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Distribution of Glycine Immunoreactivity in the Brain of Adult Sea Lamprey (*Petromyzon marinus*). Comparison With γ-Aminobutyric Acid

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ABSTRACT

The distribution of glycinergic cells in the brain of nonmammalian 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 γ-aminobutyric acid (GABA)-ergic populations. Most glycine-immunoreactive neurons were found at midbrain and hindbrain levels, and most of these cells did not exhibit GABA immunoreactivity. We describe glycine-immunoreactive 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-immunoreactive 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 observed in only 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-immunoreactive cells changed notably from lamprey to mammals, with a decrease in glycinergic populations in the forebrain and a specialization of brainstem 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 brains of other nonmammalian vertebrates. J. Comp. Neurol. 507:1441–1463, 2008.

Indexing terms: glycinergic; GABA; immunocytochemistry; confocal microscopy; colocalization; agnathans

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 associated primarily with neurons, although recent studies

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have shown that GLYT1 is also expressed in neurons (Cubelos et al., 2005). Glycine is also a modulator coagonist 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 coagonists (for revision see Wood, 2005).

Studies on the brain distribution of glycinergic neurons and/or fibers have focused mainly on mammals. Early studies of the glycinergic system examined in vivo accumulation of labelled 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; Álvarez-Otero et al., 1996; 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 distributed mainly 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 nonmammalian 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 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. As far as we are aware, there are no studies on 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 (Meléndez-Ferro, 2001; Rodicio et al., 2005; Robertson et al., 2007) lampreys. 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 (Vesselkin et al., 1995, 2000; Shupliakov et al., 1996; Birinyi et al., 2001; Villar-Cerviño et al., 2008), indicating that inhibitory effects on postsynaptic cells can be mediated by corelease 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 for 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 func-

Abbreviations

DCN	dorsal column nucleus
DIG	dorsal isthmic gray
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	spinooccipital 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

tional studies on glycinergic transmission in the spinal cord and brainstem 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 analyze 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 via double-immunofluorescence methods.

MATERIALS 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 0.05 M 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 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 4°C with rabbit polyclonal antiglycine 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 0.05 M 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 antifade 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 described above and stained with a cocktail of polyclonal rabbit antiglycine (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 nonprotein 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, 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 antiglycine antibodies revealed the same pattern of glycine-ir cell populations in both the brain and the 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 analyzed and photographed with a fluorescence microscope fitted with an Olympus DP 70 color 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 confocal figures, stack projections were converted to gray scale, inverted, and then adjusted for brightness and contrast in Corel Photo-Paint (Corel, Ottawa, Ontario, Canada). Similarly, fluorescence color photomicrographs were converted to gray scale, 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 ± SD.

RESULTS

Both antiglycine 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 (Figs. 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 (Figs. 1A, 3A), far from the glomeruli, extending caudally to the limit with the telencephalic lobes. These cells exhibited long, nonbranched 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 consists mainly of an evaginated lateral pallium with a thick pallial wall, and a medial pallium that faces the midtelencephalic 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 (Fig. 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.

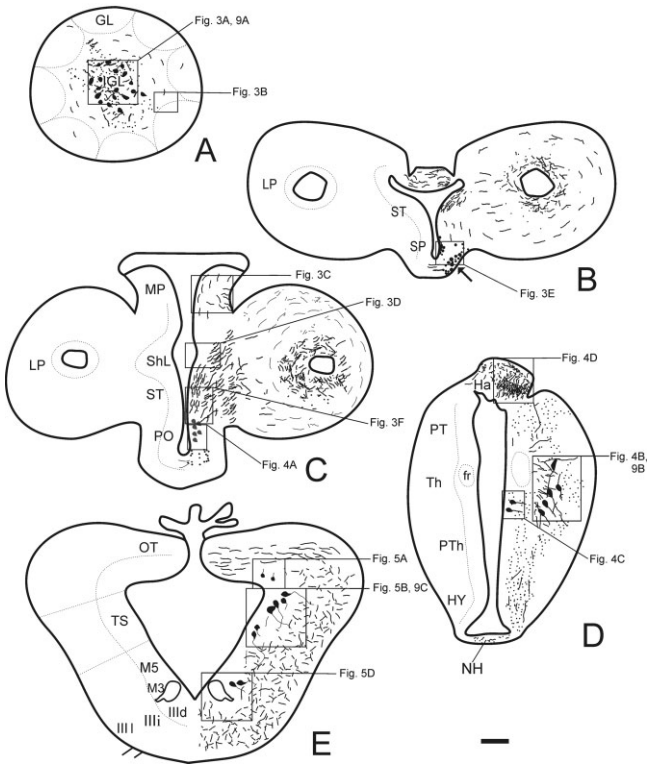

Fig. 1. Schematic drawing of transverse sections of the adult sea lamprey brain showing the distribution of glycine-immunoreactive cells and fibers (right) along with main brain structures (left). The level of sections is indicated at upper right. The size of glycinergic cells was doubled for better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

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 observed only 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

