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"Disruption of retinogeniculate afferent segregation by antagonists to NMDA receptors"

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TABLE 1   Axons in normal and in APV treated animals

	No. of animals	No. of axons	Mean arbor area in μm^2 (s.e.)	Mean sublamina index (s.e.)
Normal 2-week-old	3	12	5250 (601)	0.70 (0.01)
Normal 3-week-old	3	16	8155 (653)	0.88 (0.05)
D-APV-treated, 3-week-old	3	12	7959 (807)	0.66 (0.03)

The arbors that were examined lay within the central two-thirds of the LGN, where the thickness of the A-laminae is roughly uniform, and their locations were judged to be between 0 and 60 degrees in azimuth and ±30 degrees in elevation[24]. In this region, in each animal, every arbor that could be fully reconstructed was drawn; we did not sample or select arbors (this procedure led to 2–7 reconstructed axons per animal in each group). The arbor reconstructions were made without knowledge of laminar borders or sublaminar halves. The axons in each of the three groups spanned similar ranges of LGN locations. In each group, there were nearly equal numbers of arbors in the binocular and monocular portions of the LGN, and there were no differences between these axons. The area of an arbor was measured as the area covered by the outermost branch tips in the two-dimensional reconstruction of the arbor. The 'sublamina index' was obtained from the position of the arbor with respect to a line bisecting liamina A or A1 parallel to laminar borders. The bisected laminar halves closely approximate sublaminae; the sublamina index varies between 0.5 and 1, being 1 for an arbor that was entirely confined to one half of lamina A or A1 and 0.5 for an arbor that was located exactly midway between laminar borders. Statistical comparisons employed the Mann–Whitney U-test and were made by: (1) pooling the axons from each animal and treating each animal as a single datum, (2) treating each axon as a single datum.

Disruption of retinogeniculate afferent segregation by antagonists to NMDA receptors

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AFFERENT activity has an important role in the formation of connections in the developing mammalian visual system[1,2]. But the extent to which the activity of target neurons shapes patterns of afferent termination and synaptic contact is not known. In the ferret's visual pathway, retinal ganglion cell axons from each eye segregate early in development into eye-specific laminae in the lateral geniculate nucleus (LGN)[3]. The dorsal laminae (termed laminae A and A1) then segregate further into inner and outer sublaminae that retain input from on-centre and off-centre retinal axons, respectively[4,5]. Thus, individual retinogeniculate axons form terminal arbors within laminae A and A1 that are restricted to one inner or outer sublamina[6]. We report here that blockade of N-methyl-D-aspartate (NMDA) receptors on LGN cells with specific antagonists during the period of sublamina formation prevents retinal afferents from segregating into 'On' and 'Off' sublaminae. Retinogeniculate axons have arbors that are not restricted appropriately, or are restricted in size but inappropriately positioned within the eye-specific laminae. NMDA receptor antagonists may specifically disrupt a mechanism by which LGN neurons detect correlated afferent and target activity[7], and have been shown to reduce retinogeniculate transmission more generally[8–10], causing LGN cells to have markedly reduced levels of activity. These results therefore indicate that the activity of postsynaptic cells can significantly influence the patterning of

inputs and the structure of presynaptic afferents during development.

