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Nitric oxide synthase and background adaptation in *Xenopus laevis*

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Abstract

Adaptation of the skin colour to the background light condition in the amphibian *Xenopus laevis* is achieved by migration of pigment granules in the skin melanophores, a process regulated by α-MSH secretion from melanotrope cells in the pituitary pars intermedia (PI). α-MSH secretion in turn, is regulated by various stimulatory and inhibitory messengers synthesized in brain nuclei, especially the hypothalamic suprachiasmatic and magnocellular nuclei and the locus coeruleus in the hindbrain.

In the present study, the roles in background adaptation of nitric oxide (NO) and NO synthase (NOS) enzyme activity were evaluated. In situ, using both immunohistochemistry with anti-human brain NOS (bNOS) serum in paraffin-embedded material and using nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry in cryo-sections, we showed NOS in neurons in the optic tectum and in the locus coeruleus. NADPH-d reactivity was also found in neurons in the lateral amygdala, the ventral hypothalamic nucleus and in fibers in the median eminence. Using a Western blot stained with an anti-human bNOS serum, we demonstrated a 150 kDa band in *Xenopus* hindbrain lysates, which is similar to the NOS protein present in the rat anterior pituitary, but which was not detectable in the lysates from both the neurointermediate and distal lobes in *Xenopus*. No differences in histochemical staining pattern or on Western blotting were observed between animals adapted to a black or a white background.

Paraffin sections of the endocrine PI and pars distalis did not reveal bNOS-like immunoreactivity. NADPH-d reactivity was observed in the endothelia of this gland. However, using a new procedure of thin cryo-sections of pituitary neurointermediate lobes, we observed bNOS-immunoreactive fibers as well as cyclic 3′,5′ guanosine monophosphate (cGMP)-accumulating fibers in the PI.

The PI may be regulated by NOergic neurons from higher brain centers. The possibility that NOergic neurons in the locus coeruleus are involved in the innervation of the PI needs further investigation. The latter neurons are probably not noradrenergic because double labeling studies show no co-localization of NADPH-d reactivity and tyrosine hydroxylase immunoreactivity in locus coeruleus neurons. © 1997 Elsevier Science B.V.

Keywords: bNOS-immunohistochemistry; NADPH-diaphorase staining; Cyclic GMP-accumulation; Pituitary pars intermedia; Locus coeruleus; Optic tectum

1. Introduction

Nitric oxide (NO), a free radical (Butler et al., 1995), has emerged as an intracellular and intercellular messenger molecule with a very wide range of biological roles. For instance, NO accounts for the biological

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activity of (or is) the endothelium-derived relaxing factor (EDRF) (Ignarro et al., 1987; Palmer et al., 1987; Garthwaite et al., 1988), acts as a retrograde messenger in memory formation (Schuman and Madison, 1991; Shibuki and Okada, 1991, Garthwaite and Boulton, 1995), is involved in cell-mediated immune responses in inflammation (Stuehr et al., 1989) and may regulate growth hormone (GH)(Kato, 1992) and luteinizing hormone (LH)(Ceccatelli et al., 1993) secretion from the pituitary. Furthermore, increased levels of NO synthase (NOS), protein and mRNA were detected in the rat anterior pituitary (AP) after gonadectomy (Ceccatelli et al., 1993) and in human pituitary adenomas (Lloyd et al., 1995), suggesting an important physiological role of NOS in the AP. In vivo NO is formed from L-arginine by each of the NOS isoenzymes, referred to as brain NOS (bNOS), endothelial NOS (eNOS) and macrophage NOS or inducible NOS (iNOS) (Garthwaite and Boulton, 1995). bNOS and eNOS are constitutively present and are activated through a calcium-dependent pathway (Garthwaite and Boulton, 1995), whereas iNOS is induced by immunostimulation and the activity of the latter enzyme is not dependent on increased intracellular calcium (Galea et al., 1992, Simmons and Murphy, 1992).

A main target for the messenger NO is to activate the cytoplasmic soluble form of guanylyl cyclase (sGC), stimulating the formation of cyclic 3′,5′ guanosine monophosphate (cGMP)(Bredt and Snyder, 1989; Knowles et al., 1989; de Vente et al., 1990; Garthwaite, 1991; Southam and Garthwaite, 1993).

Recently, the neuroanatomical distribution of NOS-immunoreactive neurons and the distribution of β-nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) positive neurons has been described in a number of amphibian (Brüning and Mayer, 1996; Muñoz et al., 1996; González et al., 1996) and reptilian species (Brüning et al., 1994; Smeets et al., 1997). Muñoz et al. (1996) suggest that the function of NO in the amphibian brain, considered to be primarily related to forebrain activities, may have been well preserved during vertebrate evolution (Allaerts et al., 1997).

Among amphibians, the South African clawed toad *Xenopus laevis* shows a particularly strong adaptive response to background light intensity. This adaptation involves activation of biosynthetic and secretory processes in the melanotrope cells of the pituitary PI, whereas these processes inactivate during white-background adaptation. Activation of the PI melanotropes results in the release of proopiomelanocortin (POMC)-derived peptides, including α-melanophore-stimulating hormone (α-MSH). In dermal melanophores, α-MSH stimulates the dispersion of the black pigment melanin, thus causing skin darkening (Jenks et al., 1993; Roubos, 1997).

