Research report

Nitric oxide synthase in the brain of a urodele amphibian (Pleurodeles waltl) and its relation to catecholaminergic neuronal structures

A. González, A. Muñoz, M. Muñoz, O. Marín, R. Arévalo, A. Porteros, J.R. Alonso

Department of Cell Biology, Faculty of Biology, University Complutense of Madrid, 28040 Madrid, Spain
Department of Cell Biology and Pathology, Faculty of Biology, University of Salamanca, Salamanca, Spain

Accepted 12 March 1996

Abstract

The neuronal structures with NADPH-diaphorase activity and nitric oxide synthase (NOS) immunoreactivity have been studied in the brain of the urodele amphibian Pleurodeles waltl by means of histochemical and immunocytochemical techniques. Both approaches resulted in the selective labeling of the same neurons and fiber tracts in the brain, except for the primary olfactory fibers that did not stain for NOS but were positive for NADPH-diaphorase. NOS-containing neurons were found in the olfactory bulbs, pallial regions, septum, caudal striatum, amygdala and preoptic area. Only a few diencephalic cells were labeled in the posterior tubercle and ventral hypothalamus. In the brainstem, abundant cells were labeled in the tectum, mesencephalic tegmentum and isthmic region. The most conspicuous cell population was found in the isthmic-pretrigeminal region. Particularly well stained cells were distributed throughout the rhombencephalon in areas related to the descending trigeminal tract, solitary tract, raphe nucleus and the mid-caudal reticular formation. In the cervical spinal cord, NOS-containing cells were present in the dorsal, intermediate and ventral grey fields. Cells in the pre optic, post optic and dorsal root ganglia were also labeled. Double labeling techniques revealed an extensive codistribution of neurons with NOS and catecholamines in the urodele brain but actual colocalization in the same cells was never observed. The organization of the central systems in urodèles with NOS appears to share many features not only with other anamniotes but also with amniotes.

Keywords: NADPH-diaphorase; Nitric oxide; Catecholamine; Amphibian; Evolution

1. Introduction

Nitric oxide (NO) has recently been shown to play an important role as neurotransmitter or neuromodulator and intracellular messenger in many parts of the central and autonomic nervous systems of vertebrates [7,72,86]. Enzymes responsible for NO synthesis constitute a family with at least three distinct isoforms: neuronal, endothelial and macrophage (inducible) NO synthase (NOS) which differ in their subcellular distribution and mechanisms of their regulation [21]. Moreover, it has been reported that NOS has NADPH-diaphorase (NADPHd) activity [13,38] which makes it possible to localize NOS with a specific histochemical method using β-NADPH as substrate and tetrazolium dyes as chromogen that produce an insoluble blue precipitate [42,74]. This technique stains selectively distinct populations of neurons in the peripheral [31] and central nervous systems (see e.g. [82-85]). However, some controversial results have been reported as to whether the precipitate of the NADPHd reaction fully colocalizes with NOS in the labeled neurons. A one-to-one correlation was observed by some authors [6,13,88], whereas others [15,37,41,66,76] reported differential distribution in certain brain regions.

In the literature, many papers dealing with the distribution of NADPHd in the mammalian brain, as a putative marker for NO, have appeared (e.g. [48,85]). Moreover, the distribution of NADPHd partially overlapped that of several other neuroactive substances [2,3,11,48,57,67,77,82,83]. In particular, a selective distribution of NADPHd within a subpopulation of monoaminergic neurons has been observed in the rat brain [39] and a role for NO in the metabolism of catecholamines suggested [59].

Although NO synthases were originally identified in mammalian tissues, there is recent evidence that NO might be a neuronal messenger of early phylogenetic origin and conserved throughout evolution, since NOS activity has been demonstrated in different tissues of arthropods, gas-
Fig. 1. A–I: diagrams of transverse sections through the telencephalon and diencephalon of Pleurodeles waltl at the levels indicated in the schematic dorsal view of the brain. NADPHd-positive cell bodies (large dots) and fibers (small dots, wavy lines) are represented in the right side of the brain of every section. ab, accessory olfactory bulb; Ap, amygdala, pars lateralis; Apm, amygdala, pars medialis; ac, anterior commissure; Ch, cerebellum; cc, central canal; cp, posterior commissure; Dp, dorsal pallium; epl, external plexiform layer; flm, fasciculus longitudinalis medialis; gl, glomerular layer; H, habenula; Hyp, hypophysis; igl, internal granular layer; Ip, nucleus interpeduncularis; Is, nucleus isthmi; ll, lateral line area; Le, locus coeruleus; Lp, lateral pallium; Ls, lateral septum; ml, mitral cell layer; Mp, medial pallium; Ms, medial septum; nDT, dorsal tegmental nucleus; nt, nervus terminalis; nVT, ventral tegmental nucleus; oc, optic chiasm; POa, anterior preoptic area; POp, posterior preoptic area; Ra, nucleus raphe; Rni, nucleus reticularis inferior; Rm, nucleus reticularis medius; rt, fasciculus retroflexus; S, septum; SC, nucleus suprachiasmaticus; so, tractus solitarius; Str, striatum; tect., mesencephalic tectum; Thd, dorsal thalamus; Thv, ventral thalamus; To, optic tectum; TP, nucleus tuberculi posterioris; v, ventricle; Vd, descending trigeminal tract; VH, ventral hypothalamic nucleus. Scale bar = 1 mm. J–T: diagrams of transverse sections through the mesencephalon, rhombencephalon and upper spinal cord of Pleurodeles waltl at the levels indicated in the schematic dorsal view of the brain. NADPHd activity is represented as in Fig. 1 A–I. Scale bar = 1 mm.

