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were added to the perfusion medium and were present for at least 15 min before tetanic stimulation. In the case of muscarine, washing out was started immediately after the tetanus had been applied. In experiments involving APV, the drug was present during and after tetanus. Traces shown were digitized and represent an average of four to ten traces.

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Prenatal Tetrodotoxin Infusion Blocks Segregation of Retinogeniculate Afferents

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In the adult mammalian visual system, ganglion cell axons from the two eyes are segregated from each other into separate layers within their principal target, the lateral geniculate nucleus. The involvement of spontaneously generated action potential activity in the process of segregation was investigated during the fetal period in which segregation normally occurs in the cat, between embryonic day 45 (E45) and birth (E65). Tetrodotoxin, which blocks the voltage-sensitive sodium channel, was used to prevent action potentials. Fetuses received continuous intracranial infusions of tetrodotoxin from osmotic minipumps implanted in utero on E42. After a 2-week infusion, intraocular injections of anterograde tracers revealed that tetrodotoxin prevented segregation. The contralateral projection filled the lateral geniculate nucleus uniformly, and the ipsilateral projection expanded to occupy most of what would normally be contralaterally innervated layer A. Thus, in the fetus, long before the onset of vision, spontaneous action potential activity is likely to be present in the visual system and to contribute to the segregation of the retinogeniculate pathway.

DURING THE DEVELOPMENT OF the vertebrate nervous system, the precise pattern of connections present in the adult often emerges from an initially diffuse set of connections. For example, in the adult mammalian visual system, inputs from the two eyes are segregated from each other into separate layers within the lateral geniculate nucleus (LGN) and into separate patches, the ocular dominance columns, within layer 4 of the primary visual cortex (1). However, the initial connections within each of these structures are not segregated. Genuiculocortical axons serving the two eyes first make intermingled functional connections that drive cortical cells in layer 4 binocularly (2). When retinal ganglion cell axons from the two eyes first grow into the LGN, they are also intermixed (3). The eye-specific layers emerge gradually as the terminal arborizations of each eye's axons expand selectively in territory appropriate to the eye of origin, while branches located in inappropriate territory are lost (4).

Although the mechanisms responsible for this process of segregation are not well understood, several lines of evidence suggest that neuronal activity, such as patterns of action potentials and synaptic transmission,

may play a role (5). Formation of ocular dominance columns in layer 4 of the cat's visual cortex, for example, can be prevented by eliminating retinal ganglion cell discharges with intraocular injections of the voltage-sensitive Na⁺ channel blocker tetrodotoxin (TTX), but not by dark-rearing (6), which does not abolish the spontaneous activity of ganglion cells. These and other findings are consistent with the notion that spontaneous neuronal discharge is important for the refinement of neural connections.

We investigated the possibility that spontaneous activity plays a role in the refinement of connections prenatally even before vision is possible. The fact that retinal ganglion cell axons make ultrastructurally identifiable and functionally competent synapses within the LGN of the cat in utero (7) suggests that spontaneous neural activity could play a role in the formation of the eye-specific layers. To test this suggestion, we have infused TTX into the fetal brain to block action potentials during the time when ganglion cell axons from the two eyes would normally segregate from each other into layers.

Tetrodotoxin was infused continuously

into the region of the brain above the optic chiasm beginning at embryonic day 42 (E42; gestation is 65 days), a time when axons from the two eyes are extensively intermixed within the LGN and have not yet begun to segregate (8). A total of ten animals was studied. Each fetus was exposed by Cesarean section for implantation of a cannula attached to an osmotic minipump containing either 300 μ M TTX (eight animals) or a control citrate buffer vehicle solution (two additional animals) (9). [Chronic infusion of TTX at this concentration and rate blocks activity in the visual cortex and its afferents over an area of about 3 mm³ (10).] The fetus was returned to the uterus for 2 weeks until E56, a time when the eye-specific layers are clearly evident in normal animals (8), and just before the minipumps ceased functioning. We used the anterograde transport of [³H]leucine injected into one eye and horseradish peroxidase injected into the other to examine the effects of the treatment on the pattern of the retinogeniculate projection (8). The concentration of TTX in the fetal brains during the infusion was estimated to be between 0.1 and 1.0 μ M in two of the TTX-treated animals by bioassay of the cerebrospinal fluid (CSF) removed by cisternal puncture at E49 (11) and was found to be between 0.1 and 1.0 μ M, levels sufficient to block the compound action potential in neonatal rat optic nerve (12).

