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Presence of Glutamate, Glycine, and γ-Aminobutyric Acid in the Retina of the Larval Sea Lamprey: Comparative Immunohistochemical Study of Classical Neurotransmitters in Larval and Postmetamorphic Retinas

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ABSTRACT

The neurochemistry of the retina of the larval and postmetamorphic sea lamprey was studied via immunocytochemistry using antibodies directed against the major candidate neurotransmitters [glutamate, glycine, γ-aminobutyric acid (GABA), aspartate, dopamine, serotonin] and the neurotransmitter-synthesizing enzyme tyrosine hydroxylase. Immunoreactivity to rod opsin and calretinin was also used to distinguish some retinal cells. Two retinal regions are present in larvae: the central retina, with opsin-immunoreactive photoreceptors, and the lateral retina, which lacks photoreceptors and is mainly neuroblastic. We observed calretinin-immunostained ganglion cells in both retinal regions; immunolabeled bipolar cells were detected in the central retina only. Glutamate immunoreactivity was present in photoreceptors, ganglion cells, and bipolar cells. Faint to moderate glycine immunostaining was observed in photoreceptors and some cells of the ganglion cell/inner plexiform layer. No GABA-immunolabeled perikarya were observed. GABA-immunoreactive centrifugal fibers were present in the central and lateral retina. These centrifugal fibers contacted glutamate-immunostained ganglion cells. No aspartate, serotonin, dopamine, or TH immunoreactivity was observed in larvae, whereas these molecules, as well as GABA, glycine, and glutamate, were detected in neurons of the retina of recently transformed lamprey. Immunoreactivity to GABA was observed in outer horizontal cells, some bipolar cells, and numerous amacrine cells, whereas immunoreactivity to glycine was found in amacrine cells and interplexiform cells. Dopamine and serotonin immunoreactivity was found in scattered amacrine cells. Amacrine and horizontal cells did not express classical neurotransmitters (with the possible exception of glycine) during larval life, so transmitter-expressing cells of the larval retina appear to participate only in the vertical processing pathway. J. Comp. Neurol. 499:810–827, 2006.

Indexing terms: glutamate; GABA; immunocytochemistry; confocal laser scanning microscopy; ganglion cell; development; retina; lamprey

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The development of the lamprey retina and eye is exceptional among vertebrates. After an initial embryonic period, when a small retina with ganglion cells and a single type of photoreceptor appears (Kleerekoper, 1972; Dickson and Collard, 1979; De Miguel and Anadón, 1987; Meléndez-Ferro et al., 2002a), retinal growth slows until the midlarval stage (starting in sea lamprey larvae of about 60 mm in body length), when the margins of the retina are transformed into a highly proliferating neuroepithelium that produces by progressive lateral expansion a large undifferentiated retina lacking photoreceptors. During the second half of the larval period, between 60 mm and transformation at 120 mm length in sea lamprey, the retina comprises a small early-differentiated central region and an extensive lateral undifferentiated zone. The layering and differentiation of the lateral retina and the appearance of the two types of adult photoreceptors occur during transformation (De Miguel and Anadón, 1987). Previous studies have shown that the expression of opsin in the central retina occurs early in prolarvae, before differentiation of outer photoreceptor segments (Meléndez-Ferro et al., 2002a). Tract-tracing studies and GABA immunocytochemistry have revealed the early appearance of ganglion cells and retinofugal projections (De Miguel et al., 1989, 1990) as well as retinopetal γ-aminobutyric acid (GABA)-immunoreactive (-ir) fibers (Rodicio et al., 1995; Anadón et al., 1998; Meléndez-Ferro et al., 2002a). Both ganglion cells and GABAergic retinopetal fibers are present in the lateral retina during the second larval period (Anadón et al., 1998). However, despite Golgi impregnation studies indicating that the major classes of retinal neurons appear during the late larval period (Rubinson and Cain, 1989), to the best of our knowledge the presence of classical neurotransmitters has not been reported in any of these cells, either in the central or in the lateral larval retina. In fact, we recently reported that cholinergic amacrine cells do not appear in the lamprey retina until early metamorphosis (Pombal et al., 2003). Previous studies thus suggest that, aside from the GABAergic fibers of central origin, all neurotransmitters are absent from cells of the larval retina. Accordingly, it is not known whether the early differentiated (opsin-expressing) central retina has functional neural circuitry.

In the present study, the putative neurochemical differentiation of neural circuitry in the lamprey retina was analyzed during the larval period by using a set of antibodies directed against several classical neurotransmitters (glutamate, GABA, glycine, aspartate, serotonin, and dopamine) and a neurotransmitter-synthesizing enzyme (tyrosine hydroxylase). For comparison, the retina of recently transformed young lampreys was also analyzed. The results provide the first demonstration of glutamate immunoreactivity in neurons of the central and lateral retina of larvae and reveal further details of the centrifugal innervation by GABA-ir fibers. The significance of these findings is discussed in the context of lamprey biology and its remarkable retinal development.

MATERIALS AND METHODS

Animals

Larval sea lampreys, *Petromyzon marinus* L., ranging from 30 to 156 mm in length, were collected from the River Ulla (Galicia, Northwest Spain) and maintained in aerated aquaria before processing. Four recently metamorphosed young sea lamprey, provided by the Estación Biolóxica de Ximonde, were also used for comparison. All procedures conformed to European Community guidelines on animal care and experimentation. Animals were deeply anesthetized with benzocaine (0.05%; Sigma, St. Louis, MO) prior to fixation. For glycine (GLY; n = 12), GABA (n = 12), glutamate (GLU; n = 8), aspartate (n = 5), and dopamine (DA; n = 10) immunohistochemistry, heads of 40 larvae and eyes of four young postmetamorphic lampreys were fixed by immersion in 5% glutaraldehyde/1% sodium metabisulfite in 0.05 M Tris buffer (pH 7.4). For serotonin (5HT; n = 10), calretinin (CR; n = 5), and tyrosine hydroxylase (TH; n = 6) immunohistochemistry, larval heads and two recently metamorphosed lampreys were fixed by immersion in cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. To visualize these antisera, samples were embedded in Tissue Tek (Sakura, Torrance, CA) and cut on a cryostat (16 μm thick). For opsin immunocytochemistry (n = 3), larval heads were fixed in Bouin's fluid, embedded in paraffin, and sectioned on a rotary microtome (10 μm thick).

Two additional larvae were fixed with 2% glutaraldehyde and 2% paraformaldehyde in Tris buffer containing 1% metabisulfite and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA). Semithin sections were obtained with glass knives on an ultramicrotome and mounted on glass slides.

Single-label immunohistochemistry

For brightfield light microscopy, specimens were processed according to the peroxidase-antiperoxidase (PAP) technique. Sections were incubated in one of the following primary antisera raised in rabbits: 5HT (Incstar, Stillwater, MN; dilution 1:5,000), DA (H.W.M. Steinbusch, Maastricht, The Netherlands; 1:900), TH (Chemicon, Temecula, CA; 1:1,000), GABA (Affiniti, Mamhead, United Kingdom; 1:1,000), GLU (Sigma; 1:2,000), GLY (Chemicon; 1:200), aspartate (Chemicon; 1:200), opsin (CERN-922 anti-bovine rod opsin provided by Prof. W. DeGrip; 1:1,000), and calretinin (SWant, Bellinzona, Switzerland; 1:1,500; see Table 1). The tissue was subsequently incubated with goat anti-rabbit immunoglobulin (Sigma; 1:100) and rabbit PAP complex (Sigma; 1:400). Other sections were sequentially incubated in mouse anti-GABA (GABA93; Martinelli; 1:50), rabbit anti-mouse IgG (Dako, Glostrup, Denmark; 1:600), and mouse PAP complex (Sigma; 1:600). The immunocomplexes were developed by immersion in 3,3′-diaminobenzidine (Sigma; 0.6 mg/ml) with 0.003% H_2O_2. Photomicrographs were obtained with an Olympus DP 12 color digital camera (Olympus, Tokyo, Japan). Images were converted to gray scale and adjusted for brightness and contrast in Corel Photo-Paint (Corel, Ottawa, Ontario, Canada). Prior to GLU, GABA, and GLY immunostaining, semithin plastic sections were deplasticized with ethanol-NaOH, rinsed four times with PB, and then immunostained following a protocol similar to that used for cryostat sections.

Double immunofluorescence and confocal laser scanning microscopy

For double immunofluorescence, sections were pretreated with 0.2% $NaBH_4$ in water for 45 minutes at room temperature. Alternate series of sections were sequentially incubated for 3 days at 4°C with a mixture of 1)

TABLE 1 Antisera Used

Antisera	Host	Source[1] and code	Dilution	Lot	Immunogen
Glycine	Rabbit	Chemicon code AB139	1:200 or 1:100[2]	24080975	GLY-glutaraldehyde-BSA conjugate
Aspartate	Rabbit	Chemicon code AB132	1:200 or 1:100[2]	24030893	L-aspartate-glutaraldehyde-BSA conjugate
Glutamate	Rabbit	Sigma code G6642	1:2,000 or 1:1,000[2]	113K4824	GLU-key limpet hemocyanin conjugate
GABA	Mouse	Holstein et al., 2004; GABA93 MAb	1:50[2]		GABA-glutaraldehyde-BSA conjugate
GABA	Rabbit	Affiniti code GA1159	1:1,000	200493	GABA-glutaraldehyde-BSA conjugate
Serotonin	Rabbit	Incstar code 20080	1:5,000	051007	Serotonin-formaldehyde-BSA conjugate
Dopamine	Rabbit	HWM Steinbusch, U. Maastricht, The Netherlands	1:900		Dopamine-glutaraldehyde-BSA conjugate
TH	Rabbit	Chemicon code AB152	1:1,000	0509011790	Denatured TH from cat pheochromocytoma
Opsin	Rabbit	Gift of Prof. W. De Grip; code CERN 922	1:1,000		Bovine rod-opsin
Calretinin	Rabbit	SWant code 7699/4	1:1,500	18299	Recombinant human calretinin

[1]Sources include Chemicon, Temecula, CA; Affiniti, Mamhead, United Kingdom; Sigma; St. Louis, MO; SWant, Bellinzona, Switzerland. BSA, bovine serum albumin.
[2]Dilution used for immunofluorescence.

rabbit polyclonal anti-GLY antiserum (Chemicon; 1:100) and mouse monoclonal anti-GABA (GABA93; 1:50; Holstein et al., 2004), or 2) rabbit polyclonal anti-GLU antiserum (Chemicon; 1:1,000) and mouse monoclonal anti-GABA (GABA93; 1:50), or rabbit polyclonal antiaspartate (Chemicon; 1:100) and mouse monoclonal anti-GABA (GABA93; 1:50). All sections were subsequently incubated with Cy3-conjugated goat anti-rabbit immunoglobulin (Chemicon; 1:200) and fluorescein-conjugated goat anti-mouse IgG (Chemicon; 1:50). Sections were observed and photographed with a spectral confocal microscope (Leica TCS-SP2). For presentation of some figures, single-channel stack projections were inverted and then adjusted for brightness and contrast in Adobe Photoshop (Adobe, San Jose, CA). All antibodies for both single and double immunolabeling were diluted in Tris-buffered saline containing 0.2% Triton X-100 and 3% normal goat serum.

