

Research report

# The neuronal form of nitric oxide synthase is required for pattern formation by retinal afferents in the ferret lateral geniculate nucleus

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## Abstract

The ferret retinogeniculate projection undergoes activity-dependent refinement of connections that become restricted to eye specific laminae and On/Off sublaminae in the lateral geniculate nucleus (LGN). We have previously shown that the developmental process by which On/Off sublaminae form requires *N*-methyl-D-aspartate (NMDA) receptors and nitric oxide (NO). In this study, we investigate the role of the neuronal form of NO synthase (nNOS) in sublaminae refinement. This isoform of NOS may be coupled with NMDA receptors at postsynaptic sites. We found that nNOS is present in the developing LGN, and that blocking nNOS during development disrupts the formation of On/Off sublaminae. Endothelial NOS (eNOS) is not expressed in the LGN until after sublaminae have formed. These results suggest that the nNOS isoform is the predominant contributor of NO during development, and support the hypothesis that NO acts downstream of NMDA receptor activation to mediate activity-dependent changes in the patterning of connections in the LGN. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Activity-dependent; On-center; Off-center; Retinal ganglion cell; Development; Visual system

## 1. Introduction

During nervous system development, neuronal activity plays a role in refining axonal projection patterns and synaptic specificity. The molecular mechanisms that sense changes in neuronal activity and lead to changes in synaptic structure have been examined in the developing visual system [10,14,23,46]. One potential biochemical pathway involves postsynaptic *N*-methyl-D-aspartate (NMDA) receptors [10,23,43]. Presynaptic axon arbors are altered in the visual pathway when target activity is altered by antagonists to NMDA receptors [23]. A retrograde message is thus required to report changes in postsynaptic activity to presynaptic axon terminals. The diffusible gas, nitric oxide (NO), has been proposed as a retrograde messenger that acts downstream of the NMDA receptor on presynaptic terminals during development [6,20,32] and during long-term potentiation (LTP) in the hippocampus [2,4,35,40,45].

NO has a developmental role in axon outgrowth, dendritic branching, and refinement of connections. In cultures of mammalian dorsal root ganglia [24] and frog retinal ganglion cells [39], addition of NO promotes growth cone collapse. NO also appears to be required for the refinement of connections in retinal projections. In the chick, an ipsilateral retinotectal projection that is normally eliminated is retained when NOS is blocked in situ [49]. NO is thus involved in several aspects of neuronal development, and its role in regulating axon outgrowth and retraction are consistent with a postulated role for activity-dependent refinement of connections.

We have previously used the ferret retinogeniculate projection as a model system in which to examine mechanisms of activity-dependent refinement. In the ferret, projections from the retina to the lateral geniculate nucleus (LGN) are initially diffuse, and by one postnatal week become organized into eye-specific layers. During postnatal weeks 3 and 4, these eye-specific layers are further subdivided into On/Off sublaminae [29]. The inner sublaminae receive inputs from On-center retinal ganglion cells, while the outer sublaminae receive inputs from Off-center retinal ganglion cells [47,50]. The formation of both eye-specific layers [37,41,46] and On/Off sublayers [13]

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requires neuronal activity. On/Off sublamination also requires NMDA receptor activation [23]. NOS activity, revealed using NADPH-diaphorase histochemistry, shows that NO is produced in the ferret LGN during the formation of On/Off sublaminae [12]. Blockade of NOS using *N*<sup>ω</sup>-nitro-L-arginine (L-NoArg) during postnatal weeks 3 and 4 disrupts On/Off sublamination [11]. Neither NMDA receptor blockade [44] nor NOS blockade [11] interferes with the formation of eye-specific layers. In the retinogeniculate pathway, NO appears to be involved only when NMDA receptor activation is also required, consistent with a role for NO downstream of NMDA receptors.

An important issue in this system is whether NMDA receptor activation is linked to NO release, and if so, what is the biochemical basis for this link. Ca<sup>2+</sup> influx specifically through NMDA receptor activation has been shown to cause NO release [5,19,22,27]. There are several different forms of NOS, encoded by different genes [7,16]. Two constitutive forms, neuronal NOS (nNOS) and endothelial NOS (eNOS), are abundant in the brain and are regulated by calcium and calmodulin binding. NOS converts arginine to citrulline and reduces NADPH. Activation of NOS downstream of NMDA receptors seems to selectively involve nNOS, the most abundant form in neurons. Electron microscopic immunohistochemistry suggests that NMDA receptors and nNOS are for the most part colocalized in the cortex [1]. Moreover, the post-synaptic density proteins PSD-93 and PSD-95 form associations with NMDA receptor subunits, and seem to direct calcium from NMDA receptor channels to nNOS [8,9].

