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Research report

Transient expression of NADPH-diaphorase/nitric oxide synthase in the paratenial nucleus of the rat thalamus

E. García-Ojeda, J.R. Alonso *, C. Crespo, E. Weruaga, J.G. Briñón, R. Arévalo, J. Aijón

Departamento de Biología Celular y Patología, Universidad de Salamanca, Avenida Campo Charro 1, 37007 Salamanca, Spain

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Abstract

The distribution pattern of nitric oxide synthesizing neurons was studied in the paratenial nucleus throughout the rat development using the NADPH-diaphorase (ND) histochemical method and nitric oxide synthase (NOS) immunocytochemistry. The onset of ND/NOS activity in the paratenial nucleus was detected in the postnatal life day 1. Until the postnatal stage 4, a quick increase in the number and staining intensity of the ND/NOS positive neurons was observed. From postnatal day 4 to postnatal day 6, these variations continued slowly, whereas an increase in the neuronal size was evident. In these stages, densely packed ND/NOS-labeled neurons were observed. From stages 6 to 10, the ND/NOS-positive elements demonstrated similar number, size, and staining intensity. These cells had medium size, variable morphology and showed reaction product in the cell bodies and, at most, their proximal dendrites. After postnatal day 10, a quick decrease in the staining intensity and in the number of ND/NOS-labeled elements was detected, although no changes were observed in their morphological characteristics. Postnatal day 15 was the last developmental stage studied in which ND/NOS-positive elements were observed. Finally, the paratenial nucleus did not present ND/NOS-positive elements in adult animals. This transient expression of the ND/NOS-activity suggests a role of nitric oxide in the reorganization of the paratenial nucleus during the first postnatal fortnight. © 1997 Elsevier Science B.V.

Keywords: Development; Nitric oxide; Ontogeny; Plasticity

1. Introduction

NADPH-diaphorase (ND) activity can be easily detected in fixed tissues using a histochemical reaction [2,22,30]. It has been reported that ND activity is produced by neuronal nitric oxide synthase (NOS) [20], and this reaction has been used to locate neurons producing nitric oxide (NO) throughout the brain (ND/NOS neurons) [6,8,23]. NO seems to be involved in particular processes during brain development [13,25] including the establishment of synapses [12,18,26], changes occurring in the last developmental stages, such as apoptosis and reorganization of cell populations [9], functional modulation of hypothalamic neurons [35], and in the maturation of motor neurons [21,38]. The diversity of suggested roles for NO during brain development is probably correlated to the variations in the distribution pattern of ND/NOS neurons during ontogeny.

The paratenial nucleus (PT) in the rat is a paired structure located in the rostral region of the thalamus. All midline thalamic nuclei, including PT, are easily identifiable in perinatal periods [4]. The nuclei of the midline thalamic region project to the ipsilateral forebrain, demonstrating a bilateral symmetry. PT receives afferents from the neocortex, and projects to the orbital cortex, nucleus accumbens, amygdala and hippocampus [5,34]. In the rat, the midline thalamic nuclei including the PT are involved

Abbreviations: AD, anterodorsal thalamic nucleus; AM, anteromedial thalamic nucleus; AT, anterior thalamus; AV, anteroventral thalamic nucleus; B, basal nucleus of Meynert; BST, bed nucleus of the stria terminalis; CM, centromedial thalamic nucleus; f, fornix; GP, globus pallidus; Hb, habenular nucleus; IAM, interanteromedial thalamic nucleus; ic, internal capsule; LD, laterodorsal thalamic nucleus; mt, mammillothalamic tract; ND, NADPH-diaphorase; NO, nitric oxide; NOS, nitric oxide synthase; P0-P30, postnatal day from 0 to 30; PC, paracentral thalamic nucleus; PT, paratenial thalamic nucleus; PV, paraventricular thalamic nucleus; Pva, paraventricular hypothalamic nucleus; Re, reuniens thalamic nucleus; Rh, rhomboid thalamic nucleus; Rt, reticular thalamic nucleus; sm, stria medullaris; st, stria terminalis; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VP, ventroposterior thalamic nucleus; ZI, zona incerta

* Corresponding author. Fax: +34 (23) 294549. E-mail: jralonso@gugu.usal.es

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in the cortico-thalamic limbic pathway, and seem to be related with learning, storage and memory mechanisms [4]. In the adult nervous system, NO has been related with these same functions [34]. In a general study throughout the adult rat brain, Vincent and Kimura [37] described a scarce number of ND-labeled elements in the thalamic region and, specifically, the absence of ND-positive fibers or neurons in the PT. However, in a preliminary study on the ontogeny of ND-activity in the brain, we observed ND-stained elements in the PT of perinatal animals, suggesting time-related changes in the ND-activity in this brain nucleus during the development.

The aim of this study is to analyze the onset of ND/NOS-stained elements in the rat PT during the development of the nervous system and to correlate these findings to morphological changes in this thalamic nucleus. The differential expression of this neuronal marker may help to understand its role in the nervous system both during the development and in the adult animal.