Specificity controls

Control sections were processed as described above, except for the omission of primary antisera. No staining was observed in these controls. Moreover, staining of antisera against neurotransmitter-BSA (glycine, aspartate, GABA, serotonin, dopamine) or neurotransmitter-KLH (glutamate) conjugates was not modified by preabsorption of the primary antiserum with BSA (Sigma) or KLH (Sigma), respectively. As positive controls for cases of negative immunostaining of the larval retina, we used the adjacent brain tissue in the same head sections and the brain and the retina of metamorphosed lampreys.

The specificity of all primary antibodies has been well-characterized by the suppliers (Table 1). According to the supplier, the antiglutamate antiserum recognizes L-glutamic acid immobilized on an affinity membrane, and no cross-reaction is observed with L-aspartic acid, L-glutamine, L-asparagine, and L-alanine. Weak cross-reactivity is observed with Gly-Asp, GABA, β-alanine, glycine, and 5-aminovaleric acid (amino acid concentration 5–10 mM). The antiaspartate antiserum recognizes L-aspartic acid immobilized on affinity membrane. No cross-reaction of this antiserum is observed with L-glutamic acid, L-glutamine, or L-alanine. Weak cross-reactivity is observed with L-asparagine, GABA, β-alanine, glycine, and 5-aminovaleric acid (amino acid concentration 5–10 mM). The cross-reactivity of the glycine antiserum determined by ELISA or RIA assays indicates that it only weakly or very weakly cross-reacts with alanine (1/100)-, GABA (1/500)-, taurine (1/1,000)-, aspartate (1/20,000)-, or glutamate (<1/20,000)-BSA conjugates with regard to the glycine-BSA conjugate. The aspartate antiserum is highly specific for the L-aspartate-BSA conjugate; the cross-reactivities determined using an ELISA test by competition experiments with GLU-BSA and GABA-BSA conjugates are much lower (1/30,000 and >1/100,000, respectively) than with L-aspartate-BSA conjugate. The mab93 monoclonal GABA antibody was tested by ELISA against BSA conjugates of GABA, 16 amino acids, histamine, serotonin, adrenaline, noradrenaline, and histamine; it showed high specificity for GABA conjugate and negligible levels of cross-reactivity with the other conjugates (Holstein et al., 2004). The specificity of

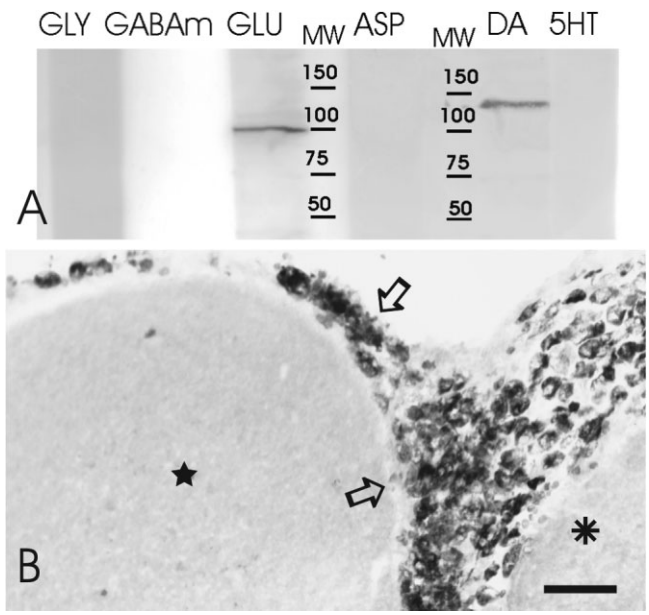


Fig. 1. **A:** Western blotting of adult brain protein extracts immunostained with antisera to the different neurotransmitters used here. Note the native protein bands stained in the glutamate and dopamine lanes. **B:** Fluorescence micrograph of a section of a paraformaldehyde-fixed adult brain stained with the antiglutamate antiserum (the photograph was inverted and converted to gray scale). Note that the only stained structures were large meningocytes (arrows), which suggests that the native protein revealed by this antibody in blots is located in these cells. Star, optic tectum; asterisk, telencephalon. Scale bar = 50 μm.

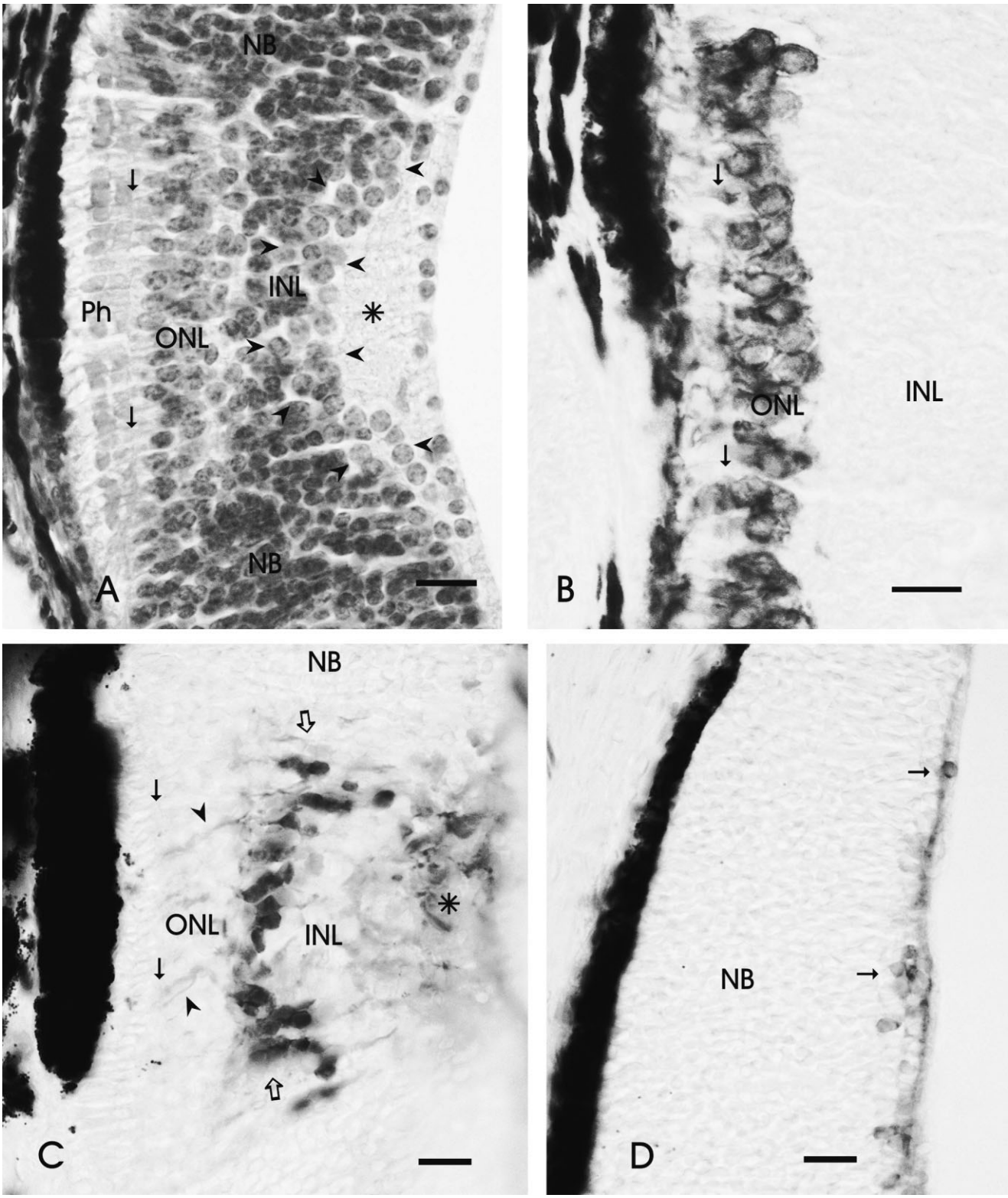


Fig. 2. Photomicrographs of vertical sections through the central retina (A–C) and the dorsal part of the lateral retina (D) of larval sea lamprey. **A:** Central retina, showing the photoreceptors (Ph), the outer nuclear (ONL) and inner nuclear (INL) layers, and the inner plexiform and optic fiber layer (asterisk). The arrows indicate the outer limiting membrane. Arrowheads point to the ganglion cell bodies located in the INL. NB, neuroblastic layer of the lateral retina. Hematoxylin-eosin staining. **B:** Photoreceptors immunostained with the CERN 922 antiopsin antibody in the central retina. The arrows point to the outer limiting membrane. **C:** Photomicrograph of the caudal pole of the central retina showing intense calretinin immunoreactivity in bipolar cells (open arrows). Note that these cells partially encircle the ganglion cells. Arrowheads point to Landolt's clubs of bipolar cells. Solid arrows indicate the outer limiting membrane. Asterisk, inner plexiform layer receiving bipolar cell CR-ir inner fibers and terminals. **D:** Photomicrograph showing that putative ganglion cells of the dorsal region of the lateral retina are moderately CR-ir (arrows). In all photographs, the vitreum is to the right, dorsal at the top, and ventral is at the bottom. Larval lengths: A, 87 mm; B, 156 mm; C,D, 114 mm. Scale bars = 12.5 μm.