These reports suggest that nNOS may have an important role in linking NMDA receptor activation to production of NO. The role of the neuronal isoform of NOS has not been examined during development; our previous studies did not examine the role of NO produced by specific isoforms of NOS. Histochemistry using NADPH-diaphorase reveals NOS activity, but does not distinguish between isoforms of NOS because all forms reduce NADPH. In addition, L-NoArg inhibits NOS nonselectively. In this study, we have used specific anti-nNOS and anti-eNOS antibodies to show that nNOS is the predominant form expressed in the LGN during On/Off segregation. Moreover, we have shown that specific inhibitors of nNOS disrupt On/Off sublamination. The effects of these inhibitors are comparable to the effects of NOS inhibition with L-NoArg, and thus NO produced by nNOS may account for most or all of the NO that is used for On/Off sublamination in the ferret LGN.

## 2. Materials and methods

### 2.1. Immunohistochemistry

Ferret kits were euthanized with > 100 mg kg<sup>-1</sup> sodium pentobarbital, i.p., and were perfused intracardially with

4% paraformaldehyde followed by 0.9% saline. Brains were removed and allowed to equilibrate in 30% sucrose in phosphate buffer, containing up to 0.5% paraformaldehyde. Brains were cut into 50 μm sections in the horizontal plane using a freezing microtome.

For immunohistochemical labeling, brain sections from different ages were run together. Floating sections were preincubated for 1 h in phosphate buffered saline (PBS) containing 10% normal goat serum (Vector laboratories, Burlingame, CA). Sections were transferred to a primary antibody solution containing anti-nNOS or anti-eNOS polyclonal antibodies (Transduction Labs; [8]), 1 to 5 μg ml<sup>-1</sup>, in PBS with 2% normal goat serum, 2.5% bovine serum albumin (BSA), and 1% Triton X-100, and incubated overnight with gentle agitation at room temperature. The primary antibody solution was rinsed with several changes in PBS, and the sections were incubated in a solution containing biotinylated goat anti-rabbit antibodies (Vector Laboratories) at a dilution of 1:200 in a solution containing 2% normal goat serum, 2.5% BSA, 1% Triton X-100 for 1 h at room temperature. The Vector ABC kit was used to label antigen-antibody complexes with HRP, and HRP was visualized using the Vector VIP substrate.

Sections processed for nNOS immunohistochemistry were analyzed for density and size of labeled somata in the LGN. Cell counts in LGN sections were used to estimate the density of labeled cells in the LGN at various ages. Stained sections were divided into six zones in an area 250 μm by 250 μm (0.0625 mm<sup>2</sup>); zones included layers A, A1, and dorsal regions of C layers, and were selected similarly for sections of different ages. Cell densities in all zones within a section were averaged and counted as a single datum. Cell body size for P21 animals was estimated by measuring the diameter of the cell body for all labeled cells within a representative zone. To account for the fact that cell bodies are irregular in shape, the diameter of a cell body was taken as the average of the longest axis and the axis perpendicular to the longest axis.

### 2.2. Blockade of nNOS

Ferret kits ages P14 to P26 were given daily injections of 7-Nitroindazole (7-NI), an inhibitor of nNOS [34], at a dose of 40 mg kg<sup>-1</sup> day<sup>-1</sup> in oil or the monosodium salt, 7-NINA [42], 20 mg kg<sup>-1</sup> day<sup>-1</sup> in distilled water. Animals received 0.1 ml intraperitoneally once each day during the treatment period. The health of animals was monitored; drug treatment did not have a detrimental effect on weight or overall appearance. The effects of 7-NI, 7-NINA and L-NoArg on NOS activity have been compared in brain tissue. In rat and mouse cerebellum, it has been shown that 7-NI and its monosodium salt, 7-NINA, inhibit NOS with similar IC<sub>50</sub> (about 0.5 μM) [42]. This IC<sub>50</sub> is similar to that of arginine analogs [33] and the doses we used were consistent with high levels of inhibition of nNOS. High doses were used in order to compare effects

of nNOS blockade to published effects [11] of NOS blockade using a nonspecific inhibitor.

