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Glycine-Immunoreactive Neurons in the Developing Spinal Cord of the Sea Lamprey: Comparison With the γ-Aminobutyric Acidergic System

VERONA VILLAR-CERVIÑO,[1] GAY R. HOLSTEIN,[2] GIORGIO P. MARTINELLI,[2] RAMÓN ANADÓN,[1] AND MARÍA CELINA RODICIO[1]*

[1]Departamento de Biología Celular y Ecología, Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela 15782, Spain

[2]Departments of Neurology and Neuroscience, Mount Sinai School of Medicine, New York, New York 10029

ABSTRACT

The development and cellular distribution of the inhibitory neurotransmitter glycine in the spinal cord of the sea lamprey were studied by immunocytochemistry and double immunofluorescence and compared with the distribution of γ-aminobutyric acid (GABA). Results in lamprey embryos and prolarvae reveal that the appearance of glycine-immunoreactive (-ir) spinal neurons precedes that of GABA-ir neurons. Throughout development, glycine-ir cells in the lateral and dorsomedial gray matter of the spinal cord are more numerous than the GABA-ir cells. Only a subset of these neurons shows colocalization of GABA and glycine, suggesting that they are primarily disparate neuronal populations. In contrast, most cerebrospinal fluid (CSF)-contacting neurons of the central canal walls are strongly GABA-ir, and only a portion of them are faintly glycine-ir. Some edge cells (lamprey intraspinal mechanoreceptors) were glycine-ir in larvae and adults. The glycine-ir and GABA-ir neuronal populations observed in the adult spinal cord were similar to those found in larvae. Comparison of glycine-ir and GABA-ir fibers coursing longitudinally in the spinal cord of adult lamprey revealed large differences in diameter between these two types of fiber. Commissural glycine-ir fibers appear in prolarvae and become numerous at larval stages, whereas crossed GABA-ir are scarce. Taken together, results in this primitive vertebrate indicate that the spinal glycinergic cells do not arise by biochemical shift of preexisting GABAergic cells but instead suggest that glycine is present in the earliest circuitry of the developing lamprey spinal cord, where it might act transiently as an excitatory transmitter. J. Comp. Neurol. 508:112–130, 2008.

Indexing terms: immunocytochemistry; confocal laser scanning microscopy; colocalization; development

Glycine is a major inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates, including lampreys (Aprison and Werman, 1965; Aprison and Daly, 1978; Homma and Rovainen, 1978; Matthews and Wickelgren, 1979; Gold and Martin, 1983; Buchanan and Grillner, 1988; Alford and Williams, 1989; Alford et al., 1990a,b; O'Donovan et al., 1992; Dubuc et al., 1993; Uematsu et al., 1993; Berki et al., 1995). The inhibitory action of glycine results from an increase chloride conductance of the postsynaptic membrane upon ligand binding to glycine receptors (Young and Snyder, 1974; Barker and Ransom, 1978; Betz, 1987; Bormann et al., 1987). Although glycine is a major inhibitory neurotransmitter in the adult CNS, it is excitatory during embryonic development and the perinatal period (Reichling et al., 1994). This is attributable to a positive chloride equilibrium potential in the postsynaptic neurons, resulting in chloride efflux upon receptor activation and in depolarization. This

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*Correspondence to: María Celina Rodicio, Departamento de Biología Celular y Ecología, Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela 15782, Spain.
E-mail: bfcelina@usc.es

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glycine receptor excitatory activity is important for synaptogenesis, because the glycine-mediated increase in intracellular [Ca^{2+}] is crucial for the correct formation of postsynaptic glycinergic membrane specializations (Kirsch and Betz, 1998). The action of glycine is terminated through reuptake by two members of the family of Na^+/Cl^--dependent neurotransmitter transporters and by the glycine cleavage system, a mitochondrial and cytosolic enzyme complex (Garrow et al., 1993). The glycine transporter GLYT1 is considered a glial transporter, whereas the GLYT2 transporter is primarily associated with neurons. Recent studies have shown that GLYT1 is also expressed in neurons and that it is associated with a subpopulation of glutamatergic synapses (Cubelos et al., 2005). Although glycinergic synapses are localized mainly in the spinal cord and brainstem of mammals, it is now well established that the glycinergic system is more widely distributed throughout the CNS (Malosio et al., 1991; Rampon et al., 1996). On the other hand, developmental studies of glycinergic neurons in the spinal cord of vertebrates are scant (*Xenopus*: Dale et al., 1986; Roberts et al., 1988; zebrafish: Higashijima et al., 2004a,b; chick: O'Donovan et al., 1992; Berki et al., 1995; mouse: Allain et al., 2006).

Lampreys are living representatives of the most primitive group of vertebrates, the Agnathans (Nieuwenhuys and Nicholson, 1998). Lampreys have a complex life cycle that begins in a river with the embryonic period (about 12 days), followed by a short prolarval stage and a very long larval stage when the animals lives by burrowing in the riverbed and filter-feeding. Through a complex metamorphosis, larvae transform into young adult lampreys that feed parasitically on fish as they grow, breed, and then die (Hardisty and Potter, 1971). The complex life cycle and early appearance in vertebrate phylogeny make lampreys critical subjects for deciphering the early history of the nervous system of vertebrates and for studying adaptive changes to markedly different larval and adult life styles.

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

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

MATERIALS AND METHODS

Subjects

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

Tissue collection and processing

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

Immunohistochemistry for brightfield microscopy

For brightfield microscopy, sections were processed by the avidin-biotin (ABC) technique. Sections were treated

with 10% H_2O_2 to abolish endogenous peroxidase, preincubated with 10% normal goat serum and incubated with a rabbit polyclonal anti-glycine antiserum (Chemicon, Temecula, CA; 1:200). The tissue was then sequentially incubated with goat anti-rabbit biotinylated immunoglobulin (Sigma; 1:100) and rabbit ABC complex (Vector, Burlingame, CA). All dilutions were done in TBS containing 1% sodium metabisulfite. The immunocomplexes were developed by immersion in a fresh solution of 3,3′-diaminobenzidine (Sigma; 0.6 mg/ml) and 0.003% H_2O_2. Photomicrographs were obtained with a photomicroscope with an Olympus DP 70 color digital camera. Images were converted to gray scale and adjusted for brightness and contrast in Corel Photo-Paint (Corel, Ottawa, Ontario, Canada).

Immunofluorescence

For immunofluorescence, sections were pretreated with 0.2% $NaBH_4$ in deionized water for 45 minutes at room temperature to quench autofluorescence. Sections were subsequently incubated for 3 days at 4°C with a mixture of rabbit polyclonal anti-glycine antibody (IG1003; Immunosolution, Jesmond, Australia; dilution 1:3000; or Chemicon; dilution 1:100) and mouse monoclonal anti-GABA antibody (GABA93; Prof. G.P. Martinelli; 1:50) in TBS with 1% sodium metabisulfite. After rinsing in TBS with 1% sodium metabisulfite, samples were incubated for 1 hour with Cy3-conjugated goat anti-rabbit IgG (Chemicon; 1:200) and fluorescein-conjugated goat anti-mouse IgG (Chemicon; 1:50) and mounted with fluorescence antifade mounting medium (Vectashield; Vector).

All reagents were diluted in TBS (pH 7.4) containing 0.2% Triton X-100 and 3% normal goat serum. For tissue processing controls, primary antisera were omitted from some tissue sections. No staining was observed in these controls. Moreover, preadsorption of GABA and glycine antibodies with BSA did not block immunostaining in lamprey. In addition, these antibodies were tested by Western blotting, with lamprey brain protein extracts (Villar-Cerviño et al., 2006; unpublished results). No protein band was stained in the blots with either glycine antisera or the GABA antibody, which strongly suggests that these antisera do not cross-react with native proteins of the lamprey CNS.

