The Journal of Neuroscience, January 1, 1999, *19*(1):229–235

NMDA Receptor-Mediated Refinement of a Transient Retinotectal Projection during Development Requires Nitric Oxide

Alan F. Ernst,[1] Hope H. Wu,[1] Esam E. El-Fakahany,[2] and Steven C. McLoon[1]

[1]Department of Cell Biology and Neuroanatomy and [2]Department of Psychiatry, University of Minnesota, Minneapolis, Minnesota 55455

A transient ipsilateral retinotectal projection is normally eliminated during embryonic development of the chick visual system. Administration of the NMDA receptor antagonist 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801) during the developmental period in which this projection normally disappears prevented its complete elimination. Previous studies showed that tectal cells express nitric oxide synthase during development, and blocking synthesis of nitric oxide also prevented elimination of the ipsilateral retinotectal projection. The effect of NMDA receptor blockade on nitric oxide synthase activity in tectal cells was assessed biochemically in chick embryos. Increasing concentrations of MK-801 resulted in a dose-dependent decrease in nitric oxide synthase activity. This result suggests that NMDA receptor activation can regulate nitric oxide synthase activity in the tectum. The degree of rescue of the ipsilateral retinotectal projection was compared in embryos treated either with MK-801 or with an inhibitor of nitric oxide synthesis, $N\omega$-nitro-L-arginine (L-NoArg). At comparable levels of inhibition of nitric oxide synthesis, no significant difference was observed in the degree of rescue mediated by NMDA receptor blockade or nitric oxide synthesis blockade. These results suggest that NMDA receptor-mediated elimination of the ipsilateral retinotectal projection is completely mediated via nitric oxide.

Key words: NMDA receptor; nitric oxide; nitric oxide synthase; retina; tectum; pattern formation; neuronal development; chick

Normal visual function depends on the proper pattern of axonal connections from ganglion cells in the retina to the primary visual centers in the brain. The adult pattern of connections arises during development by refinement of an approximately ordered embryonic pattern (e.g., McLoon, 1982; O'Leary et al., 1986; Nakamura and O'Leary, 1989; Simon and O'Leary, 1992). Refinement results in the elimination of a number of transient projections. In chick, for example, a transient ipsilateral retinotectal projection is normally eliminated during development, resulting in a completely crossed projection from each retina to the contralateral tectum (McLoon and Lund, 1982; O'Leary et al., 1983; Thanos and Bonhoeffer, 1984). Mammals exhibit a similar ipsilateral retinotectal projection, which is only partially eliminated during development (Land and Lund, 1979; Cowan et al., 1984). The process by which transient projections are eliminated is incompletely understood.

Refinement of a number of retinofugal projections is known to require NMDA receptor activation (for review, see Constantine-Paton et al., 1990). In the three-eyed frog, blockade of NMDA receptors disrupted the segregation of retinal axon terminals into eye-specific stripes (Cline et al., 1987). In ferret, blockade of NMDA receptors disrupted the segregation of retinal axon terminals into "On" and "Off" sublaminae in the lateral geniculate nucleus (Hahm et al., 1991). In rodent, NMDA receptor blockade prevented the formation of topographically appropriate connections in the superior colliculus (Simon et al., 1992). Although these experiments implicate NMDA receptors in the process of refinement, it is unclear what downstream signal transduction events are required for NMDA receptor-mediated refinement of neuronal connections.

Refinement of retinofugal connections mediated by NMDA receptors may require nitric oxide (NO). Nitric oxide was shown to be released by certain cell types *in vitro* in response to NMDA receptor activation (Garthwaite et al., 1988; Bredt and Snyder, 1989). Furthermore, in ferret, administration of either NMDA receptor antagonists or inhibitors of NO synthesis prevented the segregation of retinal inputs into sublaminae in the lateral geniculate nucleus during early postnatal development (Hahm et al., 1991; Cramer et al., 1996). Although these data suggest that both NMDA receptors and NO are involved in refinement, the role of NO in NMDA receptor-mediated refinement is not known. A quantitative comparison of the effect of blocking NMDA receptors and NO synthesis might give some insight into this question.

The chick ipsilateral retinotectal projection is ideally suited for a comparison of the effects of NMDA receptor blockade and NO synthesis blockade because it is quantifiable. Our previous study showed that NO is required for elimination of the ipsilateral retinotectal projection in chick (Wu et al., 1994). In the present study, the relative roles of NMDA receptor activation and NO synthesis in the developmental refinement of the ipsilateral retinotectal projection of the chick were compared quantitatively. Three major findings are reported here. First, NMDA receptor antagonists prevented elimination of the chick ipsilateral retinotectal projection. Second, NMDA receptor blockade *in vivo* reduced cytosolic nitric oxide synthase activity in tectal cells, indicating that NMDA receptor activity can regulate NO production. Third, no significant difference was observed in the magnitude of preservation of the ipsilateral retinotectal projection elicited by

Received Jan. 29, 1998; revised Oct. 9, 1998; accepted Oct. 13, 1998.

This work was supported by National Institutes of Health Grants EY07133 and EY11926.

Correspondence should be addressed to Dr. Steven C. McLoon, Department of Cell Biology and Neuroanatomy, University of Minnesota, 4–144 Jackson Hall, 321 Church Street Southeast, Minneapolis, MN 55455.

Copyright © 1998 Society for Neuroscience 0270-6474/98/190229-07$05.00/0

blockade of either NO synthesis or NMDA receptors, suggesting a common signal transduction mechanism. These results strongly suggest that NMDA receptor-mediated refinement of the ipsilateral retinotectal projection is completely mediated via the downstream signaling molecule NO.

