INFLUENCE OF THE TARGET ON DEVELOPMENT OF THE FERRET RETINOGENICULATE PROJECTION

by

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ABSTRACT

The focus of this thesis is to delineate the normal development of the retinogeniculate projection in the ferret's visual pathway, and examine the role of afferent-target interactions in the formation of specific connections between retinal afferents and target cells in the lateral geniculate nucleus. Specifically, the studies presented here address the influence of the target on afferent arbor structure, both at the level of the entire retinal projection and at the level of individual retinal ganglion cell axon arbors. Target influence on afferent structure has been investigated in two ways, first by reducing the level of activity of the LGN neuron during a dynamic period of afferent segregation, and second by reducing the size of the entire LGN to determine how target size affects afferent size.

The ferret's retinogeniculate projection was chosen as the model of study because it is extremely specific in organization, with segregation of both eye-specific and On and Off afferents. This additional segregation permits the process of

development to be investigated in different stages. The ferret visual system resembles that of the cat in organization and developmental timecourse, but the ferret is more attractive for studies of development, being born three weeks earlier in gestation than the cat.

In the first set of experiments, the normal development of the retinogeniculate axon arbors was determined. The results indicate that retinal afferents undergo two phases of segregation to achieve the adult form. On the day of birth (P0), retinal afferents from the two eyes are overlapped in the LGN. Individual retinogeniculate axons do not have a terminal arbor, but have side branches along the length of the axon which extend into both presumptive contralateral and ipsilateral target zones. By one week of age (P7-P8), afferents have begun to segregate into eye-specific laminae, and single axons have formed terminal arbors largely restricted to an ipsilateral or contralateral eye-specific layer. Side branches have largely disappeared. At two weeks of age (P14-P15), the LGN has clearly become segregated into the characteristic A, A1 and C layers. Retinogeniculate axons have arbors which are still spread across the extent of one eye-specific lamina. By the third postnatal week (P19-P21), laminae A and A1 have further segregated into inner and outer leaflets or sublaminae corresponding to On and Off cells, and axon arbors of retinal afferents are restricted to a single sublamina. By P28-P35 the LGN appears adult-like, and axon arbors also take on adult-like characteristics as evidenced by clusters of terminal boutons on arbor branches. Thus, retinal afferent segregation is achieved in two stages, first into eye-specific laminae, then into On and Off sublaminae.

The second set of experiments addressed the role of activity during the period of afferent segregation into On and Off sublaminae. In this study, synaptic transmission between retinal afferent and target cell was blocked by introducing an antagonist for the NMDA subtype of glutamate receptor, an excitatory amino acid

that has been shown to mediate synaptic transmission in the visual system generally and particularly at the retinogeniculate synapse. The results indicate that retinogeniculate axon arbors are indeed affected by blockade or reduction of target cell activity; axon arbors are inappropriately localized within the A and A1 laminae such that they extend across presumptive sublaminar borders rather than being confined within them. Arbor size, however, is relatively unaffected, indicating that reduction of target cell activity does not inhibit normal arbor growth.

In the third set of experiments, the influence of target size on afferent size was examined. The LGN was induced to shrink by ablating primary visual cortex at birth. In the sample of X retinogeniculate axons that were recovered, results suggest that size of arbors is regulated by the size of the LGN. These results, together with those of earlier studies from this laboratory, indicate that location and arbor size of both X and Y retinogeniculate axons are subject to extrinsic influences. For X axons there appears to be an early critical period during which change in LGN size has an affect on arbor size.

These studies have defined the development of retinogeniculate axons in ferrets, and provide evidence that target influences such as activity and overall size regulate the size and location of retinogeniculate axon arbors within the lateral geniculate nucleus.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

The visual system of higher mammals is characterized by a marked degree of specificity, in which inputs or cells of like type are grouped together and segregated from those of different type (see Rodieck, 1979; Sherman & Spear, 1982 for review). For example, retinal ganglion cell axons from the two eyes are kept separate in the first synaptic target, the lateral geniculate nucleus (LGN) (Hickey & Guillery, 1974), and this separation continues to be maintained in the projection from the LGN to the primary visual cortex (area 17). These connections, in addition, are well ordered according to site of origin in the retina; retinotopic representation of the visual field is established in the LGN and in area 17. In some animals, the visual system undergoes further segregation; for example in mustelids, the A layers of the LGN are divided into longitudinal sublaminar leaflets (Levay & McConnell, 1982; Linden et al., 1981; Cucciaro & Guillery, 1984), the inner leaflet representing On-center inputs and the outer leaflet Off-center inputs (Stryker & Zahs, 1983).

The segregated and well-ordered nature of adult structures does not arise from the onset of formation of these connections. It is now well established that in the mammalian visual system, afferents from differing inputs are intermixed when first growing into target tissue, then become segregated according to input origin through a phase of fiber sorting (see Sretavan & Shatz, 1986 for review). Such a process has been shown to occur during eye-specific segregation of the retinogeniculate projection in rodents (Bunt et. al., 1983; Lund & Bunt, 1976; So et. al., 1978, Frost et al., 1979), opossum (Cavalcante & Rocha-Miranda, 1978), and carnivores (Shatz, 1983; Linden et. al., 1981; Cucchiaro & Guillery, 1984). The geniculocortical projection in cats (Levay et. al., 1978) and in monkeys (Rakic, 1979) also achieves final structure through such a sorting process.

The nature of the mechanism underlying the process of segregation has not yet been clearly determined, but there is much data to indicate that interactions between the afferent and target are critically involved. Disruptions of normal interactive processes lead to abnormal development of both afferent and target structures. Most studies that have examined the role of interactions in visual system development have focused on afferent influences on target structures, or on interactions between different sets of afferents (e.g. Rakic, 1981; Shatz & Sretavan, 1986; Sherman & Spear, 1982; Sur, 1988; Shatz, 1990 for review). The role of target influences in the development of afferent structures has not been well documented. In the set of experiments that are presented in this thesis, I have focused on the role of afferent-target interactions in the development of segregated connections within the ferret's retinogeniculate pathway. Specifically, the influence of the target on the development of retinal afferent structure has been examined, at the level of the entire retinal projection and of the single retinogeniculate axon. The role of the target has been assayed in two ways: by interrupting synaptic transmission between retinogeniculate afferents and LGN cells with specific antagonists to postsynaptic receptors, and by altering the size of the LGN through induced retrograde degeneration. These studies yield some insight on the contribution of the target to normal developmental processes within the central nervous system.

Target Influences on Afferent Development: The Neuromuscular Junction

The idea that a target can influence the developing afferent, while not new, derives mainly from studies of the development of motoneuron innervation of muscle. Hamburger (1977) first showed that motoneurons are dependent on their target for survival in embryonic life. This has been interpreted to indicate the

presence of a target-derived trophic factor, necessary to ensure neuron survival and promote axon elongation at certain periods in development. In this mechanism, a trophic factor is synthesized by the muscle in limited amounts; the ingrowing fiber recognizes and takes up the factor, which in some way contributes to survival of that neuron. In addition, synaptic activity is known to play a role in neuromuscular connectivity. Pittman and Oppenheim (1978) found that blocking neuromuscular activity in chick embryos prevented motoneuron death that normally occurs after target innervation. These results led to the proposal that muscle production of neurotrophic factor is interrupted by synaptic activity. Inactive muscle would thus produce more trophic factor, permitting more motoneurons to survive. If a similar mechanism is present in the visual system, it could be proposed that retinal afferents compete for some target-derived factor (Schneider, 1981), produced in limited amounts by the geniculate cells. Activity blockade may hence be promoting axon outgrowth by permitting uninterrupted production of trophic factor.

Unfortunately, the data from the many studies on the effect of muscle activity on target-derived factors are far from conclusive, and do not present a clear direction to follow for research in the visual system (see Oppenheim, 1985 for review). In a study in which motoneurons were co-cultured with curare-treated or normal chick limb muscles, no increase in survival promoting activity was found for the curare-treated muscle (Tanaka, 1987). On the other hand, Henderson et al., (1986) found that there is increased neurite-promoting activity in muscles of the genetic mutant 'paralyse' mouse, and tenotomised rats. Dahm and Landmesser (1988a) have shown that intramuscular branching of motoneuron axons in chick hindlimb muscle increases after activity blockade with curare or alpha-bungarotoxin. However, it has also been shown that motoneurons are directly affected by curare treatment, so that the results may not be due to a target-dependent mechanism (Landmesser & Szente,

1986). Landmesser et al. (1988) suggest that any factor that disrupts normal production of adhesion molecules which are crucial for muscle innervation will cause increased axonal branching, and that activity blockade may be one such factor. Oppenheim (1989) suggests that 'access' to trophic factor rather than "supply" may be the limiting factor regulating taret influences on afferents. However, it has been shown that synapses, which are proposed to be sites of trophic factor uptake, do not form until the end of the cell death period (Dahm & Landmesser, 1988b). These results indicate that, in the neuromuscular system, much more work is needed before a clear picture is realized of the role of activity and target-derived trophic factor on afferents. These studies still may offer clues to the mechanism subserving segregation in the visual system.

Role of Activity in the Development of the Visual System

A critical factor which has received much scrutiny in visual system development is the role of activity. In the cat, visual experience is known to have a profound effect on visual system organization (Movshon & Van Sluyters, 1981; Sherman & Spear, 1982; Sherman, 1985; Shatz, 1990 for review). Wiesel and Hubel (1965) first showed that occlusion of one eye by monocular lid suture during a critical period led to diminished ability of that eye to drive cortical cells. Such deprivation led to increased representation in the visual cortex of the open eye, in the form of expanded ocular dominance columns in layer 4 of primary visual cortex (Hubel et al., 1977). These and other studies which altered patterned input to the cat's visual system (e.g. by inducing strabismus, Hubel & Wiesel, 1965) indicated that visual experience, and hence patterned activity of neurons, was involved in forming connections within the brain.

A more direct approach to studying the effect of neural activity is to block action potentials during development. Tetrodotoxin (TTX), a compound which blocks voltage-gated sodium channels and hence all action potentials, has been used in a number of studies to disrupt impulse activity. Ocular dominance stripes, which can be induced into the normally completely crossed retinotectal pathway of the frog (Rana pipiens) by surgically attaching a "third" eye primordium to the forebrain of embryos (Constantine-Paton & Law, 1978), are blocked by binocular TTX injections (Reh & Constantine-Paton, 1985). Similarly induced ocular dominance columns in goldfish tectum have been blocked with intraocular TTX injection (Meyer, 1982; Boss & Schmidt, 1984). The ocular dominance columns of kitten visual cortex do not form when binocular TTX injections are administered (Stryker & Harris, 1986), nor is there a shift in ocular dominance following monocular deprivation when TTX is infused into the cortex (Reiter et al., 1986).

In the kitten retinogeniculate projection, Shatz and Stryker (1988) found that TTX infusion into the region above the optic chiasm before onset of segregation prevented formation of eye-specific layers. Further, the axon arbors that formed after such blockade were highly abnormal, unlike any seen in normal development. Arbors were extremely wide, sometimes covering almost the entire width of the LGN, quite diffuse, and generally spread over a greater area than normal (Sretavan et al., 1988). Eye injections of TTX postnatally, when appropriate connections have already formed, also disrupted normal synaptic connectivity. Abnormal synaptic connections were formed between retinal afferents and LGN cells to create cells that responded to both On and Off input (Archer et al., 1982; Dubin et al., 1986). In addition, structural development of retinogeniculate synapses was blocked by TTX (Kalil et al., 1986). Furthermore, postnatal TTX eye injections induce retinogeniculate axons to sprout into "wrong" layers (Sur et al., 1985).

Further support for the role of activity in afferent segregation and topography formation comes from studies showing that the spontaneous activity of cells from one eye is likely to be correlated with cells from within the same eye (Meister et al., 1991). It has also been found that neighboring ganglion cells are more likely to be correlated in their firing pattern than distant ganglion cells (Arnett, 1978; Arnett & Spraker, 1981; Ginsberg, 1984; Mastronarde, 1983a, b; 1989). That activity may play a developmental role is supported by the finding that spontaneous retinal ganglion cell activity has been demonstrated in prenatal rats (Galli & Maffei, 1989). It has also been shown that there are functional synapses in the LGN during the period of fiber segregation (Shatz et al., 1982), and that synaptic transmission can occur during this time (Shatz & Kirkwood, 1984).

There are a number of ways by which activity blockade may disrupt the normal organization process. For instance, TTX blockade may directly affect the ingrowing afferent, altering its activity and potentially changing cellular functions critical to axon outgrowth. Activity blockade may affect synaptic transmission, thereby changing the activity level of the postsynaptic cell. In this case, the effect on the presynaptic afferent would be indirect, caused by a reduction of activity in the target cell. One way this might happen is that an afferent would detect the efficacy of its synaptic terminal, perhaps by detecting changes in extracellular transmitter content or the change of some postsynaptic factor, altered as a result of transmission blockade. Alternatively, some message might be relayed back to the presynaptic terminal, through the existence of a retrograde signal which indicates some change in the postsynaptic cell.

It has been suggested that activity blockade studies, particularly those employing TTX, should be interpreted with caution. In most studies using TTX, both pre- and postsynaptic components are affected, resulting in complete silencing

of the connection. Frank (1987) has argued that blocking all activity is such a drastic alteration of a given cell's normal state that it may cause the cell to extend processes indiscriminately. Indeed, in cultured Helisoma neurons, activity inhibited neurite outgrowth and growth cone motility (Cohan & Kater, 1986). However, there is evidence to indicate that activity does not have such an inhibitory effect on vertebrate neurons (see Schmidt & Tieman, 1989, for review). In cultured rat retinal ganglion cells neurite outgrowth was suppressed by nicotinic antagonists but not by activity. Increasing activity by depolarizing the cells with potassium, glutamate or kainate did not decrease neurite outgrowth, leading to the conclusion that the effect of nicotinic antagonists was mediated by some factor other than activity (Lipton et al., 1988). In addition there is evidence to indicate that activity mediated through NMDA receptors can promote neurite outgrowth. Pearce et al. (1987) have shown that removal of endogenous glutamate from cultures of rat cerebellar granule cells inhibited neurite outgrowth, as did introduction of the glutamate antagonist kynurenate and the NMDA antagonist 2-amino-5-phosphonovaleric acid (APV). In goldfish, TTX treatment in vivo slightly delayed the outgrowth of regenerating ganglion cell axons (Edwards & Grafstein, 1983). There is additional evidence that speed of growth cone movement is increased with calcium influx, which occurs when the cell is activated (Kater et. al., 1988).

NMDA Receptors

Focus on neural activity in regulating synaptic connectivity has sharpened in recent years because of the functional characteristics of one receptor type for glutamate, and the role this receptor type has been shown to play in inducing a phenomenon known as long-term potentiation (LTP). There are at least four known types of glutamate receptors in the brain (Cotman & Monaghan, 1989); this

discussion will be limited to the two types present in the central visual structures. These are categorized as N-methyl-D-aspartate (NMDA), named for the preferentially bound agonist, and non-NMDA, of which there are two sub-types, preferentially binding quisqualate and kainate, respectively. Quisqualate and kainate receptors are permeable to sodium and potassium ions. The NMDA receptor is a voltage-gated, ligand binding ionophore complex. The complex is active when two conditions are met: ligand is bound to the recognition site, and the cell membrane is depolarized enough to relieve a magnesium block across the channel opening (Nowak et al., 1984). Once open, the channel permits entry of sodium and calcium ions. A neuron which is gated by glutamate receptors typically shows a two part response to stimulation: a short, fast EPSP believed to be mediated by non-NMDA receptors, and a long, slow EPSP which is gated by NMDA receptors (Cotman et al., 1988).

NMDA receptors are of interest because their capacity to induce long-term potentiation may constitute one mechanism by which synapses are stabilized. In the CA1 region of hippocampus, a burst of high frequency stimulation can lead to a long period of enhanced postsynaptic potentiation, termed LTP (Bliss & Lomo, 1973). It has been shown that the induction of LTP is dependent on postsynaptic depolarization, mediated through the NMDA receptors, and dependent on intracellular rise of calcium levels in the postsynaptic cell (Teyler & DiScenna, 1987, for review). The phenomenon of LTP fits well with the Hebbian theory of synaptic stabilization. Hebb (1949) first postulated that a synaptic contact would be more likely stabilized if there was temporal conjunction of pre- and post-synaptic activity. Further, he proposed that a synapse from an afferent which was more effective in changing the activity of the postsynaptic cell would more likely be maintained than one which was ineffective. Changeux and Danchin (1976) proposed a similar mechanism of "selective synapse stabilization" whereby the synaptic activity is one

important factor, interacting with the developmental programs of afferent and target, which determines maintenance of certain synaptic contacts. Additionally, the permeability of NMDA receptors to calcium may influence synaptic strength, as calcium is an intracellular second messenger that impinges on many cellular functions (Kennedy, 1989).

The distribution of NMDA receptors in the central nervous system shows that they are most heavily concentrated in areas involved in learning and memory, and sensory processing. As yet no receptor binding studies have been done on the distribution of NMDA receptor sites within the ferret central nervous sytem, but studies of kitten and cat brain have shown that the distribution pattern is similar to that of rodents, in which the most comprehensive binding studies have been conducted (Greenamyre et al., 1985; Monaghan & Cotman, 1985; Cotman et al., 1987; Fosse et al., 1989). At adulthood, NMDA receptors are densest in the CA1 and CA3 regions and layers I-III of cerebral cortex. There is also heavy binding in the basal ganglia, basal forebrain, and sensory structures including thalamic nuclei. In young animals, binding is heaviest in the inner layers of the cortex, globus pallidus, basal forebrain and subthalamic nucleus (Young et al., 1988). Physiological studies in our laboratory indicate NMDA receptors are present in ferret LGN as early as eight days after birth, the earliest age examined (M. Esguerra, unpublished results).

Role of NMDA Receptors in Development and Synaptic Plasticity in the Visual System

Because of their functional capacities, NMDA receptors may be regarded as prime candidates as arbiters of synaptic plasticity and development of connections in the visual system. What is not clear is whether the unique characteristics of NMDA

receptors necessarily dictate a special role for their involvement in these processes (see Shatz, 1990, for review). It is possible, for example, that NMDA receptors play a special role in development, and that they are more important traditionally during development than in adulthood. Alternatively it is possible that NMDA receptors are involved in normal synaptic transmission, that any role they play in development is a consequence of their function in synaptic transmission, and that their role is similar in general to any other transmitter receptor system. Data indicating their role in development, and in support of both aforementioned positions are discussed below.

Blockade of NMDA receptors in the tectum by implant of Elvax, a slow-release polymer, impregnated with the antagonist AP5, caused induced ocular dominance stripes in frog retinotectal projection to desegregate, whereas application of NMDA caused the stripes to sharpen (Cline et. al., 1987). In a somewhat counterintuitive finding, Cline and Constantine-Paton (1990) have also shown that retinotectal arbors are modified to result in reduced branch density as compared with normal when AP5 or NMDA is applied.

Normal development of the frog retinotectal projection is also disrupted by NMDA blockers. In Xenopus, the normal topographic matching between binocular inputs (direct contralateral input; ipsilateral input relayed through the nucleus isthmus) is blocked when APV or CPP is applied to the tectum (Scherer & Udin, 1989). What is unknown in the frog retinotectal system is the role of NMDA receptors in synaptic transmission; hence these studies may have to be reinterpreted as an effect of synaptic blockade.

In kitten visual cortex, research has focused on the formation and plasticity of cellular ocular dominance. It has been reported that NMDA antagonists infused during the critical period disrupted the shift in ocular dominance that normally occurs

after monocular lid suture (Kleinschmidt et al., 1987; Bear et al., 1990). Furthermore, the ocular dominance reversal that occurs following reverse suture is blocked when NMDA antagonists are applied (Gu et. al., 1989).

There are several lines of evidence to indicate that NMDA receptors are more involved in synaptic transmission in the young visual cortex as compared with the adult's. Firstly, the number of NMDA receptors increases rapidly in the kitten visual cortex between 2-4 weeks postnatal and is elevated throughout the critical period (Bode-Greuel & Singer, 1989; similar findings for glutamate receptors, Fosse et al., 1989). Second, LTP can be induced more easily in kitten visual cortex during the critical period (Komatsu et al., 1988). Third, NMDA receptors have been reported to be more effective at mediating visual transmission in kittens than in adults (Tsumoto et al., 1987; Hagihara et al., 1988).

However, this latter finding has been challenged by Miller and colleagues (Miller et al., 1989), who found that response to visual inputs was profoundly suppressed in adult cat cortex when APV was applied. These results have been further supported by Fox and colleagues (Fox et al., 1989). Another study which, albeit indirectly, disputes the special role of NMDA receptors, demonstrated that the increase in intracellular calcium induced by glutamate is transient in immature cells but permanent in mature cells (Wahl et al., 1989). These results could be interpreted to suggest that the potential for calcium to affect cell functions as a second messenger is thus not as long-lived in developing cells, converse to what would be expected if calcium entry through NMDA receptors plays a special role in young cells. It must be taken into consideration, however, that this study used cortical neurons cultured for different lengths of time and hence the results may not be directly extrapolated to in vivo studies. Although there clearly needs to be more research in this area, it is evident that NMDA receptors are important mediators of transmission in the visual

cortex. Thus, the developmental effects seen after blockade of NMDA receptors (described above) need to be interpreted with caution, for blocking NMDA receptors in the cortex would reduce synaptic transmission and hence the activity of postsynaptic cells. Reduction of postsynaptic activity could affect geniculocortical via mechanisms other than through a specific action of NMDA receptors (see Chapter 3).

In the cat retinogeniculate pathway, research from several laboratories including our own have shown that NMDA and non-NMDA receptors are both involved in normal transmission of visual input. In vivo studies in the LGN have demonstrated that iontophoretic application of either NMDA or non-NMDA receptor antagonists block the visual response of LGN relay cells (Hartveit & Heggelund, 1990; Heggelund & Hartveit, 1990; Sillito et al., 1990a, b; Kwon et al., 1991). In isolated ferret and cat LGN preparations in vitro, LGN cell EPSP's evoked by optic tract stimulation are reduced by blockade of both NMDA and non-NMDA receptors (Esguerra et al., 1989, and submitted; Scharfman et al., 1990). Furthermore, all components of visual responses and optic tract EPSP's including early and late components, are sensitive to NMDA receptor blockade. Importantly, NMDA receptors are found to mediate postsynaptic responses in ferret LGN cells in slices as early as two weeks postnatal (Esguerra, White & Sur, unpublished observations). These data suggest that NMDA receptors are involved vitally in normal synaptic transmission. Thus, any special function for these receptors in development of the visual pathway is an open question.

Regardless of a specific a special role for NMDA receptors in development, the most intriguing possibility presented by the studies of NMDA receptor blockade on neural plasticity (including those described in this thesis), is that specifically blocking or reducing the activity of the postsynaptic cell affects the structure and

connectivity of the presynaptic afferent. Although currently there is no direct evidence, NMDA receptors are believed to be localized on the postsynaptic cell. Thus, unlike TTX blockade, all experimental manipulations to date involving glutamate antagonists can be presumed to be affecting only the postsynaptic cell, but having an effect on the presynaptic afferent. Several mechanisms could be postulated for such an effect. First, there could be altered production of or access to a target-derived trophic factor, as proposed in the neuromuscular junction. Second, such an effect could be a consequence of the presynaptic afferent's requirement that a certain number of synapses be made, with the afferent having the capacity to detect the efficacy of its synapses. Third, there could be an actual retrograde signal carrying information about postsynaptic activity. Currently, no such signal has been identified, although arachidonic acid (Dumuis et al., 1988) and nitric oxide (Garthwaite et al., 1989; Gally et al., 1990) has been shown to be released from rat striatal neurons in which glutamate receptors had been stimulated. However, given the ubiquitousness of arachidonic acid, and the fact that its release is coincident with locus of synthesis, make unclear what specific role it could have in coordinating the segregation of presynaptic afferents. Additionally it is not understood what role nitric oxide has in normal cellular functions. Still, recent experiments on LTP in the CA1 region of the hippocampus (Malinow & Tsien, 1990; Bekkers & Stevens, 1990) indicate a presynaptic locus for maintenance of LTP after postsynaptic induction, suggesting that a retrograde signal may indeed play a significant role in at least certain forms of plasticity in the central nervous system.

Effect of Target Size on Afferent Arbors

Another issue of interest is to determine how the size of the target affects the developing morphology of the afferent. This question has been extensively studied

in many afferent-target circuits in the peripheral nervous system (Landmesser, 1980; Purves, et al., 1988, for review). In the central nervous system, the majority of studies have largely been focused on the visual system. In the vertebrate visual system, the effect of target alteration on the development of afferents has been studied by removing portions of the target during development or regeneration of the retinotectal projection (Udin & Fawcett, 1988, for review). Removal of half the tectum causes the entire retinal projection to compress onto the remaining target in an orderly, topographic fashion (Gaze & Sharma, 1970; Yoon, 1971; Udin, 1977; Schneider & Jhaveri, 1974; Finlay et al., 1979). In the hamster retinotectal projection, this is achieved with little loss of retinal ganglion cells (Wikler et al., 1986; but see Udin & Schneider, 1981), and no change in receptive field properties of the remaining tectal cells (Pallas & Finlay, 1989). It has been proposed that this mismatch of afferent to target is compensated by one of two mechanisms, an elimination of collaterals or reduction of arbor sizes (Pallas & Finlay, 1991). These studies also suggest that there is communication from the target to the afferent, because the afferent must be able to detect the change in overall size of the target to locate its terminations accordingly. A wholly afferent-dependent mechanism would result in a partial visuotopic map such that only those afferents that would normally project to the remaining tectal fragment would form terminals. This issue of target size and its influence on afferent size will be addressed in the last chapter of the thesis.

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CHAPTER 2

THE DEVELOPMENT OF INDIVIDUAL RETINOGENICULATE AXONS DURING THE PERIOD OF LAMINAR AND SUBLAMINAR SEGREGATION IN THE FERRET'S LATERAL GENICULATE NUCLEUS

ABSTRACT

In this study we have detailed the normal development of the retinogeniculate pathway in the ferret's visual system. The projection from the retina to the lateral geniculate nucleus (LGN) was examined at different postnatal ages, both at the level of the entire retinofugal projection and of individual retinogeniculate axon arbors. At birth (P0), axons do not have a terminal arbor, are sparsely branched and have side branches along their length which extend into presumptive ipsilateral and contralateral target zones. At one week of age (P7-P8), eye-specific laminae are beginning to form and terminal arbors have formed on the retinogeniculate axons. By two weeks (P14-P15) the characteristic A, A1 and C laminae are evident and single axon arbors have become more clearly confined to within one eye-specific lamina. At three weeks of age (P19-P21), retinal afferents in the A layers have segregated into inner and outer leaflets, or sublaminae, that correspond to On- and Off-center cells, respectively. Individual axon arbors are confined to an inner or outer sublamina. In the following weeks (P28-P35) the LGN assumes an adult-like form. The retinogeniculate axons similarly become adult-like and form terminal boutons on branch endings. Thus, retinal afferents go through two phases of segregation in the formation of the retinogeniculate pathway, first into eye-specific laminae, then into On or Off sublaminae; this is reflected in the morphological changes in the axon terminal arbor that occur during these stages.

INTRODUCTION

The development of the mammalian visual system has been the focus of a great deal of study, as experimenters have sought to determine how an initially mixed set of afferents and mass of undifferentiated cells is transformed into a structure of precise order and exquisite detail. The formation of the specific connections which comprise the visual pathway has been shown to be achieved via processes in which mixed inputs become segregated into separate zones in the target structure. In the retinogeniculate projection, afferents from the two eyes initially overlap extensively within their target structure, the lateral geniculate nucleus (LGN), before sorting into eye-specific cell layers or laminae (Bunt et al., 1983; Cavalcante & Rocha-Miranda, 1978; Rakic, 1979; Shatz, 1983; So et al., 1978; see White & Chalupa, 1991, for review). Segregation of binocular input is maintained in the projection from the LGN to the primary visual cortex (Levay et al., 1978, 1980; Rakic, 1976). In mustelids such as ferrets and minks, the visual pathway is further stratified in that ON and OFF inputs are also segregated within the LGN and primary visual cortex (Levay & McConnell, 1982; McConnell & Levay, 1984; Roe et al., 1989; Stryker & Zahs, 1983; Zahs & Stryker, 1986, 1988), a separation also seen in the retinogeniculate pathway of tree shrews (Conway & Schiller, 1983) and macaque monkeys (Schiller & Malpeli, 1978).

In the ferret, as in the cat, retinogeniculate afferents from both eyes also display initial overlap followed by a gradual segregation (Linden et al., 1981; Cucchiaro & Guillery, 1984). The ferret visual system is much like that of the cat in both organization and development; indeed the timetable of developmental events tracks that of the cat almost on a day-by-day basis in terms of days postconception (Guillery, 1970; Shatz, 1983; Linden et al., 1981; Cucchiaro & Guillery, 1984).

However, because the ferret is born earlier (42 days of gestation vs 65 days for cat), all of the critical developmental events occur postnatally, making the system much more amenable to experimental study.

In the present study, we examined the structure of single retinogeniculate axons at different stages in the development of the ferret's retinogeniculate pathway, particularly the ages when eye-specific and On-Off segregation take place. We were interested in the morphology of single axons during development, as the structural changes that occur at different stages may give insight to the critical factors that underlie the segregation process. In the cat, the study of single retinogeniculate axons indicates that morphological changes in their arbors underlie changes in the overall pattern of the retinogeniculate projection (Mason, 1982; Sretavan & Shatz, 1984, 1986a). We paid special attention to the size and location of axon arbors within the LGN at different ages, to discern the developmental sequence as well as possible mechanisms that might be involved in the two (eye-specific and On-Off) segregation processes. A preliminary report of these data has appeared in abstract form (Hahm & Sur, 1988).

