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Nitric Oxide Synthase and NADPH Diaphorase Distribution in the Bullfrog *(Rana catesbeiana)* CNS: Pathways and Functional Implications

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Key Words

Nitric oxide synthase · NADPHd · Brain · Amphibian · Frog · Histochemistry · Immunocytochemistry · *Rana catesbeiana*

Abstract

The gas nitric oxide (NO) is emerging as an important regulator of normal physiology and pathophysiology in the central nervous system (CNS). The distribution of cells releasing NO is poorly understood in non-mammalian vertebrates. Nitric oxide synthase immunocytochemistry (NOS ICC) was thus used to identify neuronal cells that contain the enzyme required for NO production in the amphibian brain and spinal cord. NADPH-diaphorase (NADPHd) histochemistry was also used because the presence of NADPHd serves as a reliable indicator of nitrergic cells. Both techniques revealed stained cells in all major structures and pathways in the bullfrog brain. Staining was identified in the olfactory glomeruli, pallium and subpallium of the telencephalon; epithalamus, thalamus, preoptic area, and hypothalamus of the dienceph-

Abbreviations used in this paper

ad	anterodorsal tegmental nucleus	lpd	lateral thalamic nucleus, pars posterodorsalis	Ri	nucleus reticularis inferior
aob	accessory olfactory bulb	lpv	lateral thalamic nucleus, pars posteroventralis	Rm	nucleus reticularis medius
apl	amygdala pars lateralis	ls	lateral septum	sc	suprachiasmatic nucleus
apm	amygdala pars medialis	mg	magnocellular preoptic nucleus	so	superior olivary nucleus
bn	bed nucleus of pallial commissure	ml	mitral cell layer	sol	nucleus of the solitary tract
cb	cerebellar nucleus	mp	medial pallium	str	striatum
cer	cerebellum	ms	medial septum	tcg	corpus geniculatum thalamicum
cg	central gray	mSC	motor field of spinal cord	tna	anterior thalamic nucleus
cn	cochlear nucleus	nB	nucleus of Bellonci	tnc	central thalamic nucleus
dcn	dorsal column nucleus	nmlf	nucleus of medial longitudinal fasciculus	tnl	lateral thalamic nucleus
dp	dorsal pallium	oc	optic chiasm	tnp	posterior thalamic nucleus
dh	dorsal habenula	ot	optic tectum	tnv	ventral thalamic nucleus
dHy	dorsal hypothalamic nucleus	pd	posterodorsal tegmental nucleus	tsc	commissural nucleus of torus semicircularis
ds	dorsal striatum	pe	postolfactory eminence	tsl	laminar nucleus of torus semicircularis
dSC	dorsal field of spinal cord	POAa	anterior preoptic area	tsm	magnocellular nucleus of torus semicircularis
ep	entopeduncular nucleus	POAp	posterior preoptic area	tsp	principal nucleus of torus semicircularis
gl	glomerular layer	ptn	pretrigeminal nucleus	vh	ventral habenula
igl	internal granular layer	ptr	pretoral gray	vHy	ventral hypothalamic nucleus
is	nucleus isthmi	pt	posterior tuberculum	vn	vestibular nucleus
lc	locus coeruleus	ptt	pretectal gray	vs	ventral striatum
lfb	lateral forebrain bundle	pv	posteroventral tegmental nucleus	III	nucleus of the oculomotor nerve
lHy	lateral hypothalamic nucleus	pvo	nucleus of periventricular organ	X	motor nucleus of the vagus
lp	lateral pallium	r	raphe nuclei	XII	nucleus of the hypoglossal nerve

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alon; pretectal area, optic tectum, torus semicircularis, and tegmentum of the mesencephalon; all layers of the cerebellum; reticular formation; nucleus of the solitary tract, octaval nuclei, and dorsal column nuclei of the medulla; and dorsal and motor fields of the spinal cord. In general, NADPHd histochemistry provided better staining quality, especially in subpallial regions, although NOS ICC tended to detect more cells in the olfactory bulb, pallium, ventromedial thalamus, and cerebellar Purkinje cell layer. NOS ICC was also more sensitive for motor neurons and consistently labeled them in the vagus nucleus and along the length of the rostral spinal cord. Thus, nitrergic cells were ubiquitously distributed throughout the bullfrog brain and likely serve an essential regulatory function.

Introduction

Nitric oxide (NO) has emerged recently as an important factor in neural signaling and synaptic plasticity [Park et al., 1998; Rentería and Constantine-Paton, 1999; Prast and Philippu, 2001; Esplugues, 2002]. For example, glutamate activation of NMDA channels induces NO production [Garthwaite et al., 1988, 1989]. In response to increasing Ca^{2+} influx through these channels, nitric oxide synthase (NOS) catalyzes the production of NO and L-citrulline from L-arginine [Brenman and Bredt, 1997]. Once released, NO can then diffuse to neighboring cells to raise levels of cGMP and stimulate various physiological responses [Bredt and Snyder, 1989; Garthwaite, 1995]. To date, this unconventional neurotransmitter has been implicated in a variety of functions in the brain, including modulation of nociception, olfaction, and food and liquid intake [Bruhwyler et al., 1993; Prast and Philippu, 2001]. Nitric oxide also participates in the release of other neurotransmitters, such as noradrenaline and dopamine, and is associated with certain neuronal disorders, including Alzheimer's and Huntington's diseases and Down syndrome [Gotti et al., 2004; Guix et al., 2005]. Because of the novelty and significant functional implications, much effort has since been invested to identify NO pathways [Bredt et al., 1991a; Panzica et al., 1994; Wang and Morris, 1996; Mantelas et al., 2003; Raceková et al., 2003; Yao et al., 2003; Ando et al., 2004; Gotti et al., 2005].

One of the evolutionarily oldest chemical messengers, NO is broadly distributed in cells of vertebrates and invertebrates. For example, NOS is present in the most primitive of metazoans, the sponges, as well as mollusks, annelids, cnidarians and arthropods [Leake and Moroz, 1996; Moroz and Gillette, 1996; Korneev et al., 1998; Giovine et al., 2001; Kitamura et al., 2001; Rast, 2001; Watson et al., 2001; Bullerjahn and Pfluger, 2003; Christie et al., 2003; Moroz et al., 2004; Kurylas et al., 2005]. NOS-immunoreactivity (NOS-ir) in non-mammalian vertebrate brains has been detected in various fishes [Brüning et al., 1995; Villani et al., 2001; Bordieri et al., 2003; Singru et al., 2003; Ando et al., 2004; Masini et al., 2005], reptiles [Brüning et al., 1994b; Smeets et al., 1997; Haverkamp et al., 2000], and birds [Brüning et al., 1994a; Garcia-Calero et al., 2002; Balthazart et al., 2003]. Amphibians are particularly important in this regard, as they provide excellent model systems for behavioral studies [Deban et al., 2001; Arbib, 2003; Woolley et al., 2004; Moore et al., 2005; Wilczynski et al., 2005]. Indeed, NOS distribution has been examined in a few representative amphibians. Nitrergic neuronal cells have been described for the crested newt *(Triturus carnifex)* [Artero et al., 1995], a caecilian *(Dermophis mexicanus)* [González et al., 2002] and three anurans *(Rana esculenta, R. perezi,* and *Xenopus laevis)* [Brüning and Mayer, 1996; Muñoz et al., 1996; Lázár and Losonczy, 1999]. Variability in the distribution of nitrergic neurons across species might indicate important differences in the function of NO, and thus the regulation of neural pathways.

Descriptive studies often supplement NOS immunocytochemistry (ICC) with NADPH diaphorase (NADPHd) histochemisty because NO synthesis requires the cofactor NADPH [Hope et al., 1991] and there appears to be a codistribution between its source, NADPHd, and NOS [Brüning and Mayer, 1996]. NOS activity reduces the electron acceptor nitroblue tetrazolium (NBT) to NBT formazan in the presence of high concentrations of NADPH, particularly for the β form [Spessert et al., 1994]. Formazan-filled neurons can then be visualized under the light microscope. This procedure provides a simple and convenient way to locate putative NOS-positive cell bodies and fibers, which are stained a Golgi-like dark blue. The NADPHd method is quite reliable, although it labels some neurons that are not NOS-ir [e.g., Kulkarni et al., 1994]. Furthermore, NADPHd staining can be sensitive to fixation level [Matsumoto et al., 1993] and appears later in development than NOS-ir in some cases [Cork et al., 2000]. In amphibians, some discrepancies exist in the olfactory system of a number of species [Brüning and Mayer, 1996; González et al., 1996, 2002; Porteros et al., 1996; López and González, 2002; Moreno et al., 2002]. Thus, it would be most prudent to apply NADPHd histochemistry as a supplement to NOS ICC rather than as a substitution.