Despite the surgical manipulations and the presence of TTX during the 2-week infusion period, fetal growth was within the normal range. Between E42 and E58 the crown-rump length of fetuses normally increases from 57 to 68 mm to 100 to 120 mm (8); the TTX-treated animals at E56 were 105 to 110 mm ($n = 3$). In addition, the gross appearance of the brain, as revealed by examining histological sections

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stained for Nissl substance, was indistinguishable from that of normal animals (Fig. 1). As in normal animals at a similar age (Fig. 1, top), the separate thalamic nuclei and overlying forebrain structures are distinct even after the TTX infusion (Fig. 1, bottom). The LGN forms a discrete cell

mass at the posterolateral edge of the thalamus (Fig. 1) surrounded by the internal capsule and the optic tract. The mature cytoarchitectural appearance of the brain in these animals indicates that the normal sequence of extensive cell migration, differentiation, and growth that occurs throughout

the brain between E42 and E56 (8) was not arrested by the TTX infusion.

In contrast to the normal cytoarchitectural maturation, the retinal projections to the LGN after TTX treatment were strikingly abnormal in all four experimental animals studied. Figure 2 compares the pattern of the retinogeniculate projection revealed by intraocular injections of horseradish peroxidase in a TTX-treated animal at E56 (Fig. 2, A and B) and in a vehicle control animal at E57 (Fig. 2, C and D). In both control animals, the LGN contralateral to the injected eye contains anterogradely transported label within developing layers A and C, whereas the ipsilaterally innervated layer A1 has almost no label. Ipsilateral to the injected eye, layer A is largely free of label, while layers A1 and the appropriate parts of C are labeled. This pattern of labeling is identical to that found in untreated animals of the same age (8), and indicates that the infusion procedure itself had no effect on the normal formation of eye-specific layers, a process largely complete by E57. In TTX-treated animals, on the other hand, label fills the LGN contralateral to the injected eye and no gap can be seen corresponding to the position of the normal layer A1. Ipsilateral to the injected eye, the usual demarcation between layers A and A1 is missing. Label extends nearly to the innermost border of the LGN (furthest from the optic tract) into a region normally never occupied by axons from the ipsilateral eye (8). This pattern of labeling indicates that as a consequence of TTX treatment, ganglion cell axons from the two

Fig. 1. Comparison of the histological appearance of the brain from a normal fetus at E54 (top) and a fetus at E56 that had received minipump infusion of TTX for the preceding two weeks (bottom). Each section is cut in the horizontal plane, with the anterior toward the top, and has been stained with cresyl violet. Note that the gross histological appearance of the TTX section is very similar to that of the normal section. The LGNs (asterisks) are in their normal position and are of normal size and shape for this age. The organization of the diencephalon, adjacent internal capsule, and hippocampus also appear normal.