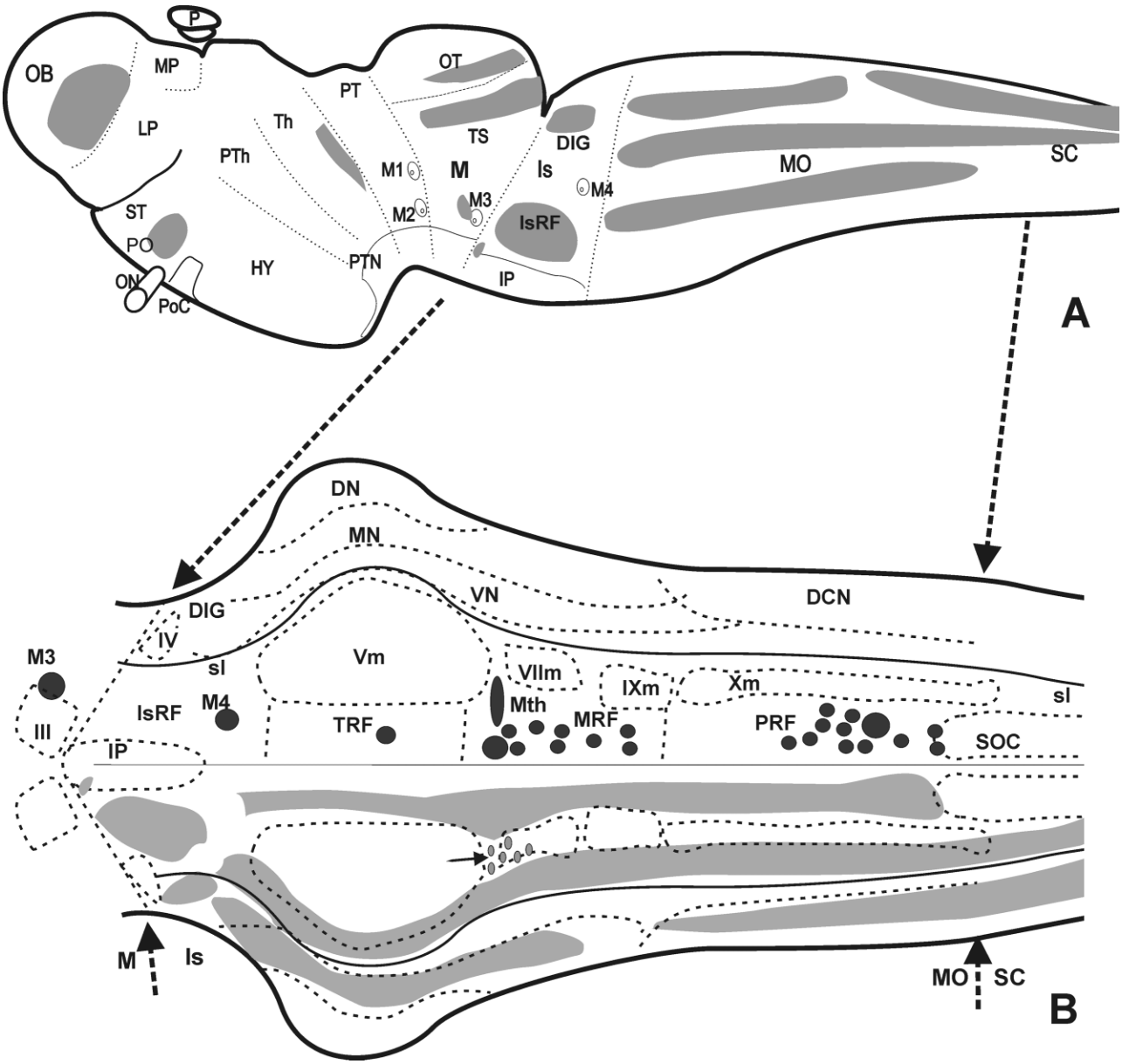

Fig. 2. **A:** Schematic lateral view of the brain showing the distribution of glycine-immunoreactive neuronal populations in the forebrain, midbrain, and hindbrain. **B:** Schematic drawing of a dorsal view of a projection of the rhombencephalon showing the distribution of the main glycine-immunoreactive 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).

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 exhibited only faint glycine immunoreactivity.

In the tuberal and mammillary regions of the hypothalamus, no glycine-ir neurons were observed. Moderate in-