Experiments were done on 32 ferret kits. Timed pregnant ferrets were either purchased from Marshall Research Animals or bred in our colony. We first examined, in normal ferret kits, the time course of segregation of retinogeniculate afferents into eye-specific laminae and 'On' and 'Off' sublaminae. At birth (embryonic day 41) axons from the two eyes overlap extensively in the LGN[3]. Axon arbors start to segregate into eye-specific laminae by the first postnatal week, and laminar segregation is essentially complete by two weeks (Fig. 1a; n = 2). Further division of laminae A and A1 into sublaminae occurs during the third postnatal week, and sublaminae can be clearly identified by three weeks (Fig. 1b; n = 3).
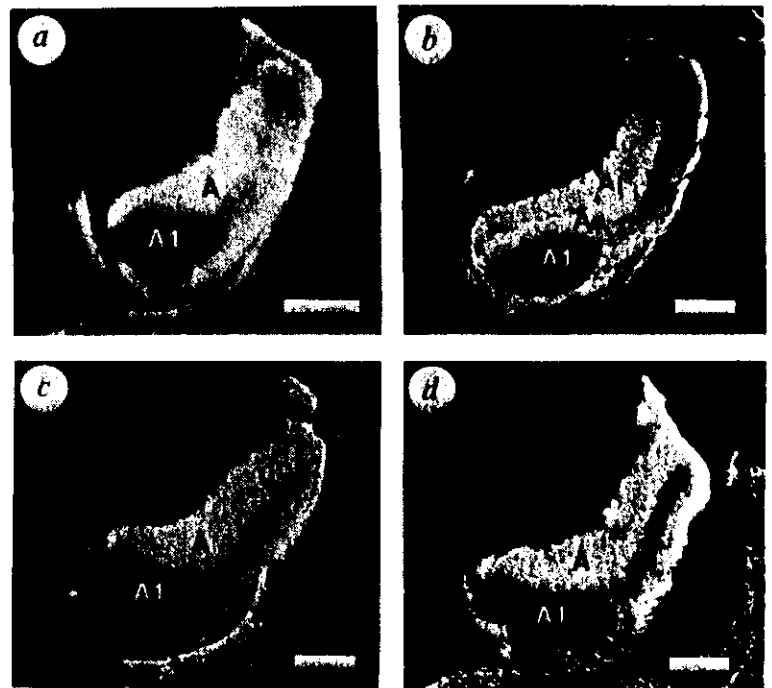
We next examined the effect on sublaminar development of blocking NMDA receptors on LGN cells between two and three weeks of age. Antagonists were delivered using osmotic minipumps either directly into the thalamus, near the LGN, or systemically using subcutaneous infusion. We first assayed the effect of the antagonists using intraocular injections of the anterograde tracer wheat-germ agglutinin conjugated to horseradish peroxidase. Blockade of NMDA receptors in the LGN with D-APV (D-2-amino-5-phosphonovaleric acid) delivered into the posterior thalamus, or with MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate) delivered subcutaneously, blocks the formation of On and Off sublaminae (Fig. 1c, d). These effects are dose-dependent. Although D-APV delivered at a minipump concentration of 0.8 mM (n = 3) blocks sublamina formation (Fig. 1c), a concentration of 0.08 mM does not (n = 2). MK-801 blocks sublamina formation at a concentration of 4.75 mM (Fig. 1d; n = 2) but not at 1.2 mM (n = 2). In contrast, thalamic infusion of 0.8 mM L-APV (the inactive isomer of APV) has no effect on sublaminae (n = 2). Similarly, thalamic (n = 2) or subcutaneous (n = 1) infusion of the saline vehicle has no effect on the formation of sublaminae.

We next examined the morphology of single retinal axon arbors in the LGN by filling optic tract axons with horseradish

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FIG. 1 Dark-field photomicrographs of horizontal sections from the right LGN in ferrets in which the left eye was injected with the tracer wheat-germ agglutinin conjugated to horse-radish peroxidase (WGA-HRP) to label retinogeniculate termi-nations. a, LGN from a normal two-week-old animal, showing that projections from the contralateral eye to the dorsal layers of the LGN are restricted to lamina A, whereas lamina A1, which receives projections from the ipsilateral eye, is free of labelled terminations. b, LGN from a normal three-week-old animal, showing that the projections from the contralateral eye to lamina A have segregated further into inner (Ai) and outer (Ao) sublaminae. c, LGN from a three-week-old animal that received thalamic infusion of 0.8 mM D-APV, a competitive antagonist of NMDA receptors, from two weeks to three weeks of age. Sublaminae within lamina A, which would normally be present by this age, now fail to form. d, LGN from a three-week-old animal that received subcutaneous infusion of 4.75 mM MK-801, a noncompetitive antagonist of NMDA receptors, from two weeks to three weeks of age. Sublaminae fail to form within lamina A. Bars, 250 μm.