teropods and echinoderms [17,18,46,55]. The evolutionary history of the NO system in the CNS of vertebrates has received increasing attention in the last few years. Thus, the fragmentary data of NO activity in the brains of nonmammalian species [44,56,62,63,70] have been documented with comprehensive studies on the distribution of NADPHd in the brain of a cyclostome [69], several teleosts [5,10,68,80], the turtle [9] and some birds [8,54]. NADPHd activity has also been confirmed in the retina, pineal organ, brain and peripheral nervous system of anuran amphibians [12,43,50,62,63].

Urodeles, and in particular salamanders, form the basis for numerous comparative neuroanatomical studies. Earlier, it was suggested [36] that the simplified arrangement of neurons and fibers in these brains represents a basic pattern common to all vertebrates [61]. However, in the CNS of urodeles most neurons are crowded close to the ventricle in a dense central grey layer, with thick dendrites that arborize in the overlying white substance. Thus, nuclei can be recognized as local condensations within this periventricular grey [35,36,53]. The outer, white zone contains well-defined pathways e.g. [51,52,87]. Immunohistochemical studies have helped to clarify and define ‘hidden’ nuclei in the urodele brain as, for instance, the detailed maps of distribution of catecholamines [22,27,29,30].

Histochemistry for NADPHd reveals Golgi-like images of particular neurons within distinct brain areas giving detailed morphological information [4]. With this technique we have mapped the distribution of NADPHd-positive cells and fibers in the brain of the urodele Pleurodeles
In order to make certain that the NADPHd histochemistry reveals neuronal structures that contain NOS, immunocytochemistry using K205 antiserum prepared against purified rat neuronal nitric oxide synthase has been performed on the same tissues. A second goal of our study was to investigate the putative sites of interaction between NO and catecholamines. Thus, NADPHd histochemistry and NOS immunohistochemistry were combined with tyrosine hydroxylase (TH) immunohistochemistry.

2. Materials and methods

The brains of 27 adult Iberian ribbed newt, Pleurodeles waltl, were obtained from the laboratory stock of the Department of Cell Biology, University Complutense of Madrid. They were kept at 22°C with a light/dark cycle of 12:12 h. The animals were anesthetized in a 0.3% solution of tricaine methanesulphonate (MS-222; Sandoz), and perfused transcardially with saline followed by a fixative mixture (4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4). The brains were removed, further fixed in the same fixative for 5–6 h, and subsequently immersed in a solution of 30% sucrose in PB for 5–8 h at 4°C (until they sank). The brains were then blocked in a solution of 15% gelatin and 30% sucrose in PB and stored for 5 h in a 4% formaldehyde solution, with 30% sucrose, at room temperature. Sections were cut on a freezing microtome at 40 μm in the frontal plane, collected in cold PB and rinsed deeply in the same buffer.

2.1. NADPH-diaphorase histochemistry

Free-floating sections were rinsed in Tris-buffered saline (TBS), 0.1 M, pH 8.0 and then incubated in a medium made up of 1 mM β-NADPH, 0.8 mM nitro blue tetrazolium and 0.08% Triton X-100 in 0.1 M TBS (pH 8.0), at 37°C for 1–2 h. The reaction was stopped by successive rinses in cold TBS. Some sections were incubated systematically in a medium without β-NADPH. A second group of control sections was heated in TBS to 70°C for 10 min. In both cases, no reaction was observed. All sections were then mounted on slides (mounting medium: 0.25% gelatin in Tris buffer, pH 7.6), dried overnight and coverslipped. In some cases, sections were counterstained with 0.1% cresyl violet.