The aim of the present study was to investigate the possible role of NO in the control of background adaptation by *Xenopus laevis*. For this purpose, we studied the presence of the NOS enzyme in the pituitary and the brain using immunohistochemistry, NADPH-d histochemical staining and Western blotting. We also evaluated NOS activity during adaptation of *Xenopus* to a black or white background. Hereby, special attention was given to the brain centers involved in the regulation of the PI, viz. the hypothalamic suprachiasmatic and magnocellular nuclei and the locus coeruleus in the hindbrain (Tuinhof et al., 1994). Recent studies in amphibians, moreover, have corroborated the noradrenergic innervation of the PI from the locus coeruleus (Jansen et al., 1997). Co-localization studies of NADPH-d reactivity and tyrosine hydroxylase immunoreactivity (TH-IR) have been performed to compare the distribution of NOergic neurons with the aminergic innervation of the PI. Finally, we compared bNOS-immunoreactivity (bNOS-IR) and cGMP-IR in the pituitary PN and PI, following the procedure of de Vente et al. (1990; de Vente et al., 1996a,b).

2. Materials and methods

2.1. Animals

Adult *Xenopus laevis* weighing ≈ 25 g were obtained from laboratory stock. The toads were kept under constant illumination on either a black or a white background for at least 3 weeks (22°C) and fed beef heart and trout pellets (Trouvit, Trouw, Putten, The Netherlands) once a week. Melanophore indices were estimated according to the protocol of Hogben and Slome (1931).

2.2. bNOS-immunohistochemistry on paraffin-embedded tissue

Toads were anaesthetized with 0.1% tricaine methane sulfonate (MS 222, Sandoz, Basle, Switzerland) and perfused via the heart with ice-cold 0.6% Ringer's solution for 3 min, followed by perfusion for 15 min with either Bouin-Hollande or Zamboni fixative (Zamboni and De Martino, 1967). Brains and pituitaries of black-adapted and white-adapted toads were dissected and post-fixed for 3 or 16 h in either fixative. Brains and pituitaries were embedded in paraplast and sagittal sections of 10–20 μm were cut with a Spencer 820 microtome (American Optical Instrument, Buffalo, NY). Sections were immunostained with the ABC method (Hsu et al., 1981), according to Ubink et al. (1994), with a polyclonal antiserum against human bNOS (Transduction, Lexington, KY; diluted 1:100) and the Vectastain ABC kit (Vector, Burlingame, CA;

diluted 1:200). Dilutions of the antiserum were made in 0.01 M sodium phosphate-buffered saline (PBS; pH 7.4) supplemented with 0.1% Triton X-100 (Sigma, St. Louis, MO) and 2% bovine serum albumin (BSA; Sigma), whereas the ABC reagent was diluted in PBS. Staining was accomplished by 30 min incubation with 0.04% diaminobenzidine (DAB; Sigma) and 0.015% H_2O_2 in PBS, followed by a 5 min rinse in running tap water. Sections were mounted with Entellan (Merck, Darmstadt, Germany).

2.3. NADPH-diaphorase histochemistry and TH-immunohistochemistry on cryo-sections

Black adapted and white adapted toads were anaesthetized and perfused as described above. Perfusion fixative was 2% (w:v) freshly depolymerized paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.4), supplemented with 0.5% (v:v) glutaraldehyde and 0.5% (v:v) picric acid after Tanaka et al. (1991). In another set of experiments, animals were perfused with periodate–lysine–paraformaldehyde (PLP) fixative after McLean and Nakane (1974), containing 2% freshly depolymerized paraformaldehyde, 0.01 M $NaIO_4$ (Merck) and 0.075 M L-lysine (Sigma) in 0.0375 M sodium phosphate buffer. The advantage of PLP fixation for cryo-immunohistochemistry lies in the cross-linking of carbohydrate moieties by lysine after oxidation by periodate (McLean and Nakane, 1974), yielding both good antigenicity and ultrastructural preservation (Allaerts et al., 1996). The brains and pituitaries were dissected and postfixed for 2 h at 4°C in the same fixative as used during perfusion, followed by cryoprotection in 30% (w:v) sucrose in 0.15 M phosphate buffer and frozen at −70°C. Sagittal and transversal sections of 15–20 μm thick were cut on a HM 500-O cryostat (Microm; Adamas, Leersum, The Netherlands) and mounted on poly-L-lysine coated glass slides. For NADPH-d staining, 20 μm thick cryosections were pre-incubated with 50 mM Tris-buffered saline (TBS or Tris–HCl; pH 7.6) for 30 min and incubated for 30 min at 37°C with 1 mM β-NADPH (Sigma) and 0.2 mM nitroblue tetrazolium (Sigma) in 0.1 M Tris–HCl buffer (pH 7.6), supplemented with 0.2% Triton X-100 (Dawson et al., 1991). Sections were washed in 0.1 M PBS and mounted in 30% (v:v) glycerol in 0.1 M PBS.

TH immunostaining was performed essentially as described before (Tuinhof et al., 1994), with minor modifications. Transversal cryo-sections of PLP-fixed *Xenopus* brains and pituitaries were immunostained using a monoclonal mouse anti-TH serum (Instar, Stillwater, MN) diluted 1:100 in PBS supplemented with 0.1% Triton X-100 and 2% BSA (PBS-T). The secondary antiserum used was a FITC-labeled donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) also diluted 1:100 in PBS-T. Co-localisation studies of NADPH-d and TH were performed on the same sections by combination of bright field and FITC-epifluorescence microscopy using a Leitz DM RB/E microscope equipped with Leica Vario Orthomat E camera system.