MATERIALS AND METHODS

Thirty-two neonatal pigmented ferrets (mustelidae putorius furo) were used for this study. The first twenty-four hours after birth was designated postnatal day (P)0. Ages studied were P0, P1, P7, P8, P14, P15, P19, P21, P28 and P35. To determine the sequence of formation of eye-specific laminae and On/Off sublaminae, two procedures were employed. In one set of animals, one eye was injected with anterograde label to visualize laminae and sublaminae. In another set of animals, single axons were labeled using an in vitro procedure to visualize axon structure.

INTRAOCULAR INJECTIONS

Procedure

Animals were removed from the mother and anesthetized with Metofane. In animals whose eyes were not yet open (P0 - P21), the eyelid was swabbed with alcohol and an incision made along the future opening. The eye was anesthetized with a drop of ophthalmic anesthetic (Proparacaine). A hole was punctured in the eye behind the sclera with a 26 gauge needle. The tip of a Hamilton syringe (10 ul, 27 gauge needle) was inserted through the hole into the posterior chamber and 5-10 ul of a mixed HRP/WGA-HRP solution (20% HRP, 2% WGA-HRP, Sigma) injected very slowly into the eye. The eyelid was closed and treated with antibiotic ointment (Tri-Thalmic). The animal received a subcutaneous injection of amoxicillin (5mg/kg) and, after recovery, was returned to the mother. After a 24-hour survival, the animal was overdosed with sodium pentobarbital (65 mg/kg) and transcardially perfused with isotonic saline followed by aldehyde fixatives (1% paraformaldehyde, 1.25% glutaraldehyde in 0.1M phosphate buffer, pH 7.4). The brain was removed and placed in 30% sucrose phosphate buffer overnight.

Histology

The brain was embedded in a mixture of albumin and gelatin. Frozen sections were cut at 50 um in the horizontal plane on a freezing microtome, placed in phosphate buffer and processed for HRP label according to Mesulam (1982) using tetramethyl benzidine (TMB) as the chromogen. The reacted sections were mounted on chrome-alum subbed slides and allowed to air dry. The slides were dehydrated in a series of alcohols, cleared in xylene or Histoclear, and coverslipped using Permount. Alternate sections were stained with cresyl violet to visualize cell bodies, and were mounted, cleared and coverslipped as above.

SINGLE AXON LABELING

Procedure

The in vitro procedure of Sretavan and Shatz (1986a) was adapted for ferret tissue. Each animal was deeply anesthetized with sodium pentobarbital (65 mg/kg) and transcardially perfused for 1-2 minutes with cold (4 deg C), oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF, pH 7.4, 1mM sodium phosphate, 10mM HEPES, 114mM NaCl, 5mM KCl, 2.5mM CaCl2, 1.15mM MgSO4, 10mM dextrose, 25mM NaHCO3) . The heart remained rhythmically contracting throughout this period. The animal was placed on a chilled platform, the skull opened, the brain quickly removed and placed in a petri dish lined with Sylgard and continuously superfused with cold aCSF. The brain was pinned ventral side down through the frontal cortex and cerebellum. The corpus callosum was cut, and each cortical hemisphere was gently lifted up and dissected away from the thalamus by cutting through the internal capsule and cutting away the basal ganglia. The exposed thalamus and midbrain were bisected midsagittally and each hemithalamus laid on its

medial surface. The pia overlying the LGN was gently peeled away to expose the optic tract.

Glass micropipettes (Borosilicate, Omega dot) were dipped in a concentrated solution of HRP and distilled water and allowed to dry, leaving a tiny pellet of HRP adhered to the pipette tip. Under an operating microscope with a fiber optic light system (Olympus, M-1), the pellet was manually placed into the optic tract below the LGN, or overlying the ventral LGN. Typically, at least three injections were placed in different regions of the optic tract. After injection, the tissue was placed in a holding chamber containing oxygenated aCSF (at room temperature) for 6-8 hours to allow the label to transport anterogradely.

Histology

After the incubation period, the tissue was immersion-fixed overnight in 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) and then placed in 30% sucrose phosphate buffer. The two hemispheres were placed with the medial surfaces apposed to each other, held in place with insect pins, and embedded in albumin-gelatin. Frozen sections in the horizontal plane were cut on a sliding microtome at 100 um, then processed for HRP histochemistry using DAB with CoCl2 intensification (Adams, 1981). Serial sections were mounted onto subbed slides, air dried, dehydrated, cleared and coverslipped with Permount.

Reconstruction

Axons were reconstructed with camera lucida on a Leitz microscope (Diaplan) using 63X and 100X objectives under oil immersion with both transmitted light and differential interference contrast microscopy. An Olympus microscope was also used for some axons, with 50X and 100X oil immersion objectives.

Photomicrographs of HRP labeling of LGN from eye injections were taken with an Olympus microscope with a 4X objective using dark-field illumination.

RESULTS

Intraocular Injections

Injections of HRP/WGA-HRP into the eye resulted in dense label of the optic tract, LGN and superior colliculus. The position of the ferret LGN changes in the first five weeks of postnatal development, changing from a relatively flat sheet of cells attached anterior in the thalamus on the day of birth, to a structure shaped like a garlic-clove in the posterior thalamus by P35. During this period the LGN also rotates such that the anterior portion is displaced laterally and the posterior segment medially (Linden et al., 1981; see also Hutchins & Casagrande, 1990). Viewed in horizontal section the nucleus changes from a thin crescent-shaped wedge of undifferentiated cells to an L-shaped, laminated structure in which the thinner monocular segment runs roughly anterior-posterior longitudinally and the binocular segment mediolaterally.

On P1 (Fig. 1a) retinal afferents from both eyes are intermixed within the LGN. In cases with the whole eye injected with HRP, there is no evidence of fibers projecting to the perigeniculate nucleus (PGN); however, in cases where single axons were labeled (see below) we observed bundles of axons which course through the LGN and continued through the PGN. No projection to non-visual structures such as the medial geniculate nucleus (MGN) or inferior colliculus is seen. By P7 segregation into eye-specific laminae has already begun and areas of separation are clearly visible. However, afferents are still somewhat intermixed (Fig 1b), especially in the presumptive A1 lamina. Interlaminar zones are not yet well established as observed in Nissl-stained material. At this age and later ages, streams of fibers can clearly be seen coursing through the PGN; these are likely to be retinal ganglion cell axons that enter the LGN through the PGN (see also Linden et al.,

1981; Roe et al., 1989). By the end of the second postnatal week (P14/P15, Fig 1c), the A, A1 and C laminae are well defined and interlaminar zones are also evident in Nissl-stained sections. During the third postnatal week (by P19-P21) the sublaminar leaflets form in the A and A1 laminae (Fig 1d). In the case shown in Figure 1d, the HRP labeling in the LGN was particularly heavy, causing the reaction product to appear purple under dark-field illumination. Thus, in the black and white photograph lamina A in the LGN paradoxically appears dark while lamina A1 as well as the PGN rostral to lamina A appear bright due to label in fibers of passage. By the fourth week (the eyes open about P28-P30) and through the fifth postnatal week (P28-P35) the LGN becomes essentially adult-like in shape, location and organization.

Single Axon Arbors

The in vitro labeling method produced axons which were well-filled and could be reliably traced back from the LGN into the optic tract. Figure 2 illustrates the location of the injection site (Fig 2a), an example of a typical injection site (Fig 2b), and the labeling that results from the injection (Fig 2c, d). Axons recovered were densely labeled, with little fading of label at branch points or at the tips of branches. Cut endings or branches were clearly distinguishable from terminal endings. Generally, axon arbors were reconstructed over a series of three or four sections, although some were recovered entirely in one section, and some spanned more than nine sections. For reconstruction, every effort was made to select axons from different levels in the LGN and from all portions of the nucleus, including the monocular segment and binocular projection zones. Axons were chosen from the middle sections through the nucleus, avoiding the most dorsal sections, in which laminar boundaries are not distinct, and the most ventral sections, which consisted

mainly of ventral LGN. An account of the major features of arbors at each group of ages examined is given below.

In the descriptions that follow, the term "parent trunk" will refer to the primary axon that arises from the optic tract, and as most often observed in our cases, enters the LGN without branching. Thus, the "trunk" of an axon in most cases refers to the single primary fiber present within the LGN parenchyma. "Collaterals" are bifurcations within the parent trunk, arising either within the optic tract or within the body of the LGN. "Branches" refer to the secondary processes that are given off from the main trunk throughout the nucleus, or form the terminal arbor.

P0-P1: Cellular laminae and interlaminar zones are not yet present within the LGN and retinogeniculate axons are relatively primitive in structure. Most axons have a single main trunk crossing the extent of the nucleus, some ending in a few major forking processes (Figure 3a). However, it is not possible to determine from their morphology whether these processes are the beginning of a terminal arbor or bifurcations in the trunk. Other axons run along the optic tract and send several collaterals into the body of the nucleus.

Axons have occasional side branches along the main trunk which protrude into both presumptive contralateral and ipsilateral zones. In addition to side branches, many axons have extremely fine fibrils studded along their extent. These fibrils appear to be less than 0.1 um in diameter, and are much thinner than the more obvious side branches, which are similar in thickness to the main trunk. Most of these fibrils are very short (<1 um), and appear to be hairlike extensions of the axon membrane rather than true axonal branches. Other fibrils, however, extend up to 100 um from the main trunk, and form some fairly elaborate networks (e.g. Fig. 3a, axons 6, 7). Side branches do not appear to be restricted to one lamina; indeed some

axons have such branches in both presumptive contralateral and ipsilateral areas (i.e. regions corresponding to the future lamina A and A1; Fig. 3b). Some axons bifurcate early in their invasion of the LGN and send out widely diverging collaterals which extend in width over a third of the nucleus (Fig. 3a, axon 6). Others bifurcate later and branch in a more limited region of the nucleus (Fig. 3a, axons 1, 4, 8, 9). These branching patterns are similar to those described in the cat (Sretavan & Shatz, 1984).

On P0-P1, a population of axons are frequently labeled which travel in the optic tract and enter the LGN, but continue on through the nucleus into the PGN. These axons are distinct from the retinogeniculate axons described earlier in several ways. First, they do not make any side branches or bifurcations within the LGN. Second, these fibers always travel in bundles which remained tightly fasciculated throughout their trajectory, a course which took them on a straight path through the LGN. Third, they tend to be thicker and more "wavy" than retinogeniculate axons. From these characteristics, it could safely be assumed that these fibers did not terminate within the LGN. They may belong to a class of non-retinal axons that travel in the optic tract (Reese, 1987), or may be optic axons on their way to the midbrain. Their ultimate destination is not clear from our material, although some could be seen continuing caudally toward the midbrain. These axons show no evidence of terminating in the PGN either (i.e., there are no branches or defasciculation of bundles, etc.). Unfortunately, they could not be followed any further because the label did not transport much beyond the PGN.

P7-P8: At this age axons exhibit the first indications of a true arbor (Fig. 4a). The main trunks have become smooth, with occasional minor side branches. The fine, hairlike fibrils have disappeared. Terminal arbors are fairly rudimentary, and the branch endings are capped with growth cones. Arbors are very widespread;

branches extend laterally in all directions from the arbor, and have not yet coalesced into the dense plexus of branches that characterize arbors at later ages.

The most notable characteristic of these arbors is that they are spread across a region approximately one-third to one-half the mediolateral extent of the nucleus. This dimension, the mediolateral distance perpendicular to laminar borders, which will hereafter be referred to as the "height" of the nucleus, is an area likely to become the presumptive height of one eye-specific lamina (see Fig. 8 for explanation of "height" measure; Fig. 1b for eye-specific lamina). Although definitive laminar boundaries are not visible at this age, the HRP label from eye injections (Fig. 1c-e) indicates that such a distance corresponds to one eye-specific layer. It must be emphasized, however, that arbors are not totally restricted to a presumptive eye-specific layer. Arbors still have branches that extend over presumptive laminar boundaries.

P14-P15: At this age cellular laminae are evident as are the interlaminar zones. Axon terminal arbors are more dense and more elaborate in the pattern and complexity of their branching (Fig. 5a). Overall, the arbor appears to become more compact. Widespread branches are infrequent, although some axons still have a few branches projecting away from the core of the arbor. Further, arbors are also somewhat shorter in height, presumably because the terminal arbor has become more confined to one eye-specific layer. Thus, any stray side branches near laminar boundaries have most likely been eliminated. In our eye injection material sublaminar leaflets are not evident at this age, and this is also reflected in the individual retinogeniculate arbors. Terminal arbors are stretched across most of the eye-specific lamina, but most arbors do not reach from one laminar boundary to the other. Branches end well within laminar borders. As an indication of sublaminar

segregation about to occur, one of the axons reconstructed (axon 2) was completely confined to the outer portion of lamina A.

P19-P21: At this age, sublaminar leaflets are well-formed, and sublaminar boundaries are evident although interleaflet zones are not yet clearly established. Examples of retinal axons are illustrated in Figure 6a. Axon trunks appear to be somewhat thicker than at earlier time points and arbors are clearly restricted to one sublaminar leaflet. The arbor framework is roughly long and narrow, with the longitudinal axis perpendicular to laminar borders. The arbor core is much more dense than previously and more confined to its target zone. There is very little encroachment over sublaminar boundaries; one or two branches may extend a short distance into another sublaminar leaflet. Most arbors have branches that extend to the edges of the sublamina.

P28-P35: Axon arbors are essentially adult-like at this age. The arbor is a narrow, dense plexus of branches, tightly confined to its target zone (Fig. 7a). Postnatal day 28 is the first age studied that terminal bouton clusters are visible on branches. They are simultaneously present with growth cones on the same arbor. By P35, growth cones have disappeared and have largely been replaced by terminal boutons.

Quantitative Measures of Arbors

In order to quantitate the changes in arbor development during the first five postnatal weeks, measurements were taken of the height and width of each axon arbor, and these sizes were compared to the size of the LGN (see Fig. 8). For all ages, we defined the beginning of the arbor at the first major branch point in the axon trunk within the LGN, a major branch being defined as at least 20 um long. The height of an arbor was determined as the extent of the arbor orthogonal to laminar borders, from the first branch point to the most distal arbor tip. Arbor width was

defined as the extent of the arbor parallel to laminar border, encompassing the outermost branch tips.

We computed the arbor height in relation to the height of the LGN where the arbor was located, in order to get an accurate portrayal of how arbor size may relate to target size. To achieve this, a line was drawn along the arbor height axis until it intersected the LGN boundaries (usually but not always perpendicular to the boundaries), and the distance between LGN borders was defined to be the LGN height for that particular axon. LGN heights varied considerably within a single section, as axons were reconstructed from the thin monocular segment as well as the thicker binocular segment. LGN width was measured from the most anterior and lateral LGN boundary to the most medial and caudal LGN boundary, along a line bisecting the nucleus parallel to LGN borders (Fig. 8). Our measurements reflect LGN dimensions at locations where arbors were found rather than a systematic sampling of LGN size at each age, and thus the values of LGN height and width obtained do not necessarily reflect average height and width of the LGN at the ages chosen.

Arbor Height: The mean height of arbors at each age are depicted in Figure 9a, and the values including the number of axons drawn at each age are listed in Table 2. Means were obtained by measuring arbors at each age and pooling the values across animals. The data indicate that the absolute height of the arbor at first declines with age, from 177 um (+/- 23.8, S.E.) at P0-P1 to 165 um (+/- 17.7) at P7-P8 (P0-P1 vs. P7-P8, $p = 0.9$, Mann-Whitney U-Test), and further to 127 um (+/- 8.8) at P14-P15 (P7-P8 vs. P14-P15, $p = .06$). In the third postnatal week arbor height increases to 156 um (+/- 8.1) (P14-P15 vs. P19-P21, $p < .05$), then again decreases slightly to 140 um (+/- 12.1) at P28-P35 (P19-P21 vs P28-P35, $p = .22$). The decrease in arbor height during the second postnatal week is not significant. The

increase in arbor height at P19 is significant, and is followed by another period when they tend to shrink.

The ratio of arbor height to LGN height (Table 2, Fig. 9b) decreases from P0-P1 to P7-P8, from about 0.45 (+/- .068) to about 0.34 (+/- .038)(P0-P1 vs. P7-P8, $p = .53$), is again reduced slightly by P14-P15 to 0.33 (+/- .034) (P7-P8 vs. P14-P15, $p = .58$), then drops further by P19-P21 to about 0.28 (+/- .015) (P14-P15 vs. P19-P21, $p = .23$). Due to the variability in arbor sizes and consequent large standard errors, these week-by-week differences were not significant. From P19 to P28-P35 the ratio drops significantly, to 0.22 (+/- .020) (P19-P21 vs. P28-P35, $p < .05$).

At ages when true arbors are present and laminar borders evident (P14 and older) arbor heights were also assessed in relation to lamina height. Lamina heights were measured in the same manner as LGN heights (Fig. 8). Figure 9c shows that at two weeks the ratio of arbor height to lamina height is 0.74 (+/- .064) whereas at three weeks the ratio is 0.59 (+/- .035), a difference which is significant (P14-P15 vs. P19-P21, $p < .05$, M-W). From P19-P21 to P28-P35 the ratio decreases even more to 0.45 (+/- .041) (P19-P21 vs. P28-P35, $p < .05$). The data indicate that arbors largely become restricted to sublaminar leaflets between two and three weeks of age.

Arbor Width: The arbor width follows the same trend as arbor height. Arbors gradually decrease in width from P0-P1 when they are 118 um (+/- 29.3) wide, to P7-P8 when they are 95 um wide (+/- 14.1) (P0-P1 vs. P7-P8, $p = 1$). At P14-P15 they are 79 um (+/- 6.43) wide (P7-P8 vs. P14-P15, $p = .56$), then increase by P19-P21 to 95 um (+/- 7.87) (P14-P15 vs. P19-P21, $p = .15$). This is followed by a period of shrinkage, as at P28-P35, arbor width is reduced to 78 um (+/- 11.46)

(P19-P21 vs. P28-P35, p = .19). None of the differences in size at any of the ages was significant.

A comparison of arbor width relative to LGN width indicates that there is gradual reduction in this ratio with age. At P0-P1 the ratio is 0.10 (+/- .024), which becomes reduced to 0.08 (+/- .014) at P7-P8 (P0-P1 vs. P7-P8, p = .86). At P14-P15 there is a further decrease to 0.06 (+/- .004) (P7-P8 vs. P14-P15, p = .32). This ratio is maintained at P19-P21 (0.06 +/- .005, P14-P15 vs. P19-P21, p = .73), then decreases at P28-P35 to 0.04 (+/- .006) (P19-P21 vs. P28/35, p = .09).

Arbor Areas: Area of LGN covered by arbor is shown in Table 4, and in Figure 11. Arbor areas were measured from the onset of arborization (P7-P8) to the oldest ages examined (P28-P35). At P7-P8 the arbor areas measured 6491 um2 (+/- 1160), and became reduced at P14-P15 to approximately 5250 um2 (+/- 601) (P7-P8 vs. P14-P15, p = .28). From P14-P15 to P19-P21, arbor areas increase dramatically, to 8155 um2 (+/- 653); this increase is significant at the $p < .005$ level. Between P19-P21 to P28-P35, arbor areas become reduced again to 6450 um2 (+/- 836), a difference which is not significant (p = .14).

DISCUSSION

In this study we have described the development of retinogeniculate axons in ferrets from day of birth to five weeks of age (eye opening is at 4 weeks). We present evidence to show that retinogeniculate afferents in the binocular segment go through several stages of development, from an early state in which axons from the two eyes are intermixed and project diffusely throughout the LGN, to an adult-like, segregated state. Segregation of retinal afferents is a two part process, with eye-specific segregation occurring first, followed by segregation into On and Off sublaminae. We argue below that different sets of mechanisms appear to be involved in the two processes. Importantly, the formation of retinogeniculate termination patterns appears to involve the developmental interplay of both retinal axon arbors in the LGN and the LGN itself.

Axons progressed from an overall immature framework to a fairly adult shape during the period of our study. On the day of birth in the ferret, axons from the two eyes are intermingled within the LGN and are fairly primitive in structure with no evidence of a true terminal arbor. As maturation progresses axons grow crude arbors, roughly confined to a presumptive eye-specific lamina, and become well-established by two weeks of age. Between two to three weeks of age, axon arbors become further restricted, from within laminar boundaries to within sublaminar boundaries. Following sublaminar segregation, arbors grow and take on fully mature characteristics as evidenced by the formation of terminal boutons.

Stages of Development of Retinogeniculate Axons

Our results show three stages in postnatal development of the ferret retinogeniculate projection: 1) Extension of simple branches with fibrils into

inappropriate and appropriate regions of the LGN; 1) During eye-specific lamina formation, arbor extent shrinks, first due to retraction or loss of side branches and fibrils from inappropriate regions of the LGN, followed by selective arborization in appropriate eye-specific regions of the nucleus. 2) During On/Off sublaminar formation, the arbor increases in extent, but the LGN grows proportionately much larger, so that the arbor occupies a relatively smaller height (and volume) of the LGN. This is again followed by a second period of slight shrinkage.

At the earliest age studied (P0), axons do not exhibit a clear specificity for target zones. Indeed, axon branches are widely spread throughout the nucleus, and are studded with side branches all along their length, protruding into both presumptive contralateral and ipsilateral zones. This stage of development which lasts several days postnatally, may be viewed as a period when axons are seeking appropriate target space. As they enter the LGN from the optic tract, axons are not fasciculated, but are widespread. This may reflect a search strategy in order to find their target, prior to forming terminal arbors.

When arborization commences, axons exhibit a specificity in terms of the specific lamina where they will ultimately localize. However, the selectivity for particular sublaminae is expressed slightly later in development. The fact that retinal axons, having entered the LGN, appear to identify and arborize within "correct" laminar target areas is in accordance with observations in the cat, in which terminal arbors were found to be fairly specific for their laminar regions from onset of arborization (Sretavan & Shatz, 1986a). Whereas in cats there is continued, gradual increase in the size of arbors, in the ferret there is a period of pruning between P7-P8 and P14-P15, during which arbors exhibit a small decrease in height and width. It should be noted that this diminution in overall size occurs simultaneously with an

increase in the density of the arbor, since branches continue to be added throughout this period.

Several factors may contribute to this phenomenon. In the early period of lamina formation, laminar boundaries are indistinct (Linden et al., 1981; cat, Shatz, 1983), suggesting that retinal axons may form arbors that at first overshoot the developing laminar boundaries and are later pruned to fit accordingly. Thus there is gross specificity for the target in terminal arborization, which later becomes more distinct. Such "sharpening" of specificity is evident in other retinofugal and geniculocortical projections (Jhaveri et al., in press; Schneider et al., 1985, 1987; Sretavan & Shatz, 1987; Schmidt, 1985; Levay & Stryker, 1978; Simon & O'Leary, 1990; Naegele et al., 1988).

Axons, having identified and arborized within their initial target, move into the next phase of development, that of confining their arbors to their final target space, a sublaminar leaflet. This is evidenced by the fact that by P15, axon arbors actually cover less of the LGN height than previously and that branch tips no longer reach all the way to laminar borders. In addition, the presence of an occasional arbor localized in the outer half of a lamina (Fig. 5) indicates that some axons have reached the second phase of segregation.

Once arbors have located their correct (sublaminar) target space, they expand again so that between P15 and P19 arbors increase in height and width, and sharply increase in area. The size of the LGN, however, increases at an even greater rate. Thus, arbors take up a smaller proportion of the LGN height at P19 than at P15 although the arbors are significantly taller. Arbor height in relation to lamina height is also reduced, from approximately 0.75 to 0.60, reflecting restriction into sublaminae (Fig. 9). The arbors become further restricted to sublaminae at P28-P35, as shown by the arbor height/lamina height ratio of 0.45. This may reflect a process

of arbor focalization into specific termination zones, similar to that seen between P8 and P15. Arbor width in relation to LGN width is first reduced from P0-P1 through P14-P15, remains constant between P14-P15 and P19-P21, then decreases again between P19-P21 and P28-P35.

The final phase of development (after P19-P21) yields adult-like morphology of axons, with tightly confined, densely innervated termination zones and terminal boutons on the branches.

Comparison with Other Systems

Distinct stages of axon growth have been described in other vertebrate systems (Holt, 1991; Morris et al., 1988; O'Leary & Terashima, 1988; Naegele et al., 1988). For example in the hamster visual system, at least two modes of growth have been proposed (Jhaveri et al., 1990, 1983; Jhaveri et al., 1991; Bhide & Frost, 1991), which are characterized by different speeds and patterns of growth, and are separated by a "waiting" period. In the first phase, elongation, retinal axons grow to their targets, the LGN and superior colliculus. They then "wait" in the optic tract for a period of two days before innervating the target nuclei. In the second phase, arborization, axons form collaterals and elaborate arbors in the target nuclei. Axon arborization can be divided into two substages, early and late focalization of terminal arbors. During early focalization, crude topographic order of the retinal projection is sharpened by elimination of branches in some areas and elaboration of terminal ramifications in others. This also results in laminar restriction of retinotectal and retinogeniculate fibers. Late focalization is a second period of restriction for exuberant arbors, in which terminal boutons increase in number while the terminal arbor area decreases. In the final phase, terminal arbors undergo differentiation into adult-like morphologies (Jhaveri et al., 1991).

The modes described are similar to phases of development seen in the ferret retinogeniculate system, although those in the ferret are not so distinct. Our period of study did not include the earliest stage, elongation, which takes place prenatally (Cucchiaro & Guillery, 1984; Sretavan & Shatz, 1986a), and in which retinal ganglion cell axons leave the eye and grow to their target. The first stages of arborization - collateral formation followed by early focalization (Jhaveri et al., 1991) - correlate with initial arbor formation and laminar segregation seen in our material (which occurs by the first postnatal week). The late focalization stage is similar to the phase of arbor contraction seen at P15. Because the ferret's retinogeniculate system undergoes further segregation into On and Off sublaminae, the two focalization stages are repeated as arbors grow into sublaminar leaflets and again slightly restrict their arbors.

Binocular Segregation of Retinal Afferents: Intrinsic and Extrinsic Factors

It has been shown that the normal development of geniculate lamination is dependent on the presence of retinal afferents from both eyes; without binocular afferents, neither cell layers nor interlaminar spaces form (Rakic, 1977; Brunso-Bechtold & Casagrande, 1981; Guillery et al., 1985). Removal of one eye early in development also disrupts the normal formation of laminae (Brunso-Bechtold & Casagrande, 1983; Chalupa & Williams, 1984; Guillery et al., 1985; Rakic, 1981). In the ferret LGN, lamination appears to be triggered by retinal afferents in that cell layers begin to form only after the afferents have begun to segregate, although completion of cell lamination is not contingent on completion of afferent segregation (Linden et al., 1981). The specific influence of retinal afferents in lamina formation is most clearly demonstrated in ferrets monocularly enucleated at birth. Whereas interlaminar zones between eye-specific laminae do not form in these animals,

interleaflet spaces between sublaminar leaflets in the A and A1 laminae of the remaining eye do form (Guillery et al., 1985). Further, the fact that afferents project to target zones where they would normally terminate and that there is limited invasion by afferents of the remaining eye into territory vacated by the missing eye suggests that although binocular afferent interaction is necessary to the formation of laminae, the location of contralateral and ipsilateral zones is not determined by such interaction. Additionally, other characteristics of adult structure, such as morphological development of geniculate cells and their relationship to target structures in the cortex are also unaffected by the absence of retinal afferents (Brunso-Bechtold & Casagrande, 1981; Brunso-Bechtold et al., 1983). The adult laminated structure thus appears to be achieved through the interplay of a number of factors, those intrinsic to the afferents and to the targets, and numerous interactions between the two (Casagrande & Brunso-Bechtold, 1985).

Our study was aimed at determining the factors that lead to retinal afferent segregation and the mechanisms by which this is achieved. The data from our single axon experiments indicate that eye-specific, laminar segregation of afferents is achieved by the formation of terminal arbors within areas appropriate for the eye of origin in the LGN. This occurs by directed terminal arbor growth into a target zone corresponding to a presumptive eye-specific lamina, coincident with the elimination of extraneous branches in inappropriate areas. Subsequently, On/Off segregation occurs by proportionately greater increase in LGN size relative to arbor size. Thus, the entire process of retinogeniculate pattern formation consists of separate mechanisms operating at distinct stages of development.

Specific arborization in the initial stage suggests that axons are intrinsically driven to form arbors of a certain size within a certain region of the LGN to achieve binocular segregation. In the cat retinal afferent arborization is also specific to

appropriate contralateral and ipsilateral zones; indeed axons appear to be even more specific, as ipsilateral axons never fully invade contralateral areas (Shatz, 1983; Sretavan & Shatz, 1986a; see however Chalupa & Williams, 1985). Further, removal of binocular interactions by very early monocular enucleation (E23), before axons reach the optic chiasm and interact with axons from the other eye, does not alter growth patterns of the remaining retinogeniculate axons (Sretavan & Shatz, 1986b). Intrinsic axonal regulation has also been postulated in other systems in which arbor size was maintained despite manipulations which altered the target by varying degrees (Schneider, 1981). However, intrinsic regulation alone cannot account for specificity of arborization. In cats if an eye is removed at E44 when binocular afferents are intermixed in the LGN, and the remaining retinogeniculate axons examined at adulthood, there is a striking abnormality in the Y class of axons, whereas X axons are affected very little (Garraghty et al., 1988a). These data suggest that intrinsic programs are important to normal development, but that timing and interactive factors have equally important influences on axon development.