### 2.3. Labeling retinogeniculate projections

On P24, animals were anesthetized with 30–50 mg kg<sup>-1</sup> intramuscular ketamine. The left eyelid was gently opened with a size 11 sterile scalpel blade, and the eye was superfused with Proparacaine to provide local anesthesia. A small hole was made in the eye through the sclera using a 26 gauge hypodermic needle. The needle of a Hamilton syringe was inserted into the eye through this hole, and after the tip of the needle was brought into view through the pupil, 10 µl horseradish peroxidase coupled to wheat germ agglutinin (WGA-HRP) was slowly injected into the aqueous humor. The eye was treated with ophthalmic antibiotic ointment. Animals recovered from anesthesia and were subsequently returned to their cages.

In some animals, direct arterial blood pressure measurements were made on P26. Animals were anesthetized with midazolam 0.5 mg kg<sup>-1</sup> i.m. followed by 50 mg kg<sup>-1</sup> ketamine i.m. The left carotid artery was exposed and cannulated with a 24 gauge catheter attached by tubing filled with sterile saline to a Gould P23 pressure transducer. Systolic, diastolic and mean blood pressure measurements were displayed on a Gould SP1405 monitor, and were recorded at intervals of 30 s to 2 min for up to 20 min. These animals received an overdose (> 100 mg kg<sup>-1</sup>) of sodium pentobarbital before recovering from anesthesia. All animals in this part of the study were perfused on P26 as described above with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed and allowed to equilibrate in 30% sucrose in phosphate buffer, containing up to 0.5% paraformaldehyde and cut into 50 µm horizontal sections. Sections were processed to detect HRP using the tetramethylbenzidine (TMB) method [30].

Sections were evaluated for On/Off sublaminations using a scoring system described previously [11,13]. Briefly, a “blind” observer rated stained sections from drug treated or control brains on a scale of zero to three, according to the extent of the stained A layer (on the right LGN) that was subdivided by a pale staining region. A score of zero indicates no sublamination, while a score of 3 indicates that sublamination is evident throughout the A layer. Scores for all sections from a brain were averaged to obtain a single score from each animal.

## 3. Results

### 3.1. Immunohistochemistry

Immunohistochemical analysis of nNOS expression revealed that nNOS is expressed transiently in LGN cells. At P14, some cell bodies were stained in horizontal sections

of LGN (Fig. 1). These cells were found in all layers of the LGN; some neuropil-like staining was seen throughout the LGN, and there was some staining in the proximal portions of cell dendrites in labeled cells. Labeling was evident in cell bodies in other regions within the same sections, suggesting that the immunohistochemistry procedure was

