EYE-OPENING DEPENDENT ELABORATION AND REFINEMENT OF THE CORTICAL PROJECTION TO THE SUPERFICIAL SUPERIOR COLLICULUS IN RATS

by

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ABSTRACT

The superior colliculus (SC) is a multi-layered midbrain structure responsible for multimodal integration and orienting behavior in mammals. The superficial layers of the SC (sSC) receive direct visual input from retinal ganglion cells (RGC) as well as input from pyramidal cells in layer V of the ipsilateral visual cortex (VC). The retinal input is refined well before eye opening (EO) and RGC axons arborize topographically to form an appropriate map of visual space. The projection from VC is still broad and unrefined at the time of EO, however. In both sSC and VC, physiological and biochemical evidence indicate considerable synaptic refinement in response to EO, which occurs naturally at the end of the second postnatal week. These studies use anterograde filling of corticocollicular axons in combination with controlled eyelid opening and reclosing paradigms to compare the corticocollicular projections of age-matched eye-opened and eye-sutured littermates. Reconstructions of individual corticocollicular axons in rat pups and statistically sampled arborization patterns across the colliculus at set times before and after controlled eye-lid opening, show that the onset of pattern vision is critical for the establishment of registration between the cortical and collicular maps of visual space. Moreover, if pattern vision is delayed by prolonging eye-lid closure the cortical projection withdraws to single axon cylinders. A latent plasticity remains, however; the corticocollicular axons can reestablish topologically appropriate arborization if eye opening occurs within at least a week of its normal occurrence.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

The retinal projection to the superior colliculus (SC) has been studied for decades as a model of axon guidance and spatiotopic mapping. This classic system has helped to elucidate the role of chemical cues such as Ephs and ephrins (Drescher, 1997) as well as the requirement for patterned physiological input in the form of retinal waves (Wong, 1999) in the precise target selection and specific axon pruning of projections. Although in rodents much of this development occurs before the end of the second postnatal week when the animal first opens its eyes (McLaughlin et al, 2003; Mrsic-Flogel et al, 2005), recently work in the Constantine-Paton lab has described biochemical and physiological changes that take place in the SC after eye opening (EO). These changes are dependent upon EO, suggesting a requirement for the onset of patterned visual stimuli in this late stage of collicular development (Yoshii et al, 2003; Lu and Constantine-Paton, 2004; Zhao et al 2006). Furthermore, although the retinal projection to the SC is refined before EO a second major input, the descending corticocollicular axon arbors, are still broad and undeveloped at this time (Lopez-Medina et al, 1989). Thus this late-developing cortical input may represent both the source and the substrate of many of these EO-dependent changes. The literature underlying axon terminal refinement in vertebrate is reviewed in the following sections to place the development of the corticocollicular projection, the topic of this thesis, in its appropriate context.

Activity-Dependent Axon Branching and Refinement

In the developing nervous system, growing axons need several different kinds of signals to locate, elaborate and refine within their appropriate targets. Chemical cues guide growth cones along specific paths, repulsive gradients turn them away from incorrect regions of termination and various activity-dependent signals stabilize appropriate connections and destabilize inappropriate ones, allowing for the formation of precise connectivity. These activity-dependent signals are often described as Hebbian (Ruthazer and Cline, 2004; Hebb, 1949); correlated activation of a pre-synaptic axon and a post-synaptic cell increases the effectiveness of the connection between those cells – in this case by promoting and stabilizing the elaboration of the axon terminal.

The mechanisms through which these Hebbian signals function are still being elucidated but many studies indicate that NMDAR activation is required for appropriate patterning (Bear et al, 1990; Cline and Constantine-Paton, 1989; Simon et al, 1992; Schlaggar et al, 1993). In fact, this developmental change appears functionally similar to the well-studied mechanisms of learning and memory, LTP and LTD. For example, studies show that changes induced by monocular deprivation occur rapidly and consist of a phase that is independent of pre-synaptic protein synthesis (Taha and Stryker, 2002).

Early deprivation or alteration of sensory input disrupts the development of appropriate functional activity in several sensory systems (Antonini and Stryker, 1993; Antonini and Stryker,

1996; Shoykhet et al, 2005; Maffei et al 2006; Lee et al, 2007; Sellien et al 2007). However, despite wide ranging evidence of functional plasticity, actual evidence of axonal rearrangement remains surprisingly sparse.

Diverse Mechanisms of Axon Growth, Elaboration and Refinement

Axon development involves a range of diverse processes. Growth cones, in addition to sensing chemical cues in the environment and using these signals to guide axon growth, may also play a role in axon elaboration, creating branch points (Zhou et al 2002) and pausing to demarcate future active regions of growth (O'Leary et al 1988; Szbenyi et al 1998). The growth cone apparatus has a variety of structures that vary not only according to axon type but also according to the substrate in the pathway along which they grow and the particular target tissue within which they elaborate (Bovolenta and Mason 1987; Kalil et al 2000; Skaliora et al 2000; Portera-Cailliau et al, 2005).

Other aspects of axon development also vary broadly. Pruning can occur via axon degeneration or via axon tip withdrawal (Lou and O'Leary 2005; Portera-Cailliau et al 2005). Portera-Cailliau et al used *in vivo,* time-lapse two-photon, imaging to study the development of thalamocortical and Cajal-Retzius neurons and showed that even though these axons shared the same target region of arborization, the manner of growth exhibited depended on the projection's source. For example, thalamocortical (TC) axons exhibit large-scale degeneration in addition to rapid growth while Cajal-Retzius interneuron axons grew more slowly and exhibited only local

branch retraction (Portera-Cailliau, 2005). Data such as this suggest different inherent growth programs at work in different cell types.

Not only do the specific mechanisms of axon development vary, the balance and timing of the different mechanisms may also differ depending on the source and target tissues. In many systems, axon terminals demonstrate a period of rapid generalized growth (frequently exuberance) followed by specific pruning (Lichtman and Purves, 1980; Sur et al 1984). However, this progression is not universal. In some cases growth and pruning occur rapidly and simultaneously (Ruthazer et al 2003; Meyer and Smith 2006). Therefore there may exist several mechanisms for branch refinement: targeted growth; a period of generalized growth followed by specific stabilization and generalized elimination; a period of generalized growth followed by specific elimination (pruning) (Ruthazer and Cline, 2004).

Furthermore, neural activity may play a different role in different growth programs. In organotypic co-cultures of the thalamus and cortex, time-lapse imaging of layer-specific TC branching revealed a reduction in axonal remodeling with pharmacological blockade of action potential activity or reduction of synaptic activity. Interestingly in the TC projection, decreasing or eliminating activity reduced branch addition in the target layer but the extension of existing branches was not affected (Uesaka et al 2007).

Thus a large number of potential mechanisms of axon development have been proposed and many of them could involve neural activity. Moreover, the mechanisms could vary not only by developmental stage, but by source and target tissue as well. This diversity is best illustrated by an overview of the role of activity in axonal rearrangement during development in several of

the most frequently examined pathways including the peripheral nervous system (PNS), the rodent barrel cortex, and several projections within the visual pathway.

Axon Development in the PNS

The neuromuscular junction is probably the most comprehensively studied and best understood synapse, and the first observations of neural activity sculpting pre-synaptic axonal structure were made in this system. At birth, muscle fibers are innervated by multiple motor neuron axons but over the first few postnatal weeks these inputs are eliminated until a one-to-one correspondence between muscle fiber and motor neuron emerges (Sanes and Lichtman, 1999; Keller-Peck et al, 2001; Fox et al 2007). The overall number of innervating cells does not change notably during this period, suggesting that axon refinement, and not cell death, is responsible for this decrease in innervation (Ostberg et al, 1986; Rich and Lichtman, 1989; Werle and Herrera, 1991; Barry and Ribchester, 1995). Furthermore, branches can be observed to detach and withdraw from individual fibers (Riley, 1977; Balice-Gordon et al, 1993; Gan and Lichtman, 1998). There is considerable evidence that the relative "strengths" of the innervating axons are important in determining which input persists and which are eliminated. Colman et al. observed that axon terminals withdrew one to two days following the development of divergence in synaptic strength at different contacts in multiply innervated muscle fibers. (Colman et al, 1997). When activity in the muscle fiber was blocked, input elimination was delayed or even lost; stimulation of the motor neuron inputs, on the other hand, accelerated the process of synapse elimination (Thompson, 1983).

This is probably the clearest and most straightforward demonstration of the importance of neural activity in the development of axon structure, yet even in this relatively simple system complications arise. A whole host of potential signals might account for this activity-dependent synapse elimination. Sanes and Lichtman propose three potential signaling schemes in which postsynaptic target muscle cells mediate axon elimination: "synaptotoxins" that promote withdrawal and are targeted towards less active inputs; "synaptotrophins" that protect active inputs from a more generalized withdrawal process and "synaptomedins" that act upon the post-synaptic apparatus of active inputs to make it more adhesive or otherwise sustaining for inputs (Sanes and Lichtman, 1999). Potential molecules (BDNF and other growth factors and proteases as neurotoxins) have been identified for all of these models at the NMJ and similar mechanisms may be at work in central synapses as well (Fox et al, 2007).

Somatosensory Systems and the Rodent Barrel Cortex:

Another well-studied site of activity-dependent development, not to mention plasticity, is the rodent whisker barrel cortex. In rodents, layer IV of the somatosensory cortex contains discrete, patterned arrays of "barrels", densely packed rings of cortical neurons with dendrites oriented in towards a cluster of thalamic afferents (Inan and Crair, 2007). These barrels are stereotypically arranged in a pattern that echoes the rows of whiskers across the rodent snout, the sensory neurons in the trigeminal nucleus ("barrelettes") and the position of the ventrobasal thalamic cells (VTB; "barreloids"). Thus, whiskers map to barrels in a one-to-one fashion (Inan and Crair, 2007).

Axon guidance molecules such as the limbic-associated membrane protein (LAMP) and cadherins, along with gradients of molecules such as Fgf8 and ephrinA5/EphA4, help direct the thalamic projections to appropriate cortical regions (Mann et al, 1998; Dufour et al, 2003; Lopez-Bendito and Molnar, 2003; Inan and Crair, 2007). The initial projection of VBT axons into the barrel cortex occurs early (E19), before the target neurons even develop (Lopez-Bendito and Molnar, 2003). In fact, the presence of these growth cone-tipped axons at the cortical plate may aid in the formation of appropriate barrel structure as, over the first two postnatal days, the whisker-specific pattern emerges even before the distinct differentiation of layer IV (Erzurumlu and Jhaveri, 1990). Even at pre-natal stages in development, thalamocortical axons can depolarize cells in the sensory plate and this depolarization is necessary for appropriate cortical innervation (Catalano and Shatz, 1998). Finally, between P6 and P12, VBT axonal arbors increase and inappropriate branches are eliminated (Inan and Crair, 2007). This elimination of inappropriate branches appears to require not only neural activity (Jensen and Killackey, 1987) but NMDA receptor (NMDAR) activation as well, for TCAs from single barreloids span areas larger than a single cortical barrel when NMDARs are blocked (Fox et al, 1996). Moreover, this requirement for NMDAR activity appears to be post-synaptic. In cortex-specific NR1 knockout mice TC axon terminals (TCAs) enter the cortical plate and form appropriate barrel patterns early. Cortical layer IV cells do not form barrel structures, however, and notably, single TCA arbors develop extensive and inappropriate arbors between P3 and P7 with appropriate focal terminals inside and exuberant branching outside the barrel region (Lee et al, 2005). This suggests a role for post-synaptic NMDAR activity in the refinement of pre-synaptic arbors in the developing whisker barrel cortex.

In addition to its involvement in barrel map development, neural activity also appears important for map plasticity. In 1973 Van der Loos and Woolsey found that trimming a row of whiskers in adult animals causes a reduction in the size of barrels in that row and an expansion of the barrels in a neighboring row (Van der Loos and Woolsey, 1973). NMDAR activity appears necessary for this form of plasticity, as chronic NMDAR blockade reduces this barrel expansion (Schlaggar et al, 1993). There is some evidence, however, that this competitive and activity-regulated adult whisker-barrel plasticity may be mediated by local inhibitory connections but there is not yet any experimental proof for a role of inhibitory networks in barrel changes in adulthood (Inan and Crair, 2007).

Visual Systems: Retinotectal, Geniculocortical and Intracortical Axon Arborization

Retinotectal Projections:

The retinotectal (in mammals, the retinocollicular) projection is one of the best-studied models of axon development and has been used to elucidate the roles of various axon-guidance and molecular gradient cues, in addition to highlighting the importance of activity in axonal refinement. The time-course of the development of the retinal input to the rodent sSC has been well described. In rats, topographic order emerges early and is essentially mature by P12; cells in the temporal retina project specifically to an appropriate termination zone (TZ) in the rostral SC and cells in the nasal retina form a topographically appropriate TZ in the caudal SC (Simon and O'Leary, 1992). This order develops from initially diffuse projections (E20-21) with regions of density at a topographically predicted TZ. The density of these TZ regions increases by P4; by P6 the TZ density increases and, importantly, the density outside the predicted TZ decreases.

This indicates that RGCs are not only targeted towards topographically appropriate regions, they are also pruned away from inappropriate regions (Simon and O'Leary, 1992).

