The Journal of Comparative Neurology 512:747–767 (2009)

Development of Glycine Immunoreactivity in the Brain of the Sea Lamprey: Comparison With γ-Aminobutyric Acid Immunoreactivity

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ABSTRACT

The development of glycine immunoreactivity in the brain of the sea lamprey was studied by use of immunofluorescence techniques at embryonic to larval stages. Glycine distribution was also compared with that of γ-aminobutyric acid (GABA) by use of double immunofluorescence. The first glycine-immunoreactive (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. J. Comp. Neurol. 512:747–767, 2009. © 2008 Wiley-Liss, Inc.

Indexing terms: ***Petromyzon marinus*; ontogeny; colocalization**

Glycinergic and γ-aminobutyric acid (GABA)-ergic neurons represent the main rapid inhibitory systems in the brain and spinal cord of vertebrates. They appear early in embryonic development, when they may be transiently involved in excitatory transmission, as suggested by the different ionic compositions of the intra- and extracellular milieu of embryonic neurons with respect to those in the adult brain (Spitzer, 2006). Despite the functional importance of glycinergic cells, few comprehensive studies of their distribution 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 nonmammalian 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 the case in the rat, also in the olfactory bulbs, the preoptic nucleus, and the thalamus. Some of these glycine-ir

Grant sponsor: Spanish Ministry of Science and Technology; Grant number: BFU2007-61056/BFI; Grant sponsor: Xunta de Galicia (to V.V.-C.).

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Received 20 June 2008; Revised 8 September 2008; Accepted 15 October 2008

DOI 10.1002/cne.21916

Published online in Wiley InterScience (www.interscience.wiley.com).

cells showed colocalization with 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).

With regard to 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 their 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.

MATERIALS 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

Abbreviations

DC	dorsal column
DCN	dorsal column nucleus
DIG	dorsal isthmic gray
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	spinooccipital 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

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 between 30 mm and 100 mm; N = 12) were collected from the River Ulla (Galicia, northwestern 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 anesthetized 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 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, sectioned on a cryostat in the transverse plane (embryos and prolarvae, 10 μm 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 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 GABA immunoreactivities, some series were treated as described above and stained with a cocktail of polyclonal rabbit antiglycine (Immunosolution, dilution 1:3,000; or Chemicon, dilution 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.