2.2. NOS immunohistochemistry

In eight cases, alternate sections were incubated for NADPH-diaphorase reaction and nitric oxide synthase (NOS) immunodetection. Thus, the sections were incubated in a sheep antiserum against neuronal NOS, diluted 1:20000 in TBS containing 0.5% Triton X-100 (TBS-T), for 48–60 h at 4°C. Rhodamine-conjugated donkey anti-sheep second antiserum (Chemicon) diluted 1:100 in TBS-T.
was applied for 1.5 h at 20°C or fluorescein-conjugated rabbit anti-sheep second antibody (Vector) diluted 1:100 in TBS-T was used. The sections were mounted on glass slides and coverslipped with Vectashield (mounting medium for fluorescence, Vector).

Immunohistochemical control experiments involved parallel incubation of alternate sections either with antiserum raised against different antigens, normal serum, or with the omission of primary antiserum. No residual immunostaining was detected. Furthermore the specificity of the antiserum has been previously described [34]. This antibody recognizes neuronal NOS using Western blotting and the immunoreactivity is abolished by absorption of the K205 antiserum with recombinant neuronal NOS protein (1 mM overnight at 4°C).

2.3. Double labeling for NADPHd and NOS

In six cases, NOS immunoreacted sections were mounted on glass slides and studied, plotted and photographed, without drying. They were then collected in TBS and rinsed profusely for 1 hour. The histochemical technique for NADPHd was subsequently performed. The comparative study of both stainings was accomplished after plotting and photographing the NADPHd reactive elements.

2.4. Double labeling for NADPHd or NOS and TH

In four cases, NADPHd-reacted sections were incubated in mouse anti-TH antiserum (Incstar) diluted 1:1000 in TBS-T for 48–60 h, at 4°C. The sections were subsequently processed with the peroxidase antiperoxidase (PAP) technique [73]. This included incubation in goat anti-mouse IgG (Dakopats) diluted 1:50 in TBS-T, for 1 h at room temperature, and then in mouse PAP (Chemicon) diluted 1:500 in TBS-T, for 1 h at room temperature. They were reacted with 0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) with 0.01% H2O2 in TBS, for 10–20 min.

In six cases, sections were simultaneously incubated in a cocktail of sheep anti-NOS (diluted 1:20000) and mouse anti-TH (diluted 1:1000) in TBS-T, for 60 h at 4°C. The sections were then incubated in biotinylated horse antimouse (Vector, diluted 1:100), for 1 h at room temperature and then in a mixture of Texas Red-conjugated streptavidin (Vector, diluted 1:100) and fluorescein-conjugated  

Fig. 2. Photomicrographs of NADPHd-positive cells and fibers in the olfactory tract and glomeruli (A), accessory olfactory bulb and rostral hemisphere (B), dorsal pallium (C) and ventral aspect of the septum (D). Scale bars = 500 μm (A,B); 100 μm (C,D).
rabbit anti-sheep (Vector) diluted 1:100, for 2–3 h at room temperature. Sections were mounted on slides and photographed with epifluorescent illumination.

2.5. Evaluation and presentation of the results

The pattern of distribution of NADPHd activity matches almost completely that of NOS immunolabeling. However, the clear picture of well-defined labeled cells and fiber structures achieved by NADPHd histochemistry was not obtained by NOS immunohistochemistry. Therefore, for the description of the cell groups and fiber systems with putative NOS activity we have based our results on the material stained for NADPHd.

The demonstration of NADPHd activity was achieved by means of a direct method using exogenous NADPH [4]. In this variant, the activity of endogenous NADPHd reduced NADPH in the presence of the dye nitro blue.

Fig. 3. Photomicrographs of NADPHd-positive cells and fibers in the anterior preoptic area (A), the area ventral to the mesencephalic tectum (B), the isthmic-pretrigeminal area (C,D), the rhombencephalon (E) and, in a higher magnification, the group of cells ventral to the solitary tract (F). Scale bars = 100 μm (A,B,D,F); 200 μm (E); 500 μm (C).
tetrazolium to form a blue insoluble reaction product that labels neuronal elements in the brain. The distribution of NADPHd positive cell bodies and fibers in the brain of *Pleurodeles waltl* was charted in Fig. 1 showing representative transverse sections of which the levels are indicated. Drawings were made by means of camera lucida in which the sections counterstained with cresyl violet facilitated the interpretation of the localization of the reactive structures. The nomenclature used in the present study is essentially the same as that used in previous studies in urodele amphibians [27,28,30].

