gested by previous studies (*12*), could represent a potential breakage point for promoting gene conversion.

We do not yet know whether the "transgene conversion" process we have found reflects a gene conversion or DNA recombination mechanism. However, the similarity of our results with studies of gene conversion in chickens and rabbits is particularly noteworthy. Furthermore, evidence suggesting a possible VDJ gene conversion event has been reported for a mouse hybridoma cell line (*13*). The relative frequency of gene conversion events in diversifying murine Ig genes might depend greatly on the availability of highly homologous V-genes and the relative location and distance between potential donor and acceptor sequences; this could explain the absence of gene conversion in some previous studies that have addressed this question (*4*–*6*). The results from our transgenic mice raise the possibility that gene conversion might contribute significantly to the diversification of some murine antibody responses.

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10. Transgenic mice were produced with the VVC_μ transgene construct as described previously (*7*). Two transgenic lines, $VVC_\mu1$ and $VVC_\mu5$, were obtained. $VVC_\mu1$ mice have ~10 to 20 copies of the integrated transgene, whereas $VVC_\mu5$ mice have ~25 to 50 copies. In both lines, breeding and Southern blot analyses indicated that the transgene copies are integrated as a tandem array at a single chromosomal site.
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14. RNAs were isolated from each hybridoma and cDNA-PCR was performed with L and C_γ primers as described previously (*11*). The L primer (5′-GTGGAATTCAAACCATGGGATGGAGCTTC-3′) hybridizes to the L exons of both the 2B4 and R16.7 VDJ regions, whereas the C_γ primer (5′-GGAATTCCGGGGCCAGTGGATAGAC-3′) hybridizes to the C_H1 exons of either $C_\gamma1$, $C_\gamma2a$, or $C_\gamma2b$. For each hybridoma, PCR products from an amplification reaction were first directly sequenced with the L or C_γ primers and the CircumVent DNA sequencing kit (New England Biolabs). Sequences for the 5I61E36, 5I72B40, 5I74B40, and 5I93D25 hybridomas were subsequently confirmed by cloning PCR products from an entirely independent cDNA-PCR reaction and sequencing PCR clones with pUC sequencing primers (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ and 5′-AGCGGATAACAATTTCACACAGGA-3′) as well as the L and C_γ primers. The sequences determined from the two independent PCR analyses were identical.
15. Sequences of the 5I61E36, 5I72B40, 5I74B40, and 5I93D25 hybridomas were determined as described in Table 1. For the remaining hybrids, cDNA-PCR products were amplified with the L and C_γ primers, PCR clones were produced and isolated, and sequences of the PCR clones were determined with pUC sequencing primers as well as L and C_γ primers. Single PCR amplifications were used in these cases because specific PCR and ELISA assays also indicated transgene conversion events in these hybridomas (see text). The 5I hybridomas were derived from an immunized $VVC_\mu5$ mouse, and the 1A and 126 hybridomas were from two immunized $VVC_\mu1$ mice. Comparing sequences from 5I61E36 with 5I117-1, 5I93D25 with 5I134-1, and 12631-6 with 12632-4 suggests these pairs might represent sister clones derived from single B cell precursors.

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Involvement of Nitric Oxide in the Elimination of a Transient Retinotectal Projection in Development

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The adult pattern of axonal connections from the eye to the brain arises during development through the refinement of a roughly ordered set of connections. In the chick visual system, refinement normally results in the loss of the ipsilateral retinotectal connections. Inhibition of nitric oxide synthesis reduced the loss of these transient connections. Because nitric oxide is expressed by tectal cells with which retinal axons connect and because reduction of nitric oxide synthesis by tectal cells resulted in a change in the connections of retinal axons, nitric oxide probably serves as a messenger from tectal cells back to retinal axons during development.

Axons from ganglion cells in the retina form synapses with neurons in the primary visual centers in a highly ordered, predictable pattern in the adult brain. Normal visual function depends on the proper pattern of axonal connections. In some species, this adult pattern arises during development by the refinement of an early, roughly ordered pattern of connections. This early projection is characterized by transient retinal connections to the inappropriate side of the brain, inappropriate brain centers, and inappropriate positions within visual centers and to the other eye. The process by which these transient projections are eliminated is incompletely understood.