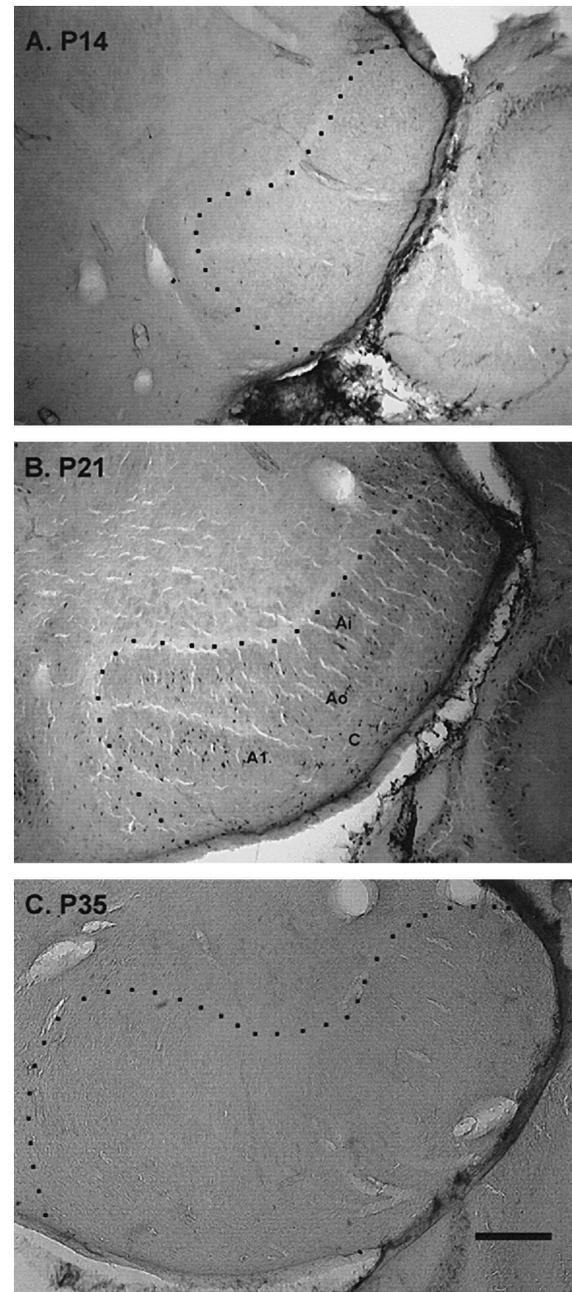


Fig. 1. Horizontal sections through the LGN at different ages, stained immunohistochemically to reveal nNOS expression. (A) At P14, a few labeled cells are visible in the LGN, outlined with dotted line. (B) At P21, nNOS staining is the most abundant. The eye-specific A (contralateral, A1 (ipsilateral), and C layers are visible, as well as the inner (Ai), or On-sublayer and the outer (Ao), or Off-sublayer. (C) By P35, no cell bodies are stained positively for nNOS. Lateral is to the right in all figures. Scale bar, 250 µm.

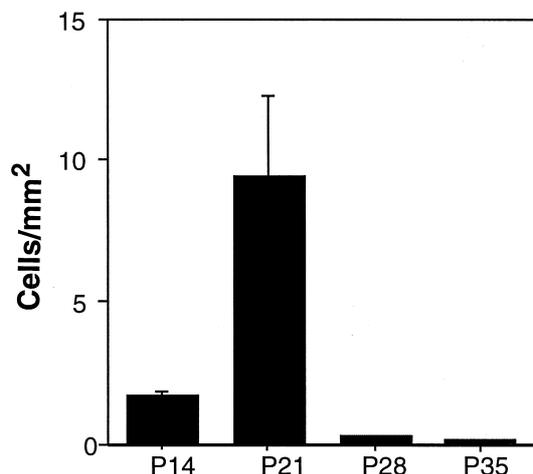


Fig. 2. Histogram showing the density of cell bodies labeled with the nNOS antibody at all the ages examined. The most dense staining is seen at P21, and staining diminishes by P28. The staining at P21 is similar to the density of labeled cells using NADPH-diaphorase histochemistry.

appropriate for this tissue. The most abundant nNOS expression was seen in sections from P21 ferrets. At this age, cell bodies in all LGN layers were stained with the antibody (Fig. 1). By P28, few, if any cell bodies were labeled in the LGN. Some neuropil labeling remained at this age. At P35, no somata were labeled in any part of the LGN (Fig. 1).

The density of labeled cells was assessed in tissue processed for immunohistochemistry. Labeled cells were counted in zones within LGN sections. At P14, the mean density of cells labeled with the nNOS antibody was  $17 \pm 2$  cells  $\text{mm}^{-2}$  (S.E.M.,  $n = 4$  zones from 2 animals). The density at P21 was  $94 \pm 28$  cells  $\text{mm}^{-2}$  ( $n = 4$  zones from 2 animals). At P28, nNOS labeled cells decreased to  $3 \pm 0$  cells  $\text{mm}^{-2}$  ( $n = 2$  zones from 1 animal). These data are summarized in Fig. 2. The changes in the density of nNOS labeled cells in the LGN are not accounted for by changes in overall cell density, as similar measurements of Nissl stained material (data not shown) indicate that there is little change in the overall cell density in the LGN over this period of development.

The developmental pattern of labeling using the nNOS specific antibody is similar to the pattern seen using NADPH-diaphorase histochemistry [12], which labels NOS [15,25]. Both methods show cell body labeling only during a few postnatal weeks, during the period when sublaminae are forming in the LGN. However, NADPH-diaphorase staining peaks at four postnatal weeks, while nNOS staining peaks at three postnatal weeks. NADPH-diaphorase labeling persists in cell bodies through the fifth postnatal week, while nNOS is largely absent from cell bodies by the fourth postnatal week. Moreover, NADPH-diaphorase labels a greater number of cells in the LGN at later ages, consistent with the view that nNOS labeled cells represent a subset of all cells that express NOS. Cortical labeling using the nNOS antibody was similar to that seen with

NADPH-diaphorase and did not seem to vary in density in the ages examined.