3. Results

3.1. Distribution of NADPH-diaphorase activity

Cell bodies, dendritic processes and thin axon arborizations with varicosities or terminal swellings were clearly revealed in all the divisions of the brain and spinal cord of *Pleurodeles waltl* and the pattern of staining was consistent between animals. NADPHd-positive cells were identified as neurons and glial cells were never stained. Cell groups with NADPHd activity were generally identified in ill-defined zones in the brain, what serves to delineate new ‘hidden’ nuclei in the CNS of urodeles (see [61]).

3.1.1. Telencephalon

The most rostrally located NADPHd-positive cell bodies were found in the main olfactory bulb (Fig. 1A). They lie close to the ventricle, confined to the granular cell layer and are weakly stained. The olfactory glomeruli of both the main and accessory olfactory bulbs are strikingly dark-labeled for the NADPHd reaction (Fig. 1A and Fig. 2A,B).

Caudally in the hemispheres, numerous positive cell bodies are distributed widely in the pallial areas (Fig. 1A–E and 2B,C). They are moderately stained and their processes are oriented parallel to the brain surface. In the medial wall of the hemispheres, weakly labeled cells are present in the medial aspect of the septum (Fig. 1B,C and 2D). A few cells are located more caudally in the ventral striatal area and a segregated group of positive cells was found at the border between the lateral pallium and the striatum. At caudal telencephalic levels, a large population of cells is labeled in the amygdala, both in medial and lateral components (Fig. 1D,E). These neurons have pear-
shaped perikarya with thick processes that enter the ventral or lateral fiber zone where they arborize.

Throughout the ventral telencephalon the fiber tracts of the nervii terminalis are labeled up to the preoptic area (Fig. 1A–C). The olfactory tracts are never stained for NADPHd.

3.1.2. Diencephalon

The hypothalamic anterior preoptic area has a conspicuous group of NADPHd-positive cells (Fig. 1E and Fig. 3A). The cell bodies are located within different rows of the cell layer that lines the anterior preoptic recess of the hypothalamic ventricle. The neurons are pear-shaped cells with a main, thick process that crosses the cell layer and arborizes profusely in the lateral fiber zone of the preoptic area. Some cells were usually observed in the innermost cell layer, just bordering on the ventricle. However, structures such as club-like protrusions, characteristic of CSF-contacting cells in this region, were never observed. More caudally in the hypothalamus, only a few cells are scattered in the posterior preoptic area and in the ventral hypothalamus, while fiber profiles are always present in the lateral aspect of the hypothalamus (Fig. 1F–I). In the infundibulum, fibers directed ventrocaudally towards the median eminence are not labeled.

Dorsally in the diencephalon, no labeled cells were found, whereas NADPHd-positive fibers innervate the asymmetrically larger, left habenula and the lateral neuropils of the ventral thalamus, i.e. the neuropils of Bellonci and the ‘corpus’ geniculatum thalamicum (see [86]) (Fig. 1F,G).

3.1.3. Mesencephalon

Numerous NADPHd-positive cell bodies were found in the mesencephalon, both in the tectum and the tegmentum. Within the optic tectum, abundant neurons, weakly labeled, were distributed in all cell layers of the periventricular grey. These are pear-shaped cells with a primary process that ascends perpendicular to the brain surface and arborizes in the deep portion of the tectal fiber layers (Fig. 1H–K). Occasionally, the cells of the mesencephalic trigeminal nucleus are moderately stained but the reaction product was always eliminated if the sections were preincubated.

From rostral mesencephalic levels, a group of darkly labeled cells lie beneath the posterior commissure, in the dorsal tegmental nucleus. These cells form a column through the midbrain and have processes that arborize into the overlying tectum and in the adjacent tegmental lateral zone (Fig. 1H–J). More caudally, in the ventral tegmental nucleus another group of positive cells whose dendritic processes distribute widely in the ventrolateral tegmentum of the mesencephalon was present (Fig. 1J,K).

In the limit between the mesencephalon and rhombencephalon at isthmic levels, a cell group was found beneath the caudalmost portion of the tectum. These cells have elongated cell bodies with dendritic processes directed toward the tectum where they arborize in the deep fiber layer among the plexus formed by the tectal cells (Fig. 1K).

3.1.4. Rhombencephalon

In the rhombencephalon, many NADPHd-positive cells are present. Probably the most conspicuous cell group in the brain is located rostrally in the rhombencephalon, in the isthmic-pretrigeminal region beneath the cerebellum.

Fig. 5. Photomicrographs of NADPHd-positive cells in the spinal cord at cervical levels (A,B) and in the preotic ganglion (C). Scale bars = 200 μm (A,C); 100 μm (B).
(Fig. 1L and 3C,D). This compact group in the lateral margin of the ventricle has a widespread fiber arborization in the lateral and ventral rhombencephalic tegmentum. Only a few cells, located more laterally, are separated from the group and their main processes are horizontally oriented toward the medial aspect of the group (Fig. 3D).