The developmental refinement of the visual projection is believed to be an activity-dependent process that involves activation of *N*-methyl-D-aspartic acid type glutamate receptors (NMDA receptors) on postsynaptic neurons in the brain (*1*). If activity in the retinal axons is blocked during the refinement period, transient projections persist (*2*). Furthermore, if NMDA receptors on the postsynaptic neurons are blocked during this period, anomalous projections also persist (*3*). The involvement of postsynaptic neurons in the refinement of retinal axon connections suggests that postsynaptic neurons must, in some way, communicate with the retinal axons. The nature of this retrograde communication is unknown.

The characteristics of nitric oxide (NO) make it an appealing candidate for a retrograde messenger in the developmental refinement of connections (*4*, *5*). Nitric oxide is synthesized and released from certain brain cells upon glutamate activation of NMDA receptors (*6*). It is able to cross cell membranes by diffusion, obviating the need for vesicular release. Moreover, NO causes an increase in guanosine 3′,5′-monophosphate (cGMP) in cells that respond to NO (*6*). In turn, cGMP can open cGMP-gated Ca^{2+} channels (*7*), and changes in intracellular Ca^{2+} concentrations influence axonal growth and retraction (*8*), essential activities for modifying patterns of axonal connections. In addition, NO appears to participate in the induction of long-term potentiation (LTP) in the adult hippocampus (*9*), and the developmental refinement of connections and LTP appear to be similar processes (*5*).

The spatial and temporal pattern of expression of nitric oxide synthase (NOS), the enzyme responsible for the synthesis of NO, is consistent with the idea that NO mediates the refinement of visual projections (*10*, *11*). In avians and rodents, the major projection from the retina is to the

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optic tectum. High concentrations of NOS were observed in the tecta of developing chicks and rats during the period in which the retinotectal projection is refined. The cells expressing NOS were concentrated in the superficial tectal layers, the layers where the retinal axons terminate. If eyes were removed or if communication between retinal axons and tectal cells was interrupted, then the expression of NOS in the tectal cells was reduced. Thus, retinal axons appear to interact with tectal cells that express NOS and that presumably produce NO.

Our study examined the effect of inhibitors of NO synthesis on the elimination of the ipsilateral retinotectal projection in the chick. This projection is from an eye to the tectum on the same side of the brain as the eye. It is present during early embryonic development and is normally eliminated during the period of refinement of the retinal connections (*12, 13*). If NO mediates the refinement process, then reduction or blockade of NO synthesis should interfere with elimination of the ipsilateral retinotectal projection.

Inhibitors of NO synthesis, N^w-nitro-L-arginine (L-NOARG) or N^w-nitro-L-arginine methyl ester (L-NAME) (*14*), were administered systemically to chick embryos daily from embryonic day 8 (E8) through E16, the period during which transient retinotectal projections are eliminated (*15*). Control embryos received saline with no drugs or N^w-nitro-D-arginine methyl ester (D-NAME), a pharmacologically inert enantiomer of L-NAME. On E16, an age by which this projection has normally disappeared, a fluorescent dye, Fast Blue (5% in 2% dimethyl sulfoxide and water), was injected into one tectum of each embryo (*13*). Fast Blue was taken up by the retinal axons that remained at this age and was transported back to the ganglion cell bodies in the retinas. Twenty-four hours after the Fast Blue injections, retinas from each embryo were fixed, whole-mounted onto glass slides, and examined with an epifluorescence microscope for the presence of Fast Blue–labeled ganglion cells.

