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Developmental regulation of brain nitric oxide synthase expression in the ferret thalamic reticular nucleus

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Abstract

We have found that cells in the ferret thalamic reticular nucleus (TRN) express brain nitric oxide synthase (bNOS) in a transient pattern during early postnatal development. Similar to our previous findings in the lateral geniculate nucleus (LGN), bNOS expression in the TRN is first observed at postnatal day 7 (P7) and continues to P35. Quantitative measures show a significant change in the relative numbers of bNOS + cells from P7–P35, and suggest there is a transition in morphology from a bipolar shape with two primary dendrites, to a more complex, multipolar arrangement. During TRN development, the pattern of bNOS expression shifts from the somatodendritic localization seen during the first postnatal month to expression within axon fibers in the adult. Expression of bNOS within TRN cells demonstrates an additional source of nitric oxide in the developing visual thalamus, perhaps indicating a common function for thalamic nitergic neurons as cellular mediators in the establishment of central topography both in the LGN and the TRN. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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The thalamic reticular nucleus consists of a flat sheet of cells that surrounds the rostral and lateral surface of the dorsal thalamus. Due to this position, corticothalamic and thalamocortical fibers must pass through the thalamic reticular nucleus (TRN) to reach their respective targets. These fibers pass through specific regions of the TRN and emit collaterals to create topographic maps within modality-specific sectors of the nucleus [3,7,15]. The TRN sends reciprocal projections to its associated nuclei in the dorsal thalamus [7,11].

The relationship between the TRN and the lateral geniculate nucleus (LGN) has been thoroughly described in the adult ferret, where these reciprocally connected nuclei generate rhythmic waves associated with sleep [21] and create an inhibitory feedback loop that may modulate visual information flow during wakefulness [19]. The relationship between the TRN and the LGN during early development is less well known, though studies have examined the early anatomical connections between the two nuclei [6,12,13]. In the ferret, TRN axons reach the LGN as early as embryonic day 23 (E23) [12], when thalamocortical axon outgrowth first begins (E23–24) [6]. The TRN axons are likely the first to arrive in the LGN since retinal and cortical axons do not reach the LGN until E27 [6]. It is unknown when corticothalamic collaterals contact TRN cells and when refinement of these connections may occur.

Though the function of the TRN during early development is unclear, it has been suggested that the early TRN fibers serve to guide initial axon pathfinding into and out of the thalamus [12]. The TRN, along with the cortical subplate, is thought to be a critical site for the axon reorganization necessary to create topographic maps that are in register between the thalamus and the cortex [1,14], although the specific mechanism is unclear. Previous studies indicate that nitric oxide (NO) influences growing retinal axons [18], and can affect segregation and refinement in the LGN [4]. However, little attention has been focused on the possible contributions of NO to corticothalamic and geniculocortical pathway development, specifically in the TRN. Here, we examined the expression of brain nitric

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oxide synthase (bNOS) in the early postnatal and adult TRN.

Ferret kits (*mustela putorus furo*) postnatal day zero (P0) ($n = 2$), P7 ($n = 3$), P11–14 ($n = 4$), P21 ($n = 4$), P28 ($n = 3$), P35 ($n = 3$), P42–43 ($n = 3$) and adult ($n = 2$) were used in this study, with 503 cells in the analysis. All experiments conformed to the guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Wake Forest University School of Medicine Animal Care and Use Committee. Ferrets were given an overdose of sodium pentobarbital then perfused with an aldehyde solution (4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffered saline [PBS]). Following fixation, brains were immersed in a cryoprotectant solution (20% glycerol, 2% DMSO in 0.1 M PBS), cut at 60 μm in the sagittal plane on a freezing microtome and collected in either 0.1 M PBS or a cryoprotectant solution (40% 0.5 M phosphate buffer, 30% ethylene glycol, 30% glycerol).

The brain, or neuronal, isoforms of NOS was investigated using a mouse monoclonal antibody (Sigma, St. Louis, MO) in every fourth section using a standard immunohistochemical (IHC) protocol. Briefly, sections were pretreated (4% MeOH, 0.3% H_2O_2 in 0.1 M PBS), rinsed, blocked (2% normal horse serum, 1% Triton-X in 0.1 M PBS), and incubated in primary antibody (1/1000 dilution with 2% normal horse serum, 1% Triton-X in 0.1 M PBS) overnight. Sections were then rinsed, incubated in secondary antibody (30 min), rinsed, and incubated in an avidin-biotin complex (ABC) solution (30 min, Vectorstain ABC kit, Vector Laboratories, Burlingame, CA). Brain NOS label was visualized using a nickel-enhanced DAB (3,3′-diaminobenzidine, Sigma) reaction product.