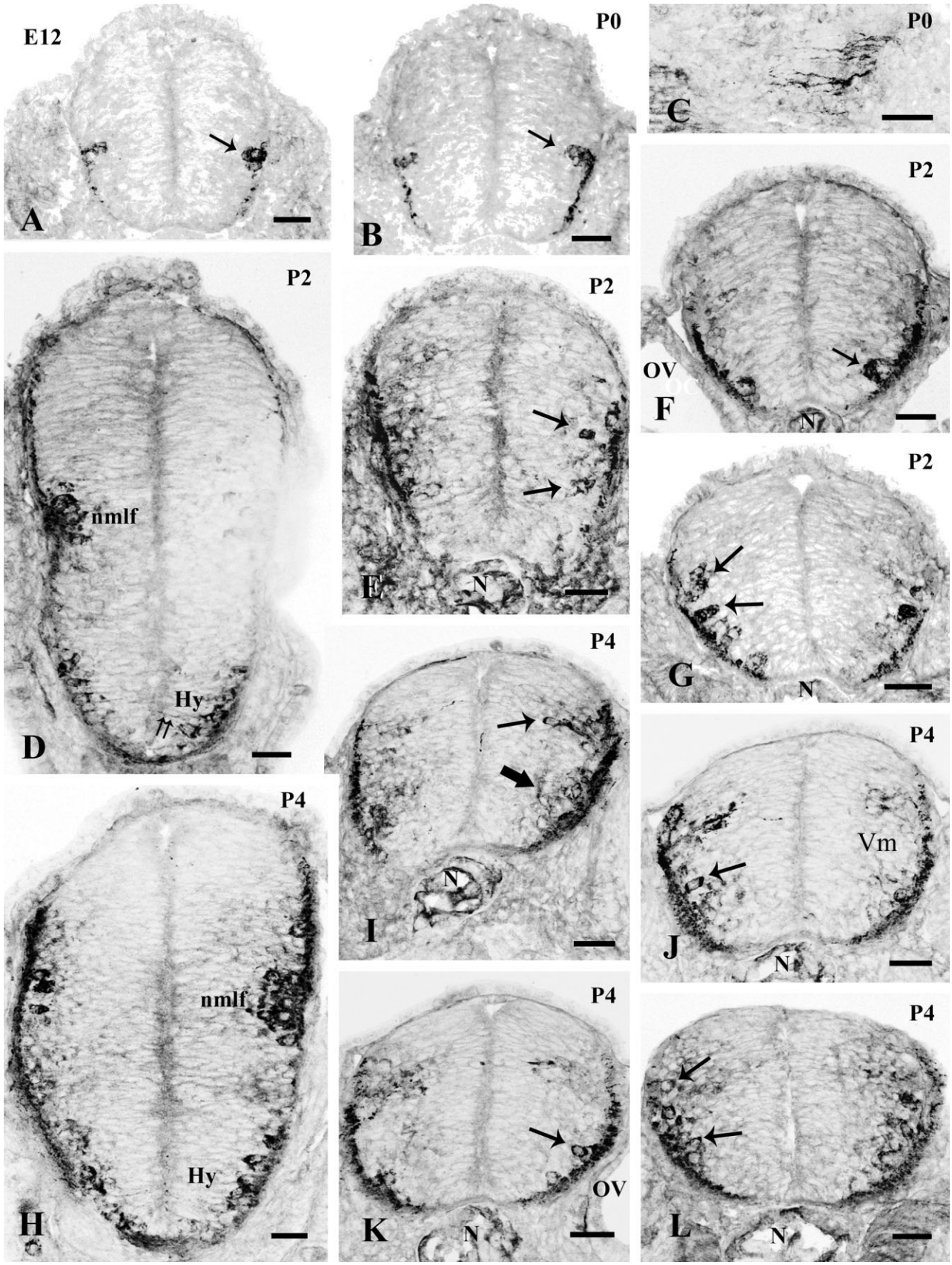
Antibodies

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 nonprotein 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, 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-bovine serum albumin (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 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, 2008b; Robertson et al., 2007). 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. 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, with animals under deep anesthesia 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 labelling 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


E12
A
P0
B
P0
C
P2
nmlf
Hy
D
P2
E
N
P2
OV
F
N
P2
G
N
P4
I
N
P4
nmlf
Hy
H
P4
Vm
J
N
P4
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K
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P4
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Figure 1

the anterior lateral line nerve (see González and Anadón, 1992). Two further larvae were injected with NB in the otic capsule, which resulted in labelling 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 labelling of the descending brain–spinal neuronal system. Larvae were allowed to survive for 2–3 days after injection, then fixed as described above. Neurobiotin was revealed with fluorescein isothiocyanate (FITC)-labelled avidin D (Vector; 1:1,000), and then single immunofluorescence was performed as described above with Cy3-coupled goat anti-rabbit polyclonal antibody (Chemicon; 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 gray scale, inverted, and then adjusted for brightness and contrast in Corel Photo-Paint (Corel, Ottawa, Ontario, Canada).

For 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 ± SD.

Terminology

Whenever possible, we used the same terminology for the different glycinergic populations of developing lamprey that 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 laboratory (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 in 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 (Fig. 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,

Figure 1.
Inverted gray-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 (arrow). **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.