METHODS. Osmotic minipumps (Alzet model 2001, infusion rate 1 μl h^{-1}) were implanted into two-week-old ferret kits. Each minipump contained one of the following (in saline): 0.8 mM and 0.08 mM D-APV, 0.8 mM L-APV, 4.75 mM and 1.2 mM MK-801, as well as isotonic saline. D-APV, L-APV and saline were infused into the thalamus, whereas MK-801 and saline were infused subcutaneously. For thalamic infusion, each minipump was connected via a catheter (a piece of polyvinyl tubing 1.5 cm long) to a 7-mm-long 28-gauge stain-less steel cannula (Plastics One). After anaesthesia with ketamine (4 mg per 100 gm body weight) and metofane (1–2%), the skin over the skull was incised and separated from subcutaneous tissue at the base of the neck to create a pocket for the minipump. A small hole was drilled in the skull, the dura punctured and the cannula inserted into the brain with the tip resting in the thalamus rostral and medial to the LGN. The cannula was glued to the skull with cyanoacrylate glue and cemented with dental acrylic. Subcutaneous infusion was done by inserting a minipump near the base of the skull. The skin was sutured and the animal returned to the mother. After six days, the animal received an injection of WGA-HRP in the eye contralateral to the thalamic implant. After another 24 h of
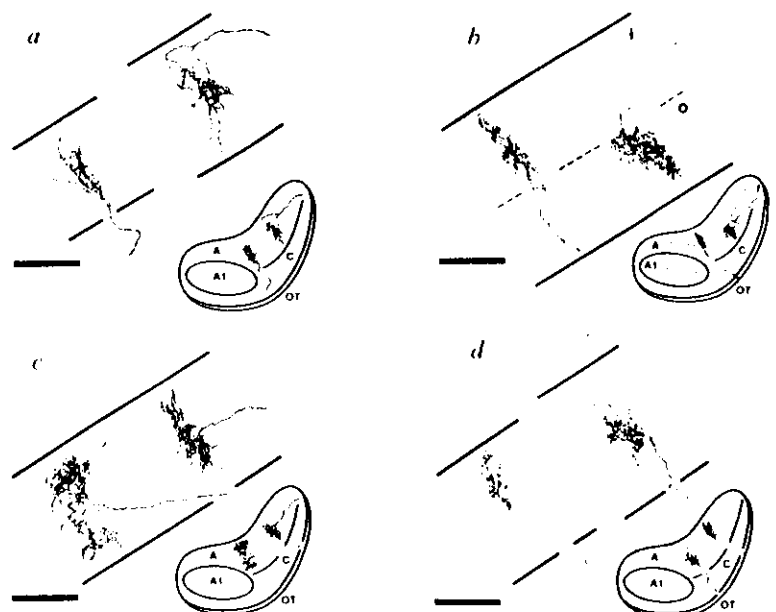
survival, animals were perfused with aldehydes, the brain cut at 50 μm in the horizontal plane and sections reacted with tetramethyl benzidine for visualizing the HRP reaction product. In animals treated with D-APV, we estimate the concentration of D-APV in the LGN as 5–10 μM, based on the distance of the cannula tip from the centre of the LGN and the dilution curve obtained by Bear et al.[15]. This concentration is adequate to selectively attenuate the NMDA component of optic tract evoked excitatory postsynaptic potentials recorded intracellularly in slices of the LGN from adult and developing ferrets (refs 22, 23; M. Esguerra, C. A. White and M.S., unpublished observations).

peroxidase in vitro. In normal ferret kits at two weeks of age (n = 3), individual retinogeniculate axon arbors span one eye-specific lamina (Fig. 2a). By three weeks (n = 3), when On and Off sublaminae have formed, individual arbors are restricted to one sublamina (Fig. 2b). Application of D-APV during the third postnatal week (n = 3) alters the morphology of individual axon

arbors, leading to arbors that differ systematically from normal three-week arbors (Fig. 2c, d). At one extreme, some arbors span the height of an eye-specific lamina and fail to restrict appropriately into On and Off sublaminae (Fig. 2c). At the other extreme, arbors restrict in size such that they would fit in sub-laminae, and some are appropriately positioned at the inner or

FIG. 2 Horseradish peroxidase-labelled retinal ganglion cell axon arbors within the LGN of normal ferrets and ferrets treated with D-APV. a, Axons in a normal two-week-old animal; arbors span nearly the entire extent of lamina A (or, not shown, lamina A1). b, Axons in a normal three-week-old animal; arbors are restricted to an inner (i) or outer (o) sublamina within lamina A (or, not shown, lamina A1). Dashed lines mark sublaminar borders. c, d, Axons in three-week-old animals treated with 0.8 mM D-APV from two weeks to three weeks of age. Some axons (shown in c) span the height of lamina A. Other axons (shown in d) are restricted in size but are positioned either normally, at the inner or outer half of lamina A, or abnormally, at the center of lamina A. Bars, 100 μm and apply to large-scale axon drawings.

