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Distribution of GABA-Like Immunoreactive Cell Bodies in the Brains of Two Amphibians, *Rana catesbeiana* and *Xenopus laevis*

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Key Words

GABA · *Xenopus laevis* · *Rana catesbeiana* · Bullfrog · South African clawed frog · Neurotransmitter · Immunocytochemistry · Amphibian

Abstract

The distribution of the neurotransmitter gamma-aminobutyric acid (GABA) is not well understood for non-mammalian vertebrates. We thus used immunocytochemistry to locate putative GABAergic cells in the brains of male bullfrogs *(Rana catesbeiana)* and South African clawed frogs *(Xenopus laevis)*. GABA-immunoreactive cell bodies were broadly distributed throughout the brains of both species with similar general patterns. In both, the greatest numbers of GABA-positive cells were found in the olfactory bulb, thalamus, and optic tectum, but virtually no major brain region lacked GABAergic cells. Species differences were also apparent. The density of GABA-immunoreactive cells was substantially higher in many areas of the bullfrog brain, compared to *Xenopus*. Bullfrogs possessed extensive cell populations in the medial pallium, preoptic area, optic tectum, torus semicircularis and tegmentum but cells were fewer in these locations in *Xenopus*. In the bullfrog hindbrain, GABA-immunoreactive cell bodies were restricted to very narrow and distinct populations. In *Xenopus*, however, cells in a similar position were fewer and spread more extensively. The distribution of GABA cells in the brain of these two species supports the hypotheses that GABA is involved in control of olfaction, audition, vision and vocalization. However, differences in the distribution of GABA between the bullfrog and *Xenopus* suggest that the extent of the GABAergic influence might vary between species.

Introduction

The amino acid γ-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates. The distribution of neural GABA in many mammals has been well established [e.g., Otterson and Storm-Mathisen, 1984; Ong and Garey, 1991; Gao et al., 1999; Gomez-Urquijo et al., 2000a, b]. The distribution of GABA in the CNS of non-mammalian species is much less well known. In birds, GABA immunoreactivity has been thoroughly mapped only in the pigeon, *Columba livia* [Domenici et al., 1988; Veenman and Reiner, 1994]. Specialized portions of the GABAergic system have also been described in the zebra finch *(Taeniopygia guttata)*, chicken *(Gallus gallus)*, and barn owl *(Tyto alba)* [Carr et al., 1989; Granda and Crossland,

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1989; Grisham and Arnold, 1994; Luo and Perkel, 1999a, b]. The distribution of GABA cells and fibers in reptiles has been focused primarily on the visual system in turtles, snakes, lizards, and crocodilians [Rio et al., 1992; Pritz and Stritzel, 1994; Kenigfest et al., 1998, 2000]. The general distribution of GABA immunoreactivity has been mapped in the forebrain and midbrain of the chameleon *(Chamaeleo chameleon)* [Bennis et al., 1991]. A few specific forebrain areas have also been investigated in two other lizards and two turtle species [Schwerdtfeger and Lopez-Garcia, 1986; Schwerdtfeger et al., 1986; Blanton et al., 1987; Schwerdtfeger and Lorente, 1988; Teruel et al., 1990; Belekhova et al., 1992]. Distribution information in teleosts is limited and focused almost exclusively on the forebrain [Martinoli et al., 1990; Medina et al., 1994; Ekstrom and Ohlin, 1995; Anglade et al., 1999; Trabucchi et al., 2000; Bosma et al., 2001]. Finally, there are a few reports of GABA distribution in the lamprey, *Petromyzon marinus*, and an elasmobranch, *Scyliorhinus canicula* [Alvarez-Otero et al., 1995; Melendez-Ferro et al., 2000, 2001, 2002; Reed et al., 2002]. Unique aspects of the distribution of GABA across vertebrate brains are thus difficult to discern due to little information.

In amphibians, mapping of GABAergic cells and fibers across the CNS is incomplete in all three orders. There are no reports on GABA distribution in any gymnophionan amphibian and the distribution in adult urodele amphibians has been well described in only three species [*Pleurodeles waltli*, *Triturus alpestris*, *T. cristatus carnifex*; Franzoni and Morino, 1989; Naujoks-Manteuffel et al., 1994]. For anuran amphibians, adult GABA-immunoreactivity has been fully mapped only in the European green frog, *Rana esculenta* [Franzoni and Morino, 1989]. The distribution of GABA in the brain of developing *Xenopus laevis* has been thoroughly mapped but expression in some brain areas is transient so the adult pattern can differ significantly [Roberts et al., 1987; Barale et al., 1996]. GABA immunoreactivity has also been described in the brainstem of developing bullfrog *(Rana catesbeiana)* tadpoles but the pattern of neurotransmitter expression across the rest of the brain was not reported [Simmons and Chapman, 2002]. Particular GABAergic systems have been studied independently in some anurans. The visual system, for example, has been the emphasis of many reports (see Discussion). Where species comparisons can be made (e.g., in the anuran optic tectum), significant differences in the pattern of GABA-immunoreactivity have been found [Rybicka and Udin, 1994; Gabriel and Straznicky, 1995; Simmons and Chapman, 2002]. Species differences might well exist in other areas of the anuran brain but this is unknown. We thus used immunocytochemistry to map the distribution of GABA-like immunoreactive cells in the brains of adult males belonging to two distantly related families of anurans.

Materials and Methods

Adult male bullfrogs (*Rana catesbeiana*; snout-vent length range 13–17 cm) were purchased from C. Sullivan Company (Nashville, Tenn., USA) and adult male *Xenopus laevis* (snout-vent length range 5–8 cm) from Nasco (Fort Atkinson, Wisc., USA). Animals were housed in the lab on a 12L:12D controlled photoperiod at 17°C in large tanks (50 × 21 × 21 cm) with flow-through water. Bullfrogs were maintained on a diet of goldfish, and *Xenopus* were fed frog brittle (Nasco). The original research reported herein was performed under guidelines established by the University of Notre Dame IACUC.

Five animals of each species were over-anesthetized by immersion in 0.02% benzocaine (ethyl-p-amino benzoate, Sigma) at room temperature. The ventral surface of the skull was removed and the brains immersed in situ in 5% gluteraldehyde and 2% paraformaldehyde in 0.1 *M* phosphate buffer (pH = 7.4). After 1 h, brains were removed from the skull, post-fixed in fresh fixative for an additional 5 h and then stored, for no more than one week, in phosphate-buffered saline (0.1 *M* phosphate buffer and 0.9% NaCl; pH = 7.5) until sectioning. Brains were embedded in butter (Land O' Lakes, unsalted), frozen at –20°C for 15 min, and sectioned frontally on a vibratome (40–50 μm sections) while immersed in ice-cold Tris-buffered saline (TBS, 0.05 *M* Tris and 0.9% NaCl, pH = 7.5). Sections were alternately placed in two vials of ice-cold TBS. Sections were stored at 4°C for up to one week.

Immunocytochemical procedures followed those of Rybicka and Udin [1994], with modification. Free-floating sections were rinsed for 5 min with TBS containing 1% BSA followed by a 5-min treatment in 1% H_2O_2 in TBS with 0.1% saponin. After two 5-min rinses in TBS with 1% BSA, sections were incubated with 10% normal goat serum (NGS; in TBS with 0.1% saponin) for 1 h and then rinsed for 10 min in TBS with 0.1% saponin. All previous rinses occurred at room temperature. The sections were then incubated with rabbit anti-GABA antibody (Sigma), 1:1000 in TBS with 0.1% saponin, 1% NGS, and 0.1% NaN_3 at 4°C for 24 h.