The development of topographic order in the projection from the retina to the tectum has been an important subject of research for many years. In the 1940s Sperry proposed gradients of "chemoaffinity cues" after observing appropriate re-sorting of retinal fibers following section of the optic nerve in fish and amphibians, regardless of the orientation of the eye or completeness of the retina, thus excluding "re-educative adjustments" (Sperry, 1963). The first identification of topographic guidance molecules did not come until the 1990s, however, when ephrin-A2 (Cheng et al, 1995) and ephrin-A5 (Drescher et al, 1995) were cloned following experiments in the Bonhoeffer laboratory in which temporal and nasal retinal axons plated on a substrate consisting of alternating stripes from anterior and posterior tectum show different patterns of growth (Walter et al, 1987). Subsequent functional and genetic studies have shown that ephrinAs act repulsively through the EphA family of receptor tyrosine kinase receptors and, at least to some extent, control the mapping of the temporal-nasal retinal ganglion axons along the anterior-posterior axis of the SC, along with other subsequently identified chemoaffinity molecular cues (McLaughlin et al, 2003). Similarly, mapping along the M-L axis is partly controlled by complementary tectal and retinal gradients of ephrinB and EphB (McLaughlin et al, 2003; Ruthazer and Cline, 2003).

These biochemical gradients alone, however, do not offer a sufficient explanation for the high fidelity mapping of topography in vivo. Nasal axons in culture do not show a preference for caudal tectal neurons (Walter et al, 1987) but nevertheless their projection to the caudal tectum is

disrupted in ephrin-A knockouts, suggesting a model in which axons compete for available target space (Ruthazer and Cline, 2003). This competition is not prevented by injections of TTX or inhibition of NMDARs, however (Huang and Pallas, 2001; Ruthazer et al, 2003; Ruthazer and Cline 2003), indicating that it probably does not require neural activity.

Neural activity is not necessary for competition for available target space to occur, but this does not exclude its importance in the development of the retinotectal projection, however. While the coarse topography of the retinotectal map is not disrupted by the presence of TTX, point-to-point connectivity is disturbed (Kobayashi et al, 1990). Moreover, studies of mutant mice that lack the β2 subunit of the nicotinic acetylcholine receptor (and thus also lack the early retinal waves that sweep across the retina during the first postnatal week, and the correlated activity patterns that these waves provide) reveal that large-scale remodeling of the retinocollicular projection does occur in rodents and that this remodeling is dependent on the correlated activity of neighboring RGCs provided by retinal waves. (McLaughlin et al, 2003). Some recovery of this topography occurs after the first postnatal week, when glutamatergic waves begin, but no further changes occur after P14 and eye opening (Chandraskaran et al, 2005). Moreover, the degree of resolution of the collicular map of visual space, as visualized through optical imaging of intrinsic signals, is disrupted in the AChβ2-/- mice (Mrsic-Flogel et al, 2005).

These studies indicate that there is a role for neural activity (though not environmentally induced visual activity) in the development of the retinocollicular projection, and earlier studies have implicated glutamate receptors – in particular the NMDAR – in this development. Chronic

TTX treatment prevents the segregation of inputs into eye-specific bands in three-eyed frogs (Reh and Constantine-Paton, 1985). The maintenance of these bands is disrupted during chronic NMDAR blockage (Cline et al, 1987) as is point-to-point order in the normal retinotectal projection with this procedure (Cline and Constantine-Paton, 1990). Furthermore, individual segregating axons, when examined with time lapse imaging, demonstrate a requirement for NMDAR activity for both branch elimination and branch stabilization in Xenopus (Ruthazer et al, 2003).

NMDAR activity is also important for appropriate mammalian retinocollicular development. When rats are treated with NMDAR antagonists during their first two postnatal weeks incorrect retinal axon collaterals are not eliminated normally (Simon et al, 1992). Moreover, in hamsters, chronic early NMDAR blockade enlarges the receptive field size of SC neurons (Huang and Pallas, 2001), suggesting that the predominant role of neural activity at this stage in development is the elimination of inappropriate connections. In addition, in rats chronic NMDAR blockade increases retinal axon synapse density and induces ipsilateral retinal axon sprouting into an scotoma within the superficial visual layers of the superior colliculus (sSC) during the eight days following a small P6 contralateral retinal lesion; chronic NMDA application produces a decrease in retinal ganglion cell sprouting and synapse density (Colonnese et al, 2001; Colonnese et al, 2005). Competition for synaptic space between retinocollicular and corticocollicular axons plays a role in these NMDAR effects. By the third post-natal week, NMDAR antagonists can only induce ipsilateral retinal axon sprouting into the sSC scotoma when the ipsilateral cortex is removed, thereby preventing corticocollicular axon arborization in the same neuropil (Colonnese et al, 2001). In addition to implicating NMDA

receptor activity in sprouting into available space, these studies demonstrate the high level of competition for space to sprout in the sSC.

Geniculocortical Projections and Ocular Dominance Columns:

Perhaps the most well studied form of early brain plasticity is the development of ocular dominance columns (ODCs), the grouping of cortical cells with different eye preferences into discrete clusters within layer IV of the primary visual cortex in binocular regions of the visuocortical map. Hubel and Wiesel initially described these columns in the 1960s using both electrophysiological recordings in the cortex and transneuronal tracers in monkey and the cat, both strongly binocular creatures. ODCs echo the eye-specific layers found within the lateral geniculate nucleus (LGN) of the thalamus. The axons of LGN neurons terminate in layer IV. Early disruptions of visual experience change the shape and distribution of ODCs, but only during a "critical period" of development. Following early monocular deprivation labeled thalamic afferents representing the deprived eye narrow while the open-eye columns widen; physiological recordings tangential to the cortical surface reflect a similar shift in eye-preference (Hubel and Wiesel, 1962; Hubel and Wiesel, 1969; Hubel and Wiesel, 1972; Shatz and Stryker, 1978a,b; Crowley and Katz, 2002). These results suggest that rearrangement of the LGN axons represent the anatomic basis for the shift in ODC physiology. Moreover, in the cat, these rearrangements take place rapidly. Branch withdrawal is observed in deprived-eye afferents after only 6 to 7 days (Antonini and Stryker, 1993) suggesting that at least some of the anatomical changes due to monocular deprivation occur nearly as rapidly as the functional changes. Interestingly, branch withdrawal of deprived-eye afferents appears to occur more

rapidly than expansion of non-deprived-eye afferents, suggesting the rapid functional change may be due to the loss of contact by the deprived eye rather than rapid axon elaboration of the non-deprived eye (Antonini and Stryker, 1996). These structural rearrangements appear to rely on the correlation between cortical and afferent activity – pharmacological inhibition of the visual cortex causes withdrawal of non-deprived eye inputs, rather than expansion, while deprived inputs remain similar to those in normal animals (Hata et al, 1999).

To further examine these potential structural underpinnings of ocular dominance plasticity Antonini et al reverse-sutured kittens so that eyes monocularly deprived for a week (originally deprived eyes: MD) were then opened for 10 days while initially non-deprived eyes were sutured (reverse sutured eyes: RS). MD eyes recover functionally after the RS occurs and RS eyes lose some (though not all) function (Antonini et al. 1998). Detailed examination of LGN arbor morphology revealed that withdrawal of RS arbors was almost as complete as that seen in arbors from deprived-eye LGN. Meanwhile, arbors from the MD eye show only partial and variable re-growth (Antonini et al 1998). These findings suggest that while axon reduction may represent the anatomical substrate of functional loss, recovery of eye-preference can occur through mechanisms other than axon regrowth. That the arbors are so sensitive to visual deprivation during this period of development, however, emphasizes the role of activity, specifically in the reduction of inactive arborization.

Most of these studies of monocular deprivation, ocular dominance plasticity (ODP) and geniculocortical arbor reorganization were carried out in cats, but ODP occurs in rodents as well (Drager, 1978; Gordon and Stryker, 1996). Rodents have very small binocular zones that are

dominated by the contralateral eye; the effects of monocular deprivation reflect this distinction. Deprived eyes lose ipsilateral function but retain the ability to drive most contralateral cells, even though non-deprived ipsilateral responses also increase substantially. (Antonini et al, 1999). Thus the most noticeable effect of monocular deprivation in mice involves the gain of (nondeprived) ipsilateral eye function over (deprived) contralateral eye function. Anatomical tracings of LGN afferents reflect this effect, showing an early expansion of non-deprived-eye arbors (though not unlimited growth) rather than a loss of deprived-eye arbors as seen in higher mammals (Antonini et al,1999).

In the mouse the initial contralateral bias clearly influences the structural plasticity resulting from monocular deprivation, but even in higher mammals input from the two eyes does not necessarily start with an even distribution of non-refined inputs. Instead, the initial establishment of ODCs takes place before the critical period. Crowley and Katz (2000) have claimed this establishment is rapid and precise and not effected by manipulations of retinal input although all temporally correlated activity was not eliminated in their study. Nevertheless, there exists the suggestion that different mechanisms may be responsible for the initial establishment of ODCs and the subsequent ODP observed during the "critical period" (Crowley and Katz, 2002)

Horizontal (Intracortical) Connections:

Intracortical connections, specifically connections between layer II/III cells, have also been well characterized and shown to display a high degree of activity-dependent plasticity (Uesaka et al, 2006; Foeller and Feldman, 2004). Pyramidal cells in layer II/III send long axon branches

across the cortex and these long branches give rise to periodic clusters of fine collateral branches in the adult (Callaway and Katz, 1990). In the cat visual cortex, these periodic collaterals provide connections between columns with similar orientation specificity (Gilbert and Weisel, 1989).

Callaway and Katz used retrograde transport of microspheres to investigate the development of these horizontal connections in the cat visual cortex and found that layer II/III projections remain dispersed and unclustered until the end of the second postnatal week, though refinement continues well into the sixth postnatal week (Callaway and Katz, 1990). Intracellular staining confirms this lack of arbor clustering in young animals. Instead, inappropriate branches are eliminated around the fourth postnatal week; this elimination is followed by extensive elaboration of fine collateral branches off of the remaining appropriate branches (Callaway and Katz, 1990).

In this system, too, activity appears to play an important instructive role. Deprivation of pattern vision via binocular lid suture has no effect on the initial establishment of crude clusters during the second postnatal week (much of it before kittens open their eyes). However, further cluster refinement does not occur in these animals (Callaway and Katz, 1991). Moreover, not just visual activity but *correlated* visual activity is important for appropriate cluster formation. When kittens are raised with an artificially induced strabismus, clusters form only within columns of cells activated by the same eye (Lowel and Singer, 1992). Furthermore, in ferrets re-wired so that their auditory system receives retinal input, horizontal connections in the auditory cortex, which normally arborize according to a map of frequency, instead form patchy,

orientation selective arborizations (Sharma et al, 2000). Functionally, however, the orientation map that is present in these re-wired ferret cortices is not as complete as that in normal visual cortex (Sharma et al, 2000). Thus sensory-driven activity clearly helps to determine the structure of developing cortical circuits, along with intrinsic, area specific signals.

Neural activity plays a role in the development of intracortical connections even before eye opening. TTX blockade prevents even the early, crude cluster formation, suggesting that this phase of refinement requires spontaneous neural activity (Ruthazer and Stryker 1996). Time-lapse imaging of single-labeled axons in organotypic slice cultures also demonstrates the activity requirement and branch dynamics of layer II/III axon arbors. Spontaneous activity emerges in these cultures around 10 DIV and rapid axon branching begins around the same time. Moreover, a reduction in neural activity, either with TTX or AMPA-blockade suppressed axon branching (Uesaka et al, 2005). Interestingly, in these organotypic cultures, NMDAR-blockade does not affect axon branching, although this may result from the lack of normal inputs due to tissue culture. The observations nevertheless emphasize the diversity of mechanisms involved in activity-dependent axon growth (Uesaka et al, 2005; Uesaka et al, 2006).

Other Learning-Induced Changes in Axon Structure:

Most of the experiments described thus far involve changes in axon structure following manipulations of a single sensory system. The range of activity-dependence present in these systems demonstrates a necessary balance of chemical cues, intrinsic programs, spontaneous

activity and sensory input for appropriate structure formation. Many of these manipulations, however, involve sensory deprivation or even a complete activity blockade and this introduces the potential confounding influence of an uneven distribution of activity (DeBello, 2001). Moreover, the topographic representation of sensory information that many of these systems achieve is important because it allows multimodal sensory convergence, yet very few experiments have actually looked at the role of activity in converging sensory maps.

The auditory-localization system of barn owls demonstrates anatomical remodeling of converging maps in response to learning. In barn owls, auditory localization cues such as interaural time differences are represented via a spatial mapping of the projection from the inferior colliculus (ICC) to the external nucleus of the inferior colliculus (IXC) (Carr and Konishi, 1988; Carr and Boudreau, 1996; Knudsen et al, 2000). This projection then converges spatiotopically with the map of visual space in the optic tectum (OT) so that the auditory and visual receptive fields of these cells are aligned (Knudsen et al, 2000). The coordination of the convergence of these two spatiotopic maps requires visual activity. Owls raised wearing prisms that shift the visual field show a corresponding shift in their auditory space map in the IXC (Miller and Knudsen, 1999). Moreover, this shift is not just functional. Anterograde labeling of the auditory map projection from the central nucleus of the inferior colliculus (ICC) to IXC in normal animals shows a process of refinement via axon and bouton elimination. This refinement involves the formation of an asymmetrical projection since there is a higher degree of axonal elimination from the caudal region of the projection. (DeBello et al, 2001). Prism-reared owls show an increase in the density of axons and boutons within the region of IXC that corresponds to the prismatic shift, whether rostral or caudal (DeBello et al, 2001). This implies not only that

the appropriate alignment of the spatiotopic maps involves refinement, but also that this process may involve both selective elaboration (rostral prismatic shifts cause bouton numbers to increase, even in this region of ordinarily high axon density) and protection from a generalized refinement (caudal processes that are normally eliminated are maintained in caudal prismatic shifts) (DeBello et al, 2001). Interestingly, these adaptive anatomical shifts persist in the adult owl, even after normal experience has been restored and normal function regained, providing a structural basis for the ability of early-conditioned animals to readapt to abnormal experiences in adulthood (Linkenhoker et al, 2005).