2. Materials and methods

2.1. Animals and tissue preparation

Fetuses, postnatal pups and adult Wistar rats were used in this study. The brains of adults, of fetuses from embryonic day 16 (E16) to 21 (E21), of pups from birth day (P0) to postnatal day 15 (P1–P15), postnatal days 20 (P20), 25 (P25) and 30 (P30) were analyzed. Six animals of each age were used for P0, P1, P5, P10, P15, P20 and P30, four animals were used for the remaining stages. Male and female adult rats were housed together for approximately 4 hr. The following 24 h after the detection of sperm were considered as embryonic day 0 (E0). The fetuses from E16–E19 were decapitated, their brains were dissected out and fixed for 20–24 h in a mixture containing 4% paraformaldehyde and 15% saturated picric acid in 0.1 M sodium phosphate buffer (PB). Fetuses from E20 and E21, and postnatal pups were deeply anesthetized with ether or ketamine and perfused through the ascending aorta with saline followed by the fixative described above. Thirty-μm coronal sections were cut using a cryostat.

2.2. NADPH-diaphorase histochemistry

The sections were processed for NADPH-diaphorase as described previously [2]. Briefly, brain sections from four animals for each development stage were incubated for 60–90 min at 37°C in a solution made up of 1 mM β-NADPH, 0.8 mM nitroblue tetrazolium, and 0.08% Triton X-100 in 0.1 M Tris-HCl buffer, pH 8.0. All reagents were purchased from Sigma. The course of the reaction was controlled by observation under the microscope. Controls for the specificity of the histochemical procedure included incubation without substrate (NADPH) or chromogen (nitroblue tetrazolium). In both cases, no reaction product was observed.

2.3. NOS-immunocytochemistry

In order to check the coincidence of ND-activity and NOS-immunoreactivity, two animals of the more critical ages: P0, P1, P5, P10, P15, P20 and P30, were processed as described above, and consecutive sections were stained for ND and NOS.

For NOS immunostaining, sections were successively incubated in (a) normal goat serum diluted 1 : 10 in PB for 30 min, (b) primary antibody (K205 sheep anti-rat neuronal NOS antibody [19]) diluted 1 : 20 000 in PB overnight, (c) biotinylated anti-sheep immuno-gammaglobulin (Vector Laboratories, Burlingame, USA) diluted 1 : 250 in PB for 90 min, and (d) avidin-peroxidase complex (Vector, Elite kit) diluted 1 : 500 in PB for 60 min. The reaction was revealed incubating the sections with 0.07% 3,3′-diaminobenzidine and 0.003% hydrogen peroxide in 0.1 M Tris-HCl buffer, pH 7.6. All steps were carried out at room temperature. The K205 neuronal NOS antibody has been fully characterized [11]. Specificity of the antibody was assessed using Western blot analysis and liquid phase pre-adsorption experiments with purified recombinant neuronal NOS [11].

Controls of the specificity for the immunocytochemical procedure were carried out as previously described [1]. No residual reaction was observed.

2.4. Quantification

For the measurement of cell sizes, in the perinatal animals only those neurons which exhibit, at least, the initial portion of one cellular process were used. In the remaining animals, only those neurons which presented two or more cellular processes were considered. In each age, 100 ND-positive neurons of two different animals were measured. The cells were plotted using a 40× planapochromatic objective connected through a digitizer tablet and optic pen to a semiautomatic image analysis system (MOP-Videoplan 2000, Kontron). The mean and S.E.M. were calculated using the corrected average for each group. The result were statistically analyzed using ANOVA. Values of $P < 0.01$ for Fisher PLSD and Scheffé F-tests jointly were considered statistically significant.

The labeling distribution was drawn using a Zeiss camera lucida and Canvas™ 3.0.6. software.

3. Results

3.1. General characteristics

The onset and evolution of the ND/NOS staining in the elements of the PT of the rat have been studied throughout

the development. In this study, the parcellation and nomenclature proposed by Paxinos et al. [29] for the developing brain, and Paxinos and Watson [28] for the adult brain were followed. The onset and evolution of ND/NOS-labeling was identical in both hemispheres. Although the distribution patterns of ND and NOS in the rat PT were generally similar throughout the development, some differences between both stainings were observed. Employing the ND-technique, in addition to the ND-stained neuronal population, blood vessels were labeled. This vascular staining can be probably explained by the ND activity shown by endothelial NOS, and the endothelial cells were immunonegative against neuronal NOS. According to the staining characteristics of ND-positive neurons, two