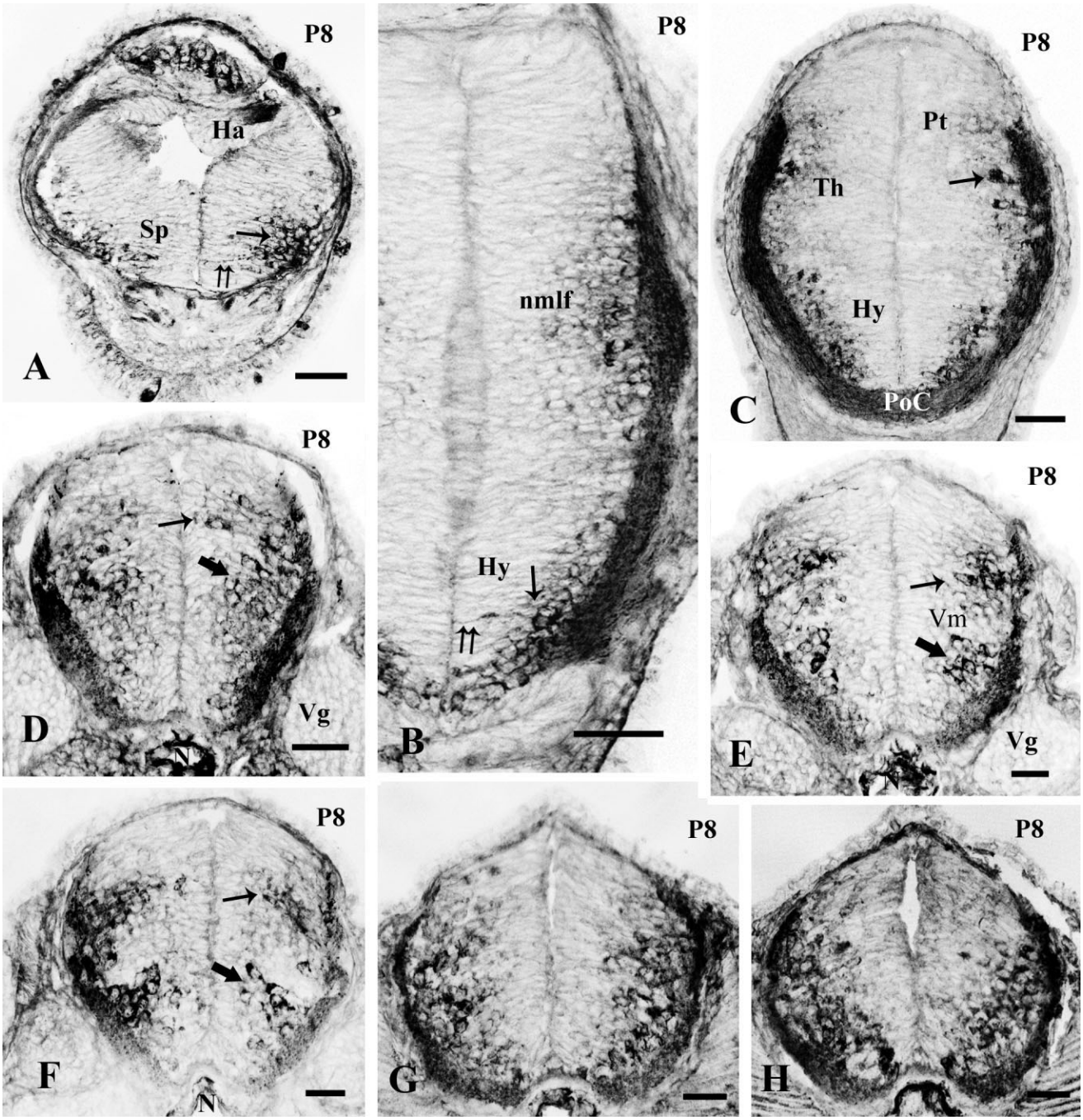

Figure 2.
Inverted gray-scale confocal photomicrographs of transverse sections through the brain of an early burrowing stage prolarvae (P8). **A:** Section through the telencephalon showing CSF-c glycine-ir cells in the septum (single arrow). Note the prolongation of the CSF-c cells to the ventricle (double arrow). **B:** Section through the diencephalon showing glycine-ir cells in the nmlf and the hypothalamus. Note hypothalamic glycine-ir cells (single 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–D; 25 μm in E–H.

may correspond to the primordium of the conspicuous dorsal isthmic gray population previously described for 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 2–5), the number of glycine-ir cells was very low at this stage, although strong glycine-ir fibers coursed in the midventral marginal layer (Fig. 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). With regard to the hypothalamic glycine-ir population, some of these cells exhibited a long dendrite that contacted the ventricle (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 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, although many only glycine-ir neurons were observed (Fig. 4A). In the preoptic nucleus, hypothalamus, and nmlf, most neurons that exhibited glycine immunoreactivity also showed GABA immunoreactivity (Fig. 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 the glycine-ir-only cells were predominant in this area (Fig. 4I).

Glycine-ir populations in the larval brain

The distribution of glycine was studied mainly in the brains of larvae of lengths of between 30 mm and 90 mm, and the following description corresponds to the distribution 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 were very scarce in, or absent from, glomerular regions.

Pallium. The larval lamprey pallium consists of three main regions, the lateral, medial, and dorsal pallium. Among 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, whereas the innervation of both the medial and the 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

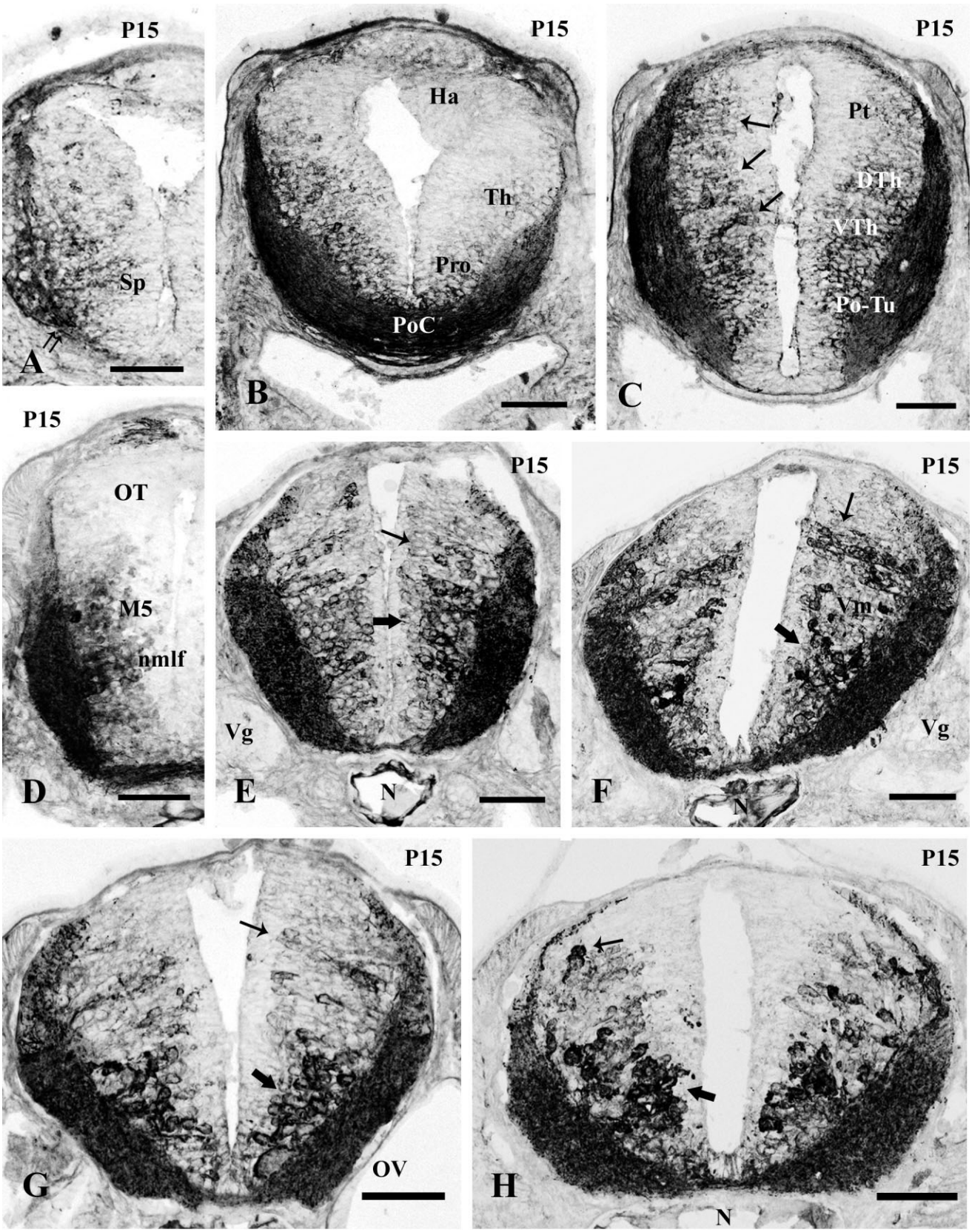
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Figure 3

