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Independent Control of Dendritic and Axonal Form in the Developing Lateral Geniculate Nucleus

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To identify mechanisms that regulate neuronal form in the mammalian CNS, we have examined dendritic development in the lateral geniculate nucleus (LGN) during the period of segregation of retinal ganglion cell axons. The tracer DiI was used to label retrogradely LGN neurons that send their axons to primary visual cortex at different ages between embryonic day 36 (E36) and E60 in the cat. LGN neurons grow extensively during this period, in concert with the progressive restriction of ganglion cell axons from the two eyes to their appropriate eye-specific layers. At E36 neurons have simple bipolar morphology; by E60 all have acquired complex multipolar dendritic trees. During this period, soma size increases by 190% and total dendritic length increases 240%. Dendritic complexity, as measured by dendritic branch points, also increases. As dendrites grow, the number of spines increases, but their density remains constant at 0.015/μm throughout this period.

Since it is known that blockade of action potential activity significantly alters the branching pattern and extent of retinal ganglion cell axonal arbors within the LGN, we also investigated whether the dendritic development of the postsynaptic LGN neurons is similarly susceptible. Following 2 weeks of the intracranial minipump infusion of TTX between E42 and E56, the morphology of LGN neurons was examined. Surprisingly in view of the striking effect of the treatment on the morphology of retinal ganglion cell axons, dendritic growth and development were essentially normal. However, the density of dendritic spines increased almost threefold, suggesting that this specific feature of dendritic morphology is highly regulated by action potential activity.

These observations indicate that normally during this period of development, the previously described changes that occur in the morphology of the presynaptic inputs to LGN neurons are accompanied by a progressive growth of postsynaptic dendrites. Because the intracranial TTX infusions have almost certainly blocked all sodium action potentials, our results suggest that the basic dendritic framework of LGN neurons can be achieved even in the absence of this form of neural activity. Moreover, since the same treatment causes a profound change in the morphology of the presynaptic axons, at least some aspects of axonal and dendritic form must be controlled independently during this prenatal period of development.

[Key words: lateral geniculate nucleus, activity blockade, dendritic development, mammalian visual system, tetrodotoxin, spines]

Neuronal form, as defined by dendritic morphology and axonal projection patterns, is a major determinant of connectivity in the nervous system. Although the morphology of neurons in the adult has been extensively described, less is known about the development of neuronal form, especially regarding the extrinsic factors that can influence the patterning of axons and dendrites. A number of lines of evidence, however, indicate that neuronal activity is likely to be an important influence at least on axonal growth and branching. Much of our understanding of the role of activity in axonal development comes from studies in the developing mammalian visual system. The primary visual pathway consists of retinal ganglion cell axons that innervate the lateral geniculate nucleus (LGN) of the thalamus, and the LGN axons that in turn project to layer 4 of visual cortex. Both the ganglion cell axons and LGN axons have restricted and characteristic branching patterns within their targets: both are segregated according to eye preference, giving rise to eye-specific layers within the LGN and ocular dominance columns within cortical layer 4. However, this specific patterning of axonal connections is not present initially during development, but gradually emerges from an unsegregated state (reviewed in Shatz, 1990).

When action potential activity in the visual system of kittens is chronically blocked by intraocular injections of TTX, geniculocortical axons representing the two eyes fail to segregate into eye-specific columns within layer 4 of visual cortex (Stryker and Harris, 1986; Antonini and Stryker, 1993). Similarly, following intracranial infusion of TTX into fetal cats, retinal ganglion cell axons from the two eyes fail to segregate into layers (Shatz and Stryker, 1988). In the case of retinogeniculate axon segregation within the LGN, the effect of TTX infusions on the branching pattern of individual axon terminals is also known: these axons have extensive and exuberant terminal branches that, unlike normals, do not follow any laminar restriction (Sretavan et al., 1988). Recent experiments involving the blockade of glutamatergic transmission imply that activation of specific glutamate receptors on postsynaptic neurons may similarly be

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necessary for the normal development and maturation of presynaptic axon morphology (Cline and Constantine-Paton, 1990; Hahm et al., 1991; Simon et al., 1992). Such evidence is consistent with the interpretation that action potential activity and synaptic transmission are important determinants of axonal form in the developing visual system and perhaps also elsewhere within the CNS.

In contrast to the cumulative evidence implying a role for neural activity in axonal development, relatively less is known about the factors that are important in specifying dendritic form. Previous work suggests that both afferent input as well as target innervation may participate in the determination of dendritic morphology. For example, studies of postnatal LGN development demonstrated that monocular eye closure, during a critical period, can profoundly affect LGN somata size and details of dendritic morphology (Friedlander et al., 1982). In addition, experiments have shown that altering peripheral target size alters the extent of dendritic branching in the superior cervical ganglion (Yawo, 1987; Voyvodic, 1989). Furthermore, deafferentation experiments such as the removal of auditory inputs to the nucleus laminaris neurons in the chick (Rubel et al., 1989) or transection of the eighth nerve input to Mauthner cells in axolotl embryos (Goodman et al., 1988) result in dendritic atrophy of the postsynaptic neurons.

In most cases, however, the mechanisms by which these perturbations cause dendritic change are not well understood. The deafferentation experiments suggest that afferent activity might be an important influence on the maintenance of dendritic morphology (Rubel el al., 1989). However, it is not known whether the resulting morphological changes are due to a lack of input activity, or to some other factor such as degeneration products following denervation, but in view of the involvement of activity in determining axonal form it is possible that activity might also be an important factor controlling dendritic development. Consistent with this suggestion are the results of *in vitro* experiments where action potential activity blockade by TTX in cultured cortical neurons or Purkinje cells results in an increase in dendritic growth and branching (Mattson et al., 1988; Schilling et al., 1991; Corner, 1992).

To investigate the possibility that neural activity is involved in the control of dendritic form *in vivo*, we have examined the morphological changes in LGN neurons in the fetal cat during the period in which their inputs, the retinal ganglion cell axons, segregate into the eye-specific layers. Since the development of retinal axon terminals in the LGN has been previously described, we could examine whether changes in dendritic morphology are correlated with phases of axonal development taking place simultaneously. Also, since it is known that intracranial infusion of TTX prevents the segregation of retinogeniculate afferent axons and has marked effects on individual axon morphology, we examined whether similar activity blockade can influence the development of dendritic form. Our observations not only reveal information about normal dendritic development, but suggest that axonal and dendritic growth in the LGN are likely to be differentially regulated during this period of development.

Materials and Methods

This study is based on a total of 163 lateral geniculate projection neurons that were retrogradely labeled from the visual cortex with DiI between embryonic day 36 (E36) and E60. Of the animals examined, 11 were normal; three received a 2 week intracranial infusion of tetrodotoxin (TTX), a drug that blocks the voltage-sensitive sodium channels in neurons, between E43 and E57; and two were infused with citrate buffer (control) between E43 and E57. The age distribution of experimental animals studied is shown in Table 1.

Table 1. Numbers of neurons analyzed at specific ages

Fetal age	Number of fetuses	Number of cells drawn
E36	3	26
E43	2	22
E50	1	13
E52	1	10
E57	2	24
E60	2	23
E43–E57 citrate[a]	2	12
E43–E57 TTX[a]	3	55

[a] In these cases, minipumps containing either TTX or citrate vehicle solution were implanted intracranially between E43 and E57.

Surgery. Cesarian sections were performed on timed-pregnant cats as previously described (Shatz, 1983). To obtain timed pregnancies, adult female cats in estrus (Scott, 1970) were placed with a male for 24 hr and the time of mating was considered E1. This method allows one to time pregnancies with 1 d of uncertainty (see Shatz, 1983, for more detail). Prior to surgery pregnant cats received a subcutaneous injection of atropine sulfate (0.05 mg/kg) followed by an intramuscular injection of ketamine hydrochloride (20 mg/kg) and acepromazine (0.2 mg/kg) to induce anesthesia. An endotracheal tube was inserted and for the duration of the procedure anesthesia was maintained with halothane (0.5–2.0%) and oxygen (1.0 liter/min). An arm vein was cannulated to allow continuous infusion of lactated Ringer's solution and the occasional injection of terbutaline sulfate (Brethine, 0.03 mg/kg) to reduce contraction of the uterus during the surgery. Heart and breathing rate, as well as expired CO_2, were monitored routinely during surgical procedures. Under sterile conditions the two uterine horns were exposed by incising the skin and abdominal musculature along the midline. The uterus and the fetal membranes were opened to allow placement of the osmotic minipumps in the cranium or to allow removal of the fetus.

Implanting minipumps. The procedure used to implant minipumps has been described fully previously (Sretavan et al., 1988) and will only be briefly described here. At E43, a sterile osmotic minipump (Alza, model 2002) containing TTX (300 μM in sodium citrate buffer; Calbiochem) or citrate buffer vehicle solution alone (320 μM, pH 4.8, sodium citrate buffer in 0.9% NaCl) sterilized by millipore filtration was sutured to the dorsal skin of the neck. A 27G needle was used to make a small penetration through the skull and Silastic tubing (Dow Corning; 1.2 mm o.d.) 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 the cranial suture bregma, a placement that delivers infusion solutions to optic tract and the thalamus. The tubing was then fixed in place with 6-0 silk and cyanoacrylate glue (Histoacryl-N-blau, Brown Melsungen). The fetuses were returned to the uterus, which was closed and the mother cat revived. The fetuses were allowed to survive for 14 d and then removed at E57, and their brains were fixed by intracardial perfusion.