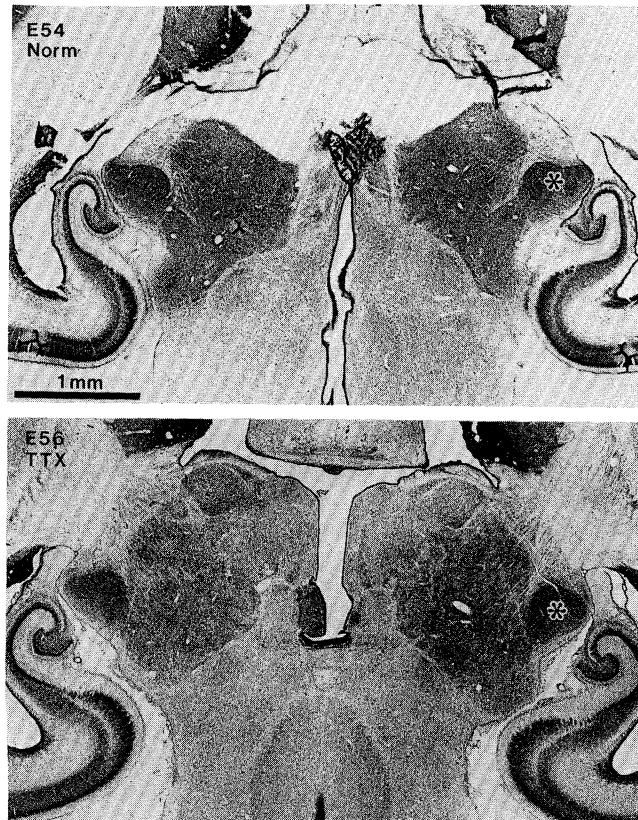
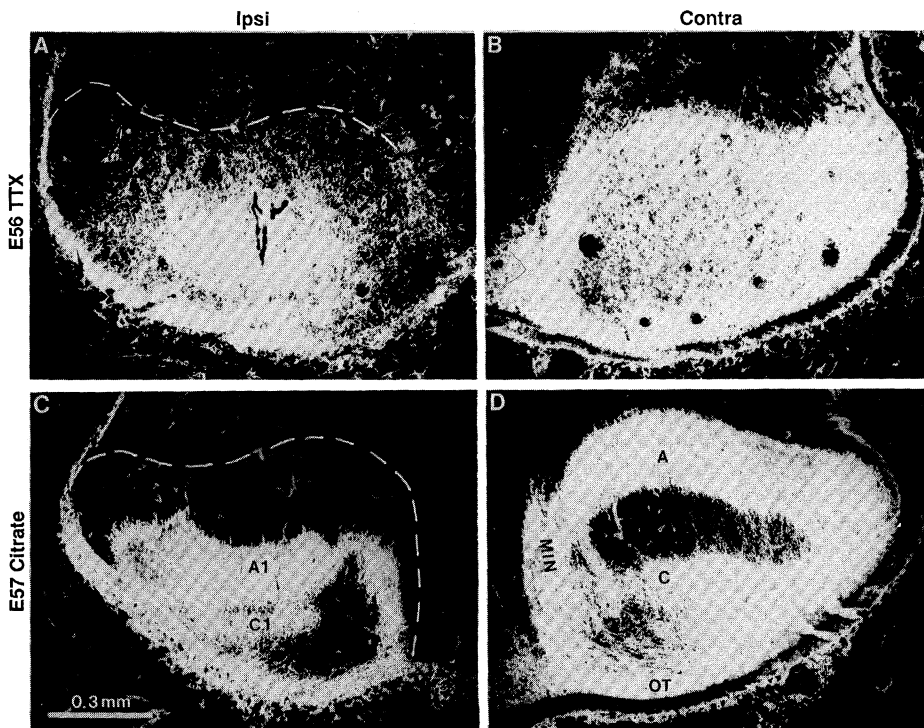


Fig. 2. Comparison of the pattern of the retinogeniculate projection to the LGN in an animal that had received a TTX infusion from E42 to E56 (A and B) and another animal that had received an infusion of citrate buffer control solution from E42 to E57 (C and D). In each case, one eye was injected with horseradish peroxidase 24 hours before the indicated age and anterograde transport was used to reveal the projection pattern ipsilateral (Ipsi, left) and contralateral (Contra, right) to the injected eye. The radioactive label appears white in these photos taken with dark-field optics. The pattern of labeling in the citrate buffer control case is indistinguishable from that seen in untreated animals at the same age [see fig. 8 in (8)] and indicates that ganglion cell axons have almost completely segregated from each other to form contralaterally innervated layer A and ipsilaterally innervated layer A1. In contrast, in the TTX-treated case, the eye-specific layers are not present: the projection from the contralateral eye occupies virtually the entire LGN (B), whereas that from the ipsilateral eye extends nearly to the innermost border of the LGN (A) (dashed white lines drawn from the adjacent sections stained with cresyl violet). In these horizontal sections, anterior is to the top and medial is to the middle of each photo. Abbreviations: A, A1, C, and C1 refer to the various LGN layers; MIN, medial interlaminar nucleus; and OT, optic tract.



eyes have not only failed to segregate from each other, but those from the ipsilateral eye have also expanded into territory usually occupied exclusively by axons from the contralateral eye. However, despite this abnormality, ganglion cell axons still appear to terminate exclusively within their normal target nuclei.

These findings show that intracranial infusion of TTX from E42 to E56 prevents the segregation of retinogeniculate afferents into eye-specific layers, a process that would normally occur during this fetal period. To explain how TTX exerts this effect, we suggest that the spontaneous activity of retinal ganglion cell axons, geniculate neurons, or both, normally operate in utero to refine an initially intermixed set of connections and that TTX treatment, by blocking this spontaneous activity, prevents the formation of the layers. This suggestion is analogous to that proposed to explain the segregation of geniculocortical afferents to form ocular dominance columns postnatally in the visual cortex of the cat (6) and the formation of experimentally induced ocular dominance stripes and the refinement of topography in the amphibian and fish retinotectal systems (13). Unlike these previous studies, in which developing or regenerating neural connections would normally be driven visually, our results imply that neuronal activity unrelated to vision plays an essential role in the refinement of connections in the visual system.