Following incubation with the primary anti-GABA antibody, the sections were rinsed, at room temperature, three times for 5 min each in TBS with 0.1% saponin and 1% NGS followed by incubation in biotinylated goat anti-rabbit IgG (Vector Laboratories; Vectastain ABC kit, Elite PK-6101), 1:200 (in TBS with 0.1% saponin and 1% NGS) for 2 h. The sections were then reacted with the Vector kit avidin-biotin complex, 1:25 (in TBS with 0.1% saponin and 1% NGS) for 2 h. Visualization was accomplished by treating the tissue with 0.05% 3-3′-diaminobenzidine in 0.05 M TBS with 0.003% H_2O_2 for 20 min. After a final 5-min rinse in TBS, the sections were quickly rinsed in diH_2O, mounted on slides, dried and coverslipped with Permount (Fisher). Specificity of the antiserum was tested on alternate sections from the same brains by replacing the anti-GABA antibody with non-immune rabbit IgG (56 μg IgG/ml).

The determination of neuroanatomical areas was based on Northcutt and Royce [1975] and Marín et al. [1997] for the olfactory bulb and Kicliter and Ebbesson [1976], Marín et al. [1997, 1998],

Table 1. Abbreviations of major neuroanatomical landmarks

a	anterior thalamic nucleus	mp	medial pallium
acc	nucleus accumbens	ms	medial septum
ad	anterodorsal tegmentum	ml	mitral layer
aob	accessory olfactory bulb	oc	optic chiasma
apl	amygdala pars lateralis	ot	optic tectum
apm	amygdala pars medialis	poa	preoptic area
av	anteroventral tegmentum	pr	principal nucleus of the torus semicircularis
c	central thalamic nucleus	ptg	pretectal gray
cb	cerebellar nucleus	ptn	pretrigeminal nucleus
cer	cerebellum	ra	raphe nuclei
dp	dorsal pallium	rt	reticular nuclei
ea	anterior entopeduncular nucleus	sc	suprachiasmatic nucleus
gl	glomerular layer	so	superior olivary nucleus
gr	granular layer	st	solitary tract
hb	habenula	str	striatum
is	isthmic nucleus	ts	torus semicircularis
lm	laminar nucleus of the torus semicircularis	vh	ventral hypothalamus
lp	lateral pallium	VIIIn	nucleus of the eighth nerve
ls	lateral septum	vl	ventrolateral thalamus
lt	lateral thalamic nucleus	vm	ventromedial thalamus

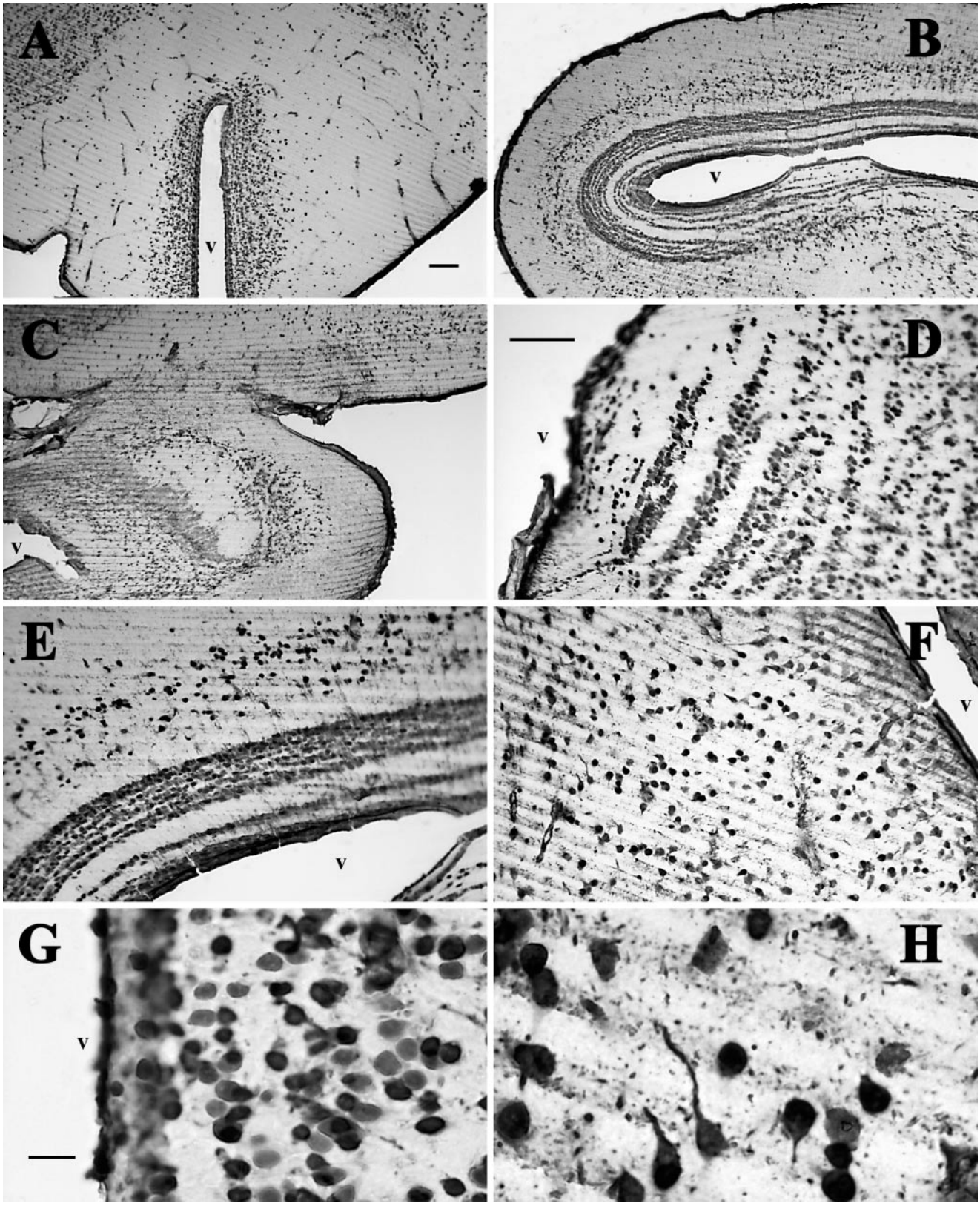
González et al. [2002], Brox et al. [2003], Moreno and González [2003], Endepols et al. [2004] for the remaining forebrain. Areas of the diencephalon and midbrain were based on Neary and Northcutt [1983], Neary [1988], Wilczynski [1988], Marín et al. [1997], Lázár [2001], and Brox et al. [2003]. Areas of the hindbrain were based on Opdam et al. [1976], Sánchez-Camacho et al. [2001], and Stuesse et al. [2001]. Neuroanatomical abbreviations are listed in table 1.

Populations of GABA-immunoreactive cells were described in relative terms. 'Dense' populations of cells were those where 60% or more of the total cells in the region were immuno-positive. 'Moderate' populations possessed between 25 and 60% immunoreactive cells of the cell total, and 'sparse' populations of GABA-immunoreactive cells had fewer than 25% immuno-positive cells from the total number of cells in the region. The total number of cell bodies in a region was determined from cresyl violet-stained sections. These designations allowed comparison of bullfrog and *Xenopus* brains even when the absolute number of cells in a given brain region differed significantly. For comparison with published reports in other species, discussion is restricted to the location of cell populations. Differences in antibody sources and/or other technical details might also influence the location of GABA immunoreactivity.