Tissue processing and DiI injections. Fetuses were perfused with 50 ml of 0.1 M sodium phosphate buffer, followed by 150–300 ml of 4% paraformaldahyde in 0.1 M sodium phosphate buffer. To label specifically the projection neurons of the LGN, we placed small crystals of the lipophilic fluorescent tracer DiI (Molecular Probes, D282; Godement et al., 1987) into the primary visual cortex. This technique enables the entire dendritic tree of individual neurons to be visualized by retrograde labeling. The DiI injected brains were stored in 4% paraformaldahyde in 0.1 M sodium phosphate buffer (with 0.05% sodium azide) in a oven at 37°C for 2–4 months to allow retrograde diffusion of the label. Horizontal sections were cut (100–200 μm) on a vibratome and coverslipped with glycerol. Labeled neurons in the LGN were visualized under rhodamine epifluorescence optics. Individual DiI-labeled neurons were drawn with a camera lucida using a 40× long-working-distance water immersion objective and a 10× eyepiece. Only neurons that contained most if not all dendritic tips within a single section were drawn.

In several cases, for photographic purposes, DiI-labeled sections were photoconverted by incubating the labeled sections in a solution of 75 mg of diaminobenzidine (DAB; Sigma) in 50 ml of Tris buffer (pH 8.2) and illuminating with rhodamine epifluorescence using either a 6.3× or 20× fluorescence objective to obtain a reaction product that can be visualized using a light microscope (Maranto, 1982).

Verification of TTX effect. In previous studies, we have measured the concentration of TTX in the CSF of fetuses following 1–2 weeks of minipump infusions and found that levels are about 1 μM, sufficient to block retinal ganglion cell action potentials (Shatz and Stryker, 1988). However, to verify the effectiveness of the TTX infusions here, in two of the fetuses receiving the TTX treatment and in one fetus receiving the citrate buffer vehicle solution treatment, one eye was injected with ^{3}H-leucine (RPN.12, Amersham; 1 mCi in 10 μl) on E56 and the fetuses were replaced *in utero* for 24 hr to permit radioactive label to be transported to the retinogeniculate afferent terminal arbors in the LGN, after which these animals were removed and perfused. Later, after all of the DiI-labeled LGN neurons in the section had been drawn (see Fig. 8), contact autoradiograms (Kodak Hyperfilm; exposure of 5–7 d at −70°C) were made from the sections labeled with ^{3}H-leucine to verify that the TTX had indeed been effective in blocking segregation of the ganglion cell axons. In addition, in one E57 normal and one E43–E57 TTX-treated case, the lipophilic tracer DiA [4-(4-dihexadecylaminostyryl)-*N*-methylpyridnium; Molecular Probes, D-3883], which fluoresces green under the Fluorescene filter set, was placed directly in the optic tract of sectioned material in which the LGN neurons had already been drawn to label individual retinogeniculate afferents entering the LGN, allowing simultaneous visualization of both the red DiI-labeled LGN neurons and the green DiA-labeled ganglion cell axons. These axons were drawn using a camera lucida and fluorescent optics (Fluorescene filter set) and compared to the axons described in Sretavan et al., 1988, to confirm that TTX had affected the morphology of retinal ganglion cells axons as expected.

Quantitative analysis. The dendritic morphology of LGN projection neurons was characterized by measuring five parameters: total dendritic length, cell soma area, number of dendritic branch points, number of dendritic tips, and number of dendritic spines. A dendritic spine was defined as a dendritic process no longer than 5 μm. Total dendritic length and cell soma area were measured using a Jandel digitizing pad and the SIGMA SCAN graphics program on an IBM AT personal computer. The other parameters were simply counted. All the data was entered onto and analyzed with a spreadsheet (EXCEL v. 2.2 program). The graphs were created on a Macintosh II with CRICKET GRAPH and MACDRAW II. To determine if there were significant differences in the various quantitative measures of dendritic form (e.g., between TTX- and normal-treated neurons) two-tailed Student's *t* tests were conducted. A difference was considered significant if $p < 0.05$ (see Fig. 10).

We were also interested in knowing whether there was any initial systematic orientation of dendrites with respect to the forming layers of the LGN. Therefore, at E36 the orientation of the LGN neurons relative to the pial surface was measured. First, we superimposed a plane with the x-axis tangential to the pial surface onto the camera lucida drawing of the LGN neurons. We defined the coordinates (0,0) as the cell body. Then we marked the x- and y-coordinates of the dendritic tips of the neurons. The dendrites were then converted into vectors with the length and orientation defined by their (x,y) coordinates. To eliminate the canceling of measurements of orthogonal dendrites, negative y-values were reflected over the x-axis. The vector sum was then calculated for each cell. The resulting vector sum was then plotted on a polar graph (see Fig. 4).

Results

The results of our experiments are presented in two parts: we first describe the normal dendritic development of LGN neurons; next the effect of TTX-mediated activity blockade on dendritic development is considered. By E36 virtually all of the LGN neurons have been generated within the ventricular zone of the third ventricle (Hickey and Hitchcook, 1984; Hitchcook et al., 1984) and many have already completed their outward migrations to form the anlage of the LGN (Shatz, 1983). Retinal axons first arrive at the LGN by about E32, and the process of ganglion cell axon remodeling leading to segregation into the eye-specific layers begins by about E43 (Shatz, 1983). Studies of the branching patterns of individual retinal ganglion cell axons (Sretavan and Shatz, 1986a,b) show that initially, as axons grow into the expanding nucleus, they are very simple and send short side branches laterally from their main trunks. Gradually, the axons develop terminal arbors in specific appropriate layers and the side branches disappear. Segregation is complete shortly after birth, but an adult-like pattern of segregation is visible as early as E54. Previous studies have also shown that the intracranial infusion of the sodium channel blocker TTX during this period (E43–E57) prevents the segregation of the retinogeniculate axons into eye-specific layers within the LGN (Shatz and Stryker, 1988; Sretavan et al., 1988).

Development of dendritic form during the period of retinogeniculate axon segregation

LGN projection neurons were labeled retrogradely by DiI injections made into visual cortex between E36 and E60. At all ages examined, such injections resulted in the labeling of neurons principally if not exclusively within the LGN. An example of the specificity of labeling is shown in Figure 1, where a large number of labeled neurons can be seen within the LGN following DiI injection into the visual cortex in an E60 fetus. Retrograde DiI labeling resulted in virtually the complete filling of the dendritic tree of the neurons, thereby allowing visualization of fine morphological details. For example, Figure 2 shows LGN projection neurons at E43 in which the DiI label was photoconverted to a permanent DAB reaction product. The dendrites of these neurons are relatively smooth, but very delicate spines are clearly evident and the dendrites are often tipped with growth cones at this age (see Fig. 2 inset). The presence of these fine morphological details makes it highly likely that we can observe most, if not all, of the dendritic tree of these neurons.

Between E36 and E60 there is significant dendritic development, as illustrated in the collection of camera lucida drawings in Figure 3. At E36 and E43, dendritic morphology is simple and consists of a few primary dendrites with few branch points. Many dendrites are tipped with growth cones at these ages. The neurons have small cell somas and there are not many dendritic spines present. Gradually, dendritic trees expand, LGN neurons acquire larger cell somas and more dendritic spines, and the dendritic arbors appear to become more extensive. These observations indicate that the dendritic tree of LGN neurons evolves from a relatively simple morphology at E36 to a highly complex one containing several higher-order dendritic branches by E60. It is also striking that even as early as E36, when the first retinogeniculate axons have just begun to invade the LGN and themselves rarely possess extensive branches, substantial dendritic outgrowth has already taken place. However, even as late as E60, we are unable to identify clearly any of the morphological classes seen in the adult (Guillery, 1966; Friedlander et al., 1981). This observation is consistent with studies of postnatal LGN neurons indicating that extensive differentiation and growth of the dendritic arbors continue to take place postnatally (Friedlander et al., 1981; de Courten and Garey, 1982; Mason, 1983; Sutton and Brunso-Bechtold, 1991).