2.4. bNOS- and cGMP-immunohistochemistry on cryo-sections

In another set of experiments, localization of bNOS-IR, as well as cGMP-IR in cryosections of the brain and pituitary were investigated according to a procedure devised for studying cGMP-IR in brain slices (de Vente et al., 1990, 1996a,b). Briefly, adult *Xenopus* (7 white-adapted and 7 black-adapted animals) were decapitated, the brains and also some neurointermediate (NILs) and distal lobes were transferred into ice-cold incubation buffer (pH 7.4), aerated with 5% CO_2/95% O_2. Subsequently, the brains were placed en bloc on a filter paper on a precooled stainless-steel table. Transverse 400 μm thick slices are cut with a McIlwain tissue chopper, and separated from each other under a microscope, while being submerged in ice-cold incubation buffer aerated with 5% CO_2/95% O_2. Brain slices and pituitary lobes were transferred into incubation vials containing ice-cold, aerated buffer and then slowly equilibrated to 22°C under an atmosphere of 5% CO_2/95% O_2. The phosphodiesterase (PDE) inhibitor isobutylmethylxanthine (IBMX; Sigma; 1 mM) was included from the beginning of the incubation. The NO-donor sodium nitroprusside (SNP; Sigma; 0.1 mM) was added after 10 min incubation. Thirty min after addition of the NO-donor, the incubations were terminated by adding ice-cold fixative solution, consisting of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4). After 30 min, slices and lobes were transferred into 4% PFA plus 10% sucrose in 0.1 M PB for another 90 min, followed by a 30 min wash in ice-cold 0.1 M PB, containing 10% sucrose. Subsequently, they were embedded in Tissue-tek (Miles, Elkhart, IN) and quickly frozen using a Fast Inverted Cryo System (FICS, Adamas). A number of 6–10 μm thick sections were cut on a cryostat (Microm, Adamas) and thawed onto chrome-alum gelatin-coated slides.

Sections were processed for immunocytochemistry as described (de Vente et al., 1996a,b; Yamada et al., 1997). Antisera used were a polyclonal sheep anti-cGMP (Tanaka et al., 1997; diluted 1:4000), a polyclonal rabbit anti-cGMP (de Vente et al., 1996a,b; diluted 1:300) and a polyclonal sheep anti-bNOS (kindly provided by Dr P.C. Emson, U.K.; diluted 1:750). Dilutions of the sera were made in filtered TBS supplemented with 0.3% Triton X-100. The specificity of these antisera in staining mammalian tissues has

been largely demonstrated (de Vente et al., 1996a,b; Herbison et al., 1996; Tanaka et al., 1997; Yamada et al., 1997). In the single cGMP-immunostaining procedure, secondary antisera used were the CY3-labeled goat anti-rabbit IgG (Jackson; diluted 1:800) or the FITC-labeled rabbit anti-sheep (Calbiochem, La Jolla, CA; diluted 1:150), depending on the host species of the primary antiserum. In the double immunolabeling procedure, rabbit anti-cGMP and sheep anti-bNOS were used as primary antisera and the secondary antisera were the CY3-labeled donkey anti-rabbit (Jackson; diluted 1:800) followed by the FITC-labeled rabbit anti-sheep IgG (Calbiochem; diluted 1:30). Co-localization was evaluated using combination of CY3- and FITC-epifluorescence microscopy (Leitz DM RB/E).

2.5. Western blotting

Homogenates of NILs and distal lobes and of the hindbrain (HB) of *Xenopus* adapted to either a black or a white background were separated by sodium dodecyl sulfate (SDS)-gel electrophoresis, according to Laemmli (1970). Four NILs and distal lobes per experiment were homogenized with a teflon homogenizer in 20 μl lysis buffer containing 0.1 μg/ml phenylmethylsulfonyl fluoride (PMSF; Sigma), 1 μg/ml trypsin inhibitor (type I-S; Sigma), 0.1% (v:v) Triton X-100, 1% (v:v) Tween-20 and 0.1% (w:v) deoxycholic acid (Sigma) in 0.05 M Hepes buffer. The HB, obtained by transversal cutting with a razor blade of the brain between telencephalon and myelencephalon, of one black- and one white-adapted toad per experiment, was also homogenized in 200 μl of the same lysis buffer. The homogenates were centrifuged at 12 500 $\times$ *g* for 5 min and diluted 1:1 with electrophoresis sample buffer containing 3% (w:v) SDS, 10% (v:v) glycerol, 0.05% (w:v) bromophenol blue and 5% (v:v) 2-mercaptoethanol (Mizutani et al., 1994) in 60 mM Tris buffer (pH 6.8). A 7.5% acrylamide gel (in 0.375 M Tris; pH 8.8) was used as the resolving gel, with a stacking gel of 4.5% acrylamide (in 0.15 M Tris; pH 6.8). After electrophoresis, the gels were used for western blotting at 10 V for 16 h to a nitrocellulose membrane (BA-S 85, Schleicher and Schuell, Dassel, Germany) in a wet transfer apparatus (Mini Trans-Blot Cell, Bio-Rad, Melville, NY), using transfer buffer containing 20% methanol, 0.192 M glycine, 0.025 M Tris and 0.1% SDS. The nitrocellulose membrane was stained using the polyclonal anti-human bNOS serum (Transduction; diluted 1:1000) and the enhanced chemiluminescence (ECL) reaction (ECL kit of Amersham Life Science, Buckinghamshire, UK)(Whitehead et al., 1979). As a positive control, a rat pituitary lysate obtained commercially (Transduction) was treated in the same way as the *Xenopus* samples and was also immunostained with the bNOS antiserum. Molecular mass markers were from a high molecular weight standard mixture (range 30–200 kDa; Sigma) and were stained with 0.5% (w:v) amido black (Merck) in 1% acetic acid.

