Organization of the Torus Longitudinalis in the Rainbow Trout (Oncorhynchus mykiss): An Immunohistochemical Study of the GABAergic System and a DiI Tract-Tracing Study

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ABSTRACT

The torus longitudinalis (TL) is a tectum-associated structure of actinopterygian fishes. The organization of the TL of rainbow trout was studied with Nissl staining, Golgi methods, immunocytochemistry with antibodies to γ-aminobutyric acid (GABA), glutamic acid decarboxylase (GAD), and the GABA<sub>2</sub> receptor subunits δ and δ2/β3, and with tract tracing methods. Two types of neuron were characterized: medium-sized GABAergic neurons and small GABA-negative granule cells. GABA<sub>2</sub> receptor subunit δ-like immunoreactivity delineated two different TL regions, ventrolateral and central. Small GABAergic cells were also observed in marginal and periventricular strata of the optic tectum. These results indicate the presence of local GABAergic inhibitory circuits in the TL system. For tract-tracing, a lipophilic dye (DiI) was applied to the TL and to presumed toropetal nuclei or toral targets. Toropetal neurons were observed in the optic tectum, in pretectal (central, intermediate, and paracommissural) nuclei, in the subvalvular nucleus, and associated with the pretectocerebellar tract. Torofugal fibers were numerous in the stratum marginale of the optic tectum. Toropetal pretectal nuclei also project to the cerebellum, and a few TL cells project to the cerebellar corpus. The pyramidal cells of the trout tectum were also studied by Golgi methods and local DiI labeling. The connections of trout TL revealed here were more similar to those recently reported in carp and holocentrids (Ito et al. [2003] J. Comp. Neurol. 457:202–211; Xue et al. [2003] J. Comp. Neurol. 462:194–212), than to those reported in earlier studies. However, important differences in organization of toropetal nuclei were noted between salmonids and these other teleosts. J. Comp. Neurol. 503:348–370, 2007.

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Indexing terms: torus longitudinalis; GABA; GABA<sub>2</sub> receptors; pretectum; optic tectum; connections; teleosts

The torus longitudinalis (TL) is a structure associated with the optic tectum only found in actinopterygian fishes; it consists of two longitudinal ridges protruding from the optic tectum midline into the mesencephalic ventricle. This late-appearing structure originates from a midline ventricular zone of the midbrain tectum that is clearly separated from the proliferating region giving rise to the cerebellar valvula (Candal et al., 2005a). The TL mostly consists of densely packed small granule-like cells. Early studies of the TL with the Golgi method (Sala, 1895; Ramón, 1899; Catois, 1901) revealed small neurons projecting to the superficial fiber layer of the tectum (“optic fiber layer” of Ramón), as well as fibers entering from the tectal commissure. More recent studies have shown a close relationship between the TL and the optic tectum (Ito and Kishida, 1978; Vanegas et al., 1979, 1984a; Grover and Sharma, 1981; Luiten, 1981; Fiebig et al.,


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Received 27 September 2006; Revised 21 December 2006; Accepted 13 February 2007

DOI 10.1002/cne.21363

Published online in Wiley InterScience (www.interscience.wiley.com).
1983; Wullimann and Northcutt, 1990; Wullimann and Roth, 1994).

These studies reveal massive projections of very thin fibers (marginal fibers) from the TL to the most superficial layer of the optic tectum, the stratum marginale, a layer found exclusively in actinopterygian tecta of relative thickness variable among the different species (Kishida, 1979). In this layer of the optic tectum, thin parallel-like fibers originating from the torus establish synaptic contacts with thorny dendrites of a special type of neurons in a way that is reminiscent of contacts of parallel fibers in the molecular layer of cerebellar cortex. These special cells have been named pyramidal cells (Ramón, 1899; Vanegas et al., 1974, 1984a) or type I cells (Meek and Schellart, 1978).

The optic tectum also projects to the TL (Luiten, 1981). Hodological studies involving direct application of neural tracer to the TL were performed in carp (Ito and Kishida, 1978; Ito et al., 2003), in an osteoglossomorph (Pantodon: Wullimann and Roth, 1994), and in holocentrids, the te-lossen family with probably the most developed TL (Kishida, 1979; Xue et al., 2003). Other tract-tracing studies have revealed torotectal connections after tracer application to the optic tectum (Luiten, 1981; Fiebig et al., 1984a) or type I cells (Meek and Schellart, 1978). Among these subunits, the δ subunit appears to be bicuculline and insensitive to baclofen) mediate the bulk of rapid inhibitory transmission in the brain. GABA_A receptors are ligand-gated heteropentameric Cl⁻ ion channels and the site of action of a variety of pharmacologically and clinically important drugs (Ernst et al., 2005). In mammals, they consist of at least 19 subunits pertaining to different families (α1–α6, β1–β4, γ1–γ3, δ, ε, η, π, ρ1–ρ3) that are expressed differentially throughout the brain. The subunit composition of GABA_A receptors affects functional properties of these receptors, including regulation by benzodiazepines (Sieghart, 1995; Scholze et al., 1996; Ernst et al., 2005). Among these subunits, the δ subunit appears to be ex-

**Abbreviations**

| AT     | anterio r thalamic nucleus of Holmgren (= nucleus glo-
merulosus) |
<table>
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<tbody>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CB</td>
<td>cerebellum</td>
</tr>
<tr>
<td>CPT</td>
<td>central pretectal nucleus</td>
</tr>
<tr>
<td>D</td>
<td>diffuse nucleus of the HL</td>
</tr>
<tr>
<td>Dc</td>
<td>central nucleus of the dorsal telencephalon</td>
</tr>
<tr>
<td>Dl+Di</td>
<td>dorsal plus lateral region of the dorsal telencephalon</td>
</tr>
<tr>
<td>ET</td>
<td>eminentia thalami</td>
</tr>
<tr>
<td>fr</td>
<td>fasciculus retroflexus</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GE</td>
<td>granular eminence</td>
</tr>
<tr>
<td>H</td>
<td>habenula</td>
</tr>
<tr>
<td>HL</td>
<td>inferior hypothalamic lobe</td>
</tr>
<tr>
<td>IN</td>
<td>interpeduncular nucleus</td>
</tr>
<tr>
<td>IP</td>
<td>intermediate pretectal nucleus</td>
</tr>
<tr>
<td>LR</td>
<td>lateral recess</td>
</tr>
<tr>
<td>LTN</td>
<td>lateral tuberal nucleus</td>
</tr>
<tr>
<td>LV</td>
<td>lateral nucleus of the valvula</td>
</tr>
<tr>
<td>MLF</td>
<td>medial longitudinal fascicle</td>
</tr>
<tr>
<td>NI</td>
<td>nucleus thalami</td>
</tr>
<tr>
<td>nMLF</td>
<td>nucleus of the medial longitudinal fascicle</td>
</tr>
<tr>
<td>nR</td>
<td>nucleus of the retina</td>
</tr>
<tr>
<td>OB</td>
<td>olfactory bulb</td>
</tr>
<tr>
<td>OC</td>
<td>optic chiasm</td>
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**The Journal of Comparative Neurology. DOI 10.1002/cne**

**THE TORUS LONGITUDINALIS OF TROUT**

1983; Wullimann and Northcutt, 1990; Wullimann and Roth, 1994).

These studies reveal massive projections of very thin fibers (marginal fibers) from the TL to the most superficial layer of the optic tectum, the stratum marginale, a layer found exclusively in actinopterygian tecta of relative thickness variable among the different species (Kishida, 1979). In this layer of the optic tectum, thin parallel-like fibers originating from the torus establish synaptic contacts with thorny dendrites of a special type of neurons in a way that is reminiscent of contacts of parallel fibers in the molecular layer of cerebellar cortex. These special cells have been named pyramidal cells (Ramón, 1899; Vanegas et al., 1974, 1984a) or type I cells (Meek and Schellart, 1978).