Results

GABA-immunoreactive cell bodies were found throughout much of the brain of both anurans. Cell somata in both species typically stained a very dark brown when positive for GABA. In many instances, GABA immunoreactivity extended beyond the soma of the cell revealing fibers in a distinctly lighter brown (figs. 1H, 2F). Populations of GABA-like immunoreactive cells were typically denser in the brain of the bullfrog than those populations found in the brain of *Xenopus* (figs. 1F, 2E). The anatomical locations of labeled cell bodies shown in figures 3–5 were consistent across individuals within a species. In both species, most cells were small in diameter (5–10 μm) and almost exclusively round, although GABA-immunoreactive cells in areas of the telencephalon including the medial pallium, septum, and amygdala often reached diameters of 12 μm. Purkinje cells were the largest observed cells with diameters up to approximately 15 μm. The distribution of GABAergic cells in the two species was similar overall. The most striking difference was that GABA-immunoreactive cells in the bullfrog brain frequently exhibited much more distinct regional laminar organization than found in *Xenopus*. This was most obvious within areas of the thalamus and the midbrain (figs. 1B, D, E, 2C). Control sections incubated in the non-immune serum showed no immunostaining (data not shown).

In the olfactory bulb of both species, dense to moderate populations of GABA-immunoreactive cells were observed in the granular layer and encircled the entire lateral ventricle (figs. 2A, 3A). These immunoreactive cells also extended into the adjacent mitral layer and ventrally into the glomerular layer, but were sparse to moderate. More caudal, GABA immunoreactivity in the accessory olfactory bulb was also sparse to moderate (fig. 3B).



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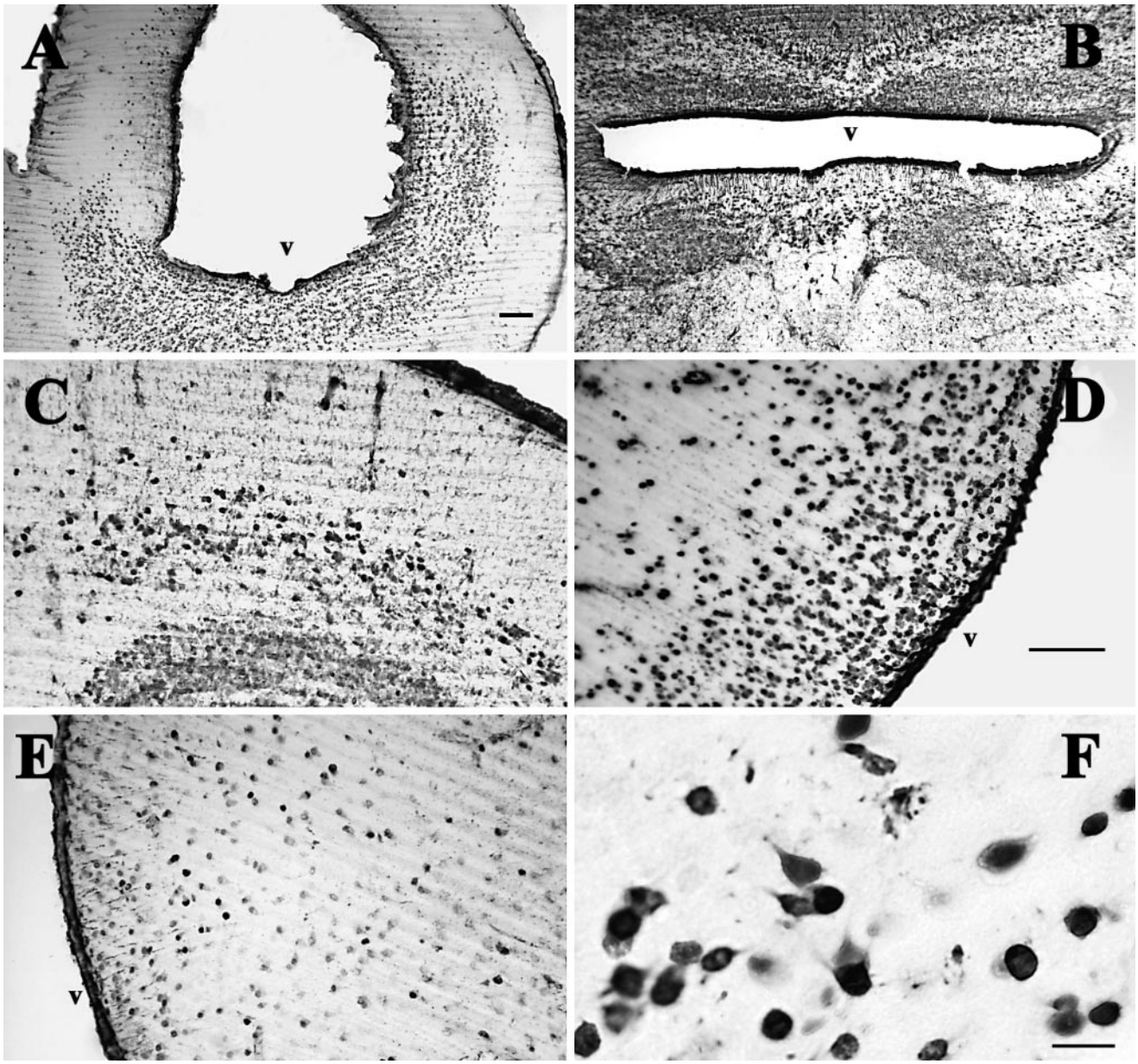

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Fig. 1. Distribution of GABA-like immunoreactivity in the brain of adult male bullfrogs (frontal sections; ventral at bottom). **A** Cells in the anterior preoptic area around the third ventricle and in the more dorsal amygdala pars medialis. **B** Clear lamination of GABA-immunoreactive cells in the optic tectum and torus semicircularis. **C** GABA distribution in the tegmentum highlighting the conspicuous absence of staining in the central portion of the isthmic nucleus. **D** Distinct lamination in GABA-positive cell distribution of the thalamus. **E** Higher magnification photomicrograph of the optic tectum showing individual layers. **F** Medial septum with large numbers of darkly staining GABA-immunoreactive cell bodies. **G** Higher magnification of the preoptic area cell layers. **H** Higher magnification of GABA-immunoreactive cell somata in the medial septum where staining of some fibers was observed. Bar in **A** = 100 μm and applies to panels **A**, **B**, and **C**; bar in **D** = 100 μm and applies to panels **D**, **E**, and **F**; bar in **G** = 20 μm and applies to panels **G** and **H**. 'v' indicates ventricle.

Fig. 2. Distribution of GABA-like immunoreactivity in the brain of adult male *Xenopus laevis* (frontal sections; ventral at bottom). **A** GABA-immunoreactive cell soma in the olfactory bulb. **B** Staining in the cerebellum and raphe nucleus. **C** Immunoreactive cell soma in the optic tectum, showing less distinct laminar organization compared to the bullfrog. **D** Preoptic area immuno-positive cells. **E** Staining in the medial septum, illustrating the fewer, more lightly-stained cells of *Xenopus* in this area. **F** GABA-immunoreactive cell bodies in the dorsal pallium. Bar in **A** = 100 μm and applies to panels **A** and **B**; bar in **D** = 100 μm and applies to panels **C**, **D**, and **E**; bar in **F** = 20 μm. 'v' indicates ventricle.

