Transient Expression of NADPH-Diaphorase in the Lateral Geniculate Nucleus of the Ferret During Early Postnatal Development

KARINA S. CRAMER, CHRISTOPHER I. MOORE, AND MRIGANKA SUR
Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

ABSTRACT

Retinogeniculate projections in the ferret are refined during postnatal development so that inputs from the two eyes become segregated into eye-specific laminae, and each eye-specific lamina is further divided into sublaminae containing inputs from on-center or off-center afferents. Segregation into eye-specific laminae and on/off sublaminae is dependent on neuronal activity; sublamination depends on activation of N-methyl-d-aspartate (NMDA) receptors. By analogy with the suggested role of nitric oxide in NMDA-mediated long-term potentiation in the hippocampus, we investigated a possible role for nitric oxide in ferret retinogeniculate development. The expression of NADPH-diaphorase, a nitric oxide synthase, was examined histologically in the lateral geniculate nucleus of ferrets at several postnatal ages. At birth, neuropil is labeled in the nucleus, although no cell bodies are visible. After the first postnatal week, some labeled cells appear, predominantly in the C laminae. By three postnatal weeks, cell bodies are clearly labeled in all geniculate laminae. Staining reaches a peak in density at about four postnatal weeks, then declines such that by six postnatal weeks labeled cells are no longer visible. This transient expression of NADPH-diaphorase activity is consistent with a role for nitric oxide in the development of mature connections within the ferret lateral geniculate nucleus.

Indexing terms: nitric oxide, thalamus, retinogeniculate connections, activity

In the ferret, much of the development of the lateral geniculate nucleus (LGN) and its connections to other areas occurs postnatally. Over the first two postnatal weeks, retinogeniculate axons segregate into eye-specific laminae (Linden et al., 1981; Hahm and Sur, 1988); these eye-specific laminae further subdivide into inner and outer sublaminae containing either on-center or off-center cells by the fourth postnatal week (LeVay and McConnell, 1982; Stryker and Zaks, 1983; Hahm and Sur, 1988). In addition, corticothalamic projections become refined during this period of development, as do projections from the superior colliculus and the brainstem (Johnson and Casagrande, 1993).

The development of on- and off-sublaminae requires the activation of the N-methyl-d-aspartate (NMDA) class of glutamate receptors. Blockade of these receptors using the selective antagonist d-2-amino-5-phosphonovalerate (APV) results in eye-specific laminae in which terminals of on- and off-center retinal ganglion cells do not segregate into inner and outer sublaminae (Hahm et al., 1991). NMDA receptors are also involved in synaptic plasticity in the adult brain; for example, their activation is required for induction of long-term potentiation (LTP) in the hippocampus (Bliss and Collingridge, 1993). In addition, NMDA receptors mediate enhancement of synaptic transmission in developing LGN cells following stimulation of retinal afferents in vitro (Mooney et al., 1993). While NMDA receptors are involved in both synaptic plasticity in the hippocampus and development of connections in the visual pathway, further similarity between the mechanisms in the two different systems remains to be elucidated. In the hippocampus, the induction of LTP occurs in the postsynaptic cell while the maintenance of LTP may involve enhanced transmitter release from the presynaptic terminal (see Bliss and Collingridge, 1993 for review; cf. Manabe et al., 1992). Hence, a

Accepted August 5, 1994.
Address reprint requests to Karina S. Cramer, Department of Brain and Cognitive Sciences, E25-618, Massachusetts Institute of Technology, Cambridge, MA 02139.

© 1995 WILEY-LISS, INC.
Fig. 1. Horizontal section from a PO ferret brain stained for NADPH-diaphorase. The background staining is light and cell bodies are not evident. In contrast to the lateral geniculate nucleus (LGN) (diagonally oriented structure at center), the medial thalamus (upper left) shows some cells stained for NADPH-diaphorase. a, anterior; m, medial. Scale bar = 200 μm.

Retrograde neuronal messenger such as nitric oxide (NO) has been implicated as playing a crucial role in the consolidation of LTP (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991; Bon et al., 1992). Other candidate molecules have also received experimental support, such as carbon monoxide (Zhao et al., 1993), arachidonic acid metabolites (Kato et al., 1991), and platelet activating factor (Kato et al., 1994). Thus, an interesting question is whether NO has a role in development analogous to its suggested role in synaptic plasticity. If NO is involved in segregation of on/off sublaminae, it must be present in the LGN during the appropriate period of development and its production might be developmentally modulated.