Antibody characterization

The specificity of primary antibodies has been well characterized by the suppliers. According to the supplier, the cross-reactivity of one of the glycine antisera (Immunosolution; code IG1003) was raised against a glycine-glutaraldehyde-porcine thyroglobin conjugate and tested in sections of retina and cerebellum from various mammals and other vertebrates as well as in dot blot immunoassays against a variety of amino acid-carrier protein conjugates, including the standard 20 amino acids found in proteins; the nonprotein amino acids D-serine, D-alanine, and D-aspartate; GABA; and the glycine-containing tripeptide glutathione, which did not yield significant reactivity. The other glycine antiserum (Chemicon; code AB139) was tested by ELISA or RIA assays, and the results indicated that it only weakly or very weakly cross-reacts with alanine (1/100)-, GABA (1/500)-, taurine (1/1,000)-, aspartate (1/20,000)-, or glutamate (1/20,000)-BSA conjugates, in comparison with the glycine-BSA conjugate. The mab93 monoclonal GABA antibody (provided by Prof. G.P. Martinelli) was raised against GABA-glutaraldehyde-BSA conjugates and tested by ELISA against BSA conjugates of GABA, 16 protein amino acids, histamine, serotonin, adrenalin, noradrenalin, and histamine; it showed high specificity for GABA conjugate and negligible levels of cross-reactivity with the other conjugates (Holstein et al., 2004).

Image acquisition

Sections were analyzed and photographed with a fluorescence microscope with an Olympus DP 70 color digital camera and/or with a spectral confocal microscope TCS-SP2 (Leica, Wetzlar, Germany). Confocal stacks were acquired and processed in LITE software (Leica). Photomicrographs were converted to gray scale, inverted, and adjusted for brightness and contrast in Adobe Photoshop (Adobe, San Jose, CA) or Corel Photo-Paint.

Quantitative analysis of cell proportions

For quantitative analysis of proportions of cell phenotypes, we counted only clearly stained glycine-ir and/or GABA-ir neurons unambiguously detected on projections of confocal stacks of alternate sections, and we did not take into account other neurons that were very weakly stained and possibly contained minimally detectable levels of glycine. For this goal, we used the "maximum projection" command of the LITE software filtered by adjusting the threshold to 80 with rescaling to 255 (eight-bit scale, 0–255 range). This threshold value was chosen because it filtered out all very faintly fluorescent cells that were judged as nonpositive above background by two different observers and left only the cells judged unequivocally as positive (Fig. 1). Only cells visible in these projections were taken into account. With this procedure, we did not take into account nonglycinergic neurons that were very weakly stained but possibly contained detectable levels of glycine (Fig. 1A,B). For each stage considered (prolarvae, larvae, and adults), three individuals and seven transverse sections of the rostral one-third of the spinal cord from each one (representing 70 μm of spinal cord per prolarva and 112 μm of spinal cord per larval or adult animal) were counted. The total number of cells analyzed in each stage was 314 cells (prolarvae), 691 cells (larvae), and 960 cells (adults).

Because our purpose was to estimate possible shifting during development in relative proportion of glycine-ir vs. GABA-ir cells in the lamprey spinal cord, and, because larger cells have a higher probability of being encountered than smaller ones in sections, a correction factor (Abercrombie's factor; T/T + H, where T is the section thickness and H the mean height of the cells; Abercrombie, 1946) was considered for calculations (Table 1). Owing to the high polarization of most lamprey spinal cells in the transverse plane of the spinal cord, H was directly estimated in sagittal or horizontal sections from 12 cells of each phenotype showing nuclei (prolarvae, larvae, and adults). For descriptive purposes, neurons with a short diameter of less than 10 μm were considered small, and cells with diameters of between 10 and 20 μm were considered as medium-sized. A roughly similar H was obtained for the GABA-ir and glycine-ir cell phenotypes in each cell population [lateral, dorsomedial, and cerebrospinal fluid-contacting (CSF-c)] within each stage, so this procedure together with the number of cells analyzed ensured enough precision in cell proportions for our goals. Cell

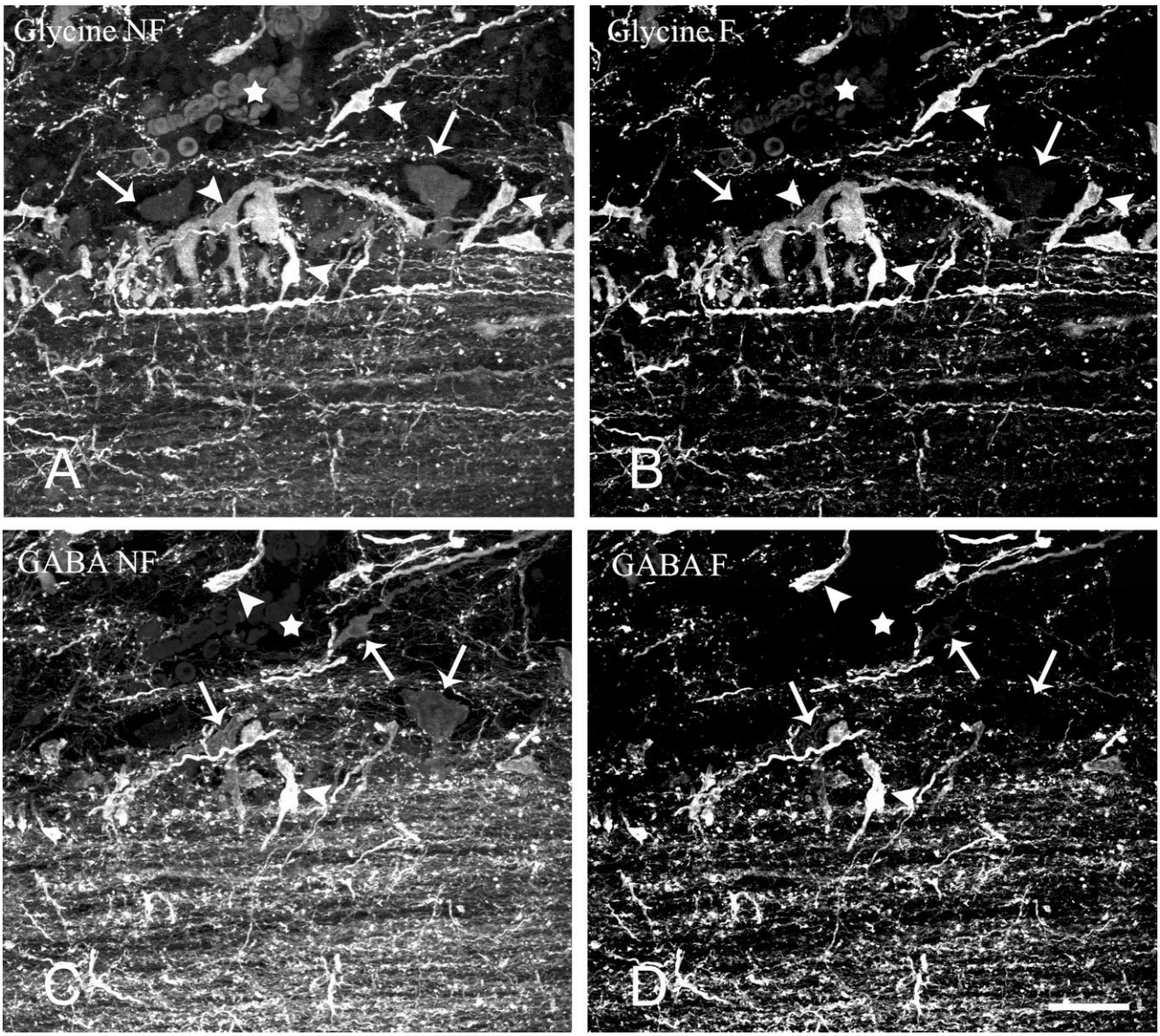

Fig. 1. Confocal projections of a section of the adult spinal cord showing glycine (**A,B**; red channel) and GABA (**C,D**; green channel) immunostaining, without (A,C) and after (B,D) threshold filtering (see Material and Methods). Arrows indicate faintly fluorescent cells considered as negative. Arrowheads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

measures were expressed as mean ± SD (Table 1). In addition, the corrected numbers of cells observed in prolarvae, larvae, and adults corresponded to both a section and a spinal cord segment (Table 2). Becausee the length of segments vary largely from embryos to adults, to calculate the segment length we used the linear function that correlates the length of spinal segments, obtained by Ruiz et al. (2004) in the sea lamprey (Galician breed). By using this function ($y = 0.0066x + 0.058$, expressed in mm), we determined the mean segment length of the prolarvae (0.11 mm), larvae (0.47 mm), and adults (4.67 mm) used for calculation.