Since many of the early LGN neurons possess simple morphologies characterized by several dendrites organized in a bipolar fashion (Fig. 3), we investigated whether the polarity of the dendrites was systematically related to the geometry of other cellular elements in the system. The bipolar form of dendrites suggested that they might be systematically oriented with respect

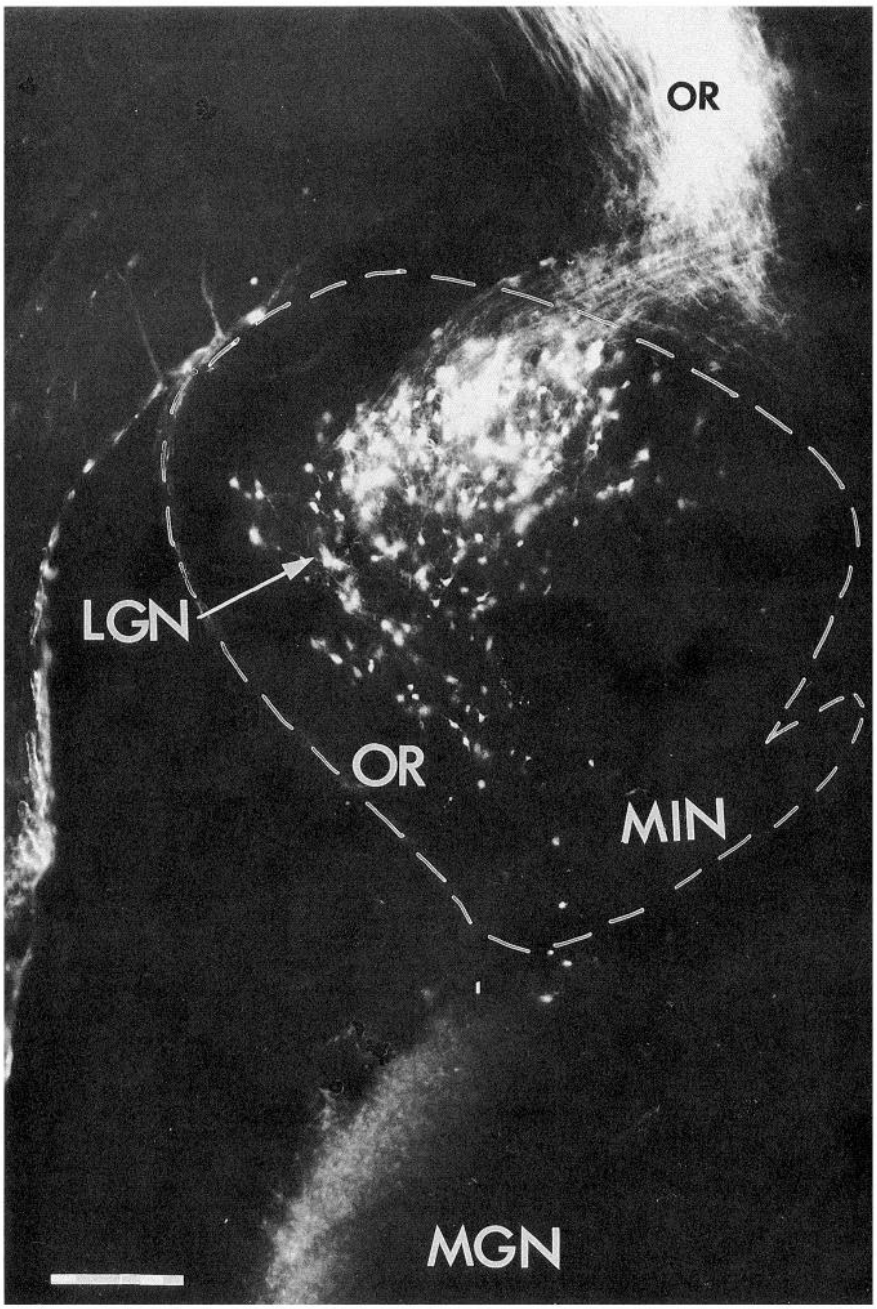

Figure 1. Neurons in the LGN are retrogradely labeled from an injection of DiI into the visual cortex at E60. Labeled cells appear as *small white dots* in this low-power photomicrograph taken with rhodamine fluorescence optics. The axons of LGN neurons as they run within the optic radiations (*OR*) toward the LGN are also retrogradely labeled. *MGN*, medial geniculate nucleus; *OT*, optic tract. This is a horizontal section: anterior is to the top, and lateral is to the left. Scale bar, 1.0 mm.

to the direction of retinal ganglion cell axon ingrowth. The first axons grow into the LGN from the optic tract in straight trajectories aligned (parallel) with the radial glia and perpendicular to the pial surface (Sretavan and Shatz, 1987). To examine whether there is a systematic relationship between dendritic orientation and the direction of axon ingrowth, at E36 the orientation and length of each dendrite were measured and the total expressed as a vector sum, as described in Materials and Methods. As shown in Figure 4, the majority of dendrites at this age, regardless of length, were oriented between 60° and 120° with respect to the pial surface at this early age. In other words, the dendrites of many LGN neurons indeed appear to be aligned with the radial glia and the ingrowing retinal ganglion cell axons. The systematic orientation of the neurons at E36 suggests that dendritic outgrowth might arise from the leading and trailing processes that these neurons used to travel along

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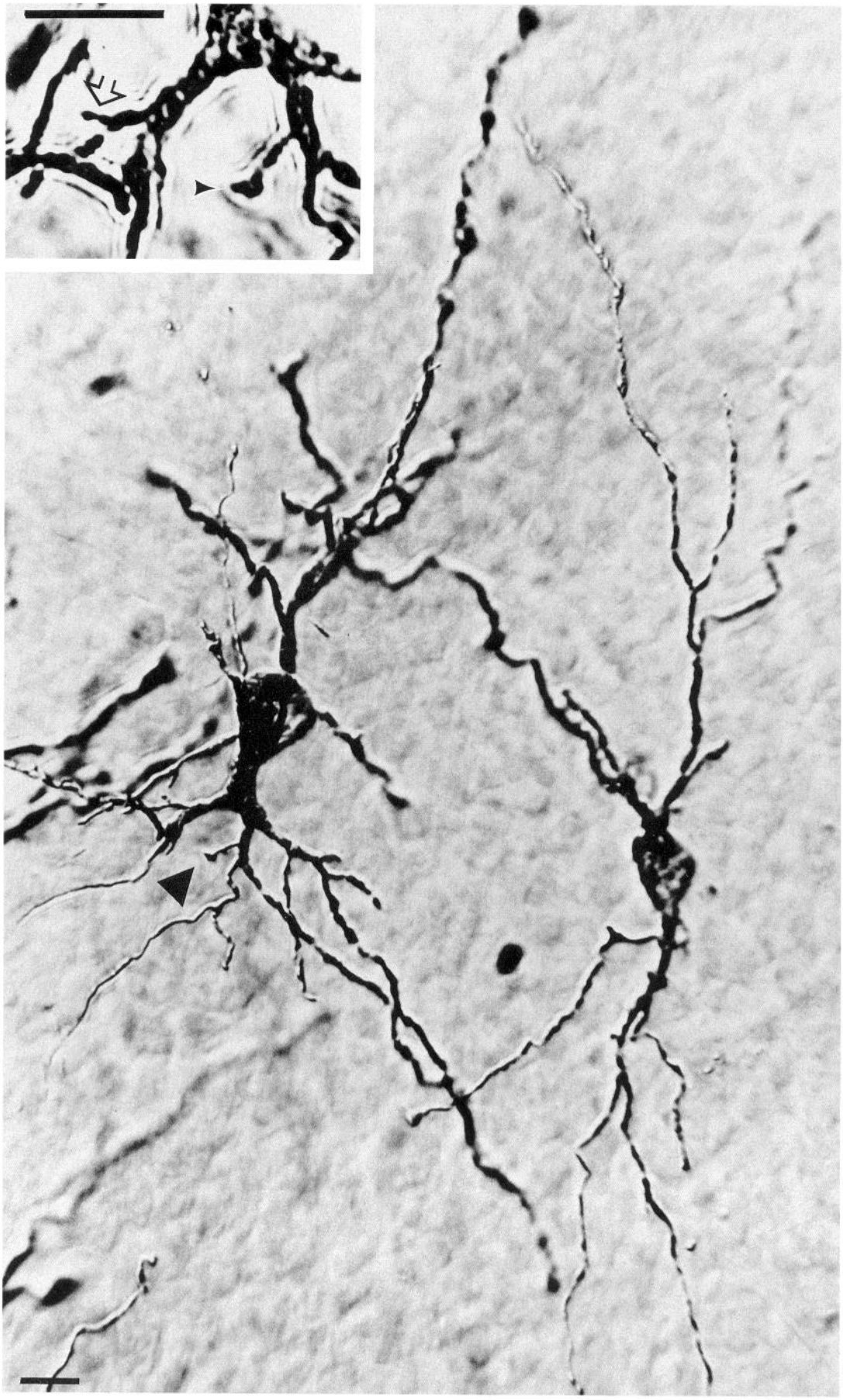
Figure 2. This composite shows two DiI-labeled neurons at E43 that have been photoconverted so that they are visible in the light microscope. *Arrowhead* indicates a dendritic growth cone on the cell to the left. *Inset* shows a magnified view of this growth cone (*arrowhead*). A dendritic spine is also indicated (*open arrow*). Scale bars, 100 μm.