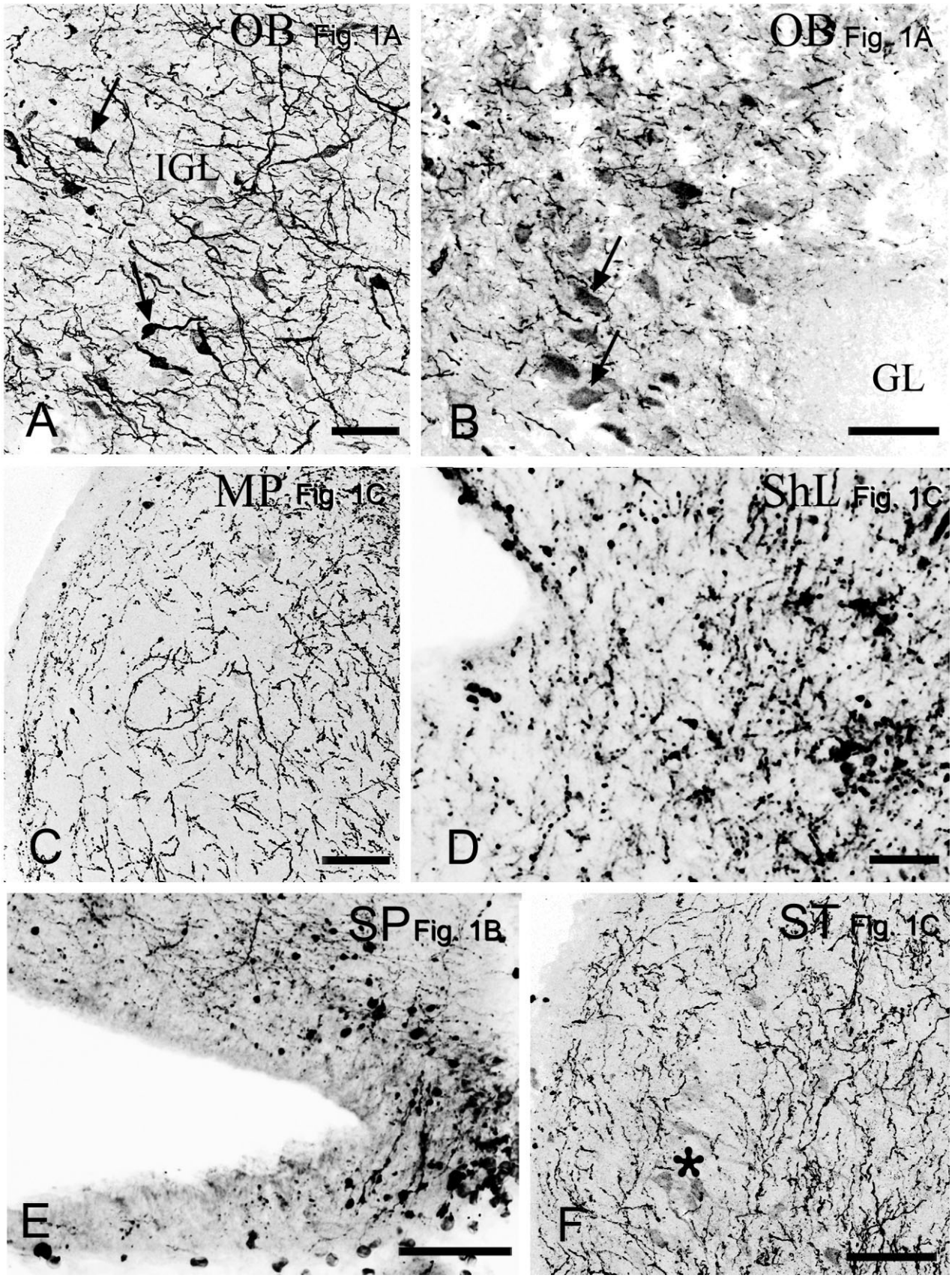

Fig. 3. Inverted gray-scale photomicrographs of transverse sections of the lamprey forebrain showing glycine-ir structures. **A:** Section of the olfactory bulb showing glycine-ir cells (arrows) and processes in the granular layer. **B:** Section of the olfactory bulb showing faint glycine-ir mitral 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 neighboring magnocellular preoptic nucleus is also shown (asterisk). For abbreviations see list. Correspondence with schemes of Figure 1 is indicated at upper right. A–C,F are confocal micrographs; D,E are fluorescence photomicrographs. Scale bars = 50 μm in A–C,E,F; 25 μm in D.