These experiments in the owl represent a rare experimental system that examines the influence of activity in the anatomical patterning of converging sensory maps. The cortical projection to the sSC offers another opportunity to study converging sensory maps in a developing system, in this case of the same sensory modality. The retinal projection to the SC is already established at the time of eye opening but the cortical input is still broad and unrefined at this time (Thong and Dreher, 1986).

The Corticocollicular Projection

The SC is a multi-layered midbrain structure whose uppermost layers receive direct visual input from the retina (Lund, 1964; Lund, 1969) as well as input from the ipsilateral visual cortex (Lund, 1964; Sefton et al., 1981) and smaller inputs from the visual thalamus and pretectum (Sefton and Dreher, 1995). These upper (superficial) layers, the stratum zonale (SZ), the

stratum griseum superficiale (SGS) and the stratum opticum (SO), together constitute the superficial superior colliculus (sSC) and are responsible for the processing and integration of visual information (Binns, 1999). Cells in the sSC then project to the deeper layers along with cortical and sub-cortical inputs from major sensory areas (Lund, 1969; Binns, 1999). These deep layers process this multi-modal information and give rise to motor output commands including control of saccadic eye movements, head orientation and positioning behaviors (Lund, 1969; Binns, 1999).

The cells of the SC are morphologically diverse. In the upper layers, cells fall into five categories: marginal cells, horizontal cells, narrow field vertical cells and wider field vertical cells (Langer and Lund, 1974). All of these SC neurons are present at birth (Labriola and Laemle, 1977) but they are not recognizable until postnatal day 4-5 (P4-5). Mature dendritic trees do not appear until P15 and continue maturing until P30 (Labriola and Laemle, 1977; Warton and Jones, 1985). Moreover, by P15-16 (a few days following eye opening) sSC lamination (SZ-SGS-SO) is distinct (Labriola and Laemle, 1977).

Considering everything that is known about the development of appropriate topography of the retinocollicular input to the sSC, surprisingly little is known about the other major afferent projection, the input from the visual cortex. Several studies have examined the time course of the development of this input in the rat and hamster, and these show that cortical axons do not reach the sSC until P3 (P5 in the hamster), do not innervate the optic fiber layer until P9 and are refined by P19 (Thong and Dreher, 1986; Lopez-Medina et al, 1989; Ramirez et al, 1990). The number of retrogradely labeled cells in layer V of the visual cortex following HRP injection into

the SC increases sharply after P6, peaks around P12 and declines between P12 and P20 (Thong and Dreher, 1986), suggesting a period of rapid growth followed by a period of refinement which, notably, occurs just after the time of EO.

While the developmental timecourse of the cortico-collicular projection has thus been approximated at the population level, it has not been examined at the level of individual cortico-collicular axon terminals. This level of detail is essential to elucidating a) the precision of the cortico-collicular topographic map, b) the relative timing of axon retraction and elongation to achieve the observed topography, c) the circuit-level plasticity of this projection during early disruptions of vision, and d) the extent of corticotectal refinement that can ultimately be achieved. These questions are critical to our understanding of the mechanisms by which the developing cortico-collicular projection comes into register with the established retinocollicular map, and the role pattern vision plays in that registration. This registration process is a vital step in visual system formation, and may be central to the orientation of mammals within their visual environment. An improved understanding of this developmental process may also provide insight into clinical problems like the recovery of functional vision following cataract removal or after early trauma to the developing visual system.

Layer V Pyramidal Cells in the Visual Cortex:

Distinct populations of layer V pyramidal cells send projections callosally and subcortically and these distinct connections are established by P3 (Koester and O'Leary, 1993; Kasper et al 1994c). All of the layer V pyramidal cells in the Visual Cortex (VC) that project to

the sSC have thick apical dendrites and a terminal tufts extending into layer I (Kasper, 1994a); they form an uninterrupted band of cells throughout area 17 with lateral extensions into 18a and medial extensions into 18b (Koester and O'Leary 1992; Kasper, 1994a).). By P5 these "thick/tufted" layer V pyramidal cells differentiate anatomically from the slender, untufted pyramidals that arborize in layer II/III and project callosally (Kasper et al., 1994c). Moreover, the "thick/tufted" cells all fire action potentials in bursts and no non-bursting cells project to the SC; most, though not all, of the cells that burst do project to the SC (Kasper, 1994a). Bursts cannot be elicited from these neurons before P15, however. In the third postnatal week this population differentiates both anatomically and electrophysiologically from other layer V pyramidal cells in the visual cortex (Kasper et al, 1994b).

In the cat, pyramidal cells in the visual cortex are known to have a high degree of direction sensitivity (Hubel and Wiesel, 1963) and in both neonatal and adult cats, lesions to VC cause a loss of direction sensitivity in cells in the SC (Berman and Cynader, 1976; Mize and Murphy, 1976). Cats open their eyes when they are approximately ten days old (Villablanca and Olmstead, 1979) and very few SC cells are affected by visual cortical cooling before P13 but shortly thereafter cortical cooling decreased the responses of cells in the SC. In addition, cells showing direction selectivity were more likely to be silenced by the cooling procedure (Stein and Gallagher, 1981).

Eye Opening and its Consequences:

Eye-opening in rodents is normally a gradual process that occurs over several days and visual stimuli can drive neuronal activity in the cortex through naturally closed eyelids even

before EO in the cat and ferret (Huttenlocher, 1967; Krug et al., 2001). Synchronized eye opening, however, causes a coordinated increase in light intensity and contrast vision and appears to induce several biochemical and electrophysiological changes that take place following controlled eye opening (EO) at P12-14. PSD-95 (an important post-synaptic scaffolding protein implicated in glutamate receptor trafficking), along with the mature form of the NMDAR that it binds are both rapidly localized to synapses following EO (Yoshii et al, 2003). A similar rapid incorporation of NR2A containing NMDARs has been reported after removal of rats from dark-rearing (Quinlan et al, 1999). In addition, increases in miniature AMPA receptor (mAMPAR) current amplitude and frequency occur following, and are dependent upon, EO. An electrophysiological analysis of the numbers of inputs per SC neuron shows that an initial rapid decrease in the numbers of inputs per neuron and an increase in the number of release sites per remaining axon are also EO-dependent (Lu and Constantine-Paton, 2004). Eye closure reduces spine density on collicular dendrites of wide field vertical neurons located in the middle SGS, where both retinal and cortical axon arborizations overlap (Phillips and Constantine-Paton, 2007; Phillips 2007). Finally, NMDAR and L-type Ca++ channel dependent LTP can be induced in sSC neurons held in current clamp within hours after, but not before, EO and experiments in which PSD-95 knock-down is accomplished in individual cells reveal that this LTP is dependent upon PSD-95 at sSC synapses (Zhao et al, 2006)

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CHAPTER 2: EYE OPENING DEPENDENT CHANGES IN THE CORTICAL PROJECTION TO THE SUPERIOR COLLICULUS

Abstract:

Here we examine the development of the visual projection between the visual cortex and the superficial visual layers of the superior colliculus. Both regions achieve maps of visual space prior to initial patterned visual experience and must establish registration of their projections after pattern vision onset, but the cellular changes underlying this process are unclear. To address this issue, we reconstructed individual rat corticocollicular axons in three dimensions and statistically sampled axon arborization patterns at set times before and after controlled eye-lid opening. We show that the onset of pattern vision is critical to the establishment of registration between the cortical and collicular maps of visual space. Moreover, if pattern vision is delayed by prolonging eye-lid closure, corticocollicular arbors disappear. A latent plasticity remains, however. Corticocollicular axons with topologically appropriate arborizations can be established late if eye opening takes place within at least a week of its normal occurrence.

Introduction:

Mapped visual projections emerge over a prolonged period during brain development. Much recent work has documented the role of adhesion, repulsion and early waves of synchronized activity (McLaughlin, Hindges et al. 2003; Mrsic-Flogel, Hofer et al. 2005;

Flanagan 2006) in this process. Comparatively speaking, the later emergence of re-enterant or convergent mapped projections in any sensory pathway has received little attention yet it is broadly acknowledged that this phase of synaptogenesis is critical to higher brain function and can extend through adolescence in humans (Edelman 1987). At these late stages, the patterns of activity necessary to establish optimum organization are likely to be more complex and to depend more on presynaptic circuitry than are the maps formed during initial synaptogenesis. Many psychiatric and neurological disabilities, including those resulting from abnormal early sensory experience, are believed to result from disruptions of these later refinement processes. Therefore, establishing systems where mechanisms of central map convergence can be manipulated should facilitate treatments for many brain disorders.

One of the most experimentally accessible converging projections is formed between layer V visual cortical (VC) neurons and the superficial layers of the superior colliculus (sSC) in rodents. Retinal ganglion cells and VC layer V pyramids both send projections to the sSC, a region where considerable synaptic refinement occurs in the third postnatal week in response to eyelid opening (EO). At this developmental time point the retinocollicular projection is retinotopic and refined (Simon and O'Leary 1992) but the corticocollicular projection remains diffuse (Thong and Dreher 1986; Lopez-Medina, Bueno-Lopez et al. 1989; Ramirez, Jhaveri et al. 1990), although both sSC and VC neurons show responses to photic stimuli (Ratto, Robinson et al. 1991; Molotchnikoff and Itaya 1993).

Cortical axons do not reach the sSC until around P5, do not innervate the optic fiber layer until P9, and are refined by P19 (Thong and Dreher 1986; Lopez-Medina, Bueno-Lopez et al.

1989; Ramirez, Jhaveri et al. 1990). Studies of retrogradely labeled layer V VC cells suggest a period of rapid corticocollicular terminal exuberance followed by a period of terminal refinement and axon retraction which, notably, occurs just after the time of EO (Thong and Dreher 1986). However, the role of pattern vision onset and its effect on corticocollicular arborizations has not been studied at the level of individual axon arbors.

Our studies use in vivo anterograde filling of corticocollicular axons, controlled eyelid opening and reclosing paradigms, and three-dimensional quantitative reconstruction techniques. We compare corticocollicular projections of age-matched, eye opened and eye sutured littermates to obtain a cellular-level understanding of corticocollicular axon refinement and begin to disentangle vision-induced, and age-dependent, developmental changes. We demonstrate a role for patterned visual activity in the retinotopic refinement of corticocollicular terminal arbors and also, surprisingly, in the maintenance of these arborizations.

Results:

Topographic labeling of corticocollicular axons:

Corticocollicular axons from the temporal-dorsal visual field of VC extend over a long distance, traveling anteriorly from the occipital cortex into the radiations and then posteriorly again along the dorsal diencephalon to terminate in posterior superior colliculus. Using superficial cerebral arteries as landmarks (Scremin 1995) (Figure 1 inset), consistently good labeling of these axons and their terminals was obtained by inserting small strands of DiI-saturated Gelfoam just beneath

the cortical surface. Strands were placed in the monocular, medial region of VC, a region known to project to posterior-dorsal sSC (Khachab and Bruce 1999) and to share visuotopic space with retinocollicular axons terminating in the region (Sefton and Dreher 1995). Labeled axons always extended to the expected posterior-dorsal third of the sSC (Fig 1b) and in animals with eyelids opened this was where most of the terminal branching was found. The size of the label site varied slightly. However, measures of DiI spread across the surface of the cortex in a sample of animals did not differ (Fig 1a). Animals lacking discrete, heavily labeled placement sites were discarded from analyses.

Normal Development of the Corticocollicular Projection shows rapid refinement and elaboration of a terminal zone (TZ).

The topographic overlap among corticocollicular axons arising from neighboring layer V VC cells and the sSC visuotopic map was established in initial studies where, in ImageJ, 150 μm-deep confocal image stacks containing an axon and its arbor were flattened (see Methods; Supplementary Fig. 1) and subsequently compared to axons and their terminals reconstructed in 3D using Neurolucida (MicroBrightField; Fig 2). Flattening fails to isolate the arbors of individual corticocollicular axons in heavily branched regions. However, after two days of patterned visual activity, axons from DiI labeled cells located near each other in the cortex arborize in the same sSC locus. This convergence invariably appeared as a higher axon terminal density in flattened arbors (Fig 2 A,C) relative to arbors of equivalent age reconstructed in 3D using Neurolucida (MicroBrightField; Fig 2 B,D).

The developmental series of 3D reconstructions (Fig 3) illustrates that at P13 (P0 = day of birth), just before eye opening (BEO), the axon terminals from VC were broad and unrefined. No specific sSC TZ was detected. Instead, long branches growing mostly mediolaterally were present along the entire anterior-posterior (A-P) length of the axons as they extended from the anterior collicular border until they ended in the posterior third of the sSC (Fig 3A). The collateral branching pattern was reminiscent of the originally described retinocollicular projection (Simon and O'Leary 1992). By P15, however, following two days of exposure to normal patterned visual activity resulting from EO on P13 (Fig 3B, left column), a region of arborization in the posterior third of the colliculus could be discerned. At this stage, branching in the TZ was more frequent and the main axon trunk was far cleaner than it was on P13, however there were still small branches emerging from the main axon cylinder throughout the more anterior colliculus.