3. Results

3.1. Immunohistochemistry and enzyme histochemistry

For paraffin immunohistochemistry, brains and pituitaries were fixed with Zamboni fluid or Bouin-Hollande. Using a polyclonal antiserum against human bNOS, we observed bNOS-like IR in neurons in the optic tectum (Fig. 1a) and bNOS-positive neurons and fibers in an area corresponding topographically to the locus coeruleus (Tuinhof et al., 1994) (Fig. 1b). However, in the paraffin-embedded pituitaries of these animals, no bNOS-like IR was found (Fig. 1c). No differences in bNOS-like immunostaining pattern of the optic tectum and locus coeruleus were observed between brains from *Xenopus* adapted to either black or white background for 3 weeks. In the brains of Bouin-Hollande fixed *Xenopus*, the bNOS staining of neurons was less conspicuous than in the Zamboni-fixed *Xenopus*.

In order to verify the distribution of bNOS-like IR in the different brain and pituitary areas, NADPH-d histochemical staining was applied to 15–20 μm thick cryosections of *Xenopus* brain and pituitary. Using NADPH-d staining, two grades of staining with the nitroblue tetrazolium reaction product were observed. Myelin-rich regions of the CNS showed no blue staining at all. A light blue staining was observed in myelin-poor areas of the brain and pituitary, whereas a dark blue staining pattern was present in neurons. The latter staining pattern was considered as NADPH-d positive staining. Darkly stained NADPH-d positive neurons occurred in the optic tectum (Fig. 2a), in the area corresponding to the locus coeruleus (Fig. 2b) and in the ventral hypothalamic nucleus (Fig. 2d). Beside the latter NADPH-d reactive areas, we recently described NADPH-d reactive neurons in the lateral and dorsal pallium and in the amygdala pars lateralis, in the nucleus anterodorsalis tegmenti and in the dorsal raphe nucleus (Allaerts et al., 1997). NADPH-d staining was negative in the magnocellular and suprachiasmatic nuclei of the hypothalamus. Except for some staining in endothelia, the pituitary neural, intermediate and distal lobes were NADPH-d negative in cryosections of animals fixed after Tanaka et al. (1991) (Fig. 2c). However, using cryosections of PLP-fixed animals (after McLean and Nakane, 1974), a slightly enhanced blue staining was noted in endothelia and some unidentified cells in the intermediate lobe (Fig. 2e). Strongly NADPH-d positive fibers were present in the median eminence (Fig. 2d). No cellular co-localization with TH

immunoreactivity was found in neurons of the locus coeruleus, but some TH-immunoreactive neurons were found adjacent to NADPH-d$^+$ neurons in this area (compare Fig. 3a,b). These double stainings indicate that NOS and TH enzyme activities occur in different neurons, at least in the locus coeruleus.

We found no differences in NADPH-d reactivity between black-adapted or white-adapted animals, either in the pituitary or in the brain.

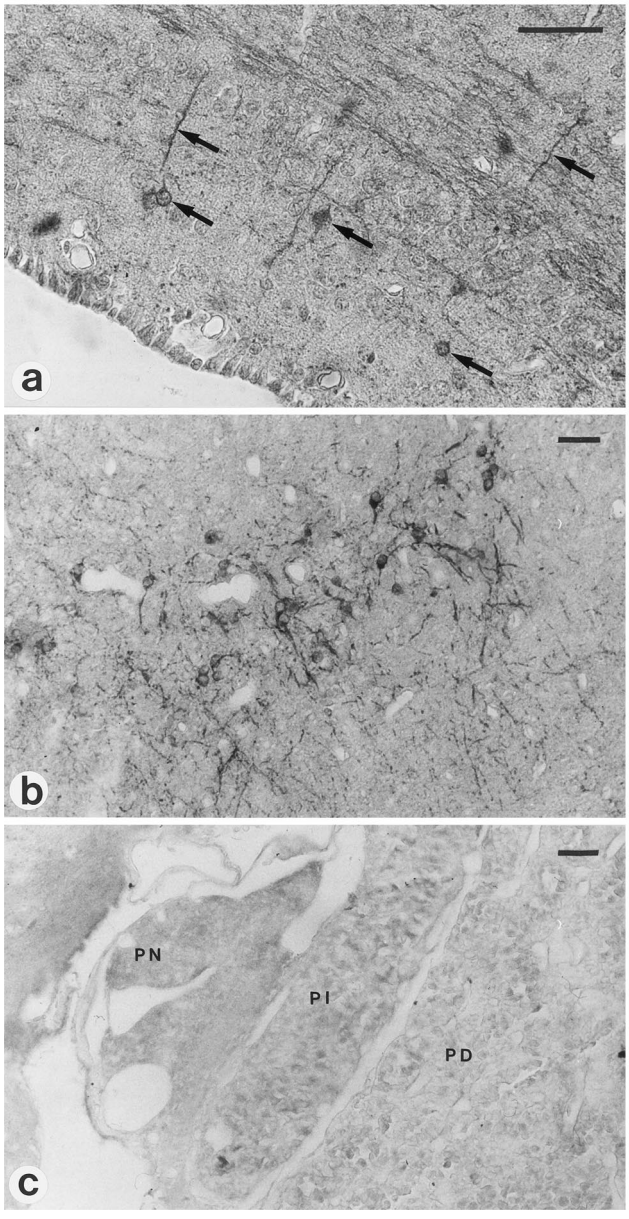

Fig. 1. Sagittal paraffin sections of black-adapted *Xenopus laevis* brain and pituitary, fixed with Bouin-Hollande (a) or Zamboni fluid (b,c) and immunostained with polyclonal anti human bNOS serum, showing: (a) bNOS-like immunoreactive neurons in the optic tectum (arrows)(× 320); (b) bNOS-like immunoreactive neurons in an area corresponding topographically to the locus coeruleus (× 160); (c) absence of bNOS-like immunoreactivity in the pituitary (× 160). PI, pars intermedia; PN, pars nervosa; PD, pars distalis (or AP).(Scale bars = 50 μm).