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pressed specifically in the cerebellum of mammals (Benke et al., 1991; Laurie et al., 1992; Wisden et al., 1992; Pirker et al., 2000) and thus might be used to identify cerebellum-like systems in other vertebrate groups. In mammals, the subunit δ confers a neurosteroid-insensitive phenotype to recombinant GABA<sub>A</sub> receptors in vitro (Zhu et al., 1996).

Biochemical and binding studies in teleosts have revealed the presence of GABA<sub>A</sub> receptors in the brain (Corda et al., 1989; Pirone et al., 2006) and retina (Lin and Yazulla, 1994). The highest benzodiazepine binding site concentration in the brain was found in the optic tectum (Corda et al., 1989; Pirone et al., 2006). The distribution of immunoreactivity to the GABA<sub>A</sub> receptor subunit β2/3 has been reported in the brain of the Atlantic salmon (Anzelius et al., 1995): strong neuropil labeling was observed in some brain regions, including the optic tectum. The distribution of the GABA<sub>A</sub> receptor α1 and α3 subunits was studied immunocytochemically in the goldfish retina (Klooster et al., 2004). The distribution of other GABA<sub>A</sub> receptor subunits in teleost brains, including the δ subunit, has not been investigated.

Teleosts are the most numerous group of vertebrates, including more than 20,000 species. Salmonids (Protacanthopterygii) are basal euteleosts considered a sister group of acanthopterygians (advanced teleosts that include the Holocentrids). Accordingly, its phylogenetic position is intermediate between this group and the cyprinids (Ostariophysean), the only groups in which the circuits of the TL system have been examined in detail. A large number of developmental, immunohistochemical, and hodological studies have focused on the brain of trout and closely related salmonids (Pouwels, 1976; Shiga et al., 1985, 1989; Oka et al., 1986; Ekström and Ebbesson, 1989; Amano et al., 1991; Holmqvist and Ekström, 1991, 1995; Vecino et al., 1991, 1992, 1995; Manso et al., 1993, 1994; Becerra et al., 1995; Yáñez and Anadón, 1996; Yáñez et al., 1996, 1997; Castro et al., 1999, 2001, 2003; Pérez et al., 2000; Díaz et al., 2001; Folgueira et al., 2002, 2003, 2004a,b, 2005, 2006; Rodríguez et al., 2003; Candal et al., 2005a,b; Kinoshiba et al., 2006), which now represents an excellent model of the brain organization of generalist teleosts. However, there have focused on the brain of trout and closely related salmonids and has also revealed new aspects of the cytoarchitectural, hodological, and neurochemical diversity of the TL in teleost lineages.

The aims of the present study were 1) to characterize the neurons and nuclear connections involved in the TL system of a salmonid, the rainbow trout and 2) to compare the cytoarchitectural and connectional patterns with those known in cyprinids and holocentrids, the only teletost groups in which these circuits have been investigated in detail. For these goals, the cytoarchitecture and connections of the TL of the rainbow trout were investigated by using Golgi methods and applications of a lipophilic fluorescent tracer in fixed brains. A further aim was to characterize immunohistochemically the GABAergic system in the TL and torus-related structures by using antibodies to GABA, GAD, and the GABA<sub>A</sub> receptor subunits δ and β2/3. This study has improved our knowledge of the organization and neurochemistry of the brain centers in salmonids and has also revealed new aspects of the cytoarchitectural, hodological, and neurochemical diversity of the TL in teleost lineages.

**MATERIALS AND METHODS**

Young adult rainbow trout (*Oncorhynchus mykiss*; 5–18 cm in standard body length) were used in experiments. These animals were obtained from a local fish farm and maintained in well-aerated aquaria for a brief period until use. Prior to experiments, trout were deeply anesthetized by immersion in a 0.05% solution of tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO). All procedures conformed to the European Community Guidelines on Animal Care and Experimentation and were approved by the Ethics Committees of the Universities of A Coruña and Santiago de Compostela.

**Fixation**

For tract-tracing experiments, 45 trout were perfused with cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion, brains were removed from the skulls and maintained in the same fixative at 4°C. For Nissl staining, two trout were fixed as above, and their brains were embedded in paraffin wax and sectioned serially on a rotary microtome (10 μm thick). For Golgi staining, 11 trout were fixed by vascular perfusion with 2% glutaraldehyde in 0.1 M PB at pH 7.4, and their brains left for 1 day in fixative before chromate impregnation. Brains were embedded in 3% agarose and sectioned on a Vibratome (100 μm thick). For GAD and GABA<sub>A</sub> receptor subunit immunocytochemistry, the brains of four trout were fixed by vascular perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS), cryoprotected in 30% sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Frozen blocks were sectioned on a cryostat. For GABA immunocytochemistry, the brains of four trout were fixed by vascular perfusion with 5% glutaraldehyde in PBS containing 1% sodium metabisulfite, cryoprotected, frozen, and sectioned on a cryostat.

**Nissl and Golgi methods**

For Nissl staining, paraffin sections were dewaxed and stained with 0.01 cresyl violet and 0.01% thionin in 0.1 M acetate buffer (pH 5.6). Golgi staining was done by using the Golgi-Colonnier (Colonnier, 1964) method. Briefly, after 1 day in fixative, brains were immersed in 25% glutaraldehyde and 3% Cr<sub>2</sub>O<sub>7</sub>OK for 3–6 days in the darkness and then immersed in 0.75% AgNO<sub>3</sub> for 3 days in the dark at 22°C. The brains were embedded in agarose and sectioned (100 μm thick) on a Vibroslice vibration microtome (Campden, Sileby, UK).

**Western blotting**

The antisera against GAD used here has been previously characterized by Western blot in dogfish and rat (Sueiro et al., 2004; Table 1). To assess the specificity of this antibody in other fishes, extracts of two trout brains were analyzed by Western blot following the same procedure as for choline acetyltransferase (Anadón et al., 2000), by using as positive control rat brain extracts subjected to identical analyses. Protein extracts of brains of dogfish (*Scyliorhinus canicula*) and sturgeon (*Acienser baeri*) were submitted to the same procedure. For Western blotting of GABA<sub>A</sub> receptor subunit δ, brains of two adult trout were mechanically homogenized; after a brief centrifugation, pellets were processed for obtaining the membranous fraction following the protocol of Nusser et al.
Polyclonal rabbit antiserum (polyclonal rabbit anti-GAD, Chemicon; polyand then incubated with 10% normal goat serum for 1

Polyclonal rabbit treated with 3% H2O2 to eliminate endogenous peroxidase

Tissue sections (16–18

For GAD and GABA receptor subunits, parallel transverse sections (16–18 μm thick) were processed by using the peroxidase anti-peroxidase (PAP) method for the different neurochemical markers. The sections were pre-treated with 3% H2O2 to eliminate endogenous peroxidase and then incubated with 10% normal goat serum for 1 hour, followed by incubation in the corresponding primary antiserum (polyclonal rabbit anti-GAD, Chemicon; polyclonal rabbit anti-GABA receptor subunit δ, kindly provided by Dr. Werner Sieghart; or mouse monoclonal anti-GABA receptor subunit β3/β3, Chemicon, Temecula, CA; dilution 1:10,000). The immune complex was visualized with a rapid electrochemoluminescent detection system (ECL Western blotting system; Amersham, Buckinghamshire, UK) and exposed onto Hyperfilm-ECL (Amersham). Precipitation of the antibody with the immunization peptide (5 μg peptide/ml; amino acids 1–44 fusion protein; kindly donated by Prof. W. Sieghart) completely abolished the staining of blots. Protein extracts of brain membranes of dogfish (Scyliorhinus canicula) and sturgeon (Acipenser baeri) were submitted to the same procedures.