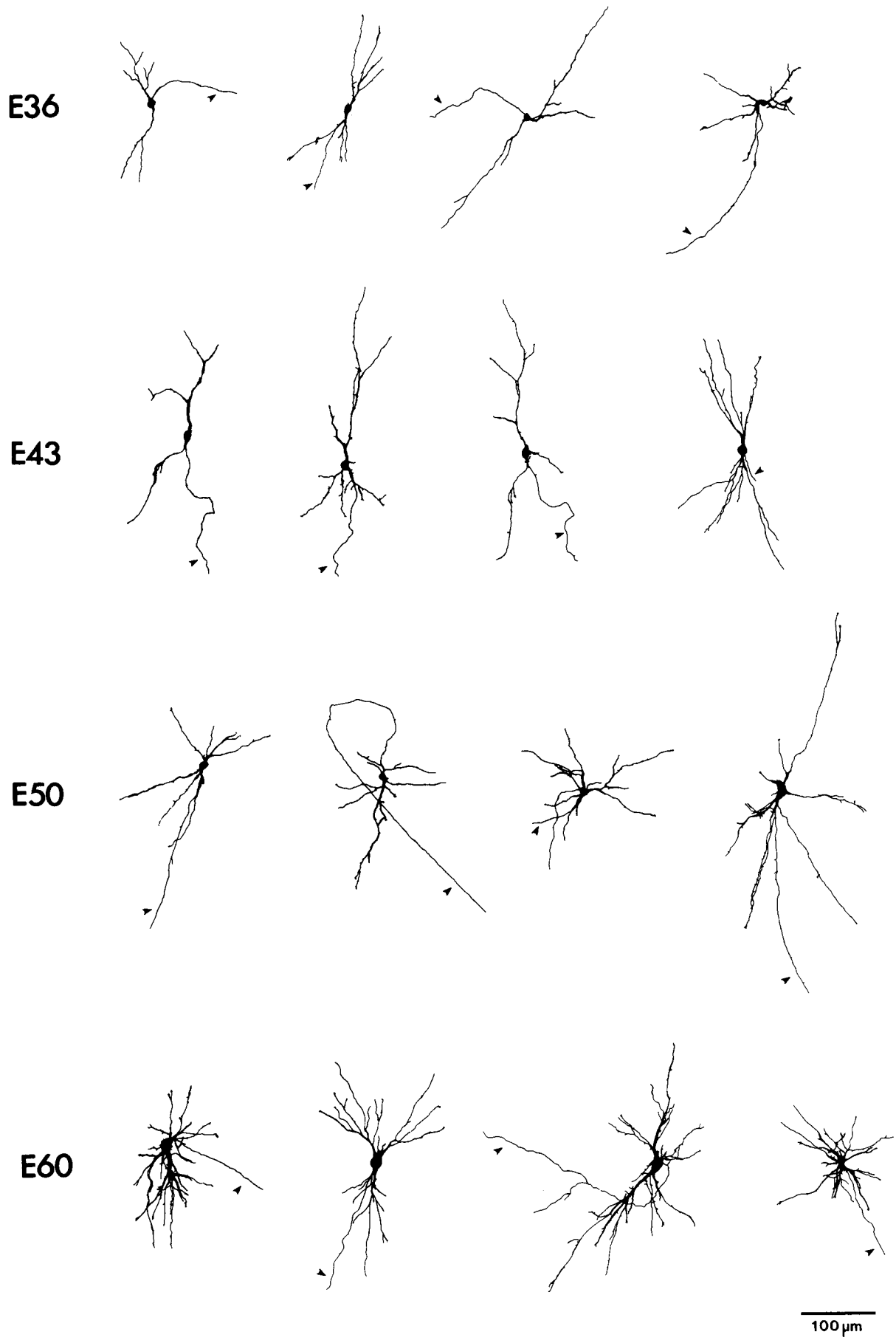


Figure 3. Camera lucida drawings of four representative neurons at E36, E43, E50, and E60. *Arrowheads* mark the axons, which were identified by tracing them into the internal capsule and optic radiations. Note the progressive increase in size and dendritic complexity of the neurons with increasing age. Scale bar, 100 μm.

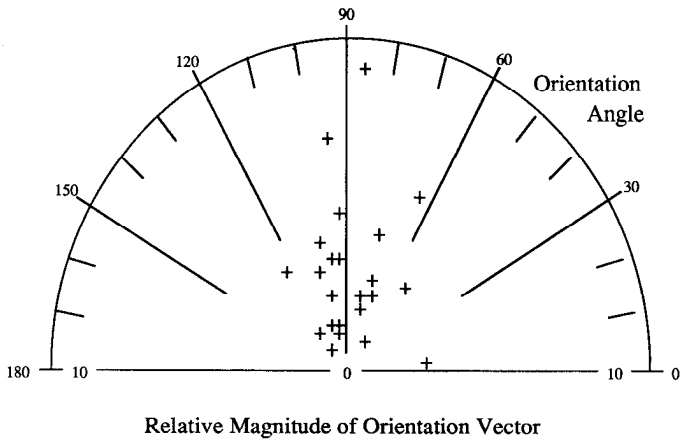


Figure 4. The orientation of the dendrites at E36 with respect to the pial surface is shown here. Each "+" indicates the orientation value for a given neuron. For example, a cell located at 90° has dendrites perpendicular to the pial surface. The extent to which the cell's dendrites are arrayed in a particular orientation is given by the distance of each "+" from the origin (e.g., the more highly polarized the dendrites, the larger the value on an arbitrary scale of 0–10). Note that the dendrites of the majority of LGN neurons are oriented roughly perpendicular to the pial surface. See Materials and Methods for more details.

the radial glia during their migration into the LGN from the ventricular zone (see Rakic, 1977).

In order to obtain a more quantitative description of the development of dendritic morphology, various parameters that serve to characterize dendritic form were measured. Cell soma area and the total length of dendritic processes were measured, allowing quantitative comparison of the overall growth and extent of cellular geometry. In addition, the number of branch points, and dendritic tips were counted, allowing quantitative assessment of the complexity of each neuron's dendritic form.

During the period between E36 and E60, there is a steady and linear increase in the size of LGN neurons. As shown in Figure 5*A*, the total dendritic length (TDL) of LGN neurons increases by approximately 240%, from 529 ± 29 μm at E36 to 1257 ± 117 μm at E60. Thus, the period of eye-specific segregation of inputs is also a period of extensive growth for dendritic arbors of LGN neurons. Moreover, as shown in Figure 5*B*, soma size increases approximately 190%, from 69 ± 4 μm^2 at E36 to 127 ± 10 μm^2 at E60.

Measurements of dendritic complexity confirm the suggestion made from inspection of the camera lucida drawings (Fig. 3) that the complexity of the dendritic arbors increases between E36 and E60. However, unlike total dendritic length and cell soma area, which both increase in a relatively linear fashion with age, dendritic branching increases in a nonlinear manner as shown in Figure 6. The number of dendritic branch points (Fig. 6*A*) and tips (Fig. 6*B*) remains relatively constant between E36 and E43. For example, at E36 the average number of branch points is 8 ± 0.6 while at E43 the average number is 7 ± 0.6. (The difference between the two is not significant.) Since total dendritic length actually increased by about 80% during this period, it appears that early in development these neurons grow primarily by extending longer neurites rather than by branching.

In contrast, after E43 the numbers of dendritic branch points and dendritic tips increase dramatically. For example, as shown in Figure 6, at E43 the number of dendritic branch points is 7 ± 0.6 while at E60 the number of branch points is 17 ± 1.5 (a

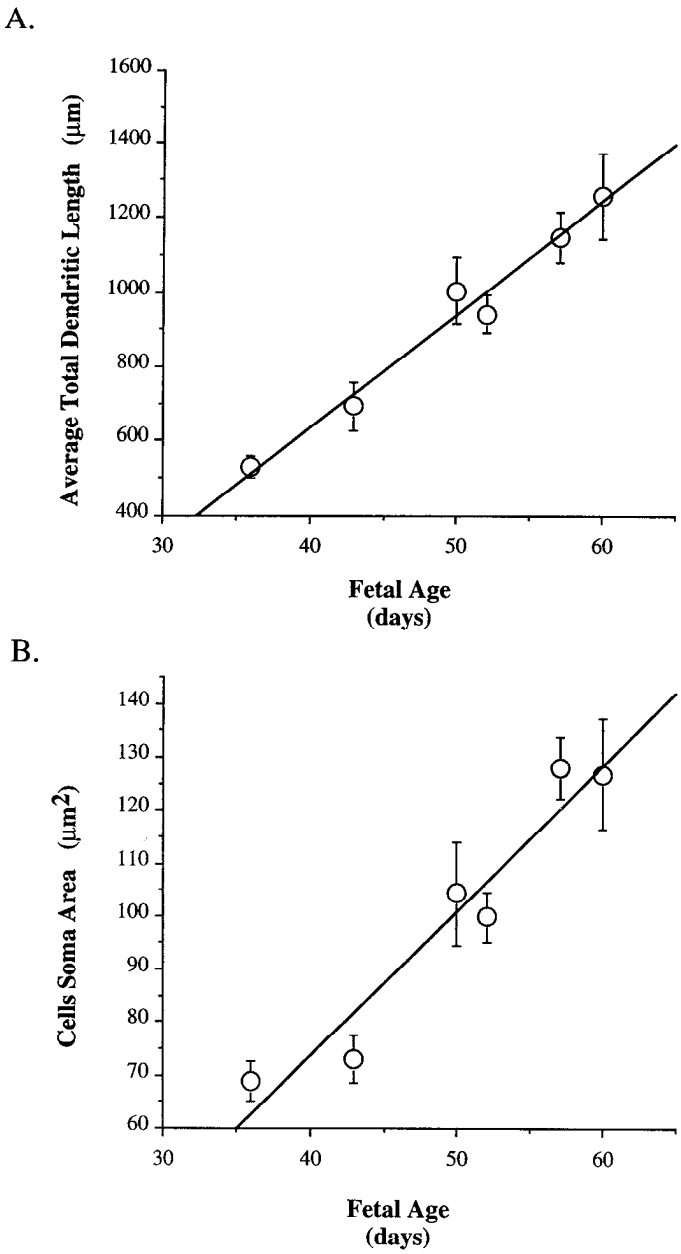


Figure 5. This composite figure demonstrates that the total dendritic length (*A*) and cell soma size (*B*) of LGN neurons increase in a linear manner between E36 and E60.

significant difference). The increased branching, however, is not accompanied by an accelerated rate of dendritic outgrowth in LGN projection neurons, since the dendritic growth rate remains linear even at these ages (see Fig. 5*A*). These observations indicate that the increase of dendritic complexity (as characterized by branching patterns) in LGN neurons begins shortly after E43, coincident with the onset of segregation of ganglion cell axons from the two eyes. Thus, the dramatic increase in complexity of the dendritic arbors of the LGN projection neurons is correlated in time with the onset of segregation of the retinogeniculate afferents.