The bullfrog *(R. catesbeiana)* is increasingly used as a model organism in neuroscience and behavior [Boyd, 1994; Wang et al., 1999; Judge and Brooks, 2001; Lin and Feng, 2001; Bee, 2003; Taylor et al., 2003]. Given the importance of NO to the generation of a variety of behaviors [Hedrick and Morales, 1999; Harris et al., 2002; Del Bel et al., 2005; Nelson et al., 2006; Panzica et al., 2006; Sanderson et al., 2006], we investigated the distribution of nitrergic cells in bullfrog brain and spinal cord. In the two comprehensive studies of NOS distribution in the ranid brain, only NADPHd was used as a marker for NOS [Muñoz et al., 1996; Lázár and Losonczy, 1999]. Variation in staining between the two species was noted and might exist also for bullfrogs [Lázár and Losonczy, 1999]. We thus mapped the distribution of NO-releasing neurons using both NOS ICC and NADPHd histochemistry.

Materials and Methods

Adult bullfrogs were purchased from C. Sullivan Company (Nashville, TN). They were maintained on a 12L:12D controlled photoperiod at 17°C in large tanks (43 × 50 × 128 cm) with flow-through water and were sustained on a diet of goldfish. Studies were conducted in August and under guidelines established by the University of Notre Dame IACUC.

Fifteen adult bullfrogs (mean snout-vent length 15.5 cm ± 0.7 cm) were anesthetized with benzocaine (Sigma). Bullfrogs were perfused transcardially with 40 ml of 4% paraformaldehyde (Fisher Chemical) in 0.1 M phosphate buffer (PB; pH = 7.4 at 4°C). Seven (2 females and 5 males) were used for NADPHd histochemistry and 8 males were used for NOS ICC. The brain and rostral portion of the spinal cord were removed, fixed in paraformaldehyde for 4 h at 22°C, and then placed in 30% sucrose in PB overnight at 4°C. For the NADPHd technique, 4% paraformaldehyde was added to the sucrose solution. Brains were then placed in Histoprep embedding medium (Fisher Chemical), sectioned frontally on the cryostat at 50 μm thick, and placed free-floating in either PB (NADPHd method) or Tris-buffered saline (TBS; 0.05 M Tris and 0.9% NaCl, pH = 7.4 at 4°C for NOS ICC method). Afterward, sections were rinsed 3 times, for 10 minutes each time, at room temperature with the appropriate buffer to clear any residual Histoprep. This rinsing procedure was followed after every incubation step for both techniques which follow.

For NADPHd histochemistry, the technique was modified from the procedure of Brüning and Mayer [1996]. Sections were incubated for 18 h at 37°C in a medium comprised of 1.2 mM βNADPH, 0.3 mM nitro blue tetrazolium, and 0.3% Triton X-100 in PB (all from Sigma). Several sections were incubated without the βNADPH to serve as controls. After incubation, sections were rinsed with cold PB, air dried, and mounted on slides with Permount (Fisher).

For the NOS ICC method, sections were incubated in 1% H_2O_2 in TBS for 15 min, washed with TBS, then exposed to 10% normal goat serum in TBS with 0.3% Triton X-100 (TBST) for 20 min prior to incubation in primary antiserum raised against NOS-I (see below), diluted to 1:500 in TBST for 59 h. Next, they were incubated for 20 h at 4°C in TBST containing goat biotinylated anti-rabbit IgG (Vector Laboratories; Vectastain ABC kit, Elite PK-6101) according to the kit recipe. Sections were washed with TBST after each incubation step (3 times for 5 min each at room temperature). Then, they were exposed to ABC reagent (Elite PK-6101 kit) for 5 h following the kit instructions, washed with TBS, and immunoreactivity revealed with 0.025% 3-3′-diaminobenzidine in 0.05 M TBS with 0.009% H_2O_2 for 30 min. After final rinsing with TBS, tissues were air dried and mounted with Permount.

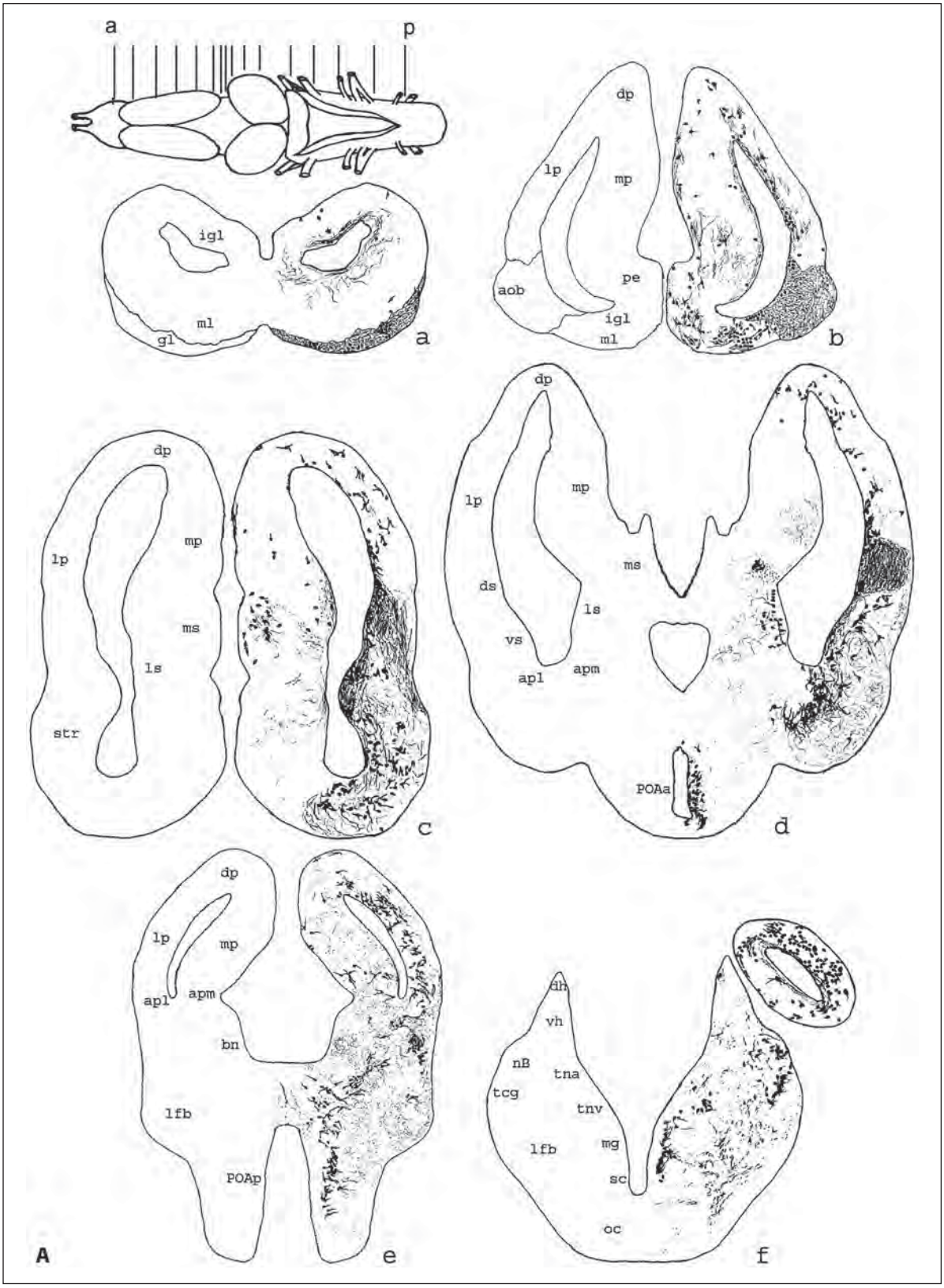
The primary antibody was raised in rabbit against amino acid residues 1414–1429 of the rat neuronal isoform of NOS [#AB1552 Chemicon, Temecula, Calif., USA; Bredt et al., 1991b]. The sequence of this peptide was FISRLRDDNRYHEDIF and it is unique to the neuronal form of the enzyme. Although the sequence of the bullfrog NOS is unknown, this region is highly conserved in two other amphibians, *X. laevis* and *X. tropicalis*. There is only a single substitution of the fourth amino acid from arginine in rat to lysine in both frogs (GenBank #AF053935 for *X. laevis* and *Xenopus tropicalis* genome project #e_gw1.340.46.1). This peptide was not commercially available for control purposes. Specificity of the antiserum was thus tested on alternate sections by replacing the NOS antibody with non-immune rabbit IgG.