MATERIALS AND METHODS

Reagents. NADPH was obtained from Calbiochem (La Jolla, CA). L-2,3,4,5-[^{3}H]arginine monohydrochloride was obtained from Amersham (Arlington Heights, IL). The ion-exchange resin DOWEX AG50W-X8 (Na^+ form) was obtained from Bio-Rad (Hercules, CA). 5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801) was obtained from Research Biochemicals (Natick, MA). *N*ω-nitro-L-arginine, fast blue, and all other reagents were obtained from Sigma (St. Louis, MO).

Animals. Fertilized chicken eggs, pathogen-free White Leghorn crossed with Rhode Island Red, obtained from the University of Minnesota Poultry Center were incubated at 37°C and 98% relative humidity. After 3 d of incubation, the embryos were removed from the shell and transferred to embryo culture chambers (Dunn and Boone, 1976). The cultured embryos were maintained in a forced-draft tissue culture incubator at 37°C, 95% relative humidity, and 1% CO_2.

Drug administration. An inhibitor of NO synthesis, *N*ω-nitro-L-arginine (L-NoArg), and/or an NMDA receptor antagonist, MK-801, were administered systemically to chick embryos daily from embryonic day 9 (E9) through E16, the period during which transient retinotectal projections are eliminated. Drugs were dissolved in 100 μl of saline at various concentrations and administered daily to the chorioallantoic membrane of the embryos. Control embryos received 100 μl of saline.

Retrograde labeling. The retinal ganglion cells that project to the ipsilateral tectum were labeled by retrograde axonal tracing on E16. A 2% suspension of fast blue in DMSO was injected into one tectum of the embryos via a pulled-glass pipette attached to an oil-filled microliter syringe. Fast blue was selected because it spreads well and is taken up by fibers of passage, allowing the maximum number of ganglion cells to be labeled. Embryos on E16 received multiple injections of fast blue along the rostroinferior margin of the right tectum. It is in this region that the retinal axons enter the tectum from the optic tract. Approximately 1.0 μl of fast blue was injected into each embryo.

Retinal whole mounts were prepared from fast blue-injected embryos on E17. The retinas were dissected from the eyes and placed in 4% paraformaldehyde/phosphate buffer, pH 7.4. After 1 hr of fixation, the retinas were rinsed in phosphate buffer, mounted whole onto glass slides, and coverslipped in an aqueous mounting medium. The number and distribution of fast blue-labeled retinal ganglion cells in whole-mounted retinas were analyzed with a microscope equipped for epifluorescence. The distribution of labeled ganglion cells in retinal whole mounts was plotted on enlarged tracings of the retinas by means of a computer interfaced with stage encoders on the microscope. Retinas ipsilateral to the injected tecta were analyzed to determine the total number and distribution of fast blue-labeled ganglion cells. Retinas contralateral to the injected tecta were also analyzed to assess the extent of the dye injection. The percentage of the contralateral retinal area with fast blue-labeled cells was determined using a Bioquant image analysis system. Because of the difficulty of routinely labeling the entire retinal projection to an injected tectum, the number of labeled cells in the ipsilateral retina was normalized based on the percentage of the contralateral retina with labeled cells. The normalized numbers of cells were used to calculate the mean and the SEM for each condition. The results of different treatments were compared with that of controls using a two-tailed Student's *t* test or were compared with each other using one-way ANOVA.

Cell death assay. Embryos were treated with either 100 μl of saline or 0.01 μmol of MK-801 in 100 μl of saline daily from E9 to E12. On E12, the peak of retinal ganglion cell death, retinas were fixed in 4% paraformaldehyde/phosphate buffer, pH 7.4, mounted whole onto glass slides, and stained with cresyl violet (Hughes and McLoon, 1979). Pyknotic cell profiles in the ganglion cell layer were counted in 25 fields distributed at regular intervals across the whole retina using a 63× objective on a Leitz microscope. The counts for each field were used to determine the average number of pyknotic cells per field. The results from five retinas for each condition were used to calculate the mean and the SEM. The result from drug-treated embryos was compared with that of the control using a two-tailed Student's *t* test.

Nitric oxide synthase assay. Nitric oxide synthase activity was assayed biochemically in homogenates of chick tectum by monitoring the conversion of arginine to citrulline using a method, with slight modifications, developed by Bredt and Snyder (1989). Tecta from E13 chick embryos exposed daily to MK-801 or saline were dissected and flash frozen in liquid nitrogen. Frozen tecta were homogenized at 23,000 rpm for 30 sec (Polytron/PT 3000; Brinkmann) in 20 mM HEPES containing 0.5 mM EGTA, 1 mM dithiothreitol, and 0.32 M sucrose, pH 7.4. Homogenates were centrifuged at 20,000 × *g* for 15 min. The resulting supernatant, which was a crude cytosol fraction, was applied to columns of DOWEX AG50W-X8 (Na^+ form) to remove endogenous L-arginine. The protein content of the arginine-free cytosolic fractions was determined using the method of Lowry et al. (1951). Aliquots of cytosol (250 μg of protein) were incubated in 20 mM HEPES buffer, pH 7.4, containing 0.5 mM EGTA, 1 mM dithiothreitol, 0.32 M sucrose, 0.5 mM Ca^{2+} (1 μM free Ca^{2+}), 200 μM NADPH, 1 μM L-arginine, and 0.1 μCi/ml L-[^{3}H]arginine. Incubations were performed for 45 min at 37°C in a final reaction volume of 300 μl. The reaction was stopped by the addition of 2 ml of chilled 20 mM HEPES and 2 mM EDTA, pH 5.5. Samples were passed through DOWEX AG50W-X8 columns (Na^+ form) to remove the unreacted L-[^{3}H]arginine, and the columns were washed with 2 ml of H_2O. The L-[^{3}H]citrulline in the flow-through and wash was quantified by liquid scintillation spectroscopy, and results were adjusted by subtraction of the counts obtained from control reactions lacking tissue. The results of different treatments were compared with that of controls using a two-tailed Student's *t* test.