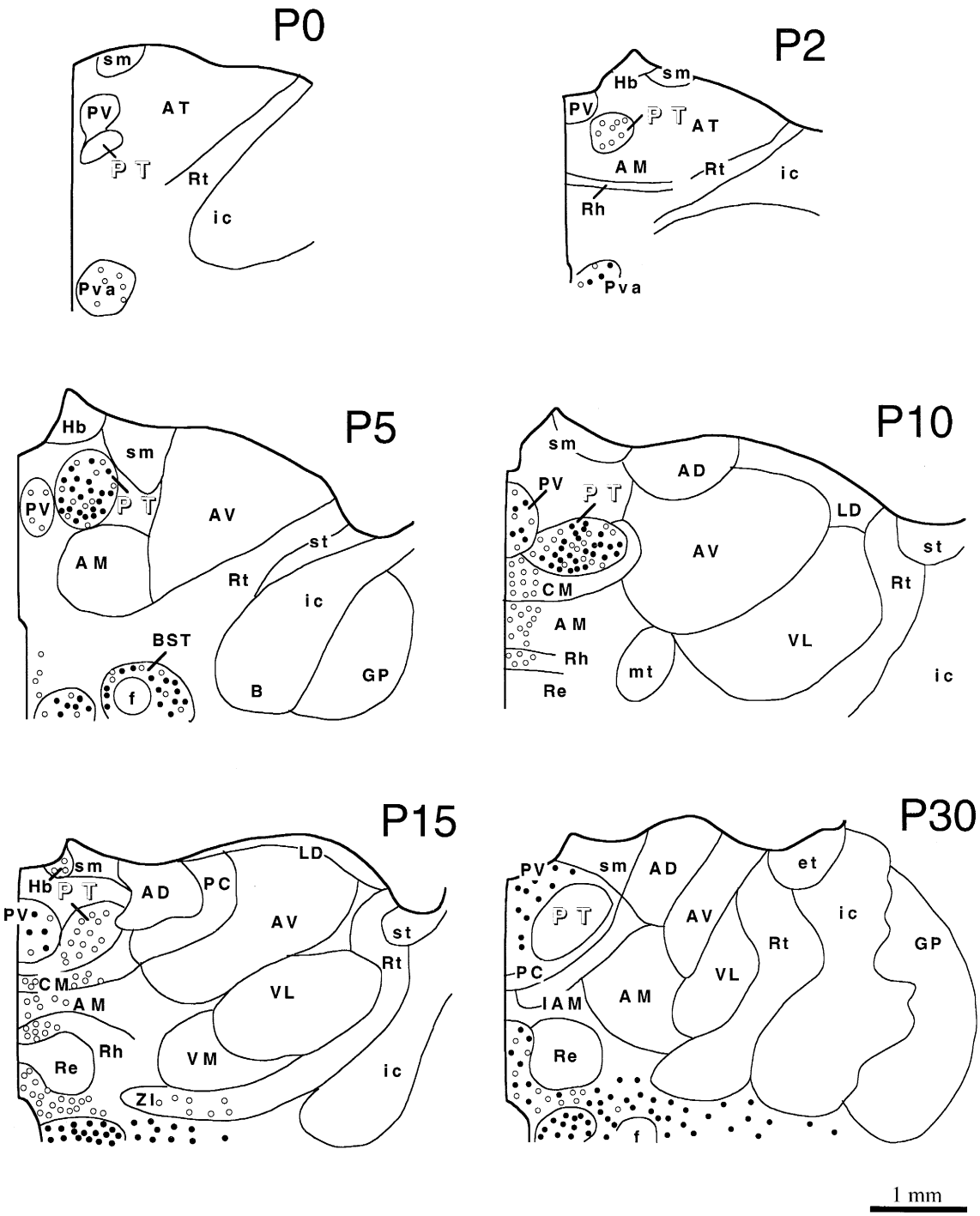

Fig. 1. Schematic representation of coronal sections of the rat thalamus showing the distribution of ND staining in the paratenial nucleus at different developmental stages. Note that in the birthday (P0) and in later postnatal stages (P30), the enzymatic activity is absent in the paratenial nucleus. Black dots represent strongly stained cells. White dots correspond to weakly stained cells.

groups of ND-labeled elements were distinguishable: neurons with a strong staining intensity in their cytoplasm that allowed to observe long portions of the dendritic tree and the initial segment of the axon, and cells with a weak staining pattern restricted to a thin line of cytoplasm surrounding the negative nucleus. It was not possible to associate any particular morphology or location to these staining phenotypes, i.e. strongly and weakly labeled neurons demonstrated similar sizes and shapes with a homogeneous distribution throughout the PT. These differences in the staining intensity were not clearly detectable using NOS-immunochemistry. Another technical difference between both stainings was that ND-stained elements demonstrated normally a more complete labeling of positive elements, including frequently portions of the dendritic tree and lengthy axons that were not observed when NOS-immunocytochemistry was used. However, as an exception, in P1, the day of the onset of ND/NOS-expression in the PT, a neuropil labeling not detectable with ND-histochemistry until a later stage was found in the NOS-immunostained sections (Fig. 4a,d).

3.2. ND / NOS staining patterns

By contrast to other thalamic structures, such as the magnocellular nucleus of the anterior commissure, the paraventricular nucleus and the periventricular fiber system, where ND-positive elements were found in prenatal stages, the ND-activity in the PT was non-existent during this period (Fig. 1).

ND/NOS-activity was detected for the first time in the PT in postnatal day 1 (P1). Using ND histochemistry, a few small neurons with round or oval cell bodies and a weak labeling restricted to the perikarya (Fig. 3a and Fig. 4a) were found, whereas with the NOS-immunochemistry in addition to these labeled neurons a weakly stained neuropil could be seen (Fig. 4b). In the following period (P2–P4), a rapid increase in the number and staining intensity of ND-positive elements in the PT was observed (Fig. 1). Similar variations occurred in the other nuclei of the rat thalamus. In P3, most ND-stained cells exhibited a weak staining intensity, although some ND-positive cells with a stronger intensity, not seen in previous stages, were evident (Fig. 3b). By contrast, only a slight increase in the cell size of the ND-labeled populations was observed between P1–P3. This increase was not statistically significant (Table 1). Weak neuropil ND-staining was also detected in the PT at P3. It was not observed in other thalamic nuclei at this stage. ND/NOS-stained neuropil in the PT remained present throughout the same period when somal labeling was found, and its intensity varied according to the intensity of the ND/NOS-positive intrinsic cells.