Inhibition of NO synthesis resulted in the persistence of an ipsilateral retinotectal projection past the developmental stage by which this projection would normally have disappeared (Fig. 1). Few Fast Blue–labeled cells were found in retinas ipsilateral to the injected tecta in normal embryos without any treatment (5 ± 1.5 cells per retina) or in saline-treated control embryos (7 ± 1.2 cells per retina). There was no significant difference between untreated and saline-treated embryos ($P > 0.05$). In contrast, the L-NOARG– or L-NAME–treated embryos had an increased number of Fast Blue–labeled cells in the retinas ipsilateral to the Fast Blue–injected tecta. There were approximately 14 times as many labeled cells in the group that received 1 μmol of L-NOARG (100 ± 14.5 cells per retina) or in the group that received 10 μmol of L-NAME (99 ± 13.4 cells per retina) as in the control group ($P < 0.001$). On the other hand, in embryos that received 10 μmol of D-NAME, few Fast Blue–labeled cells (8 ± 1.2 cells per retina) were found in the ipsilateral retina, with no significant difference from the control group ($P > 0.05$).

The number of Fast Blue–labeled cells present was dose-dependent in both L-NOARG– and L-NAME–treated groups. The higher the dose of NOS inhibitor, the more Fast Blue–labeled cells were found (Fig. 2). The effect of 1 μmol of L-NAME was completely reversed by co-administration of 5 μmol of L-arginine (3 ± 1.6 cells per retina), which indicates that L-NAME exerts a specific effect. Histological sections of these retinas verified that the Fast Blue–labeled cells were present in the ganglion cell layer, which would support the conclusion that these are ganglion cells. These results suggest that NO mediates the refinement of retinal connections in the developing chick.

The activity of NOS was assayed in the tectal tissue of treated and control embryos. The conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was measured in homogenates of tectal tissue according to established protocols (*16*). The activity of NOS was inhibited in embryos treated with L-NOARG or L-NAME but not in control embryos. The maximum effect, 82 ± 5% reduction in activity, was seen in embryos treated with 1 μmol of L-NOARG (Fig. 3). The effect was dose-related, and there was a correlation

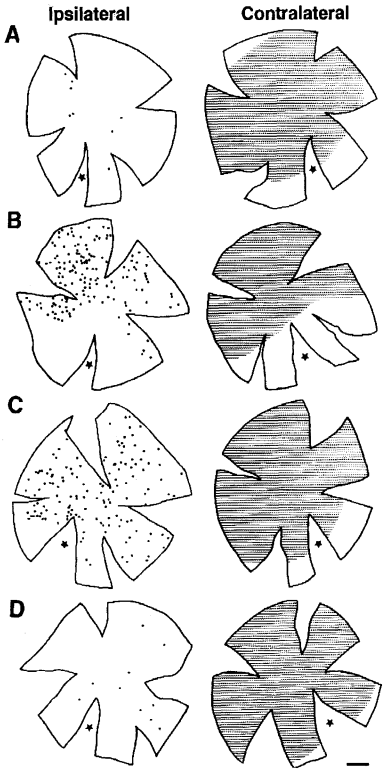

Fig. 1. Plots of Fast Blue–labeled ganglion cells in retinal whole mounts. The retinas on the left are ipsilateral to the tecta injected with Fast Blue. The dots indicate Fast Blue–labeled cells in the ganglion cell layer. The retinas on the right are contralateral to the injected tecta. The shaded areas represent areas with a high concentration of labeled ganglion cells. (**A**) Retinas from an embryo treated with saline; (**B**) retinas from an embryo treated with 1 μmol of L-NOARG; (**C**) retinas from an embryo treated with 10 μmol of L-NAME; (**D**) retinas from an embryo treated with 10 μmol of D-NAME. The star indicates optic fissure. Scale bar, 2 mm.

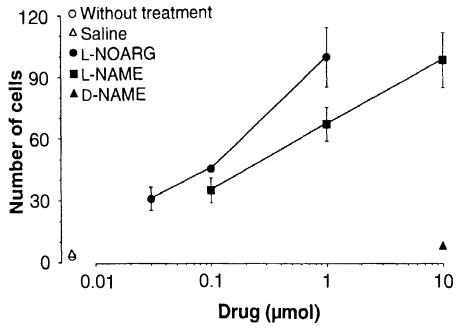

Fig. 2. The dose-related effect of inhibitors of NO synthesis on the persistence of ipsilaterally projecting ganglion cells. The graph shows the number of labeled ganglion cells found in retinas ipsilateral to Fast Blue–injected tecta for the different treatment groups. Each point represents the mean, plus or minus the standard error of the mean (n = 5 embryos per treatment). Embryos received either 150 μl of saline or 150 μl of saline with a drug at the stated concentration daily from E8 to E16.