RESULTS

NMDA receptors and refinement of the ipsilateral retinotectal projection

The first aim of this study was to determine whether NMDA receptors are involved in elimination of the chick ipsilateral retinotectal projection. This transient projection is present during early embryonic development and is normally eliminated during a discrete period of development by a process of refinement. To address this issue, we treated embryos systemically with MK-801, a noncompetitive NMDA receptor antagonist. Embryos received MK-801 daily from E9 to E16 to block NMDA receptor function during the period in which the ipsilateral retinotectal projection normally disappears. Control embryos were treated with saline. There was no significant difference between experimental and control groups in body weight or the length of the tectum, beak, or toe, indicating that general development proceeded normally in drug-treated embryos (data not shown). On E16, the fluorescent retrograde tracer fast blue was injected into the right anterior tectum to label all ganglion cells with axonal projections to the injected tectum. On E17, retinas were dissected from the embryos and whole mounted. Fast blue-labeled ganglion cells were quantified in retinas ipsilateral to the injected tectum.

NMDA receptor blockade resulted in persistence of the ipsilateral retinotectal projection past the developmental stage by which this projection would have normally mostly disappeared. In saline-treated control embryos, few fast blue-labeled cells were found in retinas ipsilateral to the injected tecta (8 ± 1.5 cells per retina; $n = 9$; Figs. 1, 2). Embryos treated with MK-801 had significantly more labeled cells in the ipsilateral retinas, and the number of labeled cells was dependent on the dose of MK-801 (Figs. 1, 2). Compared with saline-treated control embryos, embryos treated with 0.01 μmol of MK-801 had ~7.5 times as many labeled cells in the ipsilateral retina (60 ± 12 cells per retina; $n = 19$; $p < 0.005$). The highest dose of MK-801 used in this study (0.1 μmol) rescued ~34 times as many ipsilaterally projecting retinal ganglion cells compared with saline-treated control embryos (274 ± 94 cells per retina; $n = 5$; $p < 0.005$). This finding indicates that NMDA receptors are involved in elimination of the ipsilateral retinotectal projection.

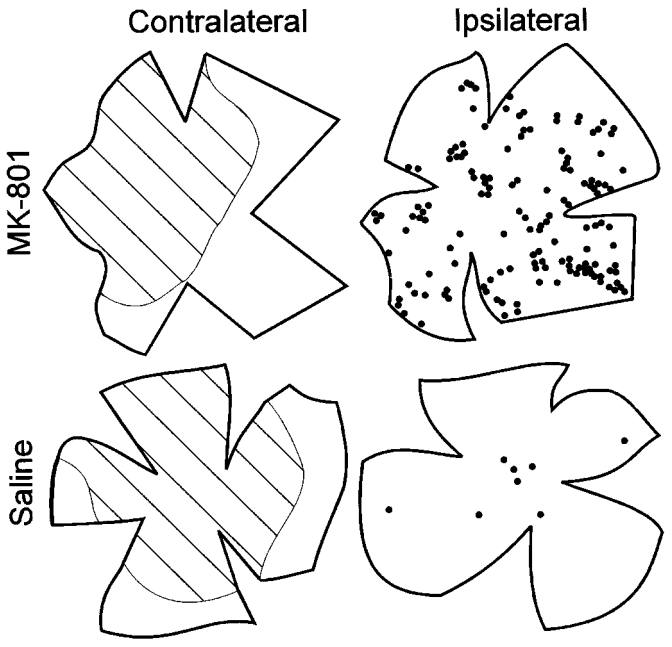

Figure 1. Plots of fast blue-labeled retinal ganglion cells in retinal whole mounts from embryos treated with MK-801 or saline. Treatment of embryos with MK-801 resulted in persistence of the ipsilateral retinotectal projection. The E17 retinas on the *left* are contralateral to a tectum injected with fast blue on E16. The *cross-hatched areas* represent regions containing high concentrations of labeled cells. The retinas on the *right* are ipsilateral to the tectum injected with tracer. The *dots* indicate individual fast blue-labeled cells. The two retinas in *each row* are from the same embryo. The retinas in the *top row* are from an embryo treated with 0.1 μmol of MK-801 daily from E9 to E16, and the retinas in the *bottom row* are from an embryo treated with saline.

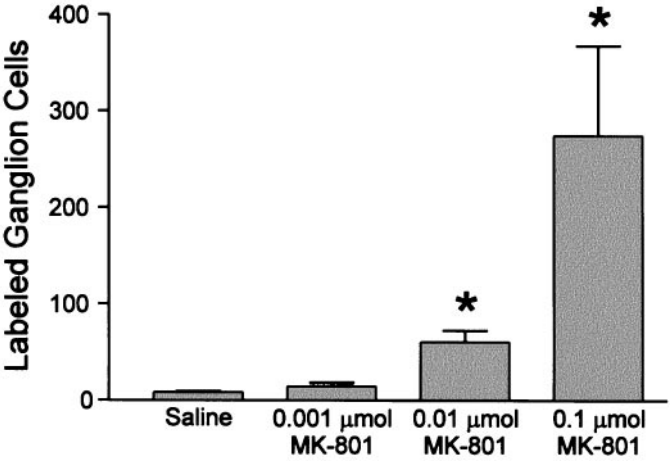

Figure 2. Dose-related effect of MK-801 on elimination of the ipsilateral retinotectal projection. Increasing concentrations of the NMDA receptor antagonist resulted in greater preservation of the ipsilateral projection. The graph shows the normalized number of fast blue-labeled ganglion cells in E17 retinas ipsilateral to tecta injected with dye on E16. Embryos received either saline or MK-801 at the stated dose daily from E9 to E16. Error bars indicate SEM; * indicates values significantly different from the saline control with $p < 0.005$.