The main variation of the ND-staining pattern in the PT at P4 was an increase in the number and labeling intensity of ND-positive cells (Fig. 2a); however, their morphological characteristics remained the same as in P3. In addition, a differential pattern of ND-positive neurons was detected in P4. In the dorso-medial portion of the PT more abundant, densely packed elements with a strong staining were observed whereas in the ventro-lateral region (Fig. 2a) they were more sparsely distributed and showed a moderate or weakly labeling. In the following postnatal stages, this pattern became more evident. After P4, a rapid increase in the size of ND-positive neurons of the PT was observed (Table 1). This dramatic variation was not similarly detected in other thalamic nuclei, where the increase in cell size occurred more slowly.

Table 1
Area (mean ± S.E.M.) (μm^2) of ND-positive neurons in the rat PT at different postnatal development stages

Stage	Area
P1	62.84 ± 1.31
P3	69.92 ± 1.63
P5 [a]	135.53 ± 2.93
P7 [a]	144.97 ± 2.62
P9 [a]	161.89 ± 3.31
P11 [a]	198.19 ± 3.33
P13 [a]	221.46 ± 4.28
P15	210.36 ± 4.01

[a] Values statistically significant in relation to that of the previous measured stage ($P < 0.01$ for Fisher PLSD and Scheffé F-tests jointly).

In the postnatal day P5, the cell size increase continued and this difference with a previous stage (P3) was statistically significant (Table 1). ND- and NOS-labelings presented similar distribution patterns (Fig. 4c,d). In P5, ND/NOS-labeled cells in the rat PT had variable morphological characteristics, including bipolar and multipolar somata. The ND/NOS-staining pattern described for the PT contrasted with those of the other midline thalamic nuclei, such as the centromedial, interanteromedial and rhomboid nuclei, where the ND/NOS-expression began in stage P4, and in P5 only a weakly stained neuronal population formed by small, rounded cells was observed. These ND/NOS positive cells did not change until the adult pattern.

From P6 to P10, variable ND/NOS positive neuronal morphologies were identified since the proximal dendrites of the labeled elements could be clearly identified (Fig. 3c). The segregated distribution pattern of ND/NOS positive neurons detected in P4 was still distinguishable in these stages (Figs. 1 and 3c). No specific grouping of ND-labeled cells was seen in the other thalamic nuclei, except for the paraventricular nucleus, where two ND/NOS-positive cell groups, one stained weakly situated rostrally and other more caudal with a stronger staining pattern were detected. From P6 to P10, no substantial variations were observed in the number, morphology or staining characteristics of the ND/NOS-positive elements in the PT. In contrast, the variation in size of the ND-labeled neurons among these stages was statistically significant (Table 1). In other areas of the thalamus, the evolu-

tion of the ND/NOS-stained elements was slower and continuous.

After the stage P10, a rapid decrease in the ND-activity of the PT and an increase in the size of its intrinsic ND-positive neurons were observed. The increase in cell size was statistically significant between P9 and P11 and between P11 and P13 (Table 1). However, the slight increase in size of the ND-stained cells from P13 to P15