The specificity of the primary antibody in ferret tissue was verified with a western blot assay and omission of the primary antibody. In addition to the monoclonal antibody, we also verified bNOS expression in the developing TRN using NADPH-d histochemistry. This revealed a qualitatively similar pattern of expression; therefore, analysis was limited to bNOS immunoreactivity.

The pattern of bNOS expression was examined in the region of the TRN adjacent to the LGN in sagittal section. Brain NOS expression in the TRN was examined for changes in the number, distribution and morphology of expressing cells across the first five postnatal weeks. All values are reported as mean number of cells per section ± standard error. The following number of sections were examined at each age: P7 ($n = 11$), P11–14 ($n = 6$), P21 ($n = 16$), P28 ($n = 17$), P35 ($n = 8$). Cutting at 60 μm yielded the following mean number of sections containing the LGN and TRN per animal: P7–14 ($n = 20$), P21–35 ($n = 40$). Changes in bNOS expression during development were evaluated using the Kruskal–Wallis analysis of ranks and the Mann–Whitney *U*-test. Changes in the morphology of bNOS + cells at specific developmental ages were evaluated with the Mann–Whitney *U*-test while changes in morphology across all developmental ages were examined using a chi-squared analysis.

At P0 there was no bNOS staining in the TRN. However, somatodendritic bNOS expression was observed in the striatum and cortex (not shown) at this age, demonstrating that

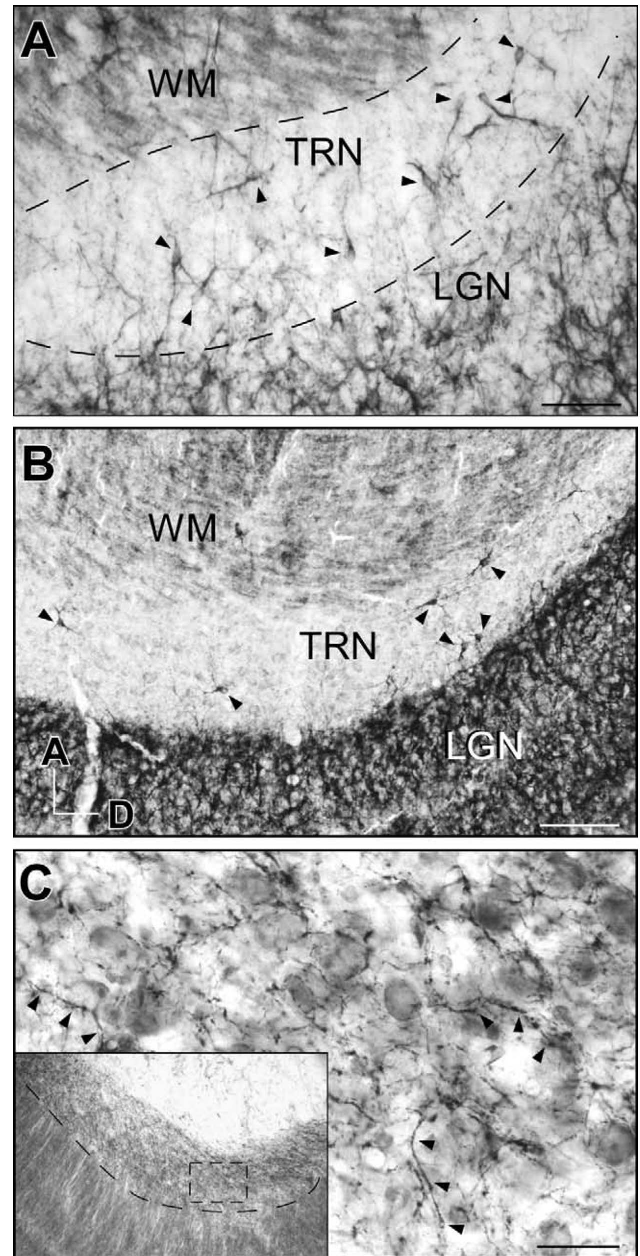

Fig. 1. Two modes of bNOS expression in the TRN. (A) Illustrates bNOS expression in the TRN at P7; and (B) illustrates bNOS expression at P21. At both ages bNOS + cells are distributed throughout the nucleus. Arrowheads indicate labeled cells in the TRN. (C) The inset shows a low magnification view of the adult TRN, and the dashed box indicates the region shown in the high magnification view. In the adult, bNOS expression shifts to fine-caliber fibers, indicated by arrowheads. These fibers are shown intermingling with somata counterstained with neutral red. WM, white matter. Scale bars: (A) 50 μm; (B) 100 μm; and (C) 30 μm.