To date, there is no direct evidence linking a molecular marker to binocular segregation, although the nature of LGN organization and afferent segregation suggests that such a marker may be involved. It is striking, for example, that afferent segregation always occurs along the axis parallel to LGN borders, and that laminae develop in a characteristic alternating fashion. Further, unlike ocular dominance columns in cortex, LGN laminae develop in a stereotyped pattern which is the same in every animal of the species, barring genetic mutation (e.g. albino ferret, Guillery, 1971; Cucchiaro & Guillery, 1984; siamese cat, Guillery, 1969; Guillery & Kaas, 1971; Hubel & Wiesel, 1971). The presence of markers in the formation of topographic connections in lower vertebrates makes speculation about such a mechanism very tempting for laminar development as well. In the chick

retinotectal projection, tectal cues such as position-specific markers or chemical gradients (Bonhoeffer & Gierer, 1984; Thanos et al., 1984; Walter et al., 1987), as well as adhesion molecules have been cited to play key roles (for review see Rutishauser, 1985; Lander, 1990). Similar mechanisms are indicated in the retinotectal system of fish and frogs (reviews, Schmidt, 1985; Udin & Fawcett, 1988; Fraser, 1991; Holt, 1991).

Recently, there is evidence to suggest that chemospecific factors may regulate geniculocortical connectivity in mammals. In LGN and visual cortex explants co-cultured in vitro, connections are formed which are specific for the appropriate afferent structure and laminar target (Yamamoto et al., 1989; Bolz et al., 1990). If such a mechanism exists in the retinogeniculate projection, one is led to speculate about how it would be manifested.

In this context, the side branches and especially the fine fibrils seen in our material could be seen as detectors of such molecular markers. It is possible that the fibrils "sample" target areas and determine which are appropriate, whereupon the axon begins to arborize in that area. However, this is a speculative hypothesis, and an alternative role for the side branches is presented below.

While intrinsic properties may have some influence on axon development, there is much evidence to indicate that axon-target and axon-axon interactions play a significant role in shaping the visual connections. The role that axon-axon interactions may play has been mentioned previously with regard to the effects of the timing of monocular enucleation on the resulting retinogeniculate arbor morphologies (Sretavan & Shatz, 1986b; Garraghty et al., 1988a). A great deal of study has been focused on axon-target interactions with particular attention paid to the role of activity. In the geniculocortical projection, activity is critically involved in the normal development of cortical organization (Hubel et al., 1977; Levay et al., 1978). There

is now good evidence to indicate that it is an important factor in retinogeniculate development as well. It is known that retinal ganglion cells are spontaneously active prenatally (Galli & Maffei, 1988), and that the retinogeniculate pathway is capable of synaptic transmission very early in development (Shatz & Kirkwood, 1984). Further, it has been shown that neighboring ganglion cells are correlated with each other in their firing (Arnett, 1978; Arnett & Spraker, 1981; Meister et al., 1990, 1991; Wong et al., 1990), so that the differences in waves of activity between the two eyes could serve as a means of differentiating inputs. A direct way to approach this issue is to silence all impulse activity in the retinogeniculate pathway prenatally, before segregation has occurred. In cats blockade of sodium channels with TTX during the period of binocular segregation prevents retinal afferents from sorting into eye-specific laminae (Shatz & Stryker, 1988), and results in grossly abnormal, nonspecific terminal arbors (Sretavan et al., 1988), indicating the importance of impulse activity in the normal maturation of retinal axons.

One question to ask is where these interactions actually take place. Sretavan and Shatz (1986a) suggest that the side branches present on axons during the period of sorting may be the arbiters of segregation. It is proposed that there is synaptic contact between afferent and target on the side branches, to determine zones where pre- and post-synaptic activity are correlated. The data in support of this view are suggestive, in that the branches are present during the process of segregation, and have largely disappeared by the time that laminar separation is evident. This is also found in our ferret material, but to an even greater extent, as the elaborate fibrils seen at P0 disappear by the time that arbors are present. Additionally, it has been shown that there are transient synapses on axons in the initially inappropriate retinal projections to target structures in both the hamster and the cat (Campbell et al., 1984; Campbell & Shatz, 1986).

Other factors which may be involved are the timing of afferent arrival and the maturational state of the retinal ganglion cells. Generally, contralateral afferents are first to arrive in the LGN, leading the ipsilateral by a few days (Shatz, 1983; Cucchiaro & Guillery, 1984). Because of this timing difference, the spread of contralateral afferents may preclude ipsilateral afferents from invading the medialmost portions of the nucleus. In the cat contralateral axons seem to mature earlier than ipsilateral axons as evidenced by more complex arbor structures (Sretavan & Shatz, 1986a). However, although the (contralateral) lamina A may be the first to show signs of sublaminar segregation [as evidenced by densely clustered label following eye injections at P14-P15 (Linden et al., 1981); in our own material, a few axons are restricted to an outer half of lamina A at P15 (Figure 5a, axon 2)], such a gradient is not well-defined in the ferret. There is a slight indication that ipsilateral axons at P7-P8 are somewhat less focalized in arbor formation, but as this is the most initial stage of arborization, such minor differences are not unexpected.

The maturational state of the retinal ganglion cell may also contribute to arbor development. In the retina, retinal ganglion cells are generated in a central to peripheral gradient (Morest, 1970), although different cell classes are born in different time windows (Walsh et al., 1983; Walsh & Polley, 1985). The varied generation of different cell classes are believed to underlie the fiber order in the optic tract (Torrealba et al., 1982; Guillery et al., 1982; Walsh & Guillery, 1985). Hence, it would not be unexpected that the more mature ganglion cells form terminal arbors earlier. In the LGN the visual field corresponding to central retina is represented medially (Kaas et al., 1972; Zahs & Stryker, 1985) thus axons found in the medial portions are likely to be older than those in lateral portions. We noted a very slight medial to lateral gradient in arbor maturity in the first postnatal week. Thus, axons 8 and 9 of the P0 cases (Fig. 3) appear to be slightly more advanced in development,

having arbor-like structures and fewer side branches and fibrils, as compared to axons in the more lateral parts of the nucleus, which have either very simple, sticklike morphologies or widely diverging bifurcating branches and elaborate fibrils. At P7-P8 (Fig. 4), axons 4, 5, and 7 have a more advanced arbor development than axons 1-3. At later ages, such a mediolateral gradient is not evident.

On/Off Sublaminar Segregation

The process of On/Off sublaminar segregation differs from binocular segregation in a number of respects. First, sublaminar segregation does not seem to be a process of arborization within a specific targeted region of the LGN, but rather a process in which afferents compete for appropriate terminal space. This is suggested by the fact that during the initial process of sublaminar segregation, axon arbor branches are not biased toward an inner or outer half of an eye-specific lamina, but are retracted away from laminar borders. Second, it is evident that segregation is achieved by simultaneous retinal afferent restriction and LGN growth.

Of course, intrinsic factors are involved to some extent in sublaminar segregation as well. The sublaminae are organized such that On cells are in the inner sublamina, and Off cells in the outer sublamina, and this pattern does not vary. This organization may arise because the target cells express some trophic factor specific to cell type which the afferents recognize. A molecular marker has been identified that is associated only with Y cells in the LGN (Hockfield & Sur, 1990; Sur et al., 1988), and a marker may exist for On or Off cells as well. Furthermore, other species (such as cats) which have On and Off-center retinal ganglion cells do not have segregated On and Off sublaminar within the LGN.

It is likely that visually driven activity plays a very important role in sublamina formation. The basis of sublaminar segregation is completely opposing

response to light, which constitutes a fundamental functional difference in the activity of the retinal afferents to the LGN. As such, the difference between correlation of corresponding presynaptic and postsynaptic activity, and non-corresponding activity, is bound to be sharp. This might provide a clearer signal in an activity correlation mechanism than slight changes in levels or timing of activity.

With such a mechanism postulated, there is evidence to indicate that visually driven synaptic transmission is present in the retina at these critical ages. In the ferret retina, it has been shown that at P15, prior to onset of sublaminar segregation, ribbon synapses indicative of bipolar cell input, and rod outer segments are present (Greiner & Weidman, 1981). Such indications of functional maturity were seen in every section studied throughout the posterior pole of the eye (Greiner, personal communication, 1991). Although the eyelids are still closed at this age, there is evidence that a signficant amount of light reaches the retina (Crawford & Marc, 1976; Loop & Sherman, 1977) and that responses to visual stimuli can be recorded in the cortex through closed eyelids (Spear et al., 1978; for review see Movshon & Van Sluyters, 1981; Sherman & Spear, 1982). The possibility that the activity of postsynaptic cells is a key mediating factor in On/Off sublaminar segregation is addressed in the next chapter.

Cell Class Determination

Our study focused solely on the morphological development of axons and did not involve any physiological determination of afferent identity. Cell class identification is normally based on a number of physiological tests (Enroth-Cugell & Robson, 1966). However, it is known that functionally different classes of retinal ganglion cells have distinctly different morphologies, not only in their soma and dendrites (Fukuda et al., 1984; Stanford & Sherman, 1984; Stanford, 1987) but also

in their axon arbor (Bowling & Michael, 1980, 1984; Roe et al., 1989; Sur & Sherman, 1982; Sur et al., 1987). Based on the characteristics described for retinal ganglion cell axon arbors in adult ferrets (Roe et al., 1989), the axons we have recovered may be of two different types. 1) These may be X axons. The axons have a single arbor terminating in lamina A or A1, and are further restricted to an On or Off sublamina. The arbors in our material are fairly narrow from the outset, and become densely branched within a compact terminal zone, which are all characteristics of X cell axons. 2) These may be A lamina terminations of Y axons. Y axons typically branch in the optic tract, and send collaterals toward their target zone in the A and C laminae. The trajectory of collaterals is quite varied; branches can be sent out from the axon trunk hundreds of microns away from each other (Roe et al., 1989). Our study was limited to arbors in laminae A and A1, as the in vitro method did not reliably label terminal arbors in the C laminae. We did not see axons with more than one terminal arbor, nor did we find branching collaterals in the optic tract. Thus, we cannot state with certainty that any of the axons we recovered were of the Y class. Of course, it is difficult to make definitive statements as to cell class based solely on morphological criteria, and we do not know whether axon morphology at very young ages, prior to eye opening, correlates to that at maturity.

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FIGURE LEGENDS

Figure 1. Photomicrographs of LGN sections in which the contralateral eye was injected with HRP/WGA-HRP. All sections are in the horizontal plane, anterior is up, lateral to the right. a) P1: Retinal afferents are intermixed throughout the extent of the nucleus; there is little indication of segregation into eye-specific zones. LGN is thin band of cells at anterior thalamus. b) P7: First clear indication of afferent segregation according to eye of origin. The LGN has become thicker mediolaterally and begun its rotation into adult position. Laminar borders are undefined, but indications that A and A1 laminae are beginning to form. C laminae have not yet formed. c) P15: Afferents well segregated into eye-specific laminae; A, A1 and the C laminae are clearly established, as are interlaminar zones. LGN has rotated such that the anterior portion is now displaced laterally, and the posterior portion has swung inward. Nucleus is more placed toward the posterior thalamus. d) P21: Axons segregated into On and Off sublaminae within the A layers. Interlaminar and interleaflet zones are distinct, LGN beginning to take on adult appearance. e) P28: LGN begins to look more adult-like. On and Off sublaminae firmly established. LGN taking on L-shape, the anterior limb running longitudinally anterior-posteriorly along thalamus, the transverse limb running mediolaterally into thalamus. f) P35: LGN organization essentially adult. Scale bar represents 250 μm.

Figure 2. Schematic and photomicrographs depicting site of injection, injection site and type of labeling obtained. a) Cartoon drawing of lateral view of thalamus. The three dots in optic tract below LGN represent locations of HRP deposits. b) Typical injection site of in vitro axon fills. This is a P35 case; injection is confined to the optic tract. c) Labeling obtained with in vitro axon label method. Axons are well-

filled and show no signs of degeneration. d) Higher power example of axon arbor. Scale bar represents 100 μm.

Figure 3. Camera lucida drawings of axons recovered at P0-P1. a) In this and all following figures of axons reconstructions, axons are depicted left to right according to mediolateral position within nucleus, left being most lateral. Optic tract at the bottom in all figures. Axons were found in all sections of the nucleus; only those from the middle sections of the nucleus were reconstructed. Axons extended to medial boundary of nucleus; many have bifurcating branches and are studded with side branches (see text for detailed description). b) Schematic showing approximate location of each axon within nucleus. The section depicts a composite of many sections within LGN. Scale bar represents 100 μm.

Figure 4. Camera lucida drawings of axons reconstructed from P7-P8 cases. a) Axons depicted lateral to medial as previously. First indication of terminal arbors, side branches still present. b) Schematic showing location of axon in nucleus. Laminar borders, thought not yet clear, are drawn in according to eye injection data for clarity. Each letter indicates position of the terminal arbor within the nucleus. Scale bar = 100 μm.

Figure 5. Camera lucida drawings of axons from P14-P15 cases. a) Axons have denser terminal arbors which appear more restricted. Side branches have disappeared. b) Schematic LGN with axon arbor locations depicted. Laminar boundaries very distinct. Scale bar = 100 μm.

Figure 6. Camera lucida drawings of axons from P19-P21 cases. a) Axon arbors now confined to sublaminar leaflets. Arbors more densely branched. b). Schematic LGN with arbor locations identified. Interlaminar and interleaflet zones clear. Scale bar = 100 μm.

Figure 7. Camera lucida drawings of axons from P28-P35 cases. a) Arbors essentially adult-like. Terminal boutons visible on many arbors. b). Schematic LGN with arbor locations noted. Scale bar = 100 μm.

Figure 8. Schematic illustrating how height and width were measured for each arbor, lamina and LGN. Arbor width (distance marked 1 in schematic) was defined as the greatest extent of arbor measured from outermost branch tips, in the axis running parallel to LGN borders. Arbor height (distance 2) was defined as the greatest extent of arbor measured from the first major branch point in the nucleus to the outermost branch tip in the axis roughly orthogonal to LGN borders. Lamina height (distance 3) was measured according to arbor height axis, and was defined as the distance between laminar boundaries at the same position in the nucleus as the arbor being measured. LGN height (distance 4) was also measured for each axon at that particular location in the nucleus, in the same axis as arbor height, defined as the distance from medial to lateral LGN boundary. The optic tract was not included in any of the LGN measurements. LGN width (distance 5) was measured for each axon and was defined as the greatest distance between LGN boundaries, extending from the tip of the anterior portion to the tip of the posterior portion.

Figure 9. Graphs of height measurements. a) Heights of axon arbors plotted as a function of age. b) Arbor heights shown as a ratio of LGN height plotted as a function of age.

Figure 10. Graphs of width measurements. a) Widths of axon arbors plotted as a function of age. b) Arbor widths expressed as a ratio of LGN width, plotted as a function of age.

Figure 11. Graphs of arbor/lamina height measurements and arbor area measurement. a) Arbor area plotted against age. b) Arbor height expressed as a ratio of lamina height, plotted as a function of age.

Figure 1

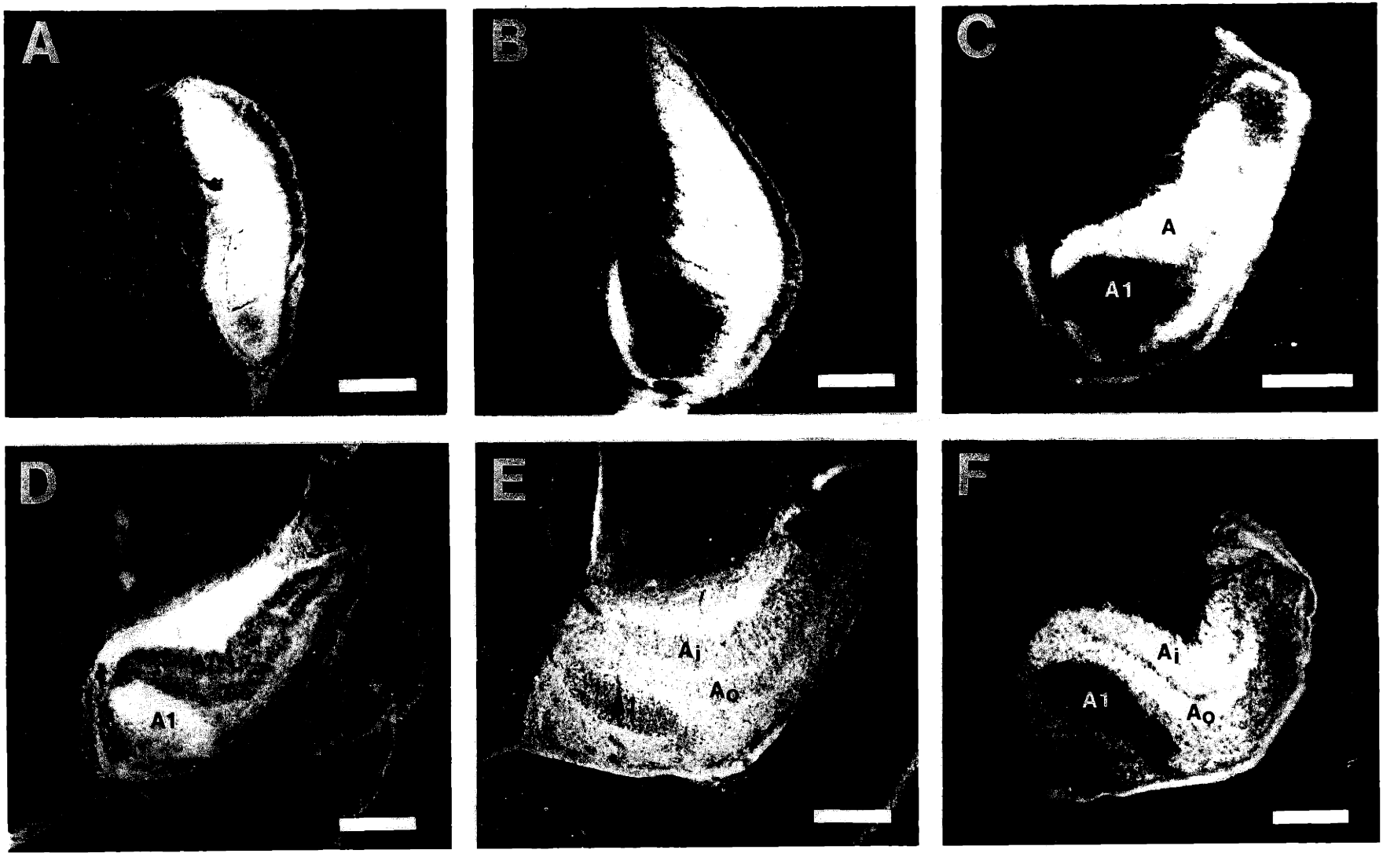


Figure 2

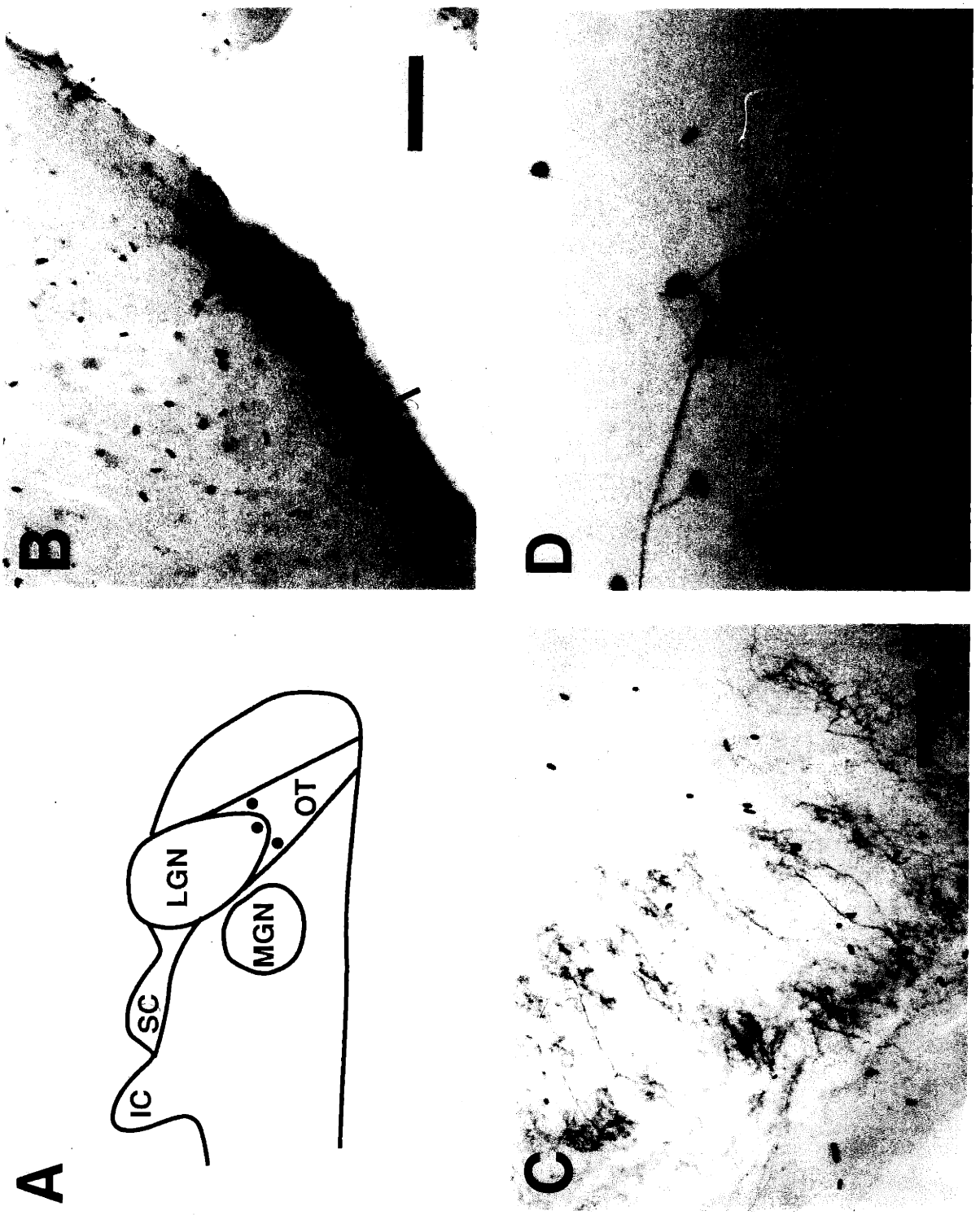


Table 1. Number of Cases

Age	Eye Injection	Single Axon
P0-P1	6	3
P7-P8	4	3
P14-P15	2	3
P19-P21	3	3
P28-P35	2	4
	17	16

Total number of animals: 33

Table 2: Axon Arbor Height Measurements

Ages	Number of Axons	Arbor Height	LGN Height	Arbor Height/ LGN Height	Lamina Height	Arbor Height/ Lamina Height
P0-P1	10	177.40 (23.82)	411.70 (24.69)	0.446 (0.068)		
P7-P8	8	165.25 (17.66)	481.63 (11.21)	0.344 (0.038)		
P14-P15	12	127.50 (8.78)	405.8 (23.91)	0.331 (0.033)	182.83 (15.86)	0.740 (0.064)
P19-P21	16	155.69 (8.08)	566.25 (18.13)	0.278 (0.015)	278.25 (19.54)	0.586 (0.035)
P28-P35	8	139.88 (12.07)	629.88 (28.60)	0.225 (0.020)	323.13 (32.04)	0.450 (0.041)

Table 3. Axon Arbor Width Measurements
Arbor Area Measurements

Age	Number of Axons	Arbor Width (μm)	LGN Width (μm)	Arbor Width/ LGN Width	Arbor Area (μm^2)
P0-P1	10	118.30 (29.29)	1258.30 (34.96)	0.096 (0.024)	
P7-P8	8	94.62 (14.12)	1171.13 (48.68)	0.083 (0.014)	6491.17 (1160.70)
P14-P15	12	79.42 (6.43)	1213.67 (48.93)	0.065 (0.004)	5250.33 (601.72)
P19-P21	16	95.19 (7.87)	1528.63 (57.68)	0.063 (0.005)	8155.48 (653.03)
P28-P35	8	78.25 (11.46)	1811.63 (83.10)	0.043 (0.006)	6450.01 (836.71)

Figure 3A

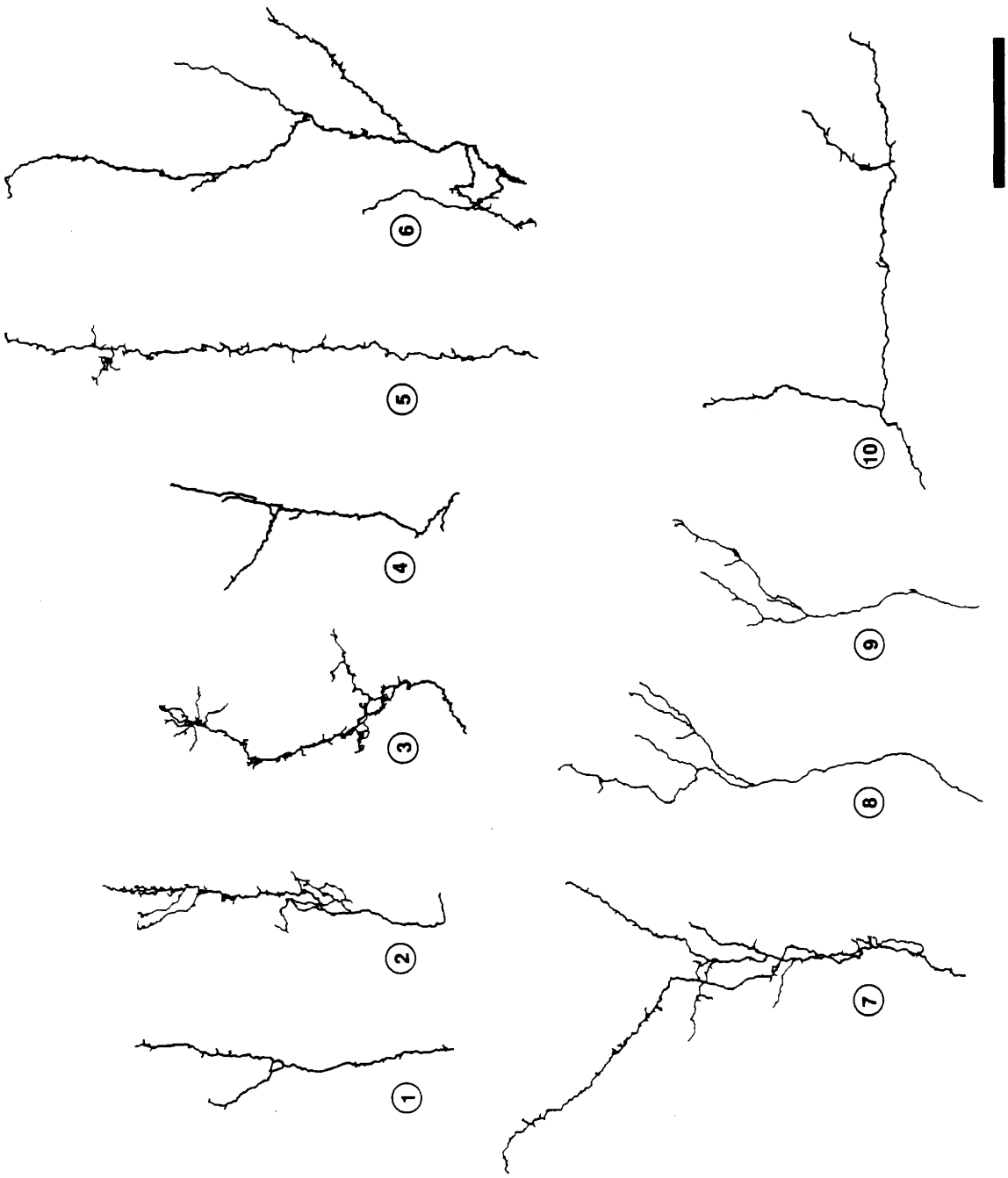
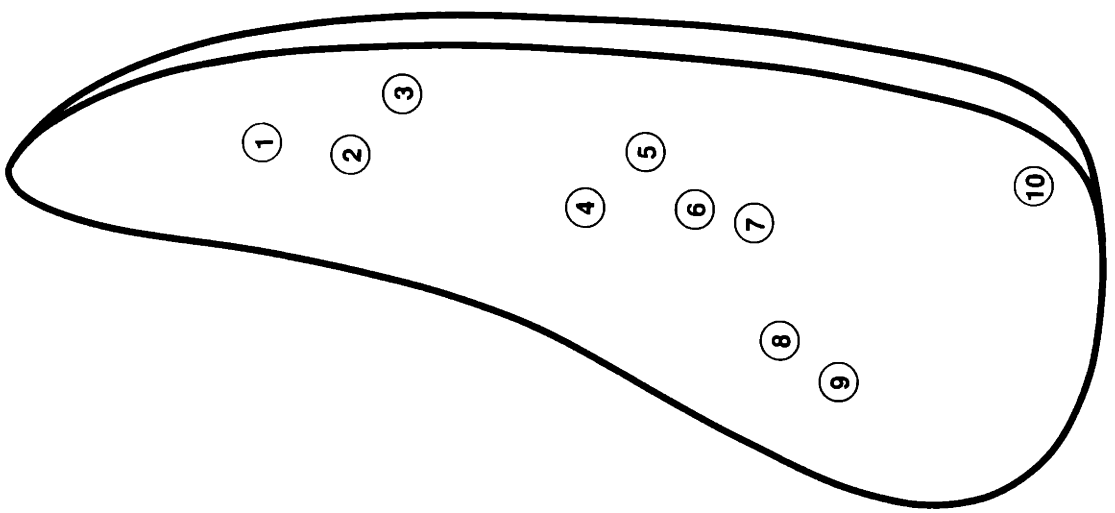