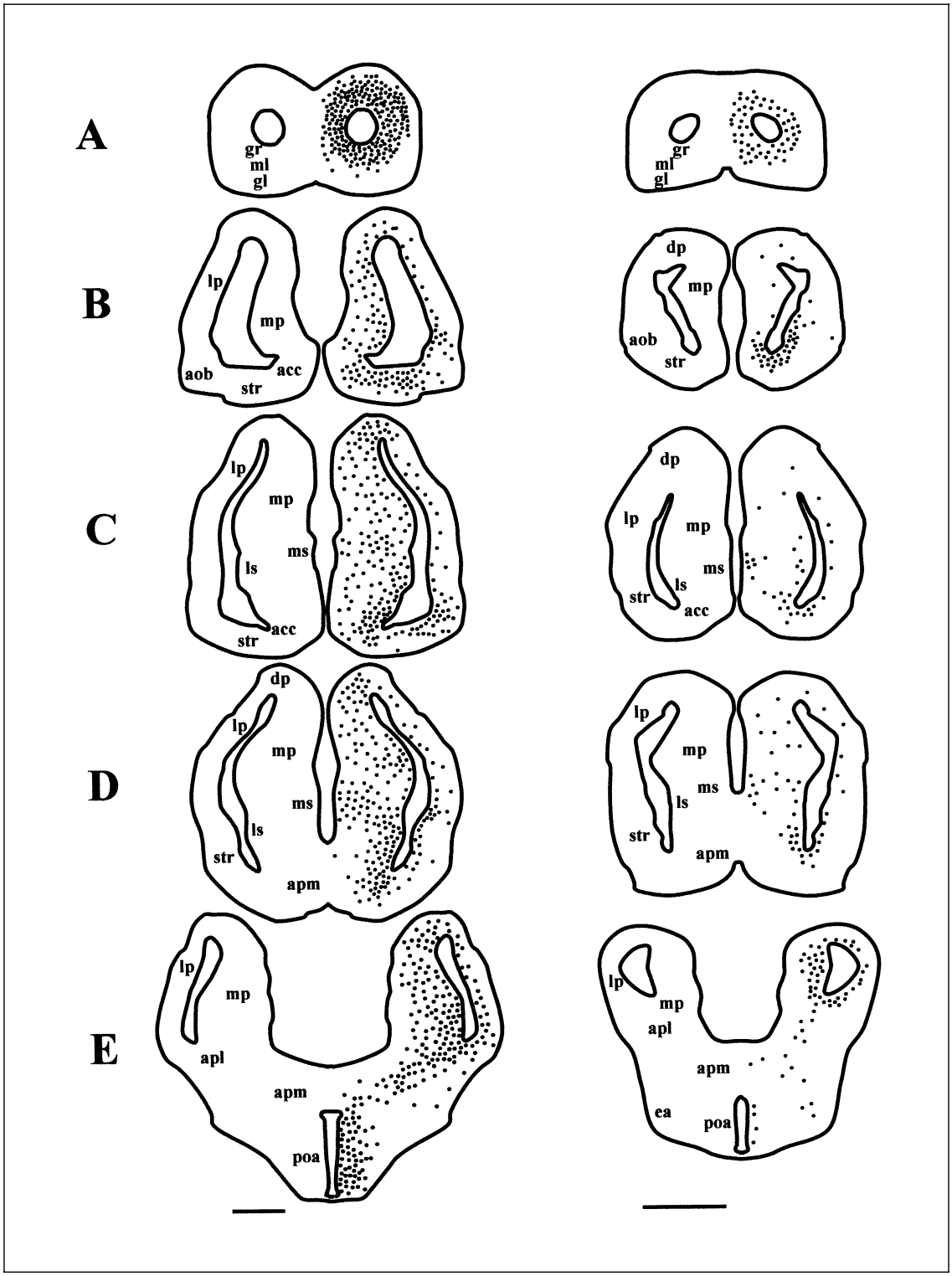

Fig. 3. Distribution of GABA-like immunoreactive cells in the telencephalon of the bullfrog (left) and *Xenopus* (right). Frontal camera lucida drawings; each dot represents about 15 immunoreactive somata. Left side of each section identifies neuroanatomical landmarks (table 1). Bars = 1 mm.