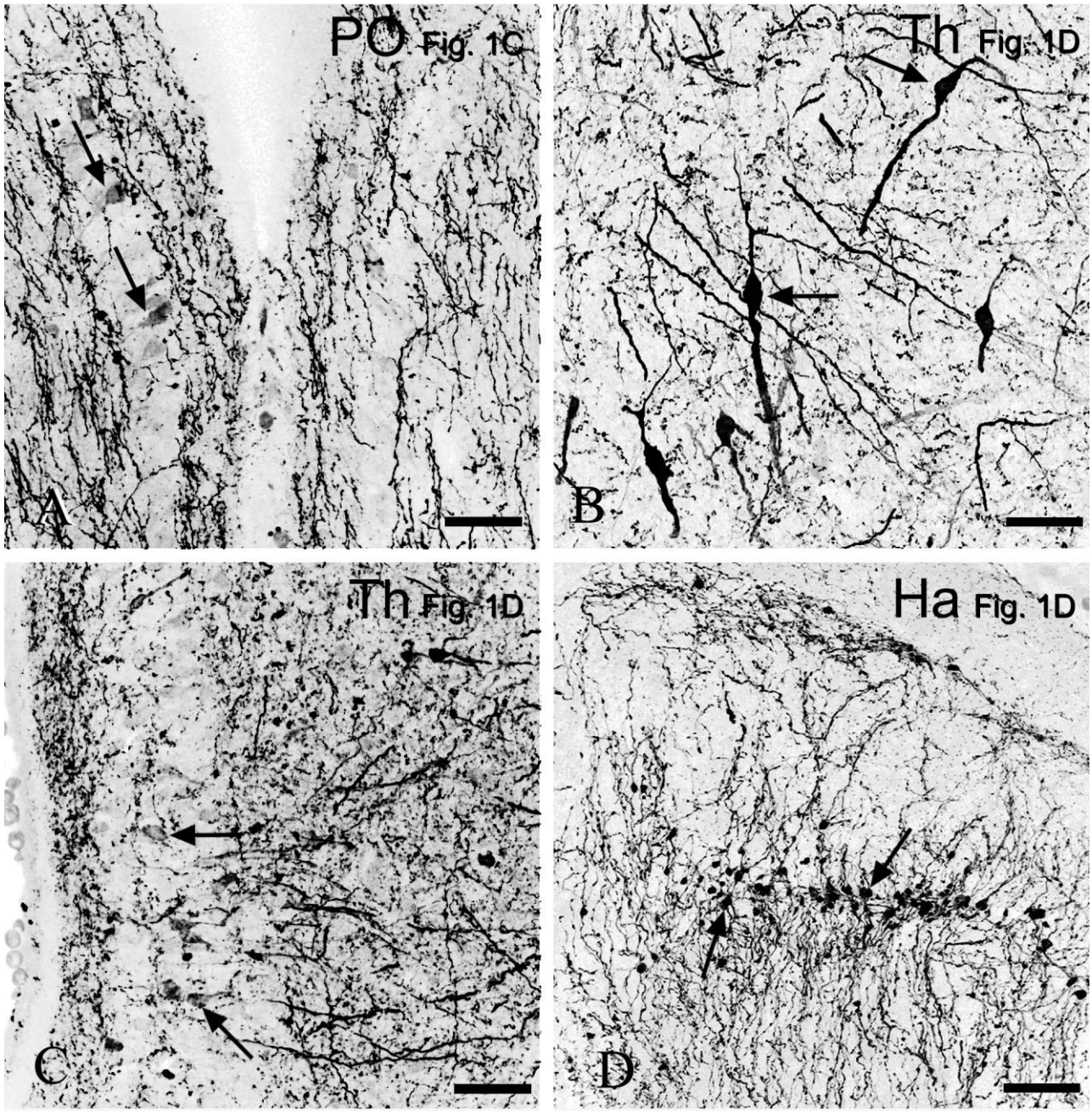

Fig. 4. Inverted gray-scale confocal photomicrographs of transverse sections of the preoptic nucleus (A), thalamus (B,C), and habenula (D) showing glycine-ir structures. **A:** Section showing faint glycine-ir neurons in the cell layer of the magnocellular preoptic nucleus (arrows) and numerous glycine-ir 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 at upper right. Scale bars = 50 μm.

nervation 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, in both the lateral and the 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 that 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 (Figs. 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 actually formed 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 (Figs. 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) toward 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 to 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).

