Inhibition of nitric oxide synthase does not alter ocular dominance shifts in kitten visual cortex

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1. Since nitric oxide has been proposed as a feedback factor in plasticity in the hippocampus, we tested whether it might also be a feedback factor in sensory-dependent plasticity in the cat visual cortex.

2. The effects of monocular deprivation were compared between eight hemispheres with infusion of a nitric oxide synthase inhibitor, and eight control hemispheres with either infusion of the inactive isomer, or no infusion. Although nitric oxide synthase activity was reduced significantly, the ocular dominance histograms were not substantially different in the two groups of animals. We conclude that the feedback factor for sensory-dependent plasticity in the visual cortex is likely to be some factor other than nitric oxide.

Monocular deprivation produces dramatic effects on the anatomy and physiology of the visual cortex during a critical period that lasts from eye opening to puberty (see Wiesel, 1982; Daw, Fox, Sato & Czepita, 1992). After a few days of monocular deprivation at the peak of sensitivity in the critical period, cells in the visual cortex are driven almost entirely by the open eye, with little input from the closed eye. However, monocular deprivation is not simply a decay of the input from the deprived eye. The prime evidence for this is that binocular deprivation also has effects on the visual cortex, but is not simply the sum of two monocular deprivations (Wiesel & Hubel, 1965). The ocular dominance histogram is dramatically shifted in monocularly deprived animals, and is comparatively normal in binocularly deprived animals, suggesting that the good eye pushes out the input from the deprived eye in monocularly deprived animals. Thus competition between inputs to the postsynaptic neuron is an important factor in the process that leads to a rearrangement of the connections in the visual cortex.

The role of the postsynaptic neuron is emphasized by experiments in which one eye is deprived of input for a particular orientation. This affects ocular dominance for cells specific for that orientation, but not for cells specific to the orthogonal orientation (Rauschecker & Singer, 1979). Since cells in the visual cortex are specific for orientation, whereas the inputs from the lateral geniculate nucleus are not, this experiment also suggests that postsynaptic cells in the visual cortex play a role in monocular deprivation, similar to that suggested by Hebb (1949) in his famous postulate for synaptic strengthening as a mechanism of learning.

While these experiments show that the postsynaptic neuron is involved in the effects of monocular deprivation, there are also effects on the presynaptic terminals. A major result of monocular deprivation is retraction of the terminals coming from the lateral geniculate nucleus (LeVay, Wiesel & Hubel, 1980). The logical conclusion is that there should be a feedback factor that is released from the postsynaptic neuron, and affects the presynaptic terminals.

The gas nitric oxide has been suggested as one candidate for such a feedback factor in the visual cortex (Gally, Montague, Reeke & Edelman, 1990). There is also evidence for a feedback factor in long-term potentiation (LTP) in the hippocampus which could be nitric oxide, arachidonic acid, carbon monoxide or some other factor (see Bliss & Collingridge, 1993). Several laboratories have reported that nitric oxide synthase inhibitors block LTP in the hippocampus (Bohme, Bon, Stutzmann, Doble & Blanchard, 1991; O'Dell, Hawkins, Kandel & Arancio, 1991; Schumann & Madison, 1991; Haley, Wilcox & Chapman, 1992). While some laboratories have been unable to replicate this result (see Bliss & Collingridge, 1993), nitric oxide remains a serious candidate for the role of feedback factor in LTP.

NADPH is a cofactor for nitric oxide synthase, and the enzyme NADPH diaphorase appears to be the same as nitric oxide synthase and can therefore be used to visualize the localization of nitric oxide synthase (Hope, Michael, Knigge & Vincent, 1991; Bredt, Glatt, Hwang, Fotuhi, Dawson & Snyder, 1991). NADPH diaphorase is found in the visual cortex. Cells that stain heavily for it are rare, localized primarily in layers II, III, VI and white matter in the macaque monkey (Sandell, 1986) and to layer VI and

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white matter in the cat (Kuchiiwa, Kuchiiwa, Mori & Nakagawa, 1994). However, there are substantial amounts of NADPH diaphorase in the neuropil in all layers (Picanco-Diniz, do Nascimento & Friedlander, 1993; Kuchiiwa *et al.* 1994). We therefore decided to investigate whether nitric oxide might be an important feedback factor in ocular dominance plasticity in the cat visual cortex, by testing whether infusion of nitric oxide synthase inhibitors would reduce or block ocular dominance shifts.