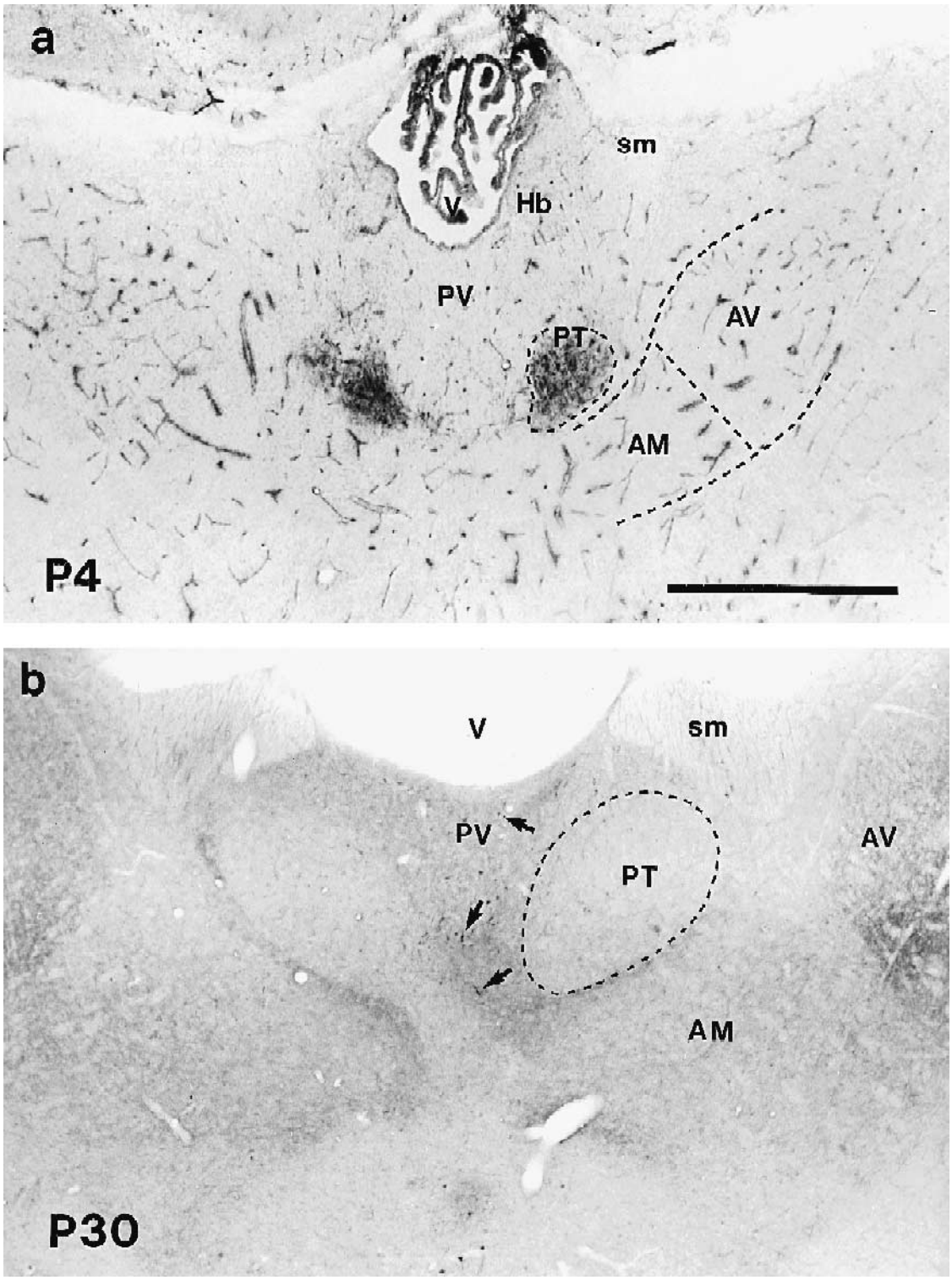

Fig. 2. Panoramic views of the paratenial nucleus after ND-staining at two postnatal stages. (a) P4, showing a strongly ND-stained neuronal population more densely packed in the medial portion. (b) Stage P30, when ND-staining has disappeared in the paratenial nucleus. Arrows indicate ND-positive cells in the thalamic paraventricular nucleus. Scale bar for both figures: 1 mm.

was not statistically significant (Table 1). Both the staining intensity and the number of ND-positive elements decreased at the same time in both hemispheres, as well as in the lateral and medial ND/NOS-positive neuronal groups. In the stage P12 (Fig. 3d), the ND/NOS-positive neuronal population of the PT was weakly stained and the number of labeled cells was reduced to approximately half of those observed in P10. P15 was the last stage studied in which reactive ND/NOS-labeled elements were observed in the PT (Fig. 1). In this stage, the cell population was reduced to a scarce group of weakly stained neurons (Figs. 1 and 4d,f). Although a loss in the intensity of their enzymatic labeling was clearly observable, ND/NOS-positive elements did not exhibit noticeable changes in their morphological differentiation from P11 to P15. This was more clearly observed in the ND-stained sections, where the morphology of positive elements could be better appreciated (Fig. 4d,f). Therefore, the size and shape of cell body, the number, length and branching pattern of the dendritic processes were similar to those observed in previous stages. The neuropil staining decreased simultaneously and, in P15, only a few weakly labeled fibers remained (Fig. 4d,f). A similar decrease in ND/NOS-activity was not found in any other thalamic region.

In the other postnatal stages studied, P20, P25, P30 and in adult animals, no ND/NOS-staining was observed ei-