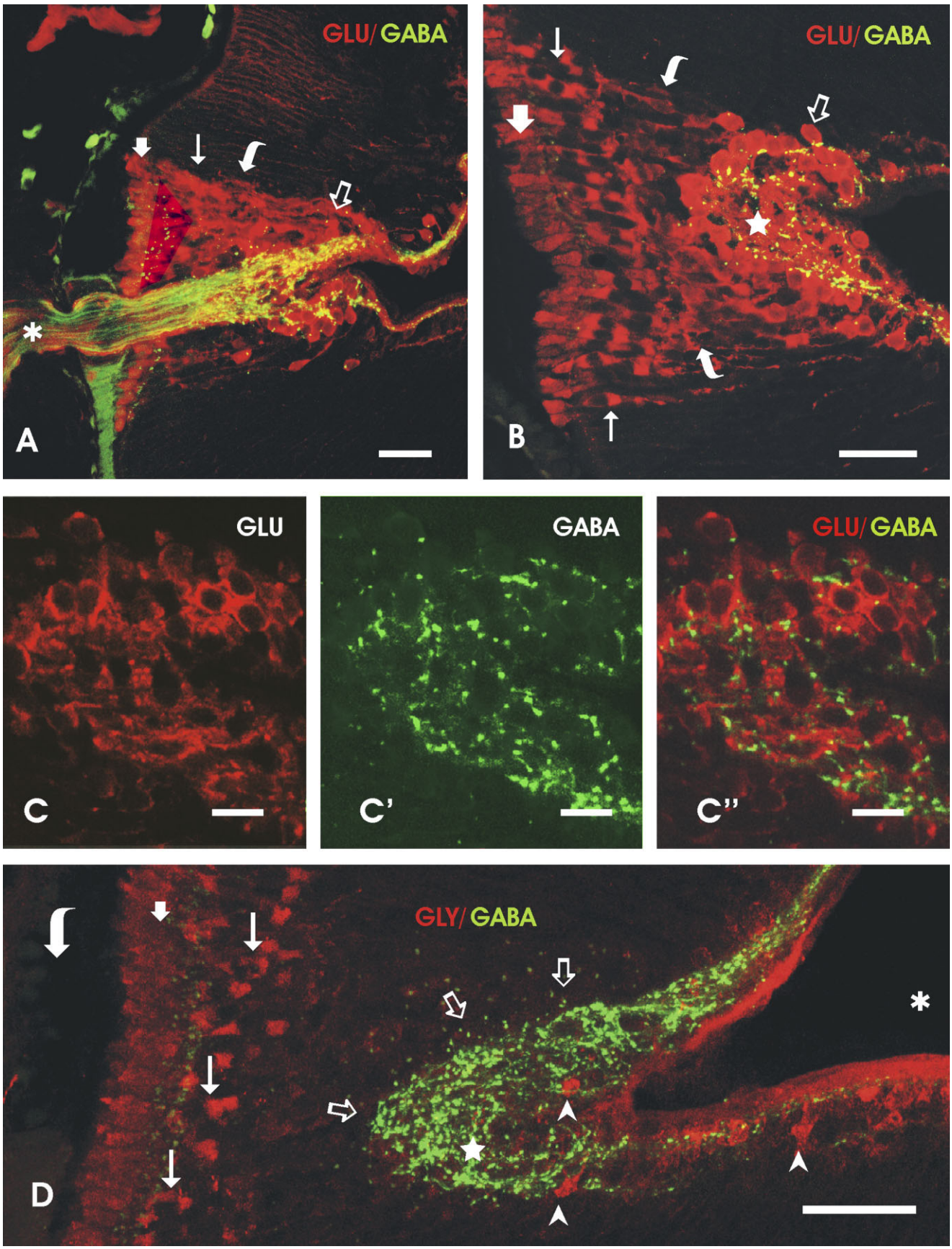


Figure 3

the rabbit anti-GABA antiserum has been extensively characterized by ELISA and immunohistochemical techniques and was adsorbed against BSA-glutaraldehyde. The dopamine antiserum does cross-react with noradrenaline for less that 10% and with other monoamines for less than 1%. The antiserotonin antibody does not cross-react with 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, or dopamine. The calretinin antiserum does not cross-react with calbindin D-28k or other known calcium-binding proteins, as determined by its distribution in the brain as well as by immunoblots. This antiserum was already tested by Western blotting in lamprey brain extracts, showing a single stained band of the appropriate molecular weight (Villar-Cheda et al., 2006). The antiopsin antiserum was shown to stain specifically photoreceptors in lampreys (García-Fernández et al., 1997; Meléndez-Ferro et al., 2002a) and teleosts (Candal et al., 2005). This immunoreaction was intended only for demonstrating photoreceptors, so characterization of opsin type specificity was not done.

As a further specificity control, antisera to glutamate, GABA, glycine, aspartate, and serotonin were analyzed by Western blotting with protein extracts of adult sea lamprey brain, as detailed previously (Villar-Cheda et al., 2006). Briefly, brains (including meninges) were homogenized at 4°C in 6 volumes of modified RIPA buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 5 μg/ml aprotinin, pH 7.4). The homogenate was centrifuged at 20,000*g*, and the supernatant was precipitated with 6 volumes of 100% methanol. The precipitate was resuspended in modified RIPA buffer, and 25 μg of total protein per lane was loaded onto 12% acrylamide gels, resolved by SDS-PAGE, and then electroblotted in a Mini-Protean 3 cell (Bio-Rad, Hercules, CA) onto 0.2-μm polyvinylidene difluoride membranes (Bio-Rad). Nonspecific binding sites on the membrane were blocked by incubating in 5% powdered nonfat milk dissolved in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour. After blocking, lanes in membranes were cut off, rinsed in TBST, and each incubated overnight with one of the neurotransmitter primary antibodies. The membranes were then rinsed in TBST, incubated with either goat anti-rabbit or goat anti-mouse HRP-conjugated antibodies (Bio-Rad; 1:15,000), rinsed again in TBST, and incubated with enhanced chemiluminescent reagent (Bio-Rad Immun-Star HRP kit). Precision Plus protein standards (Bio-Rad) were used as molecular weight (MW) markers.

In Western blots, no protein band was stained with the glycine, GABA, serotonin, or aspartate antibodies (Fig. 1A), strongly suggesting that these antisera do not cross-react with native proteins of the lamprey CNS. The anti-glutamate antibody stained a protein band of about 100 kDa in blots (Fig. 1A). To investigate the distribution of this glutamate-like immunoreactivity, cryostat sections of lamprey brain fixed in buffered 4% paraformaldehyde were submitted to the immunofluorescence procedure described above. In these sections, strong staining was observed in large, ovoid meningeal cells, whereas neurons and nerve fibers of the adjacent nervous tissue were not stained (Fig. 1B). Likewise, in sections fixed in buffered 5% glutaraldehyde, these large meningocytes were also stained. Because neurons and fibers were not stained in these paraformaldehyde-fixed controls, together these results support the idea that the native protein that cross-reacts in Western blots with the antiglutamate antibody is located in meningocytes and, accordingly, does not interfere with immunocytochemical analysis of glutaraldehyde-coupled glutamate in the nervous tissue. A protein band of about 135 kDa was stained in blots with the dopamine antiserum (Fig. 1A). However, demonstration of DA immunoreactivity in the brain and retina was strictly dependent on fixation with glutaraldehyde and addition of metabisulfite to incubation solutions. That distribution of dopamine immunoreactivity revealed with this antiserum in brain and retina matched with that of tyrosine hydroxylase (Pombal et al., 1997; Abalo et al., 2005; present results) strongly suggests that the substance demonstrated in lamprey is DA.

Additional material

Series of larval lamprey heads from our collection stained with hematoxylin-eosin were used for topographic landmarks.

RESULTS

General organization of the larval eye and retina

With hematoxylin-eosin staining, the eyes of larval lampreys between 60 and 156 mm in length showed the two retinal regions characteristic of the second half of larval life (De Miguel and Anadón, 1987): a central retina with differentiated photoreceptor and outer nuclear and inner nuclear/ganglion cell layers (Fig. 2A) and a lateral region consisting primarily of a thick neuroepithelium in which only a thin optic fiber/inner plexiform layer (IPL) is distinguishable in the innermost region. This latter layer enlarges considerably near the optic nerve head. The central retina is located mostly dorsal to the optic nerve, which has an asymmetrical location in the eye.

Opsin and calretinin immunoreactivities

The CERN-922 antiopsin antibody revealed the presence of opsin-ir photoreceptor cells in the central retina of

Fig. 3. Projections of stacks of 0.5-μm-thick confocal microscope optical sections of double-labeled retinae of larva (A–D). **A:** Vertical section at the level of the optic nerve entrance (asterisk). Most GLU-ir structures are in the central retina, whereas the adjacent neuroblastic layer of the lateral retina lacks significant immunoreactivity. The thick solid arrow indicates the photoreceptor layer, the thin arrow the photoreceptor perikarya, the curved arrow the layer of bipolar cells, and the open arrow ganglion cells. **B:** Detail of the GLU-ir ganglion cells at the level of the optic nerve entrance. Note the rich GABA-ir innervation of the IPL (star) and numerous GABA-ir boutons outlining ganglion cell perikarya. All puncta are uniquely stained in single confocal image planes; the yellow color in some boutons is due to superposition of other structures in the image stacks. C: Detail of a single confocal 0.5-μm-thick section of the region containing ganglion cells showing the distinct glutamate (in red in **C**), GABA (in green channel in **C′**), and double GLU/GABA immunofluorescence (in **C′′**). **D:** Section just caudal to the optic nerve entrance showing GLY-ir photoreceptors (thick arrows) and perikarya in the ONL (thin arrows) and some GLY-ir cells (arrowheads) in the inner part of the IPL that extend in the lateral retina. Note the position of GLY-ir cells with regard to the plexus of GABA-ir retinopetal fibers. Open arrows indicate the region containing ganglion cells in the differentiated retina. Curved arrow points to the pigmented retinal epithelium. In all panels, the vitreum is to the right (asterisk in D), dorsal is at the top, and ventral is at the bottom. Scale bars = 20 μm in A,B,D; 10 μm in C.