Measurement of axons

For measurement of axonal diameters, confocal micrographs of a transverse section of the rostral spinal cord of an adult lamprey were used. The white matter was divided into four regions: dorsomedial (DM), ventromedial (VM), dorsolateral (DL), and ventrolateral (VL). DM and VM correspond to the dorsal and ventral columns of Van Dongen et al. (1985), respectively, whereas the lateral column of these authors was subdivided into dorsal and ventral parts. In total, 451 cross-sectioned glycine-ir axons of more than 1 μm in diameter were measured with LITE software (DM, 29 axons; DL, 127 axons; VL, 169 axons;

TABLE 1. Minor Diameters (μm) of Glycine-ir and GABA-ir Neurons in the Major Populations of the Spinal Cord and Abercrombie's Factor (CF) Used for Correction of Cell Numbers[1]

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

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

TABLE 2. Proportions (%) of Glycine-ir Only, GABA-ir Only, and Doubly Immunolabeled Neurons in the Major Populations of the Spinal Cord in Prolarval, Larval, and Adult Lamprey

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

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

VM, 126 axons). These values are representative of the number of axons of more than 1 μm in diameter coursing longitudinally in these quadrants. For comparison, the mean diameter of GABA-ir axons coursing longitudinally was obtained from measures of 40 axons of diameters higher than 1 μm.

RESULTS

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

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

The appearance of GABA-ir followed a developmental pattern rather different from that observed with glycine. The only GABA-ir neurons appearing in embryos were occasional CSF-c neurons (Kolmer-Agduhr cells), and these cells were the only GABA-ir spinal cells detectable until P3 prolarvae. GABA-ir in perikarya of intermediate and dorsal bands appeared gradually after this stage (Meléndez-Ferro et al., 2003; present results). Colocalization studies in the P10–P15 prolarvae indicated that almost all glycine-ir cells were GABA negative (98.2% in the ventral column and 94.3% in the dorsal plus intermediate columns; see Table 2).

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

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

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

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

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

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

Colocalization with GABA was observed in some glycine-ir spinal populations (Figs. 3A–C, 5A–C), although the proportion of double-labeled cells varied greatly among populations. It was scarcer in the gray matter regions but high in the CSF-c population. In the dorsomedial population, only about 11% of total immunoreactive neurons showed colocalization of glycine and GABA. The lowest proportion (2%) of double-stained cells with respect to all immunoreactive somata was observed in the lateral population, whereas the highest proportion (23%) was observed in the CSF-c cells (Table 2). Most of these CSF-c cells exhibited strong GABA-ir (94.4%). In addition, a few of the glycine-ir edge cells showed also GABA-ir.

In the larval spinal cord, the most abundant glycine-ir fibers coursed in the dorsolateral, ventrolateral, and ventral white matter columns, whereas the dorsal column contained only scarce glycine-ir fibers (Fig. 3A,B). Numerous glycine-ir fibers coursing in the ventral column crossed the midline ventral to the central canal (Figs. 4I, 6D). Likewise, GABA-ir fibers in the spinal cord were fairly numerous in the white matter, with the notable exception of the dorsal column. The highest GABA-ir fiber density was observed adjacent to the dorsal column, itself showing very few GABA-labeled processes (Fig. 3A,C). GABA-ir fibers crossing in the ventral commissural region are very scarce.

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

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

The intensely glycine-ir dorsomedial cells showed small to medium-sized pear- or spindle-shaped perikarya (Fig. 7A,A′,D,D′) and long, slender, poorly branched dendrites that coursed laterally or dorsally in the dorsal column or crossed the midline just dorsal to the central canal. In horizontal or parasagittal sections, tripolar or more complex morphologies were also observed (Fig. 7B,C). These perikarya (16.4 ± 3.2 μm in diameter in adults) were located most commonly in the border between the gray and the white matters, and rarely within the dorsal col-