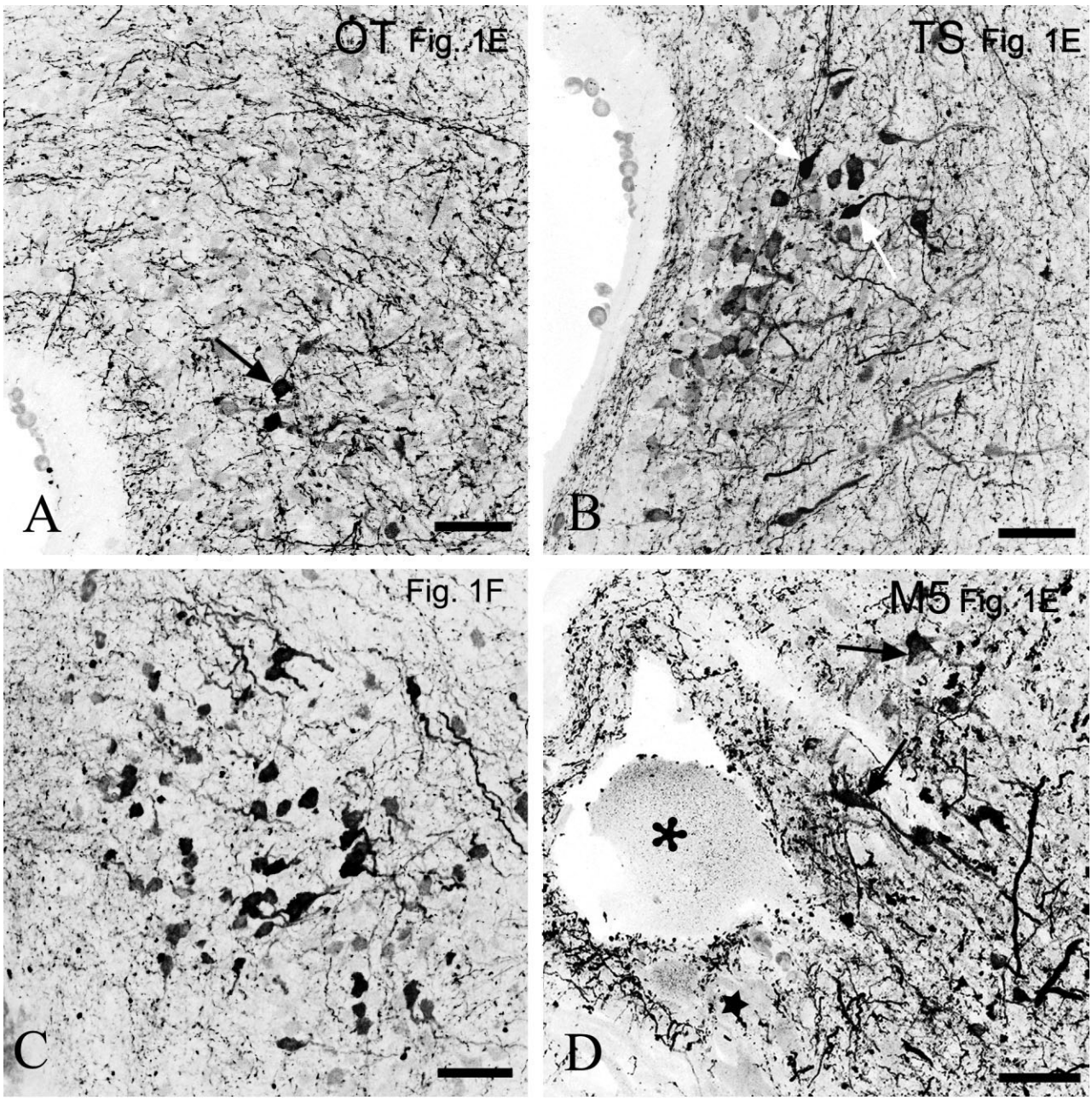

Fig. 5. Inverted gray-scale confocal photomicrographs of transverse sections through the mesencephalon showing glycine-ir structures. **A:** Section through the lateral region of the optic tectum showing small glycine-ir cells (arrow) in the stratum cellulare periventriculare. Note also the abundance 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 at upper right. Scale bars = 50 μm.

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 for sea lamprey with immunohistochemistry