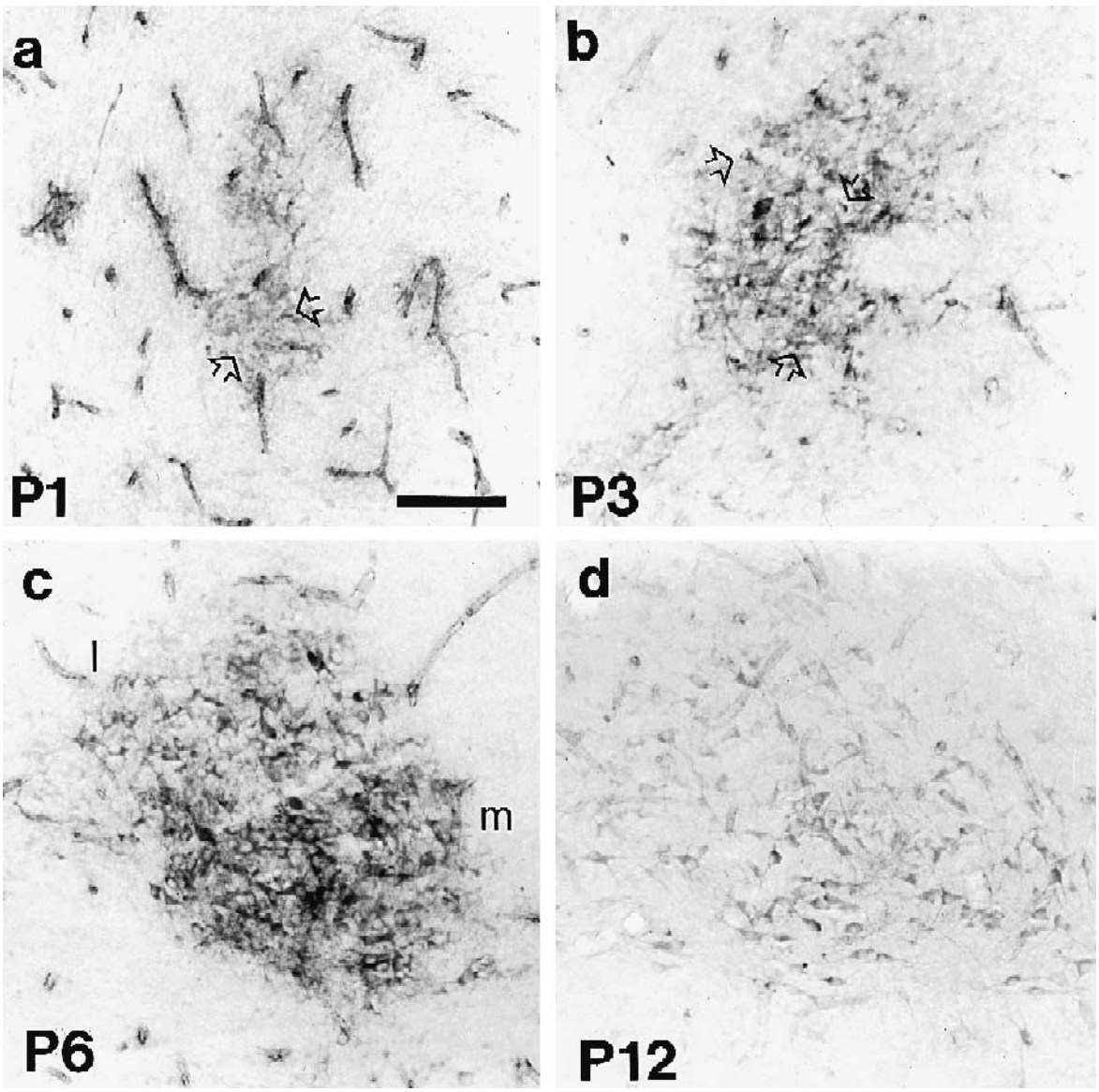

Fig. 3. Photomicrographs showing the evolution of the ND-expression in the paratenial nucleus throughout several postnatal stages. (a) P1, the day of the ND-activity onset. Open arrows point go ND-labeled neurons. (b) P3, ND-labeled neuropil and an increase in the number of neurons can be observed. Open arrows indicate ND-positive cells. (c) P6, note the strong ND-staining intensity and the two well differentiated groups of ND-neurons located in the medial (m) and lateral (l) portions. (d) P12, note the decrease in the staining intensity and number of ND-positive neurons. Scale bar for all figures: 100 μm.