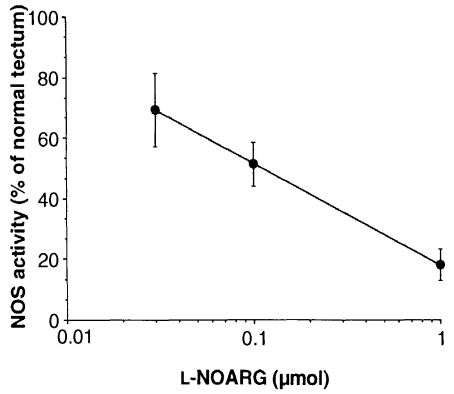

Fig. 3. Dose-related effect of L-NOARG on the inhibition of tectal NOS activity in E13 embryos. The graph shows NOS activity in tectal tissue from embryos treated with L-NOARG compared to the activity in tissue for normal embryos. Each point represents the mean, plus or minus the standard error of the mean (n = 6 embryos per treatment). Tissue for these assays was harvested 4 hours after drug administration. The drug effect was shown to last at least 24 hours.

between the level of inhibition of NOS and the degree of preservation of the ipsilateral projection.

It is possible that systemic administration of inhibitors of NO synthesis could have broad physiological effects on developing embryos. Thus, the changes observed in the ipsilateral retinotectal projection could have been due to indirect effects. Systemic administration of L-NOARG or L-NAME at the doses reported here, however, did not appear to interfere with the general development of the chick embryos. Embryos receiving these drugs appeared healthy during the entire period of the experiment. No differences between experimental and control embryos were found in wet body weights, length of the tecta in the long axis, lamination of the tecta, length of the beaks, or length of the third toes (Table 1), common indicators of chick embryo development (*17*). It has been observed that these inhibitors attenuate resting cerebral blood flow in adult animals (*18*). No change, however, was observed in the diameter of chorioallantoic vasculature upon administration of any of these drugs.

Although inhibition of NO synthesis increased the number of ipsilaterally projecting ganglion cells over 1000% compared to the number in control groups, these treatments did not completely preserve this projection. A maximum of 151 ipsilaterally projecting ganglion cells was found in a retina of an embryo treated with an inhibitor of NO synthesis, approximately 10% of the original projection. There are several possible explanations for why all of the ipsilaterally projecting cells were not rescued. During normal development, approximately half the ganglion cells die, including approximately 60% of the ipsilaterally projecting cells (*13*). It is possible that NO has no role in this cell death and that inhibition of NO synthesis would not rescue cells normally destined to die. It is also possible that sufficient concentrations of NO were generated to allow partial refinement and thus only partial elimination of the ipsilateral projection. It is likely that some NO was still produced in the experimental embryos because the most effective drug treatment reduced NOS activity in the tectum only 82%. Finally, it is certainly possible that multiple mechanisms are active in the refinement process and that not all of these mechanisms involve NO.

The site of action of inhibitors of NO synthesis relative to refinement of the visual projection is not known with certainty. Nitric oxide synthase is expressed in the adult retina (*19*). On the basis of diaphorase staining and a biochemical assay of NOS catalytic activity, NOS is not expressed at significant levels in chick retina until after the refinement of the visual projection (*20*). High concentrations of NOS, however, are expressed in the retino-recipient layers of the tectum during the period in which the projection is refined (*10*). On the basis of these observations and the fact that the number of ipsilaterally projecting cells eliminated during the period of refinement was directly proportional to the level of NOS activity in the tectum, it seems likely that the site of action was the tectum.