NO is synthesized by NO synthase from arginine in a calcium/calmodulin and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction (for review, see Garthwaite, 1991; Vincent and Hope, 1992; Bredt and Snyder, 1992). The biochemical activity of NO synthase is developmentally regulated in rat brain (Matsunoto et al., 1993). During the first postnatal week, cerebral homogenates have higher enzymatic activity than cerebellar homogenates; in adults this relationship is reversed. In the present study, we have examined the expression of NO synthase during development in the ferret LGN using a histochemical assay for NADPH diaphorase (NADPH-d). NADPH-d and NO synthase copurify (Hope et al., 1991) and cells labeled with NADPH-d colocalize with those...
stained with NO synthase immunohistochemistry (Dawson et al., 1991). In addition, kidney cells transfected with cDNA encoding NO synthase are stained by NADPH-d (Dawson et al., 1991). Our results show that NADPH-d is developmentally regulated in the ferret thalamus and labeled cells appear in the LGN between the second and fifth postnatal weeks, with a peak in expression at about four postnatal weeks; staining is substantially diminished after six postnatal weeks. These results are consistent with the hypothesis that NO is involved in postnatal development of connections in the ferret LGN.

MATERIALS AND METHODS

Ferret kits ranging in age from postnatal day 0 (P0) to 8 postnatal weeks were included in this study. Kits were deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer. Brains were extracted and immersed in 30% sucrose in phosphate buffer, which contained up to 2% paraformaldehyde. Brains were sectioned in the sagittal or horizontal plane at 50 μm. Sections were washed in 0.1 M phosphate buffer and incubated in staining solution (0.3% Triton X-100, 0.06% NADPH, and 0.69% nitro blue tetrazolium, in 0.1 M Tris buffer, pH 7.4) at 37°C for 45 to 60 minutes. In some cases sections from different ages were processed together. Sections were rinsed three times in phosphate buffer for at least 30 minutes, dehydrated in a graded series of alcohol, cleared, and mounted onto slides.

Tissue was analyzed using brightfield microscopy. Cell density measurements were obtained from at least six random square (0.0625 square mm) regions in each of 2 or 3 LGN sections through the center of the nucleus. The average sampled area was 1.4 square mm, and included the A and C laminae. Cell density was also measured in Nissl-stained sections from a P27 ferret. Cells were counted if nuclei were visible. The sampled area included two strips of 0.06 mm × 0.30 mm (0.018 square mm) through the center of the LGN, and included both A and C laminae; samples were taken from 3 sections. Soma diameter measurements were taken from camera lucida drawings of labeled cells at three, four, and five postnatal weeks (the ages at which NADPH-d staining was most pronounced). The diameter was taken as the average of the longest axis and the axis perpendicular to the long axis. Measurements were made from all labeled cells in an area containing A and C laminae of the LGN. Similarly, soma diameter measure-
Fig. 4. **Left:** Sagittal section from a P21 ferret brain stained for NADPH-diaphorase. At this age there is clear staining of cell bodies and proximal portions of processes. Stained cells are distributed throughout the LGN. **Right:** Higher power view of the same section. Cells with bipolar and multipolar somata are labeled. a, anterior; d, dorsal. Scale bars = 200 μm on left, 50 μm on right.
Fig. 5. **Left:** Sagittal section from a P29 ferret brain stained for NADPH-diaphorase. As in the P21 sections (Fig. 3), there is staining of cell bodies and processes throughout the LGN. Eye-specific laminae (A, A1, and C) are evident. **Right:** The same section viewed under higher magnification. Again, there is clear staining of several different cell types, including cells with a range of soma sizes and bipolar and multipolar somata. The density of stained cells reaches a peak at this age. a, anterior; d, dorsal. Scale bars = 200 μm on left, 50 μm on right.
EXPRESSION OF NADPH-DIAPHORASE IN LGN

Fig. 6. Cerebral cortex in horizontal sections of ferret brains stained for NADPH-diaphorase at ages P0 (A), P29 (B), and P42 (C). At each age, some cortical cells are intensely stained; staining extends into the dendrites. Scale bar = 50 μm.

ments were made from Nissl-stained sections from three and four postnatal weeks.

