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NITRIC OXIDE SYNTHASE IN THE BRAIN OF THE CLAWED TOAD *XENOPUS LAEVIS*: IS THERE A RELATIONSHIP WITH THE VISUAL SYSTEM?

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Abstract. Nitric oxide (NO), a free radical, has emerged as an intracellular and intercellular messenger molecule with many biological functions, including a role in memory. The neuroanatomical distribution of the enzyme nitric oxide synthase (NOS) is described in the brain and pituitary of *Xenopus laevis*, using immunohistochemistry with a polyclonal antiserum against human brain NOS, and using the nicotinamide adenine dinucleotide phosphate – diaphorase (NADPH-d) histochemical staining. NOS-containing neurons were found in the telencephalon, the diencephalon, the mesencephalon and the metencephalon, and were especially numerous in the pars lateralis of the amygdala, the lateral and dorsal pallium, the deep periventricular layers of the optic tectum and the locus coeruleus. The distribution of NOS-containing neurons in *Xenopus* is very similar to the distribution of NOS-immunopositive neurons as reported in several amphibian and reptilian species, and is also very reminiscent of the distribution of targets of the visual input system in amphibians. Therefore, a literature survey of tract tracing studies of the visual system in amphibians was performed, particularly referring to the thalamo-tectal, thalamo-telencephalic and crossed tecto-bulbar pathways. Beside a possible role of NO in the control of background adaptation in *Xenopus*, the present data, in combination with data reported in literature, suggest that NOergic neurotransmission is involved in the processing of visual information in amphibians.

Key-words: Nitric oxide synthase immunoreactivity, NADPH-diaphorase activity, neuroanatomy, amygdala, optic tectum, locus coeruleus, *Xenopus laevis*.

INTRODUCTION

Among the many proposed functions of nitric oxide (NO) in biological systems and especially in the central nervous system (GARTHWAITE & BOULTON, 1995), the role of NO as a «retrograde messenger» in memory (BARINAGA, 1991) remains elusive. This elusiveness is reflected for instance in the question of how the macroscopic phenomenon of memory is realized at the microscopic and/or molecular level. Different models for memory as well as for the role of NO in memory have been suggested. According to SCHUMAN &

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MADISON (1991) NO signaling is required to activate the process of long-term potentiation (LTP) underlying memory, whereas others have demonstrated an involvement of NO in memory through the process of long-term depression (LTD) (GARTHWAITE *et al.*, 1988; SHIBUKI & OKADA, 1991). Also in astrocytes cultured *in vitro*, it was shown that long-lasting changes of calcium oscillation frequency, induced by repeated glutamate stimulation, depended on the activity of nitric oxide synthase (NOS) (PASTI *et al.*, 1995), and these authors proposed this effect as a cellular model for LTP. On the other hand, NO was called a less specific and less controllable chemical compound than most neurotransmitters or hormones (ANBAR, 1995). Nevertheless, the ubiquitous presence of NO in many organs and also in many taxonomic groups, points to a remarkable function well conserved in evolution (ANBAR, 1995) and to unique chemical properties of this free radical (BUTLER *et al.*, 1995). Given the low molecular weight, neutral electric charge and limited interaction with water, ANBAR (1995) concluded that NO diffuses rapidly through cytoplasm and biomembranes. Through this ability to affect many biochemical functions simultaneously, NO was suggested to act primarily as an intracellular synchronizing chemical messenger (ANBAR, 1995). Thus, an ultra-fast messenger for function synchronization, and a messenger for long-lasting response modulation (in the brain), were two basic molecular mechanisms inferred to explain some of the actions of NO at the macroscopic level.

We recently suggested also a role for NO signaling in the brain of the clawed toad *Xenopus laevis* in the process of skin colour adaptation to background light intensity (ALLAERTS *et al.*, 1997). This conclusion was primarily based on two observations: (**1**) the ability of NO to modulate a-MSH secretion from the pituitary pars intermedia (PI), and (**2**) the presence of NOS in neurons of the locus coeruleus. This area of the hindbrain is supposed to be involved in the control of background adaptation in *Xenopus* (TUINHOF *et al.*, 1994). We identified NOS activity in this brain area using immunocytochemistry with a polyclonal anti-human brain NOS (bNOS) and using the nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemical reaction on brain sections. According to DAWSON *et al.* (1991) a close relationship, if not an identity, exists beween NADPH-diaphorase and bNOS. We also found NOS-immunopositive neurons in the optic tectum and NADPH-d reactivity in the amygdala (ALLAERTS *et al.*, unpublished), two brain areas which have not been implicated in the light mediated control of background adaptation. On the other hand, recent neuroanatomical and neuropharmacological studies have demonstrated the involvement of NOS in the visual system of birds (WILLIAMS *et al.*, 1994) and mammals (CUDEIRO *et al.*, 1994).

In the present study, we closely examine the neuroanatomical distribution of NADPH-d positive neurons in *Xenopus laevis*. Since the distribution of NADPH-d positive neurons was very reminiscent of the distribution of the targets of visual inputs for the brain of amphibians, the question was raised whether a relationship exists between the NOergic neurons and the visual system in such animals. Following a literature survey of tract tracing studies of the visual system in amphibians (see discussion), the distribution of NOS-containing neurons (NOS-immunoreactive and/or NADPH-d reactive) is compared with the functional brain areas in orientation and in visual recognition, two functions that appear to be related in amphibians (NORTHCUTT & KICLITER, 1980). The amphibian brain, and especially the telencephalon, reflects a primitive level of organization compared to all

amniota and even some of the anamniote classes, as shown for instance by the brain-body and telencephalon-body ratios which are similar to *Latimeria*, the sole living crossopterygian (NORTHCUTT & KICLITER, 1980; BUTLER & HODOS, 1996). During the evolution of the tetrapods, the amphibian telencephalic ground form, the so-called laminar organization, has served as a base form for several radiations of telencephalic hypertrofy and differentiation (BUTLER & HODOS, 1996). Despite their relative primitivism, it appears that amphibian mechanisms of orientation and visual recognition are not well understood. And, similar to the existing discrepancy between different paradigms for memory (see above), a unifying theory of the operation of the amphibian visual system has not been formulated*.

MATERIAL AND METHODS

Abbreviations used in text and figures

A	anterior thalamic nucleus
ac	anterior commissure
Ad	nucleus anterodorsalis tegmenti
Apl	amygdala, pars lateralis
Apm	amygdala, pars medialis
Av	nucleus anteroventralis tegmenti
BN	bed nucleus of the pallial commissure
bNOS	brain nitric oxide synthase
C	central thalamic nucleus
Cb	cerebellum
cbn	cerebellar nucleus
DA	dopamine
dp	dorsal pallium
eNOS	endothelial nitric oxide synthase
GABA	γ-aminobutyric acid
Hd	dorsal habenula
HRP	horseradish peroxidase
Hv	ventral habenula
Ip	interpeduncular nucleus
Is	nucleus isthmi
LA	lateral thalamic nucleus, pars anterior
LC	locus coeruleus
lfb	lateral forebrain bundle
lp	lateral pallium
Lpd	lateral thalamic nucleus, pars posterodorsalis
Lpv	lateral thalamic nucleus, pars posteroventralis
LTD	long-term depression
LTP	long-term potentiation
mfb	medial forebrain bundle
Mg	magnocellular nucleus
mp	medial pallium
a-MSH	α-melanophore-stimulating hormone
NADPH-d	nicotinamide adenine dinucleotide phosphate - diaphorase
NB	nucleus of Bellonci
NPv	nucleus of the paraventricular organ
NPY	neuropeptide Y

*After the first submission of the present manuscript, our attention was called to some recent publications that had not yet been listed in the medline bibliographical system at that moment. These publications describe the neuroanatomical distribution of NADPH-d reactive and/or immunoreactive neurons in the urodele amphibian *Pleurodeles waltl* (GONZÁLEZ *et al.*, 1996), in the frog *Rana perezi* (MUÑOZ *et al.*, 1996), in the clawed toad *Xenopus laevis* (BRÜNING & MAYER, 1996) and in the lizard *Gekko gecko* (SMEETS *et al.*, 1997). These studies largely are in line with our own observations in *Xenopus laevis*, and are schematically represented in Table 1.