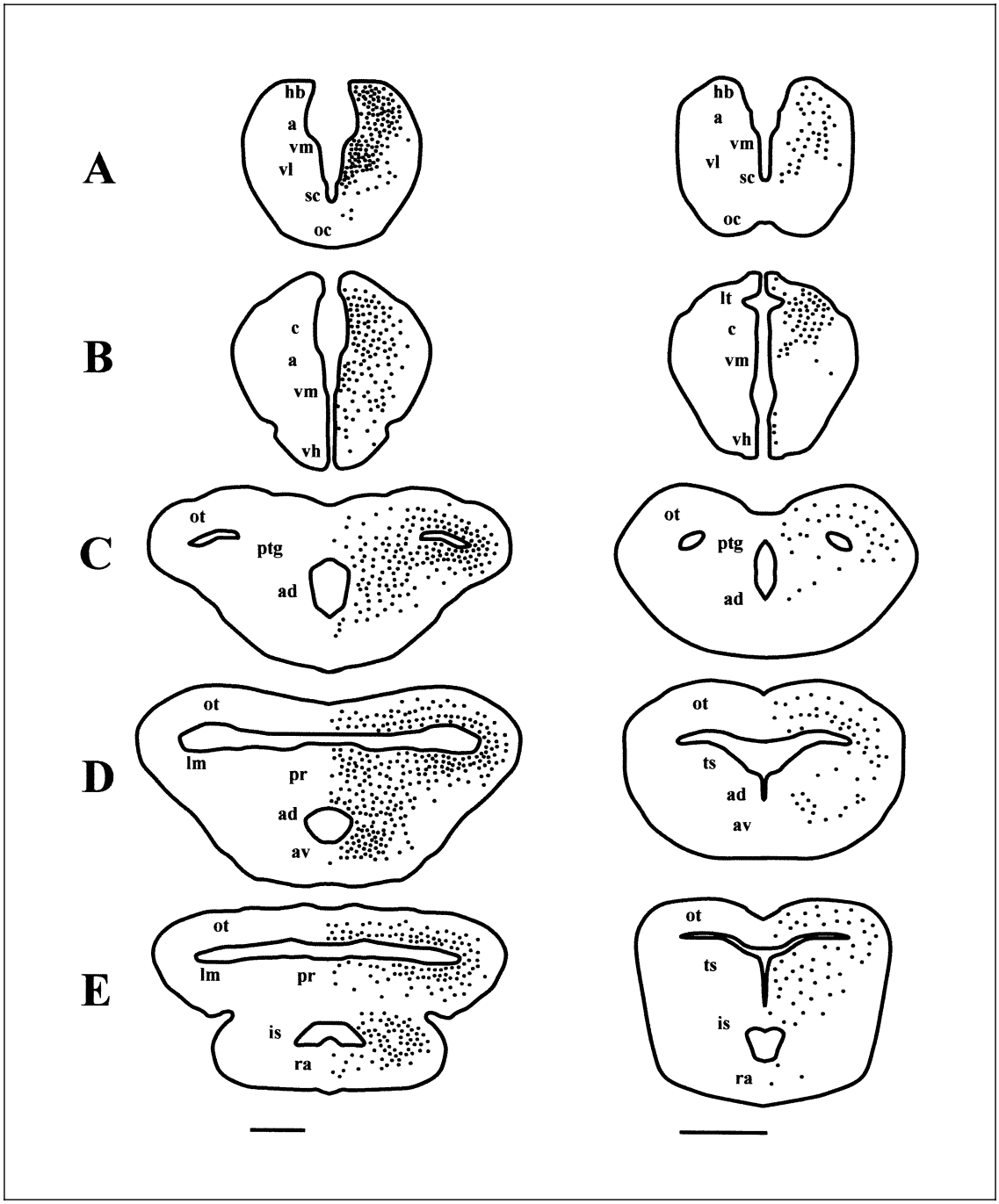

Fig. 4. Distribution of GABA-like immunoreactive cells in the diencephalon and midbrain of the bullfrog (left) and *Xenopus* (right). Frontal camera lucida drawings; each dot represents about 15 immunoreactive somata. Left side of drawings identifies neuroanatomical landmarks. Bars = 1 mm.

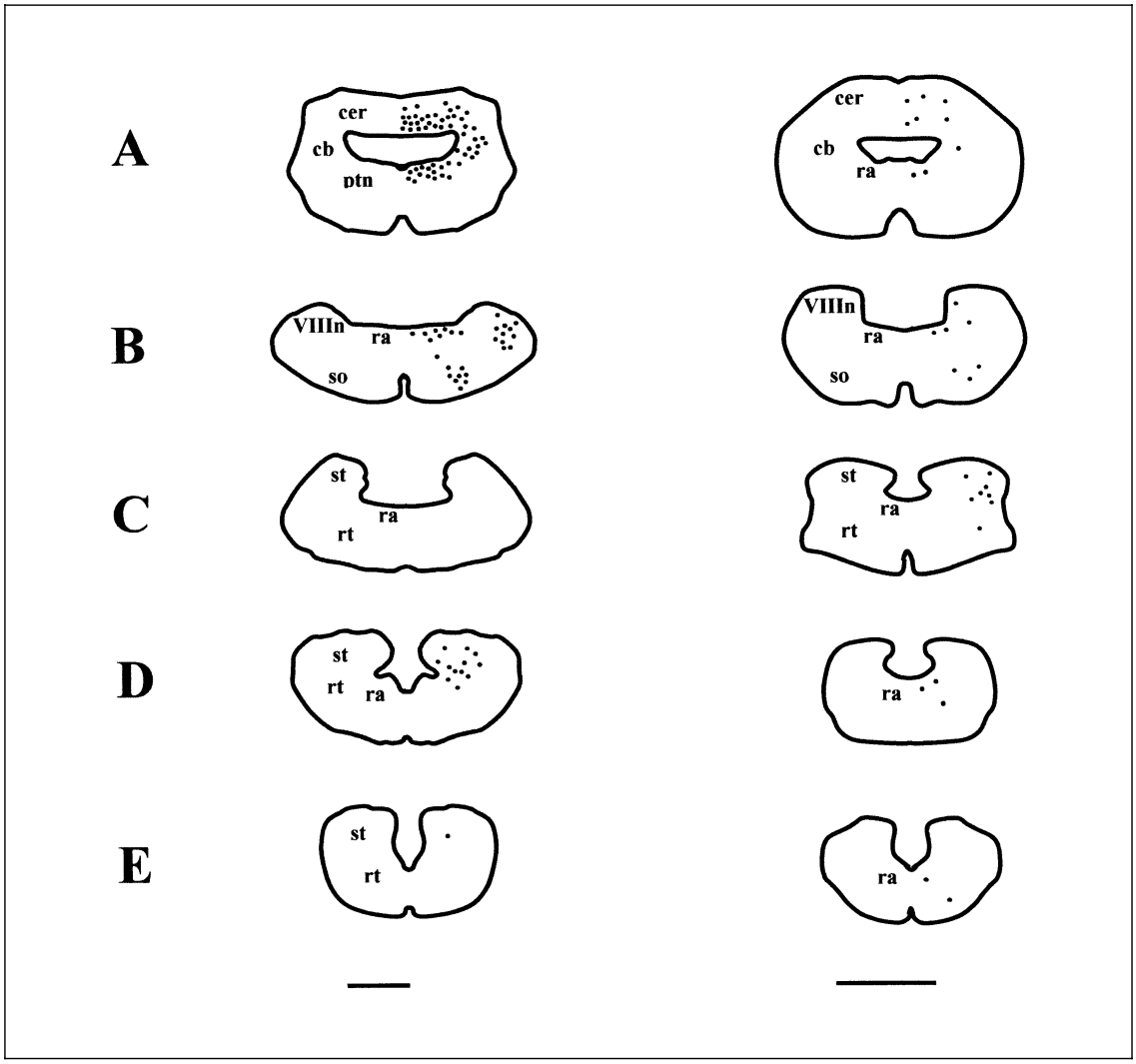

Fig. 5. Distribution of GABA-like immunoreactive cells in the cerebellum and hindbrain of the bullfrog (left) and *Xenopus* (right). Frontal camera lucida drawings; each dot represents about 15 immunoreactive cell somata. Left side of drawings identifies neuroanatomical landmarks. Bars = 1 mm.

GABA-immunoreactive cell bodies were found throughout the telencephalon of both species (fig. 3A–E). Sparse, scattered cell bodies were found in the dorsal (fig. 2F) and lateral pallium of the bullfrog and *Xenopus*. The medial pallium had moderate *(Xenopus)* to dense (bullfrog) populations of GABA-immunoreactive cell bodies and had a relatively high occurrence of cell somata with observable associated fibers (fig. 3B–E). In *Xenopus*, the densest distribution of GABA-immunoreactive cell bodies in the medial pallium occurred caudally, whereas the bullfrog medial pallium was dense with GABA-immunoreactive staining throughout. The striatum had sparse staining in *Xenopus* but a moderate population of immunoreactive cell bodies was observed in this region of the bullfrog. Similarly, the medial and lateral septal nuclei were characterized by moderate staining of GABA-immunoreactive cell bodies in the bullfrog and by sparse staining in *Xenopus* (figs. 1F, 2E, 3C, D). In addition, both species showed sparse to moderate populations of cells in the nucleus accumbens, and the amygdala pars lateralis and medialis, although the nucleus accumbens had one of the densest populations of GABAergic cells found in *Xenopus* (fig. 3B–E).

Finally, in the bullfrog, the preoptic area had a dense population of GABA-immunoreactive cell bodies in distinct columns of cells (figs. 1A, G, 3E). These columns extended from the extreme ventral portion of the preoptic area to the dorsal portion of the preoptic area and remained very dense, with the columns of cells becoming more ambiguous dorsally. Also in the bullfrog, the GABA-immunoreactive population of cell bodies in the preoptic area extended laterally from the dorsal region of the preoptic area to the amygdala pars medialis, where the population was moderate (fig. 1A). In *Xenopus*, only sparse GABA-immunoreactive somata were found in the preoptic area and these cell populations were not continuous with the GABA-immunoreactive cell bodies of the amygdala pars medialis (figs. 2D, 3E).