Over the next few days the projections appeared to refine by reducing the complexity of, and shortening branches along, the axons in anterior SC regions. Thus by P17-P19 the axon trunk in anterior SC supported only short collaterals and a TZ sometimes as long as 165.8 to 234.5 μm in the A-P dimension. The TZ covered a tangential area ranging from 4.8 mm^2 to 25.4 mm^2 (Fig. 3C-D); also see movie Supplemental Fig 2). In fact, the smallest volumes of TZ measured were the volumes from the P17 and P19 colliculi, suggesting that this apparent retraction of processes occurred four to six days after the onset of pattern vision. As can be seen in the reconstructions, even the oldest animals showed considerable variation in the density of branching between arbors drawn from colliculi examined on the same day after eye opening. The reason for this is not clear. It could be that these differences reflect normal variation but

there are also two potential sources of artifacts. First, it is possible that DiI did not completely fill all processes. This seems unlikely, given subsequent analyses to be presented below. Second, it was impossible to follow all of the arborizations into adjacent 200μm thick sections because confocal stacks with small branches from adjacent sections could never be unambiguously aligned. Therefore, partial capture of the arbor in a reconstruction might give rise to a potential artifactual reduction in arbor density. Nevertheless, central axon cylinders of reconstructed terminals at all ages stayed well within the section and notations of a branch leaving either the top or the bottom optical section in the confocal stack from one slice revealed no difference between sparse arbors and more densely branched arbors.

Corticocollicular Terminal Arbors Appear Abnormally Sparse Following Eyelid Closure.

To distinguish between age-related developmental changes and those tied specifically to EO, eye lids were sutured and glued closed on P13, just BEO. This manipulation allows diffuse light to pass through eyelids but blocks high contrast vision of the kind that would robustly activate visual cortical neurons (Krug, Akerman et al. 2001). On P15, following two days of prolonged eye closure (EC), a normal TZ had not developed, however the broad, unrefined arbors seen in the P13 BEO projections were not observed either (Fig. 3F). This suggests that robust pruning of exuberant projections occurred in the visually deprived condition; no elaborations of cortical axon arbors were detectable even after longer EC (Fig 3F-I). Occasionally end branches would show enlargements at their termini (Fig. 3G inset) reminiscent of the retraction bulbs observed with time lapse recording of dynamically imaged axons both *in vitro* and *in vivo* (Niell, Meyer et al. 2004), but similar enlargements were seen at termini of

branches in EO colliculi and retraction bulbs could not be unambiguously counted and compared between the EO and EC conditions as discussed below.

Quantification of EO dependent changes

To quantify these EO dependent changes in the corticocollicular projection, and utilize data from the large number of DiI-filled axons that did not remain within a single thick section, samples of a fixed 160 mm^3 volume of tissue were taken at regular intervals along the A-P length of the sSC (see Supplementary Fig 3). Within the sample volumes the numbers of branch points (BPs) and end points (EPs) per labeled axon branch were counted to yield a measure of the density of axon arborization in that region. In the EO condition (Fig 4A-D; white bars), as compared to the EC condition (black bars), the numbers of both BPs and EPs rose significantly in only the posterior fifth of the sSC. There were no significant differences in BPs or EPs in EO and EC conditions in any of the four more anterior collicular regions, and only the P15 data failed to show a significant increase in branching in the EO condition between the fourth and fifth posterior regions. These data were consistent with the complete reconstructions showing broader TZs at P15 (Fig 3B-E).

Delayed EO postpones, but does not prevent, TZ development.

On P13 eyes were sutured, and then re-opened on P17-P18 to allow delayed exposure to patterned visual activity. By P19-20, after a four to five day delay of normal visual onset followed by two days of normal visual experience, the projections were broad with no obvious TZ (Fig 5A), in marked contrast to the bare axon trunks observed at the same age following six

full days of prolonged eye closure (Fig 3I). In fact, the broadly arborizing terminals seen two days after delayed EO appear to resemble the branching pattern seen at P13 just before normal EO (Fig 3A). In animals that experienced patterned visual activity for four days following the two day delay of EO, corticocollicular axons began to resemble those in the normal P19 condition – the exuberant projections along the main trunk were reduced to short infrequent collaterals and clearly defined, topographically appropriate TZs were present (Fig 5B).

Corticocollicular projections remain plastic at least through the third postnatal week.

Next we investigated whether the corticocollicular terminals that appeared well refined at P16/17 were sufficiently stabilized to remain in that condition after an additional four days of eye reclosure. The four day interval was chosen because previous studies of the level of the mature glutamate receptor scaffolding protein PSD-95 showed a return to the baseline levels in the sSC and VC after eye reclosure at P16 and examination at P20 (Yoshii, Sheng et al. 2003). We found that eye reclosure on P17/18 following EO at the normal time (P13) resulted in terminals that showed a variable morphology. Some had axon trunks almost devoid of axon collaterals in the anterior sSC with only a small sparse arbor in the topographically appropriate zone. Other arbors showed refinement typical of terminals reconstructed on P19 following normal EO on P13 (Fig. 6A). Four days after reclosure on P17-18 the reconstructed terminals (P21-P22) showed more consistent absence of collateral branches (Fig 6B). However, this sparse branching was generally not as severe as that seen in axons deprived of vision for a similar duration (4 days) without ever having their eyes opened (Fig. 3H). Perhaps after several days of normal-onset pattern vision, some synapses in the corticocollicular arbors become

stabilized. These may support the arbor to collateralize, even after a period when activity dependant retinotopic information has been eliminated.

DiI transport appears similar in the EO and EC conditions.

The results presented here could potentially be due to a failure of the DiI label to fill distal axon processes rather than from physical withdrawal of branches. Despite the wide use of DiI to label axon terminal structure and topographic position in the rodent retinocollicular pathway even when retinal activity is perturbed (see McLauglin et al., for example), it was important to address this alternative interpretation, particularly for the current data where axon terminals appear to rapidly regrow "pruned" arbors when pattern vision is present after periods of eye closure. Lipophillic dyes such as DiI initially diffuse in the plasma membrane. However, when labeling is performed over days *in vivo* these dyes are endocytosed and transported in both anterograde and retrograde directions as bright vesicles (Vercelli, Repici et al. 2000). Thus the failure of DiI to label distal processes in the EC condition could simply reflect a failure of axonal transport to distal processes while the axon membrane remains intact. In this situation it is unlikely that active synapses would remain on the isolated axon segments and the functional effect of pruning or cessation of transport might be the same. Nevertheless, the mechanism of synapse elimination could be different under the two scenarios. Consequently, we quantitatively examined the pattern of axon ending in both EO and EC conditions in an attempt to distinguish these possibilities

In serial optical sections through the most distal branches of the terminals (arrowheads, Fig 7A,B) endings were qualitatively indistinguishable. For both EO and EC groups some

terminals ended in particularly bright, large granules of DiI (Fig 7 A,B, open arrowheads), which might reflect axon retraction bulbs. We also examined the three dimensional Neurolucida data (Fig 7C) of identified distal branch diameters for all reconstructed corticocollicular arbors. Average end-branch diameters of terminals reconstructed using 200x magnification were plotted as separate frequency histograms for EO and EC terminals. The numbers of endings in the EC group was invariably much smaller than those in the EO group. Therefore, each set of data was normalized as a percentage of total end-branches and binned at 0.1μm (Fig. 7D). There was no significant difference between the population of EC and EO endings. Similar histograms were plotted for 17 arbors reconstructed using 400x magnification where the pixel resolution was greater. In this analysis we compared EO and EC arbors from colliculi of the youngest and the oldest ages examined after P13 (Fig 7 E). Again there were no significant differences between EC and EO end-branch populations in either age group. This quantitative similarity of end-branches suggests that DiI indicates the true endings of the arbors in both EO and EC conditions and not the dwindling of label that might be expected if eye closure disrupted transport to the axon tip.

Discussion:

EO marks the onset of an eventful period in the development of the visual pathway. Rapid physiological and biochemical changes occur in response to, and are dependent upon, EO. The Membrane Associated Guanylate Kinase (MAGUK), PSD-95, together with the mature NR2A-rich NMDA receptor (NMDR), rapidly localize to visual synapses following EO (Yoshii, Sheng et al. 2003). PSD-95 is an important post-synaptic scaffold for glutamate-mediated signaling

molecules (Kim and Sheng 2004) and synaptic reinforcement (Futai, Kim et al. 2007). In addition, in the sSC, increases in miniature AMPAR current amplitude and frequency occur following – and are dependent upon – EO. Additional analyses reveal significant refinement of inputs to individual collicular neurons in the same post-EO interval (Lu and Constantine-Paton 2004). Conversely, prolonged eye closure reduces spine density on dendrites of GFP-tagged vertical neurons with dorsally weighted dendrites located in the middle stratum griseum superficiale SGS, where both retinal and cortical axon termination zones overlap (Phillips, Brown and MCP, submitted). Finally, NMDAR and L-type Ca++ channel LTP can be induced in sSC neurons within hours after, but not before, EO (Zhao, Phillips et al. 2006) and *in vivo* PSD-95 knock-down with siRNA reveals that this LTP is dependent upon PSD-95 (Zhao, Murata and MCP, in preparation). All of these changes could simply be the result of increases in activity in the visual pathway following an increase in the intensity of light. However, a hypothesis that attributes corticocollicular refinement to broad, non-topographic activity is not consistent with the present observations suggesting that pattern vision brings the cortical map of visual space into register with the previously established retinocollicular map of visual field position.

The initial formation of topographic sensory maps has been extensively studied in several systems, but few studies address how multiple sensory maps come into register when at least two converge on the same target. A multi-model sensory map has been extensively studied in barn owls where the auditory-localization system must achieve registration with the retinocollicular localization system to allow the birds to accurately spatially target sound in darkness. In owls the alignment of these two spatiotopic maps can be altered by goggle-rearing that shifts the active visual field projection in the tectum. The functional auditory map to the tectum then shifts

to retain convergence of the two sensory field positions (Knudsen, Zheng et al. 2000; DeBello, Feldman et al. 2001). Unlike the inputs converging in the owl however, the retinal and cortical axons converging in the rat superior colliculus represent the same modality, albeit with different levels of processing. Topographic information from the retina is already established BEO in the rodent sSC but the VC activity is specifically driven by pattern vision. Thus prolonged or reintroduced eye-lid closure after the time of normal EO does not merely shift, but rather eliminates, functional retinotopic activity in the later developing corticocollicular terminals. The present data suggest that, in the absence of any position in the sSC where the topographically organized retinal axons carry activity that is correlated with the developing cortical projection, corticocollicular axons cannot potentiate and maintain nascent synapses and this causes the observed collapse of entire terminal arbor.

This removal of corticocollicular arbors in the EC conditions also suggests that activity-dependent competition for sSC synaptic space occurs during the post-eye-opening period. The NMDAR has frequently been implicated in the mediation of such competition and synaptic refinement in developing brains (Constantine-Paton, Cline et al. 1990; Li, Erzurumlu et al. 1994; Katz and Shatz 1996). Through mechanisms similar to those involved in NMDAR-dependent LTP and long-term synaptic depression (LTD), NMDAR activity reinforces coincidently active inputs while eliminating inputs that cannot cooperatively activate target cells. Earlier studies support this role for NMDARs (Cline, Debski et al. 1987; Rittenhouse, Shouval et al. 1999). During synaptic refinement in the sSC pharmacological blockade of the NMDAR prevents activity-dependent removal of many exuberant retinal projections (McLaughlin, Hindges et al. 2003) causing the retention of non-topographic retinocollicular arbors (Simon, Prusky et al.

1992). In contrast, chronic application of NMDA itself to the sSC retards the development of glutamate excitation in the sSC (Shi, Aamodt et al. 2001), a finding consistent with NMDA induced LTD in hippocampus (Lee, Kameyama et al. 1998). These treatments also produce anatomical effects consistent with competition. Application of NMDAR antagonists causes an increase in retinal axon synapse density in normal retinocollicular projections and induces ipsilateral retinal axon sprouting into an sSC scotoma during the eight days following a small P6 contralateral retinal lesion. In contrast, chronic agonist (NMDA) application produces a decrease in retinal ganglion cell synapse density during this same early period (Colonnese and Constantine-Paton 2001; Colonnese and Constantine-Paton 2006).

Also consistent with NMDAR mediated competitive interactions are findings that neonatal enucleation causes expansion of the cortical projection in the colliculus, as well as an increase in cortical bouton density in upper regions, where the dense retinal projection normally resides (Garcia del Cano, Gerrikagoitia et al. 2002); anopthalamic mice lacking an optic nerve show a similar wide-spread sprouting of corticocollicular axons across the colliculus (Khachab and Bruce 1999). In addition, by the third postnatal week, removal of the ipsilateral cortex is necessary for NMDAR antagonists to induce ipsilateral retinal axons to sprout into the sSC scotoma produced by a small contralateral retinal lesion (Colonnese and Constantine-Paton 2001). Therefore, without patterned visual information, the cortex cannot maintain arbors in the sSC, but without the retinocollicular projection the corticocollicular projection cannot refine.