**Immunocytochemistry**

For GAD and GABA receptor subunits, parallel transverse sections (16–18 μm thick) were processed by using the peroxidase anti-peroxidase (PAP) method for the different neurochemical markers. The sections were pre-treated with 3% H2O2 to eliminate endogenous peroxidase and then incubated with 10% normal goat serum for 1 hour, followed by incubation in the corresponding primary antiserum (polyclonal rabbit anti-GAD, Chemicon; polyclonal rabbit anti-GABA receptor subunit δ, kindly provided by Dr. Werner Sieghart; or mouse monoclonal anti-GABA receptor subunit β3/β3, Chemicon; dilutions of 1:1,000, 1:50, and 1:100, respectively) overnight (see Table 1 for characteristics of these antibodies). The sections were then successively rinsed in PBS at pH 7.4 (two 10-minute rinses), incubated in goat anti-rabbit (Sigma; 1:100) or goat anti-mouse serum (Sigma; 1:30) for 1 hour, rinsed twice in PBS (10 minutes each), and incubated in rabbit or mouse PAP complex (Sigma, 1:400; 1:500) for 1 hour. The immunoreaction was developed with 0.005% diaminobenzidine (DAB; Sigma) and 0.003% H2O2. All dilutions were done in PBS containing 0.2% Triton X-100 and 2% bovine serum albumin (BSA), and incubations were done in a humid chamber at room temperature.

For GABA immunocytochemistry, we used a polyclonal rabbit anti-GABA antibody (Affiniti, Mamhead, UK; Table 1). The procedure used was similar to that for GAD. The sections were treated with H2O2 and incubated with 10% normal goat serum (Vector, Burlingame, CA) and then with the polyclonal anti-GABA antibody, diluted to 1:1,000, overnight. After rinsing the sections were sequentially incubated with a goat anti-rabbit immunoglobulin (Sigma; 1:100) and PAP (Sigma; diluted to 1:400) and developed with DAB and H2O2 as indicated above. All antibody and rinsing solutions were done in TBS, pH 7.4, containing 0.2% Triton X-100, 3% normal goat serum, and 1% metabisulfite. After developing, sections were rinsed in TBS, dehydrated, and coverslipped. This polyclonal rabbit anti-GABA antibody was previously used for demonstrating the GABAergic systems in a number of species, including amphibious, lampreys, dogfish, and trout (Yáñez et al., 1997; Anadón et al., 1998; Meléndez-Ferro et al., 2002; Sueiro et al., 2004).

**Tract-tracing**

For tracing experiments, the lipophilic carboxyamine tracer 1,1-dioctadecyl 3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) was applied to 45 paraformaldehyde-perfused brains according to the following four procedures:

1. In cases of tracer application to externally accessible areas, a small crystal of DiI was directly applied by
using an electrolytically sharpened insect pin under visual control with a stereomicroscope. The brain areas accessed by this method were the torus longitudinalis (10 cases) and the rostral (3 cases) and caudal lateral optic tectum (3 cases).

2. In seven cases, the optic tectum was carefully separated from the brain and inverted, and then DiI was applied to the TL from its inner side.

3. For DiI application to less accessible areas, the brain was previously embedded in 3% agarose and sectioned on a Vibratome under the stereomicroscope until the appropriate level was reached. The tracer was then applied as above. With this third procedure, DiI was applied to the pretectum (central, intermediate, and paracommissural nuclei; six total cases, two cases each), the TL (four cases), the eminentia thalami (two cases), and the torus semicircularis (four cases). In all these cases, the application point was sealed with melted agarose and brains were maintained in darkness in frequently renewed fresh fixative for 4–to 10 weeks at 37°C.

4. To label the pyramidal cells of the optic tectum, a solution of 3% DiI in dimethyl sulfoxide and ethanol (40:60) was applied to brains (n = 6) with a micropipette by using an electrophoresis source (Kation Scientific, Minneapolis, MN; time: 1–5 seconds; intensity: 0.5–0.8 µA), and the brains were left in fixative for 1–4 days. After incubation, transverse sections (50 µm) were cut on a Vibratome, mounted on slides, and examined with a Nikon E-1000 fluorescence microscope equipped with a rhodamine filter set.

Photography

The sections stained by Nissl and Golgi methods and by immunocytochemistry were photographed with a microscope equipped with a color digital camera. Most DiI-labeled sections were photographed by using black-and-white negative film, and selected film frames were scanned and digitalized with a film scanner. Some DiI-stained sections were photographed with a color digital camera (DXM1200, Nikon, Tokyo, Japan). Some other DiI-stained sections were photographed with a Leica spectral confocal microscope (Leica, Wetzlar, Germany) and processed with LITE software (Leica). The images were inverted to print them as positives, and their contrast and brightness were adjusted with Adobe Photoshop (Adobe Systems, San Jose, CA). Plates were assembled and lettered with Corel Draw (Corel, Ottawa, Canada).

RESULTS

Cytoarchitecture of the torus longitudinalis

The TL of rainbow trout extends along the tectum midline from just caudal to the posterior commissure to the caudal tectum. It consists of two cords of numerous small cells and scarce neuropil that are joined dorsally to the midline of the optic tectum (Fig. 1A,B). Numerous fiber bundles course between the torus and the tectum in this junction area, which apparently forms the only connection of the torus with other brain structures (Fig. 1A). In adult trout, Nissl staining reveals that the TL consists of at least two types of neuron according to cell size, the most numerous being small granule-like cells distributed throughout the torus, whereas somewhat larger cells were observed mainly in central and dorsal regions. Golgi staining of the TL revealed spherical or pear-shaped small cells with short, thin dendrites (Fig. 1C,D). In some instances, somewhat larger cells were stained in inner regions of the TL. These cells showed thicker dendrites and a short, profusely branched axon (Fig. 1E).

Western blotting and immunocytochemistry

The TL and related structures were investigated immunocytochemically with antibodies against GABA, GAD, and GABA<sub>δ</sub> receptor subunits δ and β2/β3. The antibodies against GAD and GABA<sub>δ</sub> receptor subunits δ were characterized by blotting. Western blotting of brain extracts of trout and rat revealed that the GAD antiserum stained doublets of 65 and 67 kD in rat and dogfish but apparently a single band of about 65 kD in sturgeon and trout (Fig. 2A). Western blotting of brain membrane extracts with antibodies against subunit δ led to labeling of single band of slightly lower molecular weight in fishes than in rat brain extracts (about 50 kD and 52–54 kD, respectively; Fig. 2B). This immunostaining was blocked in blots after preabsorption of the primary antibody with the amino acids 1–44 fusion protein used for immunization. Moreover, the immunostaining pattern of this antibody in trout and rat cerebellum was similar, mainly revealing granule cell processes in the granule cell layer, as previously reported in rat (Benke et al., 1991). These immunoblotting and immunocytochemical results suggest that the antibody against the rat subunit δ recognizes homologous proteins in trout, although the possibility that in the trout it recognizes an unrelated protein(s) cannot be ruled out. Accordingly, the staining produced by this antibody will be considered here as subunit δ-like immunoreactivity.