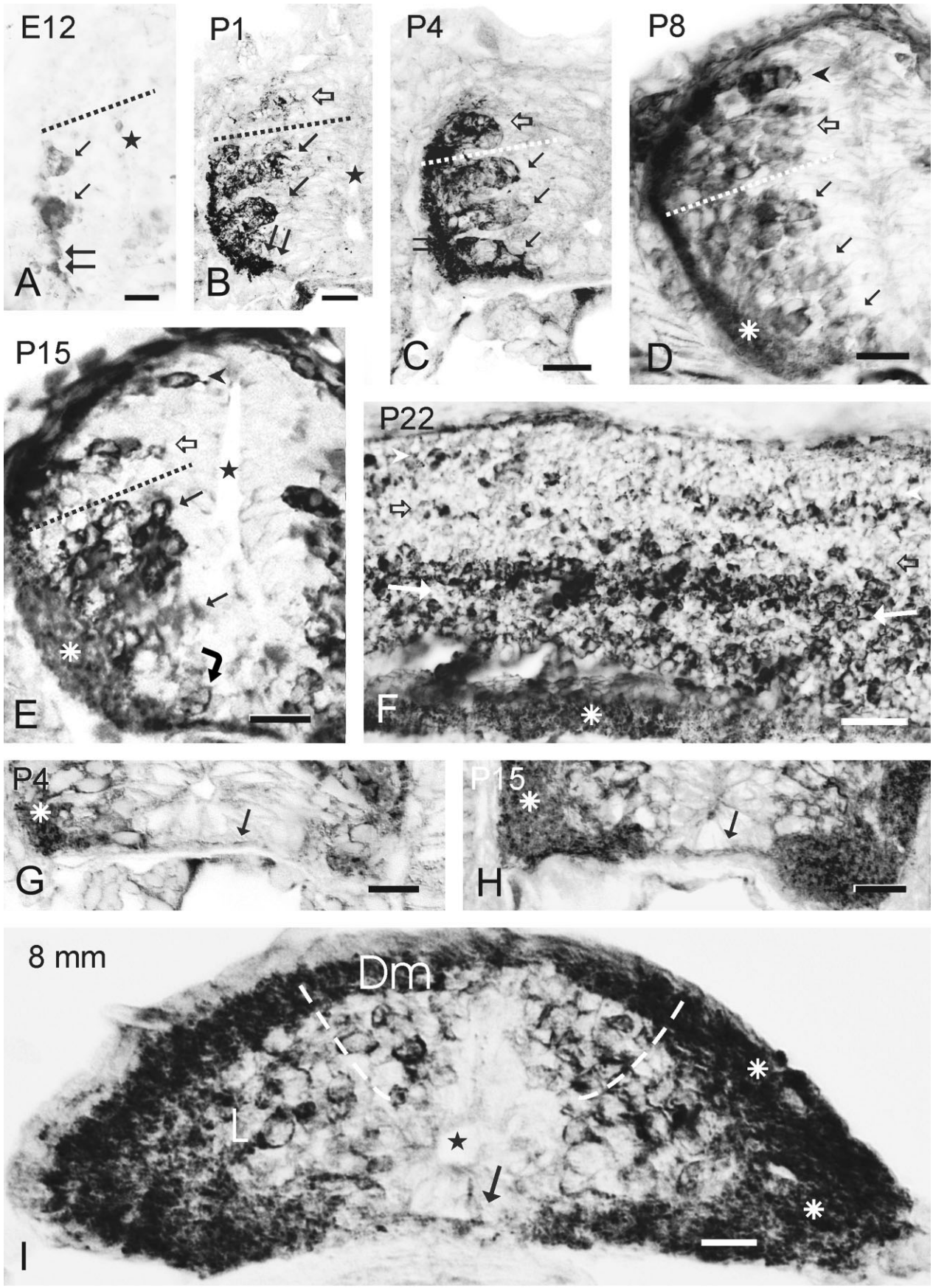

Figure 2

umn. Some spindle-shaped and tripolar neurons showed long processes coursing in a longitudinal or oblique direction (Fig. 7C). The same region contained a population of GABA-ir neurons with similar morphologies (Fig. 7A,A′′,C), although, as a mean, GABA-ir perikarya were smaller (12.8 ± 2.8 μm) than those of the glycine-ir neurons. Double immunofluorescence in upstream-migrating adults revealed colocalization of GABA and glycine in 23.7% of these dorsomedial neurons, whereas 34.8% of all immunoreactive cells were GABA-ir/glycine-negative and 41.4% were glycine-ir/GABA-negative (see Table 2).

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

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

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

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

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

In the rostral spinal cord of upstream-migrating adult lamprey, glycine-ir fibers coursing in the four white matter regions were thicker than the GABA-ir fibers, and most traveled longitudinally in the white matter (Fig. 8B–F). The most abundant glycine-ir longitudinal fibers were found in the dorsolateral, ventrolateral, and ventral columns, whereas the dorsal column and the adjacent region of the spinal trigeminal tract neuropil (located just laterally to the dorsal column) had far fewer glycine-ir longitudinal fibers (Fig. 8B–F). Similarly, glycine-ir fibers coursing through the gray matter were rather scarce in comparison with the number of GABA-ir fibers traversing the same region. In the white matter columns, glycine-ir longitudinal fibers were rather thick (dorsolateral: 6.5 ± 2.9 μm, n = 127; ventrolateral: 7.1 ± 3.6 μm, n = 169; ventromedial: 5.4 ± 2.3 μm, n = 126) and showed a continuous spectrum of diameters (Fig. 9). A few fibers exceeding 20 μm in diameter were observed in the ventral and ventrolateral columns (Figs. 8D,F, 9). In the dorsomedial column, glycine-ir fibers were scarce (Fig. 8B) and rather thin (mean diameter, 3.4 ± 1.1 μm, n = 29; maximum diameter, 5.8 μm). GABA-ir fibers were rather abundant in white matter regions (Fig. 8B), especially in the dorsal region adjacent to the dorsal column (spinal tract of the trigeminal nerve), whereas they were rather scarce in the dorsal column caudal to the dorsal column nucleus. By comparison, GABA-ir fibers were rather thin and generally did not exceed 2–3 μm in diameter, most being about 1 μm thick. Only two GABA-ir fibers of the ventromedial column were comparatively rather thick, about 10 μm in diameter (Fig. 8D). Double immunofluorescence showed that most of these coarse longitudinal axons were either glycine-ir or GABA-ir (Fig. 8B,C), and colocalization of these transmitters was not observed in those of the ventromedial column (Fig. 8D). In the gray matter, GABA- and glycine-ir was observed in some thin fibers and boutons, but GABA-ir boutons were far more numerous (Fig. 8E). Colocalization of these transmitters was observed in some thin fibers and boutons in the gray matter, but no attempt to quantify the proportion of boutons/thin fibers showing colocalization of glycine and GABA was made.

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

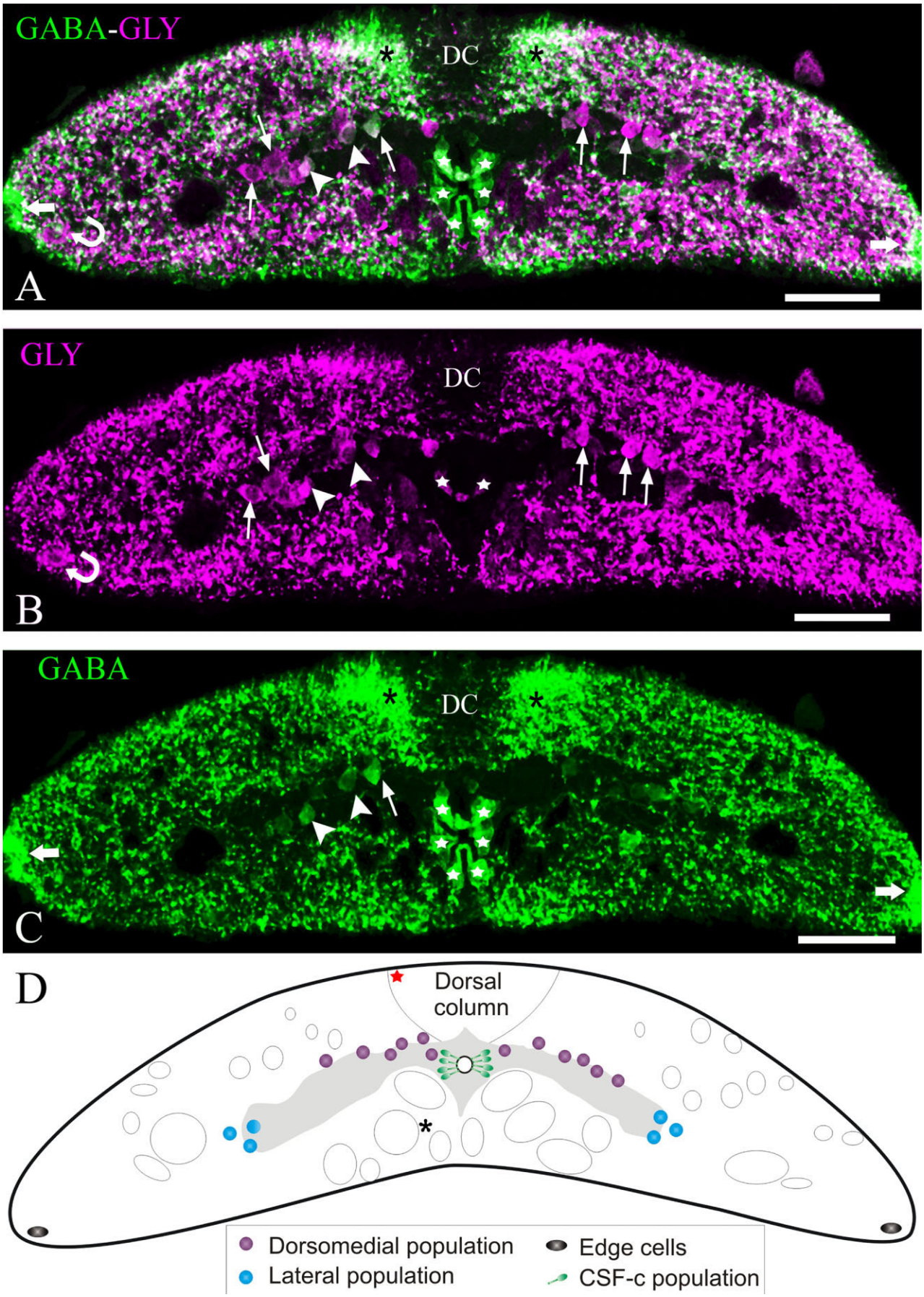

Fig. 3. **A–C:** Confocal photomicrographs of a transverse section of the spinal cord of a larva (50 mm in length) showing glycine and GABA immunofluorescence. A: Overlay; B: glycine (magenta); C: GABA (green). Note the abundance of GABA-ir fibers close to the dorsal column (DC; black asterisks) and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

Moreover, GABA-ir boutons/thin fibers were abundant among the giant reticulospinal fibers. These giant fibers showed no or very faint glycine immunofluorescence (Fig. 8D).