Results presented for NADPHd histochemistry also apply to NOS ICC, unless otherwise noted, because the staining pattern was largely similar for both methods. Neurons (and a few blood vessels) were exclusively labeled and staining was never observed in the control sections. Cells were distinguished in terms of staining intensity, size, and morphology. Lightly stained cells consisted of a labeled cytoplasm but clear nucleus, whereas darkly stained cells were uniformly dark. Neurons were classified as either small (<15 μm), medium (15–25 μm), or large (>25 μm) based on the diameter of their perikarya. A variety of cell morphologies was detected, mainly either round or piriform shape (e.g., fig. 2b), with a few fusiform (e.g., fig. 3c), triangular, and multipolar (e.g., fig. 4f) neurons in certain regions. Many regions contained small round and piriform cells of various staining intensities with thin light fibers. We thus refer to these as 'typical' cells and these characteristics should be assumed unless otherwise noted. The nomenclature was based primarily on Muñoz et al. [1996] with a few supplementary terms from Lázár and Losonczy [1999] and the pretrigeminal nucleus from Boyd et al. [1992]. All photographs are from male animals.

Results

NADPHd Histochemistry

Exquisitely fine detail of NADPHd labeled neuronal cell bodies and processes enabled precise localization of putative NO releasing populations and pathways throughout the bullfrog brain. Labeled cells and fibers were present in abundance as early as the olfactory bulb in all layers although the most striking was in the glomerular layer (fig. 1Aa), which contained large neurons that extended their processes into the mitral cell layer. In the internal granular layer, cells clustered around the



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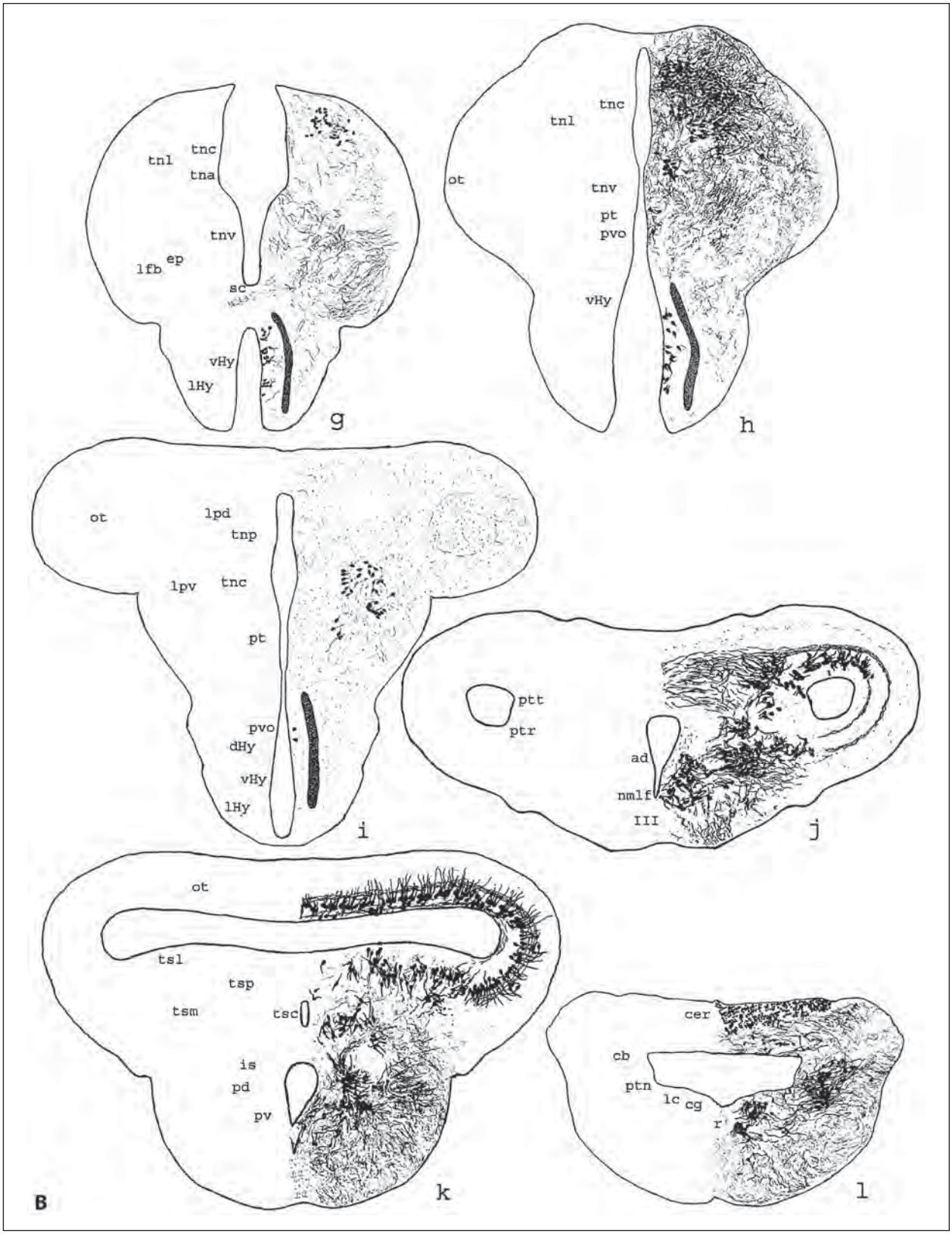

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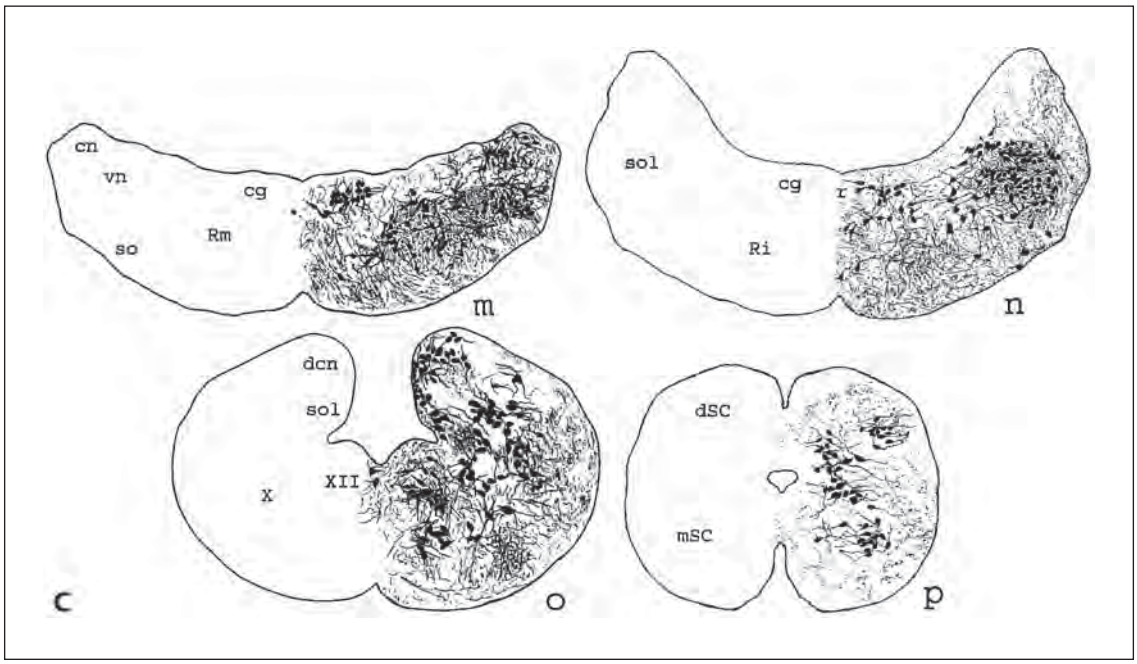
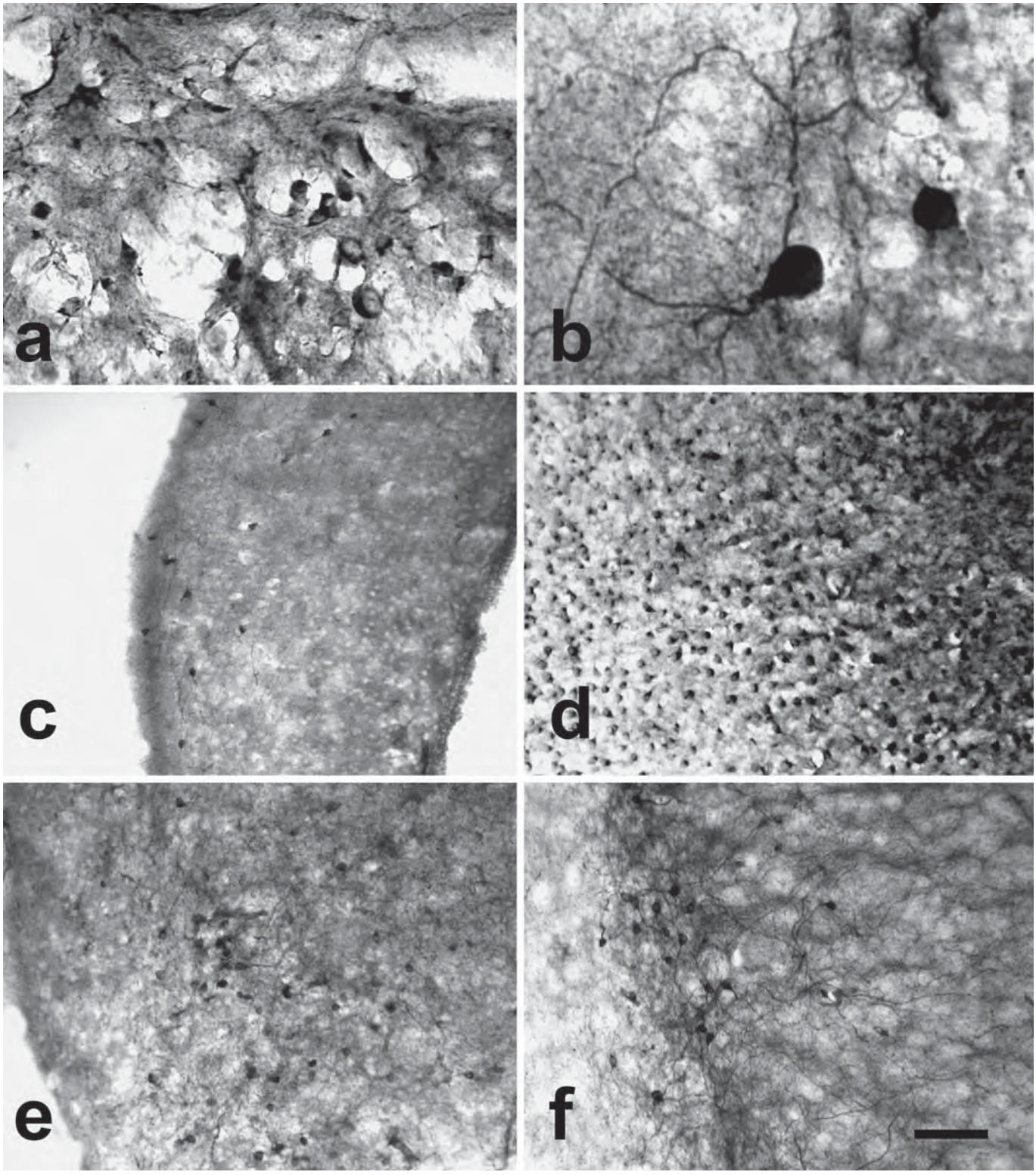


