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Short Report

Embryonic and larval development of NADPH-diaphorase/nitric oxide synthase reactivity in the brain of the amphibian *Pleurodeles waltl*

NEREA MORENO, JESÚS M. LÓPEZ, CRISTINA SÁNCHEZ-CAMACHO, MIGUEL CRESPO, MARGARITA MUÑOZ and AGUSTÍN GONZÁLEZ*

Departamento de Biología Celular, Facultad de Biología, Universidad Complutense, Madrid, Spain

ABSTRACT The distribution of NOS-containing cells was studied in the newt *Pleurodeles waltl* during ontogenesis by means of nitric oxyde synthase (NOS) immunochemistry and NADPH-diaphorase histochemistry. Embryonic and larval stages were studied. The first positive neurons were observed at embryonic stage 30 in the ventrolateral area of the caudal rhombencephalon. Subsequently (stage 33b), weakly reactive cells appeared rostrally in the mesencephalic tegmentum, isthmic tegmentum. A general caudorostral gradient of newly expressing NADPHd reactivity cells was observed through larval life. As in the adult, the nitrergic cells in the CNS of the newt are widely distributed during development, pointing to important roles of nitric oxyde through ontogenesis. The sequence of appearance of nitrergic cells revealed a first involvement of this system in reticulospinal control likely influencing locomotor behavior. As development proceeds, cells in different sensory systems express progresively the enzyme NOS in a pattern that shows many similarities with other anamniotes.

Introduction

In the last few years, experimental studies have demonstrated that nitric oxide (NO) plays a role in aspects of neural development such as synaptic plasticity, long-term potentiation and development and refining of neuroanatomical connections (Cudeiro and Rivadulla, 1999; Ernst *et al.,* 1999; Contestabile 2000). The importance of NO in early stages of avian and mammalian brain development has been progressively confirmed. Moreover, in a recent study in a teleost fish it was observed that NO producing cells are also present at early stages of development in certain brain regions. Important differences were noticed, however, in the development of the nitrergic system between fish and amniotes, although it seems that in all vertebrates nitrergic cells might be needed for developmental processes in the brain.

Most studies dealing with the localization of NO producing cells in the brain have used the relatively simple NADPH-diaphorase (NADPHd) histochemical method and NADPHd was identified as a marker for neuronal nitric oxide synthase (NOS). However, particular examples in which NADPHd staining is not partially or totally coincident with that of NOS have been reported. Therefore, it has been suggested that to study the nitric oxide-synthetizing elements it is recommendable to compare the results of the NADPHd technique with other methods for the localization of NOS.

Among vertebrates, amphibians constitute a class that marks a crucial point in evolution i.e., the transition from an aquatic to a terrestrial life style. The use of amphibian brains in neuroanatomical studies was early adopted by authors that rapidly noticed that within these relative "simple" brains it was possible to recognize most of the major brain features present in all vertebrates, including humans (Herrick, 1948). The CNS of amphibians is often considered to be primitive. This was particularly thought for urodeles where the degree of cell migration from the periventricular zone is extremely reduced. However, careful studies by Roth and co-workers (summarized by Roth, 1987) showed that even the salamander brain possesses virtually all the anatomical and functional properties found in tetrapods. It was proposed that the urodele CNS is charactrized by a secondary simplification, which gives the impression that the brains of newts and salamanders are more primitive than their phylogenetic position, as tetrapods, implies.

Several studies have demonstrated a wide distribution of NOS containing cells and fibers in the brain of adult amphibians (see Alonso *et al.,* 2000). The pattern of organization of nitrergic elements in the amphibian brain was not limited to a particular functional system and showed many similarities with those of amniotes and fish.In particular, the organization of the nitrergic system in the adult brain of the urodele amphibian *Pleurodeles waltl* was investigated by means of NADPHd histochemistry and NOS immunohistochemistry (González *et al.,* 1996). Although both methods yieled essentially a similar staining, a discrepancy was found in the olfactory bulbs where abundant NADPHd reaction was present and no NOS immunoreactive structures were observed. In this study we present a detailed spatio-temporal analysis of the location of NOS containing cells and fibers in the brain of *P. waltl* through embryonic and larval stages. The NADPHd histochemical method was used. In order to confirm that the NADPHd histochemistry reveals nitrergic neuronal structures in the developing brain, NOS immunohistochemistry was used on the same sections.

Materials and Methods

Pleurodeles waltl embryos and larvae, ranging from stage 30 to stage 52, were used (Gallien and Durocher, 1957). At appropriate times, embryos and larvae were anesthetized in a 0.3% solution of tricaine methanesulfonate (MS222, Sandoz; pH 7.3) and, subsequently processed for NADPH-diaphorase histochemistry or NOS immunohistochemistry. Briefly, for the NADPHd method, free-floating sections were rinsed in fresh PB and incubated in a medium made up of 1 mM ß-NADPH (Sigma), 0.8 mM nitro blue tetrazolium (Sigma)

***Address correspondence to:** Agustín González. Departamento de Biología Celular, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain. e-mail: agustin@eucmax.sim.ucm.es