Although the development of a refined corticocollicular projection requires visual experience, cell surface molecules are likely involved as well. For example, the stabilization of

corticocollicular axon branches by activity that is coincident with the retinal inputs to the same locus does not explain the capacity of remaining axon cylinders to re-sprout arbors once eyes are opened late (Fig 5). Semaphorins and their receptors, the plexins, as well as other adhesion systems (Bruses and Rutishauser 2001; Futai, Kim et al. 2007), have been widely implicated in axon repulsion, pruning and synaptic plasticity (Low and Cheng 2005; Tran, Kolodkin et al. 2007). PSD-95 colocalizes with both semaphorins and their coreceptors, neuropilins, and both semaphorins and plexins have been shown to alter neuronal activity and modulate the efficacy of synapses (Waimey and Cheng 2006). The semaphorin system is particularly interesting because exuberant, long projections of layer V neurons to the corticospinal tract and the inferior colliculus remain inappropriately unpruned in mice where Plexins A-3 and A-4 are knocked out (Low, Liu et al. 2008). Perhaps repulsive mechanisms such as those mediated by plexins and semaphorins are responsible for the disappearance of cortical axon terminal branches with EC. In this case robust synaptic activity could suppress the repulsive interactions and, at the same time, protect corticocollicular synapses thereby supporting terminal arbor elaboration.

Conclusion:

The corticocollicular pathway is a major pathway mediating orientation of mammals within the visual environment. Establishing precision in this map, the relative timing of axon retraction and elongation to achieve appropriate topography, and the plasticity of this pathway following disruptions of vision have implications for the potential recovery of functional vision after cataract removal or trauma to the visual pathway. This work describes early plasticity of

this pathway and provides a basis for subsequent studies exploring the potential prolongation of this plasticity into adulthood.

Methods:

Animals: Animal care and procedures followed approved MIT IACUC protocols. Pregnant Sprague-Dawely rats or rats with litters of a known birth-date were ordered from Taconic. Pregnant rats were monitored daily to determine day of birth (P0). On P12 or early P13 (before EO) pups were randomly assigned to experimental or control groups. The control group's eyes were gently pulled open with mild tension to ensure synchronous EO. The experimental group was anesthetized with vaporized isofluorane (isofluorane; Samuel Perkins, MA), both eyes were sutured with several small stitches using fine sutures (6-0 nylon black monofilament, Ethicon Inc.). The eye closures were reinforced with a thin layer of tissue adhesive (Vetbond, 3M). Eye closures were checked daily and any pups with premature eye opening or infection were excluded from the study. In the case of eye re-opening experiments, eyes were not sutured but merely glued with Vetbond and checked at least twice daily for any sign of early eye-opening. On the appropriate date this glue was removed gently while the animal was lightly anesthetized with isoflurane. In the case of the delayed eye closure experiments, animals were anesthetized and lids were sutured in the same manner as described above.

Corticocollicular Axon Labeling: After unsuccessful attempts (with horseradish peroxidase, biotinylated dextranamine, B fragment of cholera toxin and GFP-adenovirus) to fill the entire corticocollicular extent of only a small patch of layer V neurons, cortical afferents to the sSC

were successfully labeled by the cortical insertion of small strands of amphiphilic-dye loaded gelatin sponge. $DiIC_{18}$ (Molecular Probes, Invitrogen) was dissolved in dimethylformamide (DMF) until the DMF reached saturation. Small threads of the gelatin sponge Gelfoam (Pharmacia) were placed in this DiI/DMF solution until saturated and dried overnight in a sterile container. P7-P20 pups were anesthetized with isoflurane and small windows were cut (<P16) or burr holes were drilled (>P16) in the medio-posterior skull, using the terminal pericallosal arterial branch as a guide (Fig 1a, inset). Small strands (2-4mm) of DiI-loaded gelfoam were carefully inserted just below the pial membrane.

Histology: Two to three days following placement of DiI-loaded Gelfoam, animals were anesthetized with isoflurane and perfused through the left ventricle with ice cold PBS pH 7.4 followed by 4% PFA (EMS) in PBS pH 7.4 (100-200 ml/animal). Brains were dissected and post-fixed in 4% PFA at 4°C for at least 24 hours. After post-fix, sequential vibratome sections of 150 or 200μm thickness were cut in the sagittal plane of the neocortex and midbrain. Sections were collected in ice cold PBS pH7.4 and mounted in the aqueous antifade medium Fluoromount G (EMS).

Confocal Microscopy: Images were collected on a Nikon PCM2000 (MVI) confocal scanning microscope with SimplePCI acquisition software (Compix). Using a 40X/1.30 oil-immersion objective or a 20X/0.75 air lens, confocal z-series (1.5μm z-step) were collected. Three consecutive images of each optical slice were collected and averaged to enhance the signal-to-noise ratio. Between 40 and 80 images were collected in each image stack, yielding a stack with a depth of 60 - 120μm. Between five and eighteen image stacks were collected for each sSC

section and images were collected starting at the anterior pole of the sSC, proceeding towards the posterior pole with small overlaps to aid reconstruction.

Axon Reconstructions: Z-stacks from the same section were exported in Tiff format and analyzed in one of two ways. To obtain flattened reconstructions, z-stacks were opened in ImageJ (NIH), converted to black-on-white images and compressed into a single 2D projection of the 3D stack. These 2D projections were imported into Adobe Photoshop (Adobe Systems) and adjacent sections were aligned to assemble a complete montage of the A-P length of the labeled sSC. Reconstructions of the arborization were made in Photoshop by zooming to a highly pixilated view and manually filling adjacent darkened pixels on a separate Photoshop layer, using the paintbrush tool (see supplemental Figure 1). To obtain 3D single arbor reconstructions the reconstruction and analysis program, Neurolucida (MicroBrightField) was applied. Image stack files were opened and aligned in three dimensions to obtain a complete reconstruction of the sSC's A-P length. Discrete axon terminals were identified during their entry into the anterior pole of the sSC and were traced in 3 dimensions, as they coursed posteriorly. Branches that exited the imaged slice were marked as such. All reconstructions were made blind to the sample treatment.

Statistical Analysis: To sample the tissue for analysis, a 40μm x 40μm grid overlay was placed over 100μm-deep image stacks and aligned to cover the A-P length of the sSC. This yielded 160mm^3 volumes of tissue for analysis. All labeled processes in every fourth volume along the A-P axis of the sSC were traced using Neurolucida. These "grid-samples" were then analyzed in Neurolucida Explorer (MicroBrightField) and measures of branch points, end points, and end

type were obtained. Two-factor ANOVAs were carried out in SPSS 11.0 (SPSS Inc.) and once significance across the conditions was confirmed *post hoc* two-tailed student's T-tests and the Tukey HSD *post hoc* test were applied to individual sample pairs.

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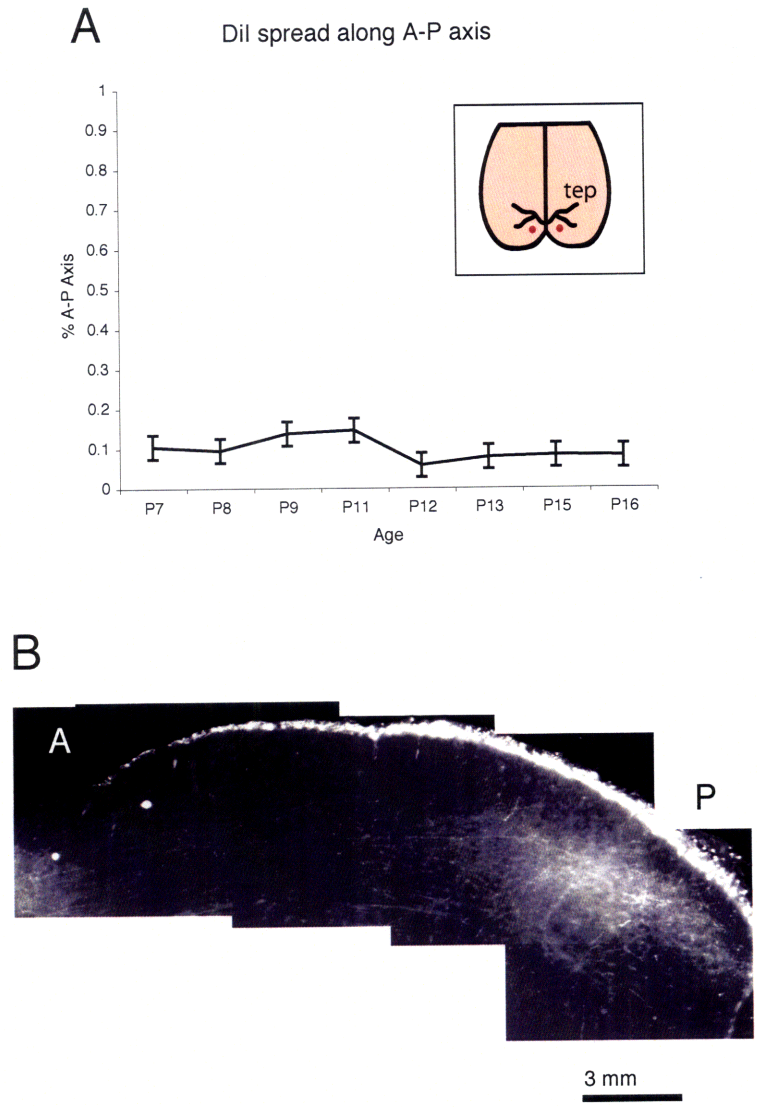


Figure 1: DiI placed in the visual cortex (VC) does not spread across the surface of the cortex and produces consistent labeling of axons in the superior colliculus (SC). (A) Anterior-posterior spread of DiI at the infusion site, as measured in sagittal VC sections. In all cases and at all ages,labeling was confined to the posterior-most cortex. Inset: Infusion sites relative to the terminal pericallosal branch (tep), a superficial cerebral artery, which was used as a landmark. (B) Labeled corticocollicular axons in section of superficial SC from a P15 rat. A=anterior end of the SC; P=posterior end of the SC. Scale 3mm.

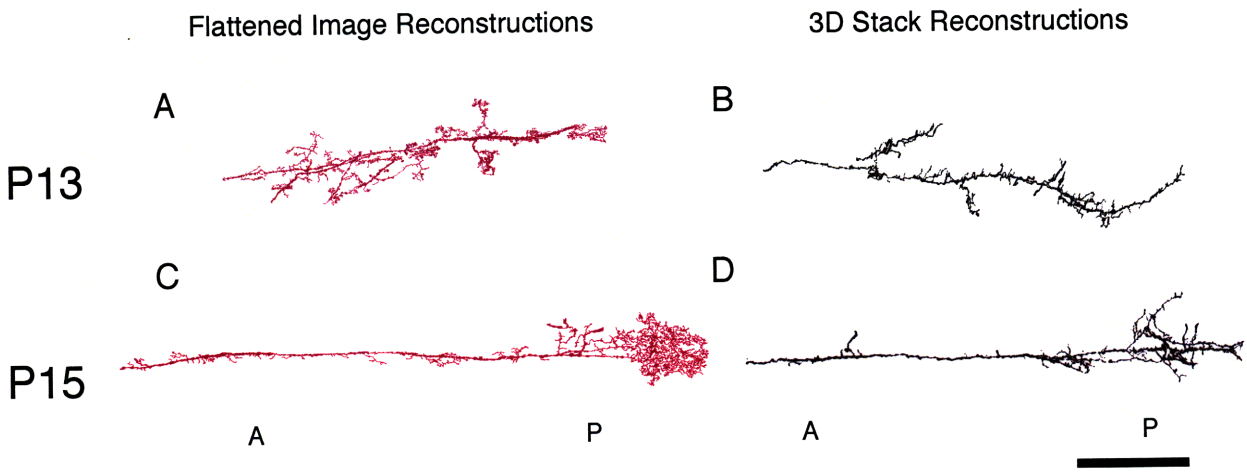
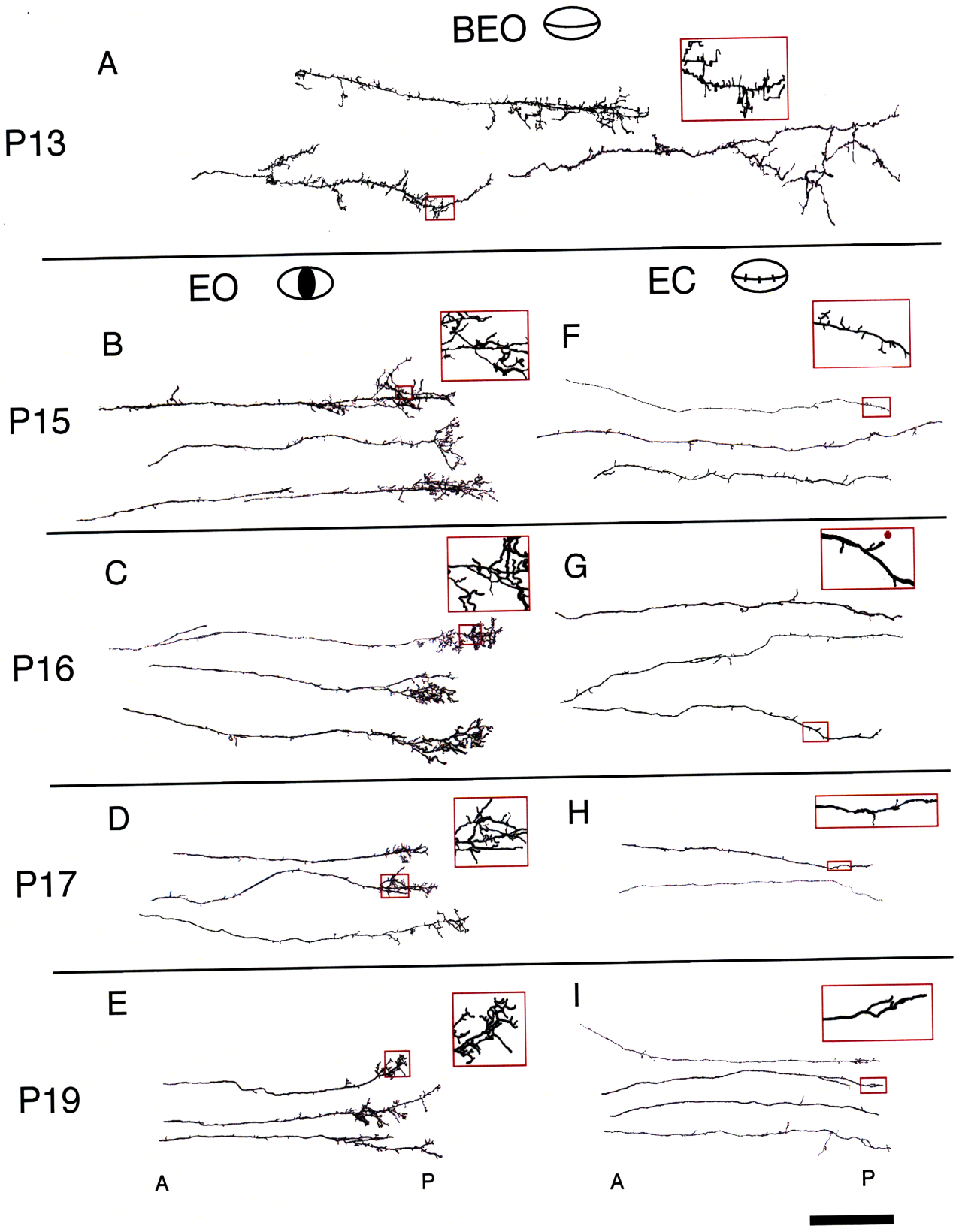