Fig. 1. A. Camera lucida drawings of NADPHd-stained frontal sections from the bullfrog brain (rostral to caudal). Cells, fibers and terminal fields are shown on the right side with neuroanatomical landmarks indicated on the left side (see List of Abbreviations). The approximate levels of the sections are indicated on the top view drawing in the upper left. **B** Continuation of camera lucida drawings from figure 1A. See figure 1A legend for details. **C** Continuation of camera lucida drawings from figure 1B. See figure 1A legend for details.

periventricular area and extended well into the rostral cerebrum (fig. 1Aa, b, 2b). Glomeruli were also stained in the accessory olfactory bulb and diffuse fibers and terminal fields were detected near the ventricle (fig. 1Ab). Typical cells and vertical fibers were also seen in the postolfactory eminence (fig. 1Ab). Similar cells were present in the mitral cell layer although fibers were scarce.

In the cerebral hemispheres, cells were few and scattered throughout the dorsal and medial pallial regions (fig. 1Ab–e, 2c), along with some terminal fields and fibers. A more concentrated group of cells and fibers lined up along the ventricle in the lateral pallium (fig. 1Ab–e). In more caudal areas, a dark band of terminal fields extended from the ventricle just dorsal of the striatum (fig. 1Ac–d, 2f). In this band, numerous thick processes visibly radiate laterally from a congregate of dark cells that became more condensed in the medial extent of the lateral amygdala, their fibers projecting among those of the lateral forebrain bundle. More caudally, cells were smaller and became more dispersed in this amygdaloid nucleus as well as in the medial amygdala. In contrast, neurons in the nucleus accumbens, nucleus of the diagonal band of Broca, and septum (fig. 1Ac–e, 2e) were less well stained (especially in the lateral septal nucleus) and virtually disappeared at more caudal levels, although a distinct bed of terminal fields was present in the medial septum. More ventrally, cells were absent in the bed nu-

Fig. 2. Nitrergic neurons and fibers in the telencephalon of the bullfrog. (**a**) NOS ICC staining of granule cells in the main olfactory bulb. (**b**) Similar cells in the internal granular layer of the main olfactory bulb stained with NADPHd histochemistry. Note these neurons demonstrate the typical piriform or round shape, found throughout the brain. (**c**) Only a few, scarce neurons are found in the medial pallium using NADPHd staining, while large numbers of cells (**d**) are found in the same region with NOS-ICC. Significant populations of NADPHd-stained neurons were also found in the medial septum (**e**) and striatum (**f**). Bar: 20 μm (**a**, **b**), 80 μm (**c–f**).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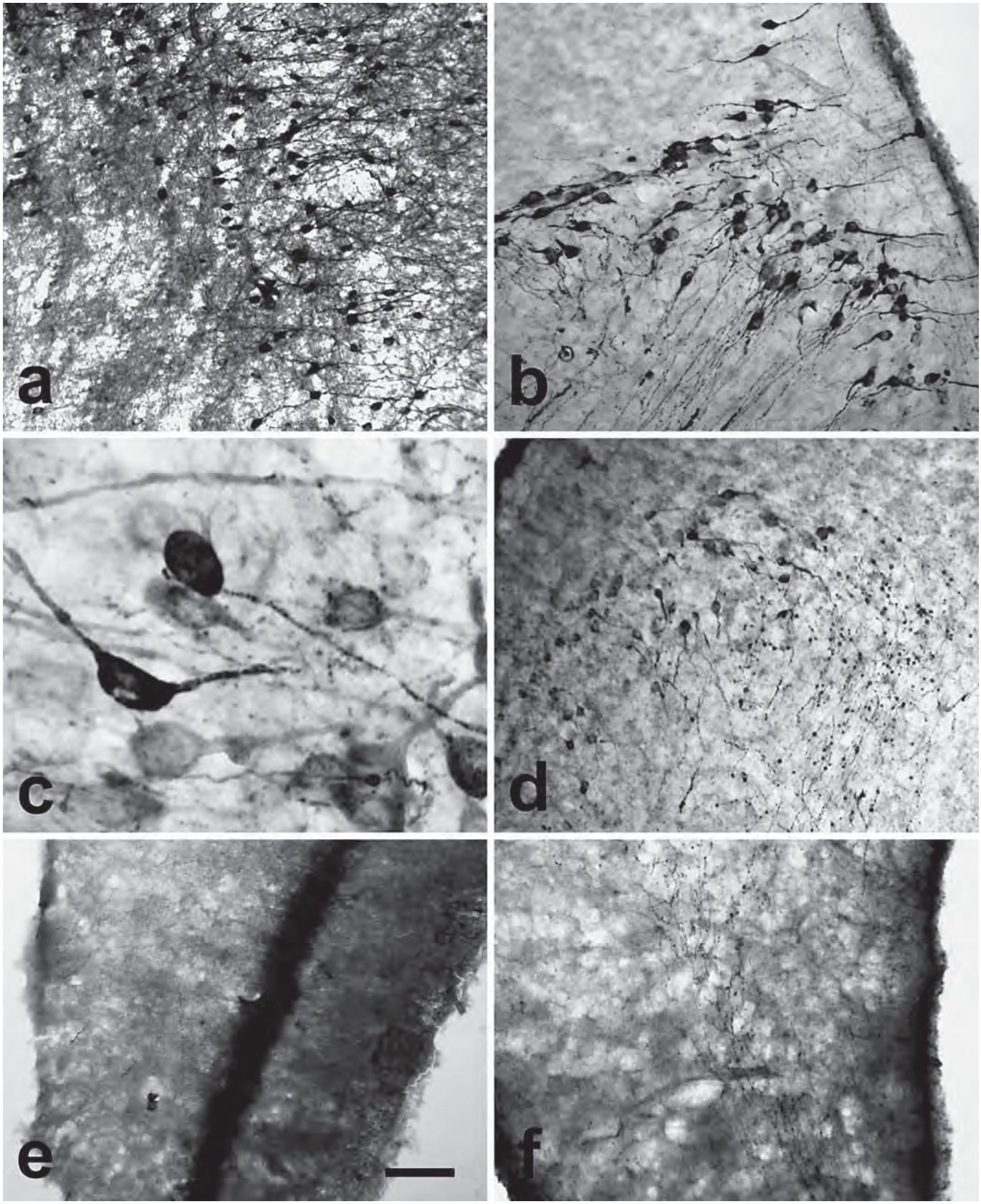
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cleus of the pallial commissure although a conspicuous cluster of terminal fields and beaded fibers was present (fig. 1Ae).