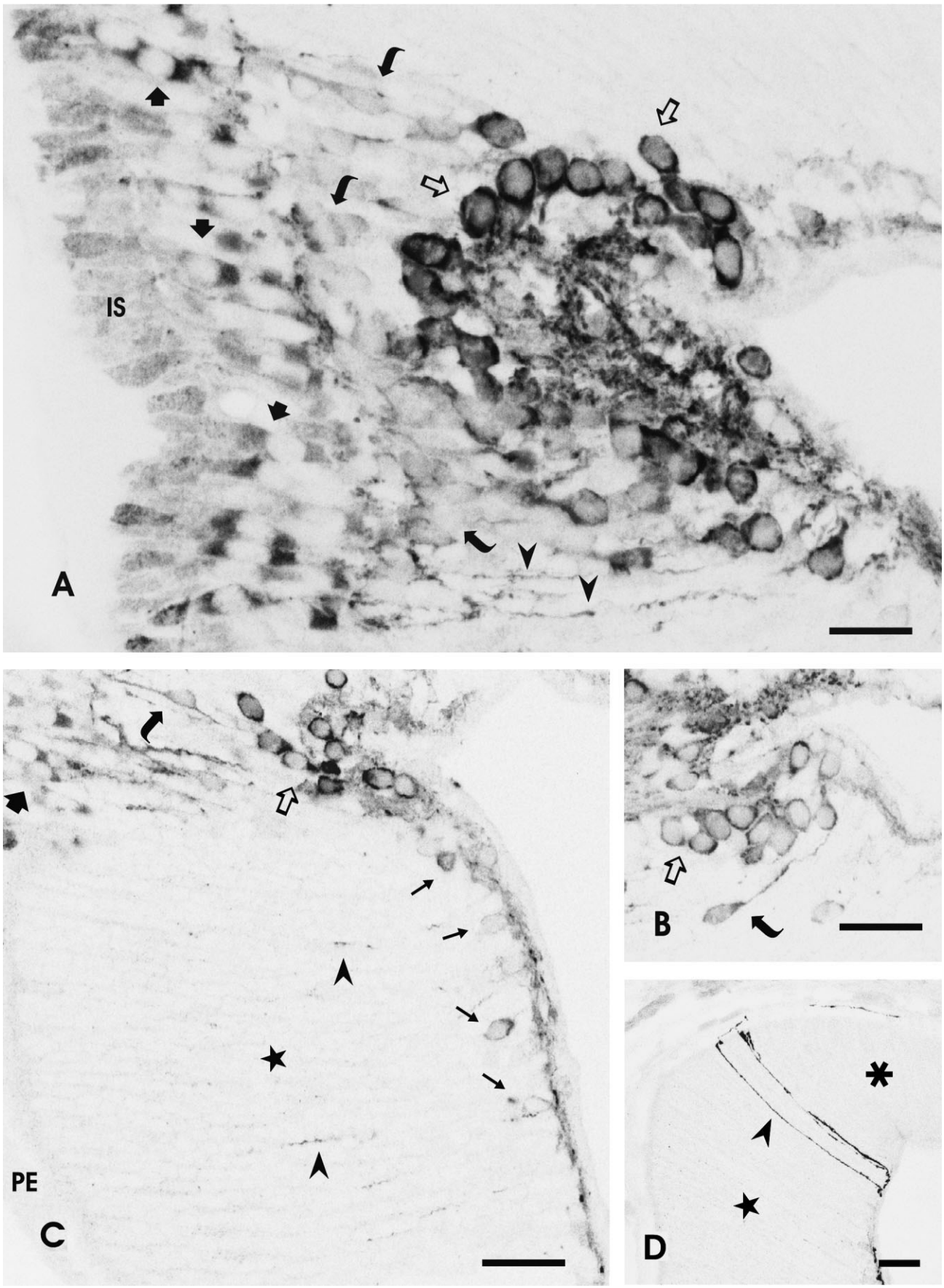


Figure 4

the larvae. The antibody stained both segments of the photoreceptors as well as their perikarya, thereby allowing the unequivocal localization of the cells to the central retina. Photoreceptor perikarya formed two rows of cells below the outer limiting membrane in larvae ranging from 38 to 156 mm in length. The cell bodies were oval and lacked any conspicuous inner processes (Fig. 2B), which is consistent with previous electron microscopic observations (De Miguel et al., 1989).

In the retina of adult sea lamprey, the calretinin (CR) antibody intensely stained two types of bipolar cell as well as some other cells in the inner nuclear layer (INL; inner horizontal cells, a few amacrine cells, and some ganglion cells; Villar-Cheda et al., 2006), which is similar to the staining reported for the retina of river lamprey (Dalil-Thiney et al., 1994). In the larval retina, intense CR immunoreactivity was observed in bipolar cells of the central retina (Fig. 2C), and faint to moderate staining was obtained in the putative ganglion cells of both the central and the lateral retinal areas (Fig. 2C,D). [The location of ganglion cells in larval sea lamprey has been demonstrated in previous tract-tracing experiments (De Miguel et al., 1989; Anadón et al., 1998).] The CR-ir bipolar cells exhibit outer and inner radial processes (Fig. 2C); the inner processes branch in the region of the inner plexiform layer. The perikarya of CR-ir bipolar cells are more numerous caudal to the optic nerve exit and are located in a central band that is well-separated from the rows of photoreceptors (Fig. 2C).

Neurotransmitter immunoreactivity in the larval retina and brain

Among the six classical neurotransmitters (glutamate, GABA, glycine, aspartate, serotonin, and dopamine) and the catecholamine-synthesizing enzyme (TH) investigated in the larval retina, we observed immunoreactivity for glutamate, glycine, and GABA only. The negative results for aspartate, serotonin, dopamine, and TH were not due to false negative results, as indicated by the positive staining of cells and fibers in the brain present in the same head sections of the larvae. Each of the antibodies yielded intense immunostaining of specific neuronal populations and of numerous fibers throughout the brain. The distribution of these GABA-, serotonin-, dopamine-, and TH-ir systems is consistent with that reported in previous studies in sea lamprey larvae (Meléndez-Ferro et al., 2002b, 2003; Abalo et al., 2005; Rodicio et al., 2005) and/or with the distributions of serotonin and dopamine in adult river lamprey (Pierre et al., 1992, 1997). A comprehensive study of glutamate and glycine systems in the larval lamprey brain is currently in progress, but further description is beyond the scope of the present report. As a positive control in the retina, we used retinas of recently metamorphosed lampreys (see below).

Glutamate immunoreactivity in the larval retina

Central retina. Glutamate immunoreactivity was observed in both the central and the lateral regions of the larval retina, although with distinctly different staining patterns (Figs. 3A,B, 4A–D). Several types of GLU-ir cells were observed in the larval central retina, including photoreceptors, ganglion cells, and putative bipolar cells (Figs. 3A,B, 4A–C). These neurons did not show immunoreactivity to GABA (Fig. 3A,B), precluding the possibility that the GLU staining reflected its role as an intermediary metabolite in the synthesis of GABA. Faint GLU immunoreactivity was observed in the inner segments of the photoreceptors, whereas the apical and basal poles of the oval photoreceptor perikarya showed intense GLU-ir, and the cell nuclei were immunonegative (Figs. 3A,B, 4A,C).

The position of horizontal cells in the INL of the central retina of the larval sea lamprey is known from Golgi impregnation studies (Rubinson and Cain, 1989). These cells can be appreciated as a loose cell region just below the outer nuclear layer in hematoxylin-eosin-stained retinae (Fig. 2A). These horizontal cells are not GLU immunostained in our material. In the middle of the INL there are small, loosely scattered, faintly GLU-ir cells with elongated perikarya (Figs. 3A,B, 4A,C). On the basis of their shape and position in the outer part of the INL, correlated with cells of similar position and appearance in Golgi stains (Rubinson and Cain, 1989), the cells were identified as bipolar cells. They were located in the same position as the bipolar cells revealed by CR immunocytochemistry.

Intensely GLU-ir neurons with rather large spherical perikarya were observed in the inner part of the INL, the IPL, and the ganglion cell layer (Figs. 3A–C, 4A–C). Based on their morphology and position in comparison with our previous tract-tracing results (De Miguel et al., 1989; Anadón et al., 1998), these cells were interpreted as ganglion cells. Some of these cells exhibited characteristic ascending processes that reached the outer plexiform layer (OPL; Fig. 4A) and occasionally branched. Ganglion cells with processes ascending to the OPL correspond to the biplexiform ganglion cells described by De Miguel et al. (1989). It has yet to be determined whether all GLU-ir ganglion cells of the central retina possess such ascending processes. GLU-ir axons of ganglion cells of the central and lateral retina course in the IPL/optic fiber layer to the optic nerve.

Lateral retina. In the lateral retina, GLU immunolabeling was observed only in cells located in the innermost retinal layer. Immunostaining was most prominent toward the central retina and very scarce, if at all present, laterally (Fig. 4C,D). GLU-ir cells were smaller than the ganglion cells of the central retina but by their size and

Fig. 4. Photomicrographs showing the morphology of GLU-ir cells and processes of the larval retina. All figures are single-channel projections of a few confocal 0.5-μm sections (inverted and converted to gray scale). **A:** Section of the central retina showing glutamate immunoreactivity in the inner segment (IS) and perikarya of photoreceptors (solid arrows), bipolar cells (curved arrows), and ganglion cells (open arrows). Note the differences in immunoreactivity among the three neuronal types. The arrowheads point to dendritic processes of biplexiform ganglion cells ascending to the OPL. **B:** Detail of a three-section projection showing ganglion cells (open arrow) and a bipolar cell (curved arrow). **C:** Section of the lateral retina showing glutamate-ir neurons (thin arrows) close to the primordial IPL, the thin layer of cells and processes close to the vitreum (below). Some of the processes of putative biplexiform ganglion cells ascend through the very thick neuroblastic layer (star). Note at the upper left the border of the differentiated central retina showing GLU-ir photoreceptors (thick arrow), bipolar cells (curved arrow), and ganglion cells (open arrow). **D:** Section through the lateral retina of a large larva showing a few conspicuous glutamate-ir processes ascending to the outer limiting membrane between the lateral (star) and the marginal (asterisk) regions. In all figures, the vitreum is to the right, dorsal is at the top, and ventral is at the bottom. Scale bars = 20 μm in A; 30 μm in B–D.