NO	nitric oxide
NOS	nitric oxide synthase
nIII	nervus oculomotorius
nV	nervus trigeminus
ON	nervus opticus
OT	olfactory tubercle
P	posterior thalamic nucleus
pc	posterior commissure
pd	hypophysis, pars distalis (in text: PD)
pi	hypophysis, pars intermedia (in text: PI)
PLP	periodate-lysine-paraformaldehyde (fixative)
pn	hypophysis, pars nervosa
POa	anterior preoptic nucleus
Rad	dorsal raphe nucleus
Rm	nucleus reticularis medius
SC	suprachiasmatic nucleus
Str	striatum
TE	thalamic eminence
tect	tectum mesencephali
tegm	tegmentum mesencephali
TH	tyrosine hydroxylase
Tor	torus semicircularis
TP	posterior tubercle
VH	ventral hypothalamic nucleus
VM	ventromedial thalamic nucleus
III	nucleus nervi oculomotorius
Vm	nucleus motorius nervi trigemini
Vpr	nucleus princeps nervi trigemini

Animals

Adult male and female *Xenopus laevis* were obtained from our laboratory stock. Animals were fed beef heart and trout pellets (Trouvit, Trouw, Putten, The Netherlands) once a week. To study a possible effect of background light condition on NOS expression, the toads were kept under constant illumination on either a black or a white background for at least three weeks, at 22 °C.

Immunohistochemistry and enzyme histochemistry

Toads were anaesthetized by immersion in 0.1 % tricaine methane sulfonate (MS 222, Sandoz, Basle, Switzerland) and perfused via the aorta with ice-cold 0.6 % Ringer's solution for 3 min, followed for 15 min with either Bouin-Hollande, Zamboni's fixative (for paraffin immunohistochemistry) or with periodate-lysine-paraformaldehyde (PLP) fixative after McLean & Nakane (1974) (for cryo-immunohistochemistry and cryo-enzyme histochemistry). Brains and pituitaries were dissected and postfixed for 2-3 hr in the same fixative. For paraffin immunohistochemistry, brains and pituitaries were dehydrated with an ethanol series (Merck, Darmstadt, Germany), isopropanol (Merck) and finally xylol (Janssen Chimica, Geel, Belgium), and embedded in paraplast. For cryo-histochemistry, brains and pituitaries were immersed in 10 % (w:v) sucrose in 0.15 M sodium phosphate buffer and after saturation frozen at -70 °C. From both paraffin-embedded and cryo-protected material we cut sagittal and transversal sections of 10-20 µm, which were transferred to poly-L-lysine-coated slides (Sigma, St Louis, MO, USA).

Sections were stained following the ABC immunostaining procedure (Hsu *et al.*, 1981) and the βNADPH-d procedure (Dawson *et al.*, 1991). Technical details of these staining procedures are described elsewhere (Allaerts *et al.*, unpublished). Antisera used in this study were a polyclonal anti-human bNOS serum (Transduction Laboratories,

Lexington, KY, USA; diluted 1:100) and a monoclonal antiserum against tyrosine hydroxylase (TH) (Instar Corporation, Stillwater, MN, USA). Sections were evaluated using bright field and epi-fluorescence microscopy with a Leitz DM RB/E microscope equipped with a Leica Vario Orthomat E camera system.

RESULTS

An overview of the brain and pituitary areas studied is represented in Figs 1 and 2. Line drawings of transverse projections are shown in Fig. 2 corresponding to the levels indicated in Fig. 1. The nomenclature follows that previously used (TUINHOF *et al.*, 1994). Figs 3 to 5 show photomicrographs of some of the transversal and also of some sagittal sections taken laterally to the medial plane (Fig. 3a, b). Results from anterograde fil-ling of the optic nerve with horseradish peroxidase (HRP) are adapted from TUINHOF *et al.* (1994).

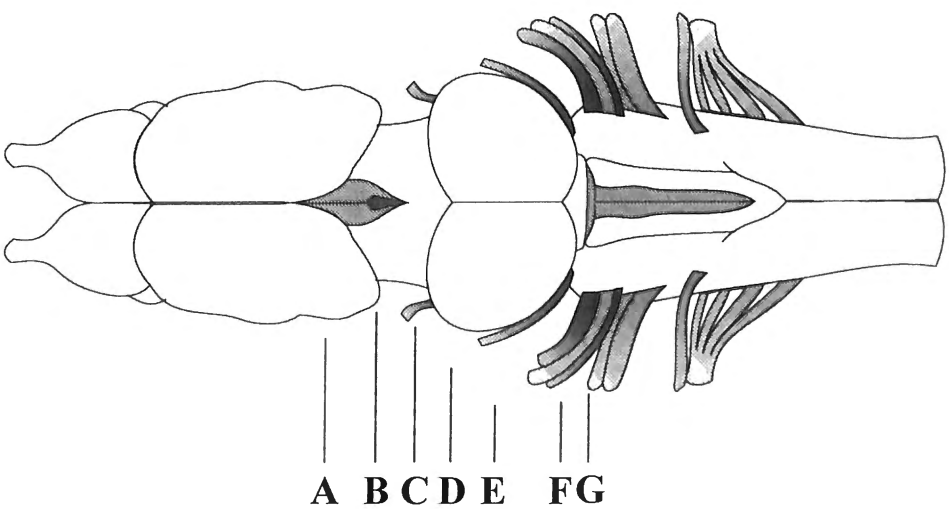

Fig. 1. – Schematic representation of the brain of the South African clawed toad *Xenopus laevis* (dorsal view), indicating the levels at which transversal sections have been presented in Fig. 2.

Telencephalon

The most rostral population of NADPH-d$^+$ neurons is found in the olfactory tubercle (OT) (Fig. 3a). In the telencephalon proper we found small populations of NADPH-d$^+$ cell bodies in the dorsal (dp) and lateral pallium (lp) (Figs 2, 4d-e). In the caudal telencephalon very strong NADPH-d reactivity is found in neurons of the amygdala pars lateralis (Apl) (Fig. 4a-c). Somata and fibers were darkly stained. Fibers from these somata cross the anterior commis-

sure to innervate the contralateral amygdala (Fig. 4c). According to NORTHCUTT & KICLITER (1980) the Apl can be regarded as an elongation of the lp, thus forming a C-shaped nucleus. The relevance of the amygdala with respect to the amphibian visual system is discussed below.