NMDA receptor blockade and retinal ganglion cell death

NMDA receptor blockade could rescue the ipsilateral projection by preventing death of ganglion cells and/or by blocking remodeling of axon terminals. Approximately 60% of the retinal ganglion cells with ipsilateral projections normally die during development, and the peak of this death is on E12 (Williams and McLoon, 1991). To determine whether MK-801 treatment affected retinal ganglion cell death, we quantified pyknotic cell profiles in the ganglion cell layer of retinas from control and MK-801–treated embryos. Embryos were treated daily from E9 through E12 with either saline or 0.01 μmol of MK-801. On E12, the retinas were whole mounted and stained with cresyl violet, and pyknotic cells were quantified microscopically. There was no significant difference ($p > 0.5$) in the number of pyknotic cell profiles in retinas from saline-treated embryos (10.61 ± 0.43 cells/field) and MK-801–treated embryos (10.27 ± 0.33 cells/field; Fig. 3). This indicates that NMDA receptor-mediated elimination of the ipsilateral retinotectal projection works via remodeling axon terminals rather than cell death.

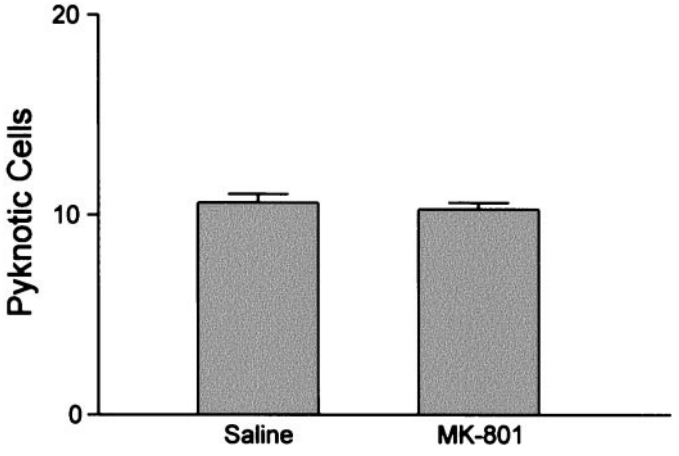

Figure 3. Effect of MK-801 treatment on cell death in the retinal ganglion cell layer. The NMDA receptor antagonist did not significantly alter cell death ($p > 0.5$). The histogram shows the number of pyknotic cells per field in the ganglion cell layer from E12 embryos that received either saline or 0.01 μmol of MK-801 daily from E9 to E12. Error bars indicate SEM.

NMDA receptors and nitric oxide synthase activity in tectal tissue

Activity of nitric oxide synthase (NOS), the enzyme responsible for synthesis of NO, was assayed in tectal tissue harvested from embryos treated with MK-801 to determine the effect of NMDA receptor activation on NOS activity *in vivo*. Embryos were treated during the period of refinement with different concentrations of MK-801. On E13, tecta were removed from the embryos, homogenized, and assayed biochemically for NOS activity. Treatment of embryos with MK-801 significantly reduced NOS activity in the tectum in a dose-dependent manner ($p < 0.005$ for all doses tested; Fig. 4). The maximum effect, a 95% reduction in activity, was observed in embryos treated with 0.1 μmol of MK-801. In addition, the dose-dependent reduction in tectal NOS activity associated with MK-801 treatment correlates with the concentration-dependent degree of preservation of the ipsilateral retinotectal projection, suggesting a link between NMDA receptor activation, NOS activity, and refinement (Figs. 2, 4).

Comparison of NMDA receptor blockade and nitric oxide synthesis blockade on refinement of the ipsilateral retinotectal projection

The effect of NMDA receptor blockade or inhibition of NO synthesis on elimination of the ipsilateral retinotectal projection

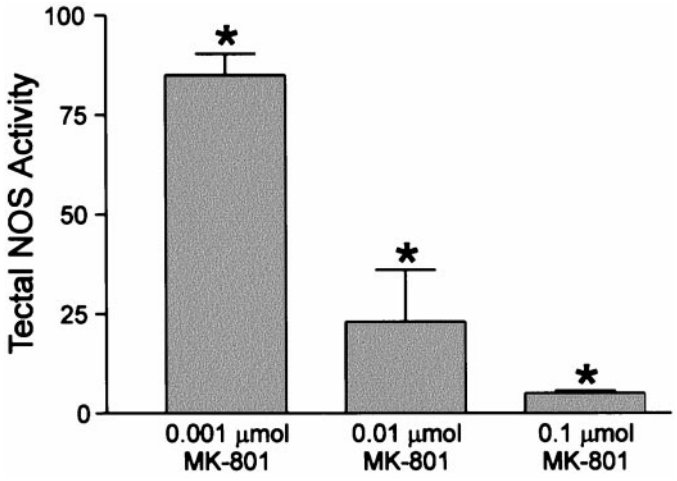

Figure 4. Dose-related effect of MK-801 on NOS activity in the tectum. Increasing concentrations of the NMDA receptor antagonist resulted in greater inhibition of NOS activity. The histogram shows NOS activity in tecta from E13 embryos after treatment with various doses of MK-801 expressed as a percentage of NOS activity in tecta from control embryos. Error bars indicate SEM; * indicates values significantly different from the control with $p < 0.005$.

was compared quantitatively to evaluate the relative roles of NMDA receptor activation and of NO with respect to refinement of the retinotectal projection. Embryos were treated with MK-801 to block NMDA receptors, L-NoArg to block NO synthesis, or both drugs simultaneously during the developmental period in which the ipsilateral projection is normally eliminated. Previous studies showed that treatment of embryos with L-NoArg had no detectable effect on general measures of development and that the chick vasculature is not responsive to NO at these stages of development (Wu et al., 1994). The selected doses of MK-801 and/or L-NoArg resulted in the same levels of NOS activity in the tectum as determined biochemically (Fig. 5*A*). Embryos were treated daily from E9 to E16 with 0.01 μmol of MK-801, 1.0 μmol of L-NoArg, or a combination of both drugs. On E16, the retinal ganglion cells projecting to the right tecta were retrogradely labeled by injections of fast blue into the anterior pole of the right tecta. The fast blue-labeled ganglion cells were quantified in the retinas ipsilateral to the injected tecta.