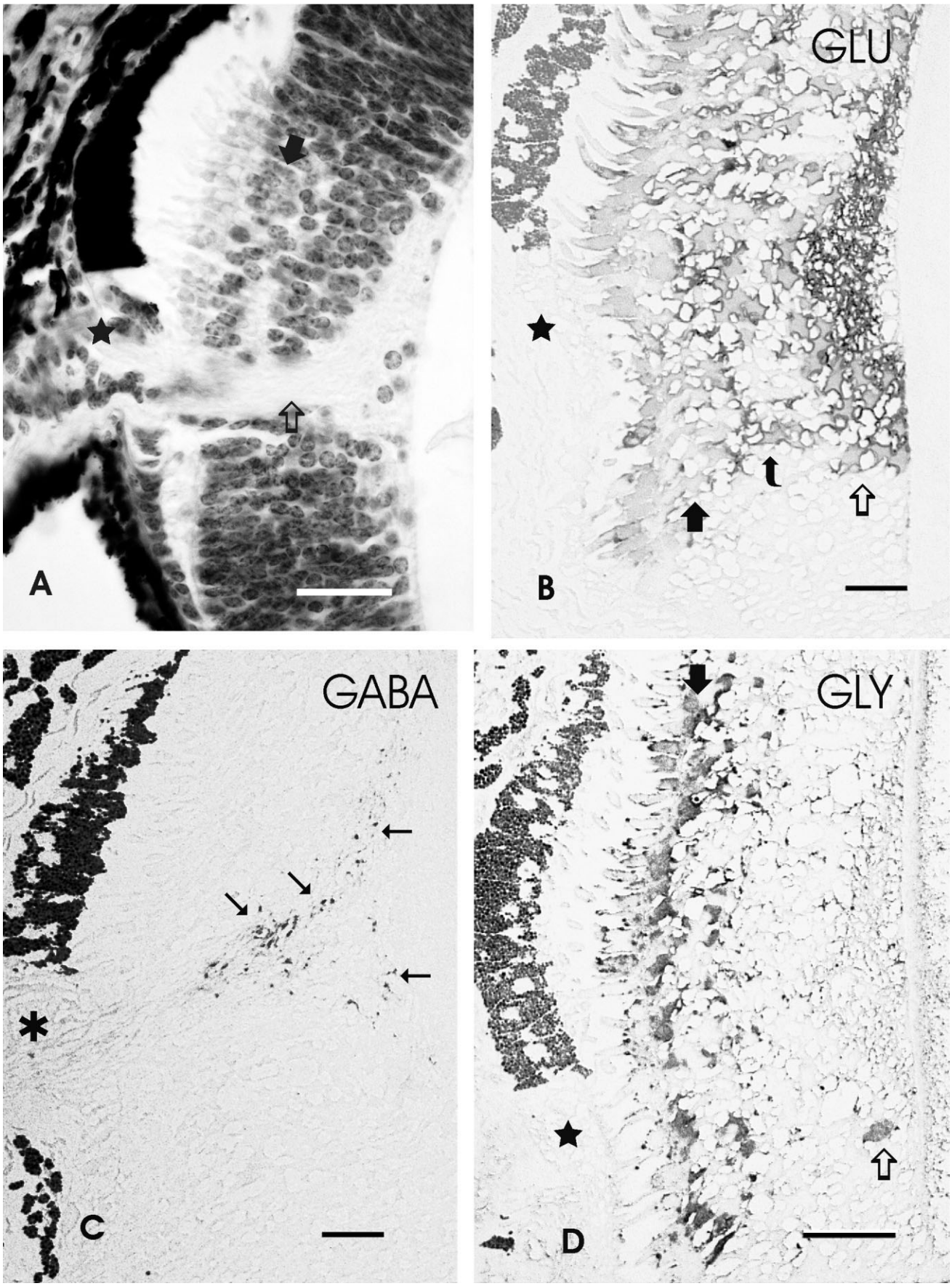


Figure 5

distribution appeared to correspond to the ganglion cells demonstrated in previous tract-tracing studies (De Miguel et al., 1989; Anadón et al., 1998). Optic fibers were also GLU immunopositive, providing additional support for the interpretation of the GLU-ir cells as ganglion cells. Near the central retina, some ganglion cells issued long radial processes directed toward the outer regions of the retina (Fig. 4C), similar to the processes of some ganglion cells of the central region. The cells of the very thick neuroblastic layer of the lateral retina did not show GLU immunoreactivity above background. In the lateral retina, occasional GLU-ir processes were observed to ascend through the neuroblastic layer, contacting the outer limiting membrane (Fig. 4D). These processes appeared at the transition between the lateral region, which contained GLU-ir structures, and the marginal region, which is exclusively neuroblastic.

Colocalization of GLU and GABA

Labeling experiments using our monoclonal anti-GABA antibody resulted in specific immunostaining of fibers in the optic nerve and retina, in close agreement with previous results from our laboratory (Anadón et al., 1998). Given the absence of any GABA-immunolabeled perikarya in the larval retina, these processes are interpreted as retinopetal fibers. Additional details of the retinopetal GABA-ir system in relation to retinal ganglion cells were obtained through double-label studies with polyclonal anti-GLU and monoclonal anti-GABA antibodies and spectral laser confocal microscopy.

The immunolabeling with the green fluorescent secondary antibody used for GABA demonstration may be confused with yellowish autofluorescent bodies, located mainly in the central retina near the outer limiting membrane but also occasionally scattered through the INL, as seen in control sections. Spectral confocal microscopy allowed us to differentiate these autofluorescent bodies from the GABA-ir fibers and boutons by means of image capture using two closely spaced wavelength ranges, one specific for the green fluorophore emission spectrum (490–524 nm) and the other covering a slightly wider range of emission (482–539 nm). With this approach, the retinopetal fibers were seen to be distributed throughout the lateral and central retina, although the density of such fibers in the central part far exceeded that in the lateral region (Fig. 3A–C). In the central retina, the GABA-ir fibers are found in both the IPL and the adjacent INL (Fig. 3B,C). The highly specific labeling of the monoclonal GABA antibody together with the high spatial resolution of the confocal microscopy revealed that GABA-ir boutons are associated with ganglion cells (Fig. 3B,C). This association is a distinctive feature of retinal ganglion cells. Analysis of 0.5-μm-thick confocal sections did not reveal clear labeling of glutamate in GABA-ir boutons, suggesting that these boutons do not accumulate glutamate above basal levels.

GLY immunoreactivity

GLY immunoreactivity was observed in differentiated cells of both the central and the lateral regions of the retina (Fig. 3D), whereas the thick neuroblastic layer of the lateral retina did not show GLY immunoreactivity above background. In the central retina, the perikarya of photoreceptors were moderately or strongly GLY-ir, whereas the inner photoreceptor segments showed fainter immunoreactivity (Fig. 3D). In addition, moderate to high intensity GLY immunoreactivity was observed in scattered cells of the outer and inner sides of the IPL characterized by its GABA-ir fiber plexus, i.e., a region also containing putative ganglion cells (Fig. 3D). However, the number of GLY-ir cells was clearly minor in comparison with that of GLU-ir cells. In addition, comparison of photographs of double immunostaining GABA/GLY (Fig. 3D) with those of GABA/GLU (Fig. 3A–C) clearly indicates that the GLY-ir cells did not form a cap over the IPL as was observed with glutamate immunostaining, the large ganglion cells of this region being only very faintly GLY-ir (Fig. 3D). Moreover, no ascending GLY-ir processes were observed, in contrast to the results obtained with glutamate immunocytochemistry. In the lateral retina, some cells close to the primordial IPL showed faint to moderate GLY immunoreactivity (Fig. 3D). As in the central retina, these cells appeared in the region containing ganglion cells. Whereas double GABA/GLY immunostaining (Fig. 3D) suggests that GABA-ir retinopetal fibers might contact GLY-ir cells processes, no specific relationship of these fibers with glycinergic perikarya was observed.

GABA, GLY, and GLU immunostaining in control semithin plastic sections

To confirm the results obtained by confocal microscopy, some semithin plastic sections were stained with antisera against GLU, GABA, and GLY. To facilitate identification of structures of these sections, a similar hematoxylin-eosin-stained section is shown (Fig. 5A). In plastic sections, GLU, GABA, and GLY immunoreactivities were observed in similar locations of the larval retina to those observed with confocal microscopy (Fig. 5B–D). GLU-ir cells were present in the regions of the photoreceptor cells, bipolar cells, and ganglion cells (Fig. 5B). In semithin sections, the monoclonal GABA antibody stained only fibers in the IPL (Fig. 5C), which were clearly identifiable as centrifugal fibers by the absence of any stained retinal perikaryon. Photoreceptors showed GLY immunoreactivity in semithin plastic sections, and a few GLY-ir cells were also observed in the innermost central retina (Fig. 5D).

Neurotransmitter immunoreactivity in the postmetamorphic retina

For comparison with the larvae and to serve as positive controls, we examined retinas from recently metamor-

Fig. 5. Hematoxylin-eosin-stained section (A) and postembedding stained, semithin plastic vertical sections of the larval retina showing immunoreactivity to glutamate (B), GABA (C), and glycine (D). **A:** Photomicrograph showing a section of the retina at a level similar to that in C. Star, ventral unpigmented part of pigment epithelium; open arrow, optic nerve entrance; solid arrow, layer of photoreceptor perikarya. **B:** Section showing glutamate immunoreactivity in cells of the vertical pathways. The thick arrow points to the GLU-ir photoreceptor perikarya, the curved arrow designates the layer of bipolar cells, and the open arrow indicates ganglion cells. The star marks the unpigmented ventral region of the pigment epithelium. **C:** Section through the level of the optic nerve (asterisk) showing that GABA immunoreactivity is restricted to centrifugal fibers (arrows). **D:** Section showing glycine immunoreactivity in photoreceptors and in scarce cells (open arrow) of the inner part of the retina. In all figures, the vitreum is to the right, dorsal is at the top, and ventral is at the bottom. Brightfield microscopy. Scale bars = 50 μm in A; 20 μm in B–D.

phosed lampreys. In these retinas, each of the antibodies against neurotransmitters revealed the presence of characteristic populations of neurons, as shown in Figures 6A–D and 7A–K. Strongly GABA-ir amacrine cells were numerous in the inner part of the INL, and processes of these cells extended to the IPL, where they formed a dense GABA-ir plexus (Figs. 6A′–D′, 7A). Faint to moderate GABA immunoreactivity was observed in the outer row of horizontal cells (HC1) and in small bipolar cells (Figs. 6A′–D′, 7A–D). GABA-ir horizontal cells exhibited short, thick dendritic trunks that gave rise to numerous delicate appendages in the OPL (Fig. 7A–D). Thick axons of these cells coursed horizontally below the layer of perikarya before ascending to branch in the OPL (Fig. 7A,D). GABA-ir bipolar cell perikarya were located either among horizontal cells or in a position intermediate between amacrine cells and horizontal cells. Some of these GABA-ir cells exhibited ascending processes that coursed to the outer limiting membrane, i.e., Landolt's clubs (Fig. 7A,B). Other retinal cells were GABA negative. Some GABA-ir fibers were also observed coursing in the bundles of optic fibers that, characteristically in lamprey, are located adjacent to the INL (Fig. 7E).

Numerous GLU-ir cells were observed in the postmetamorphic retina. Photoreceptors were faintly or very faintly GLU-ir, whereas inner horizontal cells (HC2), large bipolar cells with perikarya at the level of HC1 cells, and ganglion cells were moderately or strongly GLU-ir (Figs. 6A, 7F). Small bipolar cells and some putative amacrine cells also exhibited faint to moderate glutamate immunoreactivity (Fig. 7F). Numerous GLY-ir cells were observed both in the INL (orthotopic amacrine cells) and in the IPL, both in its innermost part and interspersed in this layer (Figs. 6B, 7G). Double-immunofluorescence confocal microscopy revealed that most GLY-ir cells in the INL do not correspond to the GABA-ir amacrine cell population, although colocalization was observed in some cells (Fig. 6B). Most GLY-ir cells in the IPL and close to the inner limiting membrane probably correspond to displaced amacrine cells. Some GLY-ir small cells of the INL showed processes ascending to the OPL (Figs. 6B, 7G, inset), suggesting they are glycinergic interplexiform cells. In addition, some GLY-ir boutons are found in the OPL just above of HC1 cells. Faintly to moderately aspartate-ir cells were observed in the postmetamorphic retina in the outer and inner parts of the INL and in the IPL (Figs. 6C,D, 7H). Some large cells of the INL can clearly be recognized as giant ganglion cells (Figs. 6C, 7H). Other aspartate-ir cells of this layer may be displaced ganglion cells, bipolar cells, and/or amacrine cells, whereas immunopositive cells of the IPL may correspond to orthotopic ganglion cells and/or displaced amacrine cells.