Figure 3B



P0 - P1

Figure 4A

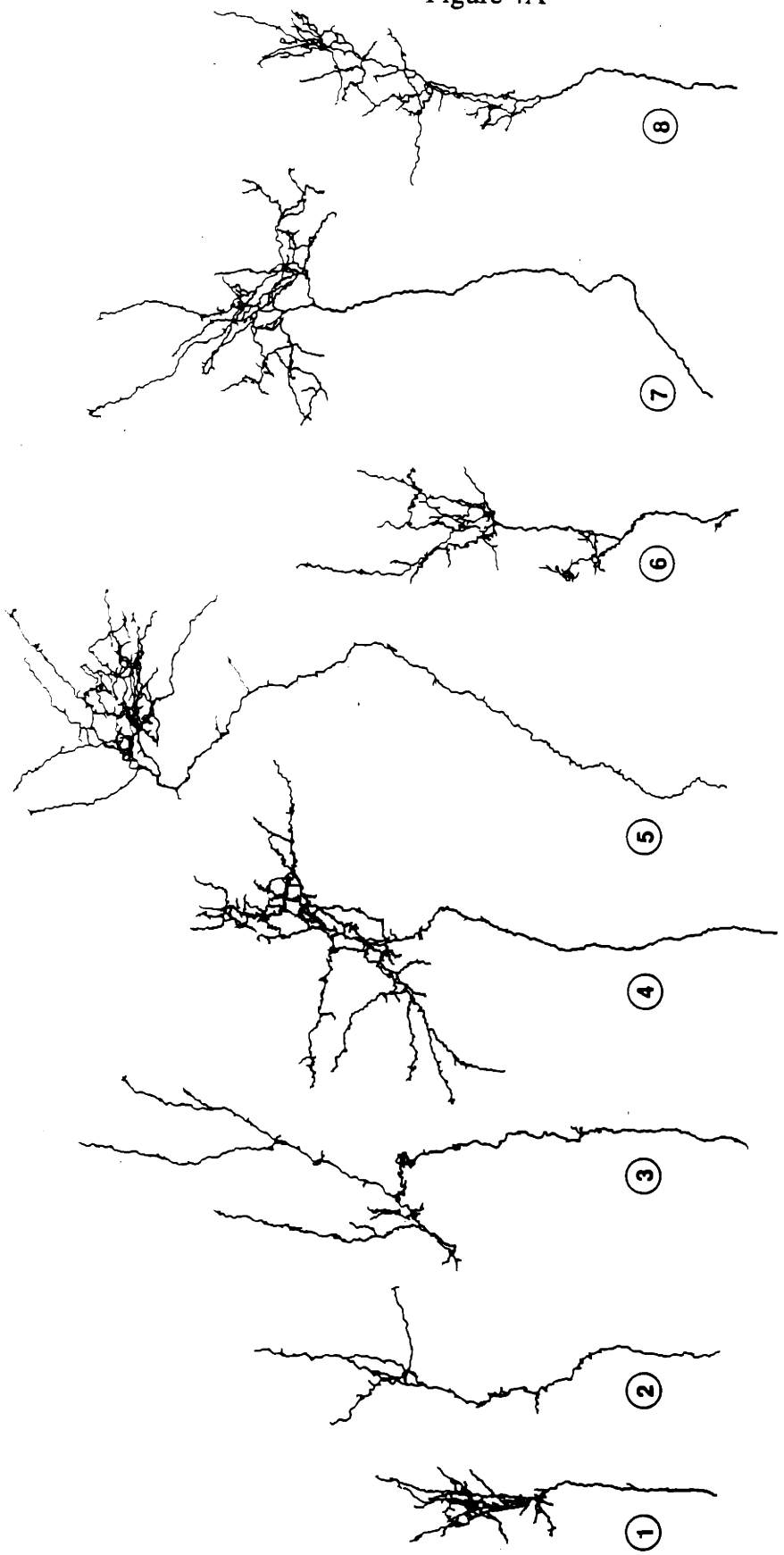


Figure 4B

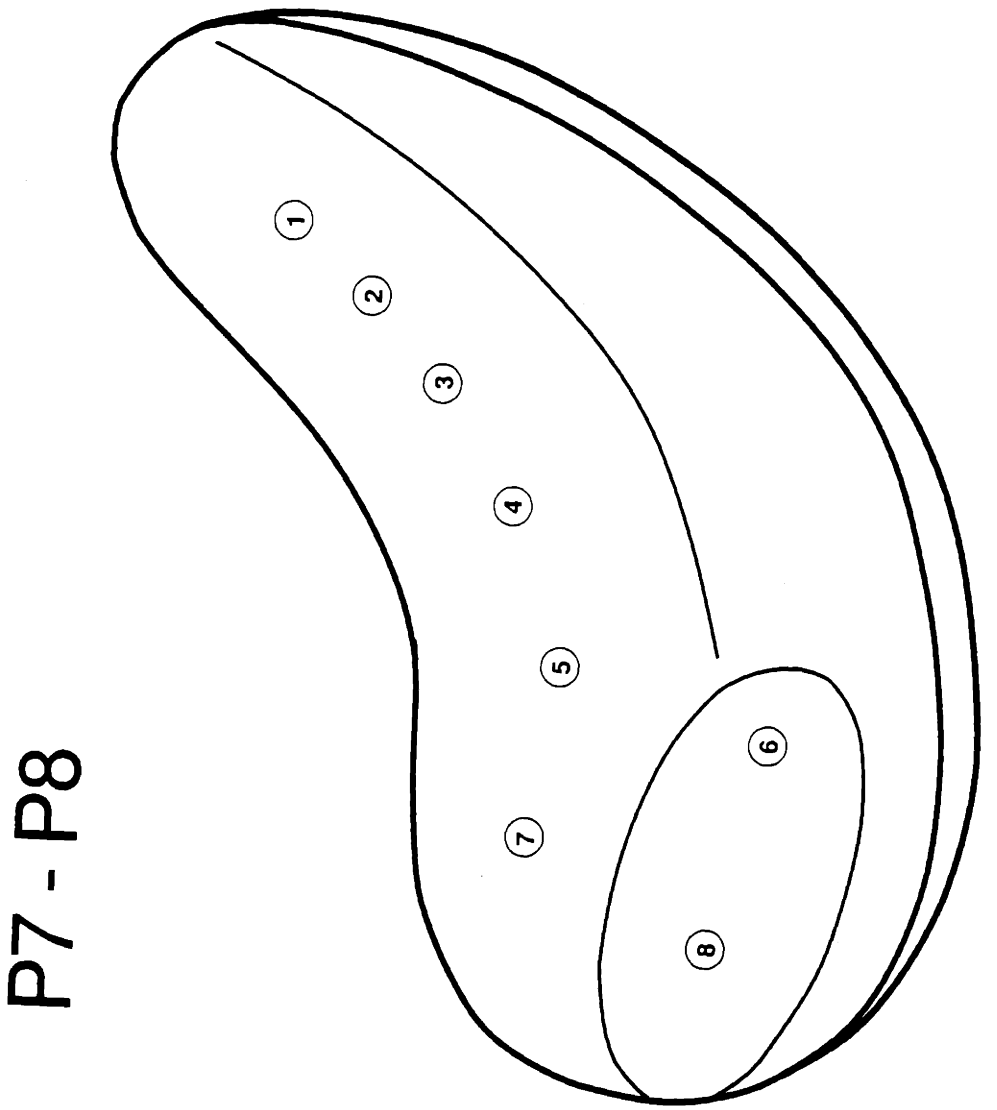


Figure 5A

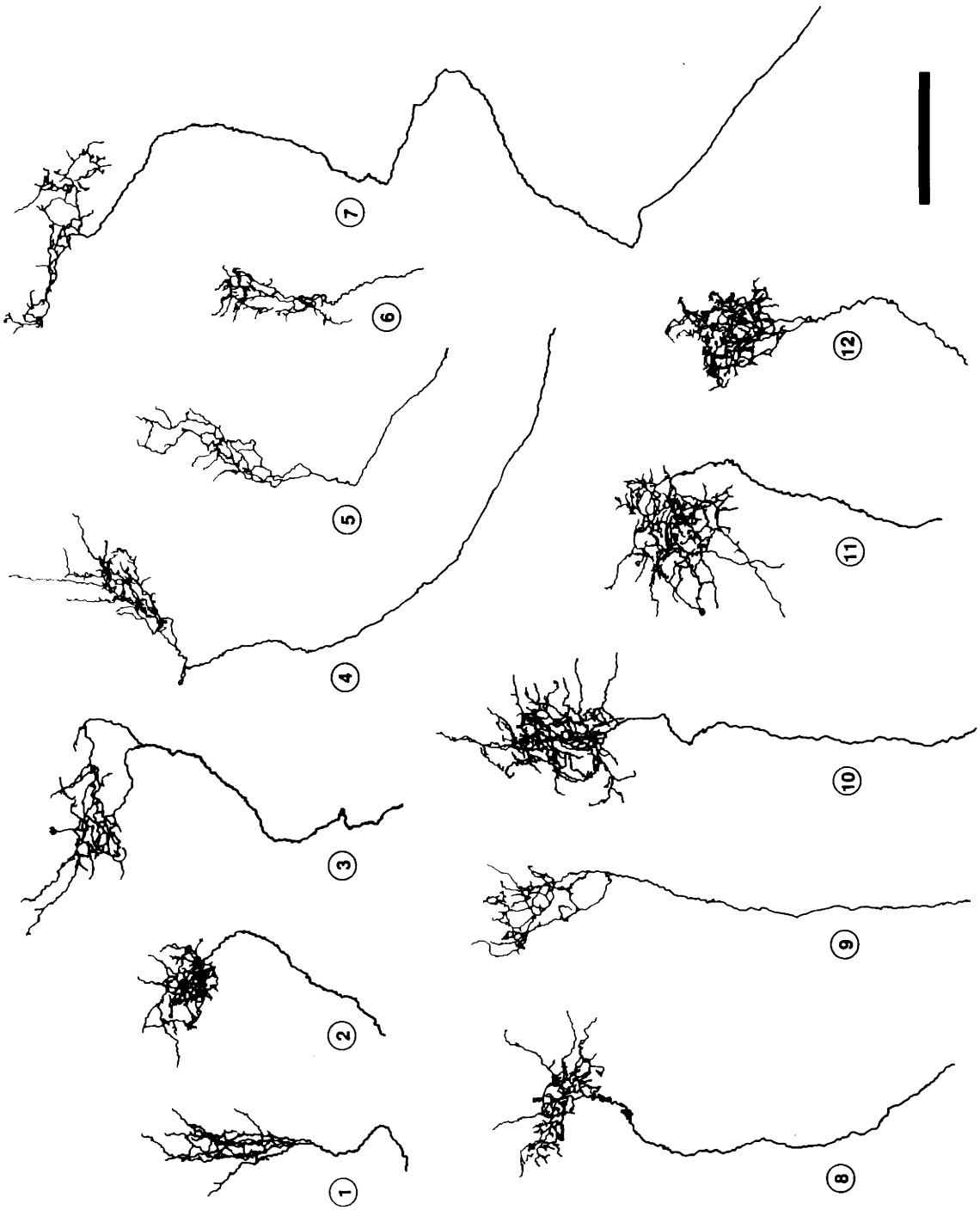


Figure 5B

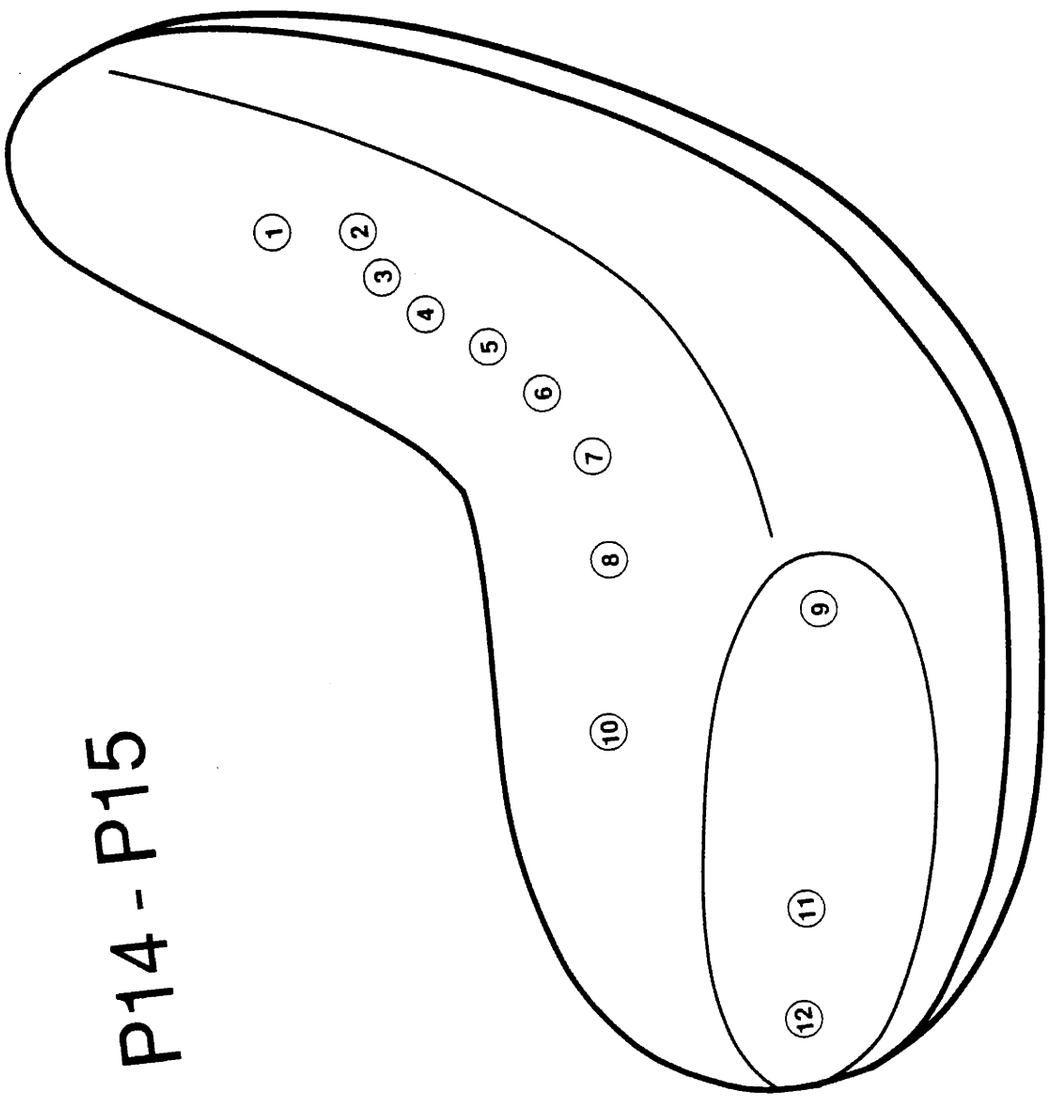


Figure 6A

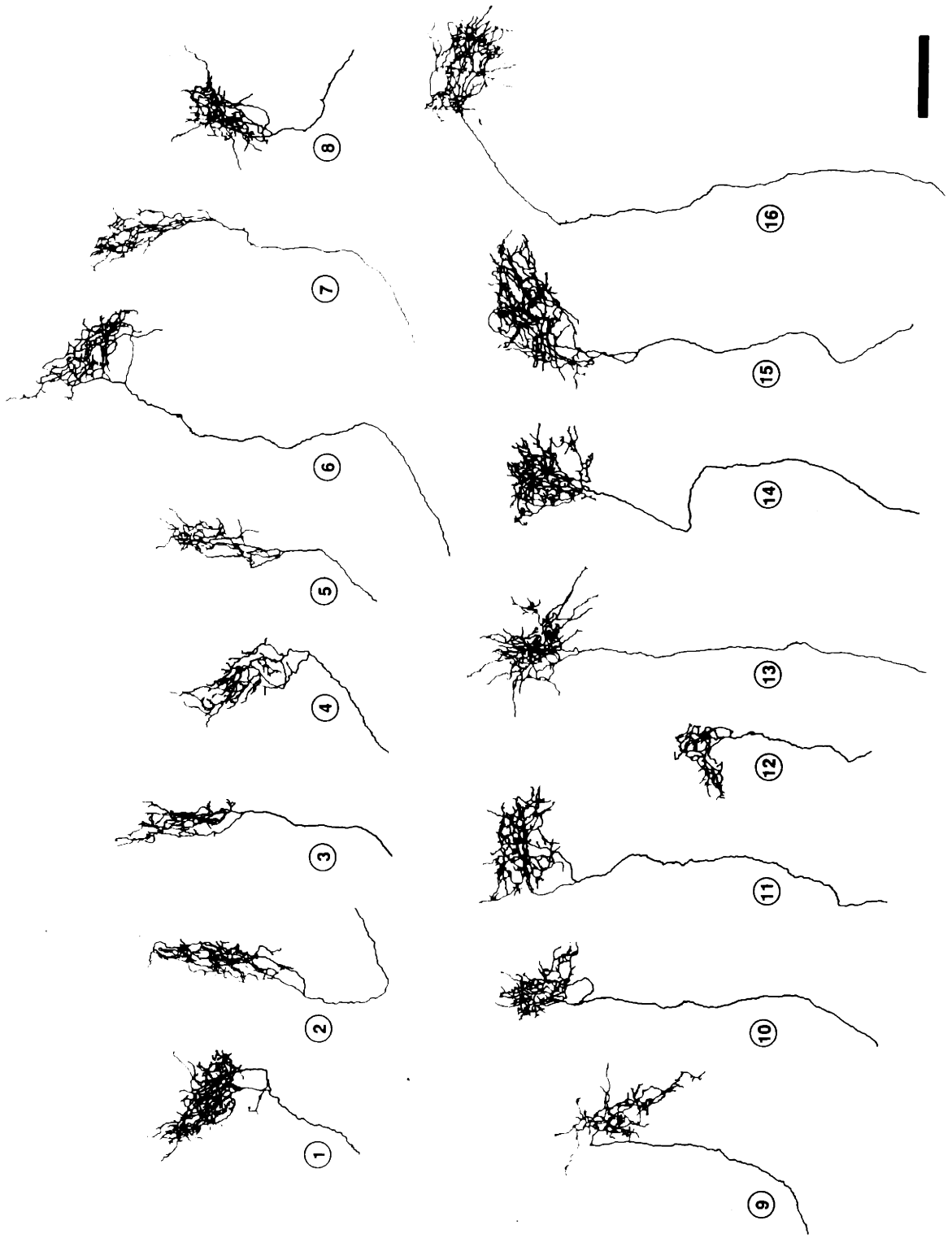


Figure 6B

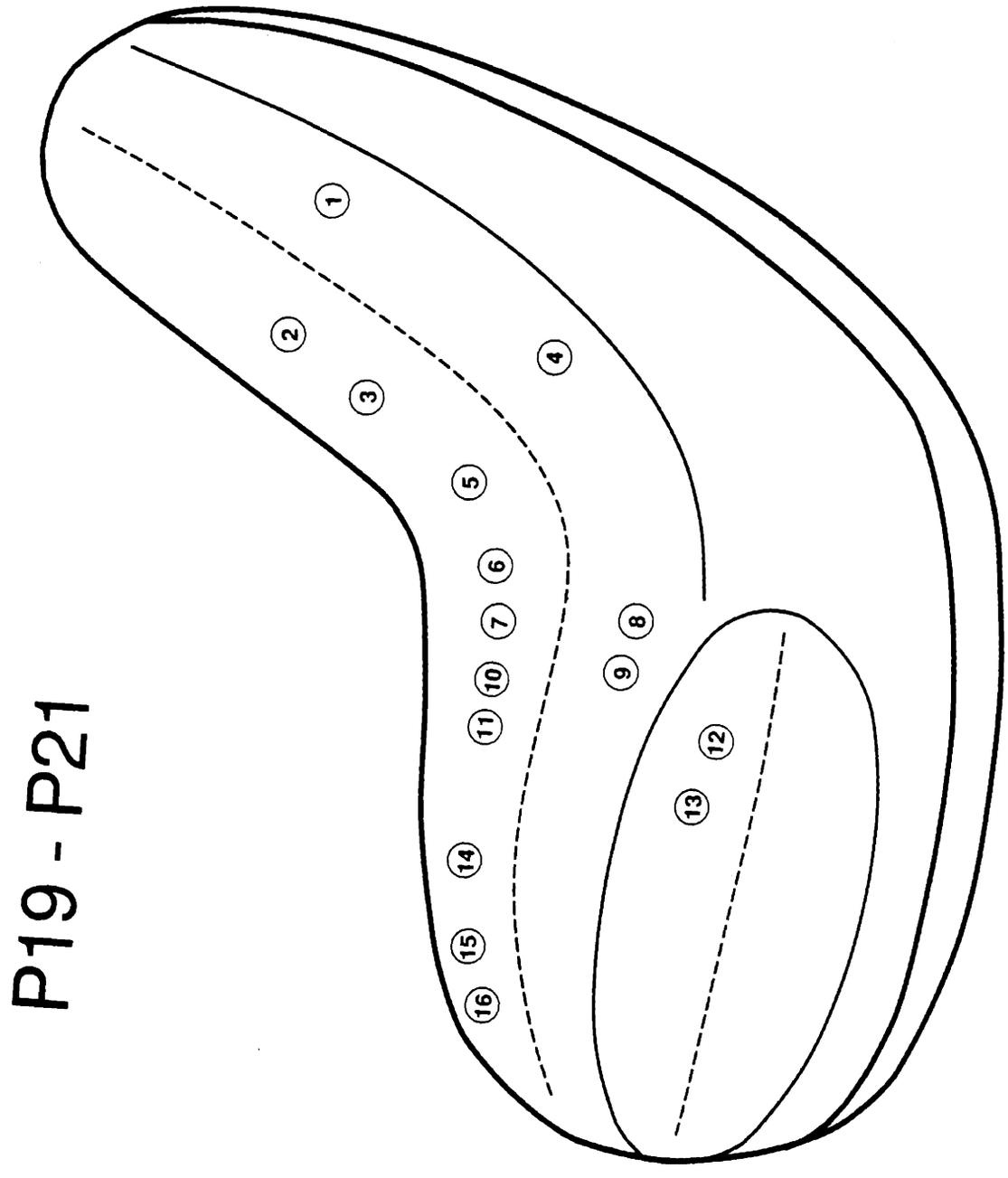


Figure 7A

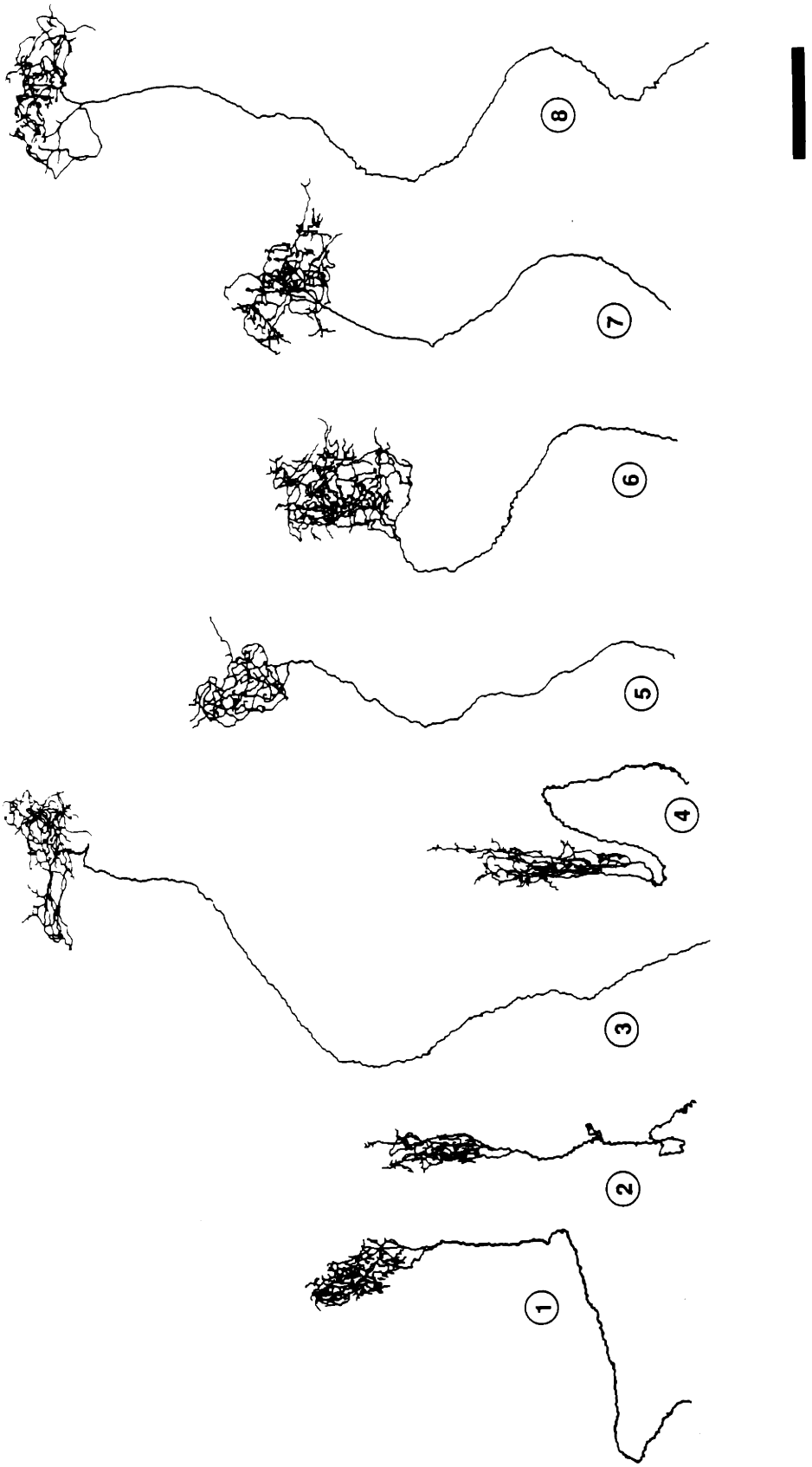


Figure 7B

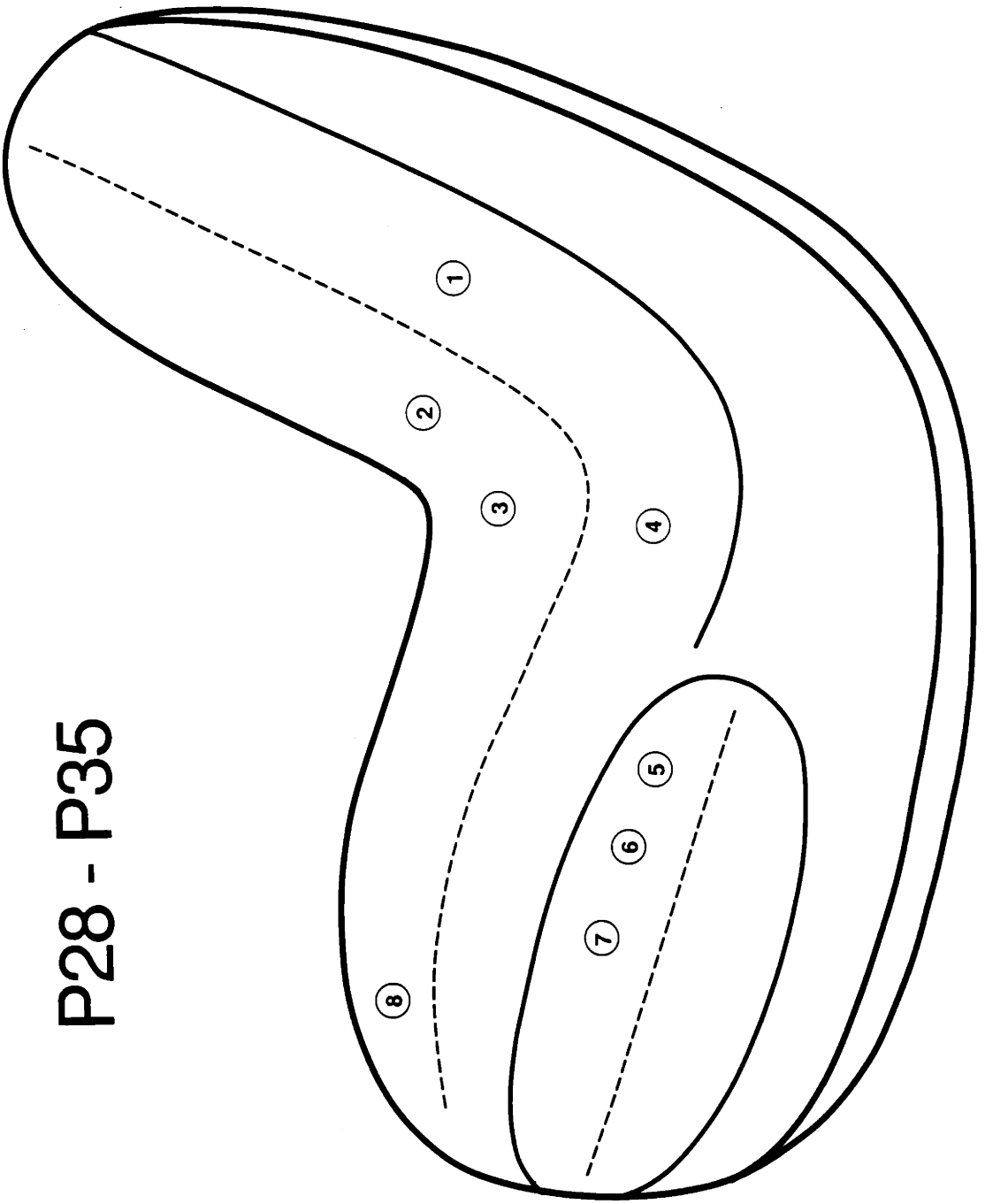


Figure 8

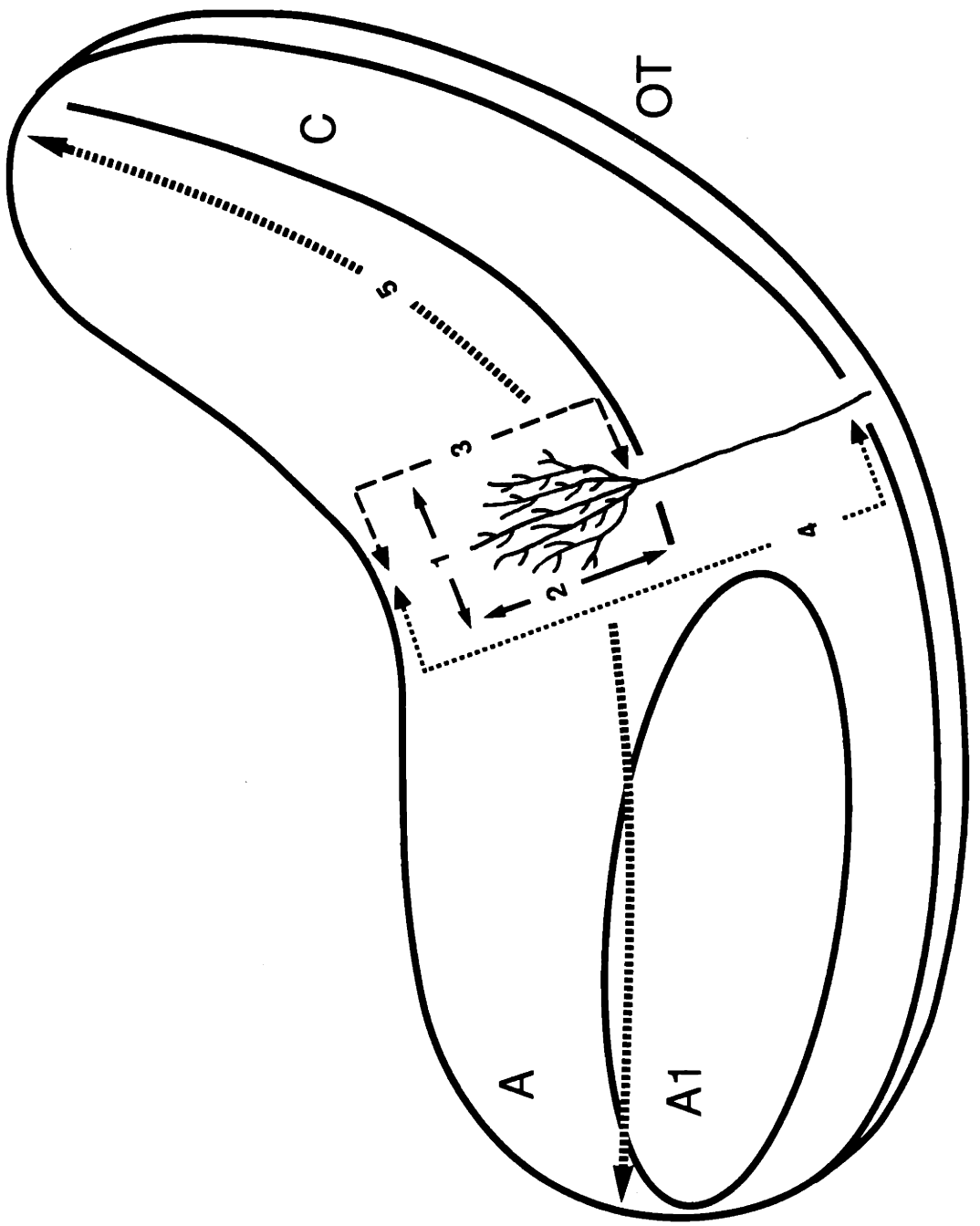


Figure 9

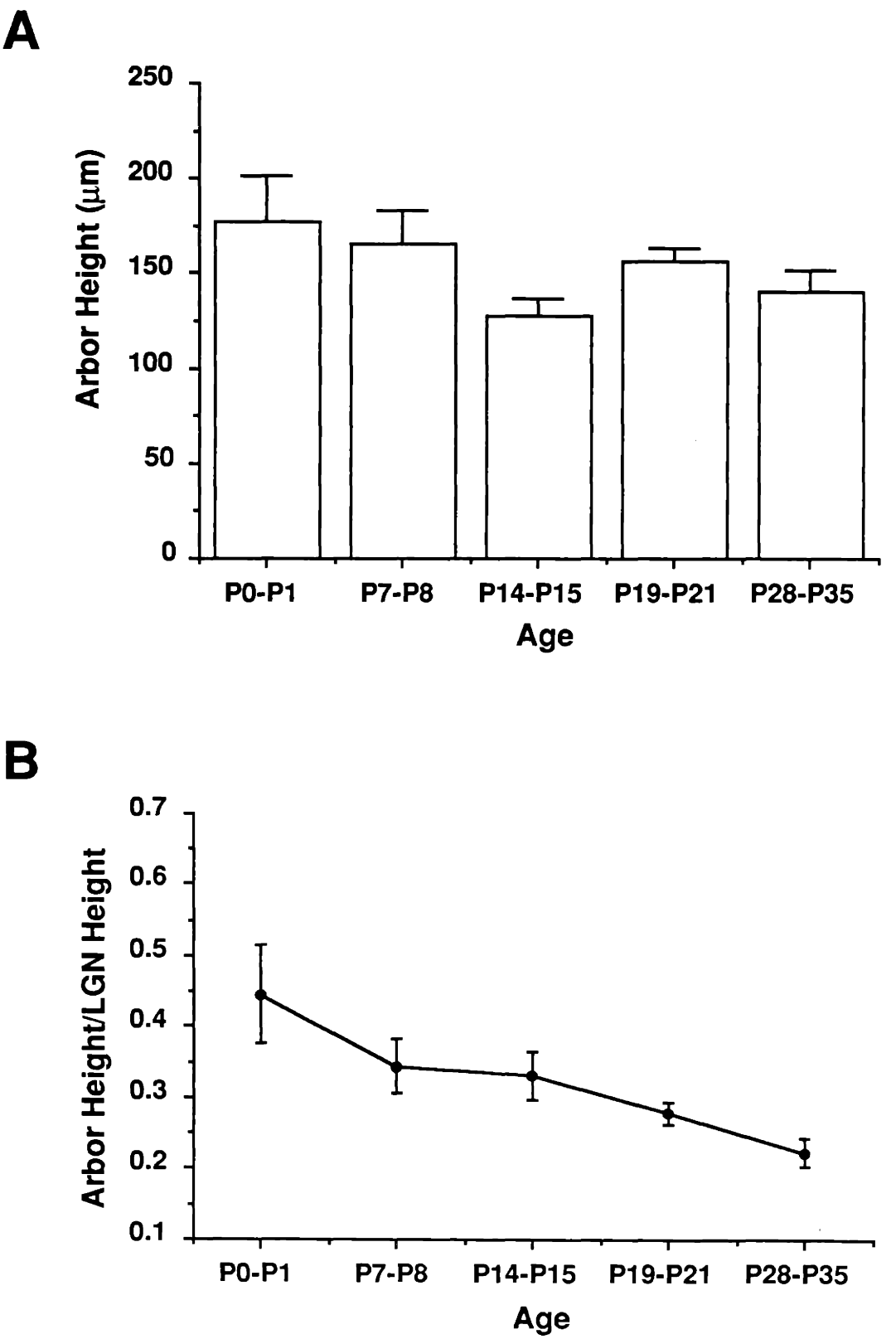

A
250
200
150
100
50
0
Arbor Height (μm)
P0-P1
P7-P8
P14-P15
P19-P21
P28-P35
Age
B
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Arbor Height/LGN Height
P0-P1
P7-P8
P14-P15
P19-P21
P28-P35
Age

Figure 10

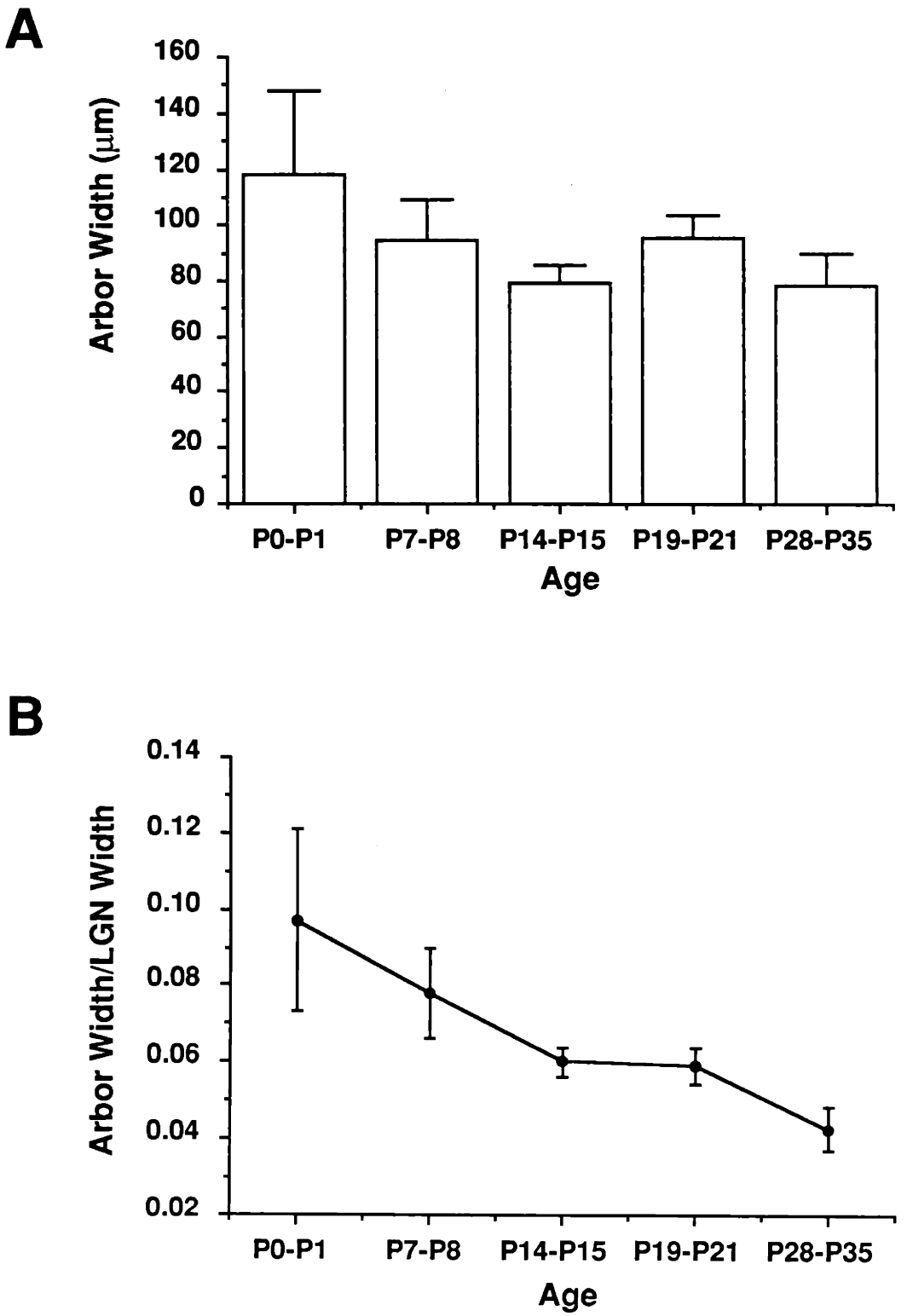
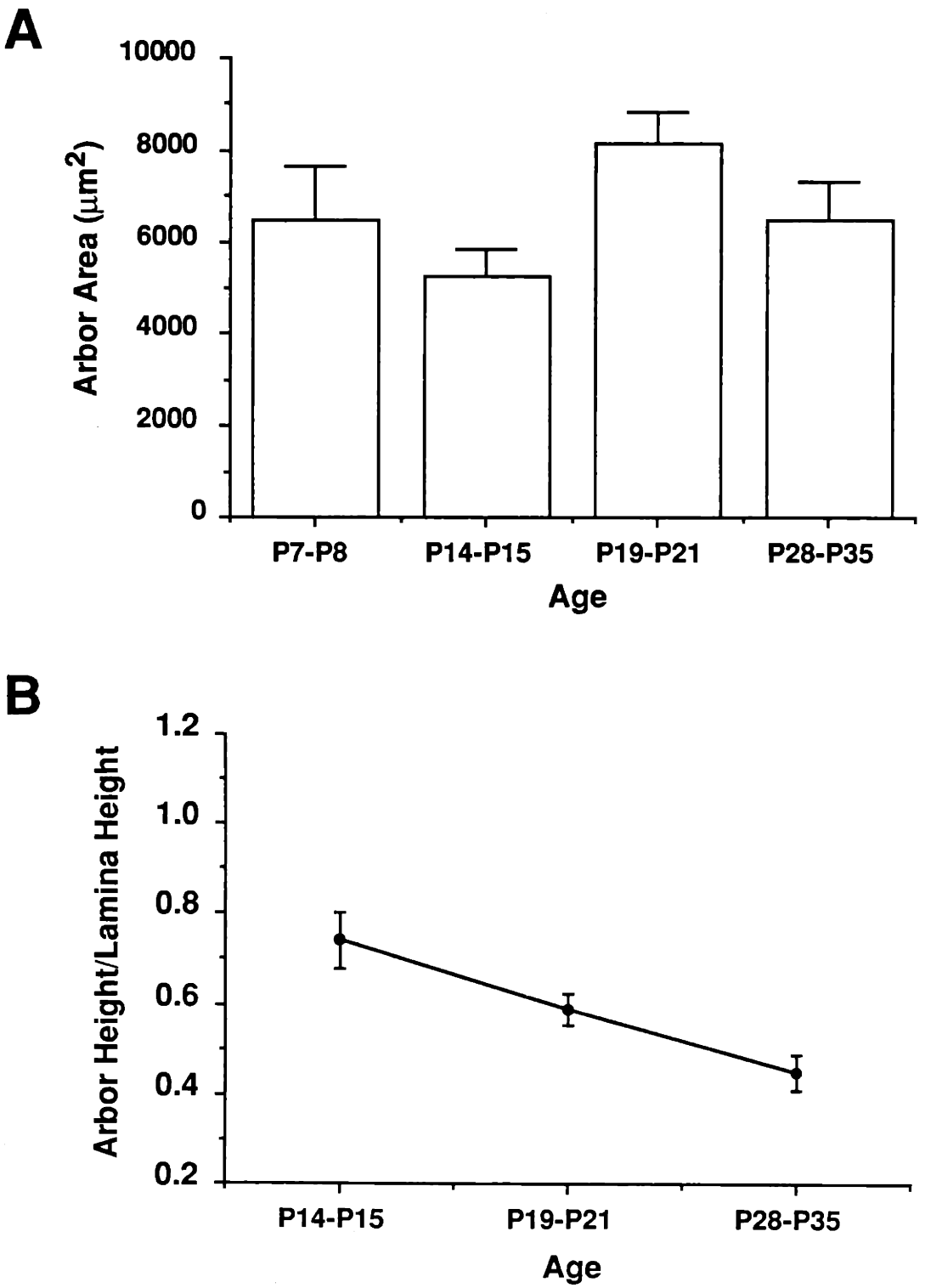

A
Arbor Width (μm)
160
140
120
100
80
60
40
20
0
P0-P1
P7-P8
P14-P15
P19-P21
P28-P35
Age
B
Arbor Width/LGN Width
0.14
0.12
0.10
0.08
0.06
0.04
0.02
P0-P1
P7-P8
P14-P15
P19-P21
P28-P35
Age

Figure 11


A
10000
8000
6000
4000
2000
0
Arbor Area (μm²)
P7-P8
P14-P15
P19-P21
P28-P35
Age
B
1.2
1.0
0.8
0.6
0.4
0.2
Arbor Height/Lamina Height
P14-P15
P19-P21
P28-P35
Age

CHAPTER 3

BLOCKADE OF NMDA RECEPTORS ON NEURONS IN THE FERRET'S LATERAL GENICULATE NUCLEUS PREVENTS NORMAL SEGREGATION OF RETINAL AFFERENTS INTO ON AND OFF SUBLAMINAE

ABSTRACT

Afferent activity is known to play an important role in the formation of connections in the developing mammalian visual system. The extent to which the activity of target neurons shapes patterns of afferent termination and synaptic contact, however, is not known. In this study the influence of target cell activity on the development of afferent arbor structure has been examined by blocking the postsynaptic receptors normally involved in synaptic transmission. N-methyl-D-aspartate (NMDA) receptors on LGN cells were blocked with specific antagonists from two weeks to three weeks of age postnatal, the period when retinal afferents normally segregate into inner (On) and outer (Off) sublaminae within the dorsal A and A1 laminae. NMDA receptor blockade prevents the retinal afferents from segregating appropriately into On and Off sublaminae. Individual retinogeniculate axons have arbors that are not restricted appropriately, or are restricted in size but inappropriately positioned within the eye-specific laminae. These results indicate that the activity of postsynaptic cells can significantly influence the patterning of inputs and the structure of presynaptic afferents during development.

INTRODUCTION

The development of the mammalian visual system is influenced by a host of factors, but perhaps the most critical and interesting is the role of activity. In the cat's visual pathway, visual experience has been found to be crucial in determining the connectivity of projections from the retina to the lateral geniculate nucleus (LGN), and from the LGN to the primary visual cortex. The disruption of afferent activity, for example by suturing the lids of one eye, has often been employed to examine the effects of visual deprivation on target cells, and been found to cause a host of morphological and physiological changes in both the LGN and the visual cortex (for review see Movshon & Van Sluyters, 1981; Sherman & Spear, 1982; Sherman, 1985). Monocular deprivation also results in changes in the pattern of connections at the level of the retinogeniculate synapse, which has been shown to result from altered morphology of retinal ganglion cell axon arbors projecting to the LGN (Sur et al., 1982).

As visual experience in terms of cellular function translates to nerve impulses, studies have examined the effects of disrupting impulse activity directly. Blockade of sodium channels with tetrodotoxin (TTX) injected either into the eye or directly into the brain has been shown to cause abnormal visual function (Archer et al., 1982; Dubin et al., 1985), as well as prevent normal segregation of retinal afferents and development of axon terminal arbors (Shatz & Stryker, 1988; Sretavan et al., 1988). Binocular TTX treatment prevents the formation of ocular dominance columns within the primary visual cortex of cats (Stryker & Harris, 1986), and blocks the formation of ocular dominance stripes in frog and fish optic tectum with input induced from a surgically attached "third eye" (Reh & Constantine-Paton, 1985; Meyer, 1982).

While the influence of afferent activity on target structure development has been studied in great detail, the influence of the target cells on afferent structure development has not been examined. It is not known, for example, whether blockade of the activity of target cells alone has the same effect on the development of axon arbors as blocking activity of the afferents. This is due in part to the difficulty of blocking target cell activity without interfering with afferent activity. TTX, which blocks sodium channels, affects the activity of both pre- and post-synaptic components in the retinogeniculate and geniculocortical projection (Stryker & Harris, 1986).

Recent evidence suggests that activation of NMDA receptors, a subtype of receptor for glutamate which is believed to be the transmitter in the visual system, is critically involved in the formation of appropriate connections within the visual system. NMDA receptors are located on the postsynaptic cell and their blockade leads to severe reduction in transmission of visual input in both the LGN (Sillito et al., 1990a, b; Heggelund & Hartveit, 1990; Kwon et al., 1991) and the visual cortex (Miller et al., 1989; Fox et al., 1989). Kleinschmidt et al. (1987) first showed that block of NMDA receptors in kitten visual cortex prevented the ocular dominance shift that usually occurs following monocular lid suture (see also Bear et al., 1990). In amphibia, it has been shown that NMDA receptor blockade desegregates ocular dominance stripes induced in the optic tectum (Cline et al., 1987; Cline & Constantine-Paton, 1990), and prevent normal binocular map formation in the tectum (Scherer & Udin, 1989). The experiments involving NMDA blockade have been interpreted to indicate a specific role for NMDA receptors in retinotectal or cortical development. If NMDA receptors are involved in transmission, however, these results require re-interpretation, as they suggest that not only afferent activity, but

also target activity is important to normal development, and further, that target activity can have a significant influence on the afferent.

The results of these studies led us to ask whether synaptic transmission mediated by NMDA receptors might not be involved in the formation of segregated pathways in the ferret's retinogeniculate pathway as well. In a study of the normal development of the ferret retinogeniculate projection, we found that retinal afferents undergo two stages of segregation in achieving the adult form: retinogeniculate axon arbors are formed first within an entire eye-specific lamina, then become confined to an inner or outer sublamina (see previous chapter). These sublaminae are segregated according to differences in cell activity - the inner sublamina consists of On-center cells and the outer sublamina of Off-center cells (Stryker & Zahs, 1983). Because the process of On-Off segregation occurs simultaneously with the maturation of retinal synaptic connections and photoreceptor outer segments (Greiner & Weidman, 1981), we wondered whether synaptic transmission through NMDA receptors might play a role in the formation of these segregated connections. It is known that functional retinogeniculate synapses are present at a very early stage in development (Shatz et al., 1982; Shatz & Kirkwood, 1984), suggesting that synaptic transmission may occur prior to retinal afferent segregation. We hypothesized that segregation of On and Off inputs, a separation based on differences in activity, would be critically dependent on both input activity and synaptic transmission between the retinogeniculate afferent and LGN cell.

In the present study we have addressed this issue by examining the development of the ferret's retinogeniculate projection following a period of activity blockade of LGN neurons. To achieve this we introduced into the thalamus specific antagonists for NMDA receptors during the period of retinal afferent segregation into