In the rostral diencephalon, the preoptic area was characterized by typical and fusiform periventricular cells with lateral and dorsolateral processes intermingling with external fibers (fig. 1Ad, e). More caudal, the cell distribution changed little although lateral fibers became more abundant, especially in the magnocellular preoptic nucleus (fig. 1Af). Likewise, a number of neurons that surrounded the infundibular recess in the ventral hypothalamus had projections toward the lateral and dorsolateral directions (fig. 1Bg, h). In the lateral hypothalamus, an intensely stained band appeared parallel to the infundibular recess (fig. 1Bg–i, 3e). Anatomical detail was obscured by this diffuse labeling, but the NOS ICC technique later revealed this structure to be a network of beaded vertical fibers (fig. 3f) that merged with the lateral forebrain bundle. These intense fibers were reminiscent of earlier processes in the optic chiasma, where even more heavily beaded fibers also radiated toward the lateral forebrain bundle. Among other hypothalamic nuclei, a few cells were also detected in the suprachiasmatic nucleus, posterior tuberculum, and periventricular organ (fig. 1Af, 1Bg–i). Closer to mesencephalic levels, cells and fibers became very scarce in this region (fig. 1Bi). Staining was not observed in the median eminence.

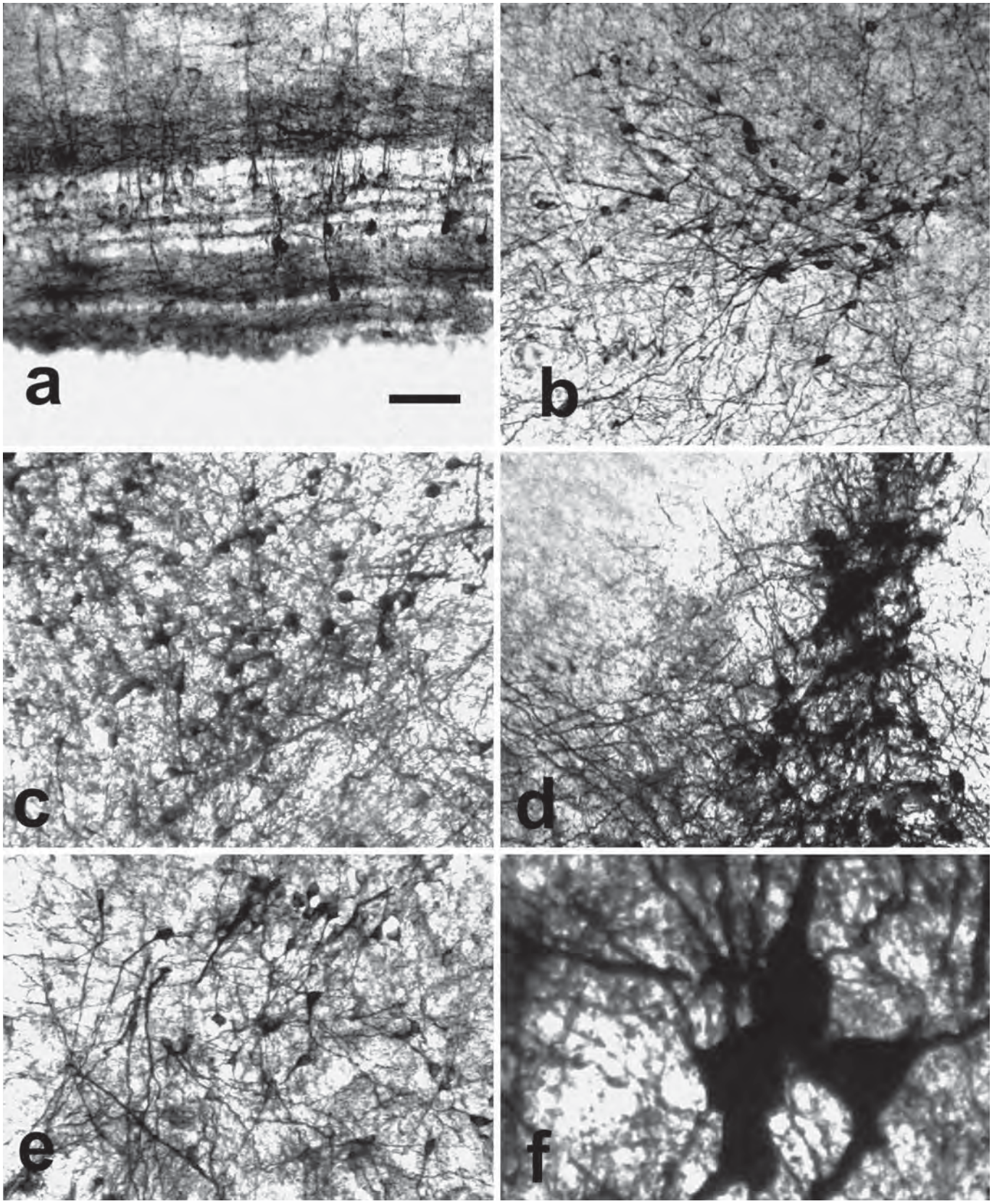
Dorsally, intensely stained terminal fields and fibers were observed in the dorsal habenula but cells were difficult to detect due to the diffuse staining (fig. 1Af). The anterior thalamic nucleus also contained no cells but beaded fibers coursed ventromedially in distinct parallel bands, a pattern which extended into the ventromedial thalamic nucleus (fig. 1Af, Bg). A cluster of lightly-stained cells also assembled in the ventrolateral region, overshadowed by more darkly-stained cells in the nucleus of Bellonci and corpus geniculatum thalamicum, which contained varicose fibers that merged with those in the lateral forebrain bundle and optic tract (fig. 1Af). More conspicuous typical and medium cells appeared within the central and anterior lateral thalamic nuclei (fig. 1Af, Bg–i, 3a). These cells appeared darker and more abundant toward the end of the diencephalon, although they became lighter and fewer again near the mesencephalon. They contained long thick processes that extended horizontally, embedded among a group of intense terminal fields and beaded fibers, which formed dark parallel bands reaching toward the ventromedial direction to the periventricular organ (fig. 1Bh).

In the mesencephalon, distinct populations of neurons were present in the pretectal and pretoral gray (fig. 1Bj). Long dark fibers in the former structure branched dorsomedially along with other varicose fibers and terminal fields. In the optic tectum, many cells were already beginning to gather near the dorsal extent of layer 5 (fig. 1Bj). As the layers became more noticeably stratified, numerous neurons assembled in layers 4 and 6 with processes extending dorsally among a network of terminal fields and fibers, which were especially intense in layers 5 and 7 (fig. 1Bk, 4a). Some cells existed in deeper layers but were scarce in superficial layers.

More ventrally, all nuclei in the torus semicircularis were labeled (fig. 1Bk, 4c). Most cells were small and piriform shaped although some medium and multipolar varieties existed as well among other beaded fibers and terminal fields. The most striking labeling occurred in the magnocellular nucleus (fig. 4c), where numerous cells were accompanied by thick fibers that reached ventrally. Nicely labeled cells were also detected in the anterodorsal tegmental nucleus (fig. 1Bj), nucleus of the medial longitudinal fasciculus (fig. 1Bj), and nucleus profundus mesencephali (fig. 4b). In contrast, the isthmic nucleus remained barren although it was encircled by fibers from an impressive assemblage of diverse cells on the medial side that appeared continuous with the posterodorsal tegmental nucleus (fig. 1Bk, 4d).