METHODS

Experiments were done on cats under anaesthesia at approximately 6 weeks of age. Twelve animals of both sexes were used, of no particular breed, ranging in weight from 550 to 710 g. The general procedure was as follows. First, a nitric oxide synthase inhibitor was infused into the visual cortex from an Alzet osmotic minipump which delivered 0·5 μl h^{-1}. Next, the eyelids of one eye were sutured together either at the same time as the minipump was implanted, or 2 days afterwards. Ten days after eyelid suture, recordings were made from the visual cortex, and a sample of single cells assayed for ocular dominance. Ocular dominance histograms from cortices infused with a nitric oxide synthase inhibitor were then compared with ocular dominance histograms from control cortices.

Implantation of the osmotic minipump

The minipump was implanted when kittens were 42–46 days old. Animals were given pre-anaesthetic doses of atropine (0·04 mg kg^{-1} I.M.; Elkins-Sinn, Cherry Hill, NJ, USA) and acepromazine (0·1 mg kg^{-1} I.M.; Fermentia, Kansas City, MO, USA). After the animal became sedated, about 10 min later, ketamine (25 mg kg^{-1} I.M.; Aveco, Fort Dodge, IA, USA) was injected. When the animal was relaxed and showed no somatosensory reflexes, a tracheal tube was inserted. Anaesthesia was then maintained with halothane in 66% nitrous oxide–34% oxygen, using a halothane dispenser that had been professionally calibrated by Anesthetic Vaporizer Services (Clarence, NY, USA). The level of halothane was gradually increased to keep the breathing rate below 50 breaths min^{-1}, and the heart rate below 170 beats min^{-1}, in a surgical plane of anaesthesia. The animal was placed in a stereotaxic instrument, and a craniotomy of 2–3 mm diameter was made over the visual cortex centred around P4–6. A hole was made in the dura, and a short-bevel hypodermic needle inserted. The other end of the needle was attached to an Alzet osmotic minipump, containing either the nitric oxide synthase inhibitor, or a control solution. The site of the craniotomy was filled with sterile bone wax, the hole covered with dental cement, and the wound sutured. Next, proparacaine hydrochloride (Alcon, Puerto Rico) was dropped onto the cornea, polymyxin B–bacitracin–neomycin (Pfizer, NJ, USA) eye ointment was applied, the eyelid margins were cut between 2 mm from the medial edge and the lateral edge, then sutured together with 4-0 thread, leaving a small aperture on the medial side for drainage. Lactated Ringer solution (2 ml kg^{-1}; Baxter, Deerfield, IL, USA) was given every hour during surgery. In the second series of experiments, where the eyelid suture was done 2 days after the minipump implantation, the anaesthetic was ketamine (20–30 mg kg^{-1}) with xylazine (0·5 mg kg^{-1}). The animal was watched during recovery. It generally awoke and started moving after 30 min to an hour. It was rotated from side to side during this time, if necessary. Proparacaine hydrochloride was applied to the lid margins if it was rubbing the sutured eye. When it was continually awake, which occurred approximately 2 h after surgery, it was returned to its mother. The animal was checked daily after that to monitor weight gain and make sure that the lid margins and head suture were healing properly.

Physiological recordings

Ten days later, recordings were made from both visual cortices. The animal was given pre-anaesthetic doses of acepromazine (0·1 mg kg^{-1} I.M.) and atropine (0·04 mg kg^{-1} I.M.), then anaesthesia was induced with 4% halothane in 34% oxygen–66% nitrous oxide in a box. A tracheal tube was inserted, then anaesthesia was maintained with 0·7–1·2% halothane, the depth being judged by heart rate (maintained at 150–170 beats min^{-1}) and absence of somatosensory reflexes. An intravenous cannula was placed into the femoral vein, the head was placed in a stereotaxic instrument, and the skull and dura were opened. Wound margins were injected with 2% lidocaine (lignocaine) hydrochloride (Butler, Columbus, OH, USA), and ear bars covered with viscous 2% lidocaine (Roxane, Columbus, OH, USA). Lactated Ringer solution was infused (1·2 ml h^{-1}) through the intravenous cannula. Following surgery, paralysis was induced with pancuronium bromide (0·35–1 mg kg^{-1} h^{-1}; Elkins-Sinn). The level of anaesthesia found to be adequate for surgery was maintained during the rest of the experiment, lasting 12–30 h, and heart rate was monitored continually, with the percentage of halothane being increased for any increase in heart rate above the range found during surgery. Body temperature was maintained at 37–38 °C by a thermostatically controlled heating pad, and end-tidal CO_2 at 3·7–4·2% by adjusting the rate and/or volume of respiration.