Caudally in the rhombencephalon, numerous positive cells are found in the nucleus of the solitary tract, the medius and inferior reticular nuclei, and in the raphe nucleus (Fig. 1M–P, 3E,F and 4). The cell bodies located ventral to the solitary tract and in the reticular nuclei have ventrally or ventrolaterally directed dendritic trunks that penetrate the fiber zone where they arborize. The group of cells ventral to the solitary tract are smaller than those in the reticular nuclei (Fig. 3E,F). Particularly striking in the rhombencephalon is a group of large NADPHd-positive cells at mid- and caudal rhombencephalic levels, coincident with the cells of the reticular nuclei (Fig. 1M–P and Fig. 4). These large neurons possess a complex dendritic arborization in the ventral tegmentum, mainly oriented in the transverse plane (Fig. 4A,B). Several of the processes cross the midline and some surround the medial longitudinal fasciculus (Fig. 4B,C). Another conspicuously labeled cell group is present just at the raphe (Fig. 4D). They are large neurons in the midline with processes that extend to both sides of the rhombencephalon. Among the profuse fiber arborizations present in the rhombencephalic tegmentum, thick fibers in the descending trigeminal tract are always labeled in the ventral or ventrolateral aspect of the medulla, and some of the small cells in the periventricular grey that extend their dendrites into this tract could represent the nucleus of the descending trigeminal tract (see [26]) (Fig. 1M,N and 3F).

3.1.5. Spinal cord

The number of cells labeled in the spinal cord was very high. In the present study, only cervical levels have been examined. From the obex, and further caudally, a NADPHd-positive cell group is located dorsolaterally to the ependyma and these neurons have very long processes that extend dorsolaterally toward the descending trigeminal tract (Fig. 1Q–S and Fig. 5A,B). In addition, abundant cells are labeled in the ventral spinal grey and their number increases caudally. All through the cervical spinal cord, labeled fibers are present in the ventrolateral funiculus.

3.1.6. Brachial and dorsal root ganglia (DRG)

Sections of the preotic (trigeminal-facial), postotic (glossopharyngeal-vagal) ganglia and DRG were stained

Fig. 6. Photomicrographs of transverse sections through the brain of *Pleurodeles walti* showing NOS-immunoreactive cells in the dorsal pallium (A), ventral to the mesencephalic tectum (B), mesencephalic tectum (C) and isthmic-pretrigeminal area (D). Scale bars = 100 μm (A–C); 200 μm (D).
for NADPHd. The ganglion cells were intensively labeled in the preotic and DRG (Fig. 5C), while in the postotic ganglion only a subpopulation of cells were moderately labeled for NADPHd.

3.2. Localization of NOS immunoreactivity

The precise localization of NOS in the brain of *Pleuronectes waltl* was achieved by immunolabeling with an antiserum that has been previously shown to be specific not only in mammalian but also in reptilian and amphibian tissues [71]. The pattern found for the distribution of NOS-immunoreactive neuronal elements was coincident with that described for NADPHd histochemistry. Not only is the distribution of labeled neurons and fibers the same but even the relative staining intensities (weak-to-heavy) were always similar (Fig. 6A–D). Moreover, the cases of double labeling in the same brain section demonstrated a one-to-one correlation between NOS- and NADPHd-labeled neurons (Fig. 7A–F). However, it should be mentioned that two exceptions were found in the primary olfactory fibers that are strongly labeled for NADPHd.

Fig. 7. Localization of NOS by immunocytochemistry (A,C,E) and NADPHD histochemistry (B,D,F). Pairs of photomicrographs illustrate identical sections where the correlation between both stainings is demonstrated in the mesencephalic tegmentum (A,B), isthmic-pretrigeminal region (C,D) and rhombencephalic reticular formation (E,F). Arrows are meant to help for identification of the same structures. Additional double labeled neurons are present out of the plane of focus. Scale bars = 50 μm.
h histochemistry (Fig. 2A,B) but lack NOS immunoreactivity and in certain structures that were only occasionally labeled for NADPHd, such as the mesencephalic trigeminal cells, were confirmed to be negative for NOS immunoreactivity. The labeling of the latter was interpreted as a failure of the technique since in all cases with preincubation of the sections (cases for double labeling experiments) they were consistently NADPHd-negative.