These data suggest that NO functions as a retrograde messenger from tectal neurons to retinal axons during development. A model of development, in which NO could serve as a retrograde messenger, has the majority of retinal terminals on any given tectal neuron arising from the contralateral retina; the ipsilateral retina contributes relatively few terminals to any one tectal cell. The major contralateral retinal projection to any given tectal cell is likely to arise from neighboring ganglion cells (*21*), and these cells have synchronous, spontaneous bursts of action potentials in the developing retina (*22*). Because a neuron must reach a certain threshold of depolarization before its NMDA receptors can be activated (*23*), the high number and synchronous firing of the contralateral terminals on a tectal cell make it likely that these terminals depolarize the cell to the point where its NMDA receptors are activated by glutamate released from the retinal terminals (*24*). The ipsilateral projection would fail to activate the NMDA receptors. Activated NMDA receptor channels allow an influx of calcium into the tectal cell. Calcium complexes with calmodulin, which in turn activates NOS in the tectal cell to produce NO (*10, 25*). Because NO can pass freely through cell membranes, it would diffuse from the tectal cell and enter all the retinal terminals on that cell. Several neuronal types express increased concentrations of cGMP in the presence of NO (*6*), but this has not yet been demonstrated for retinal ganglion cells or their terminals. Retinal ganglion cells do express a cGMP-gated calcium channel (*7*), so it is likely that the retinal terminals respond to the presence of NO with an influx of Ca^{2+}. Changes in intracellular Ca^{2+} concentrations lead to changes in axon growth and retraction (*8*) and may also modulate subsequent release of transmitter substances, including glutamate, from the retinal terminal (*26*). This release could change the efficacy of a synapse; such a change has been linked to developmental refinement processes (*27*).

It is unclear how different responses of the ipsilateral and contralateral retinal terminals to NO would result, because both terminal types on a given tectal cell would be exposed simultaneously to NO. The spontaneous activity recorded in developing retinal ganglion cells is characterized by bursts of firing followed by periods of up to 2 min of inactivity (*22*). It is likely that the axons from the contralateral retina at some point have bursts of activity during the inactive periods of the ipsilateral projection. Thus, the active contralateral terminals and the inactive ipsilateral axons would be exposed to NO. It is possible that NO may have a detrimental effect on inactive terminals or a stabilizing effect on the active terminals. The mechanism for such a phenomenon remains to be determined.

Table 1. Inhibitors of NO synthesis did not interfere with general development. Embryos were treated as described in the text. Several anatomical features of the embryos were measured as indicators of general embryological development. The data are presented as the mean (n = 8 embryos per treatment) plus or minus the standard deviation. A one-way analysis of variance indicated that no differences between the groups were present in wet body weight ($P > 0.7$), length of the tecta in the long axis ($P > 0.7$), length of the beaks ($P > 0.4$), or length of the third toes ($P > 0.3$).

Treatment	Body weight (g)	Length of the		
		Tectum (mm)	Beak (mm)	Toe (mm)
No treatment	14.3 ± 0.8	6.4 ± 0.2	4.8 ± 0.2	14.4 ± 0.7
Saline	15.0 ± 0.6	6.6 ± 0.2	4.8 ± 0.2	14.9 ± 0.4
L-NOARG (1 μmol)	15.0 ± 0.8	6.4 ± 0.2	4.4 ± 0.2	14.8 ± 0.3
L-NAME (10 μmol)	15.5 ± 0.6	6.5 ± 0.1	4.6 ± 0.2	15.6 ± 0.4
D-NAME (10 μmol)	14.4 ± 0.8	6.6 ± 0.1	4.7 ± 0.2	14.7 ± 0.3

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Activation of the Sphingomyelin Cycle Through the Low-Affinity Neurotrophin Receptor

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The role of the low-affinity neurotrophin receptor ($p75^{NTR}$) in signal transduction is undefined. Nerve growth factor can activate the sphingomyelin cycle, generating the putative-lipid second messenger ceramide. In T9 glioma cells, addition of a cell-permeable ceramide analog mimicked the effects of nerve growth factor on cell growth inhibition and process formation. This signaling pathway appears to be mediated by $p75^{NTR}$ in T9 cells and NIH 3T3 cells overexpressing $p75^{NTR}$. Expression of an epidermal growth factor receptor–$p75^{NTR}$ chimera in T9 cells imparted to epidermal growth factor the ability to activate the sphingomyelin cycle. These data demonstrate that $p75^{NTR}$ is capable of signaling independently of the *trk* neurotrophin receptor ($p140^{trk}$) and that ceramide may be a mediator in neurotrophin biology.