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 about 120–150 mm in length), located just dorsal to the fasciculus retroflexus and ventral to the GABAergic pretectal population (see 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 in 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 (1964) and the other 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-labelling 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 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-labelling experiments after application of NB to the eye orbit (Fig. 7B). Both lateral and intermediate oculomotor subnuclei were innervated by scarce glycine-ir fibers, whereas 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 gray" 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-labelling 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-labelling 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-labelling experiments after application of NB to the eye orbit (Fig. 7D). In this nucleus, glycine-ir, oval perikarya (5.3 ± 0.9 μm in diameter) that sent thin dendrites to the fiber layer were found in both the periventricular layer and 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-labelling 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 labelled 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 for the dorsal column nucleus, although these glycine-ir cells did not receive ascending fibers from the dorsal column that became anterogradely labelled after application of NB to the spinal cord (Figs. 5J, 7E).

Figure 3.
Inverted gray-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 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.

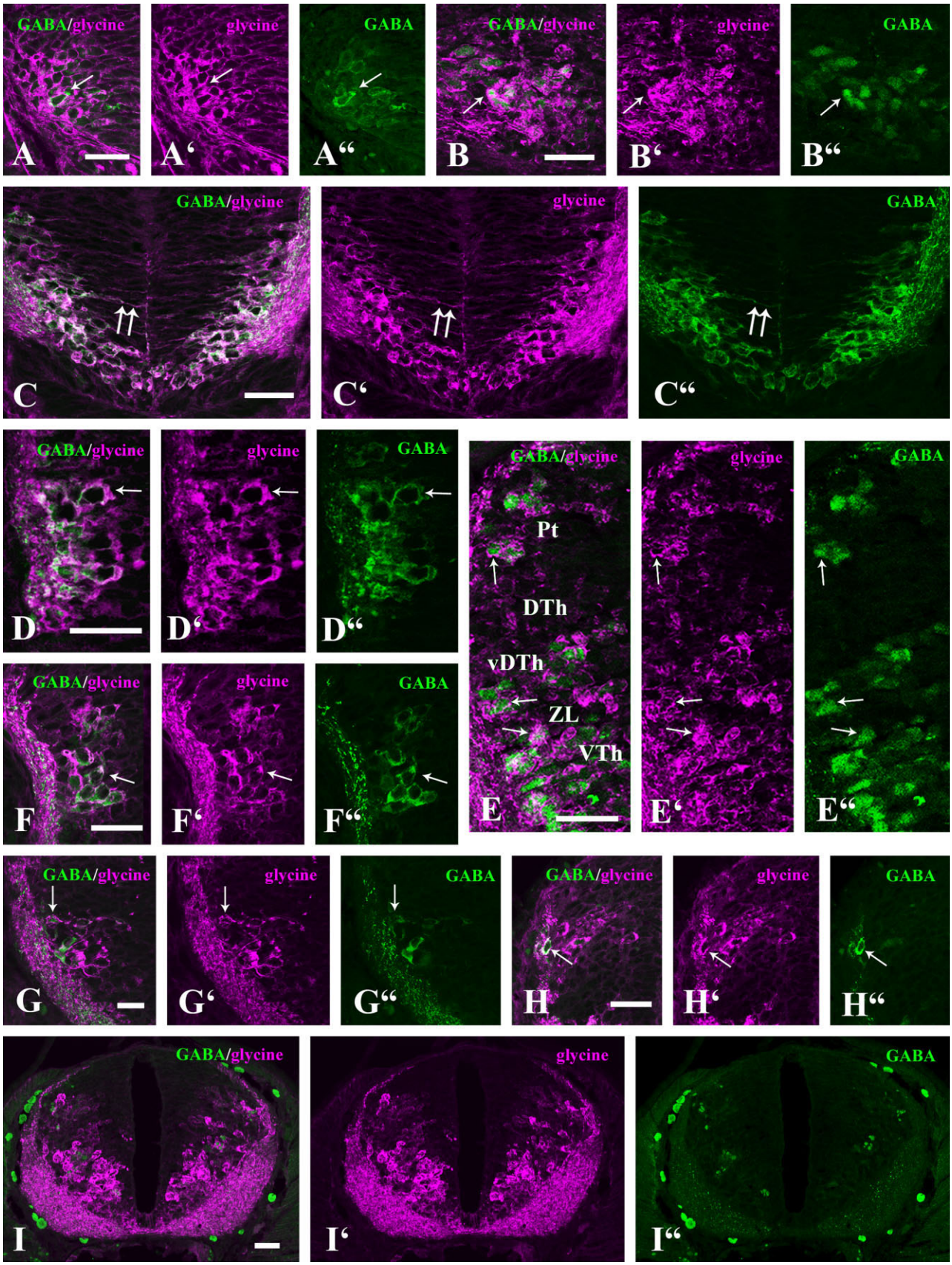
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Figure 4

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 toward the adjacent neuropil (Fig. 6J). They were located in the same region containing large and medium-sized reticulospinal neurons, as demonstrated by triple labelling, 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, intensely glycine-ir cells (5.8 ± 0.7 μm in diameter) was 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.