All three treatments resulted in a similar preservation of the ipsilateral retinotectal projection (Fig. 5*B*). The number of fast blue-labeled cells in retinas ipsilateral to the injected tecta in embryos treated with 0.01 μmol of MK-801 was 60 ± 12 ($n = 19$), the number of ipsilaterally projecting ganglion cells in retinas from embryos treated with 1.0 μmol of L-NoArg was 73 ± 13.3 ($n = 17$), and the number of ipsilaterally projecting ganglion cells in retinas from embryos treated with both 0.01 μmol of MK-801 and 1.0 μmol of L-NoArg was 59 ± 17.3 cells ($n = 7$). ANOVA indicated no significant difference in the number of ipsilaterally projecting retinal ganglion cells among the three treatment groups ($F = 3.24$; $p = 0.92$). The similarity between the effect of blocking NMDA receptor function and NO synthesis and the finding that the two treatments are not additive suggest that NMDA receptor-mediated elimination of the chick ipsilateral retinotectal projection requires the NO signal transduction pathway.

DISCUSSION

The initial pattern of axonal connections from the retina to the central visual nuclei lacks precision in warm-blooded species and includes numerous projections that are inappropriate for the adult. The adult pattern of connections arises via a process of refinement during development. Transient ipsilateral retinotectal projections are normally eliminated by refinement in avians and mammals (Land and Lund, 1979; McLoon and Lund, 1982; O'Leary et al., 1983; Cowan et al., 1984; Thanos and Bonhoeffer, 1984). The first aim of this study was to examine the role of NMDA receptors in elimination of the ipsilateral retinotectal projection during chick development. Retinal ganglion cells are glutamatergic (Kalloniatis et al., 1994; Dye and Karten, 1996) and make synaptic connections with cells that express NMDA receptors (Esguerra et al., 1992; Cline et al., 1994; Fohr et al., 1995; Guido et al., 1997). To determine whether retinotectal communication mediated by NMDA receptors is important in elimination of the ipsilateral projection, we treated embryos with an NMDA receptor antagonist, MK-801, during the period of refinement. Increasing doses of MK-801 rescued increasing numbers of ipsilaterally projecting retinal ganglion cells at an age by which this projection would normally have been mostly eliminated. This result complements previous studies in other species, showing that blockade of NMDA receptors during development disrupted refinement of retinofugal projections (Cline et al., 1987; Cline and Constantine-Paton, 1989; Hahm et al., 1991; Simon et al., 1992).

NMDA receptor activity in the developing chick visual system may regulate NO synthesis. The NMDA receptor is a cation channel that is highly permeable to Ca^{2+} when activated (Scatton, 1993). Nitric oxide synthase is activated by increases in intracellular Ca^{2+} (for review, see Schmidt et al., 1992; Oh, 1995; Ignarro, 1996). Previous studies showed that activation of NMDA receptors resulted in the synthesis of NO in cultured cerebellar granule cells (Garthwaite et al., 1988; Bredt and Snyder, 1989). Because both NMDA receptors and NOS are expressed in the developing tectum (Esguerra et al., 1992; Cline et al., 1994; Williams et al., 1994; Fohr et al., 1995; Guido et al., 1997), it is likely that activation of NMDA receptors in the tectum leads to synthesis of NO. The present study implies a link between NMDA receptor activation and NO synthesis by showing a reduction in NOS activity in tectal cells after treatment of embryos with MK-801 *in vivo*. This reduction in NOS activity was likely attributable to a decrease in the amount of the enzyme present in the tissue, because NOS activity was measured in an activated state in the presence of Ca^{2+}. MK-801 treatment also resulted in a reduction in NADPH-diaphorase staining in tectal cells (A. F. Ernst and S. C. McLoon, unpublished observations). NADPH-diaphorase staining in formaldehyde-fixed tissue is a histochemical marker for NOS in neurons (Bredt et al., 1991; Dawson et al., 1991; Hope et al., 1991). It is generally believed that NO synthesis by the "constitutive" isoforms of NOS in the brain is regulated by altering the activation of the enzyme, not by modulating levels of NOS expression (for review, see Dawson, 1995). There is, however, evidence that the level of expression of the constitutive type of NOS is altered after nerve injury or treatment with inhibitors of acetylcholinesterase (Yu, 1994; Zhang et al., 1994; Cuadra and El-Fakahany, 1997). In cultured cerebellar granule cells, blockade of NMDA receptors resulted in an increase in NOS expression (Baader and Schilling, 1996). The finding that NMDA receptor blockade reduced levels of the NOS enzyme in the present study