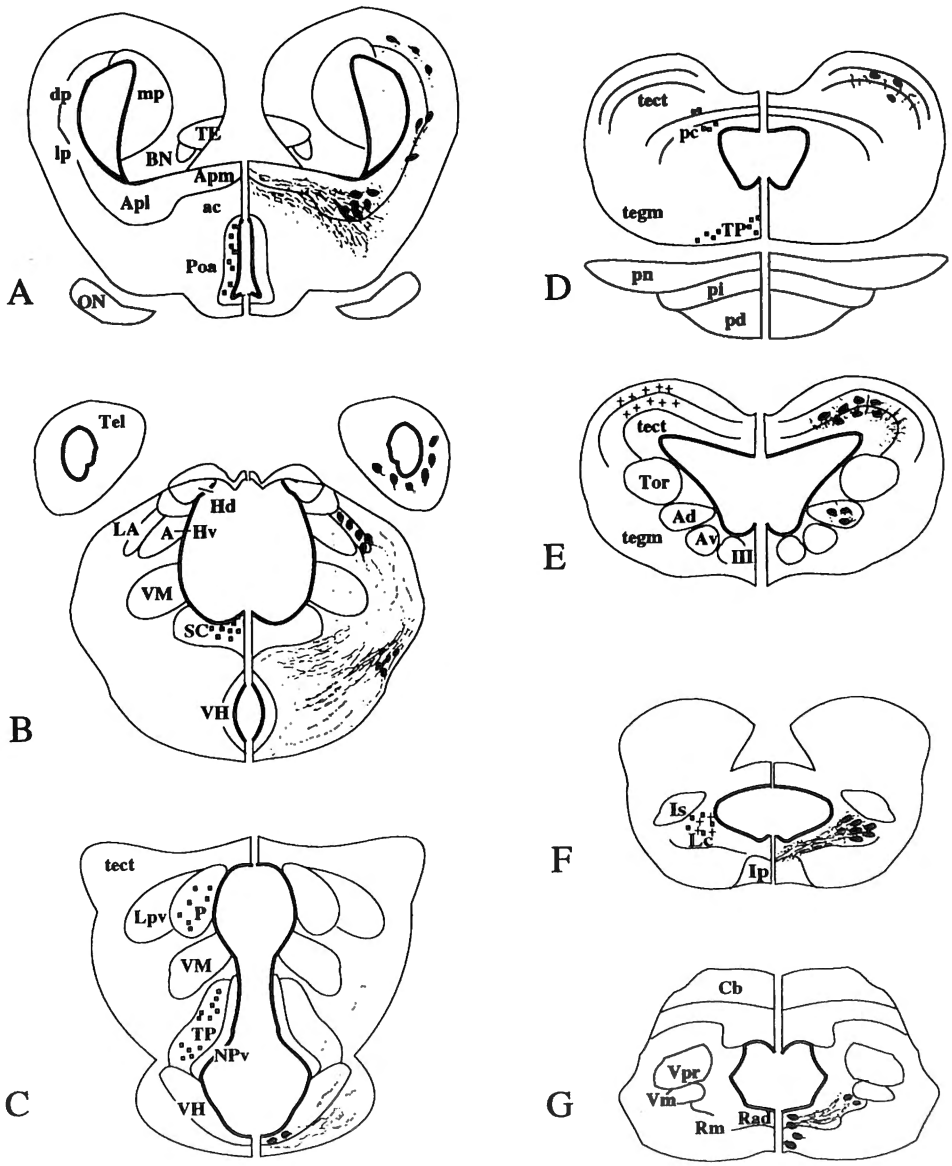

Fig. 2. – Diagram of transverse sections through the brain of *Xenopus laevis*, at levels indicated in Fig. 1. Dots and curved lines on the right indicate NADPH-d reactive neuron somata and NADPH-d reactive fibers, respectively. Plus signs on the left indicate bNOS-immunoreactive neuron somata. Squares on the left indicate TH-immunoreactive neurons (TUINHOF *et al.*, 1994).

Diencephalon

Following anterograde labeling of the optic nerve with HRP, we previously described HRP-reactive fibers contacting neuropeptide Y (NPY)-immunoreactive neurons in the nucleus suprachiasmaticus (TUINHOF *et al.*, 1994). Moreover, we found bundles of HRP-reactive fibers running parallel to the geniculate body and the neuropil of Bellonci, whereas terminal fields were observed in the optic tectum (see below). In the present study, NADPH-d$^+$ neurons were present in the pars anterior of the lateral thalamic nucleus (LA), medio-dorsal to the neuropil of Bellonci (NB), and in the ventral hypothalamic nucleus (VH) (Fig. 2). NADPH-d reactivity was absent from the magnocellular (Mg) and suprachiasmatic nuclei (SC). Furthermore, NADPH-d$^+$ fibers and small NADPH-d$^+$ cells occurred at the ventromedial edge of the optic tract (Fig. 5a). Some of the NADPH-d$^+$ fibers ran similar to the course of NOergic fibers observed at the edge of the optic tract in the turtle *Pseudemys scripta*, which would correspond to tectothalamic and thalamotectic fibers (BRÜNING *et al.*, 1994).

Mesencephalon

Using both immunostaining with anti-bNOS and the NADPH-d reaction we found NOS-reactive neurons in the optic tectum (tect) (Fig. 3c-d). NADPH-d reactivity was localized in somata of deep tectal neurons and also in some fibers projecting towards the superficial tectal layers (Fig. 3c). The NADPH-d$^+$ somata occurred mostly in layer 6 and also in layer 4 (see also HUGHES, 1990a,b and discussion). bNOS-like immunoreactivity was present in some of the neuron somata within the same layers and also in some fibers travelling across the deep medullary layer (POTTER, 1969) towards the superficial tectum and in fibers projecting to the posteroventral tegmentum (Fig. 2). Some NADPH-d$^+$ neurons, moreover, were found in the nucleus anterodorsalis tegmenti (Ad) (Figs 2 and 5b).

Metencephalon

In sagittal (Fig. 3b and inset) and coronal (Fig. 5c) cryo-sections, we encountered a dense cluster of NADPH-d$^+$ neurons (somata and fibers) in the isthmic area corresponding topographically to the locus coeruleus (LC). Paraffin sections of this area also show bNOS-immunoreactive neurons. The locus coeruleus was also identified immunohistochemically using anti-TH. Moreover, using double labeling with TH antiserum and the NADPH-d reaction we demonstrated intermingling and also close juxtaposition of TH$^+$ and NADPH-d$^+$ neurons, but no double-labeled cells. NADPH-d$^+$ neurons in the LC send fibers into a rostroventral direction (Fig. 3b: inset) towards the ventral tegmental area. Other LC neurons have axons that follow more medial and caudal directions and seem to innervate the anterior part of the dorsal raphe nucleus (Rad) (Fig. 5c).

Pituitary

We did not find bNOS-immunoreactivity in paraffin sections of the pituitary of *Xenopus laevis* fixed with Zamboni, whereas in PLP-fixed cryo-sections some NADPH-d activity occurred in endothelia of the pars intermedia (PI) and pars distalis (PD), probably corresponding to endothelial NOS (eNOS).