Cell body size of anti-nNOS labeled LGN cells was estimated by measuring diameters of labeled cells within

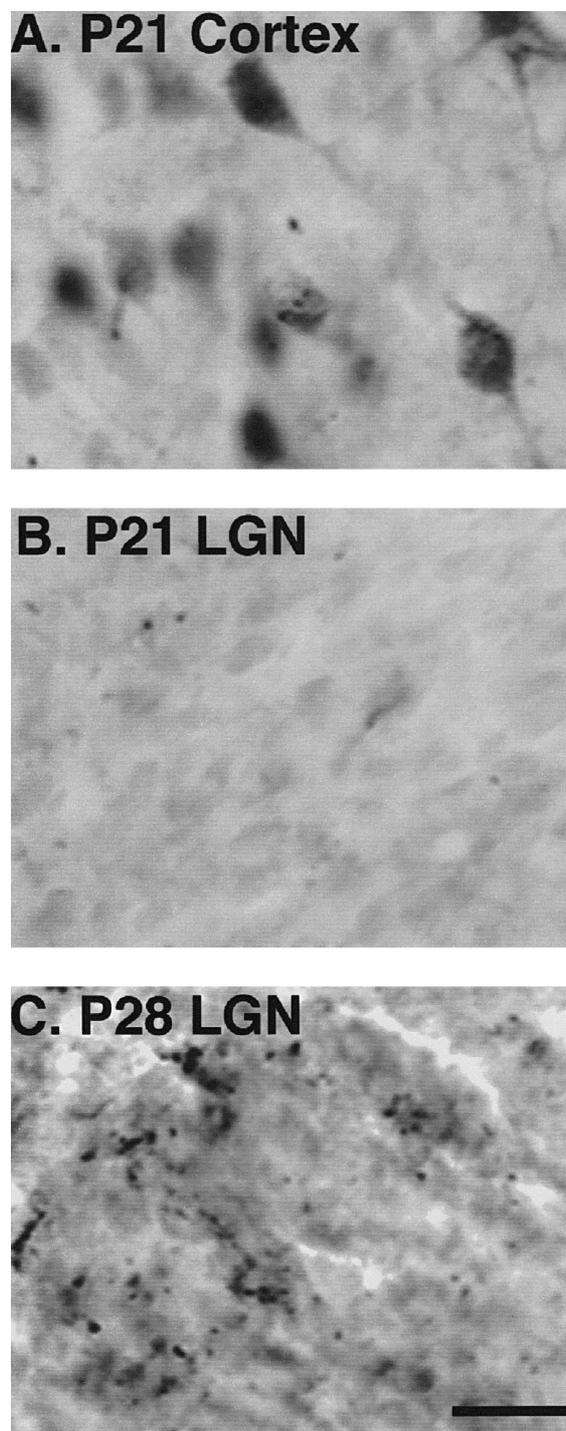


Fig. 3. Immunohistochemical staining for eNOS in horizontal sections. (A) Cortical staining at P21, showing the presence of eNOS in neuronal cell bodies at this age. (B) The same section as A, but in a region of the LGN. At this age, there is little if any cell body labeling in the LGN. (C) Section through the LGN at P28, showing that some cell bodies are labeled at this age. Scale bar, 25  $\mu\text{m}$ , applies to all panels.

sample zones at P21, when staining was most abundant. The mean diameter ( $\pm$ S.D.) was  $8.35 \pm 1.46 \mu\text{m}$ , and ranged from  $6 \mu\text{m}$  to  $12 \mu\text{m}$ , suggesting that NOS, like NADPH-diaphorase, is expressed in a variety of cell types within the LGN. The mean soma diameter for NADPH-diaphorase labeled cells was about  $9 \mu\text{m}$  [12], and ranged from  $6 \mu\text{m}$  to  $14 \mu\text{m}$ , which was similar to the range for Nissl stained cells. The similarity in size and range suggests that nNOS, like NADPH-diaphorase, stains a variety of cell types and is not restricted to relay neurons or inhibitory interneurons.