In the cerebellum, small round cells were evenly distributed in abundance throughout the molecular layer although some cells and fibers were also dispersed in the granular layer (fig. 1Bl, 5c). A profusion of horizontal fibers dominated the Purkinje cell layer, accompanied by a scatter of Purkinje cells (fig. 5e). Just ventrally, a group of small cells and fibers in the cerebellar nucleus (fig. 1Bl) intermingled with larger multipolar neurons in the pretrigeminal nucleus (fig. 1Bl, 4f). In this nucleus, an extensive field of arborizations approached various regions, including the reticular formation, which contained a mul-

Fig. 3. Nitric oxide releasing cells and fibers in the diencephalon of the bullfrog revealed by NADPHd reactivity (**a**, **e**) and NOS-ir (**b–d**, **f**). NO producing structures were found in the central thalamus (**a**), magnocellular nucleus of the preoptic area (**b**), ventromedial thalamus and lateral forebrain bundle (**d**), and ventral hypothalamus (**e**, **f**). Notice the fusiform (left) and gigantic (lower right) neurons stained in the magnocellular nucleus of the preoptic area (**c**) and the well-labeled fiber tracts revealed by the NOS antibody in the hypothalamus (**f**) that appeared simply as an obscure dark band by NADPHd reactivity (**e**). Bar: 80 μm (**a**, **b**, **d–f**), 20 μm (**c**).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titude of labeled cells of its own. In fact, staining of reticular nuclei, which incorporates the tegmentum, central gray, locus coeruleus, raphe, as well as more ventral medullary nuclei, was among the most striking staining observed (fig. 1Bk–l, Cm, n). Long reticular processes formed an elaborate web of transmission that could be easily traced toward their target correspondents, such as the vagus motor nucleus, where copious amounts of terminal fields were detected (fig. 4e, 5d). An unexpected finding was the presence of labeled motor neurons in the vagus nucleus and rostral spinal cord (fig. 1Co, p, 5d, f) [identification as motor neurons based on Mayhew and Momoh, 1974]. Similarly, labeled populations were obtained in the octaval, trigeminal, superior olivary, dorsal column, and most notably, solitary tract nuclei (fig. 1Co). Fiber tracts extended into the spinal cord where a multitude of labeled cells, fibers and terminal fields dwelled in the dorsal and motor fields (fig. 1Cp, 5f).

NOS Immunocytochemistry

Although patterns of staining were predominantly identical between NOS ICC and NADPHd histochemistry, a few essential differences should be highlighted. In general, better quality was achieved with the NADPHd procedure, especially for visualizing fibers. However, the two methods seemed to be complementary in terms of sensitivity of staining in certain brain regions. NOS immunoreactivity was present in more cells than NADPHd in the internal granular and mitral cell layer of the main olfactory bulb (fig. 2a, b), septum, and pallium, especially in the medial pallium, where NOS-ir cells were plentiful and ubiquitously distributed (fig. 2d) but NADPHd reactive cells appeared more scattered (fig. 2c). In the Purkinje cell layer of the cerebellum, more cells were also labeled with the NOS antibody (fig. 5a), although fibers showed much more NADPHd reactivity. Remarkably, motor neurons in the vagus nucleus (fig. 5b) and spinal cord (fig. 5f) were also better stained for NOS-ir and consistently appeared in most brains. On the other hand, NADPHd staining provided much better resolution in some subpallial regions, such as the striatum and amygdala.

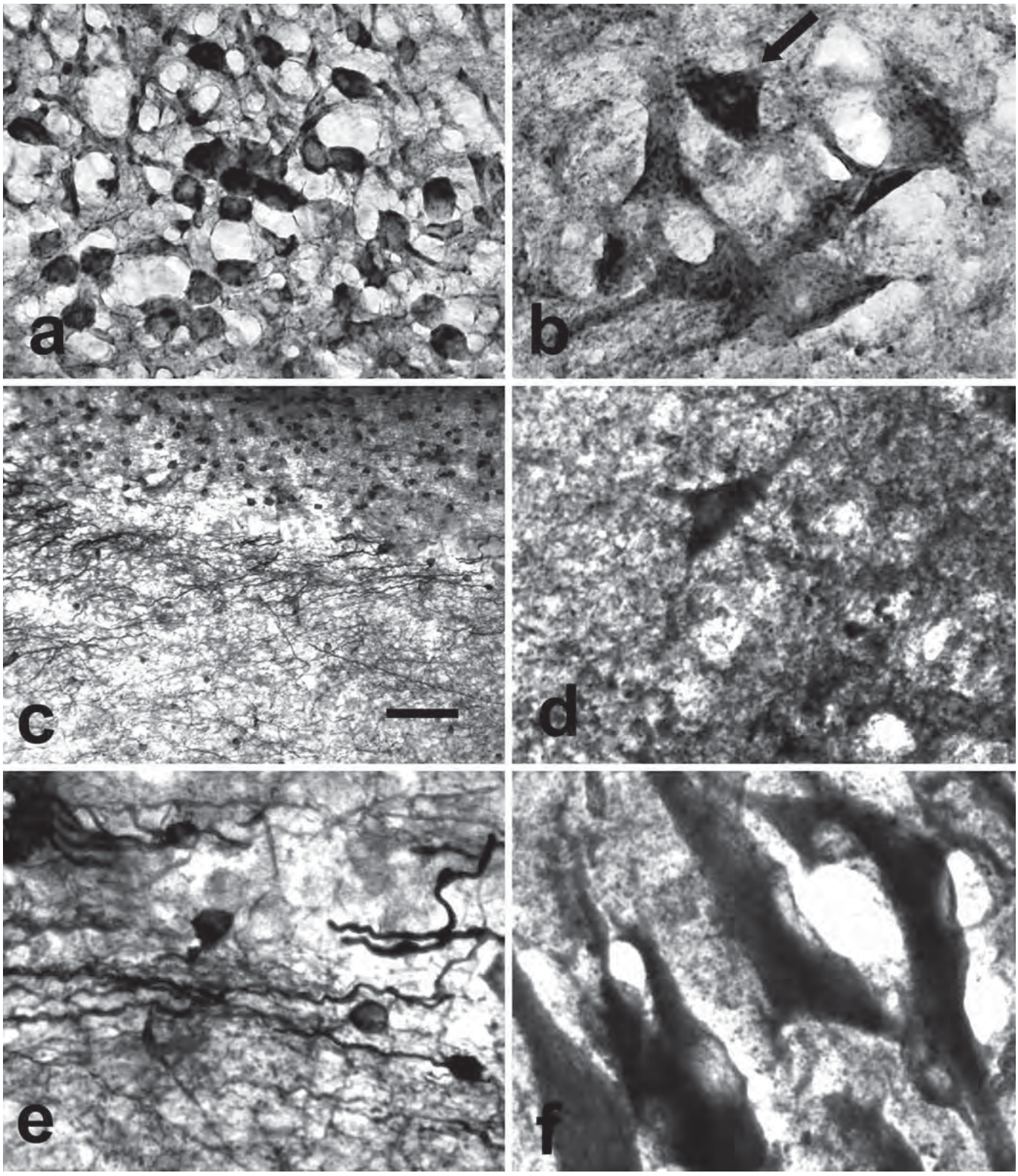
Among the most impressive labeling, however, was that observed in the diencephalon, where numerous heavily beaded fibers traversed toward the lateral forebrain bundle. Cells and fibers in the magnocellular preoptic nucleus (fig. 3b, c) were especially dark and well-stained. The habenula and hypothalamus, by contrast, were poorly stained. Unexpectedly, a distinct labeled population of cells was found in the ventromedial thalamus with fibers that radiated towards the lateral forebrain bundle (fig. 3d), whereas only fibers were labeled with the NADPHd technique. More caudally, NOS-ir cells and fibers were arranged in parallel rows in the anterior thalamic and ventromedial thalamic nuclei. The cells were quite lightly stained, however, so it seems that they were probably overlooked by NADPHd reactivity, although fibers were well-labeled. The discriminatory nature of NOS-ir for beaded fibers in the hypothalamus, though, revealed finer detail of labeled structures, in contrast to the mysterious dark band that resulted from NADPHd reactivity (fig. 3e, f).

Fig. 4. Nitric oxide releasing cells and fibers in the mesencephalon and metencephalon of the bullfrog. Populations well-stained with NADPHd histochemistry were located in the: optic tectum (**a**), nucleus profundus mesencephali (**b**), magnocellular nucleus of the torus semicircularis (**c**), posterodorsal tegmentum (**d**; note the lack of staining in the nucleus isthmi in the upper left corner), reticular formation (**e**; note the extensive branching ventrally toward motor nuclei), and pretrigeminal nucleus (**f**; note the large multipolar neurons characteristic of this nucleus). Bar: 80 μm (**a**–**e**), 20 μm (**f**).