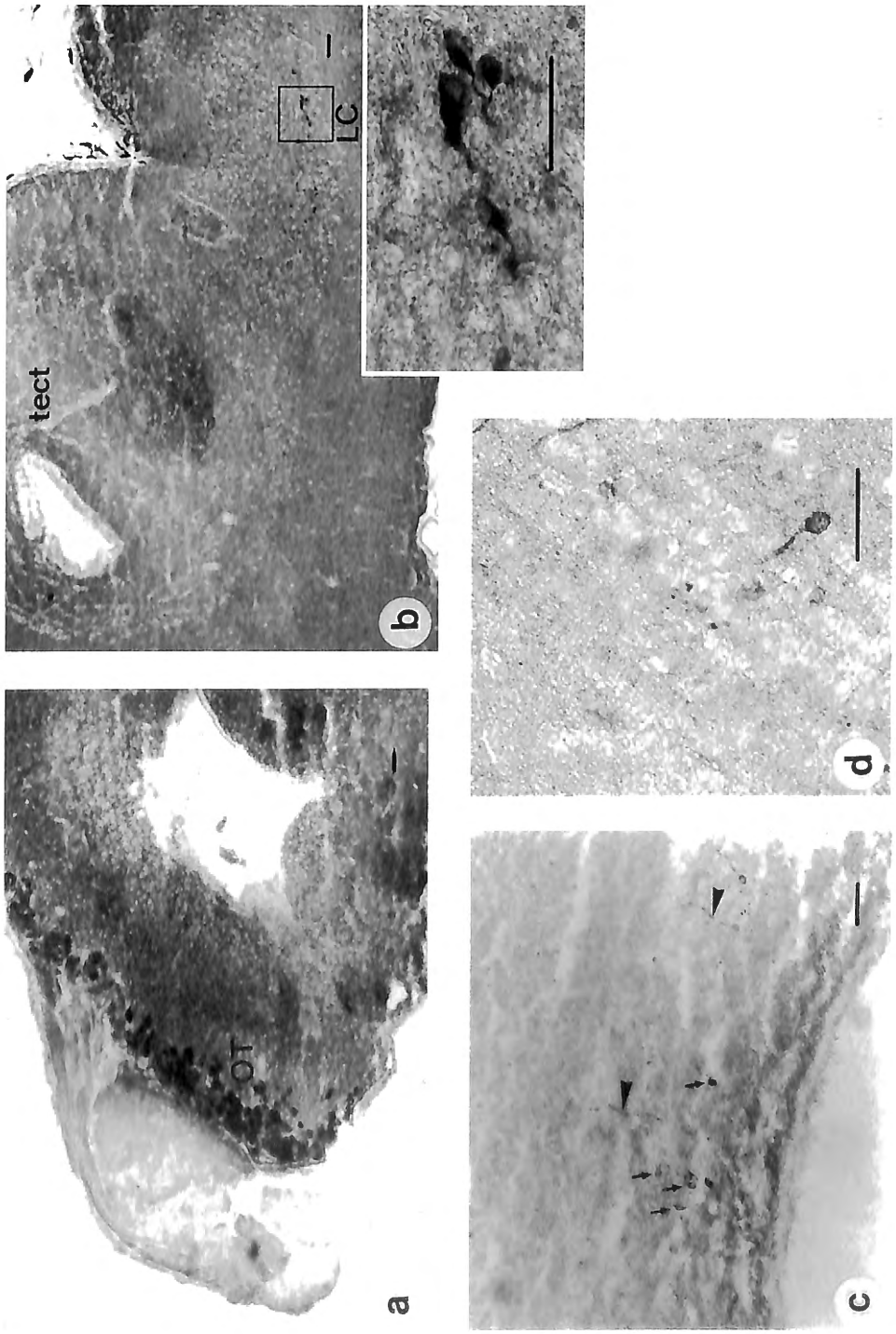

Fig. 3

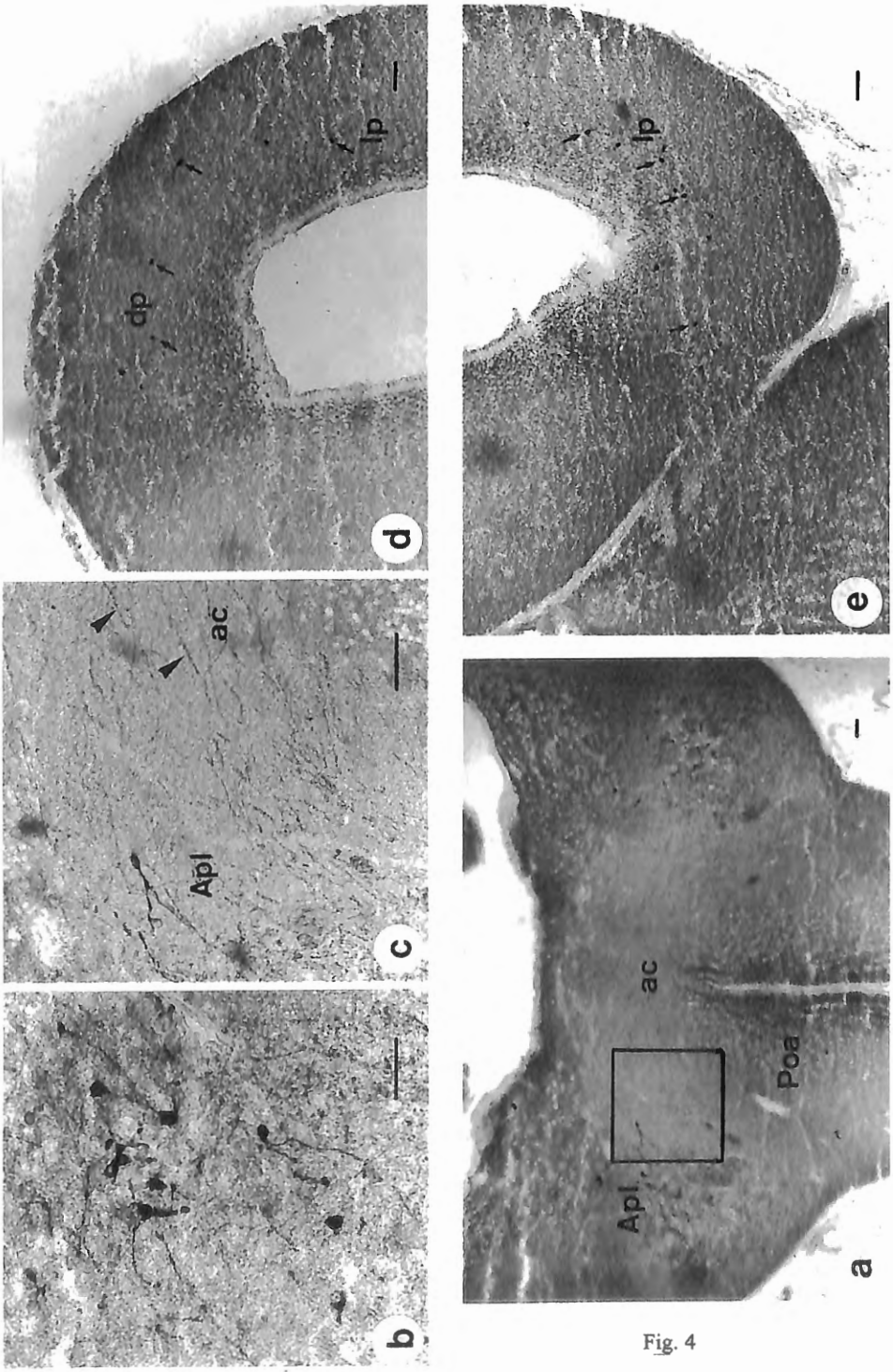

Fig. 4

Fig. 3. – Cryo-sections of *Xenopus laevis* brain fixed with PLP and stained with the NADPH-d histochemical reaction **(a-c)** and detail of a paraffin section of Zamboni-fixed *Xenopus* brain immunostained with polyclonal anti bNOS serum **(d)**. **a:** Sagittal section through the rostral telencephalon, indicating NADPH-d$^+$ somata in the olfactory tubercle (OT)(x50). **b:** Sagittal section through mesencephalon and methencephalon, showing NADPH-d$^+$ neurons in the optic tectum (tect) and the locus coeruleus (LC)(x50). A higher magnification of the framed region in the locus coeruleus is shown in the inset (x400). **c:** Transversal section of the periventricular layers of the optic tectum (x126). Note the NADPH-d$^+$ neurons (small arrows) in the deep tectal layers, and some projections towards the superficial tectal layers (arrowheads). **d:** Detail of sagittal section of optic tectum showing bNOS-immunoreactive neuron in deep tectal layer (x320). All sections are from *Xenopus* adapted to black background. (Scale bars = 50 μm).