ON and Off sublaminae. A shorter form of these results has been published elsewhere (Hahm et al., 1991).

MATERIALS AND METHODS

A total of 20 ferret kits aged 14 to 21 days of age were used in this study. Animals were obtained from timed-pregnant jills either purchased from Marshall Farms (North Rose, NY) or bred in our colony. Day of birth was considered postnatal day 0 (P0).

Drug Delivery:

Each animal was implanted with an osmotic minipump (Alza Corporation, Alzet model 2001, 1 ul/hr, 1 week infusion) containing one of the following (in saline) solutions: 1) D-2-amino-5-phosphonovaleric acid (D-APV): 0.8mM, 0.08mM; 2) L-2-amino-5-phosphonovaleric acid (L-APV): 0.8mM; 3) ((+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate (MK-801): 4.75mM, 1.2mM; 4) control vehicle (isotonic saline). D-APV, L-APV, and saline were infused directly into the thalamus, while MK-801 and saline were infused subcutaneously.

For intrathalamic infusion, each minipump was connected via a catheter (1.5cm length of polyvinyl tubing, medical grade) to a 7mm long, 28-gauge stainless steel cannula (Plastics One, model 3280p). The minipump was implanted subcutaneously and the cannula inserted into the brain. For subcutaneous infusion, the minipump alone was implanted subcutaneously.

Surgery:

All surgical procedures were performed under sterile conditions. At two weeks of age ferret kits were removed from the mother and prepared for surgery.

Animals were anesthetized with ketamine (4 mg/100 gm body weight), and supplemented with methoxyflurane (Metofane 1-2%) as necessary. Atropine (0.04 mg/kg) was administered to prevent congestion. For subcutaneous infusion, the skin over the skull was incised and separated from subcutaneous tissue at the back of the neck to create a pocket for the minipump. The minipump was slipped into the pocket, the skin sutured and the animal allowed to recover. For infusion directly into the thalamus, after the skin pocket was created for the minipump, connective tissue was cleared away from the top of the skull with a dry cotton swab. A small hole was drilled in the skull overlying rostral thalamus, approximately 2mm caudal and lateral from the lambda sutures. The dura was punctured with a 26 gauge needle. After placing the minipump into the skin pocket, the cannula was inserted into the thalamus and glued to the top of the skull with cyanoacrylate glue (Locktite), then held in place with dental acrylic. The skin was then sutured, the animal allowed to recover and returned to the mother. The animal received daily injection of a wide-ranging antibiotic (amoxicillin, 5-10 mg/kg) for seven days until it was sacrificed.

In placing the cannula within the thalamus, care was taken to avoid positioning the cannula tip too close to the medial border of the LGN. The area targeted for placement of the tip was the anterior thalamus, approximately 3 mm rostral and medial to the LGN, and 1 mm below the thalamic surface. At this distance, there was no danger of damaging the geniculocortical fibers in the optic radiations which may have affected the target cells. Further, this distance is believed to be enough to avoid nonspecific effects of drug release and, according to the dilution curve calculated by Bear et al. (1990), appropriate to deliver a drug concentration adequate to achieve receptor block in these young animals (Esguerra, White & Sur, unpublished observations).

Eye Injections:

After six days of chronic infusion, the animal received an injection of horseradish peroxidase agglutinated to wheat germ (WGA-HRP) in the eye contralateral to the thalamic implant. For consistency, animals receiving subcutaneous infusion were injected in the left eye. This procedure has been detailed (see previous chapter) and will be only briefly described here. The animal was first anesthetized with methoxyflurane and the eyelid incised. The eye was anesthetized with ophthalmic anesthetic (Proparacaine), the sclera behind the punctured with a 26 gauge needle and the tip of a 10 ul Hamilton syringe inserted. Approximately 5-10 ul of HRP (20%, 2% WGA-HRP in sterile saline) was slowly injected into the posterior chamber of the eye. The eye was then treated with ophthalmic antibiotic (Tri-Thalmic), the animal allowed to recover and returned to the mother. Following a 24-hour survival, the animal was perfused transcardially with saline followed by mixed aldehyde fixatives (1% paraformaldehyde, 2% glutaraldehyde). The brain was removed and left in 30% sucrose phosphate buffer (0.1 M) overnight.

Each cannula was tested after perfusion to ensure that drug delivery was free-flowing. Animals in which drug delivery was impaired in any way, i.e. due to blocking of the cannula tip or disconnection of the catheter from the minipump, were not considered to have been drug-treated and were considered as surgical controls.

Single Axon Labeling:

To examine the structure of retinogeniculate axon arbors, an in vitro procedure was used to label individual fibers within the optic tract (Sretavan & Shatz, 1986). The details of this procedure have been described in the previous chapter and will only be briefly recounted here. The animal was deeply anesthetized with sodium pentobarbital (65 mg/ml, 100 mg/kg body weight), and transcardially

perfused with cold (4° C) artificial cerebrospinal fluid (aCSF). The brain was quickly removed, placed in a petri dish lined with Sylgard and continuously superfused with aCSF. The cortices were dissected away and the remaining thalamus and midbrain bisected; each hemithalamus was pinned medial side down with the LGN and optic tract facing up. Small pellets of HRP adhered to the tip of glass micropipettes were inserted into the optic tract just ventral to the LGN. At least three deposits were made in different loci in the optic tract. After the injections, the tissue chunk was maintained in aCSF at room temperature for 3-4 hours to allow the HRP to transport. The brain was immersion-fixed in aldehyde fixatives and allowed to sink in 30% sucrose phosphate buffer.

Histology

For the eye injection cases, frozen sections were cut at 50 um in the horizontal plane on a sliding microtome and placed into 0.1M phosphate buffer. Sections were reacted for HRP histochemistry using tetramethylbenzidine (TMB) as the chromogen (Mesulam, 1982). Reacted sections were mounted on chrome-alum subbed slides, air dried, dehydrated, cleared and coverslipped.

For the single axon labeling experiments, sections were cut at 100um on a freezing microtome and reacted for visualization of the HRP using the diaminobenzidine and cobalt intensification protocol of Adams (1981). Sections were mounted, cleared and coverslipped as above.

Axon Reconstruction

Individual axons were reconstructed using the Leitz Diaplan microscope and camera lucida with 63X and 100X oil immersion objectives. Sublaminar leaflets

were visualized with dark-field illumination using a 4X objective on an Olympus microscope.

RESULTS

A summary of the cases in this study is presented in Table 1.

Effects of NMDA Receptor Blockade: Intraocular Injections

Figure 1 shows dark-field photomicrographs of the right LGN of animals which had received an injection of HRP in the left eye. The LGN of a normal three-week old animal is depicted in Figure 1a, illustrating that retinal afferents have segregated into inner (On) and outer (Off) sublaminae in the contralateral lamina A (also see previous chapter). Figure 1b shows the LGN of a control animal which received intrathalamic infusion of saline (L-APV similar effect, data not shown) from two weeks of age to three weeks of age. The retinal afferents are segregated into sublaminar leaflets.

Following treatment with 0.8 mM D-APV (Figure 1c, retinal afferents fail to segregate into the characteristic inner and outer sublaminae. Treatment with 0.08 mM D-APV, however, does not have such an effect (Figure 1d). Systemic infusion of 4.75 mM MK-801 also prevents retinal afferents from segregating into sublaminae (Figure 1e), whereas a dose of 1.2 mM does not prevent such segregation (Figure 1f). Thus, blockade of sublaminar segregation is dose-dependent: high doses of NMDA antagonists prevent segregation, while low doses do not.