3.3. Colocalization of NOS and catecholamines

The pattern observed for the distribution of NOS-containing neurons in the urodele brain points to an extensive

Fig. 8. Double labeled sections for NOS immunocytochemistry and TH immunoreactivity. The co-distribution, but not co-localization in the same neurons, of both stainings is illustrated for the mesencephalic tegmentum (A,B), the isthmic-pretrigeminal region (locus coeruleus area) (C,D) and nucleus of the solitary tract (E,F). Scale bars = 100 μm.
codistribution with catecholaminergic cells [29]. Thus, in the olfactory bulb, preoptic area, mesencephalic tegmentum, locus coeruleus region, and nucleus of the solitary tract intermingled NOS and CA cells were found. However, in sections stained simultaneously for TH immunoreactivity and NADPHd histochemistry it became clear that colocalization in the same neurons was absent. With this technique, the blue NADPHd reaction product of NOS cells and the brown DAB reaction product for TH cells are distinguished in separate populations of neurons. However, sometimes the two labelings are difficult to discern, mainly in areas where dark-blue neurons could mask the putative brown staining. In order to clarify the actual lack of colocalization between catecholamines and NOS in the same neurons, double fluorescent immunolabeled sections were studied and, in spite of the high degree of codistribution found, colocalization of both substances was absent in all neurons. Thus, in the main olfactory bulb, while TH-positive cells were found in almost all bulbar layers, NOS-positive cells were only found in the inner portion of the granular layer and no colocalization was present. Similarly, in the anterior preoptic area, the conspicuous NOS positive cell group lacks TH immunoreactivity, and even the periventricular NOS cells are clearly distinct from the CSF-contacting, TH-positive cells. In the mesencephalic tegmentum, NOS-immunoreactive neurons occupy a dorso-lateral position with respect to the catecholaminergic population (Fig. 8A,B). In the locus coeruleus and in the nucleus of the solitary tract, the NOS- and TH-containing cell populations are also segregated. The noradrenergic cells in subcerebellar regions, termed here locus coeruleus (see [29]), are slightly medially located to the conspicuous group of NOS positive cells situated in the isthmic-pretrigeminal zone and, although some cells intermingle, colocalization of both substances in the same cell bodies was never observed (Fig. 8C,D). Similarly, in the nucleus of the solitary tract, although both cell populations are intermingled, the NOS-immunoreactive cells are located closer to the ventricle, while TH-positive cells form a band of cells more closely related to the fibers of the solitary tract (Fig. 8E,F).

4. Discussion

Our present observations showed the topographical localization of NADPHd-positive cells and fibers in the brain of a urodele amphibian. As noted in the Introduction, a one-to-one correlation between NADPHd histochemistry and NOS immunoreactivity has been described in various sites in the CNS [6, 16, 32, 38, 41, 66, 85]. However, discrepancies between both methods have been noted in some areas of the nervous system [9, 38, 41]. In particular, studies of the distribution of NOS in the teleost brain have shown that the main difference between NADPHd histochemistry and NOS immunoreactivity was mainly due to NADPHd staining of numerous glial structures that did not show any NOS immunostaining [37]. Moreover, a recent study in the goldfish [10] has revealed that the NADPHd technique needs a preincubation of the sections as a prerequisite for demonstrating NOS-specific NADPHd-staining. Our results in Pleurodeles waltl show that the histochemical procedure used to reveal NADPHd activity is highly sensitive and specific for neuronal structures. Thus, cells of the endothelial wall and glial structures were never stained, what contrast with the results obtained in amphibians using different histochemical protocols [12]. In addition, in our study NOS immunocytochemistry and NADPHd histochemistry produced staining of the same neuronal elements in all major brain areas. However, in line with the results of Brüning et al. [10], two main discrepancies were observed: the glomeruli of the olfactory bulbs and the ganglionic neurons of the trigeminal mesencephalic nucleus were clearly positive for NADPHd, while they were negative for NOS immunoreactivity. It should be noted that after preincubation, the staining of the trigeminal mesencephalic cells was absent but the labeling of the olfactory glomeruli prevailed (see below).

The present study shows NOS-positive cells and fibers to be distributed in a unique manner, distinct from that of any other previously described neurochemically defined cell populations in the brain of urodeles. In the following topographically ordered discussion we comment on similarities and discrepancies between the distributions of NOS in different vertebrates. In addition, when possible, the relationships of the distribution of NOS to other neurotransmitters in the urodele brain is considered. Finally, the codistribution of NOS-containing cells and catecholaminergic neurons is dealt with.