Discussion

Nitric oxide-releasing cells and fibers were found widespread in the bullfrog CNS, from the olfactory bulb to the rostral spinal cord. Although the distribution of nitrergic structures was substantially similar to that in other amphibians, some important differences were also observed. In general, NADPHd histochemistry provided quite a faithful representation of NOS distribution. We found a ubiquitous distribution of NADPHd staining that is consistent with the prevalent role of NO as a neurotransmitter [Bruhwyler et al., 1993; Prast and Philippu, 2001; Esplugues, 2002; Thippeswamy et al., 2006] and other comparative studies[Brüning, 1993; Panzica et al., 1994; Artero et al., 1995; Ando et al., 2004; Gotti et al., 2005]. Furthermore, this histochemical technique provides simplicity and clarity in staining that surpasses that of NOS ICC.

However, differences in staining in bullfrog brain suggest that NADPHd histochemistry and NOS ICC are complimentary techniques. For example, NOS ICC detected more cells in the olfactory bulb and pallium, but was less sensitive in subpallial regions. On the other hand, in bullfrogs both methods were comparable in the olfac-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tory glomeruli, in contrast to the inconsistency observed in some amphibians [González et al., 1996; Porteros et al., 1996; López and González, 2002; Moreno et al., 2002]. Another novel finding was motor neurons in the vagal and spinal nuclei, which were well-labeled with the NOS antibody but were scarce with NADPHd histochemistry. This low NADPHd reactivity might account for some differences between bullfrogs and other ranids [Muñoz et al., 1996; Lázár and Losonczy, 1999]. Thus, interspecies variability might be partially explained by technical differences. In addition, NADPHd histochemistry could overlook some NOS positive cells and should therefore be applied with caution.

The broad distribution of NO releasing cells and fibers in bullfrog brain implies the involvement of this neurotransmitter in many functions. We therefore focus on functional implications and on physiological effects of NO in amphibians. Detailed phylogenetic comparisons of NO distribution in amphibians have been determined by others [Muñoz et al., 1996; Lázár and Losonczy, 1999; González et al., 2002].

Sensory Pathways

Olfaction. Interestingly, the staining patterns for NADPHd and NOS-ir cells and fibers were similar in the olfactory system of the bullfrog, although more NOS-ir cells were labeled in the internal granular and mitral cell layers. These findings contradict sharply with those in other amphibians, where often intense NADPHd reactivity, but lack of NOS-ir, is found in olfactory glomeruli [González et al., 1996; Porteros et al., 1996; López and González, 2002; Moreno et al., 2002]. This pattern is also observed in some other vertebrates [Brüning et al., 1994a; Alonso et al., 1998]. On the other hand, in *X. laevis*, the main and accessory olfactory bulbs did not contain any structure stained by either method [Brüning and Mayer, 1996], similar to a lack of staining in the glomeruli of goldfish [Brüning et al., 1995] and the lizard *Gecko gecko* [Smeets et al., 1997]. In the caecilian *D. mexicanus*, neither NADPHd nor NOS-ir staining is observed in the glomeruli of the main and accessory bulbs, although numerous lightly stained cells occur in the internal granular layer and fibers in the mitral cell layers of both structures [González et al., 2002]. Thus, there is considerable variability in the finding of nitrergic features in the olfactory system of amphibians.

It is likely that at least some of the variations are due to technical differences. For example, we noticed NADPHd staining in the olfactory glomeruli of *X. laevis*, even among lightly stained sections [unpublished data], in contrast to Brüning and Mayer [1996]. In the case of NOS ICC, our antibody was different from those used in other amphibian studies. Discrepancy between histochemical and ICC results has been attributed to interactions with cytochrome P-450, a protein with considerable homology to NOS [Porteros et al., 1996]. When we compared the sequence of the peptide used to generate our antibody with the sequence of *X. laevis* cytochrome P-450 (#BAA09124), however, we found an overlap of, at most, 3 amino acids. It therefore seems unlikely that this NOS antibody cross-reacts with the bullfrog cytochrome P-450. Lastly, anatomical differences detected with the two methods might be due to the presence of proteins that interact with NOS. Such proteins alter the activity and location of NOS in rats [Jaffrey and Snyder, 1996; Dreyer et al., 2004; Xia et al., 2006] and homologs are present in at least one amphibian (*X. tropicalis*; Accession number NM 203698.1).

The presence of NOS-ir in main olfactory afferents and glomeruli implies that NO participates directly in the transmission of odor information from the olfactory epithelium. Some reactivity was also detected in the glomeruli of the accessory olfactory bulb although, in contrast to those in the main bulb, few cells were detected by either method. In addition, the lateral amygdala, which is the main target of accessory olfactory input [Moreno and González, 2003; Moreno et al., 2005; referred to therein as the medial amygdala], also contained stained cells and fibers in bullfrogs, as in some other frogs [Muhlenbrock-Lenter et al., 2005]. Therefore, NO probably participates in multiple destinations in the olfactory pathways. Olfactory receptor neuron activity is indeed affected by NO regulation of cGMP in two amphibian species [Lischka and Schild, 1993; Schmachtenberg and Bacigalupo, 1999, 2000] but, to date, nothing has appeared on the central regulation of olfactory processing by NO in amphibians.

Vision. The presence of nitrergic cells in visual processing brain areas is a common feature in amphibians.

Fig. 5. Nitrergic neurons and fibers in the cerebellum, caudal brainstem and spinal cord of the bullfrog. NOS ICC was noteworthy in demonstrating greater numbers of Purkinje cells in the cerebellum (**a**) and motor neurons in the vagal motor nucleus (**b;** one soma indicated by the arrow), compared to NADPHd histochemistry. The NADPHd technique did label all layers of the cerebellum (**c**) and provide exquisite morphological detail for some cerebellar Purkinje cells (**e**). Likewise, some vagal motor neurons (**d**) and spinal motor neurons (**f**) could be observed with NADPHd histochemistry alone. Bar: 20 μm (**a**, **b**, **d–f**), 80 μm (**c**).

In the bullfrog, many cells were detected in the thalamus and in layers 4 and 6 of the optic tectum, along with fibers in efferent pathways that include tectotegmental, tectodiencephalic, and tectoisthmal tracts. A similar pattern is found in other amphibians [Artero et al., 1995; González et al., 1996, 2002; Muñoz et al., 1996; Lázár and Losonczy, 1999]. For example, in *X. laevis,* nitrergic cells are located in the optic tectum [Brüning and Mayer, 1996], as well as the locus coeruleus and hypothalamic suprachiasmatic and magnocellular nuclei [Allaerts et al., 1997b]. It is likely that NO participates in higher level visual processing in these areas, as it does in other vertebrates [Cudeiro and Sillito, 2006]. For example, presence of NO-releasing cells in the ventral striatum of bullfrogs suggests a role in visuomotor coordination [Buxbaum-Conradi and Ewert, 1999]. Functionally, NO participates in the remodeling of retinotectal projections during ontogeny in *X. laevis* [Rentería and Constantine-Paton, 1996; Cogen and Cohen-Cory, 2000] and might act broadly to regulate synaptic plasticity at adulthood. It was hypothesized that NO functions in background adaptation through its effect on the release of α-melanocyte-stimulating hormone from the pituitary via retino-suprachiasmatic projections. However, no difference in NOS expression in brain and pituitary is found between frogs adapted to white or black background [Allaerts et al., 1997a]. Lastly, NO clearly plays a role in signal transduction in retinal ganglion cells [Hirooka et al., 2000] and photoreceptors [Tsuyama et al., 1993; Kurenni et al., 1994; Noll et al., 1994] of amphibians.

Audition. The expression of NOS in the auditory pathway of phylogenetically diverse organisms strongly suggests a conserved role in this sensory system [Smeets et al., 1997; Reuss et al., 2000; Ando et al., 2004]. In bullfrogs, NOS-releasing cells and/or fibers were localized in all five nuclei of the torus, as well as many of the torus's afferent sources (including octaval nuclei) and projection targets [Feng and Lin, 1991], consistent with other amphibian mappings [Brüning and Mayer, 1996; González et al., 1996, 2002; Muñoz et al., 1996; Lázár and Losonczy, 1999]. Additionally, NO increases GABA release and might possibly have a mediating role in GABA's function in spatial unmasking [Lin and Feng, 2003; Li et al., 2004]. This hypothesis awaits direct support for amphibians, although NO has been shown to participate in auditory processing in the inferior colliculus of the rat [Grassi et al., 1995].