DISCUSSION

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

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

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

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

The present results in lamprey reveal important differences from the distributions of GABA and glycine in the developing spinal cord of the chick and mouse (Berki et al., 1995; Allain et al., 2004, 2006). Studies in chick and mouse embryos indicate that GABA-ir cells mature earlier (4 days and 1 day, respectively) than glycine-ir cells (Berki et al., 1995; Allain et al., 2006), whereas in the lamprey spinal cord most glycine-ir cells are detectable several days earlier than GABA-ir cells, except those of the CSF-c cells, which exhibit little glycine-ir overall. Moreover, studies utilizing glutamate decarboxylase (GAD; the GABA-synthesizing enzyme) and GABA in developing chick and rodent spinal cord have revealed a progressive loss of GAD/GABA-ir in early developing GABAergic ventral cells (Berki et al., 1995; Phelps et al., 1999; Allain et al., 2004), leading to the hypothesis that some spinal neurons are only transiently GABAergic. Similarly, for the rabbit outer retina, it has been suggested that GABA is replaced by glycine during the ontogeny of some cells (Messersmith and Redburn, 1992, 1993). However, some studies in developing rat suggest that the observed decrease in perikaryal GAD/GABA-ir may be due to selective transport of GAD to axons in mature neurons rather than to the loss of the GABAergic phenotype (Tran et al., 2003). Our results also indicate that the proportion of double-labeled GABA-ir/ glycine-ir neurons in the dorsomedial and lateral spinal populations increases between larvae and adults and that the proportion of the dorsomedial and lateral spinal cells that exhibit glycine immunoreactivity only diminishes between larvae and adults. These results do not support the notion that the GABAergic phenotype is progressively replaced by the glycinergic phenotype during development, as suggested in chick and mammalian studies.

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

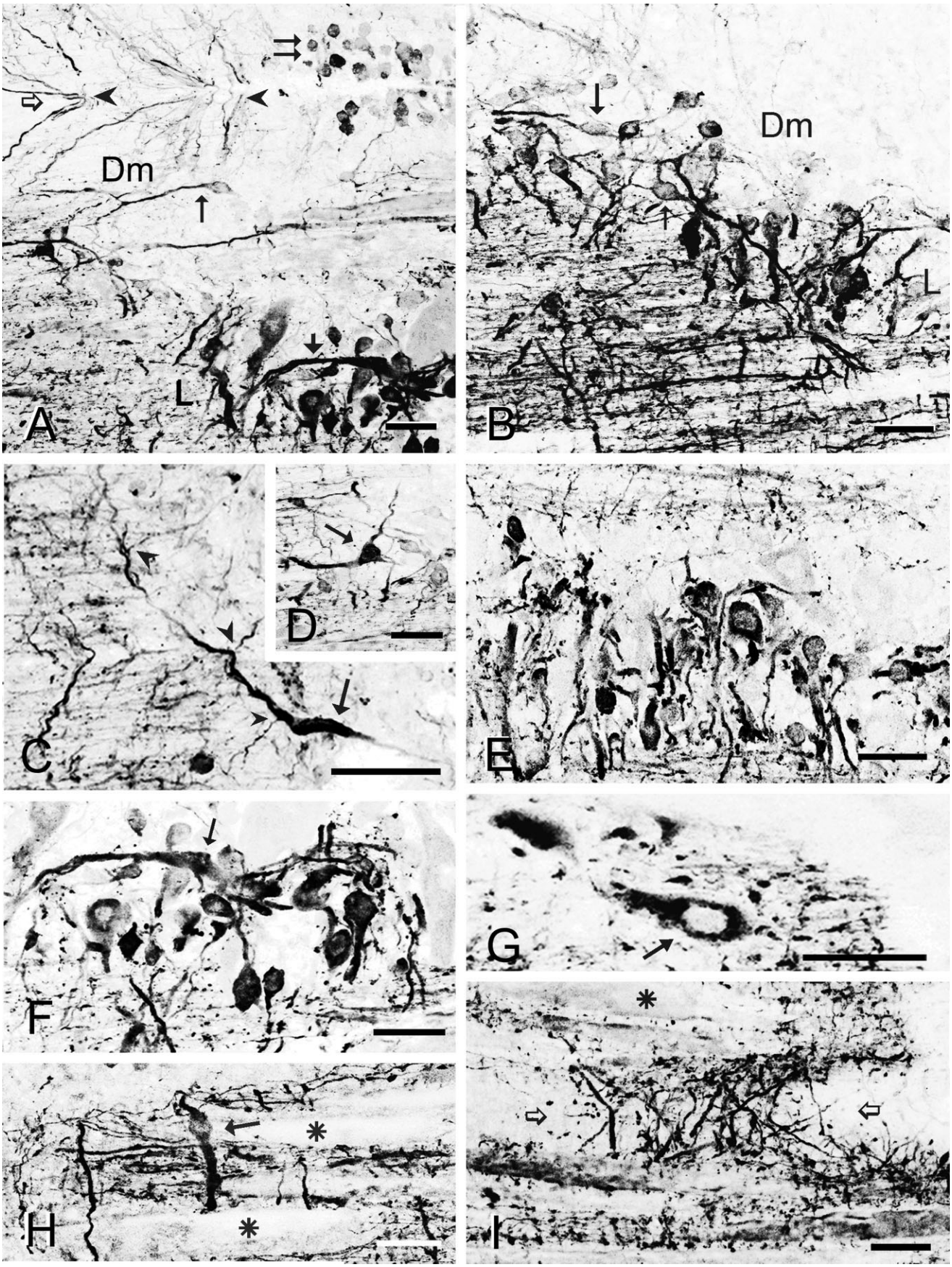

Figure 4

As regards the order of appearance of glycine-ir populations in spinal longitudinal zones of lamprey, first in the ventral column (putative basal plate) and then in the alar plate, it appears similar to that in the mouse, where basal plate populations are the first to appear (Allain et al., 2006). However, dorsal and ventral populations appear at the same day in chick (Berki et al., 1995). Moreover, in mouse, the dorsal horn population shows a notable increase in number, whereas the ventral horn cells are reduced in number at birth (Allain et al., 2006), which is unlike the case in lamprey. These differences are probably related to the much larger development of primary sensory circuits in mammals than in lampreys, including a profusion of spinal nerve fibers and terminals. Moreover, developing glycine-ir cells become scattered throughout the dorsal and ventral horns in chick and mouse (Berki et al., 1995; Allain et al., 2006), whereas in lampreys they are arranged roughly as a cell sheet on the dorsal side of the characteristic wing-like expansions of the gray matter (as seen in transverse sections), although the lateral glycine-ir neurons become interspersed among other types of neurons, as is observed in the mouse. This differential developmental pattern probably reflects the fact that many lamprey spinal cells extend long dendrites to the "white matter" (Vesselkin et al., 2000), whereas in birds and mammals the dendrites of spinal neurons are primarily restricted to the gray matter.