The diencephalon of both species was marked with moderate to dense populations of GABA-immunoreactive cell bodies throughout the different areas of the thalamus (figs. 1D, 4A, B). In the mid-diencephalon, strong column-like lamination of immunoreactive cells was seen. These cells extended ventrally from the habenula, through the anterior, ventrolateral, and ventromedial thalamic nuclei, through the magnocellular nucleus, and finally extended into the suprachiasmatic nucleus (fig. 4A–B). In *Xenopus*, thalamic populations of immunoreactive cell bodies were separate from a very sparse population of GABA-immunoreactive cell somata in the ventral hypothalamus (fig. 4B). However, in bullfrogs, thalamic populations were continuous with a sparse population of immunoreactive cell bodies in the ventral hypothalamus that lacked the lamination seen in the more dorsal regions. Additionally, the bullfrog had a sparse population of GABA-immunoreactive cell bodies lateral to the ventral hypothalamus, whereas *Xenopus* lacked these GABA immunoreactive cells (fig. 4B).

The midbrain of both species had GABA-immunoreactive cell bodies throughout (fig. 4C–E). In the rostral midbrain, a sparse *(Xenopus)* to moderate (bullfrog) population of GABA-immunoreactive cell bodies was observed in the pretectal gray (fig. 4C). GABA immunoreactivity in the the torus semicircularis differed between species. Rostrally, the torus semicircularis had GABA-immunoreactive staining. The laminar nucleus of the torus semicircularis had a moderate population of immunoreactive cell bodies. This population was continuous with the layered cells of the optic tectum that were immunoreactive for GABA as well (figs. 1B, E, 4D, E). This lamination was more pronounced in the bullfrog than in *Xenopus.* The principal nucleus of the torus semicircularis of the bullfrog had moderate populations of GABAergic cell bodies that extended ventrally to the anterodorsal tegmentum. This pattern of staining continued through the midbrain of the bullfrog. In *Xenopus*, the laminar nucleus of the torus semicircularis had moderate populations of GABA-immunoreactive cell bodies and, instead of appearing in tight rows as in the bullfrog, cells were more scattered (fig. 4D, E). However, this population also was continuous with the optic tectum. The principal nucleus of the torus semicircularis of *Xenopus* had moderate populations of immunoreactive cell bodies with the densest populations of GABAergic cells occurring caudally.

The optic tectum of both species had extensive populations of GABA-immunoreactive cell bodies (figs. 1B, E, 2C, 4D, E). Overall, moderate GABAergic cells of the bullfrog optic tectum identified its distinct cell layers throughout the midbrain. In both species, layers 2, 4, 6, 8, and 9 all displayed immunoreactivity. Layers 2, 4 and 6 had dense populations of stained cells with layer 6 possibly having the densest distribution of GABA-immunoreactive cell bodies in the entire brain. The GABA-immunoreactive cell bodies of layers 8 and 9 were scattered rather than in distinct rows. Layers 3, 5, and 7 were, for the most part, sparse in GABA immunoreactivity. Layers were not as distinct in *Xenopus* brain (fig. 2C).

The tegmentum (figs. 1C, 4D, E), especially the anteroventral portion in the bullfrog, had moderate populations of GABA-immunoreactive cells. In *Xenopus*, GABAergic cells were found mainly in moderate populations in the anterodorsal tegmentum of the central midbrain and in the anteroventral tegmentum. In the caudal midbrain of the bullfrog, moderate to dense regional staining of GABA-immunoreactive cell bodies occurred in the isthmic nucleus and raphe nuclei (fig. 4E). However, centrally in the isthmic nucleus, there was a noticeable lack of immunoreactive staining (fig. 1C). In *Xenopus*, these regions all had sparse immunoreactivity (fig. 4C–E).

Posterior to the midbrain, the cerebellum (both molecular and granular layers and the Purkinje cell layer) and pretrigeminal nucleus of both species showed GABA-immunoreactive cell bodies (figs. 2B, 5A). As in the medial pallium, GABA-immunoreactive somata in the cerebellum had distinct, associated fibers. In *Xenopus*, the immunoreactive cells of the cerebellum had the darkest (and thus the most distinctly stained) somata. However, the cytoarchitecture of *Xenopus* cerebellum was much less apparent than that of the bullfrog. GABA-immunoreactive cells in the cerebellum of *Xenopus* were substantially fewer and much more scattered than observed in the bullfrog. By contrast, staining of GABA-like cells in the bull-

frog cerebellum was dense in the granular layer and moderate in the molecular layer. Distinguishing the separation of molecular and granular cell layers were the GABA-immunoreactive cells of the Purkinje layer, which occurred in both species. In *Xenopus*, however, both the granular and molecular layers showed sparse GABA immunoreactive cells. Interestingly, the molecular layer was denser in GABA-immunoreactive cells than the granular layer, where most cerebellar cells are located.

The hindbrains of both species had sparse populations of GABAergic cells in very distinct regions (fig. 5B–E). In the most rostral hindbrain, just caudal to the cerebellum, GABA-immunoreactive cell bodies were restricted to sparse populations in the raphe nuclei in both the bullfrog and *Xenopus*. This population of cells extended dorso-laterally in both species. Also, the nucleus of the eighth nerve (acoustic and vestibular portions) and the superior olivary nucleus of both species had sparse to moderate populations of immunoreactive cells as well. More caudally, only sparse, scattered cells were observed in the raphe nuclei and reticular nuclei of both species, and, in the bullfrog, in the solitary tract.

Discussion

There were GABA-immunoreactive cell bodies distributed throughout the brains of both the bullfrog and *Xenopus*. Immunoreactive cell somata in both species were found in distinct brain regions and were often stratified in discrete rows or columns. The bullfrog brain, however, typically had greater immunoreactive cell density, more regionally distinct distribution, and more profound laminarization. The control procedure in which the antiserum was replaced with non-immune serum indicated that the staining technique was specific.

Evidence for GABA involvement in amphibian olfaction is strong. In the olfactory bulb, GABA-immunoreactive cell populations of both species were concentrated mainly within the internal granular cell layer, reflecting patterns of distribution in other amphibians [Franzoni and Morino, 1989; Kratskin et al., 1989; Hamilton, 1992], as well as other vertebrates [Domenici et al., 1988; Medina et al., 1994]. The $GABA_A$ receptor has been found in both the granule and mitral cell layers of frogs. In *R. ridibunda*, application of the $GABA_A$ receptor antagonist, bicuculline, to the granule cell layer increases the excitability of the mitral cell layer, suggesting GABAergic inhibitory control of mitral cells [Duchamp-Viret et al., 1993]. In *R. esculenta*, the $GABA_A$ receptor agonist, [^{3}H]muscimol, shows high density binding in the mitral cell layer [Tavolaro et al., 1993]. Our findings indicate that the source of GABA in the olfactory bulb is likely local. The distribution of GABA found in major cell layers of the olfactory bulb suggests a role for GABA in olfaction in both the bullfrog and *Xenopus*. Other olfactory-processing brain areas were also GABAergic. The accessory olfactory bulb of both species was sparse to moderate in GABAergic cell bodies. GABAergic cell bodies have also been found in the accessory olfactory bulb of *R. esculenta* [Franzoni and Morino, 1989]. The accessory olfactory bulb of urodeles and anurans then projects to the amygdala pars lateralis [Northcutt and Royce, 1975; Schmidt and Roth, 1990]. The amygdala pars lateralis of the bullfrog contained a moderate population of GABA-immunoreactive cell bodies, contrasting with the sparse cell immunoreactivity found in *Xenopus* (this study) and *R. esculenta* [Franzoni and Morino, 1989]. Thus, the role of GABA in olfaction in anurans might also occur, to different degrees between species, via the vomeronasal system.