Fig. 4. – Transversal cryo-sections of *Xenopus laevis* brain (PLP fixation) stained with the NADPH-d histochemical reaction: **(a-c)** through the telencephalon at the level of the anterior commissure (ac); **(d-e)** low magnification of lateral telencephalic wall at the level of the preoptic area (Poa). **a:** Overview of basal telencephalon (x50). **b:** Detail of amygdala pars lateralis (Apl) showing dense cluster of NADPH-d$^+$ neurons (x200). **c:** Higher magnification of frame in **(a)** showing Apl and anterior commissure (ac), with NADPH-d$^+$ fibers coursing through the ac (arrowhead) (x200). **d:** NADPH-d+ neuron somata (arrows) in the dorsal (dp) and lateral pallium (lp)(x80). **e:** NADPH-d$^+$ neuron somata (arrows) in ventral part of lp (x80). All sections are from *Xenopus* adapted to a white background. (Scale bars = 50 μm).

Fig. 5. – Transversal cryo-section of *Xenopus laevis* brain (PLP fixation) stained with NADPH-d histochemical reaction: **a:** Low magnification of the optical tract in the diencephalon: NADPH-d reactivity is found in fibers of the optical tract and in small cells at the ventromedial edge of the optical tract (arrowheads)(x80). **b:** Detail of the lateral thalamus showing NADPH-d$^+$ neurons in the nucleus anterodorsalis tegmenti (Ad)(x200). **c:** Cluster of NADPH-d$^+$ neurons in the locus coeruleus (LC) and NADPH-d$^+$ fibers projecting towards the dorsal raphe (arrowhead)(x140). Sections in **a** and **b** are from white-adapted, the section in **c** is from a black-adapted *Xenopus*.(Scale bars = 50 μm).

DISCUSSION

On the basis of NADPH-d histochemistry and bNOS immunohistochemistry we here present a neuroanatomical description of the NOergic neurons in the brain of *Xenopus laevis*. NADPH-d positive neurons were found in several nuclei of the telencephalon, diencephalon, mesencephalon, and metencephalon. These data largely confirm previous neuroanatomical descriptions in *Xenopus laevis* (BRÜNING & MAYER, 1996; ALLAERTS *et al.*, unpublished) and in other amphibian and reptile species (BRÜNING *et al.*, 1994; GONZÁLEZ *et al.*, 1996; MUÑOZ *et al.*, 1996; SMEETS *et al.*, 1997) (see Table 1 for comparison of the descriptions in the amphibian species). DAWSON *et al.* (1991) have shown that in the rat NADPH-d activity correlates to NOS activity both in the brain and in peripheral tissues, although some tissues like the adrenal cortex and the liver display NADPH-d activity in the absence of NOS. Also in *Xenopus laevis* (BRÜNING & MAYER, 1996) and in the urodele amphibian *Pleurodeles waltl* (GONZÁLEZ *et al.*, 1996) a close correlation was found between NADPH-d activity and NOS immunoreactivity except for the olfactory nerve (NADPH-d activity in the absence of NOS immunoreactivity). Using Western blotting of *Xenopus* hindbrain homogenates, we previously demonstrated an approximately 150 kDa

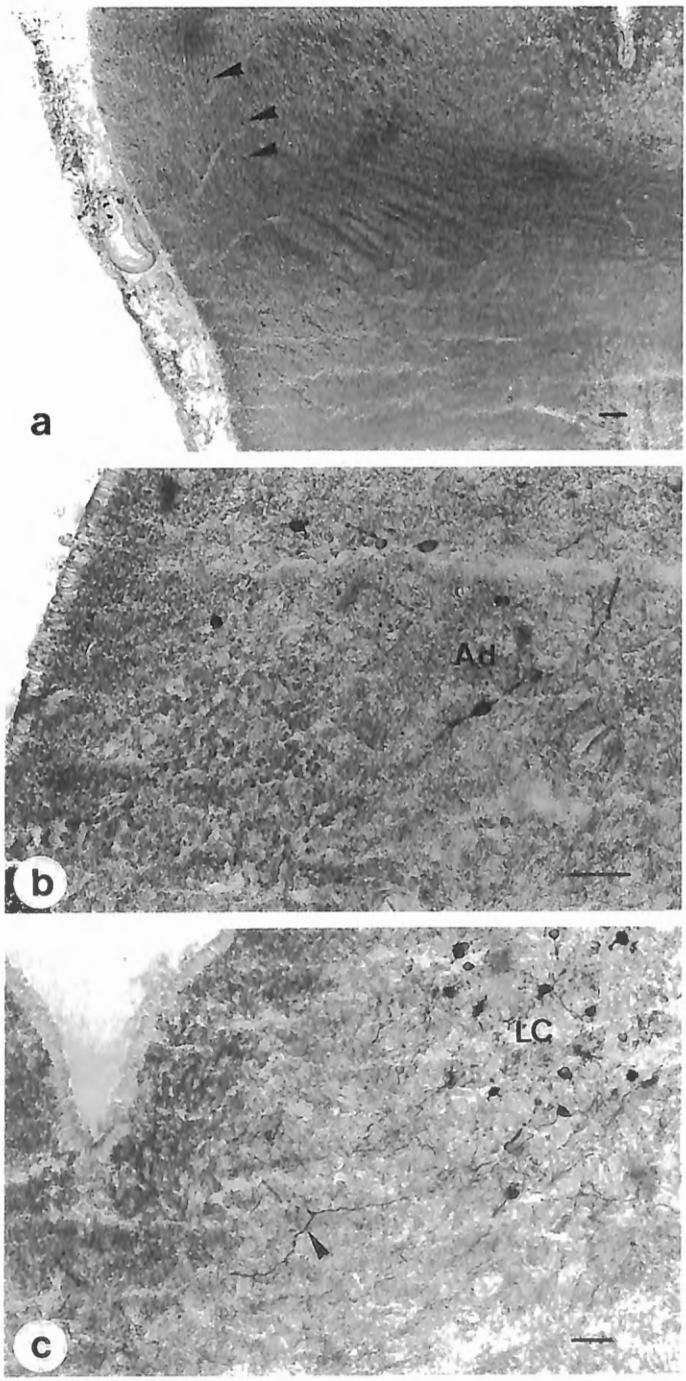

Fig. 5

band immunostained with a polyclonal bNOS antiserum, confirming the presence of bNOS in *Xenopus* hindbrain (ALLAERTS *et al.*, 1997).

Functional relevance of a brain NOergic system in Xenopus?

Previously we have shown differences in activity of hypothalamic nuclei under different conditions of background light intensity (TUINHOF *et al.*, 1993,1994). On the basis of a variety of methods (immunocytochemistry, retrograde labeling, etc.) we proposed that a small number of brain centers was responsible for the regulation of melanotrope activity in the process of background adaptation. In our view the most important regulatory mechanism is provided by the inhibition of α-MSH secretion via the hypothalamic suprachiasmatic nucleus (SC) (TUINHOF *et al.*, 1993), which contains neurons that simultaneously produce NPY, dopamine (DA) and γ-aminobutyric acid (GABA) (DE RIJK *et al.*, 1992). Another important hypothalamic centre is the magnocellular nucleus (Mg) that may have a mild stimulatory effect on the melanotropes through the release of thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH) (TUINHOF *et al.*, 1994). The third centre involved in background adaptation is the locus coeruleus (LC) in the metencephalon, for it was shown that noradrenaline (NA)-containing fibres innervate the PI, whereas NA-positive neurons are localized in the LC (TUINHOF *et al.*, 1994; JANSEN *et al.*, 1997).