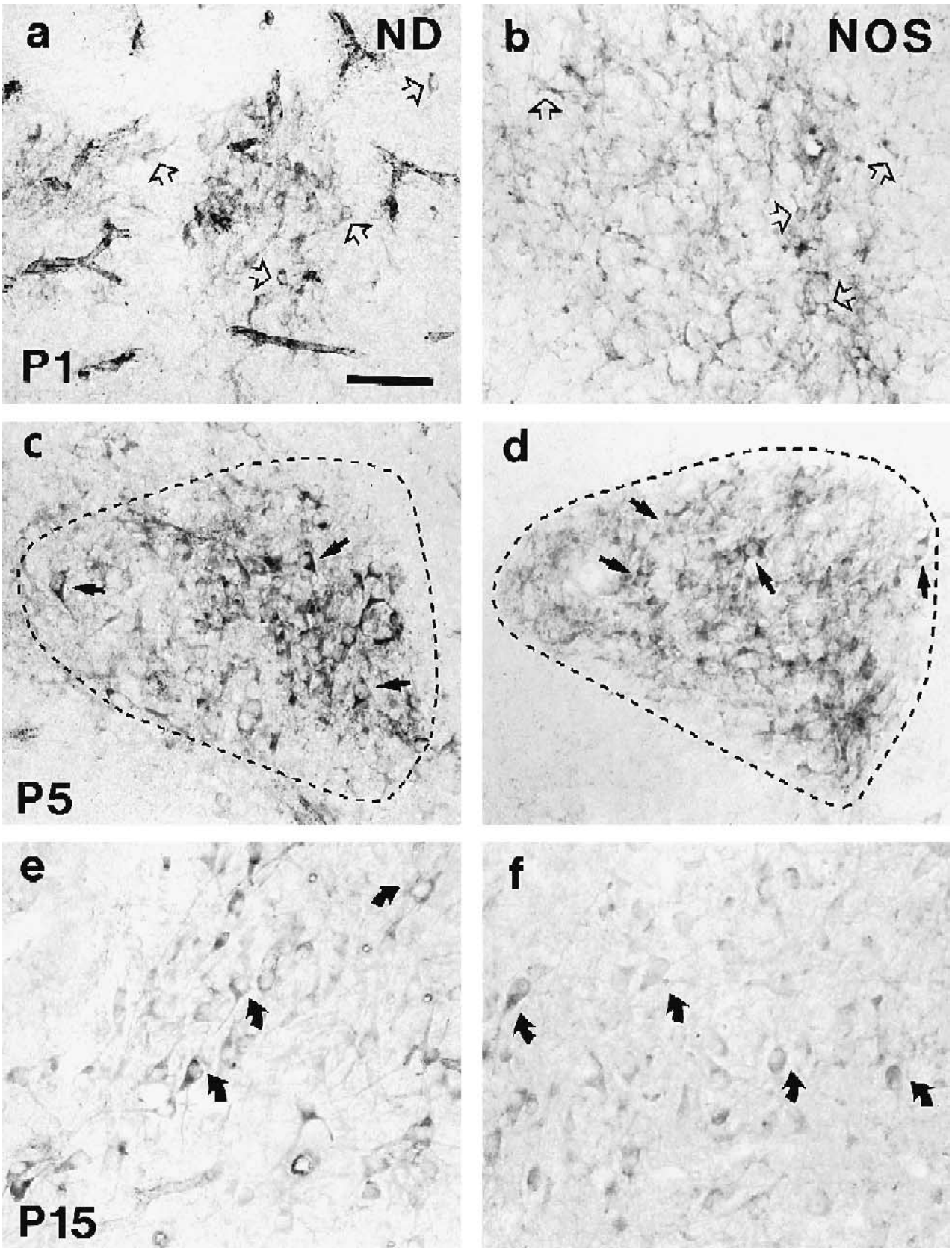

Fig. 4. ND- and NOS-labeled elements in consecutive sections of the paratenial nucleus at different postnatal stages. (a), (c), and (e) ND-histochemical reaction. (b), (d), and (f) NOS-immunohistochemistry. Note the similar evolution of the staining in both techniques. In P1, ND- and NOS-positive cells are weakly stained. In P5 both, ND- and NOS-labeled neurons, have increased in number, size and staining intensity. P15 was the last postnatal period in which ND-positive neurons were observed. Arrows indicate NOS- and ND-stained neurons. Scale bar for all figures: 50 μm.

ther in the neurons or in the neuropil of the PT (Figs. 1 and 2b). In contrast, neuropil ND/NOS-staining was observed in other thalamic nucleus from P10 onwards. This fiber staining increased in later stages and achieved the adult pattern after P30. In these stages (P20, P25, P30, and adult), the PT was clearly identifiable as two rounded clear structures located between the stronger background staining of the remaining thalamic nuclei (Fig. 2b).

4. Discussion

This study describes the existence of a postnatal transient expression of ND-activity in the PT of the rat thalamus. Our results in adult animals are in good agreement with previous studies on the distribution of ND-staining throughout the brain [37] demonstrating that there are no ND-positive elements in the adult PT. There are no previous detailed descriptions of the ontogeny of this enzymatic activity in the rat thalamus. In previous works, three ND-staining phenotypes are frequently established in the brain of adult animals: types I, II and III [2,27]. In the PT, by contrast, no neurons were observed with positive labeling in the whole or most of the dendritic tree, a characteristic of type I neurons. This is coincident with the transient ND-labeling in the neurons of the lateral geniculate nucleus of the ferret, where only types II and III ND-positive elements were detected [12]. Our interpretation is that no neurons contained sufficient ND enzyme to generate the massive deposit of formazan reaction product present in type I neurons. Since other morphological characteristics such as size and shape were not distinctive in our material, only strongly and weakly labeled neurons were differentiated.

Our data on the onset of the ND/NOS-labeling are in agreement with previous reports in other brain areas indicating that the ND/NOS-stained cells were initially observed relatively late, after cell bodies ceased dividing and extended their processes [1,10,12,18,24,36]. Comparison with previous studies indicated that the ND/NOS-expression appeared in the PT later than in the cerebral cortex [9], spinal ganglia [9,19,21,38], or the tegmental nuclei [33], where it appeared in prenatal stages, but before than in other regions such as the lateral geniculate nucleus [12], the ventrogeniculate nucleus (P3) [17] or the retina (P3) [26]. Thus, ND/NOS-expression in rat brain shows different time sequences in different brain structures.