In a final set of experiments, using 6–10 μm thin sections of neurointermediate lobes, we observed clearly bNOS-positive fibers in the PI co-localized with cGMP-IR fibers (Fig. 4a,b). When compared to the brain, the bNOS-IR neurons and fibers appeared to be distinct from but often in the immediate vicinity of the cGMP-IR neurons and fibers (compare with Fig. 4c,d). The distribution of cGMP-IR neurons in the *Xenopus* brain is described elsewhere (Allaerts and de Vente, unpublished observations).

3.2. Western blotting

A Western blot stained with the polyclonal anti-human bNOS is shown in Fig. 5. Optimal separation was obtained using a 7.5% acrylamide gel and the optimal dilution of the bNOS antiserum for staining of the blot was 1:1000. Homogenates of NIL, distal lobe and hindbrain of *Xenopus* adapted to white and black backgrounds are electrophoretically separated in lanes 3–8. A commercial lysate from rat anterior pituitary (AP) was used as a positive control (lane 2). A mixture of six mass markers with MW ranging from 29 to 205 kDa is shown in lane 1. A high MW band corresponding to $\approx$ 150 kDa was found in the rat AP lysate and in the *Xenopus* lysates of hindbrain (lanes 7 and 8). This high MW band was absent in the lysates of the *Xenopus* NILs and distal lobes. No effect of adaptation to a white (lanes 3, 5, 7) or a black background (lanes 4, 6, 8) was observed. An unidentified cross-reacting band at lower MW was stained in lane 2, i.e. the commercial rat AP lysate, which staining, however, was absent in the other lanes corresponding to *Xenopus* material.

4. Discussion

In the present study, we used immunohistochemical and enzyme histochemical staining procedures to detect NOS enzyme activities in the brain and pituitary of *Xenopus laevis* adapted to a black or a white background. We also compared the bNOS-IR in sections of conventional paraffin-embedded material with bNOS- and cGMP-IR in thin cryosections of brain slices and pituitaries, processed according to a procedure devised for immunohistochemical visualization of cGMP-accumulation in brain slices (method of de Vente et al., 1990, 1996a,b). The latter procedure was found to be qualitatively superior to the paraffin method, with regard to both antigenicity and tissue preservation at the light-optical level, as for instance indicated by the lack of dehydration artefacts due to ethanol incubation. Using Western blotting of homogenates of NIL, distal lobe and hindbrain (including the metencephalon) of either black or white adapted *Xenopus*, we obtained