We recently also compared bNOS-activity in the brain and pituitary of *Xenopus laevis* adapted to either a black or a white background (ALLAERTS *et al.*, 1997) and no differences were observed. The occurrence of bNOS activity in neurons of the LC, and the capability of NO to stimulate α-MSH secretion from single melanotrope cells *in vitro*, suggest a role of NO in the control of background adaptation (ALLAERTS *et al.*, 1997). However, our present data show the absence of NOS-activity in the Mg and SC, and especially the SC receives a direct input from the optic nerve (TUINHOF *et al.*, 1994). The intermingling of NOS-containing and TH^+ neurons without a cellular co-localization of bNOS and TH in the LC may also indicate a role in the presumed effect of stress on background adaptation. The involvement of the LC in the regulation of stress-induced responses has been documented in the rat (TILDERS & BERKENBOSCH, 1986). Alternatively, the role of NO may reside in the higher visual centers that regulate background adaptation. In this respect it is interesting to note that CUDEIRO *et al.* (1994) have demonstrated that suppression of the visual responses of relay cells in the dorsal lateral geniculate nucleus of the cat *in vivo* is induced by iontophoretic administration of N^G-nitro-L-arginine, a competitive inhibitor of NOS. This suppression of visually or N-methyl-D-aspartic acid (NMDA) evoked responses thereby seemed independent of an increase of cyclic guanosine-3',5'-mono-phosphate (cGMP), and the function of NO at this level of the visual system was called permissive (CUDEIRO *et al.*, 1994). In another model system, the chicken embryo, WILLIAMS *et al.* (1994) found a correlation of NOS expression with changing patterns of axonal projections in the developing optic tectum. They concluded that NOS is involved in the development and refinement of the proper pattern of connections in the chicken retinotectal system (WILLIAMS *et al.*, 1994).

Comparison of the distribution of NOS-containing neurons in the amphibian brain with literature data of tracing studies of targets of the visual system in amphibians shows a striking overlap between NOergic neurons and parts of the visual system. We here speculate on a possible link between NOergic neurotransmission and the visual system in amphibians. For an overview of this system we refer to NORTHCUTT & KICLITER (1980), who especially document the telencephalic connections of the visual system (see below), as well as to the tracing studies of the optic tract by HERRICK (1925, 1948), LÁZÁR (1969), HUGHES (1990a,b) and to the textbook of *Comparative Vertebrate Neuroanatomy* by BUTLER & HODOS (1996). The suggestion of a role of NOergic transmission in the visual and possibly also orientation systems in the amphibian brain, as for instance suggested by the NOS-containing neurons in the optic tectum (see below), does not preclude other functions of NOergic transmission, such as indicated by the NOS activity in the olfactory tubercle (BRÜNING *et al.*, 1994; Table 1 and present data). BRÜNING *et al.* (1994) have shown that NADPH-d reactivity in the olfactory tubercle is correlated with b-NOS immunoreactivity in the turtle *Pseudemys scripta*, but obviously these NADPH-d$^+$ neurons in the olfactory tubercle have no relationship to the visual system. Also SMEETS *et al.* (1997) remarked that NADPH-d activity and NOS immunoreactivity are not confined to any functional sensorimotor system or neurotransmitter system. Otherwise, at least a permissive role of NOergic transmission in parts of the visual system in mammals (CUDEIRO *et al.*, 1994) and birds (WILLIAMS *et al.*, 1994) has now been functionally demonstrated. However, experimental studies using selective NOS inhibitors will be necessary to substantiate the functional relevance of NOergic neurotransmission in the amphibian brain *in vivo*.

Telencephalic connections relevant to the amphibian visual system

The «classical» viewpoint on the amphibian telencephalic organization, as expressed in *e.g.* HERRICK's monography (1948), considers the amphibian telencephalon as a simple, unspecialized web of nervous tissue that receives mainly secondary or tertiary olfactory connections, and of which the efferents carry primarily olfactory information to the hypothalamus or midbrain, to become integrated with ascending gustatory information (cited in NORTHCUTT & KICLITER, 1980). According to NORTHCUTT & KICLITER (1980) several arguments can be raised against this «classical» viewpoint, favouring an integrative role of the amphibian telencephalon, including the integration of visual, auditory and somatic information. Although some of the references cited in NORTHCUTT & KICLITER (1980) may seem rather old, comparison of these references with recent reviews (*e.g.* BUTLER & HODOS, 1996) strengthens their validity, and moreover, little new information on tracing studies in the amphibian telencephalon has become available since. Below we summarize some of the arguments listed by NORTHCUTT & KICLITER (1980)(see also the schematic illustration in Fig. 6).

1) The medial pallium in amphibians not only receives connections of the lateral pallium (the main target of the olfactory input), but also receives direct thalamic projections carrying visual and somatic information, and afferents from the preoptic area (POa) and ventral thalamus as shown by HRP labeling studies (NORTHCUTT & KICLITER, 1980; BUTLER & HODOS, 1996). The medial pallium is connected via the medial forebrain bun-