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

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

The approach employed in the present study allows us to distinguish the shapes of the cells and some part of the extent of their dendrites, in addition to transmitter phenotype. We found that most glycine-ir spinal cells were small neurons, and a portion of them may correspond to the small inhibitory ipsilateral neurons described by Buchanan and Grillner (1988). Some glycine-ir cells of the dorsomedial group send long dendritic processes contralaterally through the dorsal gray commissure and may also send processes to the dorsal funiculus, suggesting that they may receive bilateral inputs. Dendritic processes of neurons of the lateral group appear to ramify scarcely within the dorsolateral and ventral funiculi, and some course longitudinally. These results suggest that these cell populations are roughly comparable to dorsal and ventral horn cell groups of the mammalian spinal cord, respectively, which is supported by the developmental results (see above). Moreover, our results indicate that less than 24% of the immunoreactive cells in these gray matter populations exhibit colocalization of GABA and glycine, the majority of cells being either glycine-ir/GABA-negative or GABA-ir/glycine-negative. These results are not in good agreement with those reported for adult *Lampetra fluviatilis* and *Ichthyomyzon unicuspis* by Shupliakov et al. (1996), who reported that more than half of the glycine-ir cells (19 cells of 32; 59%) contained GABA immunoreactivity. Instead, in the adult sea lamprey, these values are 23.7% for glycine-ir cells of the dorsomedial population and a mere 18.8% for those of the lateral population. These differences probably are due to the small number of cells counted by these authors, although the existence of between-species differences cannot be ruled out. An ultrastructural immunocytochemical study of the spinal cord in river lamprey reported colocalization of GABA and glycine in about one-third of the synaptic boutons contacting motoneuron dendrites exhibiting glycine and/or GABA (Vesselkin et al., 2000). This value is also higher than that observed by us in cell perikarya, but percentages observed in perikarya and in synapses can be compared only if glycine-ir and GABA-ir cells bear the same number of terminals per cell. Moreover, the presence of descending reticulospinal axons could disturb any conclusion.

As regards the glycine-ir cells of the gray matter, they appear somewhat heterogeneous with respect to their size, morphology, and orientation of their processes. This heterogeneity is clearly observed in the spinal cord of larvae but is more evident in that of upstream-migrating adults. The presence of small inhibitory interneurons, probably glycinergic, has been characterized physiologically by Buchanan and Grillner (1988). They also injected identified cells with horseradish peroxidase, allowing them to reconstruct their morphology. The inhibitory in-

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

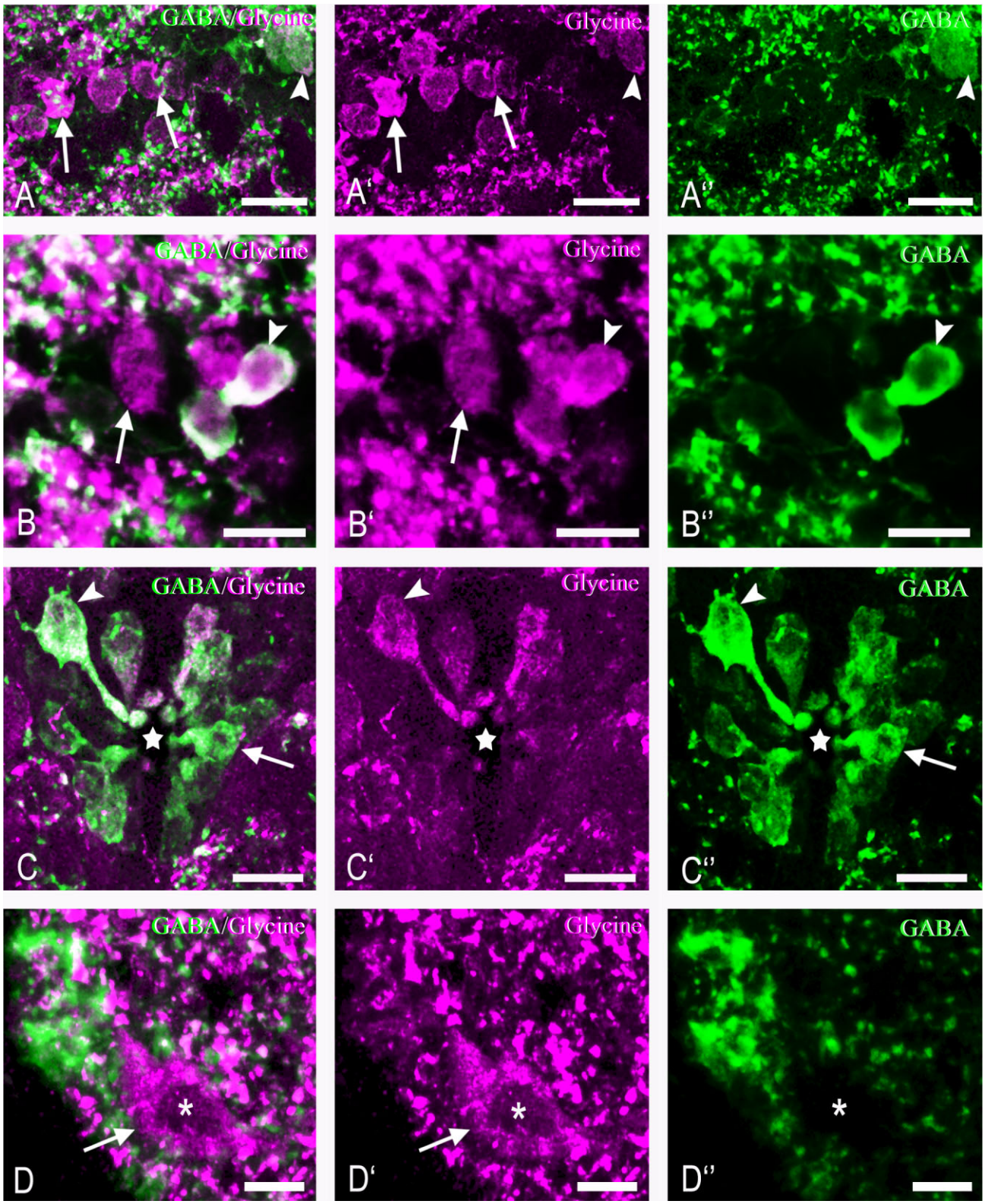

Fig. 5. Confocal photomicrographs of details of transverse sections of the larval spinal cord gray matter showing cells with glycine and/or GABA immunofluorescence. Note cells with single (arrows) and double immunolabeling (arrowheads). Left column, overlay (double channel); central column, glycine (magenta);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