Scarce dopamine-ir amacrine cells were observed in the inner part of the INL, their processes coursing in the IPL (Fig. 7I). No DA-ir processes were observed in the OPL or the ONL, indicating that the dopaminergic cells of the sea lamprey are amacrine cells. The appearance of these cells and distribution of their processes closely correspond to the TH immunoreactivity observed in the postmetamorphic retina (not shown). Serotonin (5HT)-ir amacrine cells were observed in the inner part of the INL (Fig. 7J) and, occasionally, close to the inner limiting membrane (Fig. 7K). They are more abundant than dopaminergic cells and have processes that ramify on both sides of the IPL (Fig. 7J,K).

DISCUSSION

The neurochemistry of the larval lamprey retina differs notably from the developing retinae of other vertebrates

One surprising result from the present study is that no GABA-ir, serotonin-ir, aspartate-ir, or dopamine-ir perikarya were observed in the larval lamprey retina, either centrally or laterally, despite the reported presence of serotonin-ir, GABA-ir, dopamine-ir, and TH-ir cells in adult lamprey (Negishi et al., 1986; De Miguel and Wagner, 1990; Versaux-Botteri et al., 1991; Rio et al., 1993; Yáñez and Anadón, 1994; present results). In addition, a recent study examining the localization of choline acetyltransferase (ChAT) noted the absence of putative cholinergic cells in the central and lateral parts of the larval retina (Pombal et al., 2003). In contrast to larval lamprey, the larvae of jawed anamniotes possess amacrine cells that are immunoreactive to ChAT, GABA, dopamine/TH, and serotonin (van Veen et al., 1984; Östholm et al., 1988; Zhu and Straznicky, 1992; González et al., 1995; Huang and Moody, 1998; Dunker, 1999; López et al., 2002). Unlike the case with the developing retinae of mammals (Redburn et al., 1992; Fletcher and Kalloniatis, 1997), we have never observed GABA immunoreactivity in perikarya of the larval lamprey retina (Anadón et al., 1998; present results). In other vertebrates, these substances (ChAT, GABA, dopamine/TH, and serotonin) are first expressed around or shortly after the time of photoreceptor differentiation, which does not occur in the central retina of the larval lamprey. Insofar as the central retina contains photoreceptors that express opsin immunoreactivity throughout the entire larval period (5–7 years long; Meléndez-Ferro et al., 2002a; present results), the absence of cells expressing ChAT, GABA, dopamine/TH, or serotonin represents a fundamental difference between the retinae of larval lamprey and other anamniote vertebrates. This difference appears genuine; immunocytochemistry with antisera against GABA, aspartate, serotonin, dopamine, TH, and ChAT produced reliable staining of numerous cells and fibers in the larval brains but not the retinae within the same head sections. Moreover, the distributions of GABA-, serotonin-, dopamine-, and TH-ir neurons observed in the brain were coextensive with those described in previous reports on larval (Meléndez-Ferro et al., 2002b, 2003; Abalo et al., 2005) and/or adult (Pierre et al., 1992, 1997; Pombal et al., 1997) lamprey. Likewise, the positive results obtained with the same protocols in the postmetamorphic retina rule out the possibility of false-negative results in larvae.

GLU and GLY immunocytochemistry reveals neurons in the retina of larval lamprey

A common point with other developing vertebrates is that the larval lamprey retina contains GLU-ir cells and GLY-ir cells. Immunocytochemistry with antisera against GLU and GLY produced staining of numerous cells and fibers in both the brain and the retina of lamprey larvae. Unpublished observations on the brain using double immunofluorescence to visualize GLU or GLY together with GABA indicate that many GABA-ir populations are GLU negative and/or GLY negative and, conversely, that many GLU- or GLY-immunolabeled structures are GABA immunonegative. These findings support the antibody specificity studies demonstrating that the monoclonal GABA

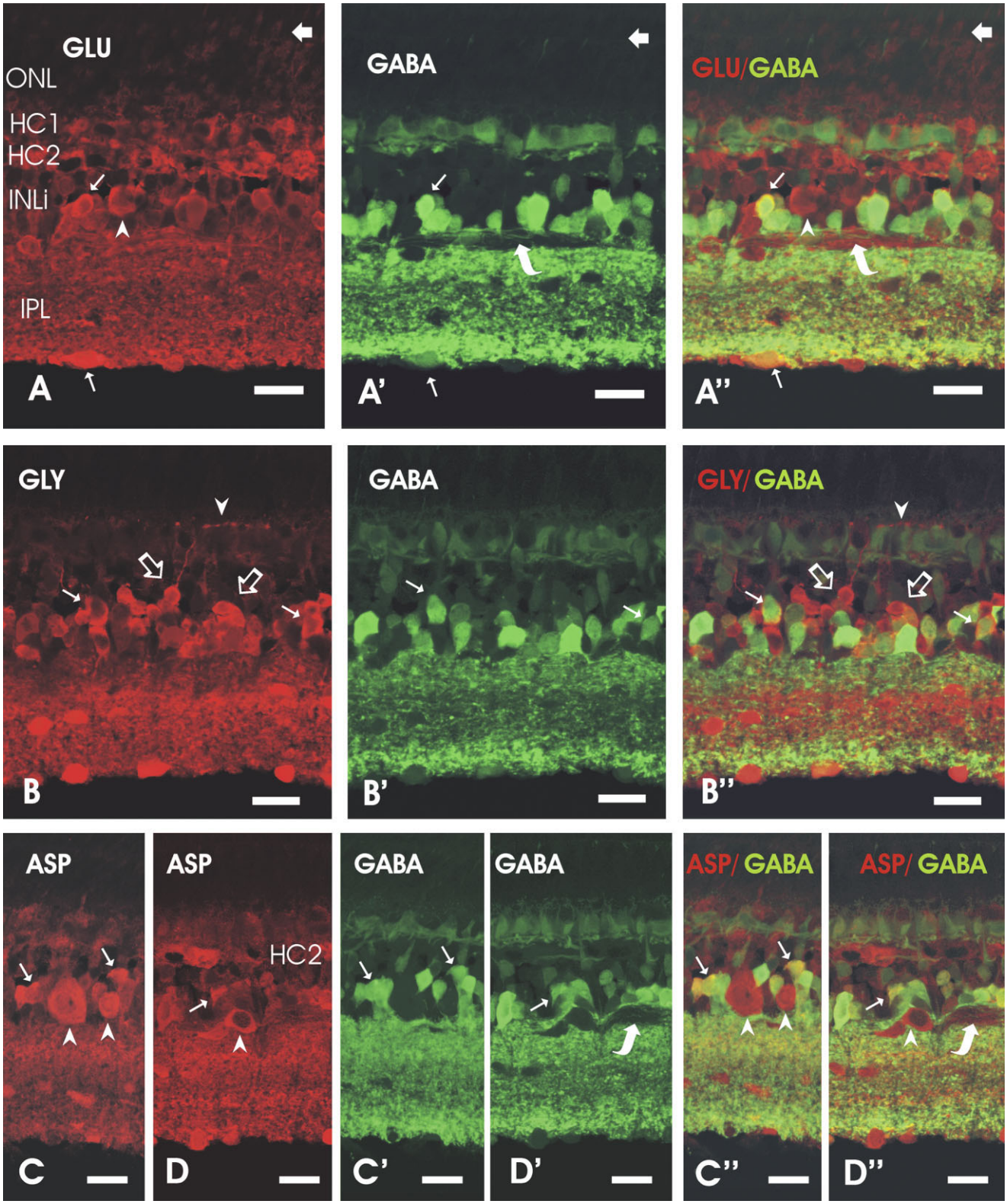
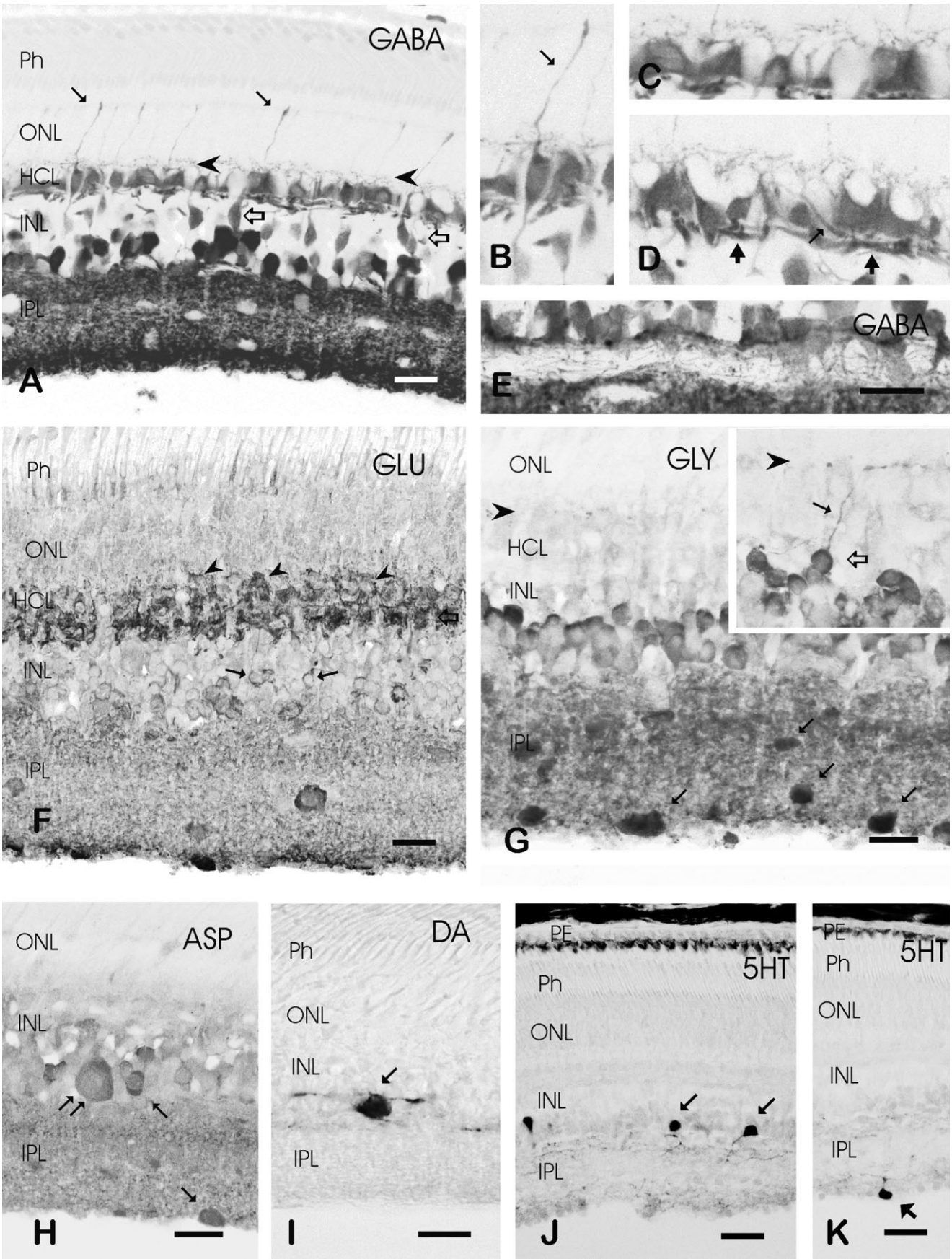


