactividad a GABA en las células horizontales externas, en algunas células bipolares, así como en numerosas células amacrinas, mientras que la inmunorreactividad a glicina se encontró en células amacrinas e interplexiformes. La inmunorreactividad a dopamina y serotonina se localizó solamente en células amacrinas. Ya que ni las células amacrinas ni las horizontales expresan ningún neurotransmisor clásico durante el periodo larvario (con la posible excepción de la glicina), parece que las células que

expresan algún neurotransmisor en este periodo están participando únicamente en la ruta vertical de procesamiento visual.

CONCLUSIONES

Tras el análisis detallado de los resultados obtenidos del estudio del sistema glicinérgico durante el desarrollo de la lamprea de mar y en la etapa adulta, de su comparación con el sistema GABAérgico, así como de la maduración neuroquímica de la retina larvaria hemos extraído las siguientes conclusiones:

1. Las primeras neuronas inmunorreactivas a glicina en el sistema nervioso central de la lamprea aparecen en el rombencéfalo y médula espinal al final del periodo embrionario, justo cuando comienzan los primeros movimientos corporales, lo que sugiere que la glicina está implicada en la generación del patrón motor en la natación. A diferencia de lo señalado en los mamíferos, las primeras neuronas espinales de lamprea inmunorreactivas a glicina aparecen antes que las inmunorreactivas a GABA.

2. La presencia de poblaciones transitorias inmunorreactivas a glicina en el cerebro de la lamprea durante la fase prolarvaria indica que la glicina podría ejercer distintos papeles durante el desarrollo.

3. Los cambios observados en las poblaciones inmunorreactivas a glicina en el mesencéfalo de la lamprea desde la etapa larvaria hasta el adulto parecen estar relacionados con la maduración durante la metamorfosis del techo óptico y el sistema visual. Los cambios observados en el rombencéfalo también indican diferencias en la organización de los centros octavos entre la etapa larvaria y la adulta.

4. Las diferencias entre las poblaciones inmunorreactivas a glicina de la médula espinal de larvas y adultos solamente implican un incremento en el tamaño y número de células, encontrándose los mismos tipos celulares en ambas etapas.

5. Al igual que lo señalado en mamíferos, las neuronas GABAérgicas se encuentran mas ampliamente distribuidas en el cerebro de lamprea que las glicinérgicas ya desde etapas tempranas de desarrollo y hasta el adulto. Además, estos dos tipos neuronales parecen representar dos poblaciones ampliamente diferentes excepto en la etapa prolarvaria, cuando la colocalización de ambos neurotransmisores en neuronas es más frecuente.

6. La presencia de algunos grupos celulares inmunorreactivos a glicina en el prosencéfalo de lamprea, así como su abundante inervación glicinérgica, indican que a lo largo de la evolución las poblaciones glicinérgicas de los vertebrados han sufrido una reducción en número tanto en el telencéfalo como en el diencéfalo.

7. La presencia de fibras y células inmunorreactivas a glicina en los bulbos olfatorios de la lamprea de mar sugieren que este neurotransmisor participa en el procesamiento de la información olfativa y que su participación en este sistema se ha reducido notablemente durante la evolución. Aún así, la gran abundancia de células GABAérgicas en los bulbos olfatorios de lamprea indica que el GABA es el principal neurotransmisor inhibidor en los circuitos bulbares de las lampreas.

8. El hecho de que el polo caudal del techo óptico reciba principalmente fibras octavolaterales y no ópticas (De Arriba, 2007), junto con nuestros resultados acerca de la distribución de células glicinérgicas, sugieren que el polo caudal del techo mesencefálico corresponde en realidad a la región caudal del torus semicircularis, lo que recuerda a la distribución de los centros visuales y acústicos en las partes rostral y caudal, respectivamente, del techo mesencefálico de mamíferos.

9. Todos los núcleos reticulares de la lamprea contienen numerosas células inmunorreactivas a glicina, lo que sugiere que este neurotransmisor debe estar implicado en los centros de control de la generación de la locomoción vía sus proyecciones ipsilaterales y/o contralaterales, siendo probablemente un sustrato para la coordinación de los distintos núcleos reticulares. La presencia de neuronas inmunorreactivas a glicina señalada en varios núcleos reticulares de otros vertebrados indica que esta característica se ha conservado a lo largo de la evolución.

10. La estrecha asociación de algunos grupos celulares inmunorreactivos a glicina con núcleos visceromotores del rombencéfalo, sugiere que estas poblaciones glicinérgicas están implicadas en la coordinación básica de circuitos visceromotores.

11. A diferencia de las retinas en desarrollo de otros vertebrados, en la retina larvaria de la lamprea de mar no se encuentran células con expresión de serotonina, GABA, dopamina o TH, ni en la región central ni en la lateral.

12. A diferencia de las larvas de otros vertebrados, sólo la porción central de la retina en desarrollo de la lamprea de mar muestra expresión de glutamato en todos los tipos celulares de la vía vertical de transmisión de la señal visual. Además, también encontramos inmunorreactividad a glicina en dos tipos celulares de esta vía en la región central, concretamente en fotorreceptores y células ganglionares.

13. La retina lateral de las larvas de lamprea de mar se encuentra formada básicamente por una capa neuroblástica sin diferenciación neuroquímica, excepto algunas células ganglionares que expresan glutamato. Por otro lado, la ausencia de expresión de neurotransmisores en células de la retina marginal denota su naturaleza únicamente proliferativa.

14. La expresión de glutamato en células de la retina central de larvas, junto con la expresión de opsinas en fotorreceptores (Meléndez-Ferro y col., 2002a), la presencia de células ganglionares que proyectan al cerebro y la existencia de fibras centrífugas (de Miguel y col., 1989; Anadón y col., 1998), sugieren que la retina central podría ser funcional en las larvas de lamprea de mar, a pesar de la ausencia de células diferenciadas neuroquímicamente de la vía de transmisión horizontal (células amacrinas y horizontales).

15. La ausencia de neurotransmisores tanto en células amacrinas como horizontales de la retina larvaria indican la inmadurez de los circuitos necesarios para el análisis de imágenes. En este sentido, el ojo de la larva, que carece de cristalino diferenciado, puede ser comparado con órganos fotorreceptivos simples como el órgano pineal, que carece de circuitos neurales horizontales en la retina y de estructuras complementarias del ojo necesarios para la formación y análisis de imágenes.