The eyes were focussed on a tangent screen at 145 cm (1 radian) by lenses of zero power and appropriate curvature. The nictitating membrane was withdrawn by a drop of 10% phenylephrine hydrochloride (Iolab, Claremont, CA, USA) and the pupils dilated with a drop or two of 0·01% atropine. A tungsten electrode (Hubel, 1957) was inserted into the cortex for recordings from single cells.

Receptive fields were characterized using a light projector moved by hand. The preferred width and length of stimulus, preferred orientation, and velocity and direction of movement were all determined, then the responses in the two eyes were compared. Two observers independently assigned an ocular dominance according to the 7 point scale introduced by Hubel & Wiesel (1962). About four penetrations were made in each cortex, spaced 0·5–2 mm apart between 0·2 and 6 mm from the tip of the needle attached to the minipump. Cells were sampled every 150–200 μm. The electrode was angled at 45 deg to the vertical, slanting anteriorly, to sample as many layers and columns as possible. Lesions (3 μA DC current for 10 s) were made so that the layer for each cell recorded could be identified.

In the first series of experiments, the brain was kept for histology and the minipump was removed before recording. At the end of the recordings, the level of halothane was raised to 4% until the heart slowed by 10%, then the chest was opened, and the animal was perfused through the heart with 100 ml phosphate-buffered saline given over 1 min followed immediately by 4% paraformaldehyde. Frozen sections of the visual cortex 60 μm thick were cut and stained with Cresyl Violet; the penetrations were then reconstructed and the layer in which each cell was recorded was determined according to the layering criteria described by Kelly & Van Essen (1974).

In the second series of experiments, the minipump was kept attached to the brain during recording and the brain was prepared for an assay of nitric oxide synthase activity. At the end of the recordings, the halothane level was raised to 4% until the heart

Table 1. Weighted ocular dominance index for cells in different cortical layers

		L-NAME-treated animals	
Layers	Control animals	< 4 mm from cannula	> 4 mm from cannula
I–III	0·93	0·74	0·69
IV	0·78	0·65	0·47
V	0·90	0·79	0·81
VI	0·89	0·93	0·89

slowed by 10%, then the chest was opened, and the animal was perfused through the heart with oxygenated phosphate-buffered saline for about 1 min, then the animal was immediately decapitated. The visual cortex was blocked off from the rest of the brain on a stereotaxic instrument. The locations of the minipump cannulae were noted, then the crown of the postlateral gyrus was removed and sliced into 1 or 2 mm-thick pieces. Each cortical slice was put into lysis buffer (300 mM sucrose, 10 mM Hepes (pH 7·5), 0·1 mM EDTA, 1% NP-40, 1 mM dithiothreitol, 10 μg ml^{-1} leupeptin, 2 μg ml^{-1} aprotinin, 10 μg ml^{-1} soybean trypsin inhibitor and 1 mM phenylmethylsulphonyl fluoride), frozen and stored at −75 °C. These methods were all approved by the Yale University Animal Care and Use Committee.