In contrast to results using the nNOS antibody, we found that eNOS labeled very few cell bodies in the LGN at P14 and P21; at these ages cortical areas had several cell bodies labeled. At P28, labeling was evident in some cells in the LGN. Results using eNOS immunohistochemistry are shown in Fig. 3. While these results are qualitative, they do point to an important difference in the develop-

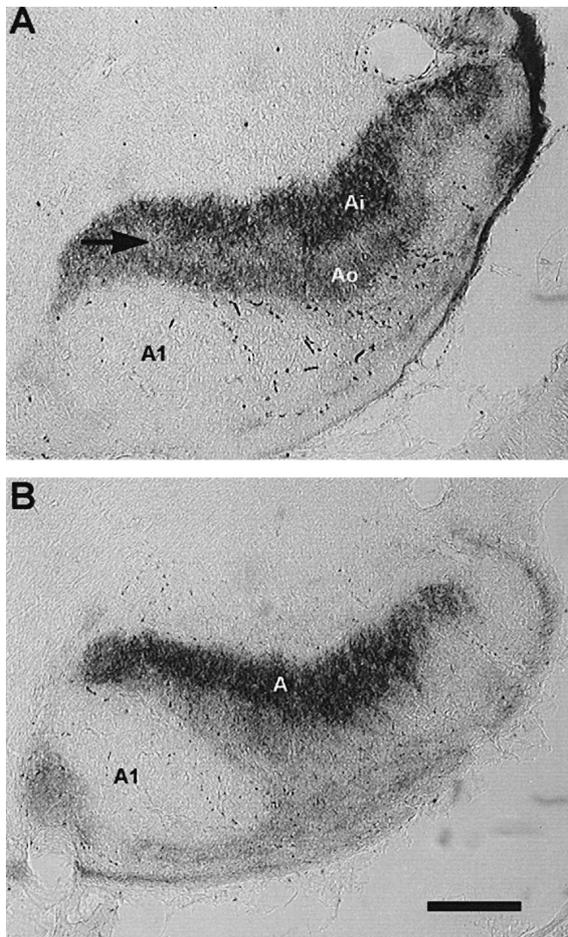


Fig. 4. Horizontal sections through the LGN of P26 animals following intraocular injection of WGA-HRP on P24. (A) Normal, untreated LGN. The eye-specific layers as well as the On/Off sublaminae (Ai and Ao) are evident. (B) LGN from an animal treated with a specific inhibitor of nNOS from P14 to P26. While the eye specific layers are visible, there is no pale staining intersublaminae region, suggesting that On- and Off-retinogeniculate axons are not segregated. Scale bar,  $250 \mu\text{m}$ .

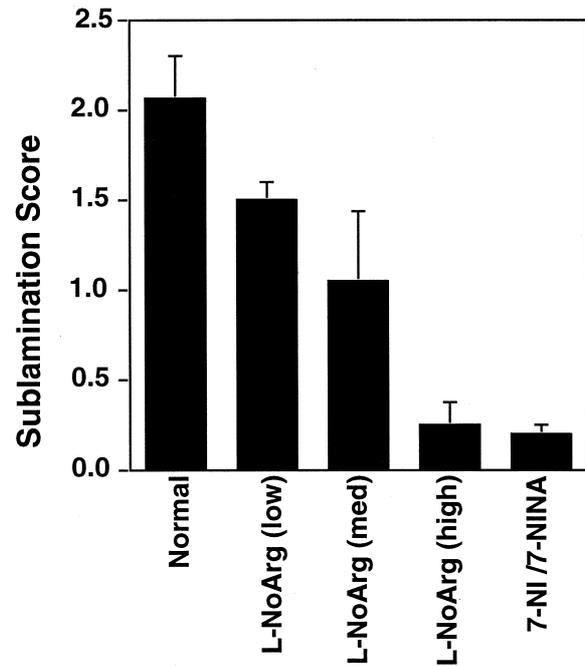


Fig. 5. Summary of sublimation scores from this study and from our previous study of NOS inhibition [11]. Sublimation scores are obtained by estimating the extent of the pale staining intersublaminae region in the labeled A layer, and are averaged across-sections so that each animal is treated as a single datum. Treatment with 7-NI or 7-NINA significantly reduces sublimation, and is comparable to the highest dose of the nonspecific NOS inhibitor, L-NoArg.

mental time course of eNOS vs. nNOS expression in LGN cells.