In the telencephalon of both the bullfrog and *Xenopus*, populations of GABA-immunoreactive cells in the striatum were found. These immunopositive cells in the striatum of the bullfrog were intensely stained, unlike those of *R. esculenta* [Franzoni and Morino, 1989]. Still, a greater difference in GABA immunoreactivity in the striatum exists between the bullfrog and *R. temporaria*, which lacks GABA immunoreactivity in the striatum altogether [Kratskin et al., 1992]. This observation indicates that although GABA is widespread in the brain of anurans, species differences might exist even within the same genera.

The areas of the pallium all had varying degrees of GABA-immunoreactive cell populations. The medial pallium had moderate *(Xenopus)* to dense (bullfrog) staining of GABAergic cells. This region projects to many areas within the telencephalon including the septal nuclei, the olfactory bulb, the lateral and medial amygdala and the dorsal and lateral pallia [Northcutt and Ronan, 1992]. All of these areas had populations of GABA-like immunoreactive cell bodies in both species, suggesting a role for GABA in many integrative functions required for responses to stimuli. The medial pallium also receives input from a number of these brain areas, including the olfactory bulb [Northcutt and Ronan, 1992]. Thus, GABA might further influence the integration of olfactory signals beyond their initial processing.

The septum of the bullfrog contained a moderate population of GABA-immunoreactive cell bodies, contrasting

with the weakly stained septum seen in *Xenopus* and previously found in *R. esculenta* [Franzoni and Morino, 1989]. In fact, there seems to be profound variation in GABA-like immunoreactivity in the septum among anuran species. In *R. temporaria*, the lateral septum is devoid of GABA immunoreactivity [Kratskin et al., 1992], whereas the bullfrog had moderate numbers of intensely stained GABA-immunoreactive somata in both septal nuclei.

The preoptic area of the bullfrog was the densest area of GABA-immunoreactive cells observed, with seemingly all cells stained. In sharp contrast, GABA-like immunoreactive somata in the preoptic area of *Xenopus* were sparsely distributed. GABA immunoreactivity in the preoptic area has also been observed in other amphibians [Franzino and Morino, 1989] and in a teleost [Medina et al., 1994]. Although GABA immunoreactivity previously found in the preoptic area of *R. esculenta* is dense, it differs from that of the bullfrog in that staining in the preoptic area of *R. esculenta* is mainly due to the staining of fibers rather than cells [Franzino and Morino, 1989]. Furthermore, the magnocellular nucleus of *Xenopus* and bullfrogs had moderate to dense populations of GABA-like immunoreactive cell bodies as well. Electrical stimulation of the preoptic area elicits vocalizations in frogs [Schmidt, 1984]. GABA could thus influence calling behavior in the bullfrog. In birds, the $GABA_A$ receptor is found in the preoptic area and can be depressed by gonadal sex steroids [Canonaco et al., 1991]. GABA cells in the preoptic area might therefore participate in endocrine and/or behavioral processes.

A sparse population of GABA-like-immunoreactive cell bodies was found in the ventral and lateral hypothalamus of the bullfrog, but was limited to the ventral hypothalamus only in *Xenopus*. GABA-like immunoreactivity in the hypothalamus of amphibians has been previously described as sparse [Franzoni and Morino, 1989]. Likewise, GABA-like immunoreactivity observed in the reptile hypothalamus has also been limited to immunoreactive fibers [Bennis et al., 1991]. Despite the relatively low abundance of observed GABA immunoreactivity, GABA might still mediate hypothalamic control over the neuroendocrine system of both the bullfrog and *Xenopus*. In both fish and mammals, GABAergic innervation of the median eminence-pituitary complex provides evidence for the GABA control of anterior pituitary cells [Kah et al., 1987a, b]. Furthermore, evidence for hypothalamic GABA regulation of the neuroendocrine system in the hypothalamus has previously been shown in mammals [Negro-Vilar et al., 1980; Vincent et al., 1982]. In goldfish *(Carassius auratus)*, the $GABA_A$ receptor agonist, [^{3}H]muscimol, stimulates growth hormone-II release, whereas the action is reversed by the $GABA_A$ receptor antagonist bicuculline [Trudeau et al., 1993]. In rats, the neurosteroid allopregnanolone decreases hypothalamic GnRH release mediated by the $GABA_A$ receptor [Calogero et al., 1998]. The hypothalamus projects to regions of the brain that include the lateral amygdala, preoptic area, dorsal thalamus, pretectum, torus semicircularis, and tegmentum. This suggests that GABA might influence multiple brain pathways, such as the limbic system and sensory systems, via the hypothalamus.

In the bullfrog, the optic tectum, which is involved in integrating the spatial aspects of vision as well as other sensory inputs, was dense with GABAergic cells. There was a very distinct pattern of distribution of GABA-like cell immunoreactivity in the optic tectum, with the heaviest staining occurring in the central and periventricular layers and the lightest staining occurring in the superficial layers. Although less profound than the bullfrog, the optic tectum of *Xenopus* possessed a relatively dense population as well. The importance of GABA to tectal function is also supported by the finding of terminals immunopositive for the GABA synthetic enzyme, glutamic acid decarboxylase (GAD), in the optic tectum of *R. pipiens* [Tyler et al., 1995]. The greatest amount of observed GABA immunoreactivity in *R. pipiens* also has been reported in optic tectum [Li and Fite, 1998]. Furthermore, the bullfrog, as well as *Xenopus*, showed the most numerous immunoreactive cells within layer 6 of the optic tectum, similar to the pattern previously reported in *R. esculenta* [Antal, 1991] and *Xenopus* [Rybicka and Udin, 1994], but in contrast to findings in *R. pipiens* [Li and Fite, 1998]. Some vertebrates, for example, the silver eel *(Anguilla anguilla)*, also possess GABAergic cells primarily in the periventricular layers [Medina et al., 1994]. On the other hand, the densest staining of GABAergic cells occurs in the superficial layers of the pigeon, *C. livia* [Domenici et al., 1988]. In the optic tectum of the chameleon, most GABA-immunoreactive cells reside within the central layers [Bennis et al., 1991]. Thus, variation in patterns of distribution of GABA immunoreactivity may exist across vertebrate classes. The optic tectum is sensitive to both light and moving stimuli with zones of both excitation and inhibition [Aleinikova and Khrenkova, 1984]. The inhibitory influence of GABA within the anuran visual system is evidence for the $GABA_A$ receptor in the optic tectum [Hickmott and Constantine-Paton, 1993; Xiao et al., 1999]. Also, an alternative GABA receptor within the frog optic tectum might exist and enhance excitatory synaptic trans-

mission [Nistri and Sivilotti, 1985; Sivilotti and Nistri, 1989, 1992].