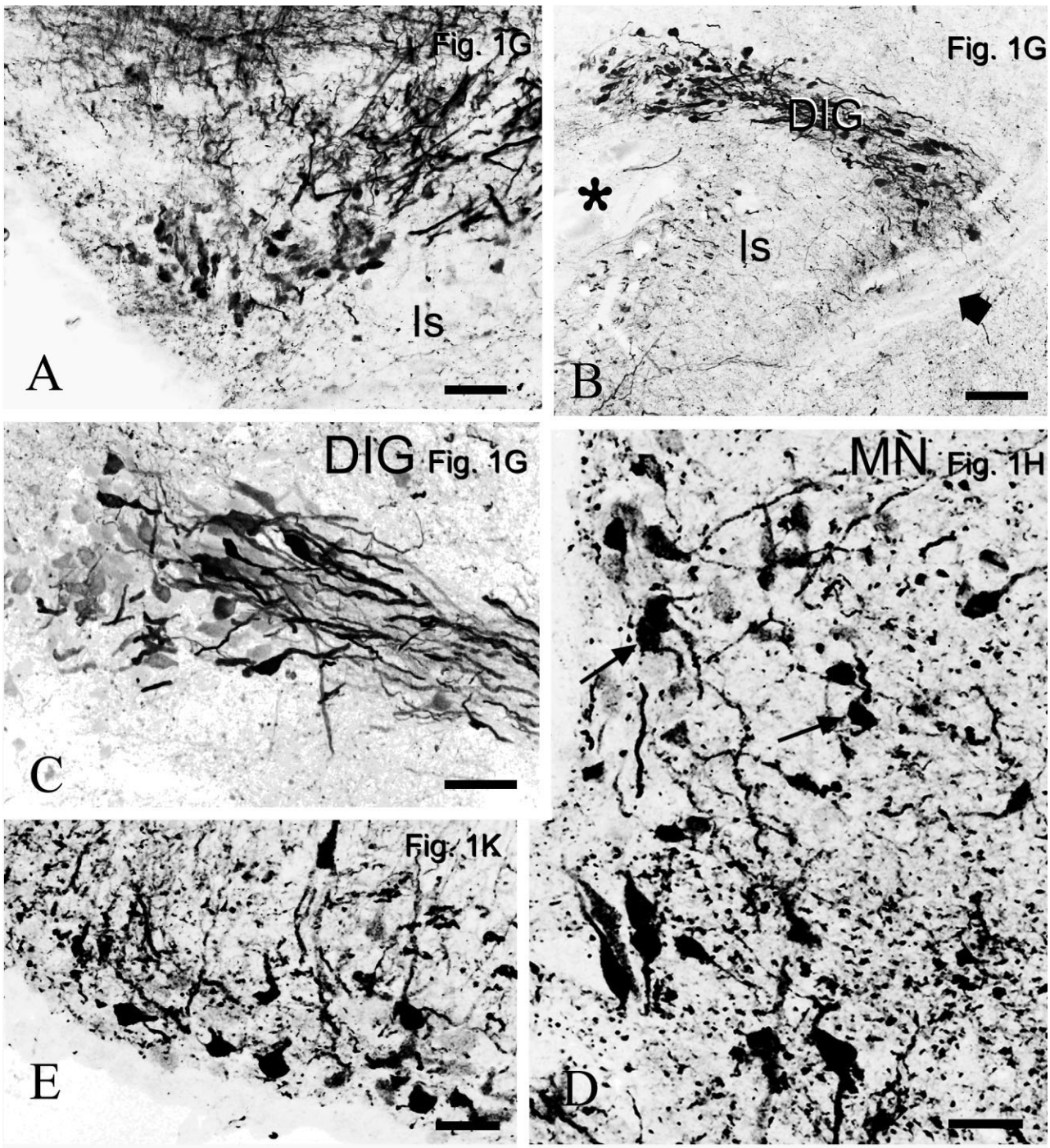

Fig. 6. Inverted gray-scale photomicrographs of transverse sections through the alar plate of the rhombencephalon showing strongly glycine-ir cells. **A:** Section through the medial region of the dorsal isthmus showing a dorsomedial population of small glycine-ir cells. Note the abundance of processes in the cerebellar plate region (top). **B:** Section showing the conspicuous dorsolateral population of strong glycine-ir cells of the dorsal isthmic gray with long and thick lateral dendrites. Asterisk, large glycine-negative reticular cells. Arrow, negative fibers of the anterior octavomotor tract. **C:** Detail of the dorsolateral glycine-ir population of the dorsal isthmic gray. **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 at upper right. A and B are fluorescence photomicrographs; C–E are confocal photomicrographs. Scale bars = 50 μm in A,C; 100 μm in B; 25 μm in D,E.

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 more laterally 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 topographic 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 the 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 the *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 neighboring 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 (Figs. 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

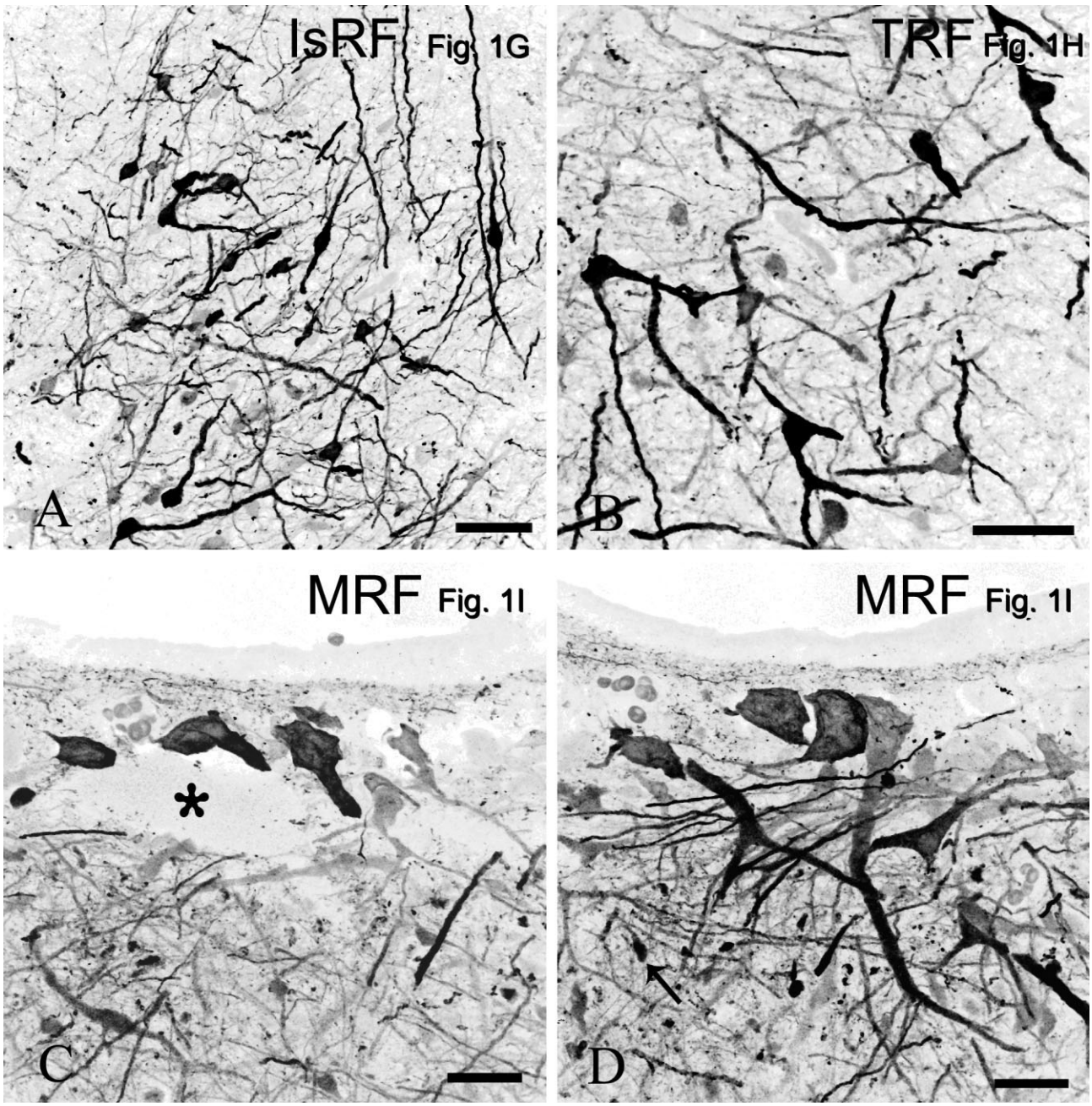

Fig. 7. Inverted gray-scale confocal photomicrographs of transverse sections through the medial zone of the reticular formation showing glycine-ir reticular cells. **A:** Section showing glycine-ir cells of the isthmic reticular formation. **B:** Section showing glycine-ir cells of the trigeminal levels 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 at upper right. Scale bars = 50 μm.