### 3.2. Blockade of nNOS

To assess the role of nNOS in the formation of sublaminae, we blocked the activity of this enzyme using 7-NI or 7-NINA [34,42]. Animals were treated with  $20$  or  $40 \text{ mg kg}^{-1} \text{ day}^{-1}$  i.p. The pattern of sublimation was assessed using intraocular injections of WGA-HRP. Treatment with nNOS inhibitors significantly reduced sublimation compared to normal control animals (Fig. 4). Treated animals had a discernible reduction in the pale staining intersublaminae zone. This pale region in normal animals correlates with reduced branching of retinogeniculate axons in the region between On and Off sublaminae [11,23].

The effects of nNOS blockade were quantified and compared to results from our previous studies. Sublimation was scored for each LGN section and sections were averaged to obtain a single score for each animal. The mean sublimation score for normal animals ( $\pm$ S.E.M.) was  $2.1 \pm 0.2$  ( $n = 6$  animals). The mean sublimation score animals treated with 7-NI or 7-NINA was  $0.19 \pm 0.05$  ( $n = 4$  animals). This score is significantly less than scores from normal animals ( $p < 0.001$ , Student's *t*-test), but is similar to scores obtained in our previous study using

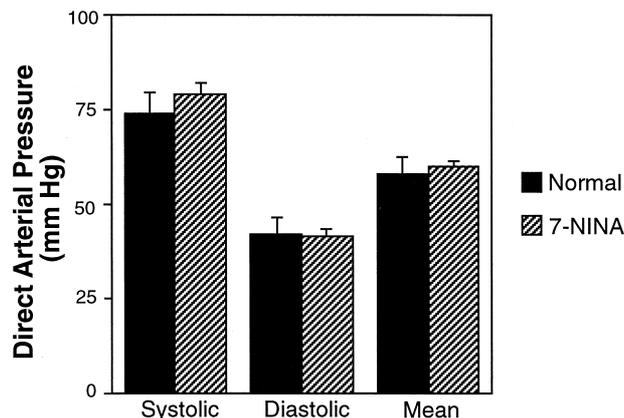


Fig. 6. Histogram summarizing blood pressure measurements on treated and normal animals in this study. Treatment from P14 to P26 with the specific nNOS inhibitor 7-NINA did not increase direct arterial blood pressure. These results support a specific effect of nNOS blockade on nNOS in the LGN, and rule out a secondary effect of systemic treatment.

systemic treatment with 4–40 mg kg<sup>-1</sup> L-NoArg ( $0.24 \pm 0.13$ ,  $n = 6$  animals,  $p = 0.76$ ). The sublamination scores, including L-NoArg data from Ref. [11], are shown in Fig. 5.

Although NOS blockade may cause substantial increases in blood pressure, nNOS blockade does not appear to have this hypertensive effect [34]. As a control, we measured blood pressure in 7-NINA treated animals to evaluate the possibility that the effect we observed on sublamination was secondary to systemic effects. We found that systemic blood pressure was normal in the treated animals (Fig. 6). The mean arterial blood pressure ( $\pm$  S.E.M.) in normal animals was  $57.6 \pm 4.6$  mmHg ( $n = 4$  animals). The mean arterial blood pressure in animals treated with 7-NINA was  $59.8 \pm 2.1$  mm Hg. These values are not significantly different ( $p > 0.7$ , Student's *t*-test). These data rule out the possibility that increases in blood pressure play a role in sublamination formation, and are consistent with results we obtained using verapamil (a pressure-reducing agent) together with a NOS blocker [11], in which blood pressure was normal during the treatment period, but sublamination was significantly disrupted. The specificity of 7-NI and 7-NINA for nNOS have been demonstrated in vitro and in vivo [3,33,34,42]; cf. Ref. [38]. Results from our blood pressure measurements support the specificity of 7-NINA for nNOS using in vivo treatment in the ferret.