Torus longitudinalis

GAD and GABA. Immunocytochemistry with GAD and GABA antibodies indicated that the TL was neurochemically heterogeneous. GAD and GABA immunohistochemistry in adult trout revealed a population of medium-sized immunoreactive neurons distributed mainly in dorsal regions of the torus (Fig. 3A–C). These cells were polygonal or fusiform. A number of small GAD-immunoreactive (ir) boutons were distributed in conspicuous cords among GAD-negative small perikarya in the ventral region of the torus. These cords were intermingled with a numerous population of smaller GABA- and GAD-negative cells (Fig. 3C,D). In the dorsal region, the GAD-ir boutons appeared smaller and did not form such conspicuous cords (Fig. 3E).

GABA<sub>δ</sub> receptor δ and β2/β3 subunits. Immunocytochemistry directed toward the GABA<sub>δ</sub> receptor subunit δ also revealed a heterogeneous organization of the trout TL (Fig. 2F,G). In most of the ventral TL region, clear immunoreactivity to this subunit was found in a number of coarse cell processes located in conspicuous cords among islands of perikarya, whereas in the dorsal region subunit δ-like immunoreactive (δ-ir) processes were fainter. This dorsal TL region was crossed by moderately δ-ir axonal bundles coursing to the stratum marginale of the optic tectum. In the ventral region, there were abundant δ-ir small perikarya among the more numerous negative cells. In the dorsal region, δ-ir puncta could sometimes be observed on the surface of medium-sized perikarya. In addi-
tion, a periventricular ventrolateral region exhibited very scarce δ-ir structures.

Immunoreactivity to GABA<sub>δ</sub> receptor subunits β2/δ3 in the TL was also heterogeneous. Cords of subunits β2/δ3-ir processes were found close to the ventral and lateral periventricular regions only in a faintly δ-ir region (Fig. 3H). In other regions of the TL, only faint immunoreactivity to subunits β2/δ3 was observed.

**Optic tectum.** The trout optic tectum exhibited a laminar organization similar to that reported in other teleosts, which, following Vanegas et al. (1974; 1984a), could be grouped, from superficial to deepest, into six layers: the stratum marginale (SM), stratum opticum (SO), stratum fibrosum et griseum superficiale (SFGS), stratum griseum centrale (SGC), stratum album centrale (SAC), and stratum periventriculare (SPV) (Fig. 4A–C).

Descriptions of the GABAergic systems in the tectum have been restricted to TL-related centers. In the SM of the optic tectum, there were scattered GABA-ir boutons and processes (Fig. 4B). GABA immunocytochemistry also revealed the presence of small GABA-ir perikarya located in this layer and in the adjacent region (SO) (Fig. 4B). Most GABAergic cells of the trout optic tectum were located in the SPV, where these cells formed a large proportion of its neurons (Fig. 4A,C). GABAergic cells were scarce or very scarce in other tectal layers.
Immunoreactivity to GABA\(_\alpha\) receptor subunits \(\delta\) and \(\beta2/\beta3\) in the optic tectum revealed a complementary pattern. Immunoreactivity to subunits \(\beta2/\beta3\) was high in the SFGS and in the outer region of the SGC, except in a conspicuous negative horizontal band of the latter. In contrast, \(\beta2/\beta3\) immunoreactivity was faint in the SM and SPV (Fig. 4E). Intense \(\delta\)-ir neuropil was found in the SM, whereas in the SFGS and SAC, immunoreactivity to subunit \(\delta\) was low, and the SPV exhibited faint immunostaining (Fig. 4F). The SO and the SAC did not show significant immunoreactivity to GABA\(_\alpha\) receptor subunits \(\delta\) and \(\beta2/\beta3\).

**Torus longitudinalis-related brain structures.** Abundant immunoreactivity to GAD was observed in some torus-related pretectal nuclei. In the paracommissural nucleus, abundant GAD-ir boutons were observed (Fig. 5A,B), although this feature did not distinguish it from the other pretectal areas that were innervated by numerous GAD-ir boutons. The richest GAD-ir areas were found in the anterior pretectal nucleus of Butler et al., 1991). Numerous GABA\(_A\)-ir. Further characterization of GABA\(_A\) receptor subunit \(\delta\)-like immunoreactivity in trout brain structures is beyond the scope of this study.

**Connections of the torus longitudinalis**

**DiI application to the torus longitudinalis.** Application of small DiI crystals to the TL labeled cells and/or fibers found mainly in the diencephalon, mesencephalon, and cerebellum. The distribution of toropetal neurons in the brain is schematically indicated in Figure 6.

**Diencephalon.** After application of DiI to the TL, retrogradely labeled cells were observed in three pretectal nuclei: the central, the intermediate, and the paracommissural nuclei (Figs. 6A–C, 7A–C). The Nissl-stained series indicated that the central pretectal nucleus consisted of a population of medium-sized neurons located among tracts of optic fibers entering the rostral part of the optic tectum, and medial to the parvocellular superficial pretectal nucleus (data not shown). Scattered medium-sized bipolar neurons were labeled in the central pretectal nucleus; they showed dendrites coursing in a wide neuropil region (Fig. 7A). The intermediate pretectal nucleus of Holmgren (1920; = accessory pretectal nucleus of Butler et al., 1991) consists of an oblique band of neurons located dorsolaterally to the anterior thalamic nucleus of Holmgren (= posterior pretectal nucleus of Butler et al., 1991). Numerous cells were labeled in this nucleus after DiI application to the TL, forming a band of bipolar or pear-shaped cells and neuropils (Fig. 7C). The Nissl-stained series indicated that the paracommissural nucleus of trout juveniles consisted of a rather compact population of medium-sized neurons located near a prominence toward the tectal ventricle and lateral to the posterior commissure. DiI application to the TL labeled abundant cells of the paracommissural nucleus (Fig. 7C). After DiI application in toto to the TL, retrogradely labeled fibers were observed coursing from the pretectal nuclei (central, intermediate, and paracommissural nuclei) to the TL, entering the torus at the level of the posterior commissure.
Fig. 3. Photomicrographs of transverse sections of the TL showing structures immunoreactive to γ-aminobutyric acid (GABA; A,B), glutamate decarboxylase (GAD; C–E), GABA_A receptor subunit δ-like (F,G), and subunit β2/β3 (H). A,B: Note the abundance of GABA-ir cells (thin arrows) in the central regions of the TL. The open arrow points to the intertectal commissure. C–E: Note GAD immunoreactivity in neuron perikarya (arrowed in C and E) and in numerous cell processes among cords of small cells of the TL (D). F: Section showing intense subunit δ immunoreactivity in cell processes coursing among TL small granular cells. Open arrow, region of entrance of fibers. G: Detail showing moderately subunit-δ-like positive perikarya in the small-celled region of the TL. H: Section showing faint to moderate subunit β2/β3 immunoreactivity in the TL small granular cells. Scale bar 100 μm in A,C,F,H; 25 μm in B,D,E,G.
At more caudal levels, intermediate between the pretectum and the torus semicircularis, some labeled cells were associated with the pretectocerebellar tract, a conspicuous round tract that also contained numerous labeled fibers after DiI application to the TL (Fig. 7D). At rostral diencephalic levels, in toto applications of DiI to the TL labeled a compact group of small pear-shaped cells bilaterally in the eminentia thalami (rostral ventral thalamus; Figs. 6A, 7E). This eminence was located periventricularly below the habenula in the rostral diencephalon. In the Nissl-stained series of juveniles, this nucleus consisted of a dense periventricular population of small neurons and a sparser lateral population of larger cells. However, cases of localized application of DiI to the TL in sectioned brains failed to label cells in the eminentia thalami. These results, together with those exhibiting reciprocal labeling, suggest that the eminentia thalami does not project to the TL (see below).