Figure 4.
Confocal photomicrographs of double-immunolabelled 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-labelled cells (arrow). **C–C′′:** Section through the hypothalamus of a P8 prolarvae where most cells exhibit double-labelled immunoreactivity. Note dendrites of the CSF-c cells (double arrow). **D–D′′:** Detail of the double-labelled 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, in which 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 bars = 25 μm.

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).

Spinooccipital motoneurons. This spinooccipital nucleus extends from just caudal to the vagal motor nucleus to the rostral spinal cord, and the neurons are located near the midline (Pombal et al., 2001). At this level, a compact group of oval, intensely 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, faintly to moderately 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 toward 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 glycine-ir cells were intermingled with facial motoneurons and gave rise to thin dendrites extending toward 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 labelled from the otic capsule region in a triple-labelling 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 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. Below we describe these populations with regard to colocalization with GABA.

Both glycine and GABA-ir cells were observed in the olfactory bulbs (Fig. 8A), thalamus (Fig. 8B), and oculomotor nu-

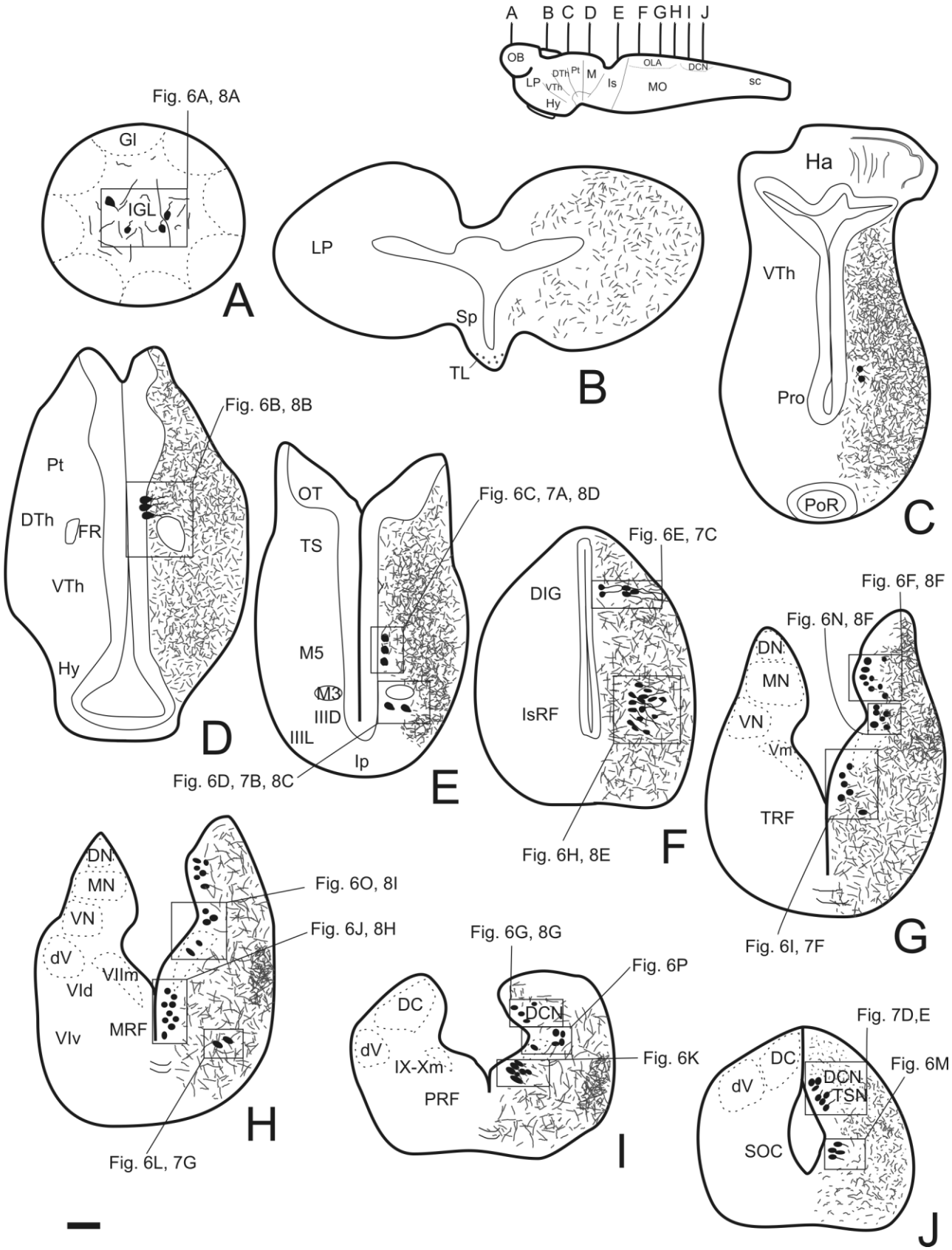

Figure 5.
Schematic drawing of transverse sections of the larvae sea lamprey brain showing the distribution of glycine-ir cells and fibers (right) and main brain structures (left). **A:** Olfactory bulb. **B:** Telencephalon. **C,D:** Diencephalon. **E:** Mesencephalon. **F:** Isthmus. **G–J:** Rhombencephalon.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

cleus 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 with regard to 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 labelled 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, whereas, 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 (Fig. 8I) nuclei, 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 spinooccipital 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 the glycinergic system in a vertebrate brain.