RESULTS

We examined brains from 2 ferrets at P0, 3 ferrets at P8, 2 ferrets at each of P14, P21–P23, P27–P29, P35, P42, P56, and one adult ferret. At P0, no cell bodies were labeled in the LGN with NADPH-d (Fig. 1). The nucleus had a diffuse blue appearance, possibly due to staining of neuropil (Fig. 2A). Neuronal staining remained present at all the ages (summarized in Fig. 2), and its intensity varied somewhat with incubation times. At P8 there were some cell bodies labeled; these were predominantly in the C laminae adjacent to the optic tract, with some labeled cell bodies in the A laminae as well (Fig. 3). Portions of neuronal processes were labeled, as well as blood vessels. Labeling in P14 ferrets appeared similar to that at P8.

At P21 and P23 clearly outlined cell bodies were labeled (Fig. 4). In many of the labeled cells the axon and primary dendrites were also labeled. The morphology of labeled cells was varied and included cells with both bipolar and multipolar somata. Most of the bipolar cells were oriented along an axis perpendicular to the boundary between eye-specific layers; however, a few were oriented parallel to this boundary. Labeled multipolar cells included cells with a range of soma sizes, including some of the largest cells in the LGN (see below).

Of the ages included in this study, NADPH-d-positive cells were most abundant in the LGN from P29 ferrets. These cells were clearly outlined, and were found in all layers of the LGN (Fig. 5). Many small and large bipolar cells were labeled along with the proximal portions of their processes (Fig. 2B). Several multipolar cells were also labeled; these cells were often among the most intensely labeled (Fig. 2C). While labeling in the LGN was at its peak density, the intensity of the staining in any given cell was much less than that seen in cortical and striatal cells in the same sections. In the intensely labeled cortical cells (Fig. 6), labeling was present in a greater extent of axons and dendrites than in LGN cells. In the LGN, labeled cells were translucent and nuclei were not stained. Other regions of the thalamus also contained NADPH-d-labeled cells at this age. In particular, the medial geniculate nucleus and ventroposterior lateral nucleus of the thalamus had labeled cells after the first postnatal week, but not in adult tissue.

At P35, the extent of labeling in the LGN was similar to that seen at P21 (Fig. 7). Some cells were clearly labeled, although their density was reduced from that seen at P29. Labeled cells were found in both A and C laminae, and were distributed rather evenly throughout the anteroposterior extent of the LGN. Many labeled cells were very pale in comparison with tissue from P29 animals; however, there were some cells labeled as intensely as those seen at P29. Again, the morphology of the labeled cells varied and included small and large bipolar cells as well as small and large multipolar cells.

By P42, cell bodies in the LGN were no longer stained with NADPH-d (Fig. 8). However, the neuropil retained a diffuse staining, and blood vessels could also be identified (Fig. 2D). At P56 and in adult LGN, cell bodies remained unstained. A pale staining delineated the LGN and cell layers. While the LGN in these older animals contained no labeled cells, cortical cells in the same sections were intensely labeled (Fig. 6), indicating that the histochemical processing was adequate. The consistency of cortical staining across age groups suggests that changes in LGN staining reflect developmental regulation and are not artifacts of variation in staining.

The density of cells labeled with NADPH-d at each of the ages examined in this study is shown in Figure 9. There was an increase in the density of labeled cells between the first and the fourth postnatal weeks, followed by a decline in staining so that by six postnatal weeks almost no cell bodies were labeled in the LGN. The highest density of labeled cells was 133 cells per square mm, at P29. The density of cell bodies in Nissl-stained sections at P27 was 1,870 cells per square mm. When these cell densities are compared, we estimate that at the peak intensity of NADPH-d labeling (at
Fig. 7. Left: Horizontal section from a P35 ferret brain stained for NADPH-diaphorase. There is a decrease in the density of stained cells. In addition to the eye-specific laminae, the inner (Ai) and outer (Ao) sublaminae of the A lamina can be seen. Right: Higher magnification of the same section. Staining is still present in some cell bodies but is less noticeable in LGN cell processes. a, anterior; m, medial. Scale bars = 200 μm on left; 50 μm on right.
Fig. 8. Horizontal section from a P42 ferret stained for NADPH-diaphorase. Blood vessels are outlined, but no cell bodies are stained in the LGN. a, anterior; m, medial. Scale bar = 200 μm.