Nitric oxide synthase assay

Coded cortical slices were placed into cold lysis buffer and rotated at 4 °C for 2 h. Lysates were centrifuged at 50 000 *g* for 30 min at 4 °C and detergent-soluble extracts were used for nitric oxide synthase (NOS) activity measurements. NOS activities were assayed by determining conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline as previously described (Sessa *et al.* 1992). Briefly, lysates (20–50 μg of protein) were incubated (total volume of 25 μl) in lysis buffer containing 1 mM NADPH, 3 μM tetrahydrobiopterin, 100 nM calmodulin, 2·5 mM $CaCl_2$, 10 μM L-arginine and L-[^{3}H]arginine (0·2 μCi, specific activity = 55 Ci mmol^{-1}) for 30 min at 37 °C. After the incubation period, the reaction was quenched by the addition of 1 ml of 20 mM Hepes stop buffer (containing 2 mM EDTA and 2 mM EGTA, pH 5·5). The reaction mix was then passed over a 1 ml column containing Dowex AG 50WX-8 (Na^+ form) resin (Sigma) (pre-equilibrated in stop buffer), washed with 1 ml of water and collected directly into a 20 ml liquid scintillation vial containing scintillation cocktail for counting. Cortical samples were split into duplicate samples and run in the absence and in the presence of 1 mM nitro-L-arginine, an inhibitor of NOS, and data presented as nitro-L-arginine inhibitable generation of L-citrulline (pmol (mg protein^{-1})).

Data analysis

Weighted ocular dominance (WOD) was calculated from the ocular dominance histograms according to the formula:

$$\frac{1/6N_2 + 2/6N_3 + 3/6N_4 + 4/6N_5 + 5/6N_6 + N_7}{N_1 + N_2 + N_3 + N_4 + N_5 + N_6 + N_7},$$

where N_i is the number of cells in ocular dominance group i (Kasamatsu, Pettigrew & Ary, 1981). WOD has the value 0 when all cells are driven by the contralateral eye, 1 when all cells are driven by the ipsilateral eye, and 0·5 when cells on average are driven equally by both eyes. The value is 0·44 in normal animals, reflecting a slight dominance by the contralateral eye (Daw *et al.* 1992). To make statistical comparisons between groups, each animal was treated as one observation, and a Student's two-tailed t test done to compare WOD values from the groups.

RESULTS

At the completion of the physiological recordings, one has a choice of perfusing the brain for reconstruction of the electrode tracks, or freezing the brain for assay of nitric oxide synthase activity. We opted, in the first series of experiments, to perfuse the brain, hoping that we would find a large effect, and that we could analyse the results to determine if the effect varied with layer. When results from the first series of experiments turned out to be ambiguous, we did a second series, in which the brains were frozen for a NOS assay to test the depletion of NOS activity by the inhibitor.

First series

Four animals had an Alzet osmotic minipump implanted into the left hemisphere. The minipump was implanted at 44–46 days, with eyelid suture on the same day, and recordings made 10 days later. This protocol was adopted so that the results could be compared with control animals that had previously been recorded using the same length of monocular deprivation, starting at the same age. In all animals the eyelids of the right eye were sutured together, and recordings were made from the left cortex, ipsilateral to the open eye. It is not valid to compare the cortex ipsilateral to the open eye with the cortex contralateral to the open eye, because one starts with a bias in favour of the contralateral eye in the normal animal which also biases the results after monocular deprivation (see Daw *et al.* 1992). The cortex ipsilateral to the open eye was used because a larger ocular dominance shift can be obtained in this cortex (WOD can shift from 0·44 to 1, rather than from 0·44 to 0), and this gives more room to compare experimental and control animals.

The minipump in the first animal contained 1 M *N*-nitro-L-arginine methyl ester (L-NAME). Some precipitate was seen, and in subsequent animals, after tests to establish the largest concentration that would stay in solution, 50 mM L-NAME was used.

There was some difference in the ocular dominance histograms taken from the control animals, and those taken from experimental animals (Fig. 1). WOD in the four control animals was 0·85, 0·815, 0·919 and 0·995. WOD in the four experimental animals was 0·76, 0·723, 0·79 and 0·70. However, when penetrations in the experimental animals were inspected for any evidence of a variation in ocular

dominance with distance from the minipump, none was seen (Table 1). The difference between treated and control animals occurred mainly in layers II, III and IV. However, there was no sign that penetrations close to the cannula (< 4 mm) had a flatter ocular dominance histogram than penetrations far from the cannula (> 4 mm): if anything, the reverse was true. We regarded this as an ambiguous result, and therefore decided to record a second series of animals to test whether NOS activity had been significantly depleted.