Effects of NMDA Receptor Blockade: Single Axon Morphology

Qualitative Differences

The morphology of retinogeniculate axon arbors in animals treated with 0.8 mM D-APV is illustrated in Figure 2. In general, the overall morphological structure

of D-APV treated axons are not greatly altered from that of normal axons. Like normal axons labeled at three weeks of age (see previous chapter) each retinogeniculate axon enters the LGN without giving rise to branching collaterals either within the LGN or the optic tract. The axon trunk within the LGN is smooth, there are no side branches along its length. Each axon has a single terminal arbor confined to either lamina A or A1.

However, in several ways D-APV treated axons differ from those of normal three-week axons. In Figure 3 the location of retinogeniculate axon terminal arbors with the A layers are illustrated for D-APV treated cases. For ease of comparison, axons from normal two-week and three-week old animals are illustrated as well. All normal data cited here are from the previous chapter. Figure 3a shows typical retinogeniculate axon arbors in the LGN of normal two-week old animals. At this age, axon arbors are confined to an eye-specific layer but do not show evidence of preferentially segregating toward an inner or outer half of the lamina. At three weeks of age (Figure 3b) the arbors are confined to an inner or outer sublamina within the A layers. Figure 3c, d shows examples of axon arbors from D-APV treated cases, and two differences from the normal are evident. First, some axons have terminal arbors which show no evidence of restriction into a sublaminar leaflet (Figure 3c). These axons span the height of an entire eye-specific lamina, with no biasing of the arbor toward an inner or outer half. Other axons (Figure 3d) are appropriately restricted in size such that they would fit into a sublamina, but are inappropriately positioned toward the center rather than in an inner or outer half of an eye-specific lamina. Still others appear to be appropriately restricted in size and location, being located close to inner or outer laminar boundaries. Thus, although the morphology of these axon arbors is not greatly changed, location of arbors is changed in the treated animals.

Quantitative Differences

We compared two characteristics of normal and D-APV treated axon arbors: terminal arbor area and degree of segregation. Arbor area of D-APV treated axons was measured in the same way as described in the previous chapter for normal axons. Briefly, the area of LGN covered by the outermost tips of arbor branches was measured. The axon data described here were pooled from several cases at each age. Data were compared two ways, by treating each axon as a single datum and by treating each animal as a single datum. The values obtained for each measure are presented in Table 2. For simplicity, the mean values for the axon population across animals will be cited here.

The measures of arbor area of normal two-week and three-week old cases and D-APV treated three-week old cases are depicted in Figure 4a. At two weeks of age, retinogeniculate axon arbors have an area of 5250 um2 (+/- 601, S.E.). By three weeks of age, arbor area has increased to 8155 um2 (+/- 653), a difference which is significant ($p < .05$ treating each animal as a single datum, $p < .01$ treating each axon as a single datum, Mann-Whitney U Test). Arbor area of D-APV treated axons is not different from normal three-week axons, being 7959 um2 (+/- 807, $p = .51$ treating each animal as a single datum, $p = .71$ treating each axon as a single datum), but is significantly different from normal two-week axons ($p < .05$ treating each animal as a single datum, $p = .01$ treating each axon as a single datum).

To assess the degree of segregation of each axon, we defined a "sublamina index" which measured the greatest proportion of an arbor lying in the inner or outer half of lamina A or A1. To do this, we bisected the lamina longitudinally, and measured the extent of the arbor on each side of this imaginary line. The larger portion was computed as a proportion of the entire arbor area, giving a ratio value between 0.5 and 1.0. Thus, a sublamina index of 1.0 represents an arbor which is

entirely confined to an inner or outer half of lamina A or A1. A sublamina index of 0.5 represents an arbor which is equally divided between the inner and outer half of the lamina, with no evidence of sublaminar segregation.

The sublamina index of normal two-week and three-week old cases and D-APV treated three-week cases are shown in Figure 4b. Between two weeks and three weeks of age in normal animals, the sublamina index increases from .70 (+/- .01 S.E.) to .88 (+/- .05), a difference which is significant ($p < .05$ comparing individual animals, $p = .01$ comparing individual axons). D-APV treated axons have a sublamina index of .66 (+/- .03), which is similar to that of two-week normal axons ($p = .38$ comparing individual animals, $p = .25$ comparing individual axons). However, it is significantly different from that of normal three-week axons, in comparisons of individual animals ($p < .05$) and of individual axons ($p < .001$). Hence, the sublamina index of D-APV treated axons indicates that arbors are less restricted to one half of the lamina than normal age-matched controls.

DISCUSSION

In this study we have presented evidence to show that specific antagonists to NMDA receptors on LGN neurons prevents the normal segregation of retinogeniculate afferents into On and Off sublaminae within laminae A and A1 in the ferret's lateral geniculate nucleus. We have shown that segregation is blocked primarily because retinogeniculate axon arbors fail to restrict appropiately into an inner or outer half of the eye-specific lamina. Axons are either not restricted in size and span the height of the entire lamina, or are appropriately sized but incorrectly positioned toward the middle of the lamina. These results strongly suggest that the activity of the target cells has a significant influence on the location of the developing afferent arbor.

Our data present further support for the critical role of activity in the development of normal connections within the visual system. More importantly, our results more specifically identify the role that activity of target cells has on the development of the afferent arbor. Blockade of target cell activity prevents the afferents from locating their arbors in "correct" areas, without interfering with terminal arbor growth. This is consistent with the findings of the next chapter, in which size of the target was altered but synaptic transmission between afferent and target maintained; retinogeniculate axon arbors are found to be located appropriately within eye-specific laminae in the LGN, and arbors are reduced in size according to the size of the LGN.

A critical point in the interpretation of these findings is whether the receptor blockade is indeed confined to the postsynaptic cell. To date there is no evidence in the retinogeniculate pathway that there are synapses on retinal ganglion cell afferents within the LGN or that NMDA receptors are located on the presynaptic afferent.

NMDA receptors, as well as non-NMDA receptors are present on cells in the retina, but they have not been shown to be located on retinal ganglion cell axon arbors within the LGN. The only known presynaptic receptor is for aminophosphonobutyrate (L-AP4), which are present on bipolar cells in the retina but are not affected by antagonists to NMDA receptors (Miller & Slaughter, 1986). A metabotropic receptor, involved in the phosphoinositide cascade and intracellular release of calcium (Sugiyama et al., 1987), also has been implicated in the plasticity of connection within the kitten visual cortex (Bear & Dudek, 1989). However, these receptors are also believed to be postsynaptically located, and further, there is no evidence that NMDA antagonists interact with these receptors.

It has been argued that NMDA receptors have a special function in the formation of connections in early development because of their capacity to induce long-term potentiation (LTP; see Teyler & DiScenna, 1987, for review) and their permeability to calcium, a regulator of many neuronal functions (Kennedy, 1989). These characteristics make NMDA receptors ideal candidates as the arbiters of synapse stabilization as proposed by Hebb (1949; Cotman et al., 1988 for review; also see Introduction). If NMDA receptors are special for development, then it would have to be demonstrated that they have a role different from that in adulthood. There is some evidence to support this view. In kitten visual cortex, the number of NMDA receptors dramatically increase in the first 2-4 weeks of life and remains elevated throughout the critical period for ocular dominance column formation (Bode-Greuel & Singer, 1989). Other studies have shown that LTP is more easily induced in kitten visual cortex as compared with the adult (Komatsu et al., 1988), and that NMDA receptors mediate visual transmission more effectively in young cortex (Tsumoto et al., 1987; Hagihara et al., 1988).

However, there is increasing evidence to show that NMDA receptors are as effective in mediating visual input in adults as in kittens (Miller et al., 1989; Fox et al., 1989). In addition, several studies, including work in our laboratory, have shown that in the retinogeniculate pathway, NMDA and non-NMDA receptors are both involved in the normal transmission of visual input. In the intact LGN, studies have demonstrated that iontophoretic application of either NMDA or non-NMDA receptor antagonists block the visual response of geniculate relay cells (Hartveit & Heggelund, 1990; Heggelund & Hartveit, 1990; Kwon et al., 1991; Moody & Sillito, 1987; Sillito et al., 1990a, b). In isolated LGN slice preparations, optic tract stimulation evoked EPSP's in LGN cells are attenuated by blockade of both NMDA and non-NMDA receptors; this was found for both ferret and cat LGN (Esguerra et al., 1989, and submitted; Scharfman, 1990). Further, NMDA receptors are found to mediate geniculate cell EPSP's in ferrets as young as two weeks of age (Esguerra, White & Sur, unpublished observations). In the retinogeniculate pathway, it is reasonable to postulate that NMDA receptors play an equivalent role in synaptic transmission as non-NMDA receptors (Kwon et al., 1991, and submitted). Finally, it has been shown that when cortical plasticity is blocked in young animals, visual transmission is also disrupted, indicating that the two phenomena are linked and that cortical plasticity is not a separate, special function of NMDA receptors (Rauschecker et al., 1990). These and other data showing that any disruption of cortical activity prevents cortical plasticity (Shaw & Cynader, 1984), suggest that any effect of NMDA receptors is through altered patterns of visual transmission, not due to specific characteristics of the receptor itself.

If NMDA receptors do not have a special function in development, the question then arises as to how their blockade, and hence blockade of target cell activity, affects the presynaptic afferent. If NMDA receptors are simply involved in

normal synaptic transmission as the data suggest, then the logic follows that blockade of other receptors involved in transmission, for example, non-NMDA receptors, or reduction of postsynaptic cell activity by other means should have the same effect on presynaptic arbors as NMDA receptor blockade. Furthermore, blockade of NMDA receptors, but elevation of postsynaptic activity to normal levels (by electrical or pharmacological means) should cause afferent arbors to differentiate normally. These issues merit further scrutiny and are being pursued in our laboratory. There is some data to suggest that non-NMDA receptor antagonists (e.g. CNQX) also prevents sublaminar segregation, but these results are very preliminary (Hahm & Sur, unpublished observations).

Regardless of the specific role of NMDA receptors in the formation of segregated pathways, our results suggest the role of a mechanism in which the target influences the afferent to locate its arbor in a certain place. Apparently there is a retrograde flow of information which allows the afferent to detect or recognize "correct" target areas. This signal or mechanism is blocked when NMDA receptors are blocked. The concept of a target-derived factor in development is not new; in the peripheral nervous system, target-derived factors have been proposed as promoting neuronal survival and neurite outgrowth (Oppenheim, 1985, 1989; Lander, 1987, for review). There is some evidence to indicate the role of target-derived factors in the central nervous system as well (Eagleson et al., 1990), in the capacity for geniculate neurons in vitro to recognize and innervate correct layers of cortex (Yamamoto et al., 1989; Bolz et al., 1990). The identity of any such signal is not known at the present time, although in rat striatal neurons, glutamate receptor stimulation results in the release of arachidonic acid (Dumuis et al., 1988) and nitric oxide (Garthwaite et al., 1989). However, there is no clear evidence at this time implicating these substances in the regulation of specific connections.

Finally, there is increasing evidence that postsynaptic activity can itself regulate the levels of intracellular signals important for synapse formation and maintenance. Calcium entry through voltage-gated T-type calcium channels may have important developmental consequences; both mature and developing LGN neurons express such channels prominently (Jahnsen & Llinas, 1982; Crunelli et al., 1990; McCormick & Feeser, 1990; Esguerra et al., 1991). Furthermore, levels of phospholipase C, a primary effector enzyme in the phosphoinositide cascade, and Ca/calmodulin, a second messenger, are also regulated by the membrane potential of cells (see Greengard, 1987, for review).

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FIGURE LEGENDS.

Figure 1: Dark-field photomicrographs of the right LGN in animals that received an injection of HRP in the left eye. All drugs were chronically infused between two weeks and three weeks of age. a) LGN of normal three-week old animal. Retinal afferents are segregated into inner (On) and outer (Off) sublaminae within lamina A. b) LGN of a three-week old animal which received either saline (similar effects with L-APV, data not shown). Sublaminae are present within lamina A. c) LGN of a three-week old animal chronically treated with 0.8 mM D-APV. Retinal afferents are not segregated into inner and outer sublaminae. d) LGN of a three-week old animal chronically treated with 0.08 mM D-APV. Retinal afferents are segregated within On and Off sublaminae. e) LGN of a three-week old animal systemically treated with 4.75 mM MK-801. Retinal afferents fail to segregate into On and Off sublainae. f) LGN of a three-week old animal systemically treated with 1.2 mM MK-801. Retinal afferents are segregated into sublaminae. Scale bar represents 250 μm.

Figure 2: Reconstruction of retinogeniculate axons recovered from three-week old animals chronically treated with 0.8 mM D-APV. Scale bar represents 100 μm.

Figure 3: Schematic illustration of retinogeniculate axon arbor location within lamina A (for simplicity, lamina A1 terminations are not shown). a) Normal two-week case: retinogeniculate axon arbors are located across the extent of an eye-specific lamina, although arbor branches no longer extend completely to laminar borders. b) Normal three-week case: At this age, arbors are confined to within an inner or outer sublamina. c, d) D-APV treated three-week case: axon arbors are either larger than normal relative to the LGN, and spread across the entire eye-specific lamina, or are

appropriately sized and would fit into a presumptive sublamina, but are inappropriately positioned in the middle of the lamina. Some appropriately sized arbors are placed correctly, toward the inner or outer half of a lamina. Scale bar represents 100 μm.

Figure 4: Histograms showing measures of arbor area and degree of segregation of retinogeniculate axons. a) Terminal arbor areas of retinogeniculate axons in normal two-week, normal three-week, and D-APV treated three-week old animals. In normal animals, arbor area increases significantly between two and three weeks of age. D-APV treated animals also show increased terminal arbor area, and are similar to normal axons. b) Histogram of "sublamina index", a measure to illustrate degree of segregation of axon arbors into inner or outer half of an eye-specific lamina (see text). A measure of 0.5 indicates complete lack of segregation, that is, arbors are placed equally in both halves of the lamina. A measure of 1.0 indicates total segregation to within an inner or outer half of a lamina. In normal two-week old animals, arbors are still not segregated into sublaminae, although the sublamina index suggests that there is a slight tendency for segregation. In normal three-week old animals, arbors are largely segregated within an eye-specific lamina. In D-APV treated three week old animals, axon arbors are not segregated to an inner or outer half of the lamina.

Table 1. Number of Cases

Drug	Eye Injection	Single Axon
Control		
Saline (0.9%)	3	
L-APV (0.80 mM)	2	2
D-APV (0.80 mM)	2	3
D-APV (0.08 mM)	1	1
MK-801 (4.75 mM)	3	
MK-801 (1.25 mM)	3	
TOTAL	14	6

Table 2. Comparison of Arbors

Single Axon Cases	Number of Animals	Number of Axons	Mean Arbor Area in μm^2 (S.E.)	Mean Sublamina Index (S.E.)
Normal 2-week	3	12	5250 (601)	0.70 (0.01)
Normal 3-week	3	16	8155 (653)	0.88 (0.05)
D-APV treated	3	12	7959 (807)	0.66 (0.03)

Figure 1

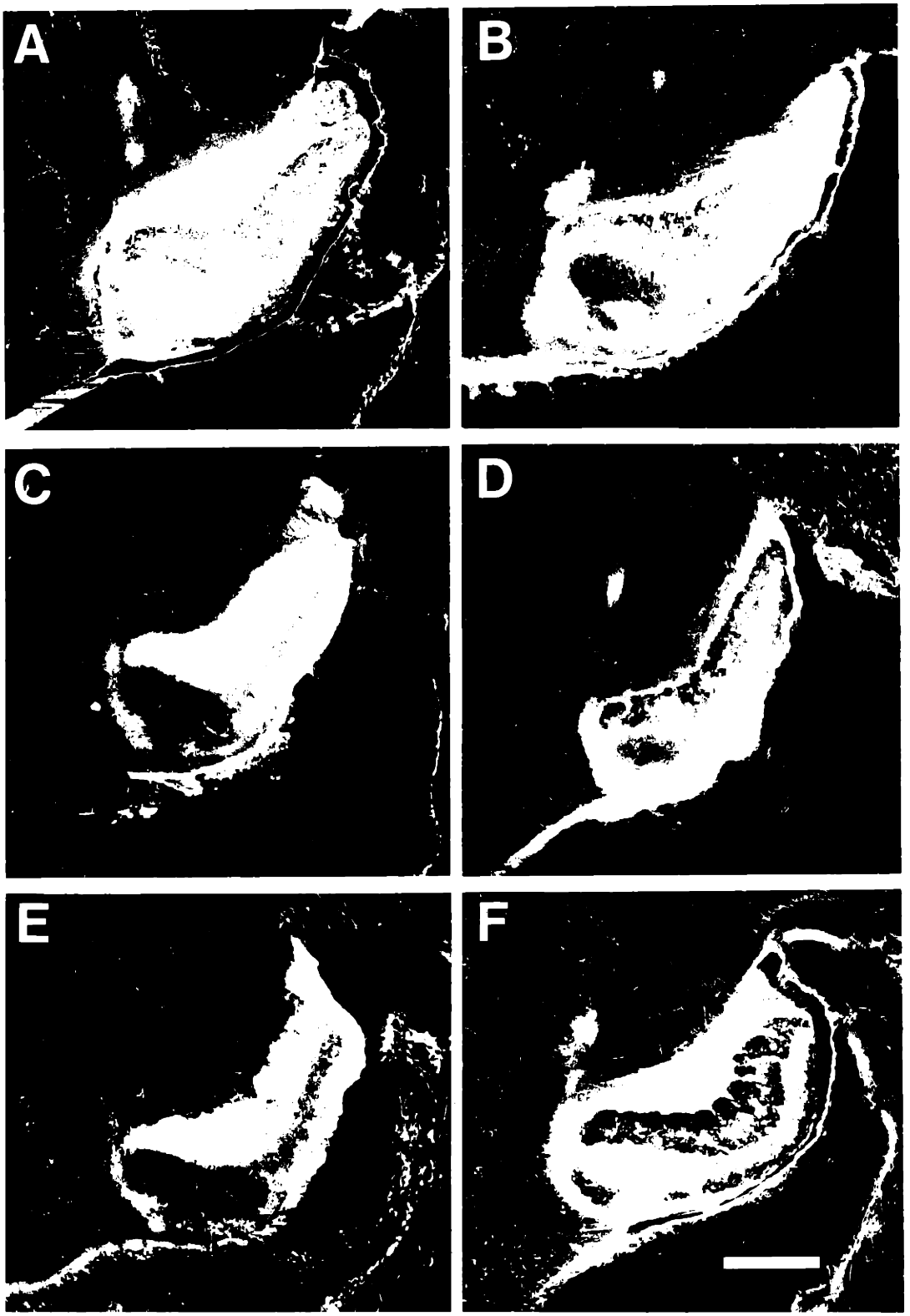


Figure 2

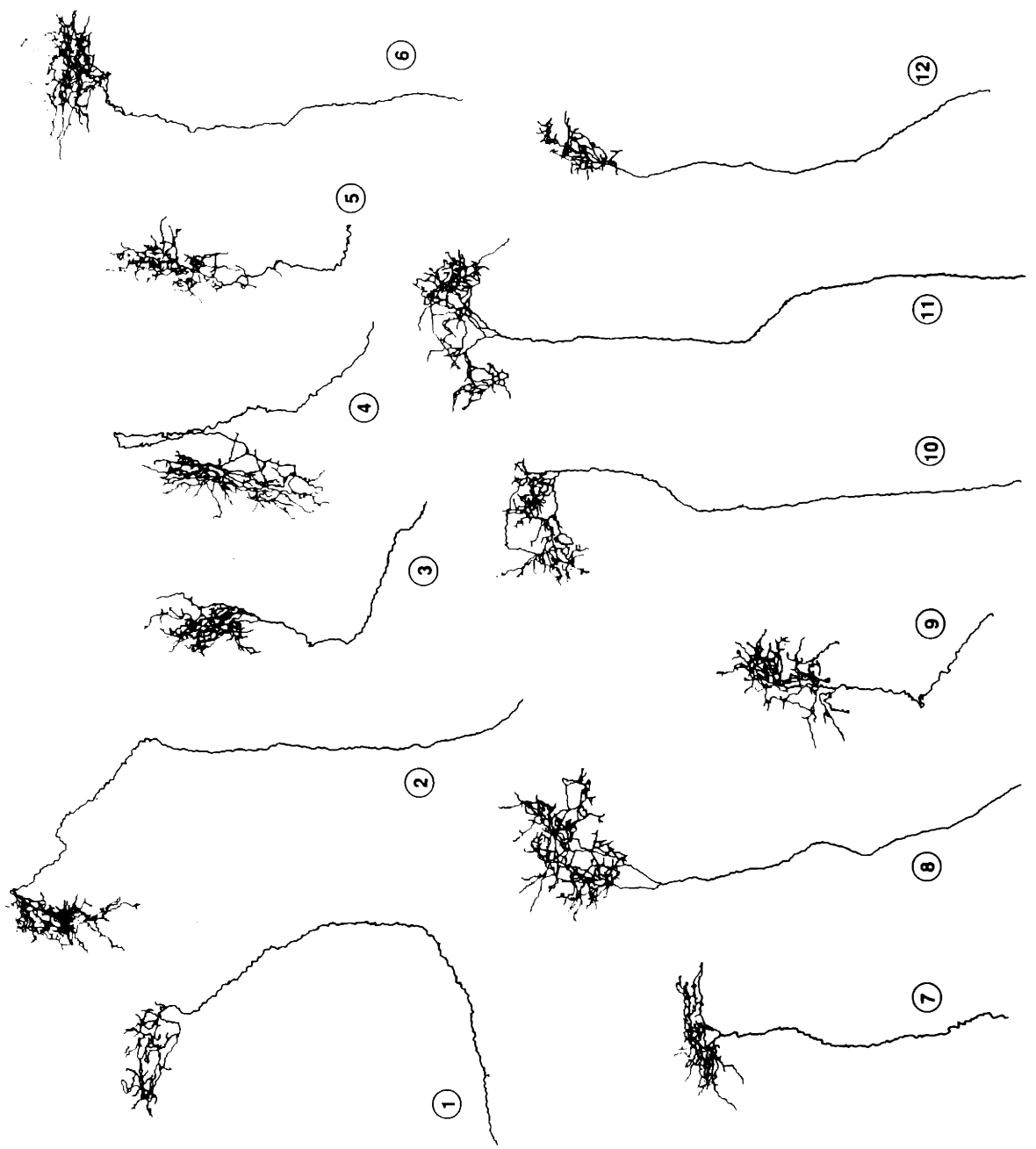


Figure 3

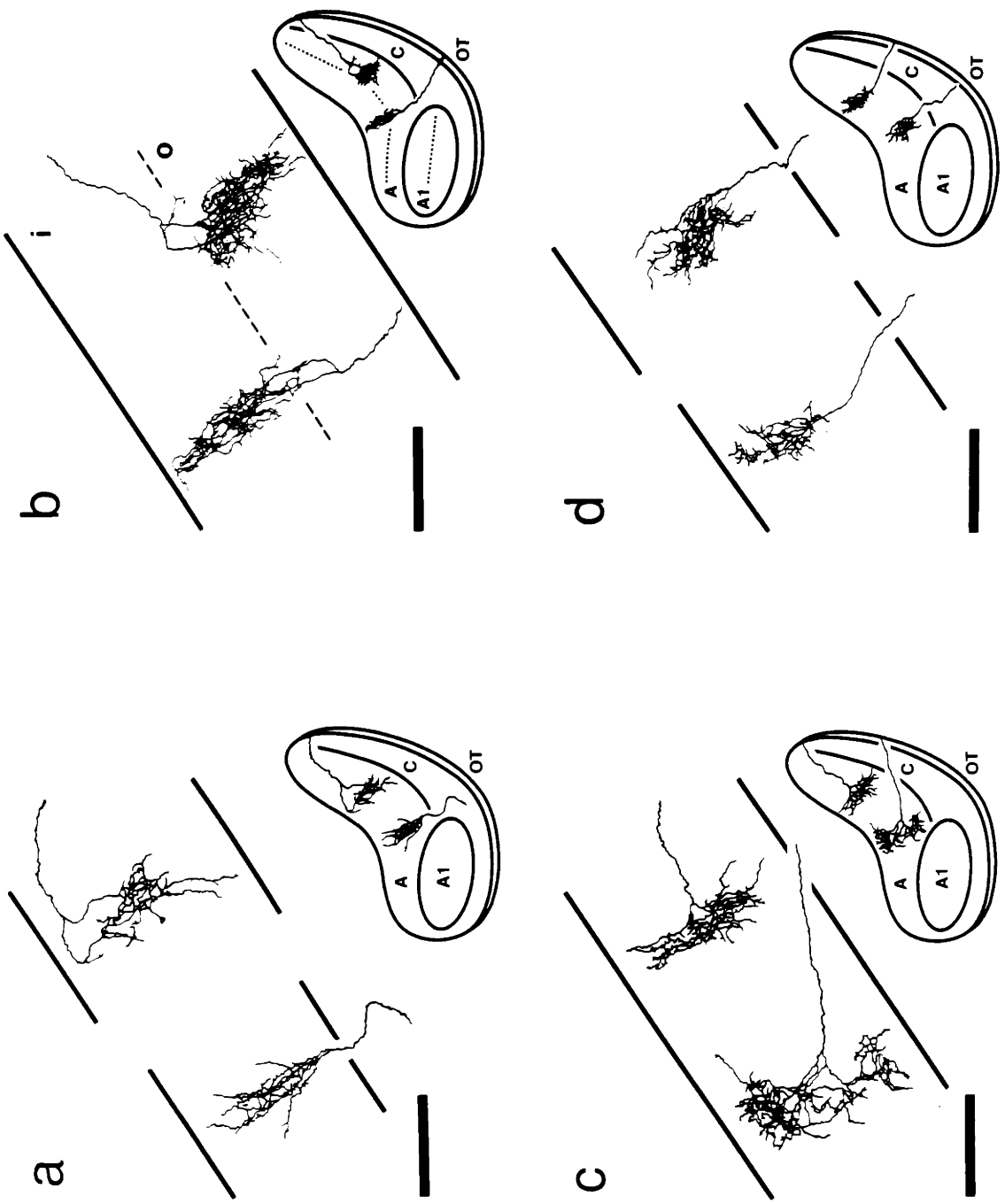
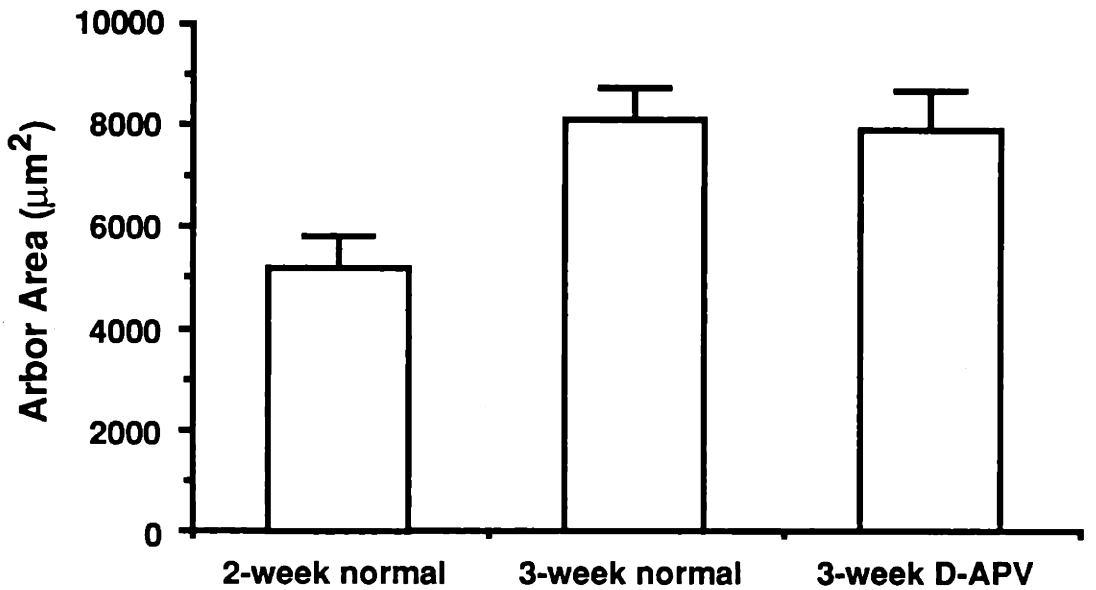
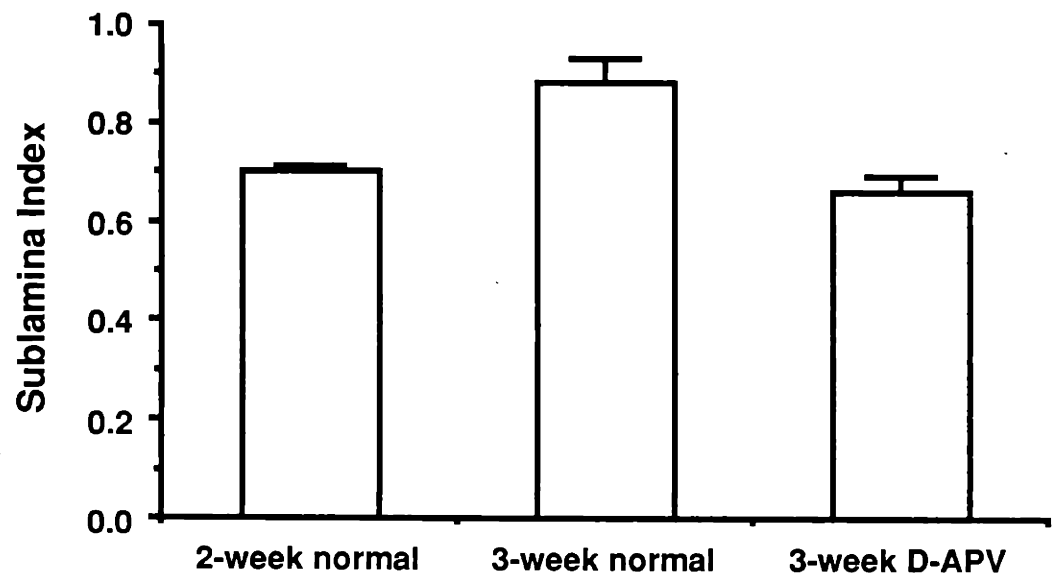


Figure 4


Arbor Area (μm²)
10000
8000
6000
4000
2000
0
2-week normal
3-week normal
3-week D-APV


Sublamina Index
1.0
0.8
0.6
0.4
0.2
0.0
2-week normal
3-week normal
3-week D-APV

CHAPTER 4

EFFECT OF LGN SIZE ON THE ARBOR SIZE OF SINGLE RETINOGENICULATE X AXONS IN FERRETS

ABSTRACT

In this study we have examined the effect of reducing size of the target on the size of the afferent arbor. At birth ferret kits received ablations of primary visual cortex, causing the lateral geniculate nucleus (LGN) to severely degenerate. At adulthood, the morphology of X retinogeniculate axon arbors was examined by intracellular recording and labeling with horseradish peroxidase (HRP). We found that X retinogeniculate axons scale the size of their arbors to the size of the LGN; a given reduction in the size of the LGN (compared with normal) resulted in a similar magnitude of decrease in the size of X axon arbors. The axons were all appropriately located in the LGN with respect to their receptive fields in visual space. These results indicate that X retinogeniculate axons are significantly influenced by the target during development of the size of their terminal arbors.