4.1. Forebrain

In Pleurodeles waltl a strong reaction for NADPHd was found in the glomeruli of both the main and accessory olfactory bulbs, as has been also reported in the salamander [65]. This contrast with recent results in Xenopus laevis where only a fraction of glomeruli in the main olfactory bulb was labeled [70]. A striking result in our study was the lack of NOS immunoreactivity in all glomeruli. In a recent paper, it has been shown that NADPHd was present in the glomeruli of the salamander due to staining of receptor cell axons however, it was proposed that NADPHd may play a role in the adult epithelium independent of NO but functionally related to the activity of carbon monoxide (CO) as modulator of cGMP [89]. CO, like NO, requires the electron donation that is generated by activity between NADPHd and cytochrome P-450 and this would explain the lack of staining for NOS immunoreactivity. This interpretation might be valid also for the case of teleosts where NADPHd labeling was described in the glomeruli [5] but NOS immunoreactivity seems to be lacking [10].
As in lung fish and frogs [50,70] the nervous terminalis of *Pleurodeles* have NOS reactivity and the course of the fibers in the ventral aspect of the hemisphere can be followed to the preoptic region and this staining matches the pathway described previously with tract tracing techniques [65].

All pallial areas of the urodele hemisphere possess a population of scattered NOS reactive cells. These cells are not arranged in any apparent stratification, what resembles the situation of anurans [50] and reptiles [9,56]. Recently, in a lizard [14] it has been demonstrated that NADPHd-positive cells in the cortex represent a distinct subpopulation of GABAergic interneurons. Although double labeling experiments should be made, the distributions of GABAergic cells [23] and NOS-immunoreactive cells in the pallium of urodeles strongly suggest a possible colocalization. Widely distributed NADPHd positive cells in several areas of mammalian cerebral cortex have been described [33,48,49]. A shared feature of amniotes and amphibians ([50]; present study) is the presence of abundant NOS in cells of the amygdaloid complex [8,9,48,54,85].

In the diencephalon, asymmetrical staining was found in the habenula where only the component of the left side, i.e. the histologically and cytologically better differentiated [75] showed NOS fiber staining. However, NADPHd-positive cells in the habenula were never observed in *Pleurodeles*, what contrasts with the results reported for teleosts with similar techniques [78–80]. Our results, however, were consistent with the lack of NOS immunoreactivity in habenular cells and this is in line with recent results in the goldfish [10].

The urodele thalamus lacks NOS-positive neurons, in striking contrast with the case of anuran amphibians where a large population of cells was found [50]. The most intensely labeled areas of the thalamus correspond to the retino-cipient neuropils, mainly lateral to the corpus geniculatum thalamicum [24,87].

The rostral hypothalamic area contains abundant NOS cells. The majority of these neurons lie in the anterior preoptic area, close to the region where vasotocinergic and mesotocinergic cells have been observed [28]. However, both the morphology of the cells and their precise location make it clear a lack of colocalization of NOS in the neurosecretory neurons. This is supported by the fact that NOS-positive fibers were absent in the median eminence. Similar results have been obtained in most vertebrates studied [9,10,50,54] with the exception of mammals where the possible function of NO as cotransmitter of magnocellular hypothalamic cells has been reported [47,64,81].

Caudally in the hypothalamus, the nucleus of the periventricular organ of *Pleurodeles* was found negative for both stainings. This fact is shared with anuran amphibians [50] and contrasts with the situation in other amniotes [10,37,68,80]. The presence of a small population of NOS-positive cells around the infundibular recess of the diencephalic ventricle seems to be a common feature between teleosts and amphibians ([5,37,50]; present study).

4.2. Brainstem

The mesencephalic tectum harbors a large population of NOS-containing neurons whose dendritic trees arborize in the deep fiber layers, leaving free of innervation the superficial retino-cipient layers [60]. The scattered cells of the mesencephalic trigeminal nucleus [26] were NOS immunonegative, although stained weakly for NADPHd. Similar observations have been described for the goldfish [10] and the reactivity for NADPHd was interpreted as a technical failure since, as in our study, after preincubation these large cells consistently lack NADPHd staining.

Abundant NOS-positive neurons have been found in the mesencephalic tegmentum. Cytoarchitectonically distinct nuclei within the tegmentum are difficult to identify [53,61]. With the NOS technique two clear cell populations are defined as ventral and dorsal tegmental nuclei (nVT and nDT, respectively). The nVT corresponds to a group of cells reciprocally connected with the pretectal neurons [51], which are NOS-negative. In turn, at least part of the nDT could correspond to the ill-defined torus semicircularis described in this region on the basis of its connections [25,45].