Second series

Four animals each had two minipumps implanted into their cortices, one into each cortex, at 42–43 days of age. In each animal, one minipump contained 50 mM L-NAME (the active isomer), and one minipump contained 50 mM D-NAME (the inactive isomer). The minipumps were coded by a faculty member not involved in the experiments (Dr T. Hughes), so that the experimenters would not know which cortex was being infused with the active isomer. Because of the asymmetry between results expected from ipsilateral and contralateral cortices, he arranged it so that

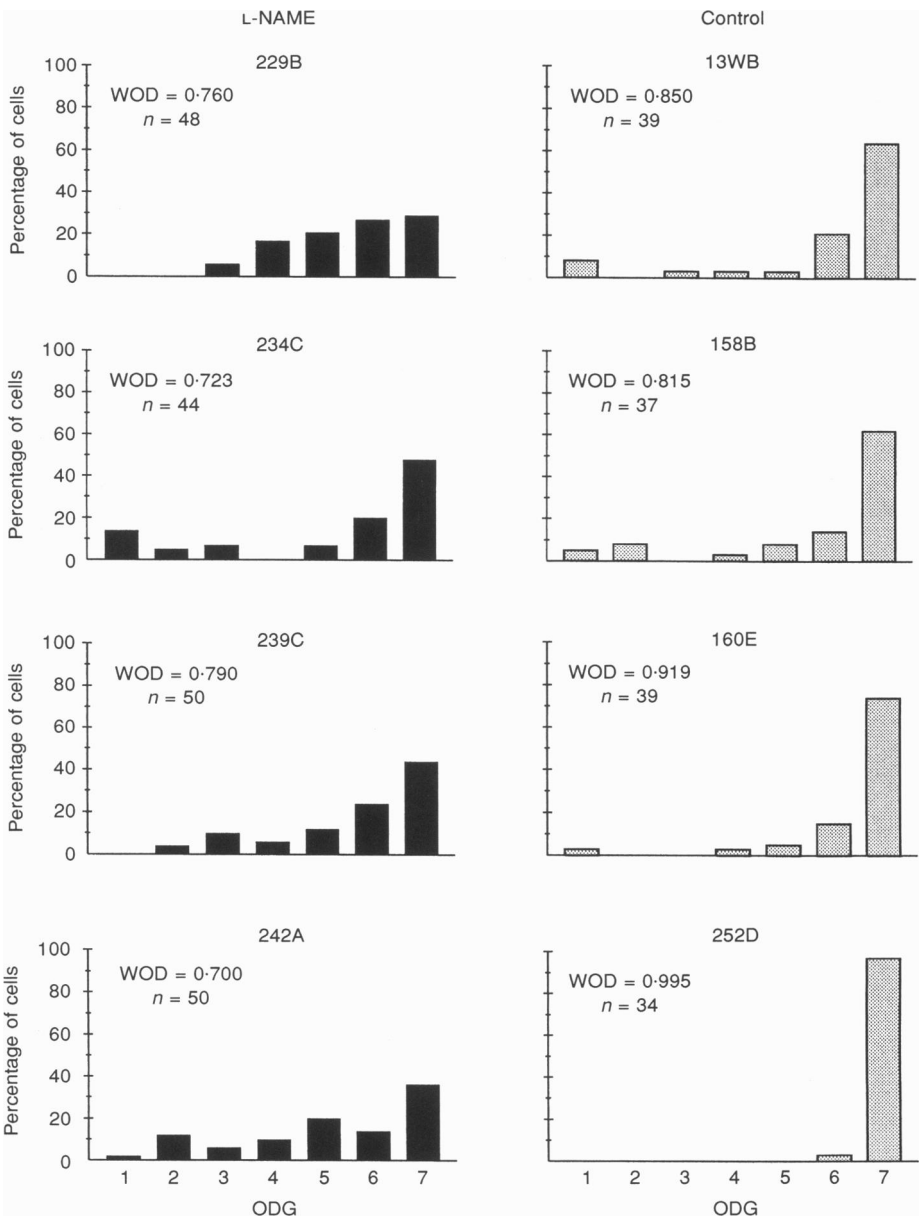

Figure 1. Ocular dominance histograms from four animals with L-NAME infused into the cortex, compared with histograms from four control animals

Ocular dominance groups (ODG) assigned according to the criteria of Hubel & Wiesel (1962). All animals were monocularly deprived for 10 days, starting at 44–46 days of age. For each animal weighted ocular dominance is given (WOD), and the number of cells (n) recorded.

the active isomer would be in the left cortex in two cases, and in the right cortex in two cases. In all cases the eyelids of the right eye were sutured together 2 days after minipump implantation, and recordings were made from both hemispheres 10 days later.