The above interpretation depends on two important assumptions: that ganglion cells are spontaneously active in utero and that TTX exerts its effect by blocking this activity. Direct evidence in support of the first assumption is provided in a recent report by Galli and Maffei (14), in which in vivo microelectrode recordings from the retina of fetal rats at similar stages (E17 to E20) indicate that ganglion cells can spontaneously generate action potentials. In addition, ganglion cells in postnatal animals are known to be spontaneously active in the dark (15), and studies of immature ganglion cells in vitro also reveal spontaneous activity (16). The second assumption has been addressed in several studies. For example, micromolar concentrations of TTX block the compound action potential in the optic nerve of neonatal rats (12). These axons are similar in their state of maturity to those of the fetal cats studied here, which can conduct action potentials and synaptically excite LGN neurons as early as E39 (7). These considerations make it likely that spontaneous activity is present in vivo and was blocked by the TTX infusion (17).

The failure of retinogeniculate axons to segregate into eye-specific layers could arise

if TTX were to act by "freezing" the axons in their immature state at the onset of treatment, or if it acted by permitting promiscuous new growth. Previous experiments have shown that intraocular TTX treatment during neonatal life, after the geniculate laminae have formed, does arrest the ultrastructural maturation of the retinogeniculate synapse (18). However, TTX treatment also produces abnormalities in the receptive fields of LGN neurons (19) and in the size and shape of retinogeniculate terminal arbors (20), suggesting that some degree of promiscuous growth can occur even postnatally (21). Prenatally, our finding that the LGN grows to roughly normal size during TTX treatment makes it unlikely that the treatment simply arrests the growth of axons and synapses. Consistent with this suggestion is the observation that the projection from the ipsilateral eye has expanded into a region of layer A in which it is normally never found (Fig. 2B), and preliminary observations that individual retinogeniculate axon arbors are abnormally large after similar TTX treatments during fetal life (22). Thus it is likely that TTX treatment prevents the formation of layers not by preventing growth but rather by permitting the proliferation and growth of inappropriate branches that would normally have been selectively eliminated.

If the TTX treatment exerts its effect by blocking spontaneous activity, then even before vision is possible, the visual system may use neural function in the form of spontaneously generated action potentials to refine initially diffuse connections into the precise adult pattern. Moreover, the development of the retinogeniculate pathway may represent just one example of a general phenomenon operating throughout the nervous system in which spontaneous activity refines connections during fetal development.

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9. Timed-pregnant cats were anesthetized with a mixture of halothane, oxygen, and nitrous oxide and Cesarean sections were performed with sterile surgical technique (8). The fetal head and neck were exposed and a sterile osmotic minipump (Alza 2002, 0.5 μ l/hour) filled with TTX (300 μ M; Calbiochem) or citrate buffer vehicle solution (320 μ M; pH 4.8; sodium citrate buffer in 0.9% NaCl) sterilized by millipore filtration was sutured to the dorsal skin of the neck. Silastic tubing (Dow Corning; 1.2-mm outer diameter) connected to the pump was inserted about 4 mm into the forebrain at 2 mm lateral to the sagittal sinus and 4 mm anterior to bregma and was fixed in place with 6-0 silk suture and cyanoacrylate glue (histocaryl-N-blau; Brown Melsungen). The fetus was returned to the uterus, incisions were closed, and the mother cat revived.
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17. The infusion procedure used in our study was designed to produce the highest concentration of TTX in the region above the optic chiasm. However, because TTX was found in CSF samples obtained by cisternal puncture (11), it is likely that high concentrations of TTX were also present in the LGN, as well as elsewhere in the brain. Thus, the activity of both ganglion cell axons and their postsynaptic targets in the LGN are likely to have been affected by the TTX treatment. An alternative interpretation, that TTX has exerted its effects on retinogeniculate segregation by blocking axoplasmic transport rather than neuronal activity, seems unlikely for several reasons. One reason is that the anterograde transport of 3 H-labeled protein or horseradish peroxidase after the intraocular injections is qualitatively indistinguishable from that of normal animals at the same age. Experiments that have examined the effects of intraocular injections of TTX in the mammalian retina have indicated that the transport of very few proteins is altered [R. V. Ricco and M. A. Matthews, *Neuroscience* **16**, 1027 (1985)]. Finally, as reported here and in (22), retinogeniculate axons continue to grow extensively in the presence of TTX.
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