In summary, LGN neurons grow extensively between E36, when some already send an axon to visual cortex (Ghosh and Shatz, 1992) and when retinal ganglion cell axons first grow into the LGN, and E60, when the axons have grown into visual

cortex, and when the inputs from the retinal ganglion cells have almost completely segregated into layers. During this period from E36 to E60, dendritic form also becomes more complex. The close correlation between dendritic branching and axonal segregation suggests that the morphological development of pre- and postsynaptic elements may be related. Since neural activity is known to be necessary for the segregation of the presynaptic retinal ganglion cell axons, we examined whether action potential activity blockade also influences the development of postsynaptic dendritic complexity and form.

Effects of TTX infusion on dendritic form

When TTX is infused intracranially via osmotic minipumps between E43 and E57, the normally occurring segregation of ganglion cell axons is prevented (Shatz and Stryker, 1988) and instead, terminal arborization within the LGN is unrestricted. Retinogeniculate axons branch throughout the LGN and are about 30% greater in total length (Sretavan et al., 1988). After an identical treatment, it was remarkable to find that the basic size and form of LGN dendrites appeared virtually unaffected. For example, Figure 7 shows camera lucida drawings of nine representative LGN neurons at E57, six of which were drawn following 2 weeks of intracranial TTX infusion. Simple inspection suggests that TTX treatment has not lead to a major reorganization of the dendritic arbor; for example, the extent and complexity of the dendritic arborizations appear normal (see also Fig. 3). In addition, even the fine morphological features of TTX-treated LGN neurons appear unaffected structurally. For instance, the photoconverted cell shown in Figure 8*A* appears to have relatively normal-looking dendrites, dendritic spines (Fig. 8, inset) and growth cones.

The interpretation that LGN projection neuron development may be independent of action potential activity depends critically on the effectiveness of the intracranial TTX treatment. We verified the effectiveness of the infusions of TTX by checking, in three of the animals in which LGN neurons had been studied, whether the segregation of retinal ganglion cell axons into layers had also been prevented. In two animals, one eye was injected with ^{3}H-leucine at E56, 24 hr prior to delivery by C-section, in order to reveal the entire pattern of the retinogeniculate projection from the labeled eye by means of anterograde transport. Following DiI labeling and our analysis of LGN dendrites, selected sections containing LGN neurons that we had previously drawn were used to make contact autoradiographs of the LGN. As shown in the autoradiograph of Figure 8*B*, layers representing the injected eye had indeed failed to form, confirming the effectiveness of the TTX treatment. In citrate buffer vehicle solution–treated controls, eye injections resulted in labeling restricted to the appropriate LGN layer (data not shown; see Sretavan et al., 1988).

Another method to demonstrate that the TTX had indeed prevented axons from segregating in the very section under analysis for dendritic development was to label several retinal ganglion cell axons within the section by placing a small crystal of DiA into the optic tract. We were able to draw these axons, since DiA fluoresces green and was therefore possible to distinguish from the red DiI-labeled LGN neurons. Examples of camera lucida drawings of axons from normal (untreated) sections of the LGN and from TTX-treated sections at E57 are shown in Figure 9. As expected from previous studies (Sretavan and Shatz, 1986a), the terminal arbors of ganglion cell axons in the untreated sections were restricted to single layers of the LGN

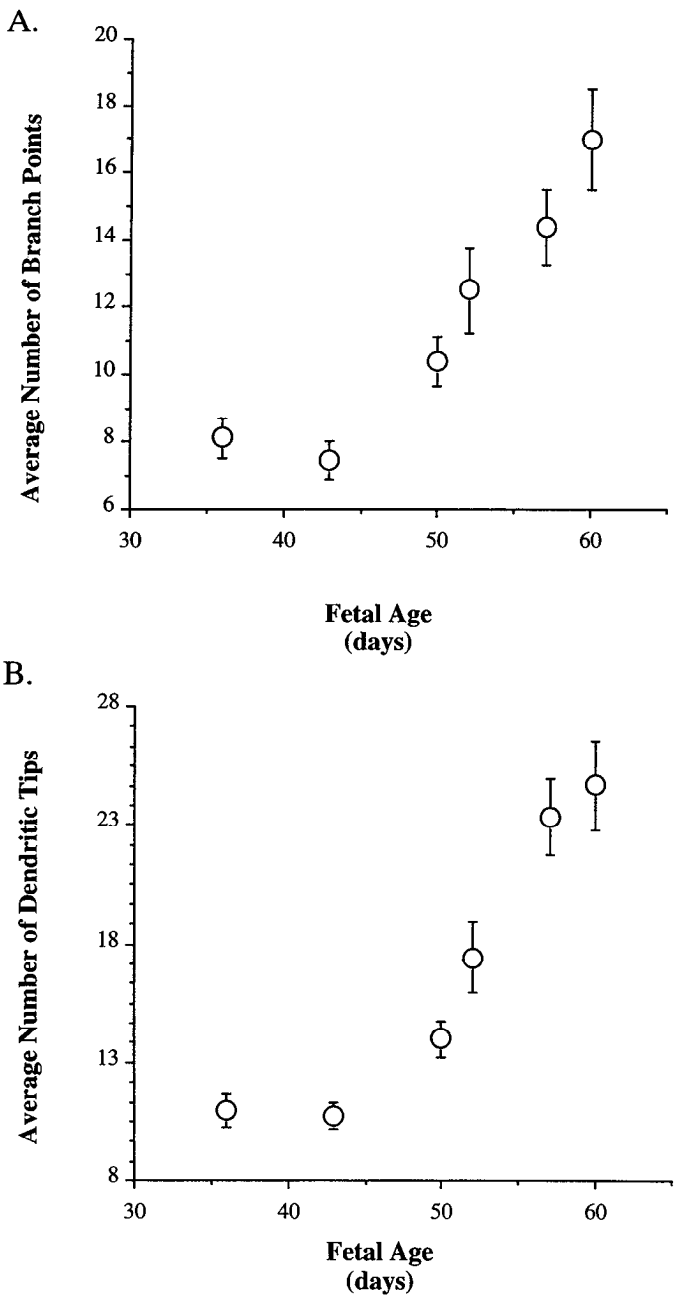


Figure 6. The progressive increase in numbers of branch points (*A*) or dendritic tips (*B*) on LGN neurons between E36 and E60 is shown. Note that there is a rapid increase in these parameters between E43 and E60.

and form a dense, compact terminal arbor (Fig. 9). In contrast, there are no layers in the TTX-treated fetuses and the terminal arbors of ganglion cell axons were found branching over a much wider area than normal, in an expanded pattern similar to that previously described following TTX treatment (Sretavan et al., 1988). The retinogeniculate axon terminals shown in Figure 9 appear somewhat less extensive than those described by Sretavan et al. (1988) because we could only inject DiA into single 200 μm sections, whereas Sretavan et al. labeled axons in an intact preparation and reconstructed them from many sections. However, when compared to the normal afferent terminal arbors, it is evident that retinogeniculate axons in our TTX-treated cases are profoundly affected in the expected fashion. Taken