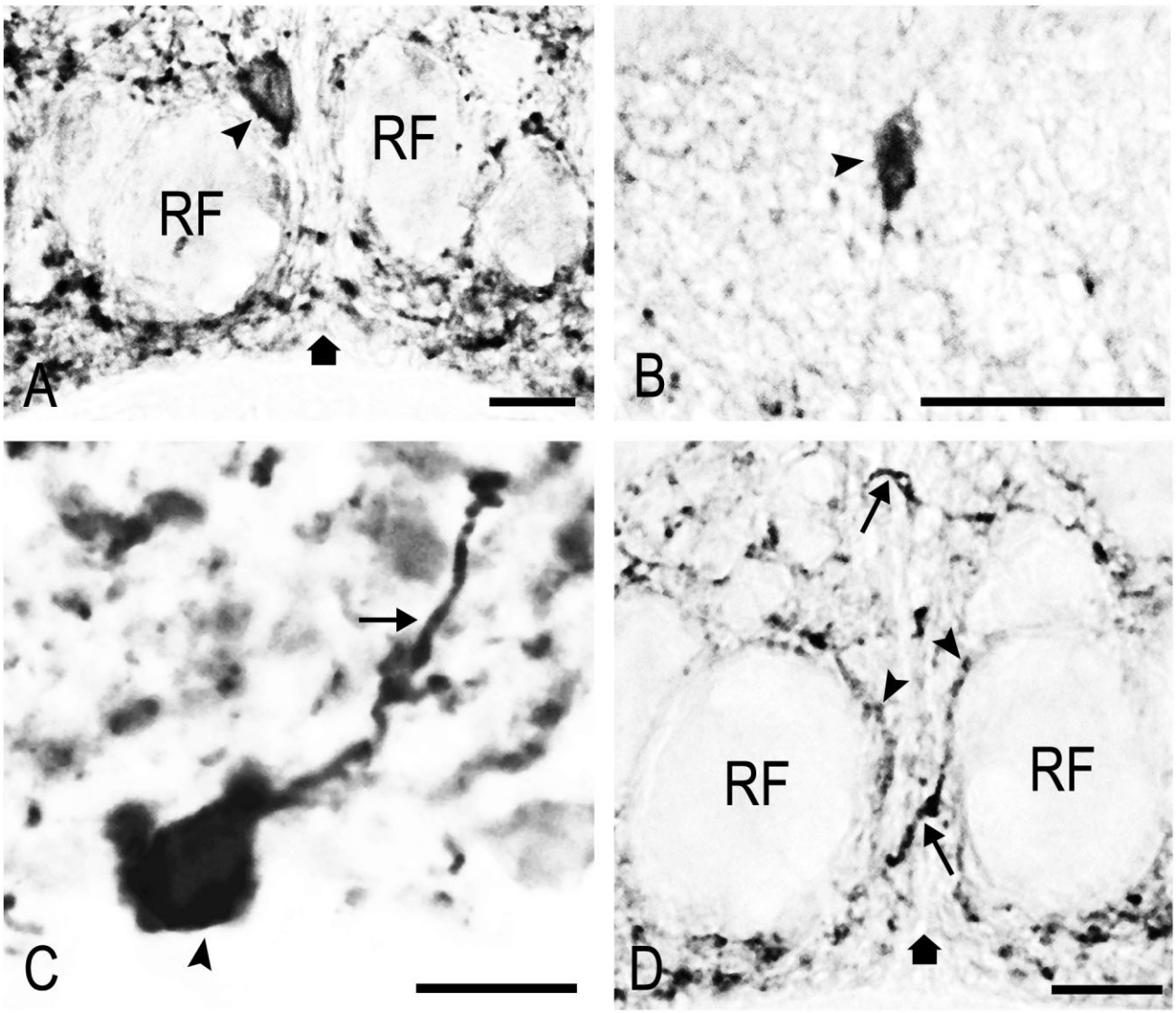

Fig. 6. Photomicrographs of transverse sections of the larval spinal cord showing glycine-ir cells (A–C) and fibers (D). **A:** Glycine-ir conic neuron (arrowhead) in the ventromedial white matter close to a giant reticulospinal axon (RF). Note the short ventral dendrite directed to the giant axon. **B:**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

terneurons revealed by these authors have a small body with relative few dendrites extending transversely toward the lateral margin and the midline, although their appearance (size, branching pattern) is not homogeneous. By their location, size, and appearance, these cells appear to correspond to some of the glycine-ir cell subtypes of the lateral population of the gray matter reported here.

Consistently, the circumventricular CSF-c cells exhibit strong GABA-ir from early stages of development (late larvae) through adulthood. Our GABA immunofluorescence results are in agreement with other studies of adult (Batueva et al., 1990; Christenson et al., 1991; Shupliakov et al., 1996) and developing (Meléndez-Ferro et al., 2003; Ruiz et al., 2004) lamprey. A subset of the CSF-c cells shows glycine-ir, and these generally occupy dorsal or dorsolateral regions around the central canal. The GABAergic CSF-c cells are considered to be the source of the rich GABA-ir plexus along the lateral margin of the spinal cord (Christenson et al., 1991), which shows very intense GABA-ir but faint glycine-ir in axonal processes (Shupliakov et al., 1996; present results). This suggests that GABA is the main inhibitory neurotransmitter released by axons in this neuropil, which is in agreement with the results of physiological studies on edge cells in lamprey (Christenson et al., 1991). Although glycine has

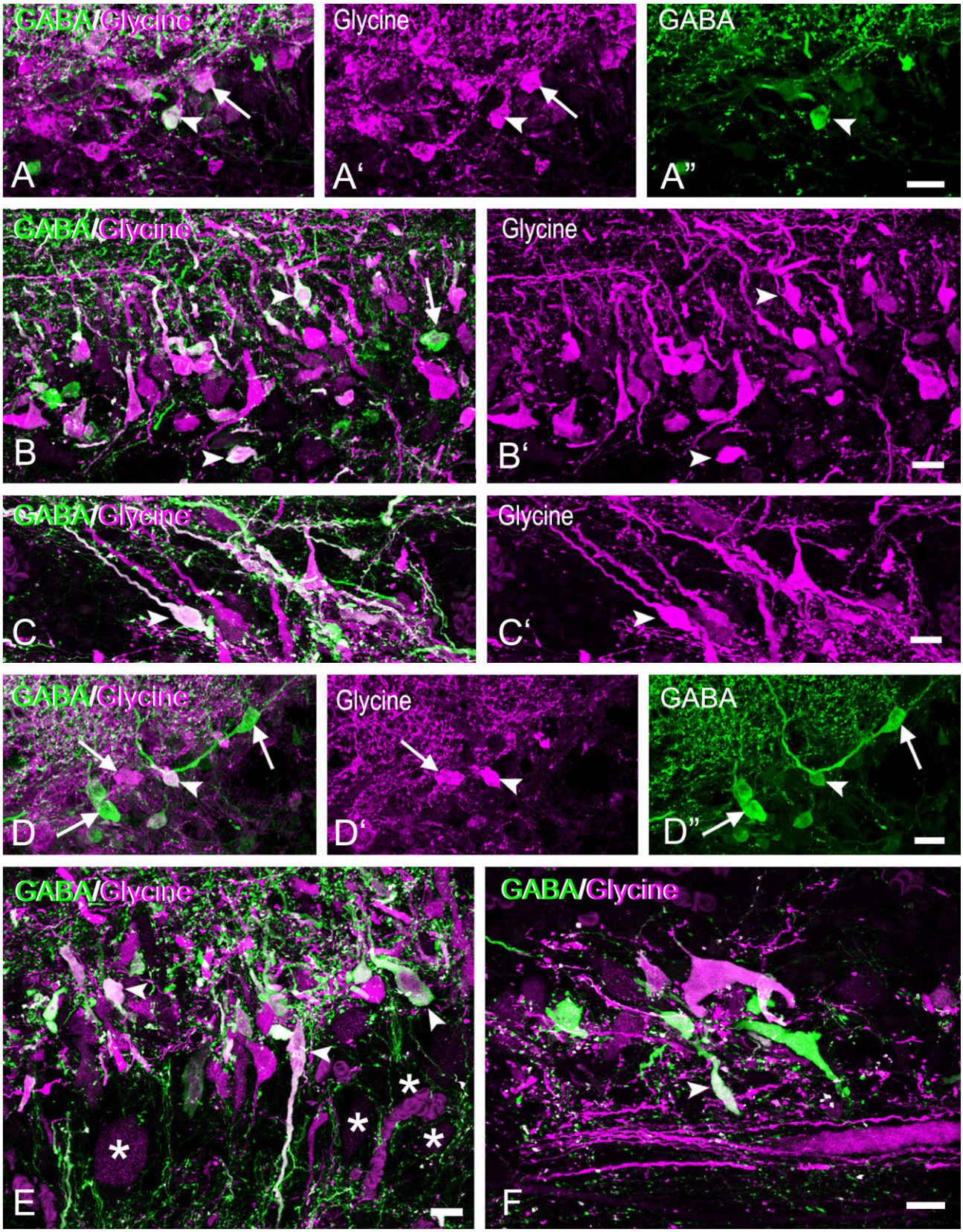

Fig. 7. Confocal photomicrographs of the spinal cord of postmetamorphic (A,D) and upstream-migrating adult lampreys (B,C,E,F) showing the distribution of glycine (magenta) and GABA (green) immunofluorescence. Cells showing double immunolabeling appear whitish to pinkish (A–F). **A:** Detail of dorsomedial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

been found to be a source of tonic inhibition of lamprey edge cells during fictive swimming (Alford et al., 1990b; Vinay et al., 1996), it is unclear whether glycine is released by axon terminals in this special neuropil or is released by synaptic boutons contacting other parts of edge cells such as the perikarya or primary dendrites.