the IHC reaction was working. Brain NOS expression in the TRN was first observed at P7 (Fig. 1A). The intensity of DAB label in stained cells was similar to that in the adjacent LGN. At this age and throughout the first postnatal month, bNOS immunoreactivity was localized to the somata and dendrites of a subpopulation of cells in the TRN and the bNOS + cells were distributed homogeneously throughout the extent of the TRN (Fig. 1A). The subpopulation of TRN cells expressed bNOS through the first five postnatal weeks. Fig. 1B illustrates the somatodendritic bNOS expression seen at P21. In the adult, the pattern of bNOS expression was distinct from that seen during early postnatal development, such that beginning at P42 bNOS expression was no longer observed within the somata and dendrites of TRN cells. Instead, bNOS expression was observed in fine-caliber fibers throughout the neuropil (Fig. 1C). The transition in the pattern of expression seemed to occur shortly after eye opening (P32). At P35, when cellular expression was decreasing, many long, thin fibers were apparent, typical of axon fibers. This early network of fiber staining, in addition to cellular label at P35, suggests overlap in somatic and fiber expression at this age.

During the period of somatodendritic bNOS expression in the TRN, two morphologically distinct groups of bNOS+ cells were observed (Fig. 2). Cells with a bipolar morphology were the predominant phenotype from P7–P14, and were observed at all ages throughout the first five postnatal weeks (Fig. 2A). Bipolar cells exhibited no consistent orientation in the TRN along the anterior-posterior or the dorsal-ventral axis. From P21–P35, the majority of bNOS+ cells had a multipolar morphology, with dendrites that exhibited an elaborate pattern of branching that was not restricted to a specific plane, and occasionally formed a radial pattern around the soma (Fig. 2B).

Using quantitative measures, we examined the developing TRN for changes in the number and morphology of bNOS+ cells. We found a significant decrease in the mean number of bNOS+ cells per section between the first and fifth postnatal weeks (Fig. 3). Examination of bNOS+ cell morphology revealed a shift in the relative numbers of cells exhibiting bipolar and multipolar morphologies. From P7 to P35 there was a decrease in the number of bipolar cells that expressed bNOS, while there was an increase in the number of multipolar cells that expressed bNOS (Fig. 3).

Our results indicate that cellular bNOS expression in the ferret TRN is a developmentally regulated event, much like that observed in the adjacent LGN [5,10]. In both structures, cellular expression is absent at P0, first observed at P7, continues to P35, and is absent in the adult. The similar pattern of bNOS expression in the developing LGN and the TRN suggest that NO in the developing visual thalamus may be important in certain developmental processes, including establishing topographically precise connections and axon refinement.

While the time course of bNOS expression in the TRN is

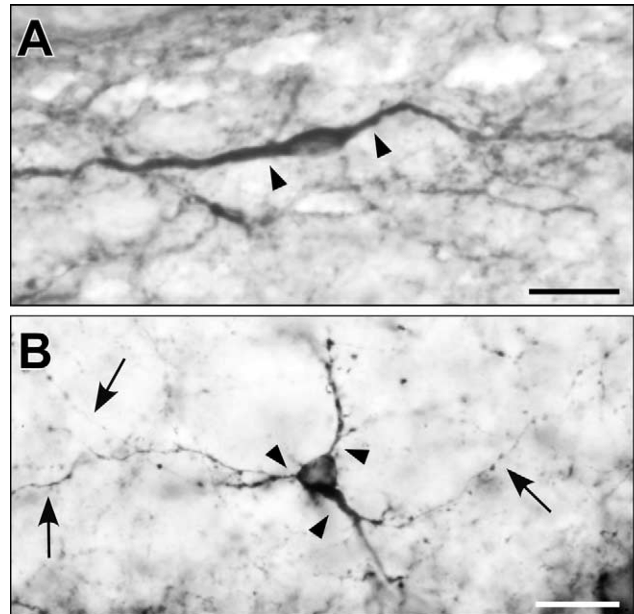

Fig. 2. Morphology of bNOS + cells in the developing visual TRN. A bNOS immunoreactive cell that exhibits a bipolar morphology at P21. These neurons have an elongated, fusiform soma with two thick primary dendrites extending from each pole (arrowheads indicate primary dendrites). Brain NOS + bipolar cells were the predominant morphological type from P7 to P14. (B) A multipolar bNOS + cell at P21. The multipolar cells had three or more thin dendrites emerging from the soma (labeled with arrowheads). The bNOS + label could be seen throughout the soma and dendritic tree, running into more distal regions of the dendrites (arrows). Thickening and swellings along the dendritic tree can also be observed. Scale bars: (A) 40 μm; and (B) 30 μm.