The most outstanding characteristic of the ND/NOS-staining in the PT was the complete disappearance of the labeling in the nucleus after two weeks of postnatal life. By contrast, in the rat central nervous system, an increase of ND-activity in the adult is found in most CNS areas [9,10,17,26]. In specific areas such as the cortex, cerebellum or the hippocampus a partial loss of ND-activity in the rat brain during development has been described [9,10,40], whereas a complete loss of ND/NOS-activity during development has not been previously reported. However, there is evidence in the rat peripheral nervous system (olfactory epithelium [9] and spinal ganglia [9,38]) and in other species (some motor ganglia of the human [18], and in the lateral geniculate nucleus of the ferret [12]) of a complete disappearance of ND-staining. In addition, Purkinje cells in the cerebellum exhibit formazan deposits during development but not in adult animals [10]. In the rat visual cortex, ND/NOS-positive neurons in layer I are only visible around P10 and not in adult animals but the labeling persisted in other layers [24], and a transient expression of ND activity was also observed in the deep layers of the rat superior colliculus [17]. Some differences were noted between our observations in the rat PT and these other reports reporting transient expression: the loss of ND/NOS-activity in the PT is complete and not restricted to particular layers (superior colliculus [17], visual cortex [24]) or particular neuronal types (Purkinje cells [10]), whereas in the lateral geniculate nucleus of the ferret [12] and in the motor ganglia of the human [18] only the somal labeling was lost and the neuropil remained stained in the adult animal. Our hypothesis is that the ND/NOS-stained fibers observed in the PT belong exclusively to the ND/NOS-positive cells, since both the neuropil and the somal staining follow a similar evolution. In the lateral geniculate nucleus [12] and in the motor ganglia [18], the persistent ND-stained fibers correspond to ND-positive afferent connections from other brain areas. Finally, in contrast to other brain regions such as the cortex [24], where some ND/NOS-active neurons exhibited symptoms of degeneration such as shrunken cell bodies and corkscrew or twisted dendrites, the loss of ND/NOS-activity in the PT was not associated with any degenerating morphology.

Although data on the ontogeny of ND/NOS-activity are relatively scarce, it is interesting to compare our observations on the midline nuclei of the thalamus and those previously reported in the cerebral cortex since the PT projects to and receives afferents from it, being involved in the thalamocortical and corticothalamic circuits [5,34]. In the cerebral cortical plate, most ND/NOS cells stain at E15–E19, with thin processes extending through the corpus striatum to the thalamus [9]. This ND/NOS-staining pattern decreases after birth and completely disappears by the 15th postnatal day [9], a phenomenon comparable to our observations in the PT.

The involvement of the ND/NOS-positive system in the brain development has been demonstrated. Inhibition of nitric oxide synthesis produced changes in the developing brain, reducing the loss of transient retinotectal connections [39]. The neurogeny of the PT cellular population takes place in prenatal stages, whereas in the neonatal stages the neurons reorganize [3]. Thus, the onset of the ND/NOS-expression in the PT appeared after its neurogenesis is complete and was coincident with the neuronal reorganization, suggesting a possible role of this enzymatic activity in this process. This hypothesis may be supported

by some recent observations in other parts of the brain. Thus, a role for NO, NO-synthesizing cells or ND-positive systems has been proposed on the establishment of the columnar organization of the cortex [9], in the production of neurotrophic factors to guide the synapses [17,26], in the modulation of the endocrine function of hypothalamic magnocellular neurons [35], in the molecular maturation of motor neurons [18,21], and in the refining of synaptic connections [12].

A latero-medial neurogenetic gradient has been detected during PT neurogenesis [3]. That is, the neurons located in the medial portion of this nucleus are formed before those located in the lateral one. However, the beginning of ND/NOS-activity occurred simultaneously in the medial and lateral PT portions. This observation confirmed that the onset of the ND/NOS-expression in the PT was independent of the neurogenetic process, but may be related with its cellular reorganization. According to our observations, ND/NOS-expression decreased after the beginning of a slight dispersion of ND/NOS-positive neurons located in the medial portion of the rat PT. On the other hand, the fact that we did not observe any degeneration or cell retraction in the latter stages in which ND/NOS-positive elements were visible in the PT, suggests that the loss of ND/NOS-activity is not due to cell death, i.e., since no degeneration signs were observed in these neurons, we assume that these cells survive to the mature state but they change their chemical phenotype.

NO synthesis has an absolute requirement for calcium and NO release by ND/NOS-positive neurons is mediated by Ca^{2+}-sensitive *N*-methyl-D-aspartate gated channels [7,16]. Calcium-buffering is mediated by calcium-binding proteins and some of them appear to be involved in brain development [14,35]. Calbindin D-28k and calretinin are two calcium-binding proteins that control intracellular calcium levels which are expressed by specific neuronal populations. In addition, both calbindin D-28k and calretinin show different degrees of colocalization with ND in several diencephalic nuclei [1]. Comparing our results to previous reports on the expression of calbindin D-28k throughout prenatal [31] and postnatal [15] thalamic development, the distribution of calbindin D-28k during PT ontogeny was different to that observed for ND/NOS. Thus, the onset of the calbindin D-28k-expression [15] occurred before (between E16 and E18) that of the ND/NOS-expression, and calbindin D-28k-immunopositive elements could be observed in the adult PT [14,15,31]. Furthermore, whereas the ND/NOS-labeled neurons did not present a specific location in the rat PT, calbindin D-28k-immunopositive cells were strictly located in the ventromedial portion of the PT [31]. The results obtained for calretinin throughout thalamus ontogeny [32] were also similar to those described for calbindin D-28k and therefore, different to our observations for ND/NOS. Selvaggio et al. [32] showed that calretinin-immunoreactivity appears in the PT in prenatal stages and it remains in this nucleus during the adult life. Thus, at the moment, the distribution pattern of ND/NOS-activity in the PT is different to those reported for calcium-binding proteins expressed in PT neurons, both during the ontogeny and in adult animals.

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