There was little difference between the ocular dominance histograms from cortices treated with the active isomer, and those treated with the inactive isomer (Fig. 2). WOD in cortices treated with the active isomer were 0·76, 0·908, 0·941 and 0·938. WOD in cortices treated with the inactive isomer were 0·856, 0·966, 0·815 and 0·867. Within each animal, the WOD in the cortex contralateral to the open eye (right hemisphere) was higher than the WOD in the cortex ipsilateral to the open eye (left hemisphere), as expected from the bias in favour of the contralateral eye. Infusion of the active isomer was never enough to overcome this tendency.

When cortices were assayed for NOS activity, this was found to be reduced in the cortex with the active isomer compared with the cortex with the inactive isomer. The amounts were (in pmol mg^{-1}): 9 (experimental) compared with 14 (control) for animal 255D; 18 compared with 36 for animal 256D; 3 compared with 13 for animal 257B and 11 compared with 31 for animal 259A. Our conclusion,

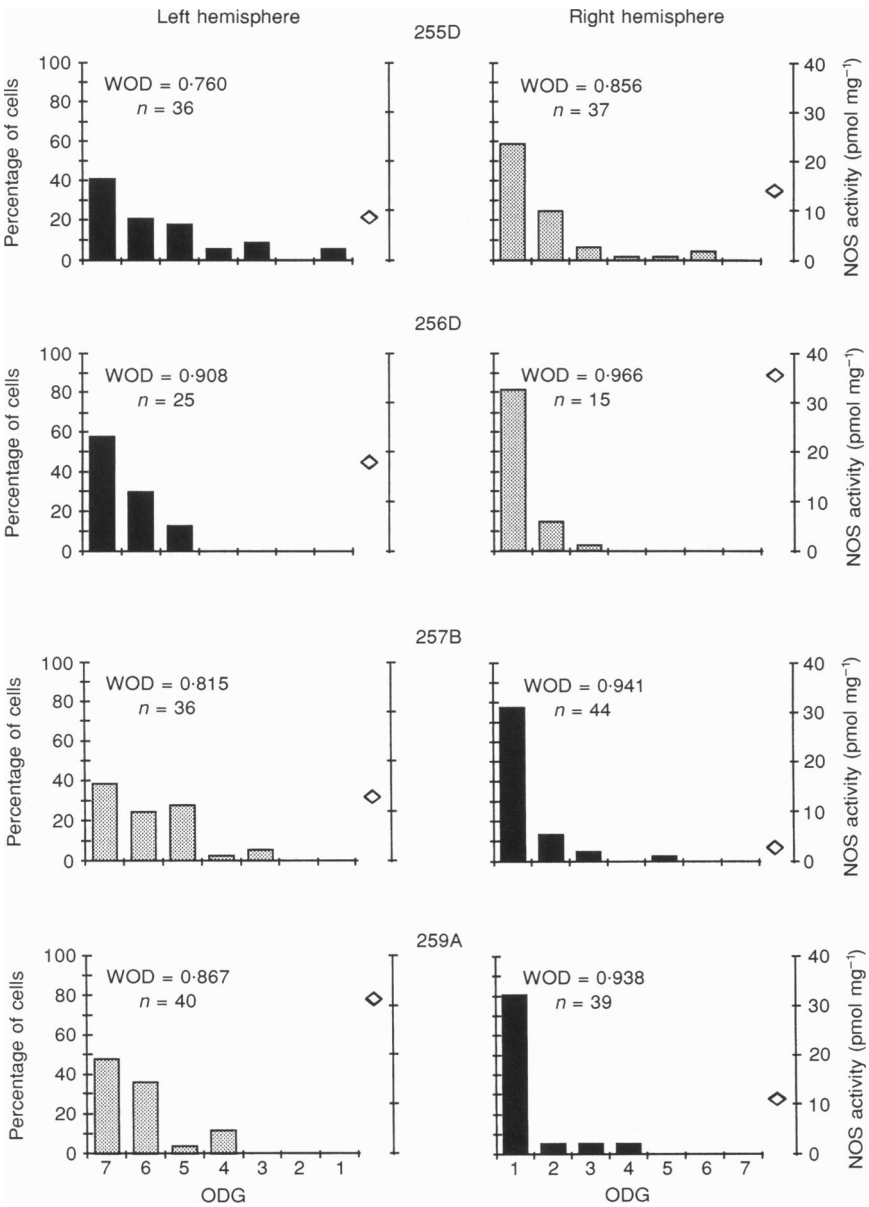

Figure 2. Ocular dominance histograms from four animals, each with L-NAME (active isomer) infused into one cortex, and D-NAME (inactive isomer) infused into the other

All animals were monocularly deprived for 10 days, starting at 42–43 days of age. Weighted ocular dominance and number of cells ae given, as in Fig. 1. NOS activity is the averaged activity within 7 mm from the minipump cannula. ■, L-NAME; ▨, D-NAME; ◇, NOS activity.

therefore, is that a treatment that reduces nitric oxide synthase activity by a substantial amount does not produce a significant change in the ocular dominance shift that normally results from monocular deprivation.

DISCUSSION

We have shown that reduction of NOS activity does not have a significant effect on the ocular dominance shifts that result from monocular deprivation. Strictly speaking, since nitric oxide is a candidate for a feedback factor in the system, one ought to look at the effect on the presynaptic terminals. The same point could be made about experiments investigating the role of nitric oxide in LTP. However, any functionally significant effect on presynaptic terminals should be measurable as an effect on the ocular dominance histogram recorded from postsynaptic cells.