similar to that seen in the LGN, the peak of somatodendritic bNOS expression is different between the two nuclei. In the LGN, bNOS expression peaks around the fourth postnatal week and then decreases toward P35 (unpublished observations, [6]). TRN expression is greatest at P7, with a gradual decline through P35. This difference may reflect differences in the afferent inputs to each nucleus and intrinsic differences between cells arising from the ventral versus the dorsal thalamus. Additionally, the density of bNOS + cells within the TRN is less than that observed in the adjacent LGN. However, the even distribution of bNOS + cells and the extent of TRN covered by bNOS + dendrites suggest that bNOS, and the resultant NO, could influence the entire nucleus.

Our data suggest that there are two morphological phenotypes expressing bNOS that intermingle within the visual sector of the developing ferret TRN. While the morphology of neurons in the adult rat, cat, and rabbit TRN has been documented [9,20], there is little data regarding the morphology of neurons in the developing ferret TRN. Since the ratio of bipolar to multipolar cells decreases over time, one possibility is that the bNOS + bipolar cells, dominant from P7–P14, are an earlier developmental precursor to the multipolar cells. Our data cannot distin-

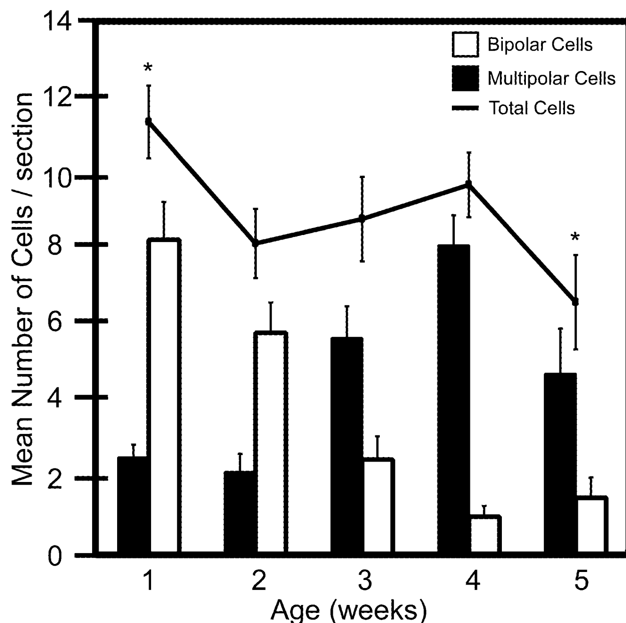

Fig. 3. Analysis of bNOS + cells in the developing TRN. The mean number of bNOS + cells in the TRN was calculated at each age between the first and fifth postnatal weeks and then separated by morphological class. At P7–P14, more cells had a bipolar morphology ($n = 119, P < 0.0002$) while from P21 to P35 round, multipolar cells were the dominant morphological type observed ($n = 131, P < 0.005$). From P7 to P35, chi-squared analysis confirmed that there was a significant change in the distribution of bipolar compared to multipolar cells ($P < 0.001$). Analysis of the total number of bNOS + cells at each age, regardless of morphology, indicated that there was an overall decrease between P7 and P35, indicated by the asterisk (P7 = 11.36 ± 0.95; P35 = 6.62 ± 1.2; $P < 0.001$).

guish this from the possibility that these types are two functionally different cell classes, since the cells we have observed are unlikely to have differentiated into mature morphological phenotypes. However, clear examples of each group could be found at later developmental stages (Figs. 2 and 3).

Brain NOS expression during nervous system development has been correlated with axonal segregation and refinement events in the ferret LGN, where NOS and the resultant NO have been suggested to participate in guiding retinogeniculate axon segregation into ON/OFF sublayers [5]. While the process of axon refinement in the TRN has not been well studied, NO may play a role in the refinement of connectivity patterns in the TRN similar to the proposed role of NO in the developing LGN.

In the adult ferret TRN, bNOS expression is localized to fibers, likely from the cholinergic, parabrachial region of the brainstem [2]. NO may act with acetylcholine to reduce oscillatory firing, as previous work has shown NO to minimize bursting [16,17]. In addition, NO may also modulate glutamatergic neurotransmission in this region of the visual pathway. Supporting this, Kurukulasuriya et al. [8] have shown that NO acts through the *n*-methyl-D-aspartate (NMDA) receptor, attenuating input at the retinogeniculate synapse but enhancing corticogeniculate input. While the functional role of NO in the developing and adult TRN is unresolved, it is clear that there are two anatomically distinct modes of bNOS expression, which may correspond to the unique demands of a developing system compared to that of adult circuitry.

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