**Midbrain and cerebellum.** At tegmental levels, in toto application of DiI to the TL led to bilateral labeling of some medium-sized cells in a nucleus situated just below the lateral nucleus of the valvula (Figs. 6E, 7F,G). In Nissl-stained sections, the cells of this nucleus formed a band close and parallel to the nucleus lateralis valvulae, from which it could be distinguished by the larger size of its cells and its projections to the TL. The trout subvalvular nucleus lay medial to the isthmic nucleus, which showed larger neurons with more conspicuous Nissl substance. These toropetal cells of trout probably correspond to the subvalvular nucleus reported in other teleosts (Ito...
Fig. 5. Photomicrographs of transverse sections of the pretectum (A–D) and cerebellum (E–G) showing structures immunoreactive to glutamic acid decarboxylase (GAD; A,B and E,F), and GABA_δ receptor subunit 6 (C,D and G). A,B: Section showing that GAD immunoreactivity is widely distributed in boutons of the pretectal region, including the paracommissural nucleus (open arrow in A) and adjacent pretectal areas (arrowhead in A). C,D: General view and detail of a section similar to that depicted in A, showing that subunit δ immunoreactivity is restricted to the paracommissural nucleus (open arrow in C). Note in D that some small perikarya are subunit δ-ir (thin arrows). The arrowhead in C points to the δ-negative region, which in A is full of GAD-ir processes. Asterisks (A,C), posterior commissure; star (A), fasciculus retroflexus.

E: Section of the ganglion cell layer of the cerebellum showing GAD immunoreactivity in Purkinje cells (arrowheads) and a Golgi cell (thin arrow). Note the outline of a large perikaryon covered with GAD-ir boutons (open arrow), probably pertaining to a eurydendroid cell (cerebellar projection cells characteristic of teleosts). F: Section of the granular layer showing small GAD-ir boutons (putative Golgi cell axon terminals; arrowheads) among cell cords. G: Section of the granular layer showing intense subunit δ-like immunoreactivity in cell processes (putative granule cell dendrites) among cell cords and faint to moderate staining in cords of granule cell perikarya (arrowheads). Scale bar = 200 μm in A,C; 50 μm in B,D–G.
Fig. 6. Schematic drawings of transverse brain section showing the distribution of labeled cells (circles) and fibers (lines) observed after tracer application to the TL (shaded area in C). Black circles indicate cells with confirmed projections to the TL; open circles indicate occasionally labeled cells; Asterisks (in the right half of sections) represent neurons labeled by in toto application of tracer to the TL but not labeled with more selective procedures (see text). Section levels are shown in the schematic lateral drawing of the brain (at the top left). For abbreviations, see list. Scale bar = 1 mm in E (applies to A–E).
et al., 2003; Xue et al., 2003). The subvalvular population was not labeled in experiments in which DiI was applied to the optic tectum (see below). Occasional retrogradely labeled cells were also observed in the lateral nucleus of the valvula itself.

In toto application of DiI to the TL labeled numerous cells in the medial nucleus of the torus semicircularis (Figs. 6D,E, 7G). However, only occasional cells were labeled after DiI application to the TL in sectioned brains. Moreover, no fibers projecting from this medial nucleus were observed in the TL in reciprocal experiments of DiI application to the torus semicircularis (see below).

After application of DiI to the TL from its inner side, fiber bundles and a number of very thin fibers were observed coursing in the SM of the optic tectum, a molecular-like layer covering the tectum in teleosts (Figs. 6B–E, 8A). Axon bundles coursed in the outermost region of this layer, whereas fine beaded fibers occupied more central regions (Fig. 8A). Owing to the small size of the DiI crystals used, sometimes terminal fibers were observed forming a discrete patch in the tectum. These axons ascended in small bundles from the deep fiber layer near the medial border of the optic tectum (Fig. 8B). A number of retrogradely labeled cells were observed in the SGC (Fig. 6B–E, 8A). The appearance of these cells was rather uniform, most of them having pear-shaped or spindle-shaped perikarya situated at different heights in the SGC, preferentially in the inner half of the SGC or near the SAC. These cells showed one or two thick ascending dendrites that branched in a thin sublayer between the SFGS and the SGC and formed a well-defined horizontal dendritic plexus (Fig. 8A). Occasional labeled cells showed
Fig. 8. Photomicrographs of transverse sections of the optic tectum showing labeled structures after application of DiI to the TL. A Photomicrograph showing numerous torofugal labeled fibers in the stratum marginale and toropetal labeled perikarya in the stratum griseum centrale. Note branching of apical dendrites of labeled neurons (open arrows) in the limit between this stratum and the stratum fibrosum et griseum superficiale, as well as the presence of crook-shaped fibers (thin arrows). Arrowheads point to thin beaded fibers coursing scattered in the inner part of the stratum marginale; double arrowheads point to compact bundles of labeled fibers in its outer region. In this case the TL was accessed for DiI application in sectioned brain. Inset: Detail of toropetal cells. B: Section of the tectum showing small bundles of torofugal fibers ascending to the stratum marginale near the TL. C, D: Sections of the optic tectum showing labeled fibers in the stratum opticum and tectotorus semicircularis tract (C) and labeled neurons in the stratum periventriculare (D) after in toto application of DiI to the TL. In A, C, and D, medial is to the right; in B medial is to the left. All photomicrographs were converted to gray scale, inverted, and printed as positive. For abbreviations, see list. Scale bar = 125 μm in A–D; 30 μm in inset to A.
perikarya situated in this sublayer. No basal dendrites of toroptetal cells were observed. The thin, varicose axon of labeled toroptetal cells was crook-shaped, ascending first to near the horizontal plexus formed by ascending dendrites, and then bending to course toward the SAC and turn toward the midline (Fig. 8A). In most toroptetal neurons, the axon arose from a dendrite. Occasional labeled cells were observed in the SPV. In toto application of DiI to the TL generally labeled other cells and fibers in the optic tectum, in addition to those observed above. These often included labeled fibers branching in the SO, numerous fibers coursing in intermediate and deep tectal levels between the tectum and the tegmentum, and labeled cells in strata other than the SGC, mostly in the SPV (Fig. 8C,D).

DiI application to the TL also occasionally labeled cells in the cerebellar body.

Reciprocal experiments. Some reciprocal tract-tracing experiments were performed in the eminentia thalami, pretectum, torus semicircularis, and optic tectum, in order to confirm the results obtained after direct DiI application to the TL.

Eminentia thalami. Application of DiI to the eminentia thalami labeled small neurons in the SPV and fibers in the SFGS and SAC of the optic tectum, but no labeled cells or fibers were observed in the TL (not shown). In turn, application of DiI to the optic tectum produced intense labeling of cells in the eminentia thalami (see below). Together, these results indicate that the eminentia thalami does not project to the TL but to the neighboring tectum.

Paracommissural nucleus. Application of DiI to the paracommissural nucleus labeled numerous fibers in the TL (Fig. 9A) and also the conspicuous pretectocerebellar tract. (For cerebellar connections of this tract in trout, see Folgueira et al., 2006.) In the TL, the thin and beaded labeled fibers coursed among the cords of cell perikarya.

Torus semicircularis. After DiI application to the torus semicircularis, anterogradely labeled fibers were observed in the ipsilateral SAC and SPV of the optic tectum. These fibers crossed the midline in the intertectal commissure, located just dorsally to the TL, to reach the contralateral optic tectum and torus semicircularis. Some retrogradely labeled cells were also observed in the contralateral torus semicircularis (results not shown).