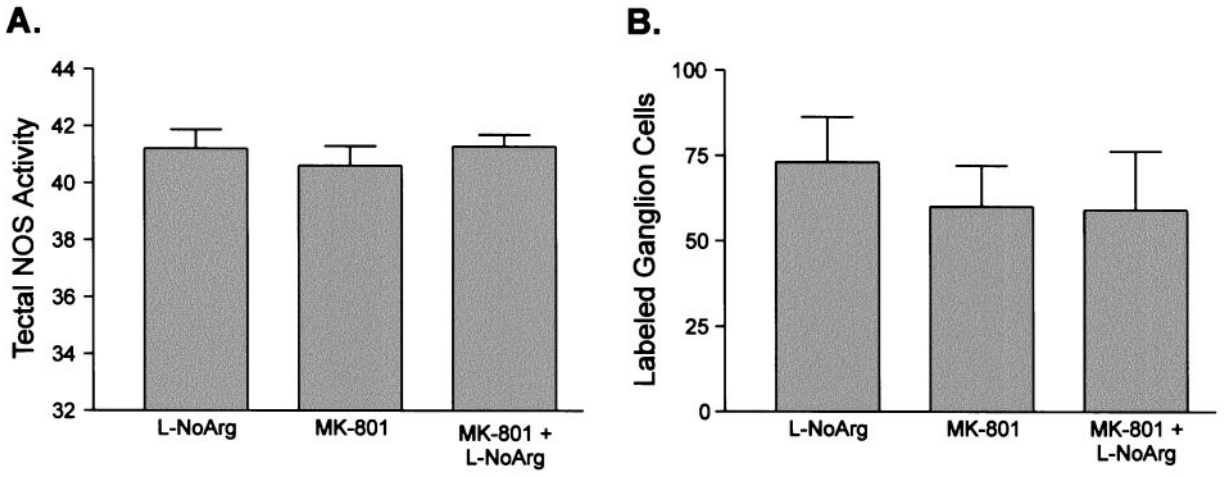

Figure 5. Comparison of the effect of MK-801 and/or L-NoArg treatments on the level of NOS activity in the tectum (*A*) and on the number of ganglion cells in a retina projecting to the ipsilateral tectum (*B*). Blocking NMDA receptor activation, NO synthesis, or both preserved the ipsilateral retinotectal projection equally. *A*, Histogram showing the level of NOS activity in tecta (expressed as 0.1 × pmol of citrulline·mg of protein^{-1}·min^{-1}) from embryos treated with 1.0 μmol of L-NoArg, 0.01 μmol of MK-801, or both 1.0 μmol of L-NoArg and 0.01 μmol of MK-801. *B*, Histogram showing the number of fast blue-labeled ganglion cells in E17 retinas ipsilateral to tecta injected with fast blue on E16. Embryos were treated daily from E9 to E16 with 100 μl of saline containing 0.01 μmol of MK-801, 1.0 μmol of L-NoArg, or both 0.01 μmol of MK-801 and 1.0 μmol of L-NoArg. There was no significant difference in the number of ipsilaterally projecting ganglion cells among the groups ($F = 3.24$; $p = 0.92$). Error bars indicate SEM.

suggests that physiological NMDA receptor activity may be responsible for maintenance of normal levels of NOS expression, possibly controlled at the level of the gene. Recent evidence linked Ca^{2+} influx, which follows NMDA receptor activation, to BDNF synthesis via regulation of gene expression (Shieh et al., 1998; Tao at al, 1998). The same mechanism could regulate NOS expression.

The finding that NMDA receptor blockade disrupted refinement and reduced tectal NOS activity suggests that NMDA receptor-mediated refinement could involve NO. By regulation of internal Ca^{2+} levels, the NMDA receptor could effect a number of downstream signal transduction pathways; multiple pathways could influence refinement. For example, the activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is regulated by Ca^{2+} influx through NMDA receptors (Colbran, 1992), and CaMKII has been implicated in refinement of retinal connections (Zou and Cline, 1996). Involvement of the tectal cell in changing connections of the retinal axons, however, implies the existence of a retrograde signal from the tectal cell back to the retinal axons. Nitric oxide is an appealing candidate for such a retrograde signal because it is capable of diffusing freely between cells (Ignarro, 1991). Furthermore, a great deal of evidence suggests that NO is capable of acting in a retrograde manner downstream of NMDA receptor activation (for review, see Zorumski and Izumi, 1993; Larkman and Jack, 1995). Administration of L-NoArg to block NO synthesis during the period of refinement in chick, like NMDA receptor blockade, prevented elimination of the ipsilateral retinotectal projection (Wu et al., 1994). Similarly in developing ferret, NMDA receptor activation and NO are involved in segregation of retinal axon terminals into On and Off sublaminae in the lateral geniculate nucleus (Smetters et al., 1994; Cramer et al., 1996). These findings do not, however, eliminate the possibility that retrograde signals other than NO are also involved in NMDA receptor-mediated refinement.

The ipsilateral retinotectal projection in chick is ideal for studying the effect of pharmacological agents on refinement because the number of cells in a retina that project to the ipsilateral tectum can be easily counted, unlike in mammals. Because virtually complete elimination of this projection takes place during a discrete period of development, any projection that persists in chick in response to various experimental manipulations can be easily distinguished from the normal projection. To test the possibility that NMDA receptor-mediated refinement requires NO, we compared quantitatively the effect on refinement resulting from application of either MK-801 or L-NoArg. If the NMDA receptor functions via multiple downstream pathways relative to refinement of the chick ipsilateral retinotectal projection, then blocking NMDA receptor activation should have a greater effect on refinement than does blocking synthesis of NO. On the other hand, if NO synthesis is an obligatory step for NMDA receptor-mediated refinement, then concentrations of MK-801 or L-NoArg that reduce tectal NOS activity to comparable levels should rescue the same number of ipsilaterally projecting retinal ganglion cells. The results from the present study demonstrated that blocking NMDA receptors with MK-801 or blocking NO synthesis with L-NoArg has the same effect on refinement of the ipsilateral retinotectal projection. Furthermore, the effect of coadministering both drugs was no more effective than using either drug individually. Taken together, these results suggest that NO is an obligatory downstream effector of NMDA receptor activation relative to refinement of the chick ipsilateral retinotectal projection. Other systems, however, may use other retrograde signals. Ocular dominance column plasticity requires NMDA receptor activation (Kleinschmidt et al., 1987; Gu et al., 1989; Bear et al., 1990; Rauschecker et al., 1990), but it does not seem to involve nitric oxide (Ruthazer et al., 1996).

Because drugs were administered systemically, it is possible that the effects observed on refinement in this study were caused by perturbations in retinal physiology. It has been reported previously that NOS is expressed in developing chick retina (Paes de Carvalho et al., 1996; Goureau et al., 1997). A number of studies, however, indicate that NOS activity levels are transiently very low during the period of refinement (Wu and McLoon, 1994; Ientile et al., 1996). It is unlikely, therefore, that systemic administration

of L-NoArg during the period of refinement resulted in major changes in NO synthesis in embryonic chick retina.