Development of glycine-ir neurons in the sea lamprey

The complex life cycle of the sea lamprey comprises five different stages: embryos, prolarvae, larvae, and 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 8 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, appearing 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 found only in the M5 nucleus of Schober and the dorsomedial oculomotor region, whereas, in adults, two new glycine populations appeared in

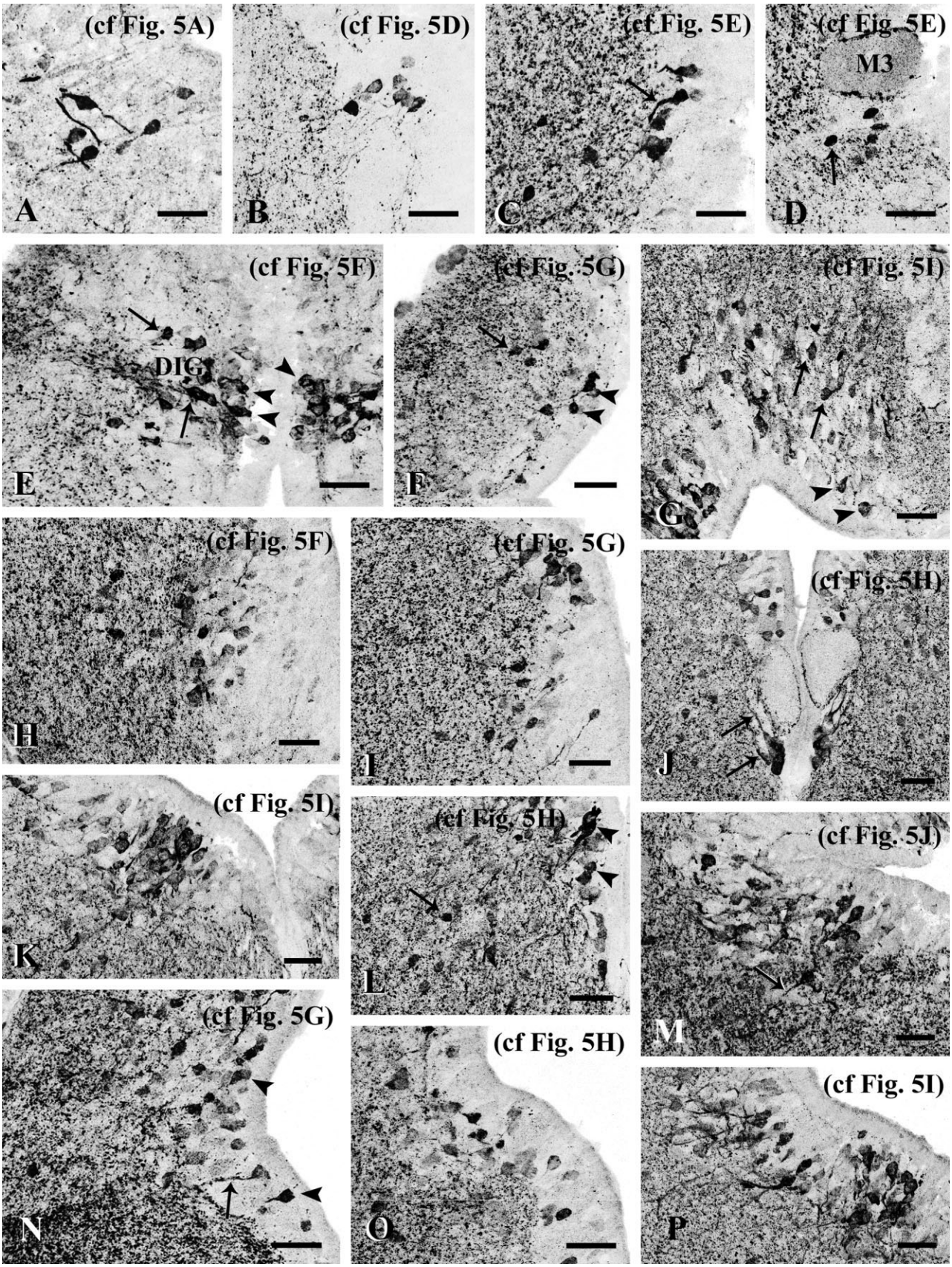
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Figure 6

the alar midbrain, one in the torus semicircularis and other in the optic tectum (Villar-Cerviño et al., 2008a; present results). Because 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), and 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 freely 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 been studied only 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 fetal life in the rat spinal cord, but 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), whereas, 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 very small numbers 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 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 projects to the rhombencephalic reticular formation, where the two 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

Figure 6.
Inverted gray-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 gray (arrows). **F:** Section through the octavolateral area showing glycine-ir cells both in periventricular regions (arrowheads) and in 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 in lateral (arrow) regions. **M:** Section through the spinooccipital 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 toward 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 boxed areas. For abbreviations see list. Scale bars = 25 μm.