Optic tectum. DiI applications were performed at different levels of the optic tectum. These reciprocal tract-tracing experiments led to the ipsilateral labeling of a number of cells and fibers in the TL (Fig. 9B). In sections with less densely labeled cases, the torus neurons were small and showed round perikarya and scarce, very thin dendrites (Fig. 9B–D). In some experiments, fibers, but not labeled perikarya, were observed in the contralateral TL. In these experiments, the trajectory of labeled fibers from the torus coursing to the SM could be followed. After their exit from the torus, the fibers coursed in the SAC for a short way before small fiber bundles were progressively deflected toward the SM.

Application of DiI to the optic tectum also led to labeling of neurons in a number of brain regions, mainly in the ipsilateral central area of the dorsal telencephalic lobes (ipsilateral; see Folgueira et al., 2004b), eminentia thalami, dorsomedial thalamus (ipsilateral), ventromedial thalamus (ipsilateral), nucleus subglomerulosus of Holmgren (1920; ipsilateral; see Folgueira et al., 2002), medial preglomerular nucleus (ipsilateral; see Folgueira et al., 2005), medial periventricular prectectum (bilateral), midbrain laminar nucleus of Ostholt et al. (1990; ipsilateral), medial nucleus of the torus semicircularis (bilateral), and nucleus isthmi (ipsilateral). A number of labeled cells were also observed in regions of the optic tectum away from the point of DiI application (results not shown). Interestingly, no labeled pretectal cells were observed in the intermediate and the paracommissural nuclei in these tectal experiments, indicating that these pretectal nuclei actually projected to the TL and not to the tectum. We have only observed occasional labeled cells in the pretectal central nucleus in our tectal DiI applications, but some cells projecting to the optic tectum from this region (termed the dorsal pretectal area) were reported by Ki-noshita et al. (2006).

Golgi staining and DiI labeling of pyramidal cells

The fibers of the SM mainly contacted dendrites of pyramidal cells (Vanegas et al., 1974, 1984a). To assess the morphology of pyramidal cells in trout, we performed a study with Golgi methods. In Golgi-stained preparations, trout pyramidal cell perikarya exhibited one or two thick apical dendritic trunks that branched three to five times dichotomously in the SM, giving rise to numerous oblique or horizontal branches studded with short dendritic appendages (Fig. 10A,B). These branches extended in a small, wide-angle, conical region around the point of entrance of the trunk in the SM. The thick descending dendritic trunk of pyramidal cells gave out short collaterals branching at one or two levels within the SFGS, before producing at its end a thinner descending dendrite that reached the middle of the SGC and branched in a horizontal sublayer. The thin axon originated from the end of the descending trunk or from other regions of the cells, coursing to and branching in the SFC. The presence of a thin descending terminal dendrite was not observed in all pyramidal cells. Golgi methods showed that perikarya of pyramidal cells may occupy different heights in the SO-SFGS, some cells exhibiting perikarya close to the SM and others at deeper levels.

For further characterization of the morphology and descending projections of trout pyramidal cells, we also performed iontophoretic application of DiI to the SM of the optic tectum followed by a short 1–2-day incubation, in addition to the DiI crystal applications intended for labeling of tectal connections. This local application of DiI labeled cells with a bipolar appearance exhibiting a thick descending dendrite that emitted short collaterals to two to three sublayers of the SFGS, and often a thinner dendrite that reached the SGC (Fig. 10C–E). These cells were clearly pyramidal cells (Fig. 10C), although in most cells apical dendrites were hardly appreciable because of the intense labeling of the SM. Very thin axons of these cells arose from the basal dendrite and coursed to a thin sublayer in the middle of the SGC, giving rise there to terminal branches (Fig. 10D–F). In these DiI applications to the SM, some radial glial cell processes (Fig. 10C) and occasional nonpyramidal cells were also labeled. Occasional cells labeled in the SGC with this procedure (Fig. 10D) might correspond to the small pyriform neurons with an axon ascending to the SM reported by Vanegas et al. (1974, 1984a).
Fig. 9. Sections of the TL showing fibers (A) and cells (B–E) labeled after application of DiI to the paracommissural nucleus (A) and optic tectum (B–E). A: Numerous anterogradely labeled beaded fibers course among cell cords of the TL (arrows). B: Large number of small neurons labeled in the ipsilateral torus after DiI application to the tectum. Note the fiber bundles passing toward the tectum (open arrow). C: Less densely labeled torus region in which the small size of labeled cells and their thin processes (arrows) are appreciable. D,E: Labeled small neuron showing short dendritic branches arising from the single common process (arrow). All photomicrographs were converted to gray scale, inverted, and printed as positive. For abbreviations, see list. Scale bar = 100 μm in A,B; 40 μm in C,D; 10 μm in E.
DISCUSSION

The present results have characterized for the first time cytoarchitecturally, hodologically, and neurochemically the TL system of a salmonid by using Golgi methods, immunohistochemistry to four GABAergic markers (GABA, GAD, and GABA_α receptor subunit β₂/β₃ and δ), and tract-tracing methods. Western blotting of trout brain extracts was also performed in order to establish the specificity of antibodies against GAD and GABA_α receptor subunit δ.

Western blots

The specificity of the GAD antibody was tested previously in our laboratory with Western blotting of brain extracts of dogfish (Sueiro et al., 2004). In blots of trout brain extracts (present results), the antibody stained a band of 65 kD, similar to that stained in rat brain extracts run in parallel. Western blot results with GAD in sturgeon also showed a band of about 65 kD. This indicates that in fishes this antibody recognizes at least one of the 67/65 GAD isoforms.
Western blotting results indicate that the antibody to the GABA A receptor subunit δ used in the present investigation, which was raised against amino acids 1–44 of the rat subunit, recognizes a single protein band of about 50 kD in extracts of trout and the other fishes, suggesting that the epitope recognized by the antiserum is highly conserved. However, the molecular mass of the band stained in rat extracts is slightly higher (52–54 kD) than in fishes. We have compared the immunostaining pattern in rat (Nusser et al., 1991; Pirker et al., 2000), dogfish (Sueiro, 2003), and trout cerebella (present results). In all cases, the subunit δ antibody densely stained granule cell dendrites near cerebellar glomeruli and more faintly granule cell perikarya. No other cerebellar cells were subunit δ-like immunoreactive in these species.

The pattern of subunit δ-like immunoreactivity in trout cerebellum is similar to that found with the GABA A receptor subunit α6 in rat, chick, and goldfish with in situ hybridization (Bahn et al., 1996) and in rat with immunocytochemistry (Jechlinger et al., 1998; Pirker et al., 2000). Bahn et al. revealed a high similarity between the fish and mammal α6 subunits. Interestingly, close inspection of the lower left panel of Figure 2 in Bahn et al. (1996) shows that subunit α6 is clearly expressed in the TL, which is seen as a cap over the cerebellar valvula, paralleling the expression of subunit δ-like immunoreactivity in the torus (present results). Coassembly of α6 and δ in the cerebellum was demonstrated by immunoaffinity chromatography (Jechlinger et al., 1998). A close relationship between expression of subunits α6 and δ in the cerebellum has been found in α6 subunit knockout mice, which results in dramatically reduced levels of the δ subunit (Jones et al., 1997). In mammals, subunit δ confers a neurosteroid-insensitive phenotype to recombinant receptors in vitro (Zhu et al., 1996). However, the present Western blot results do not rule out the possibility that in fishes the subunit δ antibody was staining a protein unrelated to this GABA A receptor subunit, although our results are rather suggestive that it is staining this protein. Subunit δ has not been sequenced in fishes, so we await further studies to test this hypothesis fully.