Even in the developing chick retinotectal system, multiple mechanisms seem to be active in refinement. Concentrations of MK-801 or L-NoArg that elicited the maximum effect on refinement rescued a maximum of 30% of the cells comprising the initial ipsilateral retinotectal projection. Thus, ~70% of the initial projection must be eliminated by other mechanisms, presumably not involving NMDA receptors or NO. Previous studies demonstrated that refinement of retinofugal projections does not always involve NMDA receptors. For example, segregation of retinal fibers into eye-specific layers in the ferret lateral geniculate nucleus was not disrupted by NMDA receptor antagonists (Smetters et al., 1994). The nature of NMDA receptor-independent mechanisms involved in elimination of the chick ipsilateral retinotectal projection is unclear, although cell death is probably involved.

There is normally massive death of retinal ganglion cells during the refinement period (Hughes and McLoon, 1979; Insausti et al., 1984). Approximately 60% of the cells comprising the chick ipsilateral retinotectal projection are normally eliminated by retinal ganglion cell death (Williams and McLoon, 1991). Neither MK-801 treatment to block NMDA receptors nor L-NoArg treatment to inhibit NO synthesis altered cell death in the ganglion cell layer. Thus, the role of NMDA receptors and NO in refinement of the ipsilateral retinotectal projection seems to involve remodeling axon terminals rather than cell death. It is possible that the availability of neurotrophins, such as BDNF, determines whether a ganglion cell lives or dies. Preliminary evidence from our laboratory suggests that administration of excess BDNF disrupts refinement of the retinotectal projection (H. H. Wu and S. C. McLoon, unpublished observations). The segregation of geniculate afferents into ocular dominance columns in visual cortex also seems to involve BDNF (Cabelli et al., 1995). It is not yet known, however, whether the role of neurotrophins in the refinement of connections is independent of the role of NMDA receptor activation. It is likely that other, as yet undiscovered, players are also involved in the refinement of neuronal connections.

REFERENCES

Baader SL, Schilling K (1996) Glutamate receptors mediate dynamic regulation of nitric oxide synthase expression in cerebellar granule cells. J Neurosci 16:1440–1449.

Bear MF, Kleinschmidt A, Gu Q, Singer W (1990) Disruption of experience-dependent synaptic modifications in striate cortex by infusion of an NMDA receptor antagonist. J Neurosci 10:909–925.

Bredt DS, Snyder SH (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci USA 86:9030–9033.

Bredt DS, Glatt CE, Hwang PM, Fotuhi M, Dawson TM, Snyder SH (1991) Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuron 7:615–624.

Cabelli RJ, Hohn A, Shatz CJ (1995) Inhibition of ocular dominance column formation by infusion of NT-4/5 or BDNF. Science 267:1662–1666.

Cline HT, Constantine-Paton M (1989) NMDA receptor antagonists disrupt the retinotectal topographic map. Neuron 3:413–426.

Cline HT, Debski EA, Constantine-Paton M (1987) *N*-methyl-D-aspartate receptor antagonist desegregates eye-specific stripes. Proc Natl Acad Sci USA 84:4342–4345.

Cline HT, McDonald JW, Constantine-Paton M (1994) Glutamate receptor binding in juvenile and adult *Rana pipiens* CNS. J Neurobiol 25:488–502.

Colbran RJ (1992) Regulation and role of brain calcium/calmodulin-dependent protein kinase II. Neurochem Int 21:469–497.

Constantine-Paton M, Cline HT, Debski E (1990) Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways. Annu Rev Neurosci 13:129–154.

Cowan WM, Fawcett JW, O'Leary DMM, Stanfield BB (1984) Regressive events during neurogenesis. Science 225:1258–1265.

Cramer KS, Angelucci A, Hahm JO, Bogdanov MB, Sur M (1996) A role for nitric oxide in the development of the ferret retinogeniculate projection. J Neurosci 16:7995–8004.

Cuadra AE, El-Fakahany EE (1997) Enhancement of maximal activation of neuronal nitric oxide synthase at muscarinic M1 receptors following prolonged agonist treatment. Eur J Pharmacol 334:107–110.

Dawson VL (1995) Nitric oxide: role in neurotoxicity. Clin Exp Pharmacol Physiol 22:305–308.

Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH (1991) Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci USA 88:7797–7801.

Dunn BE, Boone MA (1976) Growth of the chick embryo *in vitro*. Poult Sci 55:1067–1071.

Dye JC, Karten HJ (1996) An *in vitro* study of retinotectal transmission in the chick: role of glutamate and GABA in evoked field potentials. Vis Neurosci 13:747–758.

Esguerra M, Kwon YH, Sur M (1992) Retinogeniculate EPSPs recorded intracellularly in the ferret lateral geniculate nucleus *in vitro*: role of NMDA receptors. Vis Neurosci 8:545–555.

Fohr KJ, Schirm T, Finger W (1995) NMDA-induced whole-cell currents and single channel conductances in tectal neurons during two stages of early development of chicken. Neurosci Lett 183:87–90.

Garthwaite J, Charles SL, Chess-Williams R (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 336:385–388.

Goureau O, Regnier-Ricard F, Jonet L, Jeanny J, Courtois Y, Chany-Fournier F (1997) Developmental expression of nitric oxide synthase isoform I and III in chick retina. J Neurosci Res 50:104–113.

Gu QA, Bear MF, Singer W (1989) Blockade of NMDA-receptors prevents ocularity changes in kitten visual cortex after reversed monocular deprivation. Dev Brain Res 47:281–288.

Guido W, Lo FS, Erzurumlu RS (1997) An *in vitro* model of the kitten retinogeniculate pathway. J Neurophysiol 77:511–516.

Hahm JO, Langdon RB, Sur M (1991) Disruption of retinogeniculate afferent segregation by antagonists to NMDA receptors. Nature 351:568–570.