Figure 2: Flattened vs. 3D reconstructions show increased focal arborization after EO indicative of converging axon terminals from the VC locus. (A and C) Flattened image reconstructions: reconstructions of labeled VC axons in the SC from rats aged P13 and P15. These were produced by collapsing 150μm confocal image stacks and tracing adjacent pixels in Adobe Photoshop. (B and D) 3D Reconstructions: Neurolucida reconstructions of single axons in the sSC from rats aged P13 and P15, respectively. A=anterior; P=posterior. Scale 250 μm.

Figure 3: Prolonged eyelid closure (EC) prevents normal developmental elaboration of the corticocollicular axons in the superficial superior colliculus. (A) Neurolucida reconstructions of corticocollicular axons before eye opening (BEO) at P13. Projections show exuberant branching along the length of the axon trunk. (B-E) Neurolucida reconstructions of corticocollicular axons after eye opening (AEO), at ages P15, P16, P17 and P19. Animals that have experienced patterned vision for 2-6 days show tight and elaborate regions of VC axon arborization. At older ages slightly less overall branching is observed. (F-I) Neurolucida reconstructions of corticocollicular axons in the EC condition. VC terminals from animals that have been deprived of pattern vision (following eye suture) are sparsely branched along the entire axonal length and lack both the elaboration and the topographic specificity seen in the corresponding eyes open (EO) age group. Note the decrease in axonal branching relative to the BEO group. A=anterior; P=posterior. Scale 250 μm. Insets show details of the branching pattern as well as the infrequent indication of a retraction bulb (*).


BEO
A
P13
EO
EC
B
P15
F
C
P16
G
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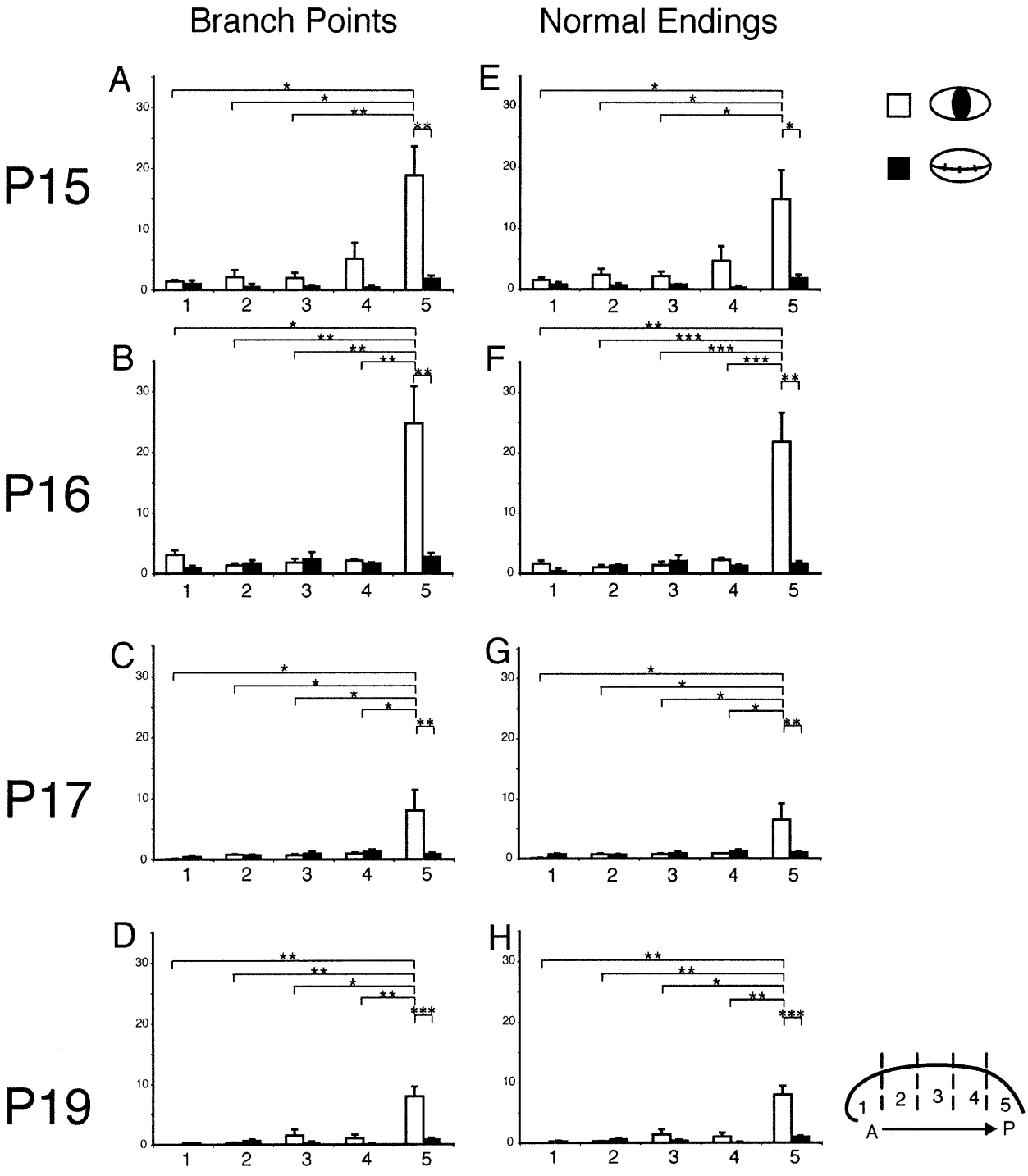
P17
H
E
P19
I
A
P
A
P

Figure 4: Quantification of EO-dependent changes in axon branching. Measurements of the number of BPs/segment (A-D) or EPs/segment (E-H) of traced axon within a fixed 160mm3 volume of tissue. The anterior-posterior length of the colliculus is divided into even fifths (diagram, lower right; A=anterior, P=posterior). (A-H) Volumes sampled in the posterior fifth of EO SC (white bars) have significantly more BPs than volumes sampled from anterior EO SC and significantly more BPs than EC SC in the posterior fifth (black bars). Volumes sampled across the anterior-posterior extent of the SC show no significant difference in the number of BPs/segment in the eye closed condition (black bars). (E-H) Volumes sampled in the posterior fifth of EO SC (white bars) have significantly more EPs than volumes sampled from anterior EO SC and significantly more EPs than EC SC in the posterior fifth (black bars). Volumes sampled across the anterior-posterior extent of the SC show no significant difference in the number of EPs/segment in the eye closed condition (black bars). (Two-Factor ANOVA $p<0.05$; Tukey HSD post-hoc test $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; Post-hoc two-tailed Student's T-test $^{*}p<0.05$, $^{**}p<0.05$, $^{***}p<0.05$).


Branch Points
Normal Endings
P15
P16
P17
P19
A
B
C
D
E
F
G
H
1 2 3 4 5
A P

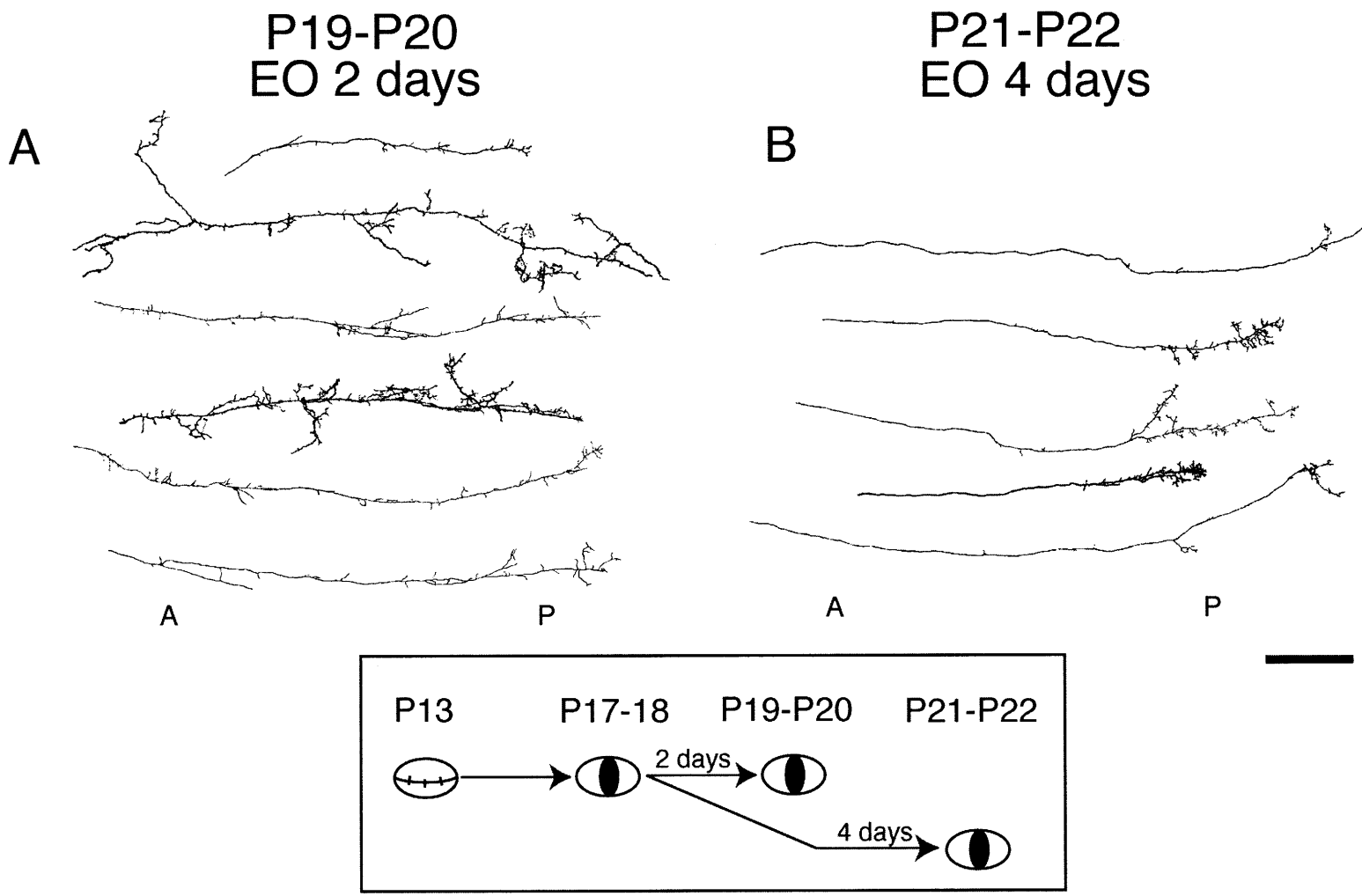


Figure 5: Delayed eye opening (EO) postpones but does not prevent target zone (TZ) development. (A) Axon reconstructions from animals that experience four days of visual deprivation (eye suture) followed by two days of normal patterned vision. Eyes were sutured on P13 and reopened on P17-18; reconstructions were made from SC aged P19-20 and appear broad and unrefined. (B) Axon reconstructions from animals that experience visual deprivation (eye suture) followed by four days of normal patterned vision. Eyes were sutured on P13 and reopened on P17-18; reconstructions were made from SC aged P21-22 and by this time exuberant projections along the main trunk are reduced and TZs are present. A=anterior; P=posterior. Scale 250 μm.