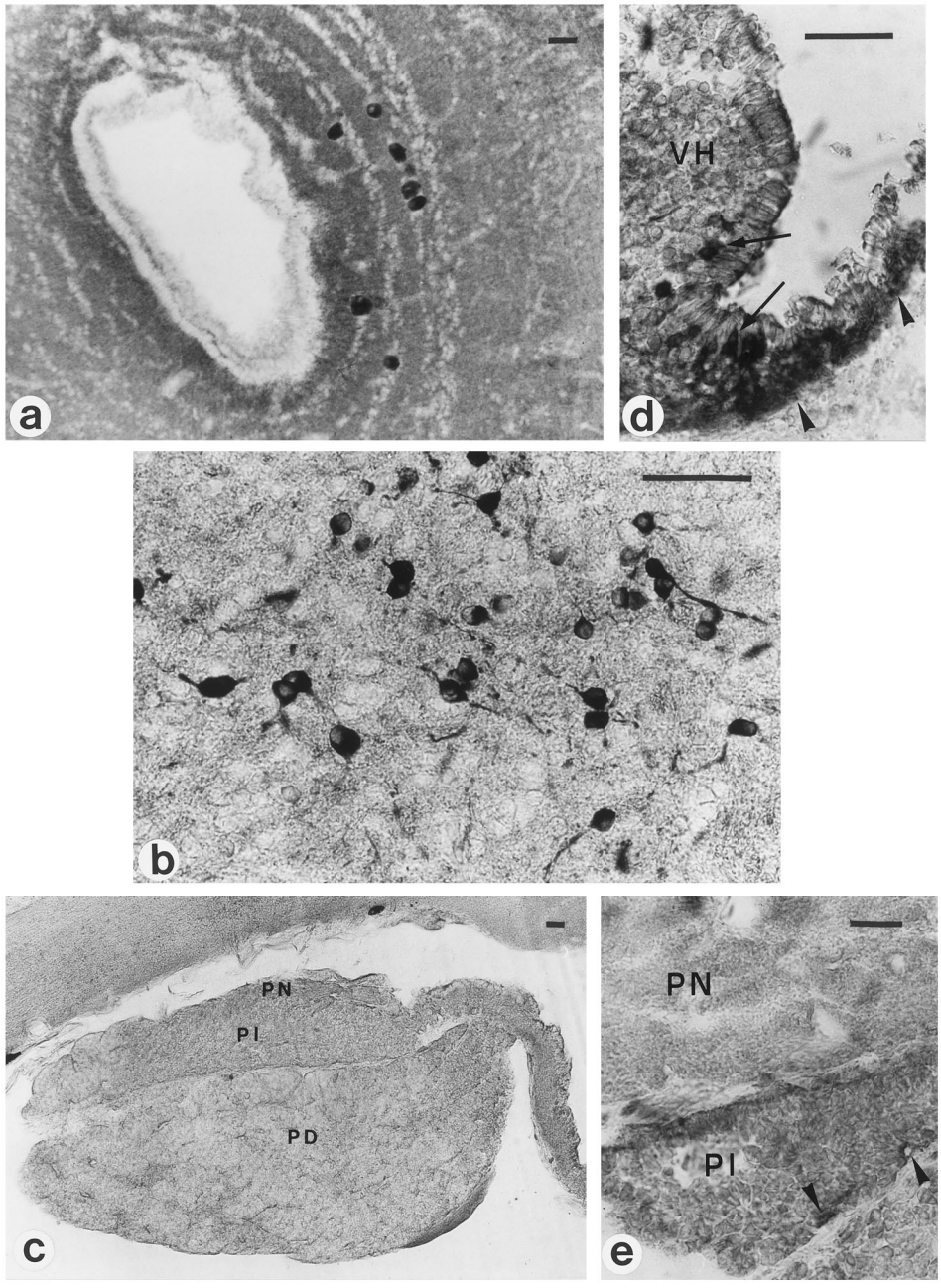

Fig. 2. Cryo-sections of *Xenopus laevis* brain and pituitary stained using the NADPH-diaphorase histochemical reaction: (a) white-adapted *Xenopus* fixed after Tanaka et al., 1991, showing NADPH-d$^+$ neurons in the optic tectum (sagittal; $\times$ 100); (b) black-adapted *Xenopus* fixed with PLP after McLean and Nakane (1974), showing NADPH-d$^+$ neurons in the locus coeruleus (transversal; $\times$ 400); (c) pituitary of black-adapted *Xenopus* fixed after Tanaka et al., 1991 (sagittal; $\times$ 63); (d) detail of ventral hypothalamic nucleus (VH) and median eminence in white-adapted *Xenopus* fixed with PLP, showing NADPH-d$^+$ cells in the VH (long arrows) and NADPH-d$^+$ fibers in the median eminence (arrowhead)(sagittal; $\times$ 320);(e) detail of pituitary from white-adapted *Xenopus* fixed with PLP, showing faint NADPH-d$^+$ reaction in endothelia and some unidentified cells (arrowheads)(transversal; $\times$ 190). (Scale bars = 50 μm).

evidence for bNOS-like protein(s) in the brain of *Xenopus*, but were unable to demonstrate a bNOS-like protein in the NIL and distal lobe. This could be due to the limited number of NOergic fibers innervating the NIL. Moreover, our observation of bNOS-IR in fibers in the PI (although bNOS was not detected in the NIL lysates on Western blotting) corroborates the view that immunohistochemical detection in cryosections is the most sensitive of our methods used (Sternberger, 1979).

4.1. Localisation of NOS-isozymes

In paraffin sections we were able to demonstrate bNOS-like immunoreactive neurons in the optic tectum

and locus coeruleus of *Xenopus*, using a polyclonal anti-human bNOS serum, whereas bNOS-IR was absent in the pituitary. In thin cryosections processed according to the method of de Vente et al., 1990, 1996a,b), apart from the regions reported bNOS-positive in paraffin sections and NADPH-d reactive in thick cryosections, bNOS-IR fibers were also detected in the pituitary PI and PN.

No differences were found in the expression of bNOS-like protein between black and white adapted animals. However, an adaptation period, of 3 weeks to a black or white background, may be inadequate to observe possibly transient changes in the bNOS expression in the brain following a change of background condition. A time series experiment will be undertaken to test this hypothesis.

Up to now, no evidence for a role of the optic tectum in the control of the melanotrope cells of *Xenopus* has been obtained. In contrast, the involvement of the locus coeruleus in the regulation of melanotrope cell activity is rather well documented (Tuinhof et al., 1994; see also below).

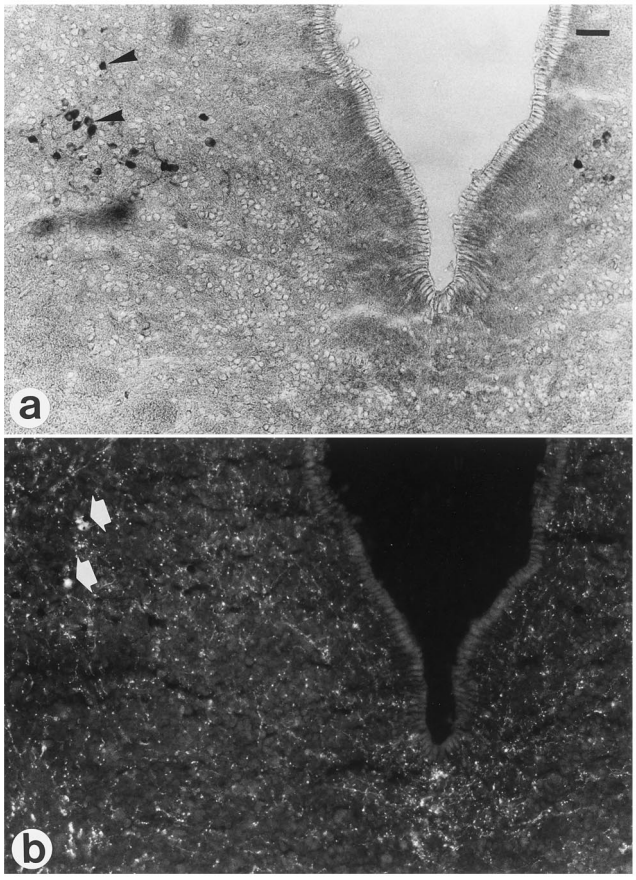

Fig. 3. Double labeling of transversal cryosection fixed with PLP of black adapted *Xenopus laevis* using NADPH-d staining (a) and anti-TH immunofluorescence-staining (b) in the locus coeruleus (× 126). Note the NADPH-d-positive neurons (arrowheads in a) and TH-positive neurons (white arrowheads in b) in close juxtaposition. (Scale bar = 50 μm).