Somatosensory Perception. Cells and fibers were well labeled in the dorsal column nuclei and dorsal field of the spinal cord of the bullfrog. Likewise, efferent projections in the medial lemniscus were stained along with some fibers in target areas of the ventromedial and ventrolateral thalamus. Surprisingly, cells in the ventromedial thalamus were detected well with NOS ICC but not NADPHd histochemistry. Similar labeling for NADPHd reactivity was also seen in other ranids [Muñoz et al., 1996; Lázár and Losonczy, 1999; Morona et al., 2006] but labeled cells were not detected in the dorsal column nucleus of a newt, although fiber afferents were present in the spinal cord [González et al., 1996]. These results, along with those from other species [Brüning et al., 1994b; Egberongbe et al., 1994; Dyuizen, 2003; Stoyanova and Lazarov, 2005], suggest that NO modulates somatosensory perception. It is likely that NO plays a significant role in proprioception [Schuppe et al., 2004; Schuppe and Newland, 2004] and spinal pain processing [Meller and Gebhart, 1993; Thippeswamy et al., 2006]. Presently, it is known that NO modulates lateral line organ sensory processing in the salamander, *Ambystoma tigrinum* [Vega et al., 2006], but data are lacking on the functional involvement of NO in other somatosensory processes.

Motor Systems

An impressive display of NO releasing structures exists in bullfrog motor networks. In the cerebellum, positive staining was observed throughout with the most cells in the molecular layer, labeled Purkinje cells and abundant fibers in the Purkinje cell layer. Similar patterns of NOS-ir occur in *R. esculenta* and *X. laevis* adults [Brüning and Mayer, 1996; Pisu et al., 2002]. In contrast, Purkinje cells were NADPHd-negative in *R. esculenta* and *R. perezi*, although a few positive cells occurred in other layers [Muñoz et al., 1996; Lázár and Losonczy, 1999]. No cerebellar labeling was found in a urodele amphibian or in *X. laevis* juveniles [González et al., 1996; López and González, 2002; Moreno et al., 2002]. In bullfrogs, the striatum and reticular formation also possessed well-labeled cells and rich arborizations that dominated the brainstem. Such a pattern has been observed consistently in amphibians [Artero et al., 1995; Brüning and Mayer, 1996; González et al., 1996, 2002; Muñoz et al., 1996; Lázár and Losonczy, 1999]. Indeed, the prominent staining of reticulospinal neurons is a general feature of anamniotes [González et al., 1996]. Thus, the effects of NO on motor systems are likely pronounced and widespread, mirroring the effects on mammalian motor behaviors [Del Bel et al., 2005].

Expression of NOS in motor neurons has been debated in amphibians. In bullfrogs, we consistently labeled motor neurons in the vagal nucleus, as well as along the ros-

tral spinal cord, using the NOS antibody. The location of stained neurons in the vagus nucleus coincides with that of laryngeal motor neurons identified from tract tracing studies in the bullfrog [unpublished data]. However, autonomic motor neurons are also dispersed in this region [Stuesse et al., 1984] so the identity of these cells cannot be confirmed without double-labeling. The NADPHd method was less sensitive and only stained spinal motor neurons well in one frog. In other amphibians, nitrergic motoneurons occur in the spinal cord of *R. perezi* [Muñoz et al., 2000] and *X. laevis* [Crowe et al., 1995]. However, no activity in motoneurons was reported in other amphibian studies [Artero et al., 1995; Brüning and Mayer, 1996; González et al., 1996, 2002; Muñoz et al., 1996; Lázár and Losonczy, 1999; López and González, 2002; Moreno et al., 2002]. This inconsistency might be attributed to an age-associated increase in NOS expression [Kanda, 1996]. Thus, the more sensitive NOS ICC technique can consistently locate nitrergic motor neurons whereas NADPHd would only detect them in aged frogs.

Central regulation of two motor systems by NO has been documented in amphibian model organisms. First, bullfrog respiratory patterns are modulated by NO and it is proposed that NO provides excitatory input to the respiratory central pattern generators (CPGs) [Hedrick and Morales, 1999; Harris et al., 2002; Hedrick et al., 2005]. The importance of NO to the activity of a variety of CPGs is emerging as a critical role of the transmitter [Scholz et al., 2001; Alford et al., 2003]. Additionally, in a toad, NO in the nucleus isthmi has an inhibitory effect on respiration during hypercapnia [Gargaglioni and Branco, 1999]. Anuran respiratory patterns can thus be altered by NO activity at multiple points in the control pathway. Second, locomotion CPGs are also modulated by NO in the amphibians, *X. laevis* and *R. temporaria* [McLean et al., 2000, 2001; McLean and Sillar, 2002]. Despite a close correlation in location of nitrergic cell populations [McLean et al., 2000; Merrywest et al., 2004], NO has different effects on swimming in the two species by acting as a metamodulator of aminergic and GABAergic neurons [Sillar et al., 2002]. Other stations in amphibian motor control pathways are also likely modulated by NO, from the striatum [Muhlenbrock-Lenter et al., 2005] to the neuromuscular junction itself [Etherington and Everett, 2004].

Regulatory Functions

The elaborate network of nitrergic cells and heavily beaded varicose fibers in the bullfrog diencephalon reflects the complex modulation and heavy traffic that traverses this region, especially in the magnocellular preoptic nucleus, suprachiasmatic nucleus and ventral hypothalamus. Fibers were found in the lateral hypothalamus and habenula as well, but no cells were well stained. The presence of NOS in the preoptic area and ventral hypothalamus seems to be characteristic of amphibians [Moreno and Gonzalez, 2005]. However, more discrepancy occurs in the habenula, where cells have been observed in a caecilian [González et al., 2002] and ranids [Muñoz et al., 1996; Guglielmotti and Fiorino, 1999] but not in *Xenopus* [Brüning and Mayer, 1996; López and González, 2002] or a urodele [González et al., 1996]. It is possible that the bullfrog habenula does contain nitrergic cells, but either staining was not adequate to show them or the expression of NOS is transient [Guglielmotti and Fiorino, 1999].

Co-localization of NOS in neurohypophysial peptide expressing cells may vary with vertebrate class. Both vasopressin and oxytocin have been co-localized with NOS in rats [Nylen et al., 2001a, b; Xiao et al., 2005]. Likewise in a teleost fish, neuronal NOS is co-localized with vasotocin [Bordieri et al., 2003; Bordieri and Cioni, 2004]. On the other hand, vasotocin is not co-localized with NADPHd staining in two bird species [Sanchez et al., 1996]. In bullfrogs, there was substantial overlap in the location of nitrergic and vasotocinergic cell bodies in the magnocellular nucleus [current results compared with Boyd et al., 1992]. Nitrergic cells were about half the size of typical vasotocinergic cells. This supports the hypothesis that cellular co-localization does not occur in bullfrogs, although double-label studies are required. In other amphibians, NOS containing cells are not distributed in regions of great overlap with vasotocin-producing cells [González et al., 1996; Muñoz et al., 1996]. Birds and amphibians might thus be similar in lacking the production of NO in vasotocin cells.

Extensive involvement of diencephalic centers in regulatory functions further underscores the significance of NO signaling, but only reproductive behavior and temperature regulation have been investigated in amphibians. First, expression of NOS in the magnocellular preoptic nucleus is temperature-dependent in the toad *Bufo arenarum* [Nicolini et al., 1998]. Nitric oxide might thus influence thermogenesis or reflect seasonal change which could induce appropriate behavioral accommodations. Second, NOS levels are correlated with amplectic clasping behavior in the frog, *R. esculenta*. In females, NOS levels decrease during amplexus [Gobbetti and Zerani, 1999]. This might be inhibitory via a pathway that decreases 17β-estradiol. The opposite NOS expression pat-

tern occurs in amplexing males and leads to increased testosterone levels [Zerani and Gobbetti, 1996]. It is also likely that changes in gonadal steroids, in a reciprocal fashion, change NO production as shown in mammals [Panzica et al., 2006]. In the bullfrog, NO-releasing cells were located in many brain regions shown to be critical to the display of sexual behavior, including the preoptic area, pretrigeminal nucleus, and laryngeal motor nucleus [Moore et al., 2005; Wilczynski et al., 2005]. The function of NO in these areas remains to be shown.

Conclusion

In general, our findings are consent with other amphibian studies and confirm the extensive NO signaling in sensory, motor, and regulatory systems of the CNS. In contrast to the current viewpoint, our results suggest that NADPHd histochemistry does not nonspecifically label structures but rather might overlook some nitrergic cells. Despite these inconsistencies, some major themes emerge. First, NO signaling is ubiquitous and possibly serves a regulatory function in the refinement and interaction of many different networks. Second, effects of NO are likely exerted at a higher hierarchical level in neuronal communication, such as the CPGs of respiratory and vocal behaviors. Third, specific effects of NO might vary among species despite similar expression patterns, as is the case for the swimming network of *X. laevis* and *R. temporaria*. Lastly, NO modulation appears to be persistent in some networks but might be more transient in others.

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