Just beneath the caudal portion of the tectum, a group of NOS-positive cells is present in the dorsal tegmentum in the zone described as nucleus isthmi [19,20,51,53,58]. If these cells are actually comparable to those of the isthmic nuclei of other vertebrates is still uncertain. Comparatively, NOS containing cells in the nucleus isthmi of amniotes have not been described [5,10,50]. Probably the most conspicuous NOS-immunoreactive cell group is found caudally to the isthmic nuclei, beneath the cerebellum. The position and degree of reactivity lead us to compare it to the mesopontine NADPHd neurons of the rat [83] and homologable nuclei of other vertebrates [8,9,44,54]. These cells have been identified as cholinergic neurons in several species [44,83] but for the case of urodele amphibians the localization of cholinergic cell groups have never been reported. However, in a recent study in the frog [50] the presence of NADPHd and choline acetyltransferase immunoreactive neurons in a comparable region have been indicated.

The population of NOS-positive cells in the rhombencephalon of *Pleurodeles* is very large. As in cyclostomes [69], teleosts [5,10] and reptiles [9,44], the majority of the NOS positive cells most likely correspond to large reticulospinal cells all through the rhombencephalon [52]. This feature seems to be a shared character of amniotes that is more restricted in reptiles and birds and almost absent in mammals [85].

Other structures with NOS-containing neurons in the brainstem of the urodele include the raphe nucleus, trigem-
inal sensory nuclei and the nucleus of the solitary tract. NADPHd activity in cells in equivalent places have been reported in amniotes. Longitudinal NOS-positive fibers were present in the solitary tract and in the descending trigeminal tract and their cells of origin have been localized in the present study in both the preotic and postotic ganglia. In accordance with this, abundant NADPHd activity was found in the nodose ganglion of the rat [1].

4.3. Spinal cord

The pattern of NOS staining in the cervical spinal cord included abundant neurons and fibers. A large population of cells was found in the dorsolateral margin of the grey that most probably represent the caudal extent of the nucleus of the descending trigeminal tract [26]. The unlabeled neurons located immediately medial to these cells would be compared to the dorsal column nucleus in anurans, but it has been demonstrated that a subpopulation of dorsal column cells in anurans do contain NADPHd activity [50]. The motoneurons were never stained for NADPHd histochemistry and NOS immunocytochemistry, as also did not stain the motor nuclei in the brainstem. NO has been reported to play a role in motoneuron development [40]. In Xenopus laevis strong NADPHd staining of motoneurons was found during development and in the adult only certain motoneurons retained their reactivity [12]. Since we have studied in the urodele only the cervical spinal segments, the possible existence of NOS-positive motoneurons caudal to them can not be excluded.

The abundant fiber staining in the spinal cord included the areas of the primary afferents (as was expected for the NOS positivity found in the DRG cells) and in longitudinal fiber tracts in the funiculi. As indicated above, long descending axons of reticulospinal cells are NOS-positive as also are the axons of the cells in the dorsal root ganglia that ascend in the dorsal funiculi towards the obex regions. This findings are consistent with results in other species and support the idea of NO being involved in spinal sensory processing.

4.4. Codistribution of NOS and catecholamines

One of our goals in the present study was to evaluate the degree of colocalization of NOS and catecholamines in the brain of urodeles. The catecholaminergic cell bodies in the brain of Pleurodeles [27,30] were found in several places where interaction with NOS-containing cells could be expected. However, we found no colocalization in the brain. In a recent study in the frog, using the method of double labeling for TH and NADPHd on the same sections, a few cells in the posterior tubercle and the locus coeruleus appeared to colocalize both NOS and catecholamines [50]. In reptiles, the situation could be clearly different since, in a lizard, numerous cells have been found to contain NOS and dopamine in the substantia nigra, ventral tegmental area and retrorubral region [71]. It should be noted that in mammals, NADPHd activity exists in numerous places of the brain within catecholaminergic cell groups; however, using double labeling techniques the distribution of catecholaminergic cells with NADPHd activity was restricted to the anterior ventral tegmental area and the periaqueductal gray [39]. Therefore, a limited degree of colocalization can be assumed to occur in amniotes. The lack of colocalization in urodeles raises the question whether NOS and CA act in completely independent neuron systems in the brain of anamniotes (with the possible exception of anurans). Further studies are needed in other classes of amniotic vertebrates.

Acknowledgements

This research was supported by grants of the Spanish DGICYT (PB91-0424 and PB93-0083) and the Junta de Castilla y León (SA 55/09/92). We thank Dr. P.C. Emson for kindly donating the K205 antibody. The critical evaluation and linguistic corrections of the manuscript made by Dr. Gloria E. Meredith are most appreciated.

References


Rettig, G., Connections of the tectum opticum in two urodèles (Salamandridae, _Salamandra salamandra_ and _Bolitoglossa subpalmata_), with a special reference to the nucleus isthmi, _J. Hirnforsch._, 29 (1988) 5–16.