Hope BT, Michael GJ, Knigge KM, Vincent SR (1991) Neuronal NADPH diaphorase is a nitric oxide synthase. Proc Natl Acad Sci USA 88:2811–2814.

Hughes WF, McLoon SC (1979) Ganglion cell death during normal retinal development in the chick: comparisons with cell death induced by early target field destruction. Exp Neurol 66:587–601.

Ientile R, Malecka B, Picciurro V, Naso A, Pedale S, Macaione S (1996) Nitric oxide synthase in chick embryo retina during development. FEBS Lett 379:82–84.

Ignarro LJ (1991) Heme-dependent activation of guanylate cyclase by nitric oxide: a novel signal transduction mechanism. Blood Ves 28:67–73.

Ignarro LJ (1996) Physiology and pathophysiology of nitric oxide. Kidney Int [Suppl] 55:S2–S5.

Insausti R, Blakemore C, Cowan WM (1984) Ganglion cell death during development of ipsilateral retino-collicular projection in golden hamster. Nature 308:362–365.

Kalloniatis M, Tomisich G, Marc RE (1994) Neurochemical signatures revealed by glutamine labeling in the chicken retina. Vis Neurosci 11:793–804.

Kleinschmidt A, Bear MF, Singer W (1987) Blockade of "NMDA" receptors disrupts experience-dependent modifications of kitten striate cortex. Science 238:355–358.

Land PW, Lund RD (1979) Development of the rat's uncrossed retinotectal pathway and its relation to plasticity studies. Science 205:698–700.

Larkman AU, Jack JJ (1995) Synaptic plasticity: hippocampal LTP. Curr Opin Neurobiol 5:324–334.

Lowry O, Rosebrough NJ, Farr L, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

McLoon SC (1982) Alterations in precision of the crossed retinotectal projection during chick development. Science 215:1418–1420.

McLoon SC, Lund RD (1982) Transient retinofugal pathways in the developing chick. Exp Brain Res 45:277–284.

Nakamura H, O'Leary DDM (1989) Inaccuracies in initial growth and arborization of chick retinotectal axons followed by course corrections and axon remodeling to develop topographic order. J Neurosci 9:3776–3795.

Oh S (1995) The generation of nitric oxide and its roles in neurotransmission and neurotoxicity. Keio J Med 44:53–61.

O'Leary DDM, Gerfen CR, Cowan WM (1983) The development and restriction of the ipsilateral retinofugal projection in the chick. Dev Brain Res 10:93–109.

O'Leary DDM, Fawcett JW, Cowan WM (1986) Topographic targeting errors in the retinocollicular projections and their elimination by selective ganglion cell death. J Neurosci 6:3692–3705.

Paes de Carvalho R, de Faria MH, do Nascimento JLM, Hokoc JN (1996) Development of the NADPH-diaphorase in the avian retina: regulation by calcium ions and relation to nitric oxide synthase. J Neurochem 67:1063–1071.

Rauschecker JP, Egert U, Kossel A (1990) Effects of NMDA antagonists on developmental plasticity in kitten visual cortex. Int J Dev Neurosci 8:425–435.

Ruthazer ES, Gillespei DC, Dawson TM, Snyder SH, Stryker MP (1996) Inhibition of nitric oxide synthase does not prevent ocular dominance plasticity in kitten visual cortex. J Physiol (Lond) 494:519–527.

Scatton B (1993) The NMDA receptor complex. Fundam Clin Pharmacol 7:389–400.

Schmidt HH, Pollock JS, Nakane M, Förstermann U, Murad F (1992) Ca^{2+}/calmodulin-regulated nitric oxide synthases. Cell Calcium 13:427–434.

Shieh PB, Hu S-C, Bobb K, Timmusk T, Ghosh A (1998) Identification of a signaling pathway involved in calcium regulation of BDNF expression. Neuron 20:727–740.

Simon DK, O'Leary DDM (1992) Development of topographic order in the mammalian retinocollicular projection. J Neurosci 12:1212–1232.

Simon DK, Prusky GT, O'Leary DDM, Constantine-Paton M (1992) *N*-methyl-D-aspartate receptor antagonists disrupt the formation of a mammalian neural map. Proc Natl Acad Sci USA 89:10593–10597.

Smetters DK, Hahm J, Sur M (1994) An *N*-methyl-D-aspartate receptor antagonist does not prevent eye-specific segregation in the ferret retinogeniculate pathway. Brain Res 658:168–178.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20:709–726.

Thanos S, Bonhoeffer F (1984) Development of the transient ipsilateral retinotectal projections in the chick embryo: a numerical fluorescence-microscopic analysis. J Comp Neurol 224:407–414.

Williams CV, McLoon SC (1991) Elimination of the transient ipsilateral retinotectal projection is not solely achieved by cell death in the developing chick. J Neurosci 11:445–453.

Williams CV, Nordquist D, McLoon SC (1994) Correlation of nitric oxide synthase expression with changing patterns of axonal projections in the developing visual system. J Neurosci 14:1746–1755.

Wu HH, McLoon SC (1994) Nitric oxide synthesized in developing chick tectum is involved in elimination of the ipsilateral retinotectal projection. Soc Neurosci Abstr 20:1089.

Wu HH, Williams CV, McLoon SC (1994) Involvement of nitric oxide in the elimination of a transient retinotectal projection in development. Science 265:1593–1596.

Yu WH (1994) Nitric oxide synthase in motor neurons after axotomy. J Histochem Cytochem 42:451–457.

Zhang ZG, Chopp M, Gautam S, Zaloga C, Zhang RL, Schmidt HH, Pollock JS, Forstermann U (1994) Up regulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in rat. Brain Res 645:85–95.

Zorumski CF, Izumi Y (1993) Nitric oxide and hippocampal synaptic plasticity. Biochem Pharmacol 46:777–785.

Zou DJ, Cline HT (1996) Expression of constitutively active CamKII in target tissue modifies presynaptic axon arbor growth. Neuron 16: 529–539.