Fig. 6. Projections of stacks of 0.5-μm-thick confocal microscope optical sections of double-labeled retinae of postmetamorphic sea lamprey showing in the left panels glutamate (A), glycine (B), and aspartate (C,D) immunofluorescence (in the red channel), in the central panels immunoreactivity to GABA (in green), and in the right panels, double immunolabeling. **A–A′′:** Section showing abundant glutamate immunoreactivity in horizontal cells and putative ganglion cells (arrowhead). Colocalization of GABA and glutamate is occasionally found in cells of the INL and IPL (thin arrows). The curved arrow points to a bundle of optic fibers close to the INL. Small arrows point to clearly double-labeled cells. **B–B′′:** Sections showing glycine immunoreactivity in amacrine cells of the INL and the IPL. Colocalization with GABA is observed in some amacrine cells (thin arrow). The open arrows point to glycinergic interplexiform cells. Arrowheads, interplexiform cell processes in the outer plexiform layer. **C–C′′,D–D′′:** Sections showing abundant aspartate immunoreactivity in some horizontal cells, in ganglion cells (arrowheads), and in some cells of the IPL. Thin arrows point to putative amacrine cells showing colocalization of aspartate and GABA immunoreactivity. In A, thick white arrows point to the outer limiting membrane. In all photographs, sclera is toward the top, and the vitreum at the bottom. Scale bars = 20 μm.


GABA
Ph
ONL
HCL
INL
IPL
A
B
C
D
GABA
E
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Ph
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INL
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F
GLY
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HCL
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5HT
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ONL
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PE
5HT
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ONL
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K

Figure 7

antibody does not recognize either of the other two amino acids (Holstein et al., 2004). In line with this evidence in the brain, no correspondence was observed between the GLU- and the GABA-ir and between the GLY- and the GABA-ir structures in double-labeling experiments in the larval retina.

GLU-ir cells of the larval lamprey retina: comparison with the retinae of other vertebrates

Developmental studies of GLU-ir cells in the vertebrate retina are very scarce: as far as we are aware, this has been addressed exclusively in mammals (Redburn et al., 1992; Pow et al., 1994; Fletcher and Kalloniatis, 1997). In the rabbit retina, GLU immunoreactivity first appears by embryonic day 20 (E20). Ganglion cells and photoreceptors are GLU-ir at birth (Redburn et al., 1992), and bipolar cells and some amacrine cells also become GLU-ir at about postnatal day 10 (P10; Pow et al., 1994). Some horizontal cells are reported to be GLU-ir at birth by Redburn et al. (1992) although not by Pow et al. (1994).

The unique retina of larval lampreys affords the opportunity to explore simultaneously the immunoreactivity to GLU and GABA during three distinct developmental phases in the same larvae. Whereas the central retina has a layered organization with photoreceptors, horizontal cells, bipolar cells, and ganglion cells, the lateral retina consists mainly of a thick neuroblastic layer as well as differentiating ganglion cells and retinopetal fibers in the innermost region, and the marginal retina is a purely neuroblastic region lacking identifiable ganglion cells or retinopetal fibers (De Miguel and Anadón, 1987; De Miguel et al., 1989; Anadón et al., 1998; Meléndez-Ferro et al., 2002; present results). The distribution of GLU in the central retina of the larval sea lamprey is reminiscent of that reported before eye opening in the vertical system of rabbit, immunoreactivity being observed in photoreceptors, some bipolar cells, and ganglion cells. These GLU-ir cells of the larval lamprey retina are not GABA-ir in the postmetamorphic lamprey, with the exception of GABA expression in a bipolar cell subtype (present results), and no GLU immunoreactivity was observed in the region of future GABAergic amacrine cells. These findings are consistent with our interpretation that GLU is the neurotransmitter utilized by neurons of the vertical pathways in the larval retina.

For the lateral and marginal retina, our results demonstrate the absence of GLU immunoreactivity above background in cells of the neuroblastic layers. In fact, the presence of GLU-ir cells in the innermost layers provides a biological marker to distinguish the lateral from the marginal regions. One interesting observation concerns the long GLU-ir processes of ganglion cells ascending through the neuroblastic layer. These processes may be viewed as either transient trailing processes of recently migrated cells, or processes of biplexiform cells awaiting the differentiation of photoreceptors and the OPL. On the other hand, the absence of GLU-ir and GABA-ir fibers and cells in the marginal region reinforces the exclusively proliferative nature of this zone.

With regard to the retinae of adult vertebrates, most studies indicate the presence of GLU in most photoreceptors, bipolar cells, and ganglion cells (teleosts: Kageyama and Meyer, 1989; Van Haesendonck and Missotten, 1990; Marc et al., 1990, 1995; Connaughton et al., 1999; amphibians: Yang and Yazulla, 1994; reptiles: Schutte, 1995; birds: Kalloniatis and Fletcher, 1993; mammals: Davanger et al., 1991; Crooks and Kolb, 1992; Jojich and Pourcho, 1996; Kalloniatis et al., 1996). GLU has also been reported in a GABA-negative horizontal cell type in goldfish (Marc et al., 1995) and in some horizontal cells of the tiger salamander (Yang and Yazulla, 1994). The colocalization of GLU with GABA or GLY has been observed in amacrine cells of tiger salamander (Yang and Yazulla, 1994), amacrine and horizontal cells of chicken (Kalloniatis and Fletcher, 1993), and amacrine cells of some mammals (Jojich and Pourcho, 1996). The current results indicate the presence of immunoreactivity to GLU in some cell types of the retina of postmetamorphic sea lamprey, including some horizontal and amacrine cells. As reported for goldfish, the GLU-ir horizontal cells of lamprey (inner row of horizontal cells; HC2 cells) do not correspond to the faint GABA-ir horizontal cells (outer row; HC1 cells). In postmetamorphic lamprey retina, photoreceptors and small bipolar cells exhibit only faint GLU immunoreactivity.

Biplexiform ganglion cells of larval lamprey are demonstrated via GLU immunocytochemistry

The presence of biplexiform ganglion cells has been reported from studies using retrograde tract-tracing in larval (De Miguel et al., 1989; Anadón et al., 1998) and adult (Fritzsch and Collin, 1990; Rio et al., 1998) lamprey retinae. Ultrastructural observations indicate that these ganglion cells, first described for the primate (Mariani,

Fig. 7. Photomicrographs of vertical sections of the postmetamorphic retina showing the immunoreactivity to GABA (A–E), glutamate (F), glycine (G), aspartate (H), dopamine (I), and serotonin (J,K). **A:** Section showing strong GABA immunoreactivity in some amacrine cells and moderate immunoreactivity in type 1 horizontal cells and in some bipolar cells (open arrows). The arrowheads point to appendages of horizontal cells in the OPL. Note Landolt's clubs of bipolar cells coursing to the outer limiting membrane (solid arrows). **B:** Detail of the Landolt's club (arrow) of a GABA-ir bipolar cell with perikaryon located among horizontal cells. **C:** Detail of dendritic appendages of GABA-ir horizontal cells. **D:** Detail of axons of GABA-ir horizontal cells coursing below the perikarya (thick arrows) and ascending to the OPL (thin arrow). **E:** Detail of several putative retinopetal GABA-ir fibers coursing in a fascicle of optic fibers close to amacrine cells of the INL. **F:** Section showing abundant glutamate immunoreactivity in HC2 horizontal cells (open arrow) and in other neurons of the INL and the IPL. Thin arrows, small bipolar cells; arrowheads, large bipolar cells with perikarya just below the outer plexiform layer. **G:** Section showing the distribution of glycine-ir amacrine cells in the INL and IPL. The arrowhead points to the outer plexiform layer showing a few GLY-ir boutons. **Inset:** Detail of a glycinergic interplexiform cell (open arrow) showing an ascending process (thin arrow) coursing to the OPL (arrowhead). **H:** Section showing a large aspartate immunoreactive ganglion cell (double arrow) and other cells showing abundant immunoreactivity in the INL and IPL (single arrows). **I:** Detail of a dopamine-ir amacrine cell of the INL. Note that DA-ir processes course in outer parts of the IPL. **J:** Serotonin-ir amacrine cells located in the INL. Note that 5HT-ir fibers mainly course in outer and inner sublaminae of the IPL. **K:** Section showing a serotoninergic displaced amacrine cell (arrow) that is close to the inner limiting membrane. A–H: Single channel projections of confocal stacks (inverted and converted to gray scale). I–K: Brightfield microscopy. In these photographs of the adult retina, the sclera is at the top, and the vitreum at the bottom. Scale bars = 20 μm in A,F–H; 20 μm in E (applies to B–E); 25 μm I–K.

1982), are postsynaptic to photoreceptors in larval (De Miguel et al., 1989) and adult (Rio et al., 1998) lamprey. Our immunocytochemical results reveal for the first time that the processes of larval ganglion cells ascending toward the OPL (in the central retina) or through the neuroblastic layer (in the lateral retina) are GLU-ir. These processes can be distinguished clearly from retinopetal fibers, which are GABA-ir and do not ascend to the OPL/neuroblastic layer (Anadón et al., 1998; present results), further suggesting that the parent cells are glutamatergic. Although biplexiform cells have been reported in the retinae of other vertebrates (anurans: Toth and Straznicky, 1989; bony fishes: Cook and Becker, 1991; Cook et al., 1992; Collin and Northcutt, 1993; mice: Doi et al., 1995), neither the contacts with photoreceptors nor the neurotransmitter(s) used by these cells have been investigated.

An intriguing observation is the presence of conspicuous GLU-ir processes ascending to the outer limiting membrane in the limit between the marginal pure neuroblastic region and the larval lateral retina. Similar processes and occasional bipolar ganglion cell perikarya resulted in labeling in the same transition region after application of horseradish peroxidase to the optic nerve of larval lampreys (De Miguel et al., 1989). The present observations preclude that these processes were retinopetal fibers, so the most plausible explanation is that they represent processes of recently differentiated ganglion cells that migrated to the ganglion cell layer that maintain transiently a process contacting the outer limiting membrane, in a manner roughly similar to the Landolt's club of some bipolar cells of the adult lamprey retina (Dalil-Thiney et al., 1994; Villar-Cheda et al., 2006).