Fig. 9. Density of cell bodies labeled in the ferret LGN using NADPH-diaphorase histochemistry at each of the ages included in the study. Error bars denote standard error when each animal is treated as one data point; n = 2 at each age except one week, where n = 3. No cells are stained at P6. The density of labeled cells increases between the first and fourth postnatal weeks; staining begins to decline in the fifth week and cell bodies are no longer stained by six weeks.

EXPRESSION OF NADPH-DIAPHORASE IN LGN

four postnatal weeks), about 7% of the cells in the LGN are stained.

The distributions of soma diameters at three, four, and five postnatal weeks are shown in Figure 10. The mean soma diameter (± standard error) was 9.2 ± .34 μm at P21 (range = 6.5 to 13.5 μm); 12.7 ± .44 μm at P29 (range = 7.5 to 21 μm); and 11.9 ± .40 μm at P38 (range = 7 to 18.5 μm). In Nissl-stained sections from animals at comparable ages, soma diameters were 7.5 ± .24 μm (range = 4.5 to 11.5 μm) at P20 and 11.0 ± .46 μm (range = 6.1 to 17.9 μm) at P27. While the mean diameters were slightly larger in the NADPH-d-stained sections compared to Nissl-stained sections, the ranges of cell body sizes were similar using both types of histology. The difference in mean diameter may reflect the fact that only some cell types are labeled with NADPH-d histochemistry; however, the mean cell body diameters may differ due to systematic differences in shrinkage during tissue processing. The similarity in the ranges of cell body diameters suggests that NADPH-d staining includes several, if not all, of the cell types within the developing LGN.

DISCUSSION

During postnatal development, neurons in the ferret LGN are transiently stained with NADPH-d histochemistry. The labeled cells represent several morphological types. The large and small bipolar cells are similar, respectively, to the presumptive class 1 and class 2 oriented cells described in the developing LGN (Sutton and Brunso-Bechtold, 1991; Rocha and Sur, 1992). The large and small multipolar cells in this study are similar to presumptive class 1 and class 2 stellate cells. A rigorous comparison would require comparison of detailed dendritic morphology, which is not adequately revealed using NADPH-d histochemistry. When cells in the developing LGN were labeled by intracellular injection of Lucifer Yellow (Rocha and Sur, 1992), many cells could be classified according to soma size and morphology by about three postnatal weeks, consistent with the present study. Because the range of soma diameters in sections stained for NADPH-d is similar to the range of Nissl sections, it is likely that NADPH-d labels several different cell types in the LGN. The diversity of cell types labeled with NADPH-d histochemistry can be further demonstrated by combining the histology with immunohisto-
chemistry. Preliminary results suggest that some NADPH-d-positive cells in the LGN are GABAergic while others are not; thus, NADPH-d may label some inhibitory interneurons as well as a subset of thalamic relay cells.

The intensity of NADPH-d staining observed in the LGN differs from that seen in other areas. The labeled cells in the LGN range from light to medium intensity; none have the opaque, dark staining characteristic of cells in cortex and striatum (see also Vincent and Kimura, 1992). The ferret LGN labeling also contrasts with that seen in the rabbit retina (Sagar, 1990), the amygdala (Sims and Williams, 1990; Pitkänen and Amaral, 1991; McDonald et al., 1993) and other areas in the rat brain (Vincent and Kimura, 1992), which contain both darkly and lightly stained cells. While we find expression of NADPH-d to be transient in the ferret LGN, Vincent and Kimura (1992) describe weakly stained cells in the dorsal LGN of adult rats. It remains possible that NO synthase levels in the LGN of older ferrets have simply decreased below detection threshold rather than disappeared completely. In normal adult cats, no NADPH-d-stained cell bodies are found in the LGN (Bickford et al., 1993), similar to our results in adult ferret. While staining of cell bodies in the LGN is transient, neuropil staining is evident in the LGN at all the ages examined. In the adult cat LGN, neuropil is also stained with NADPH-d, and represents cholinergic parabrachial innervation (Bickford et al., 1993). Further study will be required to test the possibility that this brainstem innervation accounts for neuropil staining in the postnatal ferret LGN.