METHODS. Minipumps filled with 0.8 mM D-APV were implan-ted into two-week-old ferret kits as described in the legend to Fig. 1. After one week of survival, animals were deeply anaesthetized with sodium pentobarbital and transcardially perfused with cold (4 °C) artificial cerebrospinal fluid. The brain was quickly removed, the cortex dissected away, and the thalamus exposed and bisected. Small deposits of horse-radish peroxidase were placed in the optic tract ventral to the LGN. The tissue was maintained in artificial cerebrospinal fluid at room temperature for 4–6 h to allow transport of horseradish perioxidase to axon terminals, then fixed by immersion and sectioned at 100 μm in the horizontal plane. Sections were reacted with diaminobenzidine for visualizing

horseradish peroxidase. Axon arbors were reconstructed from serial sections under a camera lucida, using a ×63 objective for transmitted light and a ×100 objective for phase contrast microscopy.

outer half of an eye-specific lamina, as in normal animals (Fig. 2*d*). Many of the restricted arbors, however, are inappropriately positioned in the eye-specific laminae, being located towards the centre of the lamina rather than in the inner or outer half (Fig. 2*d*).

In normal animals, arbor areas increase between two and three weeks of age (Table 1, $P < 0.05$, Mann-Whitney U-test, treating each animal as a single datum; $P < 0.01$ treating each axon as a single datum). In animals treated with D-APV, arbor sizes are intermediate between two-and three-week-old normal animals. We defined a 'sublamina index' as the proportion of an arbor's area lying in the half (either inner or outer) of lamina A or A1 containing the greatest portion of that arbor. A sublamina index of 0.5 thus represents a total absence of sublamination (equal division of an arbor between inner and outer halves), and 1.0 signifies complete restriction to a single sublamina. The index increases between two and three weeks in normal animals (Table 1, $P < 0.05$ comparing individual animals; $P < 0.01$ comparing individual axons), consistent with the segregation of arbors into On and Off sublaminae during this period. In D-APV treated animals, the index is similar to normal two-week-old animals but lower than in normal three-week-old animals ($P < 0.05$ comparing individual animals; $P < 0.005$ comparing individual axons), consistent with arbors that vary from being less restricted in size with respect to the A-laminae to being restricted but located inappropriately within the laminae.

The effects of NMDA receptor blockade on the development of axon arbors have not been examined previously in the mammalian brain, or in any other system in which the receptor is involved directly in synaptic transmission and in regulating the level of postsynaptic neuronal activity. In amphibia, NMDA receptor antagonists disrupt the segregation of eye-specific stripes in experimentally created retinal projections to the tectum[11]. Individual retinotectal afferents in these animals have arbors that are more dispersed than normal[12] and have reduced branch density[13]. Physiological effects of NMDA receptor blockade during development include disruption of the alignment of maps from the two eyes in the amphibian tectum[14], and prevention of ocular dominance plasticity in the kitten visual cortex[15] (see, however, refs 16-19). Infusion of tetrodotoxin into the thalamus alters the morphology of retinogeniculate terminations in fetal cats[20,21], indicating a role for activity-dependent mechanisms in shaping afferent arbors. Tetrodotoxin, however, acts by blocking action potentials, and would do so both pre- and postsynaptically when infused intrathalamically.

NMDA receptor antagonists in the developing ferret LGN may specifically disrupt a mechanism detecting temporal correlations between retinal axon and LGN cell activity[7]. More generally, blockade of NMDA receptors during development reduces retinogeniculate transmission and the activity of postsynaptic LGN cells. Iontophoresis of NMDA antagonists in adult cats severely reduces the visual and background responses of LGN cells[8-10]; NMDA receptors contribute substantially to the optic tract evoked excitatory postsynaptic potential in cells recorded *in vitro* from the LGN of adult[22,23] and developing ferrets (M. Esguerra, C. A. White and M.S., unpublished observations). Thus the effects we observe on retinogeniculate axon arbors suggest strongly that the structure of retinal afferents in general, and the restriction of retinal afferents into On and Off sublaminae in the ferret LGN in particular, is regulated significantly by the activity of LGN cells during development.

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