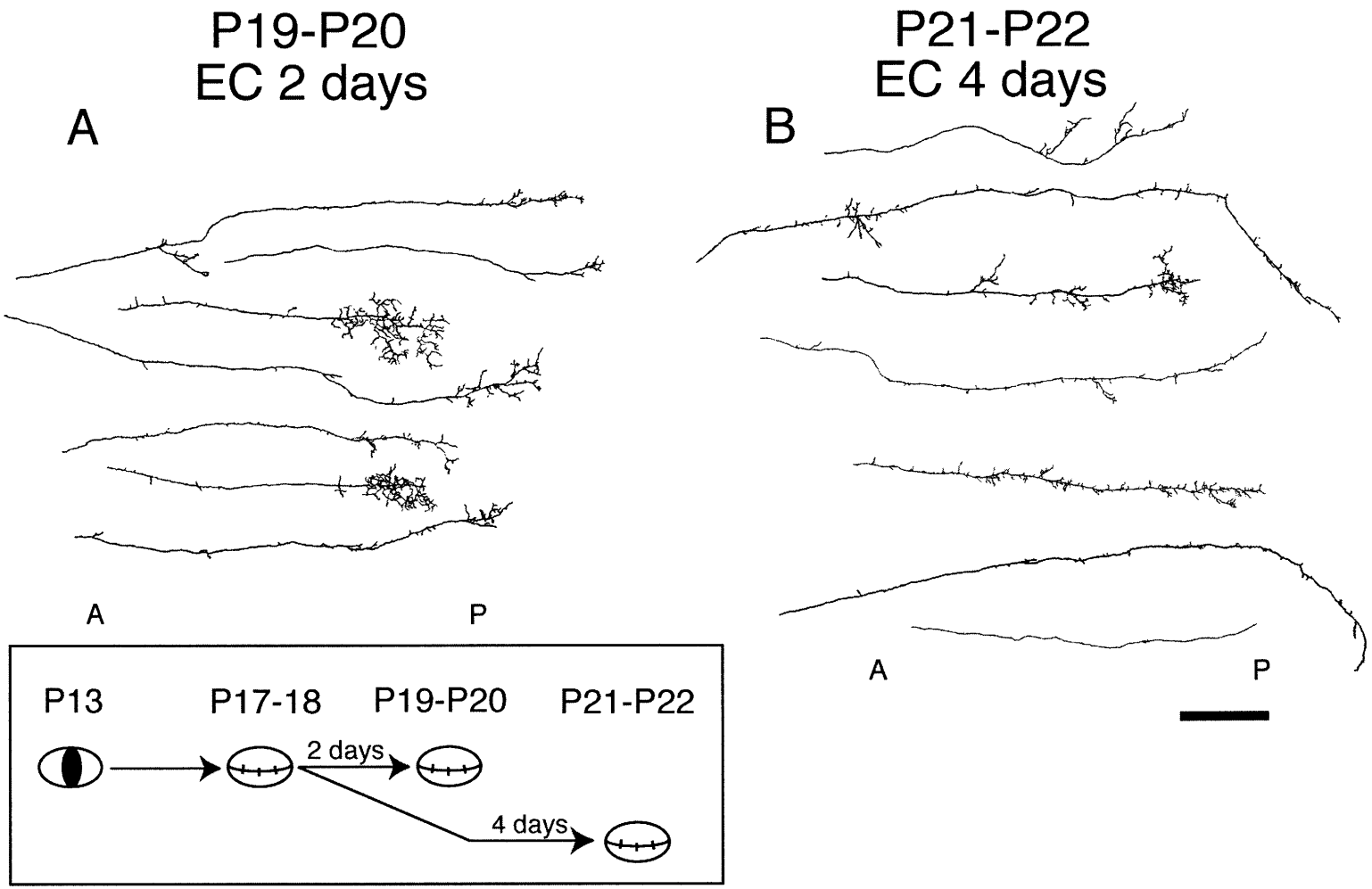


Figure 6: Eye-reclosure destabilizes the target zone (TZ). (A) Axon reconstructions from animals that experience visual normal patterned visual activity followed by two days of visual deprivation (eye suture). Eyes were opened on P13 and sutured closed on P17-18; reconstructions were made from SC aged P19-20 and show a range of morphologies. Some show only sparse arbors in the posterior superior colliculus (SC) while others show refinement typical of TZs in animals without eye re-closure. (B) Axon reconstructions from animals that experience normal patterned visual activity followed by four days of visual deprivation (eye suture). Eyes were opened on P13 and sutured on P17-18; reconstructions were made from SC aged P21-22 and by this time all axons show significant branch retraction. A=anterior; P=posterior. Scale 250 μm.

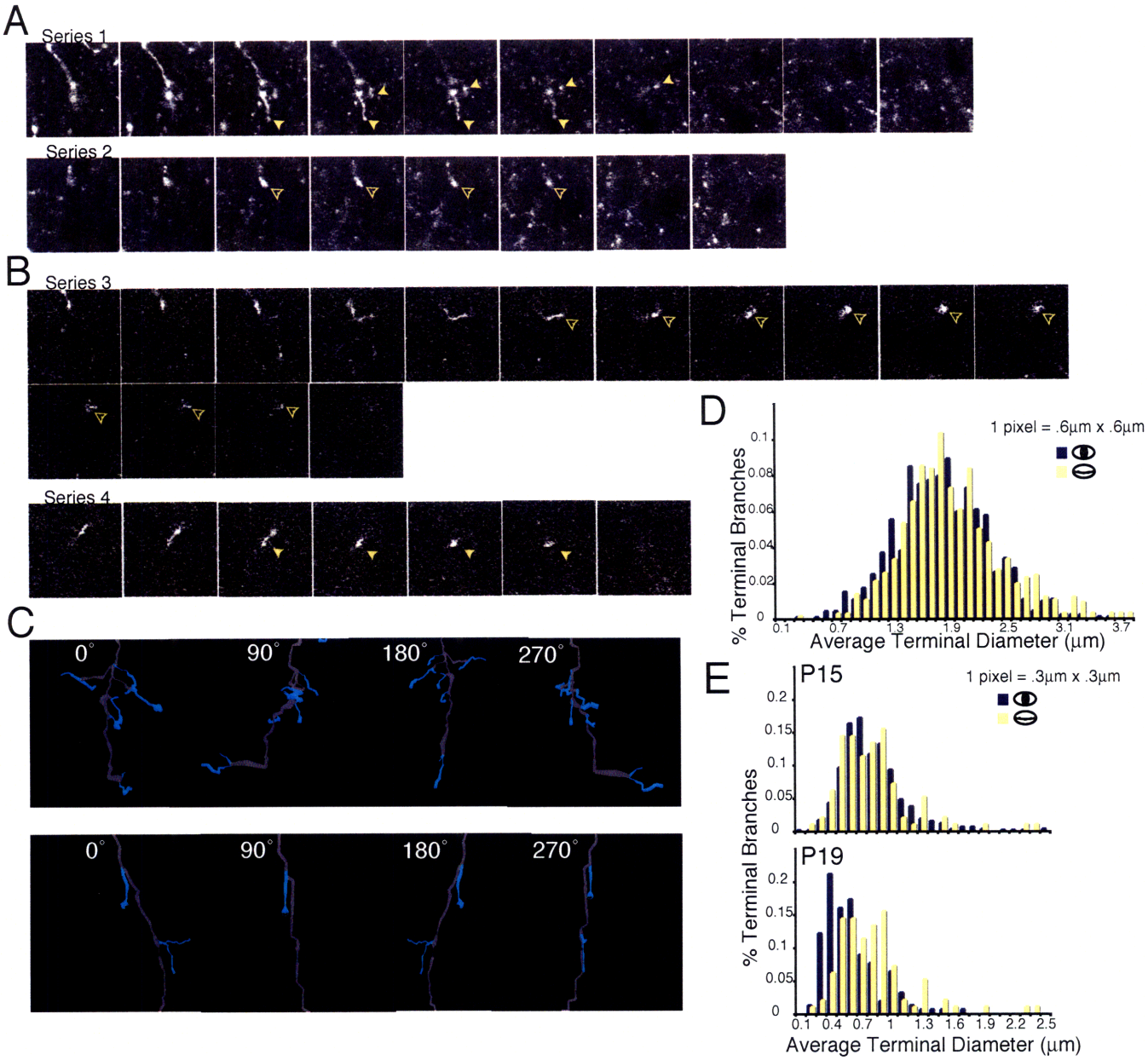
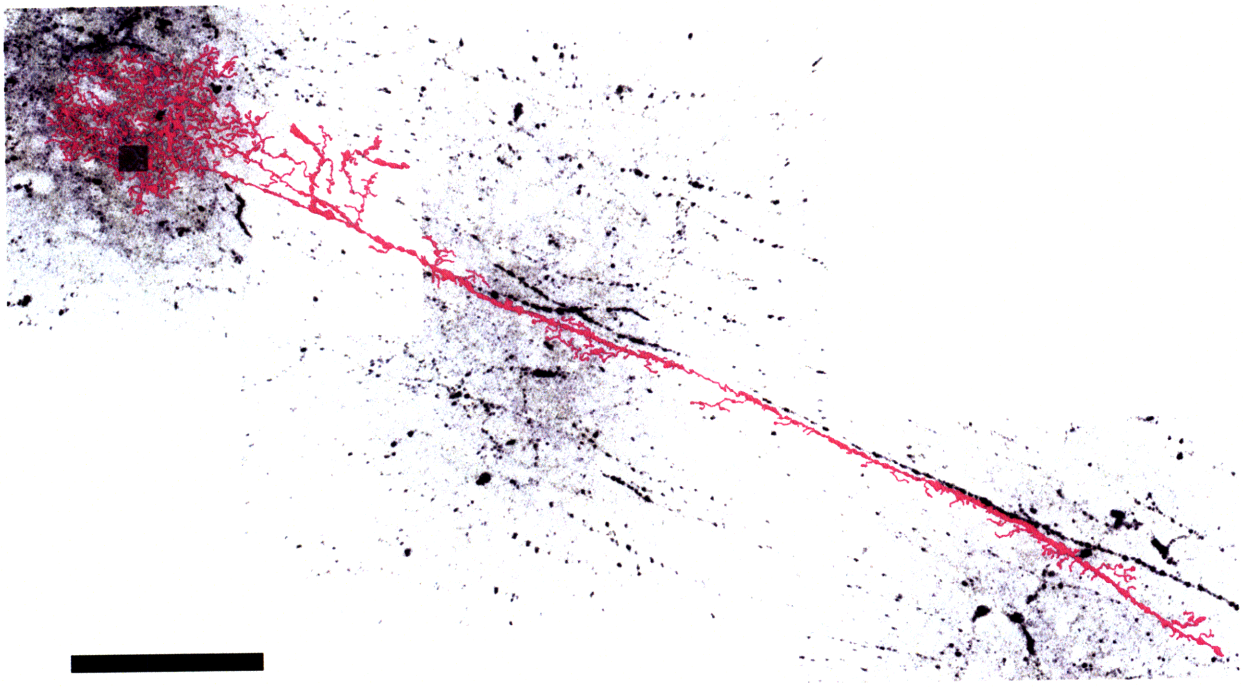
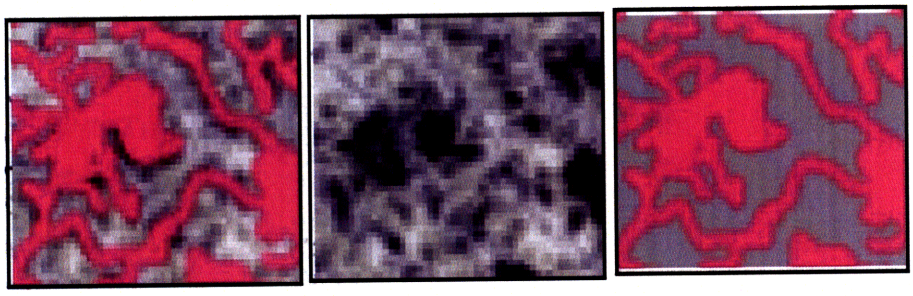


Figure 7: Terminal ending average is not different in the EO and EC conditions. (A) Representative series showing two axon terminal endings in P15 EO sSC. (B) Two series of axon terminal endings in the P15 EC condition. All series are from confocal z-stacks taken at 1.5µm intervals with 400 power maginification, and show the presence of small terminals (closed arrowheads), sometimes ending with a characteristic end bulb (open arrowheads). Each frame is 30x30µm. (C) View of representative axon terminal endings (in light blue) as reconstructed in Neurolucida, shown at approximately 90º successive rotations. The morphology of the endings appears similar in both cases. (E) Histogram showing the percentage of all reconstructed terminal branches (light blue) at each average diameter, traced using a 20x objective. There is no significant difference between the EO population (blue bars) and EC population (yellow bars; Mann-Whitney Test $p<.05$; EO n=19 reconstructed arbors; EC n=34). (F) Histograms of age-matched average terminal diameters as reconstructed by Neurolucida, showing the percentage of all measured terminal branches at each average diameter, traced using a 40x objective at P15 and P19. There is no significant difference between the EO populations (blue bars) and the EC populations (yellow bars) at either age (Mann-Whitney Test $P<.05$; P15 EO n=5; P15 EC n=4; P19 EO n=4; P19 EC n=4).