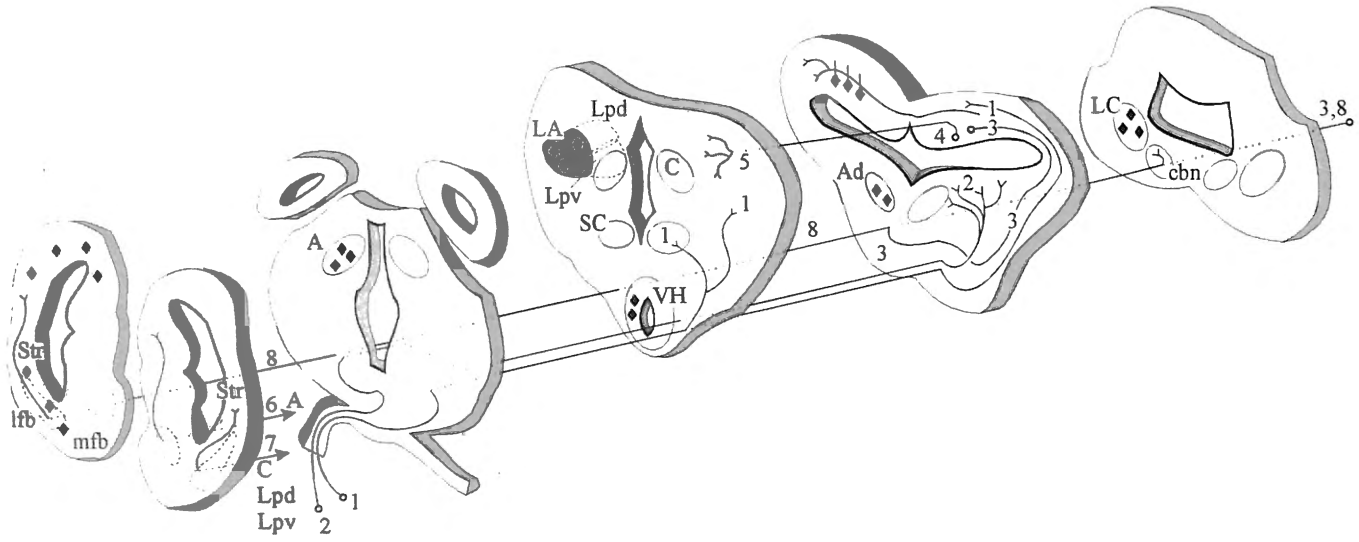

Fig. 6. – Schematic representation of the connections of the visual system in the amphibian brain, adapted from tracing studies by LÁZÁR (1969), SCALIA & GREGORY (1970), KICLITER & NORTHCUTT (1975), KICLITER (1979) (reviewed in NORTHCUTT & KICLITER, 1980). The terminology of diencephalic nuclei follows the terminology of NEARY & NORTHCUTT (1983). The brain is represented by thick sections from rostral to caudal position comparable to thin sections B, C, E, F in Fig. 2 as well as a mid-telencephalic thick section (rostral to Fig.2, A). Black diamonds represent NADPH-d active and/or bNOS immunoreactive neurons as described in this study (see also Fig.2). **1-2:** retino-tectal and retino-thalamic pathways observed following HRP-labeling of the optic nerve; **3-5:** efferent pathways of the optic tectum found by anterograde degeneration studies (e.g. LÁZÁR, 1969): tecto-isthmic connection and crossed tecto-bulbar pathway (**3**), fibers in mesencephalic commissure (**4**) and tecto-diencephalic connection (**5**); **6-7:** connections of mfb (**6**) and lfb (**7**) to dorsal thalamic nuclei (see text) (thalamo-frontal tract, NORTHCUTT & KICLITER, 1980); **8:** telencephalic-medullar connection to the cerebellar nucleus (cbn), as revealed by HRP-labeling (KICLITER, 1979).

dle (mfb) (Fig. 6) to the anterior thalamic nucleus (A) (terminology adapted in NEARY & NORTHCUTT, 1983).

2) The amphibian striatum (str) receives both visual and auditory information from dorsal thalamic centers (already suggested by HERRICK, 1948), as demonstrated by anterograde neuron degeneration studies (KICLITER & NORTHCUTT, 1975). NORTHCUTT & KICLITER (1980) suggest that the amphibian striatum (str) may be linked to the optic tectum via the lateral forebrain bundle (lfb) and ipsilateral relay centers located in the lateral dorsal thalamus (SCALIA & GREGORY, 1970), but not by relay centers in the anterior thalamic nucleus (BUTLER & HODOS, 1996) (Fig. 6).

3) The amphibian amygdala pars lateralis receives projections via the lfb from the hypothalamus and the caudal dorsal thalamus (namely the central nucleus [C] and lateral nuclei [Lpv and Lpd]), which areas in amphibians are linked with the optic tectum (see argument 2) (NORTHCUTT & KICLITER, 1980).

4) HRP labeling studies of the lateral amygdala revealed afferent cell groups of the ipsilateral dorsal thalamic nuclei (connected via the lfb) as well as HRP-labeled cell groups in the ipsilateral rostral medulla of the brainstem (KICLITER, 1979), identified as the cerebellar nucleus (cbn) according to NIEUWENHUYS & OPDAM (1976) and situated immediately caudal to the LC (Fig. 6). The lfb carries visual information to the str and possibly also to the Apl (GRUBERG & AMBROS, 1974), as already shown by tracing studies of LÁZÁR (1969). Interestingly, BRÜNING & MAYER (1996) observed dendrites of NOS-positive neurons in the anterior entopeduncular nucleus (lateral from amygdala) of *Xenopus laevis*, intermingling with fibers of the lfb (see below).

Several lines of evidence, obtained from anatomical, histochemical and embryological studies, have led to NORTHCUTT & KICLITER's conclusion (1980) that the amphibian lateral pallium and pars lateralis of the amygdala (which are topologically contiguous regions of the lateral telencephalic wall) are homologous to the reptilian dorsal ventricular ridge and lateral cortex. However, in sauropsids (reptiles and birds) the different regions of the dorsal ventricular ridge became segregated so that different regions of the primary pallial region received only one single sensory modality, whereas in amphibians several modalities converge on the striatum, i.e., the subpallial region of the lateral telencephalic wall (NORTHCUTT & KICLITER, 1980). Whether the amphibian striatal cells are also multimodal, however, remains to be clarified (NORTHCUTT & KICLITER, 1980). The relevance of the medial telencephalic wall (pallium and striatum) for the amphibian visual system was proven experimentally by showing evoked potentials recorded upon electrical stimulation of the optic nerve (KARAMIAN *et al.*, 1966), but beside visual afferents the medial telencephalic wall also receives auditory and somatosensory afferents (KARAMIAN *et al.*, 1966).

In our study, NADPH-d activity in the telencephalon was found in the pars lateralis of the amygdala, in the lateral and dorsal pallium and in the olfactory tubercle (see Table 1 for comparison with other studies in amphibians). Studies in reptiles (BRÜNING *et al.*, 1994; SMEETS *et al.*, 1997) moreover, have shown bNOS-immunoreactivity in neurons of the basal ganglia complex, the basal amygdaloid nucleus and the dorsal ventricular ridge, and in fibers coursing in a tract connecting the basal amygdaloid nucleus with the hypothalamus, corresponding to the stria terminalis. Besides the homology between amphibian and reptilian subpallial regions, also the NOergic neurotransmission seems to be con-

TABLE 1

Comparison of literature data on the bNOS immunoreactivity and the NADPH-d reactivity in the brain of three amphibian species.

Pleurodeles waltl[1]	*Rana perezi*[2]	*Xenopus laevis*[3]
Telencephalon		
primary olfactory fibers (d)		terminal nerve (d)
olfactory bulb (+)	olfactory bulb (d)	olfactory lobe (-)
pallium (+)	pallium (d)	pallium (+)
septum (+)	septum (d)	septum (very few)
caudal striatum (+)	striatum and nucleus of diagonal band (d)	striatum (+)
		nucleus accumbens (+)
amygdala (+)	amygdala (d)	amygdala pars lateralis and anterior entopeduncular nucleus (+)
Diencephalon		
preoptic area (+)	preoptic and infundibular recesses of 3rd ventricle (d)	preoptic nucleus (+)
ventral hypothalamus (+)	suprachiasmatic and magnocellular nuclei (d)	ventral hypothalamus (+)
posterior tubercle (+)		posterior tubercle (+)
	ant.,lat., centr. and lateral posteroventral thalamic nuclei (d)	lateral thalamic nuclei (lat., ant. & post. thalamic nucl.) and post.entopeduncular nucl.(+)
Brainstem		
mesencephalic tegmentum (+)	pretectal area (d)	
optic tectum (+)	optic tectum (d)	optic tectum (+)
	torus semicircularis (d)	magnocell.nucl. of torus semicircularis (+)
isthmic-pretrigeminal region (+)		
isthmic region (+)	isthmic nucleus (d)	isthmic nucleus (-)
	locus coeruleus (*)	locus coeruleus (+)
Rhombencephalon		
descending trigeminal tract (+)	sensory trigeminal nuclei (d)	descending nucleus of trigeminal nerve (+)
	octaval area (d)	
solitary tract (+)	nucleus of solitary tract (d)	dorsal to solitary tract (+)
raphe nucleus (+)	raphe nucleus (d)	
mid-caudal reticular formation (+)	reticular nuclei (d)	inferior reticular nucleus (+)
	dorsal column nucleus (d)	

[1] GONZALEZ *et al.*, 1996: distribution of bNOS immunoreactivity and NADPH-d activity.
[2] MUÑOZ *et al.*, 1996: distribution of NADPH-d activity.
[3] BRÜNING & MAYER, 1996: distribution of bNOS immunoreactivity and NADPH-d activity.
+ : bNOS immunoreactivity and NADPH-d activity are co-localized.
d : only NADPH-d activity.
- : reported negative for both NADPH-d and bNOS staining.
* : co-localization of NADPH-d with tyrosine hydroxylase immunoreactivity.

served in both vertebrate groups, indicating a well conserved and important role for NO in the functioning of this brain region. However, the multimodal or multi-functional character of the amphibian medial telencephalic wall may hamper the characterization of individual functional units (NORTHCUTT & KICLITER, 1980), so making the functional characterization of the NOergic transmission in the amphibian telencephalon difficult.