The present study shows that in the ferret LGN, the greatest density of labeled cell bodies occurs at about four postnatal weeks, with clear labeling evident from P8 to P35. During this period of development, many of the projections to the LGN are being refined. Eye-specific laminae arise from more diffuse retinogeniculate projections during the first and second postnatal weeks (Linden et al., 1981; Hahn and Sur, 1988). These projections are further refined during the next two postnatal weeks (Hahn et al., 1991) such that axon arbors are restricted into sublaminae containing either on-center or off-center afferents and target cells (Levay and McConnell, 1982; Stryker and Zahs, 1983;
EXPRESSION OF NADPH-DIAPHORASE IN LGN

Roe et al., 1989). The timing of LGN layer development depends on afferent activity (Casagrande and Condo, 1988), and the afferents forming the on/off sublaminae fail to segregate normally when NMDA receptors are selectively blocked (Hahm et al., 1991). Thus, the peak in NO synthase activity occurs in LGN cells during a period which requires afferent activity and postsynaptic NMDA receptor activation.

The activation of NMDA receptors has been linked to NO release. NO synthase activity increases in response to NMDA receptor activation in cerebellar slices (Bredt and Snyder, 1989) and in cerebral cortex synaptosomes (Montague et al., 1994). These systems are also linked during development in the rat cerebellum (Southam et al., 1991). NO has been implicated in NMDA-mediated LTP in the hippocampus (Bohme et al., 1991; O’Dell et al., 1991; Schuman and Madison, 1991, 1994a,b; Bon et al., 1992; cf. Williams et al., 1993) and in NMDA receptor-mediated excitotoxicity, in which NO-producing cells are selectively spared from damage by excitotoxins (Koh and Choi, 1988). Thus, the developmental modulation of NADPH-d expression is consistent with a role for NO in the NMDA-dependent segregation of on/off sublaminae in the ferret LGN.

While there are several examples of how NMDA activation correlates with NO synthase activity, an additional possibility is that NO has a role in development independent of NMDA activation. Because at least some cells express NADPH-d at P8, it remains possible that NO is involved in the development of eye-specific layers, a process which appears not to require the activation of NMDA receptors (Smetters et al., 1994). Moreover, while NMDA receptors are involved in the plasticity of binocular connections in cat striate cortex (Bear et al., 1990), such plasticity appears to be unaffected when NO synthase is blocked in cortex (Gillespie et al., 1993). In our material, NADPH-d was not developmentally modulated in the cortex, in contrast with our results in the LGN. It is therefore likely that at least some of the actions of NMDA receptors and NO in development remain distinct.

In other developing systems, there is evidence that NO has a role in refining projections. For example, in cultured rat dorsal root ganglia, NO inhibits process outgrowth (Hess et al., 1993). In addition, NO appears to be required for removal of a transient retinotectal projection in the chick; blockade of NO synthase during embryonic development permits the normally transient projection to remain (Wu et al., 1994).

The developmental changes we find in NADPH-d expression may reflect the involvement of NO in the refinement of other connections in addition to that of retinogeniculate axons. While on/off sublaminae in the LGN are present only in the A laminae, NADPH-d-positive cells are present in all laminae. The LGN receives projections from visual cortex, superior colliculus, and several brainstem areas. Several of these projections develop during the period examined in the present study (e.g., Stein et al., 1985; Johnson and Casagrande, 1993). However, because inhibition of NO synthase disrupts on/off sublamination in the developing ferret LGN (Cramer and Sur, 1994), we are currently examining how NO is involved specifically in the refinement of the retinogeniculate projection.

ACKNOWLEDGMENTS

The authors thank Suzanne Kuffler, Udaya Liyanage, Alex Polonsky, and Dr. Francisco Clasca for assistance with histology, and Dr. Sacha Nelson for helpful comments on the manuscript. This work was supported by NIH Grant EY07023.

LITERATURE CITED