Organization and neurochemistry of the torus longitudinalis system of trout

On the basis of Golgi, electron microscopic, and tracetracing studies (Ito, 1974; Ito and Kishida, 1978; Schroeder et al., 1980; Ito et al., 2003; Xue et al., 2003), the TL has long been considered a specialized structure intimately related to the optic tectum. The TL projects thin fibers to the most superficial tectal layer, the SM. These fibers arise from a large population of granule cells, in a way that is reminiscent of the parallel fiber system of the cerebellum, and they contact pyramidal cell apical dendrites that are studded with short thorny appendages (Ito and Kishida, 1978; Schroeder et al., 1980; Meek, 1981; Xue et al., 2003). Electron microscopy of the TL reveals that small cells are closely packed and that there are synaptic glomeruli similar to those of the cerebellar granular layer (Ito, 1974). Observations with Nissl staining and electron microscopy have indicated the presence of cells of different sizes in the TL of holocentrids and carp (Ito, 1974; Ito et al., 2003; Xue et al., 2003), although the significance of this observation is not known.

Here we show for the first time that the trout TL consists of at least two types of neurons with different neurochemical profiles, small and medium-sized, the latter being located mainly in dorsomedial regions of the torus. Our immunocytochemical results also reveal that medium-sized cells express both GABA and GAD immunoreactivity, markers of GABAergic cells. These cells are the most probable origin of the rich plexus of GAD/GABA-ir fibers found in the TL and hence are probably local inhibitory neurons. Our results also show for the first time that the toral tectal neurons observed here are not GABAergic and that the extratectal toroptic nuclei do not appear to contain GABAergic cells. It is also probable that toral GABAergic cells correspond to short axon cells revealed with Golgi methods (present results), suggesting that they may be compared with cerebellar Golgi cells. In fishes, as in land vertebrates, Golgi cells form inhibitory synapses on the outer part of the cerebellar glomeruli (Alvarez-Otero et al., 1995).

In regard to the toro-recipient region of the trout optic tectum, the SO exhibits small GAD-ir cells and numerous boutons, whereas the adjacent SM contains scattered GAD/GABA-ir boutons and occasional cells. The existence of GAD-ir boutons in the SM of the optic tectum has also been reported recently in the zebrafish (Castro et al., 2006), suggesting it is a shared characteristic. These results, and the rich expression of the GABA A receptor δ subunit observed in this layer of the trout optic tectum (see below), support the presence of local inhibitory GABAergic circuits in the SM. The density of GABAergic boutons in this layer is much less than in deeper tectal layers (Castro et al., 2006; present results), but it is significant. Most studies of the optic tectum have considered that its marginal layer consists only of parallel fibers arising from the TL and of pyramidal cell dendrites, although the presence of some ascending axons to this layer has been noted in Golgi studies (Schroeder et al., 1980). Our results suggest for the first time that these cells with ascending axons might be GABAergic, being part of a local (columnar) inhibitory feedback on the marginal layer. In regard to GABAergic structures of other tectal layers, some GABAergic cells are located in the SPV of trout, as also reported in the eel (Medina et al., 1994) and the zebrafish (Castro et al., 2006). The SO, the SGC, and the SFGS exhibit a rich GABAergic innervation in these three species, suggesting widespread actions of GABAergic neurons in the teleost optic tectum.

The present results reveal for the first time close neurochemical similarities between the cerebellum and the TL in regard to the expression of GABA A receptor subunits (see above). Moreover, comparisons of the pattern of expression of the δ subunit in the cerebellum and other brain regions of trout (present results) with its expression in rat brain (Benke et al., 1991; Laurie et al., 1992; Wisden et al., 1992; Pirker et al., 2000) suggest a conserved expression pattern of GABA A receptor subunits between teleosts and mammals in the cerebellum and cerebellum-related systems. In regard to the TL, our results indicate that small granular cells of the TL exhibit immunoreactivity to the δ subunit of the GABA A receptor, which in mammals and trout is abundantly expressed in the granular layer of the cerebellum (Laurie et al., 1992; present results).

Our results on the expression of GABA A receptor β2/β3 subunits in the torotectal system and other brain regions are in good agreement with those of Anzelius et al. (1995) in a related salmonid species. Interestingly, significant
immunoreactivity to β2/3 subunits was observed in the TL, in the SGC and SFGS of the optic tectum, and in the paracommissural nucleus of both species. In regard to the distribution of the GABA_α receptor δ-like subunit, it has not been described previously in any fish species. Immunoreactivity to this subunit is found in TL-related nuclei such as the optic tectum and paracommissural nucleus. Comparison of the distribution of the δ subunit (present results) with that of the β2/3 subunits (Anzelius et al., 1995; present results) in the optic tectum reveals important differences. The most notable difference is the strong expression of the GABA_α receptor δ subunit in the SM, whereas this layer does not express β2/3 subunits. Our results also reveal for the first time that the paracommissural nucleus contains numerous GAD-ir boutons in a proportion similar to adjacent pretectal periventricular nuclei (unpublished results). However, the strong expression of the GABA_α receptor subunit β2/3 and δ immunoreactivity in the paracommissural nucleus clearly distinguish it from adjacent pretectal nuclei (Anzelius et al., 1995; present results), indicating differential actions of GABA on these structures. This paracommissural nucleus probably relays telencephalic information to the cerebellum, whereas this layer does not express β2/3 subunits.

In regard to the main neurotransmitters in the torocerebellar system, immunoreactivity to glutamate has been reported in granule cells of the TL of goldfish (Kageyama and Meyer, 1989), which would be in agreement with presumed neurotransmitters of cerebellar granule cells. These cells also take up and accumulate [3H]aspartate injected into the marginal layer of the optic tectum, suggesting that marginal (parallel) fibers use aspartate and/or glutamate as neurotransmitters (Poli et al., 1984).

Connections of the torus longitudinalis

Tracer application to the TL of the rainbow trout reveals for the first time retrogradely labeled cells in three pretectal nuclei: the central, intermediate, and paracommissural nucleus. These trout pretectal nuclei were also shown to project to the cerebellum (Folgueira et al., 2006). Pretectal neurons projecting to the TL were also described in tilapia (Imura et al., 2003), carp (Ito et al., 2003) and in four species of holocentrids (Xue et al., 2003), but important differences seem to exist between these species and trout. The projection from cells in the paracommissural nucleus to the torus seems to be shared by all of these species (Imura et al., 2003; Ito et al., 2003; Xue et al., 2003; present results). On the other hand, cells projecting to the TL have not been described in the central pretectal or intermediate pretectal nuclei (pretectal area) of tilapia, carp, and holocentrids (Imura et al., 2003; Ito et al., 2003; Xue et al., 2003). However, it is noteworthy that the torocerebellar projection is not known, but it might be ancillary to the pretectal circuits projecting to both the TL and the cerebellum. These pretectal circuits might contribute via the optic tectum and cerebellum to modulation of premotor and motor centers (midbrain reticular formation, oculomotor nuclei) that control body and eye movements under the influence of tectal and cerebellar projections (Torres et al., 1995; Luque et al., 2007).