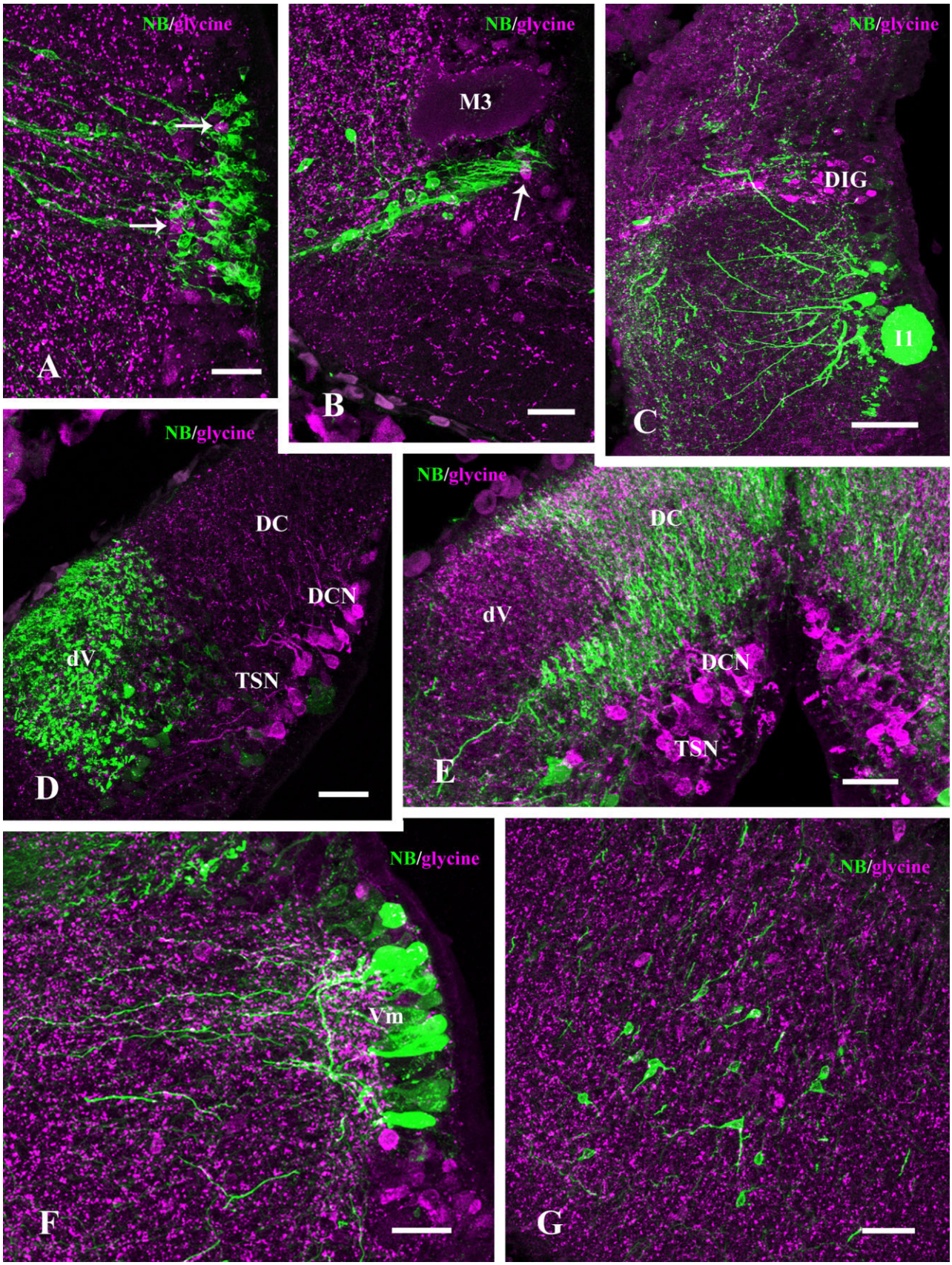
NB/glycine
A
NB/glycine
M3
B
NB/glycine
DIG
I1
C
NB/glycine
DC
DCN
dV
TSN
D
NB/glycine
DC
dV
DCN
TSN
E
NB/glycine
Vm
F
NB/glycine
G

Figure 7

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 present only 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 double-labelled 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-stage 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 gray 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 were 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-labelled neurons were observed from prolarvae to adulthood. 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 that at 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, whereas 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 located mainly 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.

Figure 7.
Confocal photomicrographs of double-immunolabelled 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 labelled after the application of NB to the eye orbit. **B:** Detail of the glycine-ir cells at the level of the oculomotor nucleus labelled 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 gray with respect to the large I1 Müller cell labelled after application of NB to the rostral spinal cord. **D:** Detail of the glycine-ir cell groups of the DCN and TSN labelled after application of NB to the eye orbit. **E:** Detail of the location of glycine-ir cell groups of the DCN and TSN labelled after application of NB to the rostral spinal cord. **F:** Section showing glycine-ir cells located ventral to the trigeminal motor nucleus labelled 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 labelled after the application of NB to the eye orbit. For abbreviations see list. Scale bars = 25 μm in A,B,D–G; 50 μm in C.