GLY immunoreactivity in the larval retina

In the larval lamprey, GLY immunoreactivity was observed in photoreceptors and in some cells in inner retinal regions containing putative ganglion cells. Presumably, the photoreceptors are the same cells that express GLU immunoreactivity, although this was not directly assessed. The distribution of GLY-ir cells of the inner retina is rather similar to that of GLU, at least in the lateral retina. For the central retina, we did not observe GLY-ir bipolar cells similar to those immunolabeled for GLU or ascending processes of putative ganglion cells. Moreover, the large, strongly GLU-ir ganglion cells detected by immunocytochemistry (present results) and HRP transport (De Miguel et al., 1989) near the optic nerve exit appear to be very faintly GLY-ir. Whether this finding indicates that ganglion cells are neurochemically heterogeneous, that some cells labeled near the IPL/optic fiber layer are in fact not ganglion cells but immature amacrine cells, or that GLU/GLY differences are due simply to the relatively low levels of GLY present in these cells/processes is not yet known.

The distribution of GLY in other larval retinas has been investigated only in tiger salamander (Yang and Yazulla, 1988; Li et al., 1990). In that system, GLY immunoreactivity is found primarily in amacrine cells, in cells of the ganglion cell layer that may be displaced amacrine cells, and rarely in bipolar cells (Yang and Yazulla, 1988). This distribution is different from that observed in larval lamprey, perhaps because of several unique features of the larval lamprey eye (see below), because GLY immunoreactivity in the postmetamorphic lamprey retina is restricted primarily to amacrine and interplexiform cells (present observations). In rabbit, GLY appears to provide an excitatory drive during early retinal development, playing a developmentally regulated role in the initiation and propagation of spontaneous retinal waves (Zhou, 2001). The existence of GLY in photoreceptors and some cells (possibly amacrine cells) of the innermost layer in the larval retina may also serve a developmental function.

The GABA-ir retinopetal fibers of larval lamprey are GLU and GLY negative

The retinopetal system of lampreys has been studied extensively (Vesselkin et al., 1980, 1984, 1989, 1996; De Miguel et al., 1989; Rio et al., 1993, 2003; Rodicio et al., 1995; Anadón et al., 1998; Meléndez-Ferro et al., 2002a). Several immunocytochemical studies have revealed the presence of GABA in retinopetal cells of the midbrain and in fibers of the IPL, both in adult and in larval lampreys (Rio et al., 1993; Vesselkin et al., 1996; Anadón et al., 1998; Meléndez-Ferro et al., 2002a). An experimental electron microscopic study in adult lamprey found that GABA-ir fibers account for 45% of all tracer-labeled retinopetal fibers but that GLU immunoreactivity is present in all retinopetal fibers (Rio et al., 2003). The present results obtained with double-immunofluorescence and spectral confocal microscopy in the larval retina confirm the presence of boutons of GABA-ir retinopetal fibers in the larval IPL/optic fiber layer, where they appear to contact ganglion cell perikarya. Moreover, these GABA-ir fibers and boutons do not show GLU or GLY immunolabeling above background level. Finally, our results regarding GLU-positive/GABA-negative retinopetal fibers in larval retinae are inconclusive, in that the numerous GLU-ir processes of ganglion cells in the IPL/optic nerve layer preclude distinguishing these retinopetal fibers with the present methods.

Neurotransmitters of the postmetamorphic lamprey retina

The retinae of recently metamorphosed sea lamprey show a layered distribution of immunoreactivities to the neurotransmitters investigated. Our results reveal numerous GABA-ir amacrine cells in the inner part of the INL, in agreement with results in other vertebrates (Mosinger et al., 1986; Osborne et al., 1986; Connaughton et al., 1999). A small portion of these cells were also GLY immunoreactive, as was also observed in a lizard (Sherry et al., 1993). Faint to moderate GABA immunoreactivity was observed in the outer row of horizontal cells, in line with results in teleost retinas (Osborne et al., 1986; Connaughton et al., 1999). Although GABA immunoreactivity was noted in some small bipolar cells of sea lamprey, similar to that reported in amphibians (Mosinger et al., 1986; Osborne et al., 1986; Yang and Yazulla, 1994; Yang et al., 2003), the significance of these cells is not well understood. It has been proposed that these GABA-ir bipolar cells corelease GLU and GABA, thereby contributing to modulation of ganglion cell responses (Yang and Yazulla, 1994).

Our results reveal for the first time the presence of GLY immunoreactivity in numerous amacrine cells of the lamprey retina. GLY-ir orthotopic amacrine cells are generally located more externally in the INL than the GABA-ir amacrine cells, and displaced GLY-ir cells are rather abundant in the innermost region of the IPL. Some small GLY-ir cells of the INL are interplexiform glycinergic

cells, as indicated by the presence of processes coursing to the OPL. The presence of GLY-ir interplexiform cells has also been observed in teleosts (Marc and Lam, 1981; Kalloniatis and Marc, 1990; Yazulla and Studholme, 1990; Connaughton et al., 1999), amphibians (Yang and Yazulla, 1988; Vitanova et al., 2004), reptiles (Eldred and Cheung, 1989; Sherry et al., 1993), and chicks (Kalloniatis and Fletcher, 1993). This GLY distribution is roughly similar to that reported in teleosts (Yazulla and Studholme, 1990; Connaughton et al., 1999). Our results also indicate that GLU immunoreactivity is abundant in cells and processes of the INL and IPL, with high levels in inner horizontal cells and in other INL cells (ganglion cells, large bipolar cells, putative amacrine cells). Colocalization of GABA and GLU was observed in some amacrine cells. The presence of GLU immunoreactivity in some GABA- or GLY-ir amacrine cells has been also reported in the retina of tiger salamander (Yang, 1996). Aspartate immunoreactivity is abundant in putative ganglion cells, including giant ganglion cells, as well as in some amacrine cells, and moderate levels were also observed in inner horizontal cells, suggesting colocalization of aspartate and GLU in some cell populations.

Our results with antibodies against both dopamine and TH do not reveal immunoreactive processes in the OPL of the postmetamorphic sea lamprey retina, confirming the identify of the dopaminergic cells as amacrine cells (Yáñez and Anadón, 1994). Although the presence of dopaminergic interplexiform cells in the sea lamprey retina has been reported with an antibody against TH (De Miguel and Wagner, 1990), this staining might be due to cross-reaction with other substances. Unlike the case in sea lamprey, many teleost retinas show a well-developed dopaminergic interplexiform cell system (Osborne et al., 1984; Yazulla and Zucker, 1988; Kalloniatis and Marc, 1990; Wagner and Behrens, 1993; Frohlich et al., 1995), although, in pure-rod teleost retinae, dopaminergic cells are amacrine cells (Frohlich et al., 1995). The serotonin-ir amacrine cells of the postmetamorphic sea lamprey retina are mostly orthotopic, and their processes branch in outer and/or inner sublaminae of the IPL. These amacrine cells are similar to those described for river lamprey (Versaux-Botteri et al., 1991).

Functional considerations

The eye of the larval lamprey is covered by a thick, nontransparent skin and has an immature lens, indicating that it is not an image-forming eye (Kleerekoper, 1972). Thus, larval lampreys must wait several years until metamorphosis to acquire a truly functional, camera-type eye. However, the larval central retina shows numerous traits suggesting functional maturation beginning in early posthatching stages, including the presence of opsin-expressing photoreceptors with well-developed outer segments (Meléndez-Ferro et al., 2003), the presence of brain-projecting ganglion cells and of centrifugal fibers (De Miguel et al., 1989; Anadón et al., 1998), and the expression of GLU in cells of the vertical pathway (present results). Together, these traits strongly suggest that at least some parts of the larval retina are functional, but perhaps as a nondirectional or broadly directional photoreceptive organ like a simple ocellus. In this regard, the absence of neurotransmitter-ir amacrine cells throughout the entire larval period may be interpreted in terms of immaturity of the image-forming circuitry, which requires opposing influences to extract the different qualities of the image (movement vs. static background, light/dark contrast, etc). In some way, the larval lamprey eye is comparable to the lamprey pineal organ, which also lacks image-forming circuitry.

It is likely that the functional roles of GLU and GABA in the retina of larval lamprey depend on the specific neuronal types and retinal regions. Although GLU and GABA are viewed as the major excitatory and inhibitory neurotransmitters in the vertebrate brain, their various actions depend on developmental stage and on the nature and subcellular distribution of pre- and postsynaptic ionotropic and metabotropic receptor subtypes(s) (Ben-Ari et al., 1990; Cherubini et al., 1991, 1998). It is well documented that GABA acts as an excitatory neurotransmitter during retinal development, because of the high membrane potential of developing neurons and/or action through elevation of Ca^{2+} levels (Redburn-Johnson, 1998).

In addition to its functions in the adult vertebrate retina, GLU appears to participate in several processes that occur during development. In the developing cat retina, stratification of ON and OFF ganglion cell dendrites depends on activity mediated by metabotropic GLU receptors (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995). In addition, responses of early fetal ganglion cells to ionotropic GLU receptor agonists before synaptogenesis in the INL appear remarkably similar to those of postnatal cells: GLU and AMPA produce fast desensitizing currents, kainate yields large steady-state currents, and the application of N-methyl-D-aspartate results in multiple channel openings (Liets and Chalupa, 2001). Ganglion cells in the developing chick retina (E5–E6 stages) begin to express ionotropic GLU receptor subunits before any distinction of an IPL has occurred (Silveira dos Santos Bredariol and Hamassaki-Britto, 2001). Insofar as the appearance of the chick embryo retina at these stages is remarkably similar to that observed in the lateral retina of larval lamprey, it is conceivable that the GLU-ir ganglion cells observed there are also GLU receptive. Although it may be assumed that larval lamprey photoreceptors, as with those of other vertebrates, are hyperpolarized by light and release GLU in the dark, the actual functions of these neurotransmitters in the retina cannot be deduced from their distributions. Moreover, the precise actions of GLU and GABA may be different in the central and in the lateral regions of the larval lamprey retina. In the lateral retina, GABA released by retinopetal fibers is probably the most influential neurotransmitter: perhaps GABA exerts some excitation on differentiating GLU-ir ganglion cells that are deprived of sensory vertical input long term. In addition, a role for GLU released by the lateral retinal ganglion cells, or by glutamatergic retinopetal fibers if they are present, on the maintenance or differentiation of this part of the retina cannot be ruled out. In the central retina, however, the glutamatergic vertical neuronal system appears functional, and it is more probable that GABA acts as an inhibitory neurotransmitter.

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