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

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

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

Glycine immunocytochemistry also reveals the presence of a number of commissural fibers crossing below the central canal. However, only occasional commissural GABA-ir fibers have been observed in developing and adult lampreys (Meléndez-Ferro et al., 2003; present results). In embryonic and larval zebrafish, studies of expression of the glycine transporter GLYT2, GABAergic markers, and neuronal tracers indicate that early glycinergic cells are of various commissural and circumferential types, whereas GABAergic neurons are cells with longitudinal axons and Kolmer-Agduhr cells (Highasijima et al., 2004b). Commissural glycine-ir cells have also been reported in the spinal cord of amphibian embryos (Dale et al., 1986; Roberts et al., 1988). Unlike the case in lampreys and zebrafish, conspicuous commissural GABAergic fiber systems have been reported in the developing spinal cord of the rat (Phelps et al., 1999) and an elasmobranch (*Scyliorhinus canicula*; Sueiro et al., 2004), which suggests the existence of important neurochemical differences in the crossed inhibitory spinal circuits among vertebrates. Comparison of hemicord preparations of lamprey spinal cord with the intact cord have provided strong evidence that the reciprocal inhibition between opposite hemisegments ensures left-right alternation in the locomotor pattern and promotes multiple action potentials per cycle in network neurons (Cangiano and Grillner, 2005). Glycine-ir fibers represent the majority of the commissural fibers with inhibitory neurotransmitters in the lamprey spinal cord, providing further morphological evidence for these crossed interactions.

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

CONCLUSIONS

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

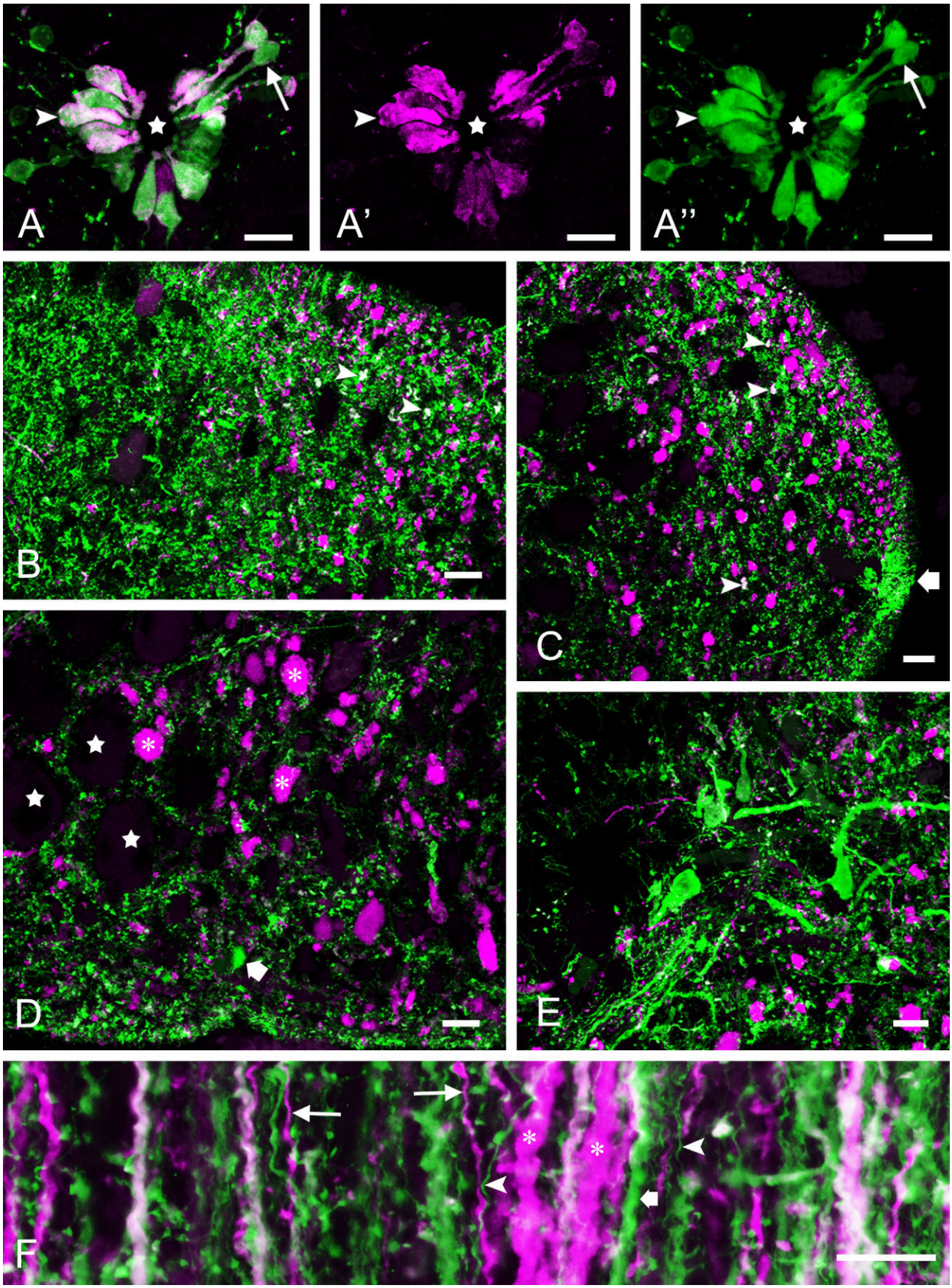

Figure 8

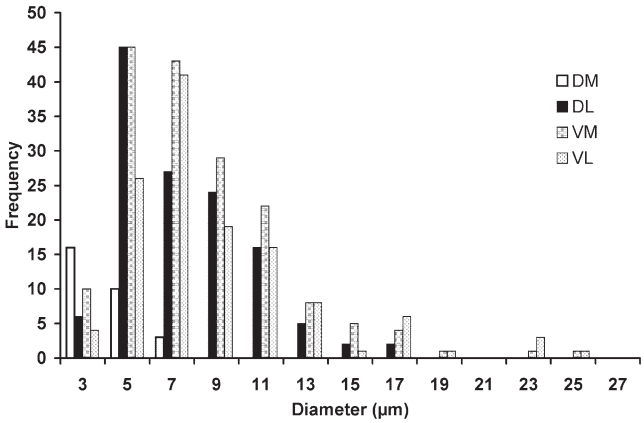

Fig. 9. Graphic representation of the diameter distribution of glycine-ir fibers coursing longitudinally in the dorsomedial (DM), dorsolateral (DL), ventromedial (VM), and ventrolateral (VL) white matter columns of the adult rostral spinal cord. Fibers less than 2 μm thick were not measured. Values in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

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

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

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