After DiI application to the trout TL, retrogradely labeled cells were observed just beneath the lateral nucleus of the valvula (LV; present results). Although labeled fibers were observed in the TL after tracer application to the lateral nucleus of the valvula (Folgueira et al., 2006), reciprocal tract-tracing experiments (present results) showed that this projection does not originate in the lateral nucleus of the valvula itself, but rather from cells placed close to the LV. These cells are located in a similar position to those labeled in the subvalvular nucleus after tracer application to the TL of carp (Ito and Yoshimoto, 1990; Ito et al., 2003) and holocentrids (Xue et al., 2003), and these nuclei may be homologous. The similar pattern observed in carp, holocentrids, and rainbow trout (Ito et al., 2003; Xue et al., 2003; present results) indicates that the nomenclature used by Ito et al. (2003) for this subvalvular nucleus is also suitable for trout. Cells from the dorsal tegmental nucleus and the LV were described as projecting to the TL in Pantodon buchholzi (Wullimann and Roth, 1994). Although these results could indicate the existence of important species differences between Pant-
odon and other teleosts regarding the projections to the TL from mesencephalic regions, it appears more probable that the toropetal cells reported in \textit{Pantodon} in the dorsal tegmental nucleus and LV in fact correspond to the subvalvular nucleus of other species, as suggested by Ito et al. (2003).

An interesting difference between TL afferents of trout and those reported in carp (Ito et al., 2003) and holocentrids (Xue et al., 2003) is the absence of afferents from the granular eminence/subeminential nucleus region. In the carp, these afferents are bilateral and numerous, arising from two types of neurons, whereas in holocentrids only a few neurons were labeled ipsilaterally in this region. The functional significance of this projection is not known. Our negative results in trout suggest either that this is a shared projection that has been lost secondarily in salmonids or that its presence in two widely separated teleost groups is the result of a convergent evolution.

Although tracer application to the TL of trout labeled cells in the torus semicircularis, reciprocal tract-tracing experiments suggest that there is no direct projection from the torus semicircularis to the TL (present results). Fibers from cells in the ipsilateral torus semicircularis cross to the contralateral side in the intertectal commissure, which is located just dorsal to the TL, so tracer application in the TL in toto probably results in en passant labeling of these fibers (de Wolf et al., 1983; present results). Similar results were also reported in carp (Echteler, 1984; Ito et al., 2003).

The tectal and extratectal connections of the trout TL are summarized schematically in Figure 11. (Compare these connections with those reported in holocentrids: see Fig. 11 in Xue et al., 2003).

**Torotectal and tectotoral projections**

The present results showed a highly specific reciprocal connection between the optic tectum and the TL in salmonids. When this manuscript was in revision, a study of efferent tectal neurons of the rainbow trout appeared online reporting labeled cells and fibers in the TL after tracer application to the optic tectum (Kinoshita et al., 2006), although the cells of origin of fibers in the TL or the tectal layers receiving projections from the TL cells were not characterized. The presence of afferent fibers to the TL from the tectum was reported in other groups of teleosts (Ebbesson and Vanegas, 1976; Grover and Sharma, 1981; Luiten, 1981; Fiebig et al., 1983; Ito et al., 2003; Xue et al., 2003), but the cells of origin of this projection have only been characterized in carp (Ito et al., 2003) and holocentrids (Xue et al., 2003). As reported in these teleosts, our results show that most of the cells projecting to the TL in trout were restricted to the SGC, but occasional labeled cells were observed in the SPV. Our results indicate for the first time that these cells are non-GABAergic, which rules them out as the origin of the GABA/GAD-immunoreactive fibers observed in the TL. These projections are probably part of a positive feedback loop between the tectum and the TL.

Comparison of the toropetal tectal cells of trout with those of carp and holocentrids reveal important differences among teleost species. Unlike trout toropetal cells, those of the carp are morphologically heterogeneous and have dendrites that are spread rather horizontally (Ito et al., 2003). Some toropetal cells are located inside the SGC, many are in the outer part of the SPV, and a few are inside or close to the SAC. Those of holocentrids are rather uniform in appearance and have a radial orientation similar to that of trout cells, but form two horizontal bands of dendrites, apical and basal (Xue et al., 2003); the basal band receives the axonal branches of pyramidal cells. In trout, toropetal neurons appear homogeneous morphologically and only show apical dendrites reaching the level where pyramidal cell thick basal dendrites end, whereas the axonal terminal field of these cells shows no obvious relationship to toropetal cells. Moreover, the crook-shaped axons of trout cells are unlike the axons of the holocentrid toropetal cells, which originate from the basal dendrite. Our results in trout strongly suggest that the relationship between pyramidal cells and toropetal neurons is not direct, as postulated in holocentrids (Xue et al., 2003). It is probable that other tectal interneurons participate in the connections between these cells in trout.

In holocentrids there is a prominent telencephalotectal projection from the central nucleus of the dorsal telencephalic area (Dc), which ends at different tectal heights, the heaviest telencephalic terminal being located at the level of the sublayer with basal dendrites of toropetal cells and axonal trees of pyramidal neurons (Xue et al., 2003). A direct telencephalotectal projection originating from the...
Our results in trout show the presence of GABAergic cells in the TL as well as in the optic tectum, mainly in its SO and SPV. Our results with Golgi methods suggest that the torus GABAergic cells may be local interneurons, but further studies are necessary to confirm this hypothesis. Many radially oriented neurons of the trout tectal SPV appear to be projection neurons (Folgueira et al., 2005; Kinoshita et al., 2006), but their ascending dendrite gives off swarm-like branches that probably represent presynaptic dendrites (Schroeder et al., 1980; Vanegas et al., 1984a), which suggests that they also are both presynaptic and postsynaptic in intratectal circuits. The presence of GABAergic boutons in the TL, as well as in the marginal and central tectal layers, indicates that the tecto-tortocellular feedback circuitry may be locally modulated by inhibitory cells present in both the torus and the tectum.

Evolutionary considerations

The TL of trout appears to be neurochemically and cytoarchitecturally heterogeneous; our results reveal for the first time the presence of an intrinsic GABAergic toral population and some cerebellum-like features of the GABAergic toral system. The involvement of GABA in TL circuits does not appear to be exclusive to salmonids, because the presence of GABAergic cells in the TL was reported in the eel (Médina et al., 1994). Our results in the rainbow trout also show that the TL has highly specific connections with the pretectum and the optic tectum, as was also noted in recent studies in two other teleost groups (Ito et al., 2003; Xue et al., 2003). The pattern of connections of the TL is roughly similar in these species pertaining to three separate radiations of teleosts (Ito et al., 2003; Xue et al., 2003; present results). However, several important differences among them were noted in the cells of the tectoral system and the nuclei involved in the pretecto-TL circuits, which is suggestive of different specializations of these circuits in the different lines of teleosts. This also suggests that the shared tecto-TL-tectal and the pretectal-TL-circuitry in these various teleosts appeared before the radiation of modern teleosts, which might be related to a important conserved functional role for the TL. A study of the laminar organization of the optic tectum in 75 teleost species revealed a wide variation among species in the relative thickness of the toro-recipient layer (SM) as well as in other layers (Kishida, 1979). Although a TL has been reported in Polyodon and other chondrosteans (primitive actinopterygians), the presence of an SM in the optic tectum is doubtful, and the possible TL circuits in these non-teleost species have not yet been investigated (Nieuwenhuys, 1998), which precludes comparison with teleost results. The teleost TL is probably related to adaptation to changing luminescence patterns in the aquatic milieu, but the selective forces guiding these adaptations or their biological significance for the different species are not known.

ACKNOWLEDGMENTS

We thank Mrs. Pilar Gómez (Piscifactoría Berxa, Mesía, A Coruña) for supplying the animals used in this study. We thank Prof. Dr. Werner Sieghart (Center for Brain Research, Section of Biochemistry and Molecular Biology of the Nervous System, Medical University Vienna) for his generous gift of antibodies to the GABA_4 receptor subunit and the control fusion protein. We also thank Dr. Thomas Hawkins (Department of Anatomy and Developmental Biology, University College London) for help with the English.
The Journal of Comparative Neurology. DOI 10.1002/cne

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