INTRODUCTION

A central issue in the study of nervous system development is to delineate the role that afferent-target interactions play in the formation of adult structures. Of particular interest is to specify how the state of one structure, for example the target, affects the developing morphology of the other, in this case the afferent. While this question has been extensively studied in many afferent-target circuits in the peripheral nervous system (for review see Landmesser, 1980; Purves et al., 1988), in the central nervous system the majority of studies have been focused on the visual system. In the vertebrate visual system, the effect of target alteration on the development of afferents has been studied by removing portions of the target during development or regeneration of the retinotectal projection (Udin & Fawcett, 1988, for review). Removal of half the tectum causes the entire retinal projection to compress onto the remaining target in an orderly, topographic fashion (Gaze & Sharma, 1970; Yoon, 1971; Udin, 1977; Schneider & Jhaveri, 1974; Finlay et al., 1979). The mechanism by which this is achieved has not been determined although some theories have been proposed (Pallas & Finlay, 1991).

The present study was undertaken with the specific aim of determining how changes in target size influence the size of an afferent arbor. We addressed this issue by examining the terminal arbor morphology of single X retinal ganglion cell axons that project to the lateral geniculate nucleus (LGN) in the ferret's visual pathway. The retinofugal projection of this particular cell class has several characteristics which make it a good model to study this issue. First, the size of the target, the LGN, can be changed by ablating the visual cortex early in development. The visual cortex is the main target of LGN efferent projections, and cortical ablation causes the LGN to degenerate significantly (Murphy & Kalil, 1979; Sur et al., 1987b; see Kalil,

1984, for review). Second, the morphology of single retinogeniculate axons can be studied via intracellular recording and labeling, such that changes in afferents due to target changes can be determined at the single axon level. Third, much is already known about the morphology of normal ferret X retinogeniculate axons, so there is a substantial body of control data with which to compare the axons projecting to the experimentally altered LGN. Most importantly, in contrast to Y axons, X axon arbors have been shown to have a very stereotyped arbor morphology with relatively little variation in arbor size or projection pattern, thus making any differences obviously recognizable (Bowling & Michael, 1980, 1984; Sur et al., 1987a; Roe et al., 1989).

It has been shown that ablation of areas 17, 18 and 19 at birth in cats causes massive degeneration of the LGN, and in particular retrograde extensive loss of X retinal ganglion cells (Murphy & Kalil, 1979; Kalil, 1984; Pearson et al., 1981; Payne et al., 1984; Tong et al., 1982; Tumosa et al., 1989). This is not unexpected because 90% of X retinal ganglion cells project solely to the LGN (Fukuda & Stone, 1974; Illing & Wassle, 1981). Furthermore, neonatal ablation of visual cortex causes more severe loss of X retinal ganglion cells than ablation performed at adulthood (Tong et al., 1982), suggesting that sustaining projections of X cells to targets other than the LGN (such as the pretectum, Leventhal et al., 1985) perhaps develop postnatally. In ferrets, large ablations of visual cortex (that include areas 17, 18 and parts of 19) at birth cause a loss of X cells in the LGN (see Sur et al., 1987b). However, cortical ablations confined to portions of area 17 alone (a procedure not described so far in cats) simply causes a shrinkage of the LGN, without noticeable loss of X cells in the LGN or medium-sized cells in the retina (Sur, Garraghty & Roe, unpublished results; A.W. Roe, Ph.D. thesis, 1991; see also below).

To examine how retinogeniculate axons are affected by alterations in target size, we examined by intracellular recording and labeling the morphology of X axon arbors in adult ferrets that had received lesions of area 17 at birth. We find, somewhat surprisingly, that the size of X retinogeniculate axon arbors is regulated substantially by the size of the target. These results have been published previously in abstract form (Hahm et al., 1989).

MATERIALS AND METHODS

Four adult ferrets which received unilateral ablations of visual cortex on the day of birth were used in this study. Animals were obtained from timed pregnant ferret jills either purchased from Marshall Farms (North Rose, NY) or bred in our colony. They were maintained on a 14:10 light cycle and fed cat food and water ad lib.

Surgery

All neonatal surgical procedures were conducted under sterile conditions and have been detailed elsewhere (Pallas et al., 1990). On the day of birth (E42/P0) kits were removed from the mother and anesthetized with hypothermia. A longitudinal incision was made in the skin overlying the skull. A triangular flap of skull was removed to expose the left visual cortex (areas 17 and 18), and the posterior pole of cortex was ablated with heat cauterization. The skin was sutured and the incision coated with topical anesthetic and antibiotic (Triple Antibiotic). The animal was given a single injection of antibiotic (amoxicillin, 5 mg/kg), allowed to recover under a heat lamp, then returned to the mother.

Physiology

At adulthood, animals were prepared for in vivo intracellular recording. The methods used for recording have been detailed elsewhere (Roe et al., 1989) and will be described here only briefly. Each animal was anesthetized with ketamine (40 mg/kg) and xylazine (3 mg/kg); atropine (0.04 mg/kg) was also administered to prevent congestion. Following anesthesia, the femoral vein was cannulated, and a tracheotomy performed to insert an endotracheal tube. The animal was placed in a

stereotaxic apparatus, paralysis was induced with an injection of gallamine triethiodiode (5 mg), and the animal artificially respired. Anesthesia for subsequent surgical procedures was maintained with halothane (1%) and a 70:30 mixture of N2O/O2. Paralysis was maintained by intravenous infusion of gallamine triethiodide (3.6 mg/kg/hr) and ketamine (10 mg/kg/hr) in 5% dextrose lactated Ringers solution. End-tidal CO2 was maintained at 4.0%, and the body temperature at 38° C; the heart rate was continuously monitored. Atropine sulphate and neosporin were administered to the eyes to dilate the pupils and prevent infection, respectively. Eyes were fitted with zero power contact lenses to focus them on a tangent screen 114 cm in front of the animal, and optic disks were plotted by reflection onto the screen.

The skin overlying the skull was incised and the muscles resected. A craniotomy and durectomy was made over the area of the LGN (centered A -1.0, L 6.0), and a cylindrical plexiglas recording chamber cemented in place. Two holes were drilled in the skull over the optic chiasm (A 5.5, L 1.5) and stimulating electrodes (a pair of insulated tungsten wires), were lowered into the holes to straddle the optic chiasm. The stimulating electrodes were cemented in place when they reached a depth that yielded a potential of at least 2-4 mV in response to a flashing strobe light.

A recording pass was made with a metal microelectrode (tungsten, parylene coated, 3-5MΩ) to locate the LGN. Subsequent recording passes were made with glass micropipettes filled with horseradish peroxidase solution (10% HRP in 0.05 M KCl-Tris), initially pulled to 160-200 MΩ on a Kopf puller and then bevelled to 98-100 MΩ. The recording glass microelectrode was initially lowered 5 mm into the brain, after which the chamber was filled with warm 3% agar and then sealed with dental wax.

Units encountered in the LGN were identified as retinogeniculate axons or geniculate cells based on the following criteria (Bishop et al., 1962; Sur et al., 1987a): 1) Axons generally have shorter latencies to optic chiasm stimulation; 2) axons have monophasic action potentials; 3) axons do not exhibit epsp's; 4) axons can follow optic chiasm stimulation of more than 100 Hz without failure or variability. Once a retinogeniculate axon was identified, its chief physiological characteristics were determined, including eye of origin, size and location of the receptive field, On/Off response to light, the extent of center and surround. A battery of physiological tests were used to determine cell class (Enroth-Cugell & Robson, 1966; Hochstein & Shapley, 1976; Roe et al., 1989), including the latency to optic chiasm stimulation, the receptive field size, response to fast-moving, center-inhibiting stimuli, and linearity of response to a counterphased grating stimulus. These criteria were used to assign the axon as X or Y. W retinogeniculate axons were encountered infrequently by our electrodes. After classification, we attempted to impale the axon with a series of small depolarizing current pulses or by advancing the electrode. Successful impalement was indicated by a 15-40 mV DC drop. Once in the axon, HRP was iontophoresed for 20-60 seconds by passing a 20 nA depolarizing current.

Following a survival time of at least 6 hours after the last injection, the animal was overdosed with halothane and sodium pentobarbital and then transcardially perfused with saline followed by aldehyde fixatives (1% paraformaldehyde, 2% glutaraldehyde) and 10% and 20% sucrose phosphate buffer. The brain was removed, blocked, and left in 30% sucrose phosphate buffer overnight.

The LGN was cut at 100 μm in the sagittal plane on a freezing microtome, placed in 0.1 M phosphate buffer and processed for HRP histochemistry using cobalt intensification of diaminobenzidine (DAB) (Adams, 1981). The sections were

mounted onto chrome-alum subbed slides, dehydrated through a series of alcohols, cleared with xylene and coverslipped with Permount.

Recovered axons were reconstructed using a 50X (Olympus) or 63X (Leitz) oil immersion objective and a drawing tube. The LGN visual field representation maps of Zahs and Stryker (1985) were used to match the recovered axons with recorded units according to the position of the receptive fields. Terminal arbor volume was determined by measuring the arbor area in single sections with the aid of a computer-assisted digitizing pad and multiplying that area by the section thickness (100 um). The number of terminal boutons was also measured for each axon. LGN volume was estimated by making a camera lucida drawing of the outline of every fifth section of the nucleus, measuring the area on the digitizing pad, multiplying that area by 500 um and summing those values. All statistical comparisons presented here are based on the Mann-Whitney U test.

RESULTS

We encountered a total of 20 axons, 12 X, and 8 Y in four animals that had received ablations of area 17 at birth. Five retinogeniculate axons were recovered from three animals. Data on recovered axons are presented in Table 1. The axons exhibited linear spatial and temporal summation, and in particular had a "null point" in response to a grating stimulus. Latency to optic chiasm stimulation ranged from 0.7-1.0 ms. Based on these characteristics, all axons were considered to be from X retinal ganglion cells (Enroth-Cugell & Robson, 1966; Hochstein & Shapley, 1976; So & Shapley, 1979; Roe et al., 1989). Receptive field locations of recorded axons are shown in Figure 1.

Qualitative Analyses of Axon Morphology

Reconstructions of recovered axons are shown in Figures 2-6, along with the location of each axon's terminal arbor in the LGN. In each figure, the axon is shown in its entirety from the point of injection.

Axon Termination Sites

All axons in lesioned animals were located in the A or A1 laminae, or in one case in which the LGN was severely shrunken and interlaminar zones not evident, in the innermost portion of the nucleus. It was not possible to determine in any of the three cases whether the terminal arbors of these axons was contained within sublaminar boundaries as sublaminar borders and intralaminar zones were not visible in the shrunken nuclei. However, interlaminar zones were evident in two of three cases, and it was possible to localize each axon to within an inner or outer half of each eye-specific lamina.

Figure 2 shows a reconstruction of an Off-center axon from the contralateral eye. Note that although the LGN is drastically shrunken (51% of normal), eye-specific laminae and interlaminar zones are still intact. The axon's terminal arbor is localized to the outer half of lamina A. Figure 3 illustrates an On-center axon from the ipsilateral eye in a case in which the LGN was 54% of normal size. This was the only ipsilateral axon recovered; it is located in the inner half of lamina A1, and it has the smallest terminal arbor of all axons in our sample. The next three figures present axons from a single case. All axons were driven by stimulation of the contralateral eye. In this animal the cortical ablation was more extensive, and the LGN is shrunken to 23% of normal size. Interlaminar zones were not visible in this case, although the axons were appropriately located in the LGN for their receptive field location in the visual field according to the visual field projection maps of Zahs and Stryker (1985). Figure 4 shows an Off-center axon, which is located within the medial third of the nucleus, where lamina A would normally be. However, it is not located right next to the LGN's medial border but positioned a short distance away. This indicates that it is localized to the outer portion of the presumptive A lamina, which would be appropriate for an Off-center axon. The axon shown in Figure 5 is an On-center axon which is located in the innermost portion of the LGN, close to the inner endge of the LGN; thus it is localized to the inner portion of the presumptive A layer. The axon depicted in Figure 6 is an On axon and is located very close to the LGN border.

Terminal Arbor Morphology:

All axons recovered with one exception have terminal arbor morphology typical of X axons. Each arbor arises from one primary axon which enters the LGN from the optic tract and, with little variability, projects straight to the target zone

where it branches to form a terminal arbor. Similar to X axons in normal animals (Roe et al., 1989), there are no collaterals either within the LGN or in the optic tract that lead to arbors elsewhere. The terminal arbor is a focal, thickly branched plexus that is densely studded with terminal boutons. One axon, illustrated in Figure 6, was sparsely branched with few terminal boutons, and larger in volume than the other arbors.

Quantitative Analyses

The volume of each axon's terminal arbor and number of boutons are listed in Table 1, along with mean values from normal axons from an earlier study from our laboratory (Roe et al., 1989). Figure 7a shows arbor volume plotted against bouton number. Axons from cortically ablated animals are significantly reduced in terminal arbor volume compared with normal, the mean arbor size is 43% of normal (range 29% to 58%, $p = .001$). Mean number of terminal boutons is also reduced to 45% of normal (range 25% to 69%, $p < .005$). Whereas normal axons have a fairly linear relationship between arbor volume and bouton number in that larger arbors tend to have more boutons ($R=.59$), axons of ablated animals do not follow this trend ($R=.03$). In Figure 7b arbor volume is plotted against eccentricity. There are no significant correlations of arbor size to eccentricity in either normal animals or ablate animals.

As mentioned above, the LGN shrinks dramatically after visual cortex ablation, ranging from 54% to 23% (mean 43%) of normal size (Figure 8a). The amount of shrinkage appears to correlate roughly with the extent of cortex ablated.

When terminal arbor volume is measured as a function of LGN volume, X axons of ablated animals occupy a slightly greater proportion of LGN space than normal axons; this difference, however, is not statistically signficant ($p = .33$). In

Figure 8b, the arbor volume as a ratio of LGN volume is plotted against bouton number. The mean ratio of normal X arbor volume to LGN volume is 6.0×10^{-4} (range 3.3 to 11.7). The mean ratio of arbor volume to LGN volume in cortically ablated cases, on the other hand, is 9.0×10^{-4} (range 3.1 to 14.7), which is 150% of normal. Thus, the relative size of X axon arbors seem to be scaled to match the size of the LGN in animals with neonatal ablation of area 17.

DISCUSSION

In this study we have presented evidence that in the ferret retinogeniculate pathway, X retinal ganglion cells survive and project to the LGN after lesions of visual cortex (area 17) performed at birth. Our results further indicate that while target reduction does not affect the general morphological structure and response properties of X retinogeniculate axons, it does significantly affect the size of axon terminal arbors. Retinogeniculate axons apparently scale the size of their arbors to the size of the LGN. Thus, a given decrease in the size of the LGN following neonatal cortical ablation results in an equivalent decrease in the size of afferent axon arbors. This decrease is reflected both in the terminal arbor volume and number of boutons, so that density of boutons and ratio of arbor volume to LGN volume is essentially normal. The resulting axon arbors are simply smaller versions of normal axon arbors, with no distinguishing differences. These data indicate that arbor reduction is significantly influenced by the size of the target.

At least partial regulation of arbor size by target size has also been demonstrated in the cat retinogeniculate pathway. In the LGN of cats monocularly enucleated at birth, the cell layers corresponding to the remaining eye increase in size. The retinogeniculate axon arbors which project to these layers are also increased in size (Garraghty et al., 1986b); this was found for both X and Y retinogeniculate axons. However, X and Y axons are differentially affected by changes in their target, a point which will be discussed in more detail below.

The notion that target size regulates afferent size is supported by studies in the hamster retinotectal projection. In this system, partial lesions of the target, the superior colliculus, cause the entire retinofugal projection to compress into the spared portions of the target (Schneider & Jhaveri, 1974), with preservation of the normal

visuotopic map (Finlay et al., 1979). This compression is not accompanied by an increased loss of retinal ganglion cells (Wikler et al., 1986; but see Udin & Schneider, 1981), and the receptive field sizes of the remaining cells in the superior colliculus are normal (Pallas & Finlay, 1989). These findings led Pallas and Finlay (1989, 1991) to propose that such afferent/target mismatches are compensated by one of two mechanisms, either a loss of collaterals or a reduction in the size of retinal ganglion cell axon arbors innervating the collicular cells. Our findings support the latter view, and there is preliminary evidence to indicate that this is also the case in the hamster retinotectal projection (Xiong et al., Soc. Neurosci. Abst., in press).

As previously mentioned, it is apparent that X and Y retinogeniculate axons are affected quite differently by changes in target size. In our study, X axons are reduced in both arbor volume and bouton number in response to target reduction. In contrast, Y axons in the cat retinogeniculate projection retain normal sized arbors after large ablations of visual cortex, and hence show a massive increase in arbor size relative to LGN size (Weber et al., 1989). At the same time that the number of boutons is decreased. These results, taken together with the findings of other studies (see Garraghty & Sur, 1988 for review), suggest that X and Y retinogeniculate axons have different developmental programs, resulting in differential responses to changes in extrinsic conditions.

In previous studies, X axons have been shown to be relatively unchanged from normal in response to changes in external factors. The characteristics of X axons, such as arbor size and laminar specificity, are relatively preserved in various paradigms (such as early monocular enucleation) in which extrinsic conditions such as binocular interactions have been altered (Garraghty et al., 1986b, Garraghty et al., 1988). Even the changes in arbor size that occur along with changes in target (lamina) size do not result in axons larger than those normally seen in development

(Garraghty et al., 1986a; Sur et al., 1984). Y axons, on the other hand, are significantly affected by changes in extrinsic conditions, exhibiting great alterations in arbor morphology and translaminar sprouting (Garraghty et al., 1986a, b, 1988; Weber et al., 1989). However, the data from the present study suggest that under certain conditions, X axons are capable of changing arbor size. Further, preliminary evidence indicates that they also have the capacity to alter arbor location. In cats, very early monocular enucleation (at embryonic day 36) results in X retinogeniculate axons which are inappropriately located with respect to eye-specific laminae (Roe et al., 1989). These data suggest that X axons as well as Y axons are sensitive to the influences of extrinsic factors, but that due to different developmental programs, the set of extrinsic factors and the time of effectiveness may be different for the two classes of axons.

One likely candidate for extrinsic determinant of arbor location is the target cell activity. In the previous chapter it was shown that blocking the receptors for synaptic transmission on the postsynaptic cell with specific antagonists caused developing retinogeniculate axons to localize their arbors inappropriately within eye-specific laminae. The size of arbors, however, was not affected. Taken together with the results of this study, this indicates that afferents must interact with the target cells in order to appropriately determine the position and size of their arbors.

Conservation of the X Pathway

The finding that many X retinal ganglion cells survive after early visual cortex lesion is somewhat surprising given previous work in cats, in which the X pathway is selectively and drastically reduced following large neonatal lesions of visual cortex (areas 17, 18 and 19). Studies have shown that there is a reduction in the number of X LGN cells (Tumosa et al., 1989), following large cortical lesions in

cats and retrograde loss of X retinal ganglion cells (Payne et al., 1984; Pearson et al., 1981; Tong et al., 1982). The Y pathway, however, remains largely intact, as evidenced by the survival of large neurons in the LGN, particularly in the C layers, and physiological identification of Y geniculate cells and retinogeniculate axons (Murphy & Kalil, 1979; Tumosa et al., 1989; Weber et al., 1989). The selective loss of the X pathway has been attributed to the lack of a sustaining collateral by X retinogeniculate axons to other subcortical structures (Tong et al., 1982; Weber et al., 1989).

In ferrets, on the other hand, smaller lesions of visual cortex (confined to area 17) preserves the retinogeniculate X pathway. Two possible explanations may account for the difference in our findings compared with those in the cat. One is age at time of lesion. The ferret, whose visual system closely matches that of the cat in organization and developmental sequence, is born three weeks earlier in development than the cat; thus at birth (E42) the entire visual pathway is in a much more immature state. Retinal afferents have not yet segregated into eye-specific laminae (Linden et al., 1981; Hahm & Sur, 1988; Shatz, 1983) and layer IV cells of the cortex are just migrating into place (Jackson et al., 1989). Further, fibers from the LGN are not likely to have invaded the cortex, as in cats it has been shown that at this age geniculocortical afferents are waiting in the subplate right below the cortical plate (Shatz & Luskin, 1986). It may be argued, then, that LGN X cells may not be specified for their targets at this early stage and may project to other targets. However, Sur et al. (1987b) have shown that this is not the case, for large lesions of visual cortex (including areas 17, 18 and parts of 19) in ferrets do cause selective loss of the X pathway.

A second possibility is that the size of the cortical lesion affects X cell survival. In the very immature state in which X geniculate cells are in at birth, axons

may be able to project to a target other than area 17. If so, a smaller lesion of cortex including area 17 alone would leave more cortex available to serve as a target for LGN X cells, and thus permit a substantial portion of the X cells to survive. Presumably, the X cells survive by projecting to remaining visual cortex including area 18. When the cortical ablation includes all of areas 17 and 18, the X pathway does not survive (Sur et al., 1987b), suggesting a limited plasticity in the geniculocortical X cell projection: some or all of area 18 must be spared for the X pathway to remain. Given our findings, previous data obtained in cats may be interpreted as a being a result of the size of the cortical lesion. What is not clear in this interpretation is whether the X pathway survives because the immature geniculate cells are able to sprout projections to a new target, or because X geniculate cells initially have a transient projection to area 18 which is sustained after area 17 ablation. Presently, it is not known whether X cells project to area 18 early in development. These issues need to be addressed with double labeling studies and with smaller lesions of visual cortex.

How Does Target Size Regulate Afferent Arbor Size?

While we have shown that reducing the size of the LGN causes a concomitant reduction in the sizes of retinogeniculate X arbors, the actual mechanism by which target size might regulate the size of afferent arbors remains open. A tentative hypothesis may be as follows. Reducing the size of the LGN might not be accompanied by a proportional reduction in the number of LGN cells, and thus lead to a reduction in LGN cell dendritic area compared to normal. Retinogeniculate axon arbors appear to be matched rather closely to LGN dendritic arbors (Bowling & Michael, 1984; Sur et al., 1987a; Friedlander et al., 1981), and a reduction in LGN

dendritic arbors would lead to a concomitant reduction in retinogeniculate axon arbors.

Several features of this hypothesis seem to be borne out in ferrets with large neonatal lesions of visual cortex. Soma sizes of LGN cells are significantly smaller than normal (Sur et al., 1987b) and cell density greater, suggesting that the number of LGN cells is not reduced proportional to LGN size. Receptive fields of X cells in the LGN of these animals are comparable to those of X cells in the normal LGN (Roe, Ph.D. thesis), suggesting that retinogeniculate convergence and divergence remains similar to normal. That is, each retinogeniculate X axon still contacts two or three LGN cells (see Sherman & Spear, 1982; Hamos et al., 1985), implying indirectly a correspondence between retinogeniculate arbor structure and LGN cell dendritic structure. It would be of interest in these animals to see if LGN cell dendritic arbors are scaled with respect to LGN size, which is what our hypothesis would predict.

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FIGURE LEGENDS

Figure 1: a) Receptive field locations within visual field. All normal axon data presented in this and the following figures are derived from Roe et al. (1989). Normal axons (n=18) represented by clear circles, VCX ablate axons (n=5) represented by black circles. b) Plot of receptive field size in relation to eccentricity. Mean receptive field size of VCX ablate arbors (closed triangle on ordinate) is significantly larger than those of normal arbors (open triangle).

Figure 2: Camera lucida reconstruction of a contralaterally projecting Off-center axon which terminates in the outer half of the A lamina. Sublaminar borders are not visible in this case. Scale bars as marked.

Figure 3: Camera lucida reconstruction of an ipsilaterally projecting On-center axon terminating in the inner half of the A1 lamina. Sublaminar borders not visible. Scale bars as marked.

Figure 4: Camera lucida reconstruction of a contralateral Off-center axon. Interlaminar zones and sublaminar borders are not evident in this case. The axon terminated in the medial third of the nucleus, but was displaced away from the medial border of the LGN, thus was located in the outer half of the presumptive A lamina. Scale bars as marked.

Figure 5: Camera lucida reconstruction of a contralateral On-center axon terminating in the medial third of the nucleus close to the LGN border. Scale bars as marked.

Figure 6: Camera lucida reconstruction of a contralateral On-center axon which terminates in the medial third of the nucleus very close to the LGN border. Scale bars as marked.

Figure 7: a) Plot of arbor volume vs. bouton number. Note mean arbor volume for VCX ablate axons (closed triangle on ordinate) is less than half of normal (open triangle). Mean number of boutons of VCX ablate axons (closed triangle) is also reduced to less than half of normal (open triangle). In normal axons there is a linear relationship between increasing arbor volume and bouton (dashed line, R=.59), but not in VCX ablate axons (solid line, R=.03). b) Plot of arbor volume vs. visual field eccentricity. There are no significant correlations in either normal or VCX ablate axons.

Figure 8: a) Mean LGN volume for normal cases (n=7), and LGN volumes of each VCX ablate case. b) Arbor volume expressed as a ratio of LGN volume, plotted relative to bouton number. VCX ablate axon arbors occupy more LGN volume (mean ratio, closed triangle) than normal axons (open triangle).

Table 1: VCX Ablate Cases

Case (n=3)	LGN Volume (mm^3)	X/Y Encounter Rate (%)	Axon Eye/Center (n=5)	Arbor Volume (X 10^4 μm^3)	Bouton Number	Arbor Vol./ LGN Volume (X 10^{-4})	Receptive Field Size (deg)
VCX	2.220	60/40	contra OFF	93.73	313	4.22	3.9
	2.328	50/50	ipsi ON	72.32	114	3.11	4.7
	0.986	80/20	contra OFF	101.96	209	10.35	11.0
			contra ON	123.55	215	12.54	6.1
			contra ON	145.09	161	14.72	5.8
Mean ± S.E.	1.844 ± 0.431			107.33 ± 12.51	202 ± 33	9. 0 ±2.3	6.3 ± 1.2
(% normal)	(43)			(43)	(45)	(150)	(166)
NORMAL * (n=7, n=18 axons)							
Mean ± S.E.	4.312 ± 0.295			251.45 ± 23.73	455 ± 26	6.0 ±0.56	3.8 ± 0.5

*Normal data from Roe et al., 1989.