The distribution of GABA throughout much of the visual pathway, in addition to the optic tectum, indicates a major role for GABA in visual integration. The prevalence of GABA in the tectum and midbrain tegmentum supports the hypothesis that GABA participates in the visually-elicited orienting behavior of anurans [Kostyk and Grobstein, 1987; Masino and Grobstein, 1989a, b, 1990]. Also, the suprachiasmatic nucleus, which receives primary retinal innervation, had moderate to dense GABA-like cell immunoreactivity in the bullfrog brain, which further suggests GABAergic function in visual integration. Interestingly, this contrasts with *R. pipiens*, that lacks GABA immunoreactivity in the suprachiasmatic nucleus altogether [Li and Fite, 1998].

The optic tectum is reciprocally innervated with regions of the thalamus of the frog [Trachtenberg and Ingle, 1974; Hofmann et al., 1990]. In particular, layers 6 and 8 of the tectum project to different regions of the thalamus [Montgomery and Fite, 1991]. These layers contained GABA-like immunoreactivity, with layer 6 being very dense in immunoreactivity. Antagonistic interaction between color-sensitive fibers exists in the tectum and thalamus when receiving multiple color receptor stimulation [Maximov et al., 1985]. The inhibitory action of GABA as a candidate neurotransmitter in the antagonistic actions of this pathway seems likely. The isthmic nucleus, reciprocally innervated with the optic tectum [Glasser and Ingle, 1978; Gruberg and Lettvin, 1980; Grobstein and Comer, 1983; Kulik and Matesz, 1997], responds to visual stimuli as well [Gruberg and Lettvin, 1980]. This region might be involved in motion detection [Caine and Gruberg, 1985; Gruberg et al., 1991]. The isthmic nucleus of both the bullfrog and *Xenopus* was sparse in GABA-immunoreactive cells, similar to findings in *R. esculenta* [Pollák et al., 1999] but in contrast to a report in *R. pipiens* which describes GABA-immunoreactive cells in this nucleus as among the densest in the brain [Li and Fite, 1998]. Despite the sparse immunoreactivity we observed in bullfrogs and *Xenopus*, GABA could still influence integrative pathways involving the isthmic nucleus and its projections to the optic tectum [Gruberg and Lettvin, 1980; Li and Fite, 2001].

In the anuran midbrain, auditory integration is performed in the torus semicircularis. The torus is considered the major auditory integrating center as it receives input from brainstem auditory centers, somatosensory systems, and even nonsensory inputs such as from the hypothalamus and preoptic area [Wilczynski, 1981]. In the bullfrog, there was a moderate amount of GABA-like cell body immunoreactivity in all three major regions of the torus semicircularis, whereas *Xenopu*s showed sparse to moderate populations of GABA-like soma immunoreactivity. Cells immunoreactive for GABA have previously been reported in the magnocellular nucleus (ventral torus semicircularis) and laminar nucleus of three anuran species including *Xenopus* [Endepols et al., 2000]. On the other hand, the bullfrog toral magnocellular nucleus has been previously described as devoid of GABA-immunoreactive somata [Simmons and Chapman, 2002]. The presence of GABA in the torus semicircularis has also been found in the auditory systems of other vertebrate classes including a teleost [Medina et al., 1994], a reptile [Bennis et al., 1991], and in the inferior colliculus (homologous to torus) of birds [Carr et al., 1989] and mammals [Vater et al., 1992]. Also, the rat inferior colliculus contains large populations of GAD-immunoreactive cells and terminals [Moore and Moore, 1987]. Thus, the possible influence of GABA in the integration of auditory signals is well conserved.

There is functional support for GABA involvement in auditory processing. In the torus semicircularis of *R. pipiens*, GABA inhibition can shape the frequency selectivity of neurons via the $GABA_A$ receptor [Hall, 1999]. However, it is unknown which cells are involved in this local inhibition. In anurans, the laminar nucleus receives input from forebrain projections of the striatum and thalamus [Endepols and Walkowiak, 1999]. GABA could influence acoustic integration in the torus semicircularis via GABAergic cells which actually reside in the striatum or thalamus. Such broad GABAergic integration occurs in the avian auditory pathway, where the $GABA_A$ receptor antagonist, bicuculline, can block superior olivary stimulated depolarization of the magnocellular nucleus, which relays phase-locked signals to the laminar nucleus [Monsivais et al., 2000]. GABA in the torus might also have a wide-ranging influence on auditory integration as the ventral torus and laminar nucleus project to the thalamus, tegmentum, and the periaqueductal gray [Luksch and Walkowiak, 1998]. Outside of the torus, the striatum, preoptic area and septum might control acoustically guided behavior in anurans as well [Walkowiak et al., 1999]. The abundance of GABA-like immunoreactivity in these regions in both species, with the exception of the preoptic area in *Xenopus*, likewise implicates GABA in the auditory processing of anurans. GABA is known to inhibit spontaneous firing and acoustically evoked responses in the mammalian inferior colliculus via the $GABA_A$ receptor and $GABA_B$ receptor [Faingold et al.,

1989; Yamauchi et al., 1989; Vaughn et al., 1996]. It is likely that GABA could serve a similar inhibitory role in the anuran auditory system.

Further evidence for a role of GABA in the auditory pathway of the bullfrog and *Xenopus* was found in the hindbrain. Although GABA-like immunoreactivity in the hindbrain of both species was relatively low, there were sparse to moderate populations of GABA-like immunoreactive cell bodies in the dorsal lateral nucleus, vestibular nuclei, and the superior olivary nucleus. In ranids, afferents from the amphibian and basilar auditory papillae terminate in the dorsal lateral nucleus [Lewis et al., 1980; Fuzessery and Feng, 1981]. In addition to projecting to the torus semicircularis, the dorsal lateral nucleus projects to the superior olivary nucleus, which is reciprocally innervated with the major auditory nuclei in the anuran brain [Feng, 1986a, b]. Our findings are consistent with previous reports in bullfrogs, *R. esculenta*, and *R. temporaria* [Franzoni and Morino, 1989; Reichenberger et al., 1997; Simmons and Chapman, 2002]. In *R. pipiens*, GABAergic inhibition via the $GABA_A$ receptor can shape the frequency tuning of neurons and modify the response properties of the superior olivary nucleus [Zheng and Hall, 2000]. Thus, GABA could influence the processing of initial input into the brainstem auditory pathway.

Both the bullfrog and *Xenopus* showed GABA-immunoreactive staining in the cell layers of the cerebellum, however, there was a striking difference in that *Xenopus* had many fewer cells stained and the layers were much less distinguishable than in the bullfrog. Immunoreactive staining of cells in the bullfrog cerebellum was similar to that in *R. temporaria* [Reichenberger et al., 1993]. In both ranids, as well as *Xenopus*, the Purkinje cell bodies and part of their primary dendrites were distinctly labeled. It is likely that GABA plays a critical role in neuronal processing in the cerebellum, as there is evidence for very high levels of the $GABA_A$ receptor within this region [Schmitz et al., 1988; Tavolaro et al., 1993].

The distribution of GABA-like immunoreactive cell bodies in the brain of the bullfrog and *Xenopus* revealed widespread immunopositive cells within each species. However, interspecific differences existed with the bullfrog possessing much greater densities of GABAergic cell somata throughout most of the brain. The ubiquitous distribution of GABA cells in both species, coupled with direct and indirect evidence of widespread GABA receptors in the anuran brain [Tavolaro et al., 1993; Aller et al., 1997; Hollis and Boyd, 2003], suggests that GABA influences many brain pathways in anurans including olfactory, visual, vocal, and auditory central systems.

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