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 den-

drites 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 toward 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 toward 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 another 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 is 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-labelling immunofluorescence experiments. The general distribution of GABA-ir perikarya and fibers observed in these double-labelling 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 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

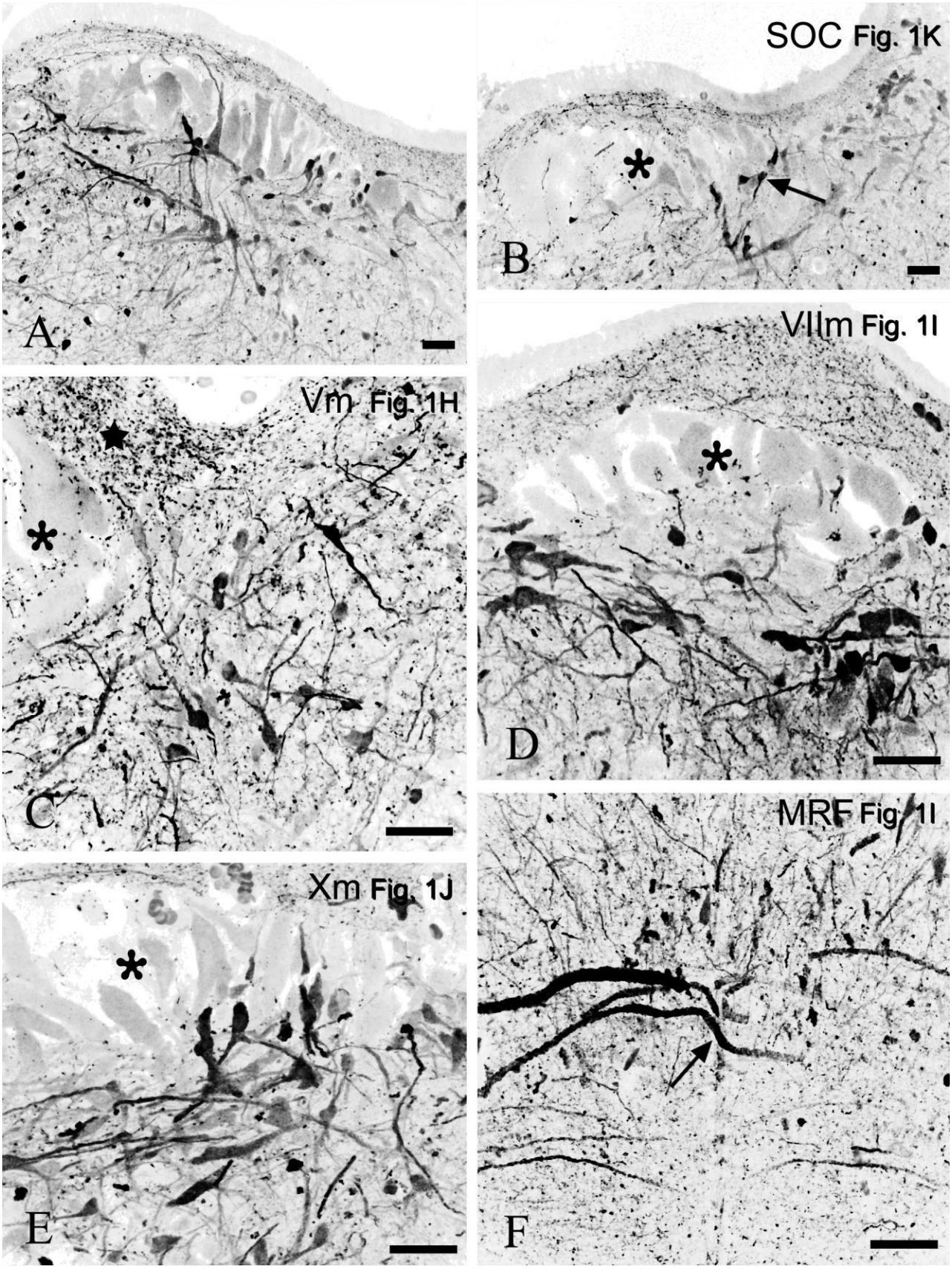

Fig. 8. Inverted gray-scale confocal photomicrographs of transverse sections through basal regions of the rhombencephalon showing glycine-ir cells and fibers. **A:** Section showing ventrally migrated glycine-ir cells in the region of the abducens nucleus. **B:** Section showing glycine-ir neurons (arrows) lateral to the spinooccipital 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 at upper right, except in A, which corresponds to a level intermediate between schemes 1I and 1J. Scale bars = 50 μm.