Our result is consistent with recent evidence on the time course of the development of NADPH diaphorase activity in the rat cortex. Yan, Garey & Jen (1994) have shown that NADPH diaphorase-positive cells develop early. The number and laminar pattern of these cells, as well as staining in the neuropil, reached adult levels by the second postnatal week. This is the period over which cells in the rat cortex are differentiating and there is active formation of synapses (Juraska & Fifkova, 1979*a*, *b*). The critical period for sensory-dependent plasticity, which involves a rearrangement of connections after they are first formed, occurs later than this (Fagiolini, Pizzorusso, Berardi, Domenici & Maffei, 1994). Thus, nitric oxide is more likely to play a role in early events in development, than in sensory-dependent plasticity.

Nitric oxide also plays a role in vascularization (Iadecola, 1993) and control of transmitter release (Montague, Gancayco, Winn, Marchase & Friedlander, 1994). Both these processes have been studied in the adult, and there is no evidence currently that they are more active in young animals at the peak of the critical period for sensory-dependent plasticity. Nitric oxide also has effects in the lateral geniculate nucleus (Cudeiro, Rivadulla, Rodriguez, Martinez-Conde, Acuna & Alonso, 1994) where it is found in presynaptic terminals of afferents from the parabrachial nucleus (Bickford, Gunluk, Guido & Sherman, 1993), and it selectively suppresses NMDA responses (Cudeiro *et al.* 1996). Our results do not address these modulatory effects of nitric oxide.

Results on the role of nitric oxide in the hippocampus have recently become controversial. While NOS inhibitors affect LTP *in vitro* (see Introduction), they do not affect spatial learning or LTP *in vivo* (Bannerman, Chapman, Kelly, Butcher & Morris, 1994*a*, *b*). These authors found more general effects on the physiology and behaviour of the animal, but effects specific to LTP and hippocampal-based learning were not apparent. Our results, and the results of Ruthazer, Gillespie, Dawson, Snyder & Stryker (1996), suggest that a similar conclusion can be reached about the visual cortex. There may be general effects on the physiology of the cortex, and nitric oxide may play a role in sensory-dependent plasticity in conjunction with some other factor, but a specific effect on plasticity, associated with a change in the nitric oxide system that correlates with the peak of the critical period for sensory-dependent plasticity, has not been established.

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