Thalamic connections relevant to the amphibian visual system

The demonstration of two telencephalic visual projections in amphibians according to NORTHCUTT & KICLITER (1980) enables a comparison between the amphibian visual system and that of the sauropsids, which also have a dual telencephalic visual system (HALL & EBNER, 1970; KARTEN & REVZIN, 1966; KARTEN & NAUTA, 1968; KARTEN *et al.*, 1973). This dual system consists of **(a)** the retino-thalamo-telencephalic system (shortly «thalamofugal» system) and **(b)** the retino-tecto-thalamo-telencephalic system (shortly «tectofugal» system) (NORTHCUTT & KICLITER, 1980). Characteristics of sauropsid visual systems provide criteria for proposing homology of the amphibian visual system. When rigidly applying the criteria for homology, according to NORTHCUTT & KICLITER (1980) neither of the two sauropsid visual projection systems is exactly represented in amphibians, although the retino-tecto-thalamo-telencephalic system comes closest to meeting the criteria. SCALIA & GREGORY (1970) already stated that amphibians do possess a well-developed retino-tectal pathway but have a comparatively small retino-thalamic system, whereas anatomical evidence for a thalamo-telencephalic projection is still scarce. RUBINSON (1968) and LÁZÁR (1969) described projections of the optic tectum in *Rana* to a region of the lateral thalamic neuropil, which does not receive direct retinal projections. Post-synaptic cells of fibers in this neuropil are located in the posterocentral and part of the posterolateral nuclei, and project to the striatum via the lfb (SCALIA & GREGORY, 1970). SCALIA & GREGORY (1970) therefore concluded that the latter pathway may represent the amphibian homologue of the geniculo-striate system in mammals, i.e., the mammalian form of the thalamofugal system (BUTLER & HODOS, 1996). A specific problem in the localization of post-synaptic neurons in amphibia, according to SCALIA & GREGORY (1970), is caused by the relatively long dendrites of nuclei with rather distant location compared to the terminal fields of the retinofugal fibers. MUÑOZ *et al.* (1996) observed terminal fields of NADPH-d$^+$ neurons in the visual recipient plexus of Bellonci and in the thalamic geniculate nucleus of the frog *Rana perezi*. A functional role of NOS in the visual relay cells of the geniculate nucleus of the cat was shown by CUDEIRO *et al.* (1994).

In conclusion, the absence of a dorsal ventricular ridge in amphibians tones down any homology between amphibian and higher vertebrate telencephalic systems, unless we may regard the lateral pallium and pars lateralis of the amygdala as a primitive, relatively unspecialized but evolutionary homologue of the dorsal ventricular ridge, as suggested by NORTHCUTT & KICLITER (1980).

NOS and the crossed tecto-bulbar pathway in amphibians

The NOS-immunopositive and NADPH-d reactive neurons were observed in layer 4 and 6 of the optic tectum (terminology according to LÁZÁR, 1969, POTTER, 1969 and

HUGHES 1990a,b). This distribution is reminiscent of the distribution of neurons that give rise to the tecto-bulbar pathway, as demonstrated using HRP-labeling in the frog *Rana pipiens* by HUGHES (1990b). The somata of these neurons according to HUGHES (1990b) are located in the superficial half of layer 6, with some dendrites ascending to the superficial tectal layers, the so-called retino-recipient layers (HUGHES, 1990a). BRÜNING *et al.* (1994) observed NOS-immunopositive neurons in the deep tectal layers of the turtle *Pseudemys scripta*, and suggested that NOS-positive fibers originating from the periventricular gray leave the tectum in a way corresponding to the tecto-bulbar and tecto-thalamic tract. Efferent pathways of the amphibian optic tectum have been demonstrated by LÁZÁR and co-workers (LÁZÁR, 1969; LÁZÁR *et al.*, 1983) and include projections to the ipsilateral dorsal thalamic nuclei and the ipsilateral isthmic nucleus (BUTLER & HODOS, 1996) (Fig. 6). Further labeling and tracing studies are necessary to demonstrate that NOergic neurons indeed are involved in the crossed tecto-bulbar pathway (as suggested by BRÜNING & MAYER, 1994) and also to demonstrate their synaptic input.

In contrast to most mammalian species studied (reviewed in HUGHES, 1990b), in the optic tectum of frogs and turtles the neurons that give rise to the crossed tecto-bulbar pathway have dendrites that extend into the retino-recipient superficial layers of the tectum. This was *e.g.* demonstrated using the HRP-labeling and the cobalt filling technique in the frog (LÁZÁR *et al.*, 1983). The latter finding is consistent with the view that the neurons that give rise to the frog's crossed tecto-bulbar pathway receive a direct retinal input, and also suggests that the neurons may receive this input from more than one type of retinal ganglion cell (HUGHES, 1990b). Moreover, a gradient-like distribution of these neurons with preferential location at the ventrolateral border has been described in many amphibians, reptiles, birds and mammals, although with some dissimilarities, indicating a fundamental role in tectal functioning (HUGHES, 1990b). HUGHES (1990b) suggests that the spatial gradient of these deep tectal neurons underlies the functional gradients in the control of orienting movements, such as demonstrated in the cat (MCILWAIN, 1982).

CONCLUDING REMARKS

In the introduction we referred to some studies suggesting a «macroscopic» role of NO in memory, through processes at a «microscopic» or molecular level, corresponding to long-lasting response modulation (in neurons) and fast function synchronization (in cells in general). When looking at the microscopic neuroanatomy of NOS-containing neurons in *Xenopus laevis*, a complex neuronal web emerges. Comparison of the distribution of bNOS-immunopositive and NADPH-d reactive neurons with the results of a literature survey of tract tracing studies of targets of the visual system in amphibians, shows that NOS is present in many (if not all) tracts and centers involved in orientation and visual information processing. The interrelationship between orientation and visual recognition suggested is based on at least two arguments: **(1)** the anatomy and ultrastructure of the optic tectum (see *e.g.* HUGHES, 1990a,b) and **(2)** the connections between the optic tectum and the pars lateralis of the amygdala, which is the main target of the vomeronasal organ, indicating a possible integration of visual and other sensory input (reviewed in NORTHCUTT & KICLITER, 1980). Despite the multi-functional character and relatively unspecialized

aspect of the amphibian telencephalic lateral wall, the overall simplicity of the amphibian telencephalon may favor both macroscopic and microscopic approaches to the study of the role of NOergic transmission in the vertebrate brain. Pharmacological *in vivo* studies (using NOS inhibitors, guanylate cyclase activators and/or specific phosphodiesterase inhibitors) and also further neuroanatomical tracing studies will be necessary to prove the suggested link between NOergic transmission and the visual system, as already shown in parts of the visual system in birds and mammals (WILLIAMS *et al.*, 1994; CUDEIRO *et al.*, 1994).

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