NADPH-d histochemical staining of cryo-sections showed darkly stained neurons in the optic tectum, the locus coeruleus, the ventral hypothalamic nucleus, the amygdala and fibers in the median eminence (present study and Allaerts et al., 1997). The distribution of NADPH-d$^+$ neurons corroborates the findings of Brüning and Mayer (1996) in *Xenopus laevis* and is also in line with recent studies in other amphibian species (González et al., 1996; Muñoz et al., 1996) and in some reptiles (Brüning et al., 1994; Smeets et al., 1997) (reviewed in Allaerts et al., 1997). In the rat, it was shown that NOS catalytic activity is responsible for NADPH-d staining (Hope et al., 1991; Dawson et al., 1991), although some tissues like the adrenal cortex and the liver display NADPH-d activity in the absence of NOS (Dawson et al., 1991). The faint NADPH-d reactivity in the PI of PLP-fixed *Xenopus* (except for the reactivity in some endothelial cells) may eventually be explained by assuming some cross-reactivity with mitochondrial cytochrome P450 reductase (Bredt et al., 1991), as was also suggested by Smeets et al. (1997) for some regions in the brain of *Gekko gecko*.

Our immuno- and enzyme-histochemical data demonstrate that NOS activity is present in some nuclei (locus coeruleus and possibly others), but not all nuclei involved in background adaptation in *Xenopus laevis*. Muñoz et al. (1996) observed NADPH-d activity in the suprachiasmatic and magnocellular nuclei in the frog *Rana perezi*, but this observation was not confirmed in studies in *Xenopus laevis* (Brüning and Mayer, 1996), nor in the urodele *Pleurodeles waltl* (González et al., 1996). Therefore, the data presented and also data from other studies indicate that the locus coeruleus neurons may be good candidates for the NOergic innervation of the PI.

4.2. Relationship between NADPH-d$^+$ and TH$^+$ neurons in the locus coeruleus

Retrograde labeling of the PI of *Xenopus* by Tuinhof et al. (1994), has shown that the area in the brainstem of *Xenopus* corresponding to the locus coeruleus in mammals, directly innervates the PI. Jansen et al. (1997) have corroborated these findings in the frog, *Rana esculenta* and the newt, *Triturus carnifex* and moreover, demonstrated colocalization of TH-IR and the retrograde label, indicating that noradrenergic neurons in the locus coeruleus are the source of noradrenergic projections to the PI. In order to test whether NOS is found in noradrenergic neurons, we performed co-localization studies of NADPH-d and TH. No co-localization of NADPH-d$^+$ with TH-IR neurons was observed in the locus coeruleus area, although both neuron types were closely intermingled, as described for *Gekko gecko* (Smeets et al., 1997). On the other hand, Muñoz et al. (1996) did find co-localization of