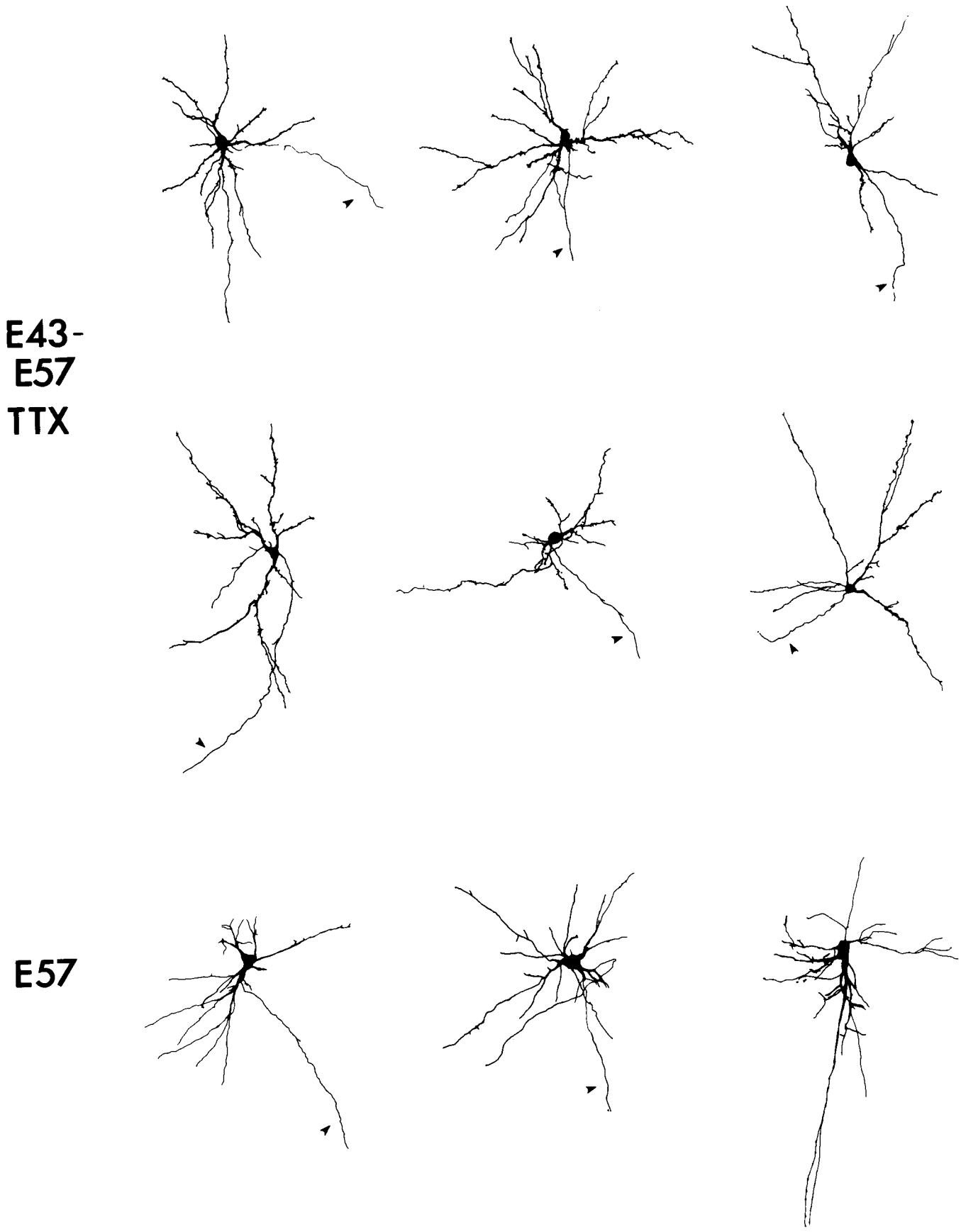


Figure 7. Camera lucida drawings of representative LGN neurons following treatment with TTX by intracranial minipump infusion from E43 to E57 (*top two rows*), as compared with normal LGN neurons at E57 (*bottom row*) are shown. Note that there is little discernable morphological difference between normal and TTX-treated cells. Scale bar, 100 μm.

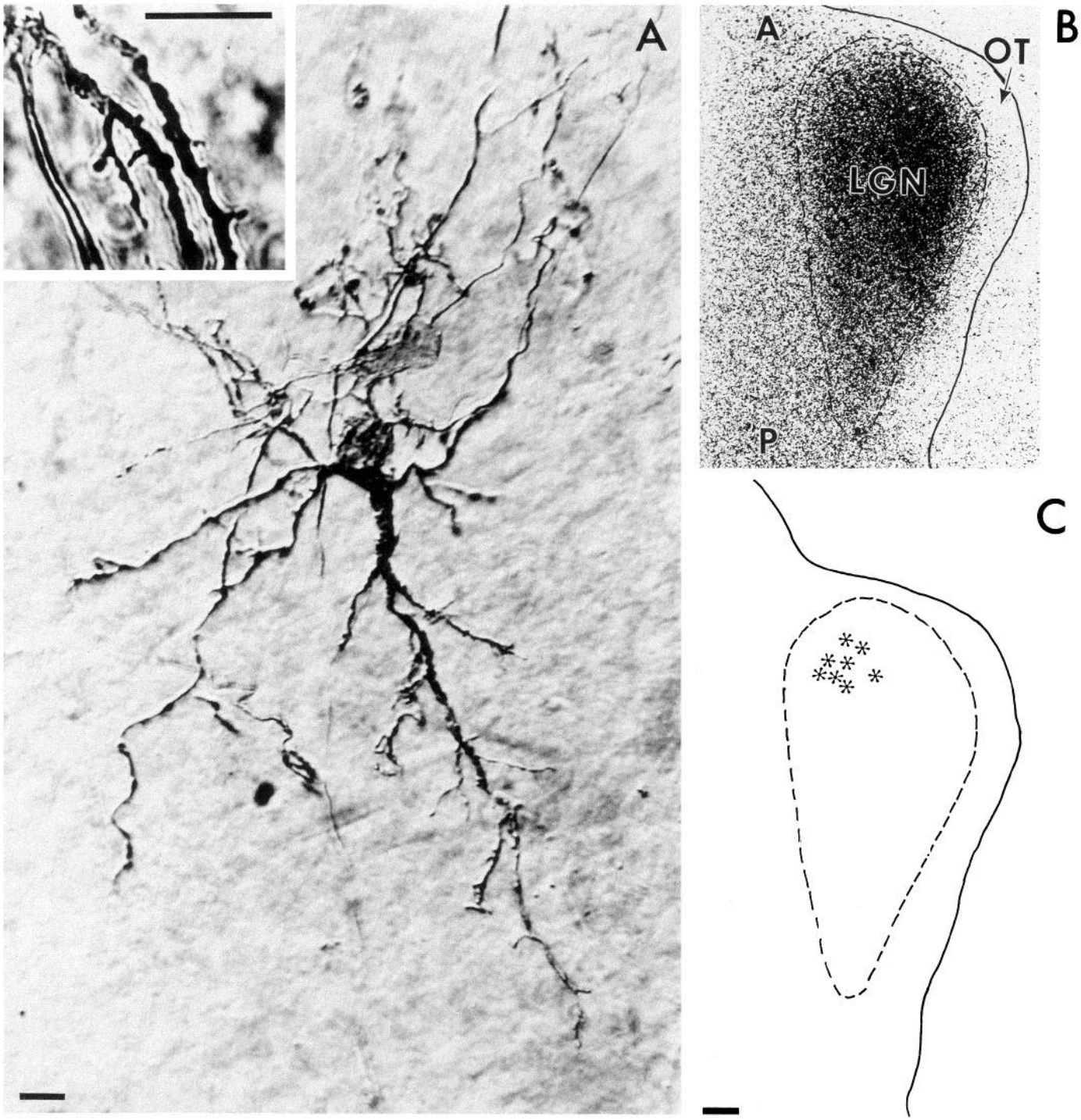


Figure 8. Composite figure from an experiment in which TTX was infused from E43 to E57. *A* shows a photomicrograph of a treated LGN neuron at E57 that appears essentially normal. The *inset* illustrates examples of abundant spines present on the dendrites of another TTX-treated neuron. The effectiveness of the TTX treatment was assessed in the same experiment by examining whether the segregation of retinal ganglion cell axons into eye-specific layers had been prevented. *B* shows an autoradiograph demonstrating the pattern of ganglion cell terminal labeling following an intraocular injection of ^{3}H-leucine at E56. Note that the radioactive label (*black grains*) is uniformly distributed in the LGN (*broken lines*) without being restricted to layers. The locations of cells in this section that were included in our analysis are shown by the *asterisks* in *C*. *OT*, optic tract. *A*, anterior; *P*, posterior, in this horizontal section. Scale bars, 100 μm.

together, these observations indicated that the TTX treatments were indeed effective as judged by the fact that afferent segregation was prevented.

To confirm our impression that TTX treatment had little effect on dendritic development within the LGN, we measured dendritic parameters of neurons after treatment with TTX from E43 to E57. First, we ascertained that the citrate buffer vehicle infusions had no significant effect on any of the parameters under study. As shown in the scatter plot of Figure 10, LGN neurons following 2 weeks of citrate buffer infusion (E43–E57) were not different in the overall range of either soma size or total dendritic length from normals at E57. Therefore, we treated these two