Nerve growth factor (NGF) controls the survival, development, and differentiation of neurons of the peripheral and central nervous systems (*1*) and interacts with two classes of binding sites. The high-affinity binding site ($K_d \sim 10^{-11}$ M) requires expression of the product of the *trk* proto-oncogene, $p140^{trk}$, which is a receptor tyrosine kinase (*2*, *3*). The low-affinity NGF receptor ($K_d \sim 10^{-9}$ M) is a highly glycosylated, 75-kD transmembrane protein that lacks kinase activity (*4*) and is termed $p75^{NTR}$ (*5*). Although $p140^{trk}$ can mediate NGF-induced effects in the absence of $p75^{NTR}$ (*6*), the functional significance of $p75^{NTR}$ in NGF signal transduction is unresolved. This uncertainty is due, in part, to the lack of evidence coupling $p75^{NTR}$ to activation of signal transduction pathways. Cytokines such as tumor necrosis factor–α (TNF-α) (*7*) or interleukin-1β (*8*) have been shown to activate the sphingomyelin (SM) cycle in a proposed signaling pathway (*7*, *9*). The activation of the SM cycle by ligands that inhibit cell growth and induce differentiation (*10*) led to the hypothesis that some of the effects of NGF may be associated with activation of SM hydrolysis. Moreover, the structural homology between $p75^{NTR}$ and TNF-α receptors (*11*) suggested that NGF might activate the SM cycle through $p75^{NTR}$. We therefore examined the effects of NGF on the SM cycle and the role of $p75^{NTR}$ in this process.

Rat T9 anaplastic glioblastoma cells undergo growth inhibition and differentiation to an astrocyte-like phenotype in response to NGF (*12*). Similar to NGF, the cell-permeable ceramide analog, C_2-ceramide (*13*), induced a dose-dependent decrease in the uptake of [^{3}H]thymidine (Fig. 1). This effect appears to result from growth inhibition and not toxicity because more than 90% of the cells remained viable as determined by exclusion of trypan blue. Similarly, C_2-ceramide also mimicked the effect of NGF on T9 cell morphology and doubled the percentage of cells bearing processes greater than one cell body in diameter (Fig. 2, A and B). To determine if ceramide may be an endogenous mediator, we investigated the effect of NGF on the activation of the SM cycle in T9 cells. Nerve growth factor (50 ng/ml, 1.9 nM) induced a 30% decrease in the amount of sphingomyelin in the cells (corresponding to 5.6 pmol per nanomole of phospholipid phosphate) within 12 min (Fig. 3A). Sphingomyelin hydrolysis was maximal (35 to 40% decrease) at NGF concentrations of 100 to 150 ng/ml (Fig. 3B). Activation of SM hydrolysis in the SM cycle should be accompanied by an increase in cellular ceramide levels (*9*). Indeed, NGF (100 ng/ml) also increased the amount of cellular ceramide to a maximum of 1.9 ± 0.2–fold (4.2 ± 0.8 pmol per nanomole of phospholipid phosphate) at 12 min (Fig. 3A). Thus, most of the hydrolyzed SM is accounted for by the generated ceramide. Both SM and ceramide levels recovered to base line within 20 min. This pattern is reminiscent of responses seen with TNF-α in leukemia cells, although NGF produced a greater relative change in SM and ceramide than did TNF-α (*7*). Thus, NGF activates an SM cycle in T9 cells at concentrations approximating the K_d of $p75^{NTR}$ (1 to 4 nM). Moreover, these concentrations correlated closely with those required for process formation (Fig. 2, A and C). On the other hand, NGF (0.26 ng/ml, 10 pM) was sufficient to induce differentiation of PC12 cells (*14*). These results suggest

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