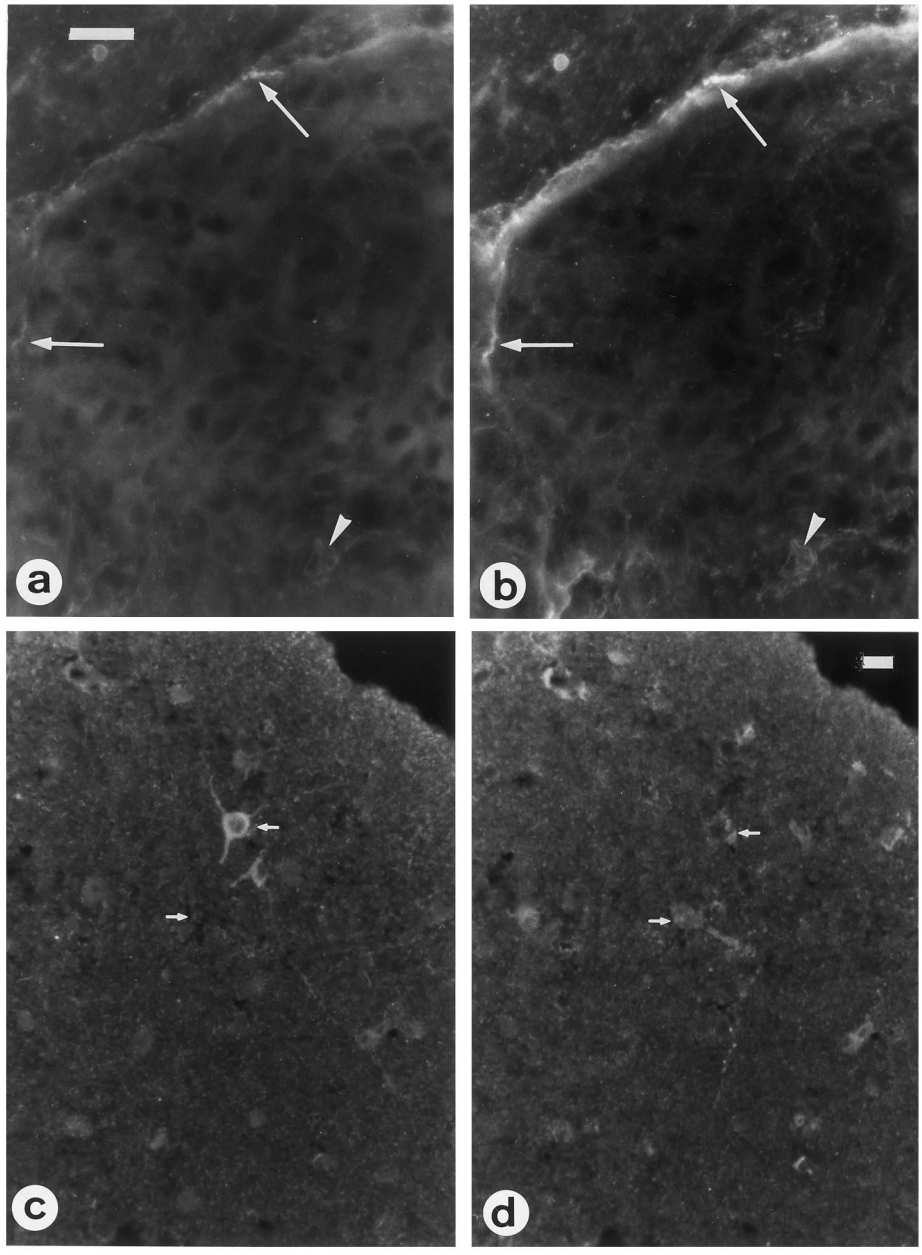

Fig. 4. Double labeling of 6 μm cryosection of NIL (a, b) and brain (c, d) of a black-adapted *Xenopus laevis* processed according to the method of de Vente et al., 1990, 1996a,b. High magnification photographs of the PI (a, b; $\times$ 500) and dorsal pallium (c, d; $\times$ 250) revealing bNOS-IR (a, c; FITC optics) using a polyclonal sheep antiserum against bNOS (gift of Dr P.C. Emson; diluted 1:750) and revealing cGMP-IR (b, d; CY3 optics) using a polyclonal rabbit antiserum against cGMP (diluted 1:300). Note the co-localization of bNOS-IR and cGMP-IR in the PI in some fibers (long arrows) and in some unidentified PI cells (arrowheads), whereas in the dorsal pallium the bNOS-IR is topographically distinct from the cGMP-IR (small arrows indicate exactly matching positions). (Scale bar = 20 μm).

NADPH-d activity and TH-IR in the locus coeruleus of *Rana perezi*. According to Smeets et al. (1997), although extensive co-localization of NADPH-d or NOS and catecholamines occurs in the midbrain dopaminergic cell groups of reptiles and birds, co-localization is only 'sparsely' present in the corresponding cell groups of amphibians and mammals. Alternatively, it may be that the amounts of TH and NOS are inversely related within the neurons innervating the PI, making a demonstration of their co-localization technically difficult. A similar situation is observed with respect to the complementary expression of acetylcholinesterase and NADPH-d in the dorsal thalamic nuclei of the rabbit (Caballero-Bleda et al., 1991).

4.3. Possible sources of NOS in the PI

Apart from the bNOS-IR fibers in the PI and PN (Section 4), in cryosections of PLP-fixed pituitaries some NADPH-d activity was observed in endothelia of

the pars distalis and PI, probably corresponding to eNOS. In the rat, Vanhatalo and Soinila (1995) found NADPH-d activity in nerve terminals and also in pituicytes in the PN and in cleft epithelial and interlobular cells (between the melanotropes) in the PI. Further research will be necessary to localize the NOergic neurons in higher brain centers that innervate the PI of *Xenopus laevis* and to investigate the NOergic input of the PI via fibers running through the median eminence. These fibers may appear similar to the NOergic nerve endings found in the mammalian PN (Kadowaki et al., 1994; Pow, 1994; Vanhatalo and Soinila, 1995), although due attention should be paid to the difference in pituitary innervation between mammals and amphibians. Also in analogy with the mammalian pituitary, a local (paracrine or autocrine) effect of NO in the PN (Pow, 1994) and the PI (Vanhatalo and Soinila, 1995) cannot be excluded, but requires further studies.

4.4. cGMP-immunoreactivity in the brain and pituitary of Xenopus laevis

In addition to the distribution of NOS-IR and NADPH-d positive neurons, we also observed cGMP-IR in the brain and pituitary of *Xenopus laevis*. The distribution of cGMP-IR neurons and fibers in the brain corroborates the distribution of targets of the presumed NOergic signaling system (Allaerts and de Vente, unpublished observations). In particular, we found cGMP-IR fibers running through the ventral hypothalamus and median eminence towards the pituitary (not shown), as well as few cGMP-IR and bNOS-IR fibers in the PN and PI. In general, the distribution of bNOS-IR and cGMP-IR neuron somata and fibers showed distinct patterns, confirming the view of Southam and Garthwaite (1993) that in the rat, the microanatomical locations of cGMP-accumulation and NOS-activity are more complementary than identical: in some areas NOS occurs in postsynaptic structures and cGMP is accumulated in presynaptic elements (also in neuron fibers and glial cells), whereas in other areas the location was reversed (Southam and Garthwaite, 1993). The co-localization of bNOS-IR and cGMP-IR fibers in the pituitary PN and PI of *Xenopus laevis*, therefore contrasts with this principle of complementarity.

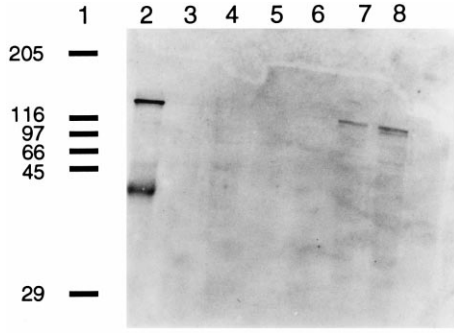

Fig. 5. Western blotting of SDS-PAGE gels with homogenates from *Xenopus laevis* NILs (lanes 3, 4), distal lobes (lanes 5, 6) and hindbrains (lanes 7, 8). Animals are adapted to a white (lanes 3, 5 and 7) or a black background (lanes 4, 6 and 8). Gels are loaded with 10 μl lysate containing one equivalent of NIL or distal lobe per lane, or 1:40 equivalent of *Xenopus laevis* hindbrain per lane. A commercial lysate from rat pituitary (4 μg protein/10 μl), is used as a positive control in lane 2. The blot is immunostained with the polyclonal anti human bNOS serum (diluted 1:1000). A high MW band corresponding to $\approx$150 kDa is found in the rat anterior pituitary and *Xenopus* HB homogenates, but is absent from the *Xenopus* NIL and distal lobe homogenates.

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