Figure 1

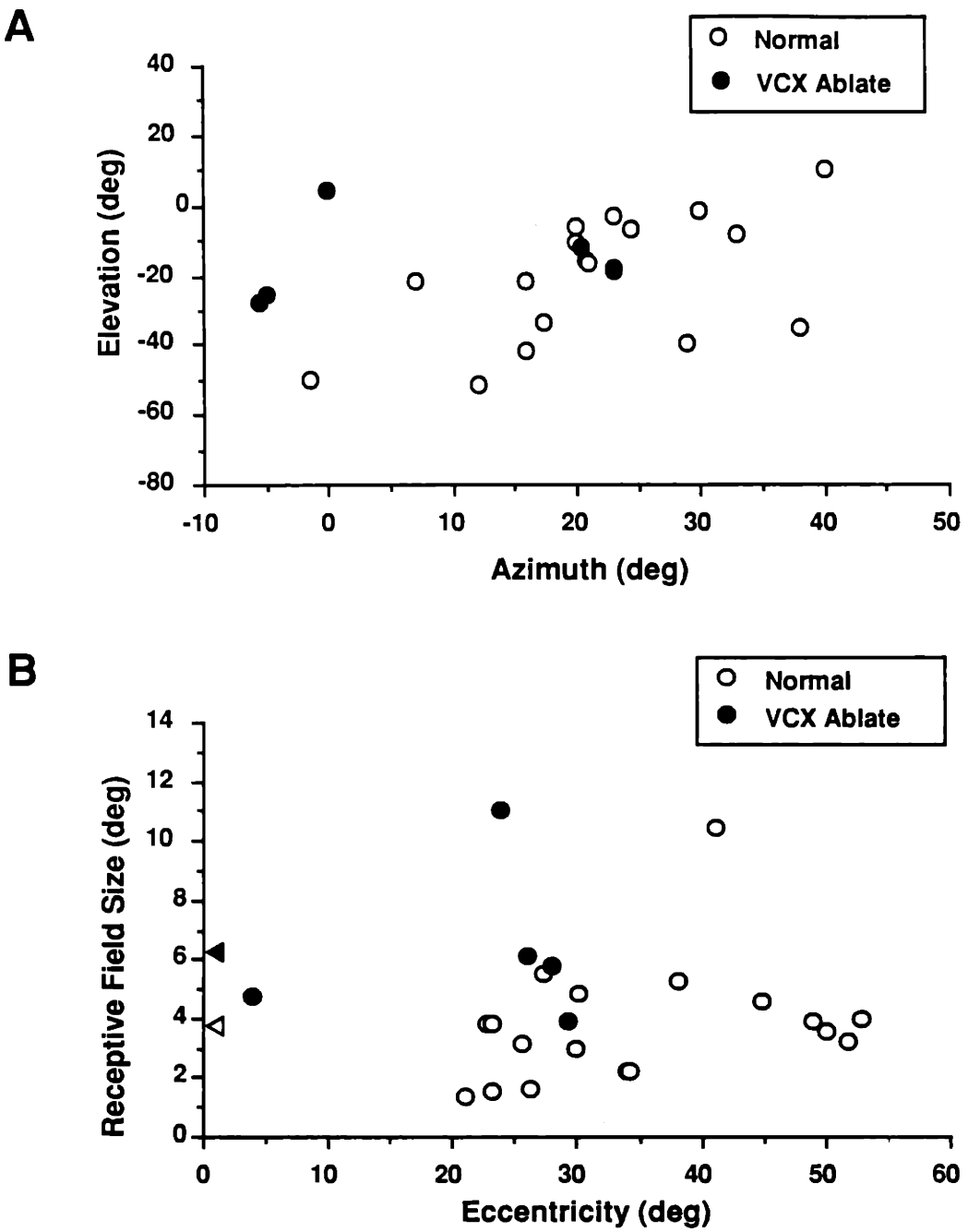

A
Normal
VCX Ablate
Elevation (deg)
40
20
0
-20
-40
-60
-80
-10
0
10
20
30
40
50
Azimuth (deg)
B
Normal
VCX Ablate
Receptive Field Size (deg)
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
Eccentricity (deg)

Figure 2

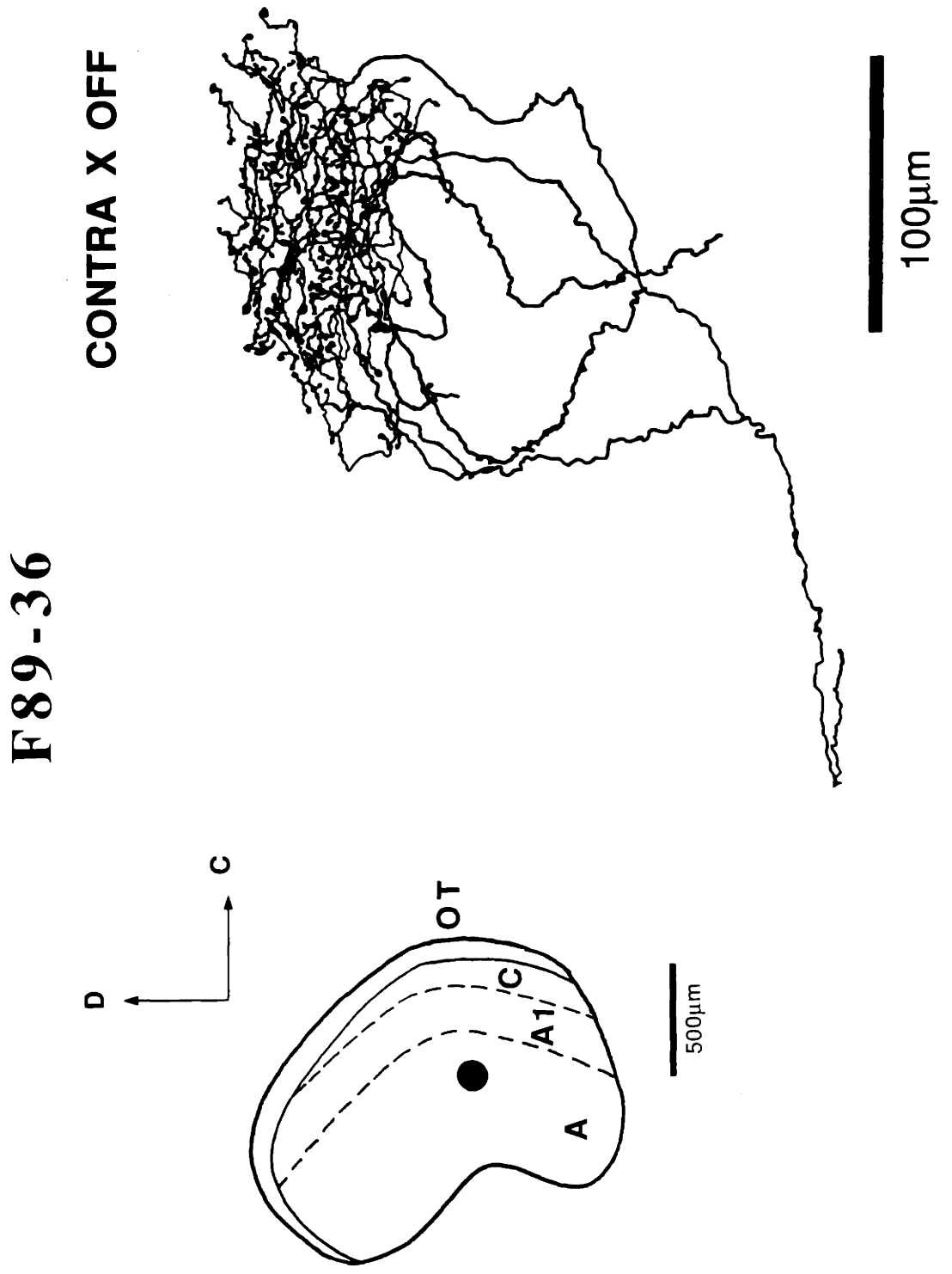
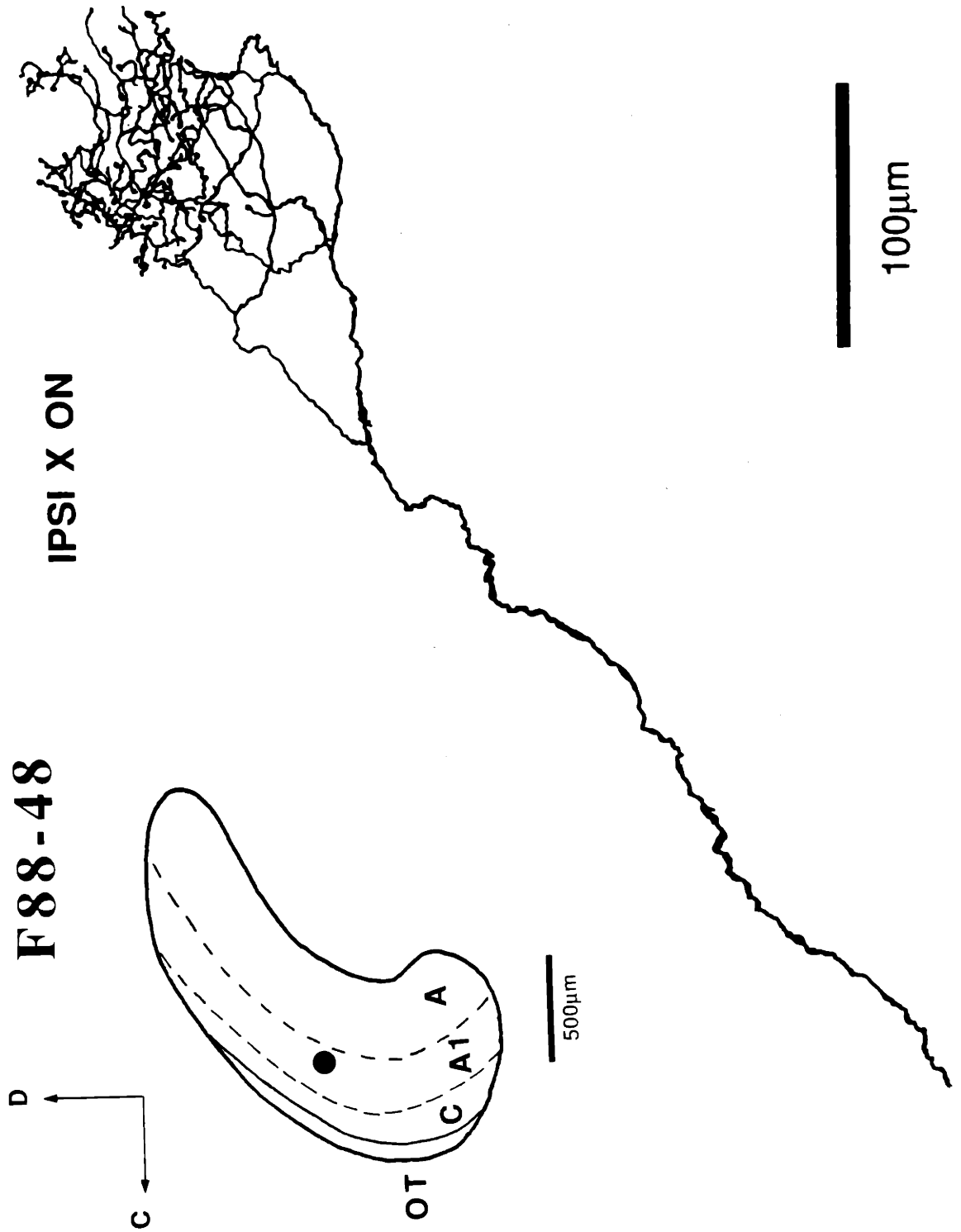


Figure 3



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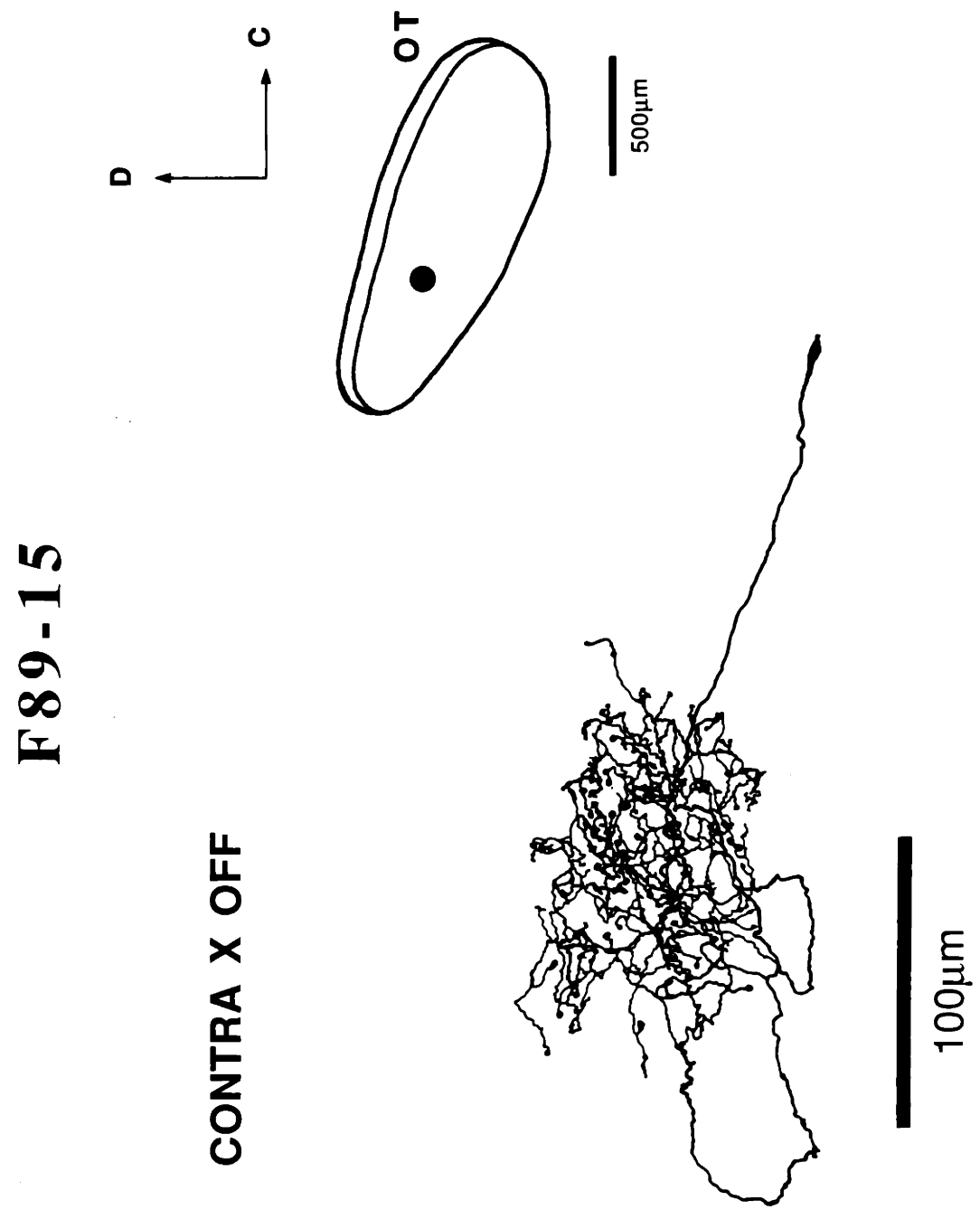
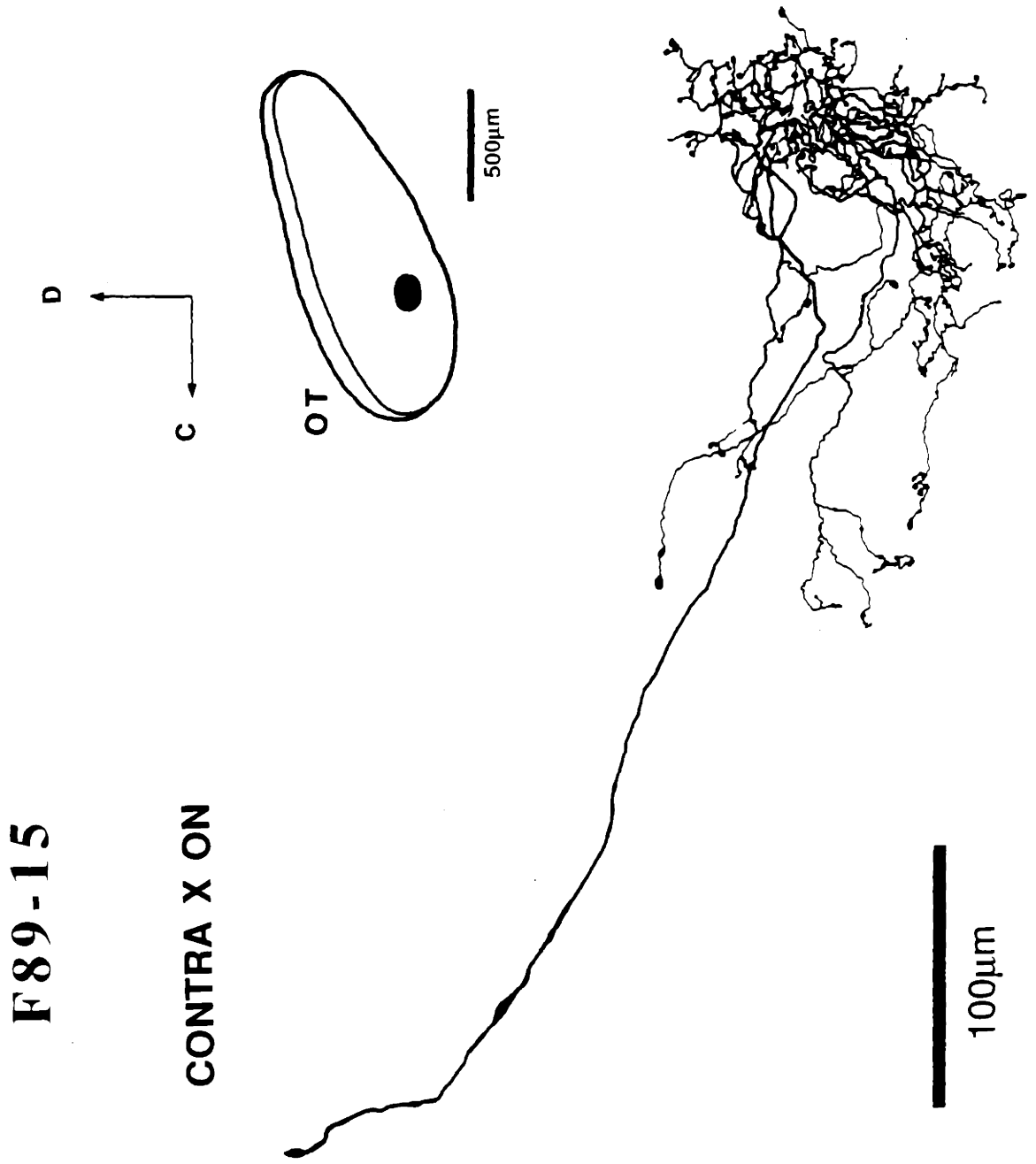


Figure 5



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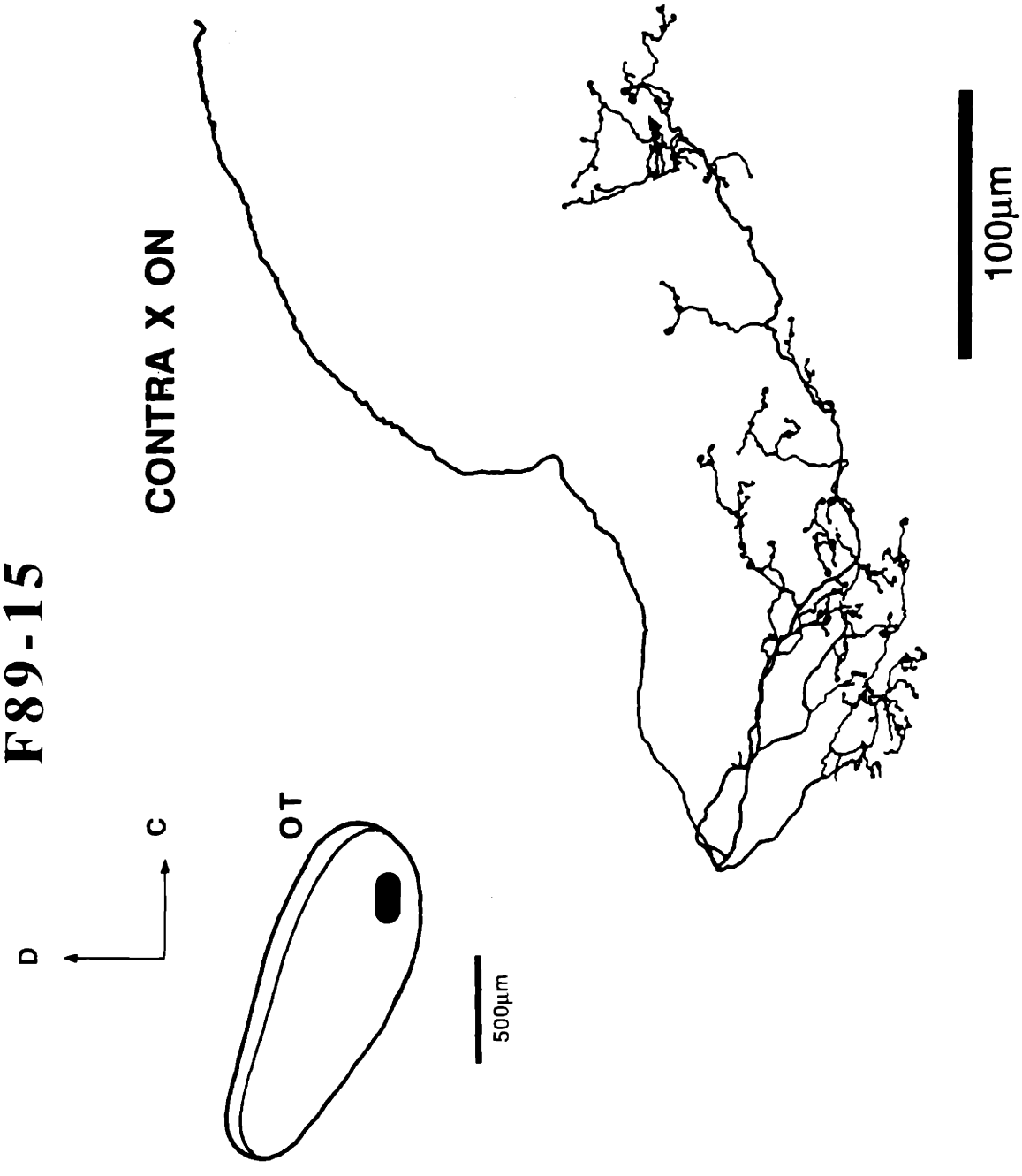


Figure 7

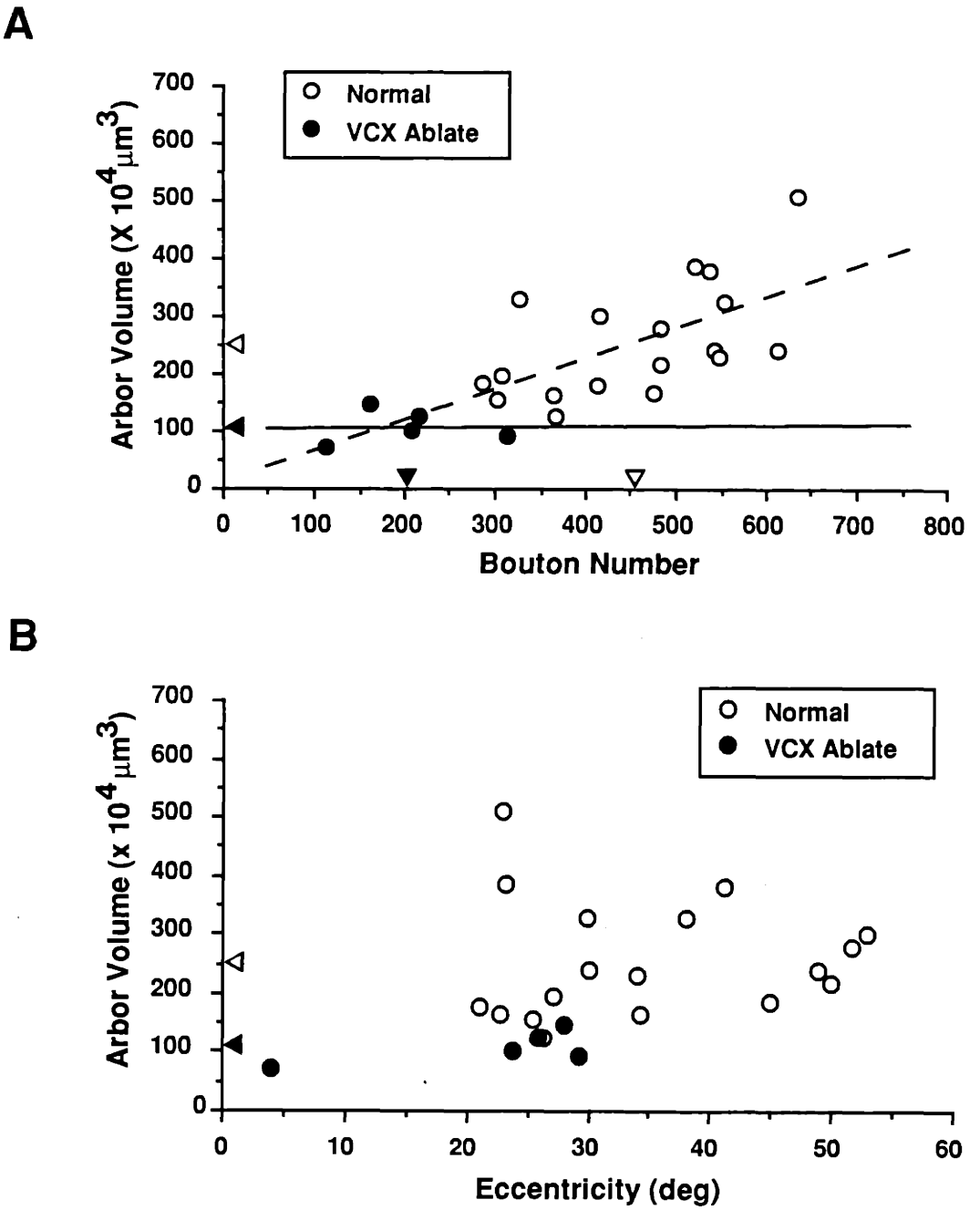
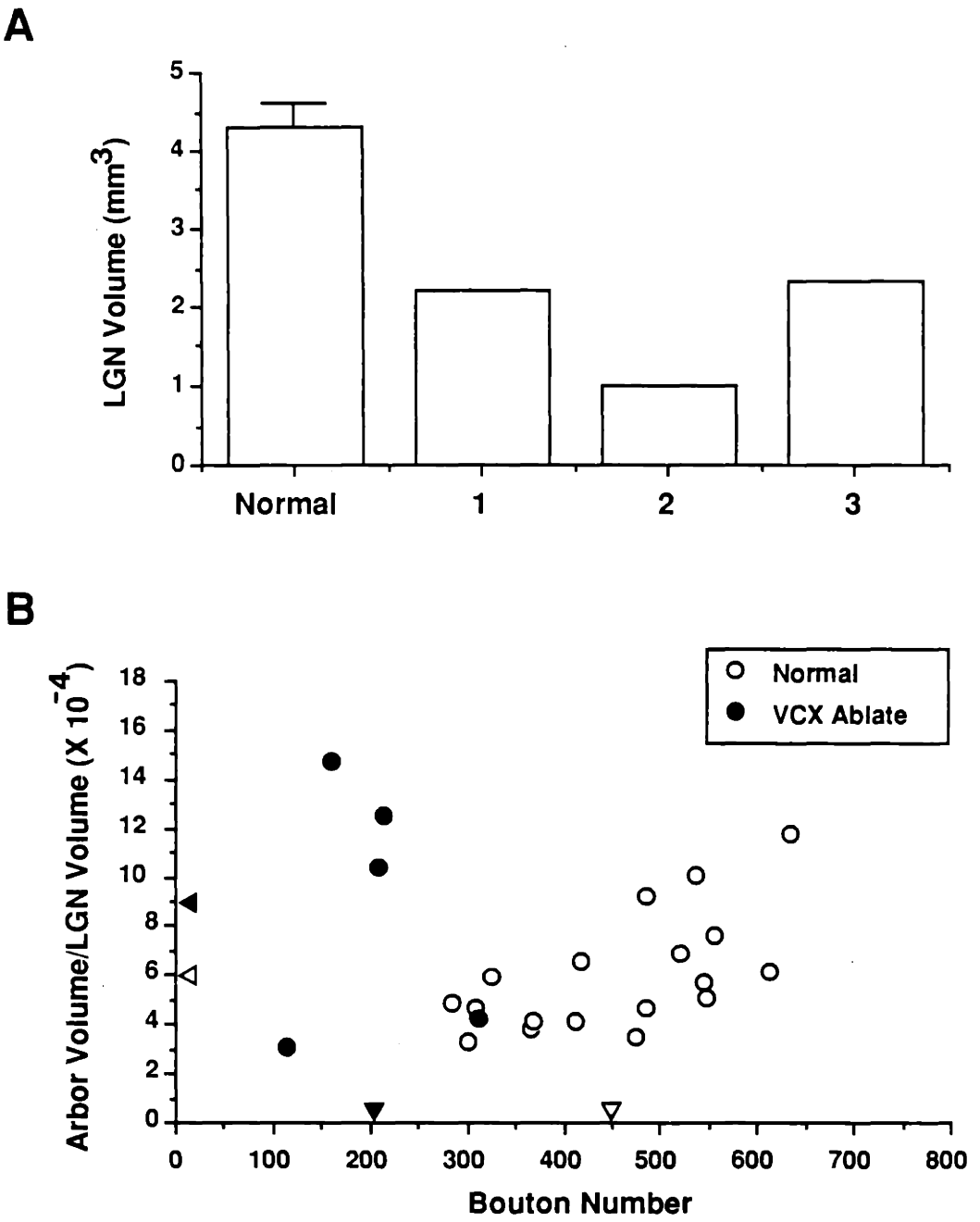

A
Normal
VCX Ablate
Arbor Volume (X 10^4 µm^3)
Bouton Number
0
100
200
300
400
500
600
700
800
B
Normal
VCX Ablate
Arbor Volume (x 10^4 µm^3)
Eccentricity (deg)
0
10
20
30
40
50
60

Figure 8


A
LGN Volume (mm3)
0
1
2
3
4
5
Normal
1
2
3
B
Arbor Volume/LGN Volume (X 10-4)
0
2
4
6
8
10
12
14
16
18
0
100
200
300
400
500
600
700
800
Bouton Number
Normal
VCX Ablate