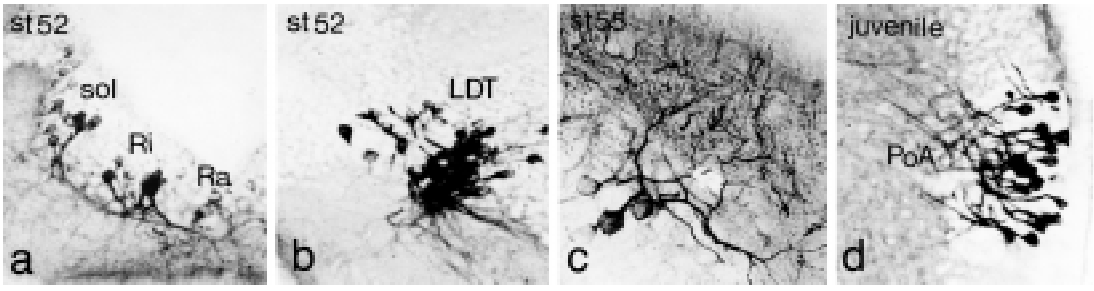

Fig. 1. Transverse sections through the developing brain of *P. waltl* illustrating NADPHd positive cells and fibers *in:* **(a)** *caudal rhombencephalic reticular formation (Ra: raphe, Ri: inferior reticular nucleus, sol: solitary tract);* **(b)** *laterodorsal tegmental nucleus (LDT);* **(c)** *dorsal field of the spinal cord;* **(d)** *anterior preoptic area (PoA).*

and 0.06% Triton X-100 in PB, at 37ºC for 1-2 hours. NOS immunohistochemistry was carried out with a sheep antiserum against neuronal NOS (K205 antibody, kindly donated by Dr. P.C. Emson), diluted 1:20,000 in PB containing 0.5% Triton X-100 (PB-T), for 48-60 hours at 4°C. Rhodamine-conjugated donkey anti-sheep secondary antiserum (Chemicon, Temecula, CA, USA) diluted 1:100 in PB-T was applied for 1.5 hours at 20ºC or fluorescein-conjugated rabbit anti-sheep secondary antibody (Vector, Burlingame, CA, USA) diluted 1:100 in PB-T was used.

Results

The localization of nitrergic structures in the developing brain of *Pleurodeles waltl* has been primarily studied by means of the NADPHd histochemical technique. However, the precise demonstration of NOS 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 (González *et al.,* 1996). Our results confirmed that the distribution of NOS-immunoreactive neuronal elements was coincident with that observed with NADPHd histochemistry. Not only was the distribution of labeled neurons and fibers the same with both techniques at any given stage, but also the relative staining intensities (weak-to-heavy) were always similar. In addition, the cases of double labeling in the same sections demonstrated a one-to-one correlation between NOS- and NADPHd-labeled neurons. However, it should be noted that a striking exception was found in the fibers of the terminal nerve and some olfactory glomeruli that were strongly labeled for NADPHd from embryonic stages up to the adult but lacked NOS immunoreactivity at all stages. Due to the simplicity of the histochemical technique to reveal NADPHd activity and the clear, almost Golgi-like images of cell profiles and fiber processes that it yields, the pictures presented were based on NADPHd-stained material (Fig. 1 a-d).

The first positive neurons were observed at embryonic stage 30. Strongly reactive cells were found in the ventrolateral area of the caudal rhombencephalon. Subsequently (stage 33b), weakly reactive cells appeared rostrally in the mesencephalic tegmentum, isthmic tegmentum and hypothalamus, and caudally in the rhombencephalon a new group of stained cells was observed in the proximity of the solitary tract. At initial larval stages (34-38), two new groups appeared and the number of cells that were detectable increased for each nitrergic cell group. In the caudal telencephalon a group of weakly positive cell bodies was found in the ventral portion belonging to the amigdaloid complex. At rostral rhombencephalic levels, a small group of weakly reactive cell bodies was found in the area of the medial reticular nucleus. During the active larval life (stages 39-55c) the nitrergic system develops significantly. At stages 39-42 reactive cells were found in the inner granular layer of the olfactory bulb, the telencephalic pallium, the pretectal region and the optic tectum. Finally, a population of nitrergic amacrine cells was observed in the retina. New populations of nitrergic cells appear during the second half of the larval period (stages 52-55). Rostrally, reactive cells were found in the telencephalic diagonal band nucleus and medial septum and in the thalamic eminence. Caudally, another change is noted in the rhombencephalon where two new populations appeared in the raphe and the descending trigeminal nucleus. The last changes occur during the juvenile period (metamorphic climax) when nitrergic activity in cells of the spinal cord and the preoptic area was first observed.

Conclusions

The early development of nitrergic system in the brain of *Pleurodeles waltl* suggests that nitric oxide play a significant role during the development. A developmental sequence of nitrergic activity reveals a first involvement of this system in reticulospinal control, likely influencing early locomotor behaviour.

The sequence of appearance of nitrergic cells reveals that NOS expression appears earlier in many areas of the brainstem than in the forebrain. This ontogenetic (and phylogenetic) character matches the postulation that pylogenetically “older” brain parts develops earlier that “newer” brain parts.

The observed development of NADPHd/NOS cells in *Pleurodeles* shares many charcteristics with amniotes suggesting a common pattern for tetrapods.

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