#### 4. Discussion

The neuronal isoform of NOS is expressed transiently and is abundantly present in the LGN precisely during On/Off sublamina formation. Both nNOS and NADPH-diaphorase labeling are developmentally regulated in LGN

cells, appearing transiently during the first few postnatal weeks. However, nNOS expression is more sharply regulated than NADPH-diaphorase expression, disappearing from LGN cells by four postnatal weeks, while NADPH-diaphorase is most abundant at four weeks and persists until 5 weeks [12]. Based on our immunohistochemical findings, it is likely that the additional, late expression of NADPH-diaphorase represents eNOS expression. We found that eNOS is expressed in the LGN after sublamination is already complete, and thus may not contribute significantly to NO production during retinogeniculate segregation. At earlier ages, NADPH-diaphorase staining and nNOS immunohistochemistry label similar numbers of cells in the LGN. Thus, at P21, when sublamination is in progress, the density of nNOS labeled cells indicates that nNOS accounts for most of the NOS seen with NADPH-diaphorase histochemistry. Moreover, the effect of specific blockade of nNOS is similar to the effect of blocking all forms of NOS with L-NoArg, according to our assessment of sublamination. Thus, while other forms of NOS may be present at the early ages, the neuronal isoform of NOS provides most or all of the NO that is used in signaling during the formation of On/Off sublaminae.

While our results support a role for nNOS in retinogeniculate refinement in the ferret, eNOS may be involved in other developing projections. In the mouse, eNOS may have a role in retinocollicular refinement, in which large patches of ipsilateral retinal projections are normally removed [31]. In these studies, mice lacking the gene encoding nNOS had normal refinement of the retinocollicular projection, while double knockout mice lacking both the nNOS and eNOS genes showed a developmental delay in this refinement. These findings suggest nNOS is not required for retinocollicular refinement, and are consistent with a role for eNOS.

The role of NOS isoforms has also been examined in synaptic plasticity. The most prevalent form of NOS in neurons is nNOS [21]. An in vivo study in rats provided evidence that CA1 hippocampal LTP is dependent on nNOS, as treatment with 7-NI strongly inhibits field potential LTP [18]. However, eNOS appears to have a role in some forms of synaptic plasticity as well. Mutant mice that lack the nNOS gene still exhibit hippocampal LTP, and this LTP remains sensitive to blockade by inhibitors of NOS, suggesting that other forms of NOS may be more important contributors of NO for LTP, or that other forms may compensate for the function of nNOS in the mutant mice [36]. Interestingly, eNOS is the most abundant form of NOS in hippocampal pyramidal neurons [17], and may thus be an important source for NO in hippocampal LTP. This postulated role for eNOS in the hippocampus is supported by experiments in double knockout mice lacking both nNOS and eNOS. These mice are deficient in LTP, suggesting that NO produced by either isoform is sufficient to allow LTP; in these experiments eNOS mutants had a more pronounced deficit [45]. These results suggest

that both nNOS and eNOS contribute to LTP in the hippocampus — however, the link between eNOS and NMDA receptor activation is not understood.

The association of NMDA receptors and nNOS by postsynaptic density proteins provides a mechanism by which NMDA receptor activation leads to rapid production of NO in a restricted region within a neuronal projection. The association of nNOS with these membrane proteins seems to be mediated by amino acid sequences known as PDZ domains [8,9]. The interaction of nNOS with PSD95 and PSD93 may be regulated by other proteins [26]. The organization of proteins in the postsynaptic membrane appears to have a complex regulation, which may involve levels of palmitoylation in PDZ-containing proteins [48]. Interestingly, NO has been shown to inhibit palmitoylation of some proteins during axon outgrowth [24]; however, it is not known whether NO regulates palmitoylation of PSD95.

The results of our study are consistent with an association of nNOS and NMDA receptors in the developing ferret retinogeniculate projection. The nNOS isoform accounts for the NADPH-diaphorase staining during the period of development in which retinogeniculate On/Off sublamination occurs, and selective blockade of this isoform prevents the formation of these sublaminae, similar to blockade of NMDA receptors. The present study provides support for the hypothesis that nNOS activation is downstream of NMDA receptor activation in a signaling pathway that refines the retinogeniculate projection. Several interesting questions remain about this pathway. The expression of postsynaptic density proteins and PDZ containing proteins in the developing ferret LGN may provide important insights into the NMDA receptor/NOS link in this system. Additionally, the identification of molecular interactions downstream of NO release [28] will lead to an understanding of how neuronal activity during development ultimately leads to specific changes in synaptic structure and function.

The results presented here also suggest that eNOS is expressed in the LGN. This additional source of NOS is expressed later and persists later in development than nNOS, and is not required for the development of On/Off sublaminae. The function of NO produced at this later stage is unknown; it may involve regulation of vascular development, development of non-neuronal components of the nucleus, or further refinements of synaptic connectivity within the LGN. Further study of these processes will help elucidate the role of NO in central nervous system development.

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