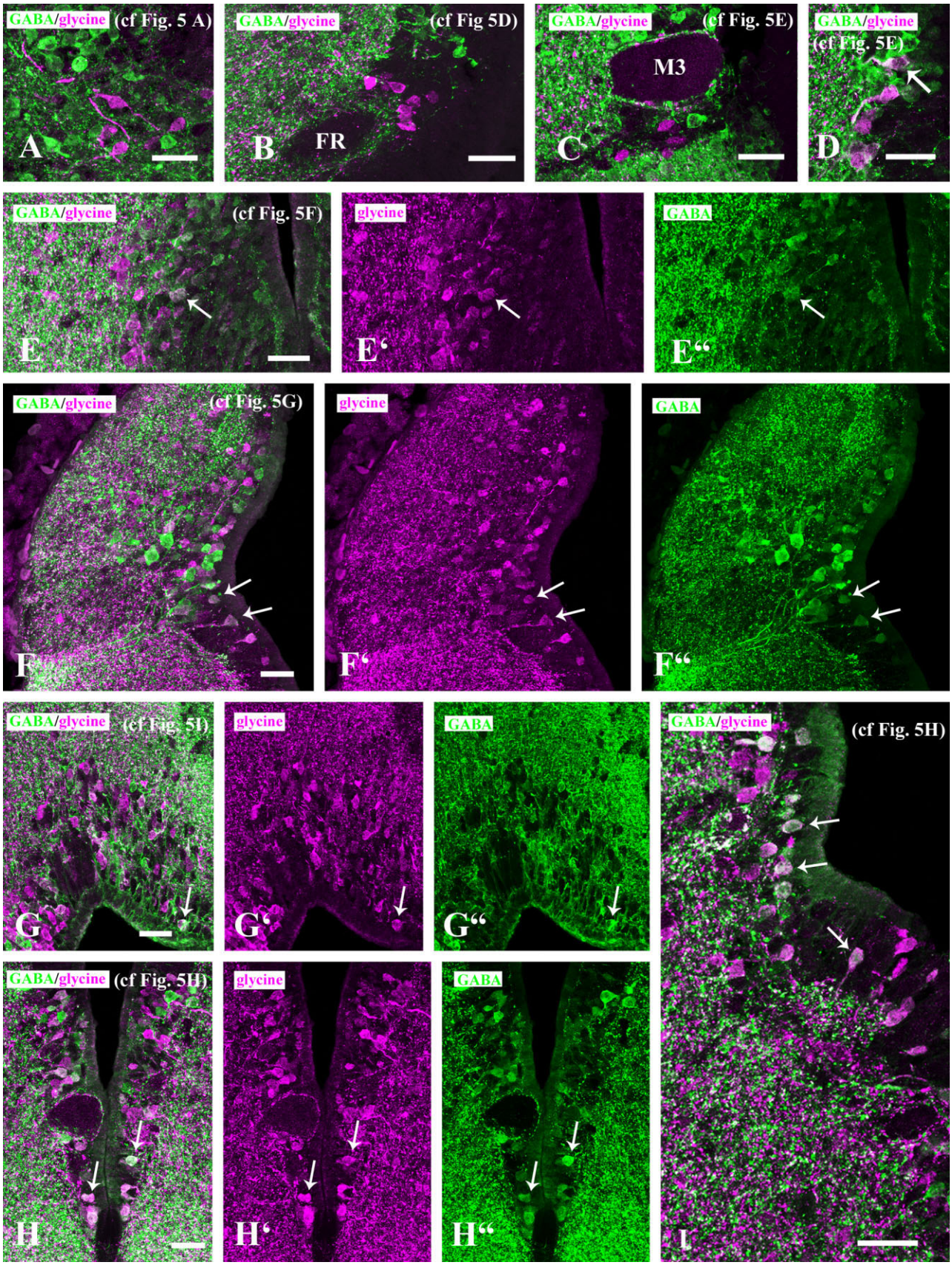
GABA/glycine (cf Fig. 5 A)
A
GABA/glycine (cf Fig 5D)
FR
B
GABA/glycine (cf Fig. 5E)
M3
C
GABA/glycine (cf Fig. 5E)
D
GABA/glycine (cf Fig. 5F)
E
glycine
E'
GABA
E''
GABA/glycine (cf Fig. 5G)
F
glycine
F'
GABA
F''
GABA/glycine (cf Fig. 5I)
G
glycine
G'
GABA
G''
GABA/glycine (cf Fig. 5H)
I
GABA/glycine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

Figure 8.

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 adulthood, 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 that of glycine in the brain of lampreys from late embryonic stages onward. 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 for 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 that at rostral levels.

ACKNOWLEDGMENTS

We thank the staff of the Biological Station of Ximonde (Galicia, Spain) for providing the lampreys used in this study. We also thank the Servicio de Microscopía Electrónica and Mercedes Rivas Cascallar (University of Santiago de Compostela) for confocal microscope facilities and assistance.

Figure 8.
Confocal photomicrographs of double-immunolabelled 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-labelled 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 doubly labelled (arrows). **G,G′′:** Section through the DCN showing some double-labelled glycine-ir cells (arrow). **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 boxed areas. For abbreviations see list. Scale bars = 25 μm.

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