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 in 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 gray") 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 localized mainly 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 labelling 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 antiglycine 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

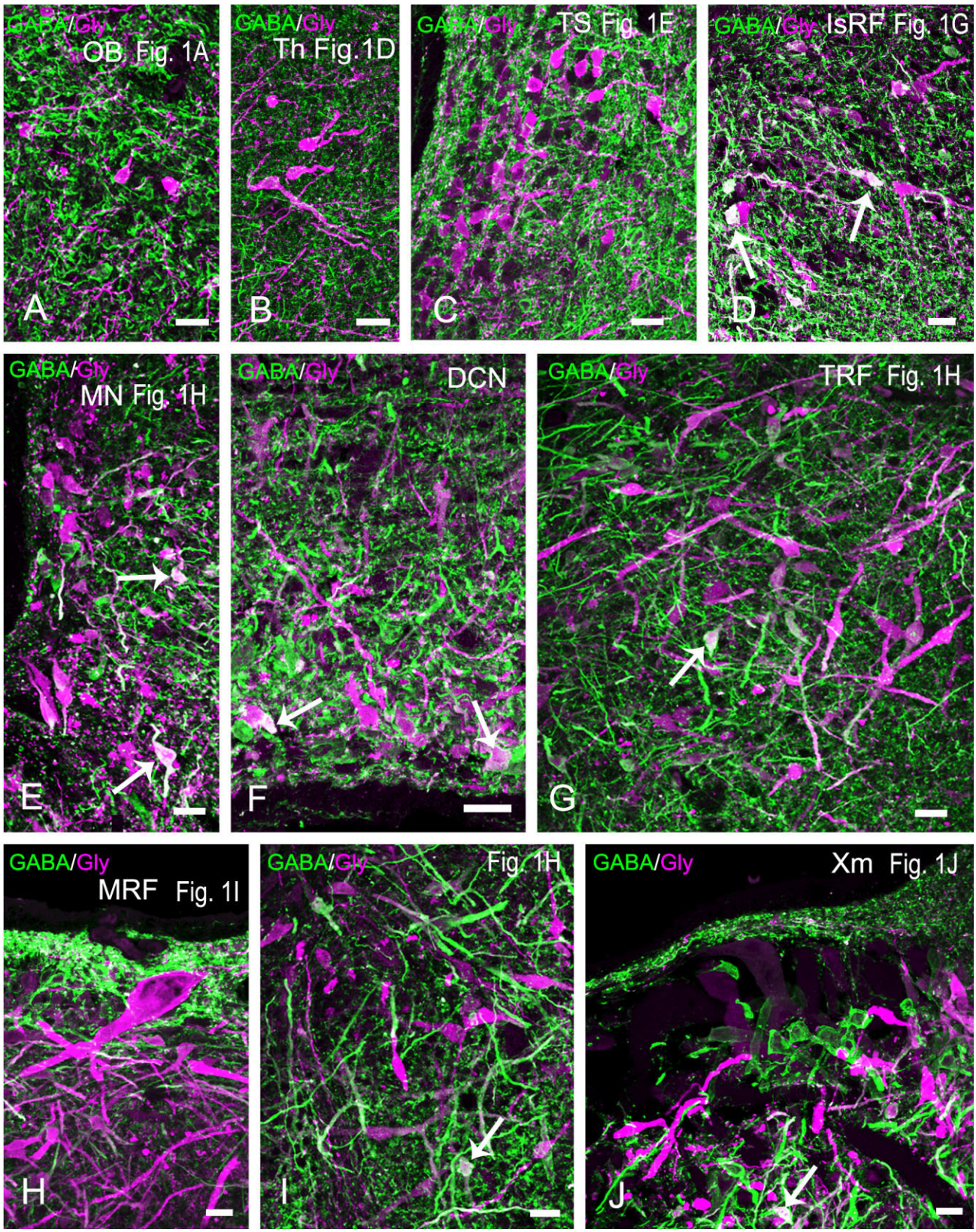

Fig. 9. Confocal photomicrographs of double-immunolabelled sections showing glycine (Gly)-ir (magenta) and GABA-ir (green) structures in several brain regions. Note that, in most regions, colocalization of GABA and glycine (white structures) is either absent (A–C,H) or present in a few cells (arrows 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 at upper right. Scale bars = 25 μm.

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-labelling 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 of 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 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 gray (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 rats 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 gray 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 gray and deep mesencephalic nuclei have not been formally described in the midbrain of lampreys, we can only 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 coexists 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 the case in unlike the 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 might 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, 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 and Dicke, 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 hyperpolarization 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 to 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), provides 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, vigor 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, 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 labelled neurons medial to this nucleus on the ipsilateral side, extending farther rostrally in the isthmic region. More of interest for the present discussion, continuous columns of labelled 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 labelled cells are notably similar to those 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 to be 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 the 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 from those releasing glycine alone is not known. In addition, glycine is a modulator coagonist of the N-methyl-D-aspartate (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 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 glyciner-

gic 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.

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