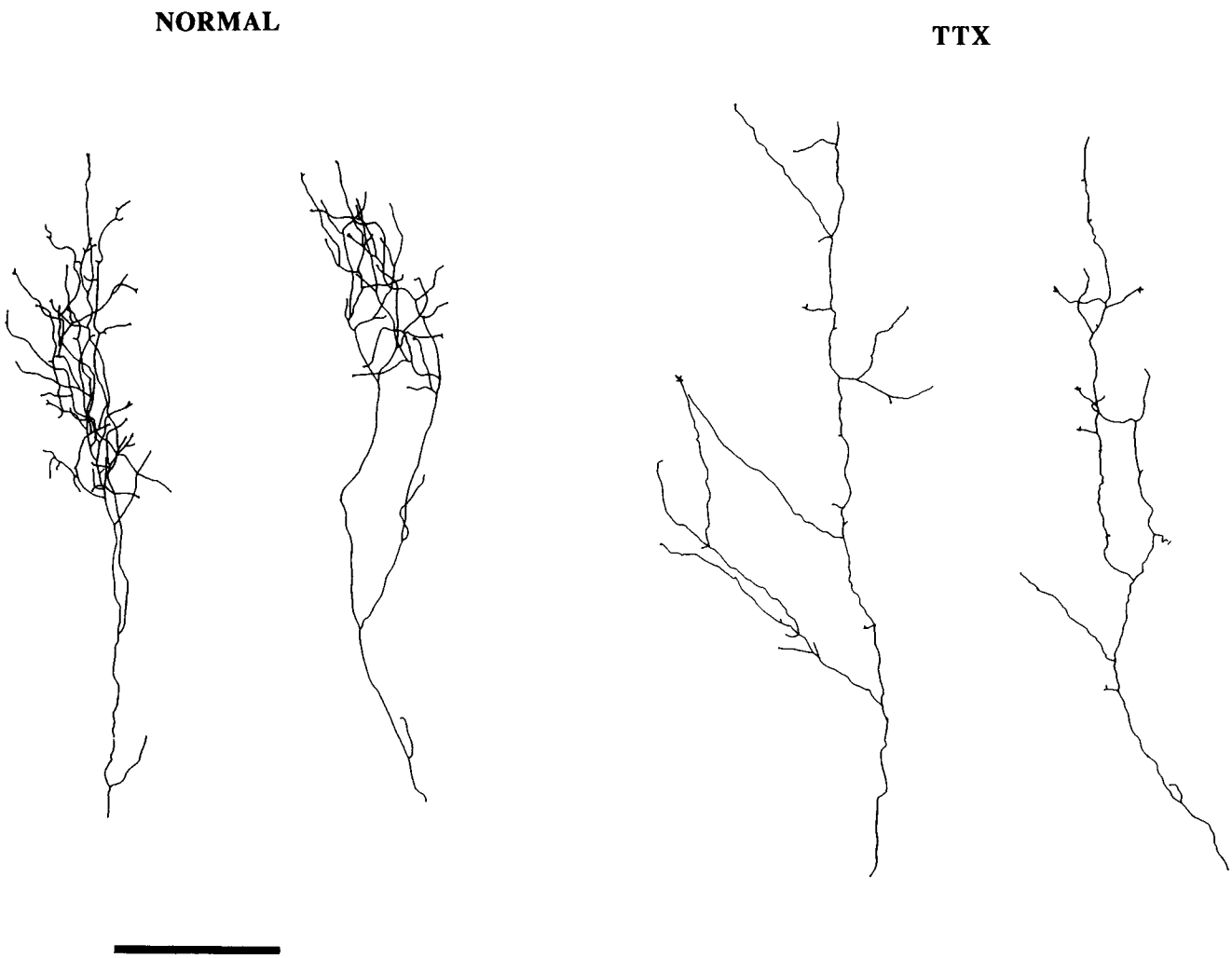


Figure 9. The camera lucida drawings in this figure illustrate the morphology of retinal ganglion cell axons located in the same sections from which the morphology of DiI-labeled LGN neurons was assessed. The axons were filled by labeling them with another fluorescent tracer, DiA, placed in the optic tract. To the *left* are two axons drawn from a normal LGN at E57. Note the restricted and dense terminal branching pattern of these axons, as expected at this age. To the *right* are two axons from a TTX experiment. Note that these axons lack a dense terminal arbor and instead branch along their entire length. The pattern of unrestricted branching indicates that the TTX treatment was effective despite the fact that LGN dendrites in the same section developed in a relatively normal fashion. Scale bar, 100 μm.

data sets as one population and compared them with TTX-treated cases. Even following TTX treatment, total dendritic length remained unchanged (Fig. 10), as did the number of dendritic branch points and tips (branch points: E57 normal and citrate treated, 12.2; TTX, 13.3, not significantly different $p < 0.1$; dendritic tips: E57 normal and citrate treated, 20.3; TTX, 19.1, not significantly different $p < 0.1$). Further, cell soma area following TTX treatment was not significantly different from either normal animals at E57 or citrate controls ($p < 0.1$). These results suggest that LGN neurons can achieve significant growth and maturity of form even after 2 weeks of TTX treatment.

Given the essentially normal appearance of LGN neurons following TTX treatment, it was surprising to find that the density of dendritic spines increased dramatically following TTX treatment when compared to both normal animals at E57 and citrate buffer controls (Fig. 11). After TTX treatment, LGN projection neurons carry almost three times as many spines per micrometer than the normal or control. This increase is not due to a normally occurring transient peak in spine density that is simply prolonged or maintained by the TTX treatment. As shown in Figure 11, spine density remains remarkably constant throughout normal development between E36 and E60. Therefore, TTX treatment may induce the formation of extra spines on LGN dendrites. Nor is this increase in spine density due to nonspecific effects of the minipump infusions or implantation itself, since in the two animals treated with citrate buffer vehicle solution, spine density was in the normal range ($0.16 \pm 0.0019/\mu$m; $n = 11$ cells; see Fig. 11). Thus, these observations indicate that while the basic morphological development of LGN neurons can proceed normally in the presence of TTX, spine production is altered.

Discussion

In this study we have used the technique of retrograde labeling with DiI to examine the development of LGN dendrites during the early prenatal period in the cat in which the afferent innervation from retinal ganglion cells segregates to form the alternating eye-specific layers, and also under the condition where afferent segregation has been prevented by the intracranial in-

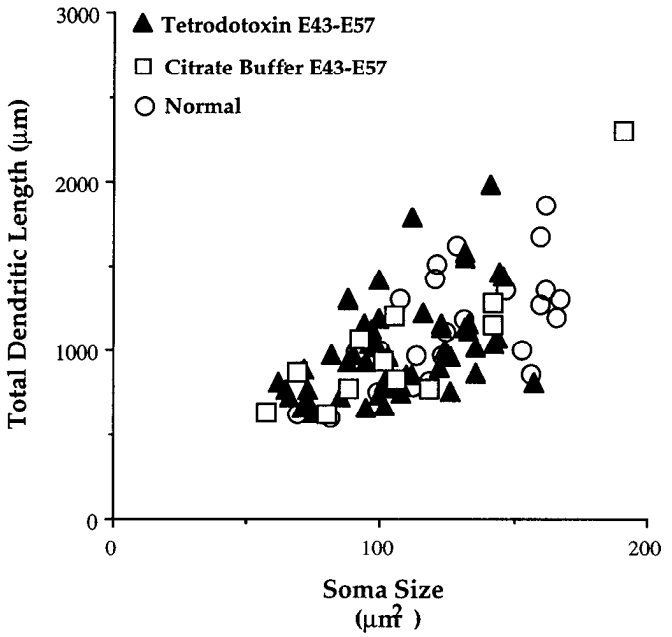


Figure 10. Scatter plot of total dendritic length (TDL) versus cell soma area showing that these features develop essentially normally following TTX treatment from E43 to E57. *Open circles* are measurements of normal (untreated) LGN neurons at E57; *open squares* are neurons treated for 2 weeks between E43 and E57 with minipump infusions of sodium citrate buffer; and *solid triangles* are measurements from neurons treated for 2 weeks between E43 and E57 with TTX. The regression lines for all three distributions are not significantly different from each other (R = 7.1, normal; R = 10.6, citrate; R = 5.5, TTX).

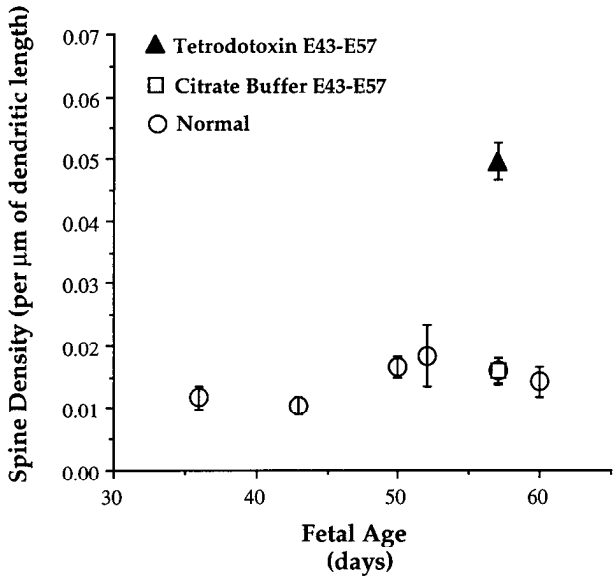


Figure 11. This graph illustrates that during normal development between E36 and E60, spine density remains constant, but following TTX treatment, LGN neurons demonstrate a marked increase in spine density. (Note that spine density of the citrate buffer infused controls is also normal.) Spine density was calculated by dividing the number of spines on each neuron by total dendritic length. These numbers were then averaged for all neurons at each age.

fusion of TTX. We have found that during this period, LGN dendrites normally grow extensively and progressively from an initially simple bipolar morphology at E36 to achieve a highly branched dendritic tree by E60. A major finding is that this basic pattern of dendritic growth persists even in the presence of TTX, which simultaneously grossly perturbs the growth and pattern of arborization of the ganglion cell axons that provide afferent input to the LGN neurons. This observation suggests that the factors that control dendritic and axonal form must differ at least during the period under study. Moreover, it appears that the dendritic growth of LGN neurons is not rigidly yoked to the growth of the presynaptic ganglion cell axons.

Normal dendritic development of LGN neurons

DiI labeling of LGN neurons indicates that as early as we can look, E36, these cells have already extended primary dendrites over several hundred micrometers. We could not look earlier because axons from the LGN have not arrived at the visual cortex, and therefore we could not definitely retrogradely label LGN neurons (Shatz and Luskin, 1986; Ghosh and Shatz, 1992). At E36, many LGN neurons have just completed their migrations to form the LGN (S. J. Eng and C. J. Shatz, unpublished observations) and retinal axons have already begun to invade from the optic tract (Shatz, 1983; Kliot and Shatz, 1985). In contrast, corticogeniculate axons will not grow into the LGN until about E60 (McConnell et al., 1994). At E36, the retinogeniculate afferents are also morphologically very simple—the majority are completely unbranched (Sretavan and Shatz, 1986). Comparison of the extent of growth of LGN dendrites with that of ganglion cell terminal arbors within the LGN between E36 and E60 shows that both have remarkably parallel, linear curves (compare Fig. 5*A* with Fig. 9 in Sretavan and Shatz, 1986). Such a temporal correlation raises the possibility that the retinogeniculate afferents might directly regulate the growth of LGN dendrites. However, as discussed below, the situation cannot be this simple because TTX treatment results in a 30% increase in the extent of ganglion cell axonal arborization (Sretavan et al., 1988) without altering the extent or pattern of LGN neuron dendritic branching.

In contrast to the chiefly linear increase in dendritic length during development, we found a dramatic nonlinear rise in dendritic complexity (as measured by number of branch points or dendritic tips) beginning at about E43, and persisting throughout the period during which ganglion cell axons from the two eyes segregate from each other to form the eye-specific layers. This period of major axonal rearrangement within the LGN is therefore accompanied by substantial development of the postsynaptic cell, but again this particular aspect of dendritic growth cannot be tightly coupled to the process of axonal segregation since it too persists in TTX when segregation is blocked.

Previous Golgi impregnation studies of the postnatal development of cat LGN dendrites had suggested that at birth, dendrites are very simple and most growth occurred postnatally (Mason, 1983). However, our present results indicate that LGN dendrites even prior to birth are much larger than previously supposed, and undergo extensive prenatal growth. Similar results using DiI labeling have also been obtained recently in studies of the ferret LGN (Sutton and Brunso-Bechtold, 1991; Rocha and Sur, 1993). One interpretation of this difference is that the Golgi technique fails to impregnate the fine distal dendrites of immature LGN neurons (whereas DiI does), thereby leading to an underestimate in size and complexity. Another possibility is that the two techniques have labeled different populations of LGN neurons: the DiI retrograde labeling technique permits us to examine only the LGN projection neurons and not interneurons, whereas perhaps the Golgi technique labels

chiefly interneurons. However, this suggestion is made unlikely by studies using intracellular HRP injections that have demonstrated that Golgi preparations can indeed label both projection and interneurons, at least in the adult (Friedlander et al., 1981). Whatever the case, we can conclude that LGN projection neurons grow extensively prenatally and in this regard the dendritic surface area potentially available for innervation by retinal ganglion cell axons may increase steadily during the period of segregation to form the eye-specific layers. However, even though there is extensive growth of dendrites prenatally, a good deal of growth and differentiation continues well into postnatal life: at E60 we could not identify LGN projection neurons according to their adult subclasses (Guillery, 1966; Friedlander et al., 1981), whereas these cell classes can be identified by 4 weeks postnatal following the additional dendritic growth needed to acquire the characteristic dendritic branching patterns (Friedlander and Tootle, 1990). Thus, this feature of LGN dendritic maturation appears postnatally (Mason, 1983).

The role of action potential activity in the development of dendritic morphology

In this study, we examined the effects of intracranial infusions of TTX on the development of dendritic form. It was remarkable to find that the basic extent and pattern of LGN dendrites formed normally during the same period when the growth and branching of their presynaptic inputs were profoundly altered. These observations suggest that many important aspects of dendritic development during this period can proceed in the absence of action potential activity mediated by voltage-sensitive sodium channels. A similar result was recently reported in axolotl by Goodman and Model (1990) for the dendrites of Mauthner neurons following activity blockade. The interpretation of our results depends critically on the assumption that the TTX infusion into fetal animals effectively blocks action potentials in the retinogeniculate pathway. Several lines of evidence support this suggestion. First, previous experiments in which the concentration of TTX in the cerebrospinal fluid was measured following the minipump infusions indicated that it was of a sufficient level (1 μM) to block completely all action potentials in the optic nerve and tract (see Shatz and Stryker, 1988). Second, *in vitro* physiological experiments in which extracellular microelectrode recordings were made from the LGN in a preparation detached from the retina revealed that although LGN neurons could fire action potentials in response to direct electrical stimulation of the optic nerve, no spontaneously generated action potentials could be recorded (Shatz and Kirkwood, 1984). This observation suggests that the action potential activity of LGN neurons derives principally if not exclusively from retinal inputs, which are themselves almost certainly silenced by the TTX infusion. We also know that the TTX treatment itself must have been effective here because it was present in sufficient amounts to alter retinal ganglion cell axon morphology in the same brain in the two cases where retinogeniculate axons were examined. Thus, the basically normal morphology of LGN neurons in TTX-treated animals is highly likely to develop in the absence of postsynaptic action potentials.

This conclusion, that dendritic form can develop without action potentials, seems at first glance to conflict with the results of previous studies during postnatal development of the LGN, in which alterations in neural activity can have a profound affect on the morphology of LGN neurons. For example, monocular eye closure or intraocular TTX injection into one eye causes a profound shrinkage of LGN somata (Guillery, 1973; Ricco and Matthews, 1987; Herrmann et al., 1992). In addition, with long-term monocular eye closure the characteristic morphology of LGN dendrites is perturbed, making it difficult to distinguish the various classes from one another based on structure alone (Friedlander et al., 1982; but see Humphrey and Weller, 1988). These experiments indicate that alterations in neural activity can indeed affect the postnatal development of LGN neurons. However, it should be noted that in these experiments, only one eye's activity was altered or blocked. Consequently, it is not possible to separate the effects on LGN dendritic development of altered *competition* between LGN neurons receiving inputs from the two eyes for their postsynaptic cortical targets from the effects of *decreased activity* derived from their presynaptic retinal inputs. In our present experiments, by blocking action potential activity within the LGN (rather than in the retina) via minipump infusions, we have eliminated the variable of intraocular competition and can focus exclusively on the effects of eliminating activity on the dendritic development of LGN neurons.

The observation that a good deal of dendritic development can occur in the absence of action potentials suggests that there may be an initial activity-independent phase to dendritic development. However, because of the confounding problem of intraocular competition mentioned above, at present it is difficult to conclude that there is a later postnatal period in which action potential activity in LGN neurons is necessary for the continued *normal* development of their dendrites. Indeed, action potential activity appears to play a relatively minor role the postnatal dendritic development of cat retinal ganglion cells. Retinal ganglion cell dendrites normally undergo considerable growth and then extensive remodeling during development: prenatally, they acquire many spines and higher-order branches that are subsequently lost postnatally before the adult dendritic form appears (Dann et al., 1988; Ramoa et al., 1988). This dendritic remodeling proceeds entirely normally even when ganglion cell firing is blocked by direct intraocular injection of TTX during postnatal life (Wong et al., 1991). It would be important to know whether bilateral intraocular injections of TTX similarly have little effect on postnatal dendritic development in the LGN.

In the case of both LGN and retinal ganglion cell dendritic development, although postsynaptic action potentials are blocked by TTX, it is worth emphasizing that many other aspects of neural function in the pathway still remain. For instance, in the retina, many interneurons do not fire Na^+-dependent action potentials and in both retina and LGN, the spontaneous release of neurotransmitter by presynaptic terminals can still occur, as can subthreshold changes in postsynaptic membrane potential. It is possible that these transmitter-evoked events are essential for dendritic development, as proposed from the results of *in vitro* studies by Mattson et al. (1988; also reviewed in Kater and Mills, 1991) and implied by recent studies demonstrating that blockade of glutamatergic transmission can indeed alter dendritic development of mammalian retinal ganglion cells (Lau et al., 1992; Bodnarenko and Chalupa, 1993) and LGN neurons (Rocha et al., 1991).

Not all aspects of the development of LGN neurons are normal following the TTX treatment: dendritic spine density is increased by almost threefold over normal. In the adult, dendritic spines are known sites of synaptic contact (Harris and Landis, 1989; Harris et al., 1989; Peters et al., 1991); if the same

holds for development (Boothe et al., 1979; Landis, 1987; Landis et al., 1989; but see also Wong et al., 1992), then the observed increase could represent an increase in the number of synapses, which would then occur in concert with the observed increase in the size of the presynaptic ganglion cell axon terminal arbor (Sretavan et al., 1988). Alternatively, it is possible that the constant spine density observed for normal LGN dendritic development is maintained by an equilibrium between spine elaboration and retraction, which is itself dependent at least in part upon postsynaptic action potentials. Thus, TTX may perturb this relationship by decreasing the rate of spine retraction.

Previous deafferentation studies have implied a crucial role for the presence afferent axons in the normal dendritic development of target cells (Purves and Hume, 1981; Purves et al., 1988; Rubel et al., 1989). If retinal ganglion cell axons indeed influence dendritic development, the results presented here imply that such effects are not likely to require action potential activity. As mentioned earlier, interactions between pre- and postsynaptic cells could still be mediated by neurotransmitter release, and in addition hormonal (Breedlove, 1985, 1986; Booker and Truman, 1987; Truman and Reiss, 1988) or neurotrophic factors could contribute (Snider, 1988; Cohen-Cory and Black, 1993). Whatever the case, our present observations on the development of LGN dendrites, coupled with previous information on the development of their presynaptic inputs, the retinal ganglion cells, have permitted a correlation of development of pre- and postsynaptic partners in the retinogeniculate pathway. While it is true that many dendritic features of LGN neurons develop in parallel with changes in the presynaptic axons, our results using TTX indicate that the two developmental processes can be uncoupled. Thus, axonal and dendritic form within the same structure are likely controlled by different cellular mechanisms during the same period of prenatal development.

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