A

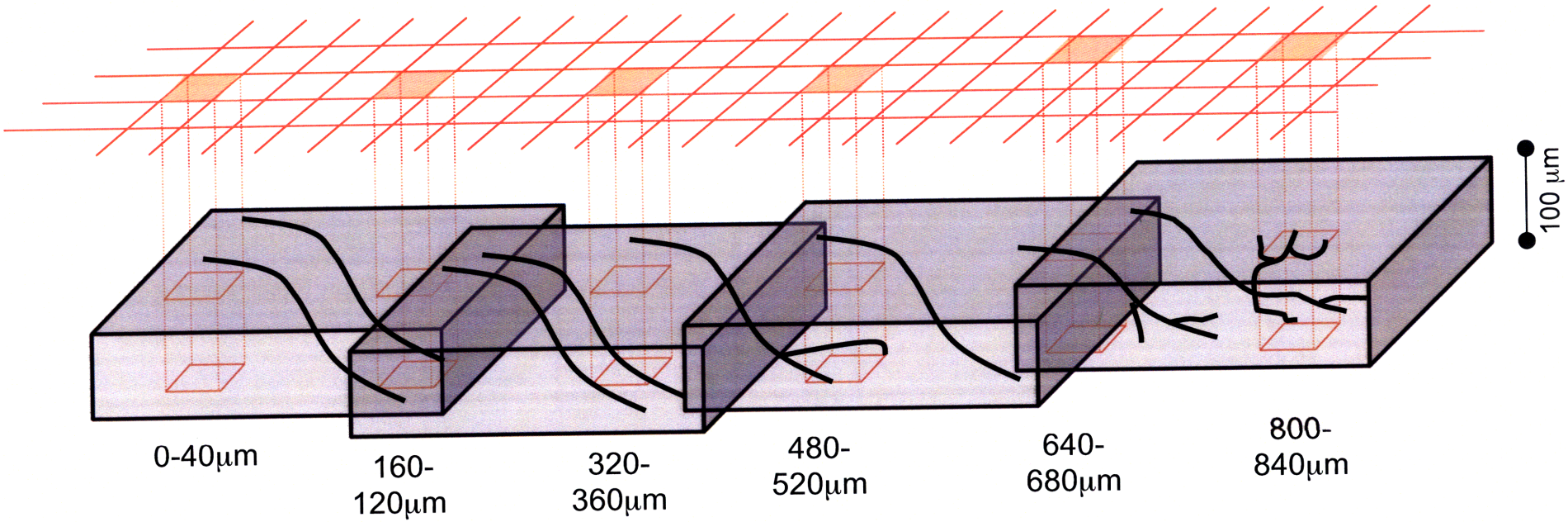


B



Supplementary Figure 1: Detailed reconstruction of flattened image stack. (A) Confocal z-stacks of labeled sSC, converted to black-on-white and compressed into a single 2D projection of the 3D stack. The reconstructed projection is filled in pink. Scale 250 μm. (B) Highly pixilated detail from the dense region of arborization shown in A. Left: overlap. Middle: labeled pixels. Right: flattened reconstruction Reconstructions were made by filling adjacent darkened pixels using the paintbrush tool.

Supplementary Figure 2: Rotations of three dimensional reconstructions of corticocollicular axons. Projections were reconstructed in three dimensions and rotations showing the three dimensional structures could be captured using Neurolucida software. (A) Selected rotation from the P13 BEO condition demonstrating non-topographic, exuberant branching along the A-P length. (B) Selected rotation from P15 EO condition showing a topographically appropriate TZ and lack of exuberant branching. (C) Selected rotation from the P15 EC condition showing only sparse branching along the entire length of the reconstruction. (Anterior=top of the frame; Posterior=bottom of the frame).



Supplementary Figure 3: Schematic of the "Grid Sampling" Method. Axons (in black) frequently run at an angle to the plane of the section montage (grey blocks) and so complete single axon reconstructions cannot be made. Instead, volumes of tissue are sampled by placing a 40x40μm grid overlay (red) atop 100μm-deep image stacks. This grid is aligned to cover the A-P length of the sSC and all segments of labeled axon that fall within every fourth 160mm^3 volume of tissue are traced and analyzed.

CHAPTER 3: GENERAL DISCUSSION

The superficial layers of the superior colliculus (sSC) represent a promising model system for the study of activity-dependent development of converging spatial maps. Afferents from both the retina and the visual cortex project to the sSC, development of the retinal projection has been well described and visual input can be manipulated via eye closure or dark-rearing. This study looks at the normal time course of the development of the cortical projection to the colliculus, as well as the effects on that projection of eye closure, delayed eye-opening and eye-reclosure. The findings imply that an eye-opening induced pattern of VC activity is necessary to retain and reinforce corticocollicular synapses and if that reinforcement is not spatiotemporally related to the previously established retinocollicular projection the corticotectal synapses are simply withdrawn. These manipulations of EO correlate well with changes observed in synaptic levels of PSD-95 following similar manipulations. Postsynaptic density protein-95 (PSD-95), an important post-synaptic scaffolding molecule implicated in glutamate receptor trafficking and NMDAR dependent LTP, together with the mature form of the NMDAR, rapidly localizes to synapses following EO in both the VC and SC. These increases occur within two hours of EO, independent of age (Yoshii et al, 2003).

PSD-95 is a MAGUK (membrane-associated guanylate kinase), one of a family of intracellular scaffolding proteins with multiple protein-protein interaction domains that bind

NMDARs and bring them into close association with many downstream signaling molecules, thus organizing signaling complexes at the post-synaptic membrane (Kim and Sheng, 2004). Another member of this family of scaffolding molecules, SAP-102, is expressed in the sSC at an earlier age than PSD-95 and shows a preferential affinity for the NMDARs containing the NR2B subunit, which is also expressed at younger ages. PSD-95, on the other hand, appears to bind preferentially to the latter-expressed NR2A subunit (Townsend et al. 2003, van Zundert et al 2004). These different subunits have distinct kinetic and pharmacologic properties. Specifically, NMDARs with NR2B subunits produce longer channel open times and longer decay times than receptors with NR2A subunits in whole cell recordings of synaptic events (Monyer et al, 1992; van Zundert et al. 2004). NR2A-knockout mice lack LTP in the sSC at P15-17, an age when LTP is normally expressed (Zhao and Constantine-Paton, 2007). In these mice, however, miniature NMDAR currents disappear and this disappearance is coincident with the increase in PSD-95 and NR2A expression in the normal animal, suggesting the displacement of NR2B-subunit expressing NMDARs anchored by SAP102 to a perisynaptic position (Townsend et al, 2003; van Zundert et al, 2004). Thus the loss of LTP in the NR2A-knockout mouse could be due to distinct NR2A subunit kinetics, to distinct signaling properties mediated by the localization of NRs within the post-synaptic membrane immediately opposed to the pre-synaptic release sites, or to distinct signal molecules associated with the PSD-95 but not the SAP102 scaffold. PSD-95 has also been implicated in synapse potentiation and has been shown to anchor stargazin/AMPAR complexes at synapses in the hippocampus and the cortex (Ehrlich and Malinow, 2004; Bats et al, 2007; Béïque et al, 2006). PSD-95 overexpression in hippocampal neurons drives maturation, clustering and activity of glutamate receptors (El-Husseini et al,

2000) while PSD-95 knockdown with RNAi disrupts maturational changes and decreases the stability of spines in hippocampal cultures (Ehrlich et al, 2007). In the sSC, increases in mAMPAR current amplitude and frequency occur 8-12 hours after the PSD-95 increase at visual synapses and show the same EO-dependence. Electrophysiological analyses using the method of shoulders reveal significant refinement of inputs to individual collicular neurons over the same interval (Lu and Constantine-Paton, 2004).

These correlations between corticocollicular refinement and PSD-95 delivery to the synapse strongly suggest that these mechanisms play a role in the growth and pruning of the cortical projection that takes place in the sSC after EO. There is evidence that is consistent with this hypothesis from recent dynamic imaging studies in zebrafish. *In vivo* time lapse imaging in zebrafish tecta shows a strong correlation between synaptogenesis and axon branch formation and stabilization (Meyer and Smith, 2006). When PSD-95 becomes localized to a previously dynamic dendritic site, this site appears to remain stable over time and becomes a node from which dendritic filopodia sprout and retract until one of the filopodial branches subsequently becomes a site of PSD-95 accumulation. (Meyer et al 2005; Niell et al 2004). Previous work from the Smith lab has indicated that this relative stabilization of a dendritic segment is complimentary to a similar relative stabilization of growth cone activity from an ingrowing retinal terminal (Meyer and Smith, 2006).

Although these zebrafish studies provide evidence for the complimentary and dynamic growth and stabilization of post-synaptic apparatuses and pre-synaptic terminals on a short time-scale (20-30 min), the level of reorganization observed in the rat corticocollicular projection in

the days after EO is striking. Perhaps the most startling observation is the degree to which the deprivation of patterned vision leads to pruning within all collateral branches. Evidence from other systems suggests that an overabundance of projections might have been expected. In peripheral muscles, activity block can delay or eliminate the selective elimination of multiply innervating motor neuron fibers (Thompson, 1993). The elimination of inappropriate thalamic projections to the rodent barrel cortex requires not only neural activity (Jensen and Killackey 1987) but NMDAR activation as well (Fox et al, 1996); a block of NMDARs in the sSC during the first two postnatal weeks prevents the pruning of topographically inappropriate RGC axon terminals (Simon et al, 1992). Furthermore, in accord with the suggestions on dendrites from the zebrafish work, the neurons of layer IV Barrel cortex fail to show distinct targeting of their dendrites to the center of the barrels, where the corticothalamic axons enter, when the NMDAR is selectively knocked out in cortex (Iwasato et al, 2000).

One potential explanation for the unexpected broad withdrawal of corticocollicular projections following EC is that, unlike projections that refine earlier, the three week old sSC synaptic space is filled with earlier projections that are already refined. Evidence of sprouting into a contralateral retinal scotoma earlier in development appears to disappear in the third postnatal week unless the cortex is removed. (Colonnese et al., 2001, 2005). These studies demonstrate the high level of competition for space to sprout in the sSC after EO and suggest that when cortical activity is normal, cortical afferents have a competitive sprouting advantage, possibly because, as has been suggested in the neuromuscular literature (Kasthuri and Lichtman, 2003), inputs that do not have many targets are better competitors for stability than competing inputs that already have other potent synapses established. The bareness of the corticocollicular

axon trunk following eye-suture, however, suggests that successful competition for available space in the neuropil requires activity that is synchronized with other converging inputs. Without such correlations corticocollicular axons lose their competitive edge.

Although the development of the corticocollicular projection clearly requires neural activity, cell surface molecules that mediate adhesion or cause retraction are likely involved. Semaphorins and their receptors, the plexins, have been widely implicated in a range of cell processes including axon repulsion, attraction, pruning and synaptic plasticity (Low and Cheng 2005; Tran et al, 2007). Membrane-associated semaphorins can bind directly to plexins but most secreted semaphorins interact with plexins via association with a neuropilin co-receptor (Waimey and Cheng, 2006). PSD-95 colocalizes with both neuropilins and semaphorins in culture and both semaphorins and plexins have been shown to alter neuronal activity and modulate the efficacy of synapses (Waimey and Cheng, 2006). Plexin signaling appears to be involved in the appropriate targeting of layer V pyramidal cell axons to appropriate midbrain and spinal cord targets as well. Plexins A-3 and A-4 (PLXA3 and PLXA4) are expressed throughout the cortex on P7 but are restricted to visual cortex by P11 (Low et al, 2008). At this age the exuberant, long projections to the corticospinal tract (CST) and the inferior colliculus (IC) begin to be pruned, leaving only the appropriate SC projection. These exuberant projections to the CST and IC remain unpruned in both the PLXA3/PLXA4 double knockout and the neuropilin-2 (NPN-2) knockout, although projections from layer V cells in the motor cortex are appropriately pruned from the SC in these animals (Low et al, 2008). The pruning of long layer V pyramidal cell tracts from inappropriate downstream targets is of a different scale from the fine activity-dependent rearrangements seen with controlled EO, of course, but the involvement of such a

large and diverse family of signaling molecules is still suggestive, especially because semaphorins and neuropilins may act through synapse modulation as well as purely activity-independent axon modifications. Interestingly, microarray screens show an increase in Sema4a mRNA in the sSC after EO but not before (Rory Kirchner, personal communication). Thus the sSC may provide a good system in which to examine the effects of the plexin-semaphorin-neuropilin signaling complex on both activity-dependent and activity-independent axonal rearrangements as well as the modifications in synaptic signaling that underlie these changes.

Prospectus for Future Work:

In fact, the work presented here suggests several interesting areas of future study. The correlation between activity-dependent axonal rearrangements and synaptic targeting of PSD-95 suggests synaptic changes brought about by PSD-95 trafficking might be necessary for signaling processes that underlie the detection of coincident activity in the sSC and VC, but further examination is needed to establish a causal link. Brain-derived neurotrophic factor (BDNF) drives PSD-95 to synapses and is in turn activated by NMDA receptor activation and signaling through the PI3-K-AKT pathway (Yoshii and Constantine-Paton, 2007). Thus it would be interesting to see if a block of BDNF in the VC or sSC would disrupt corticocollicular axon refinement.

The effects of eye-closure on layer V pyramidal cells in the VC also presents a promising area for future study. The layer V pyramidal cells in the VC that project to the sSC all have thick

apical dendrites, terminal tufts extending into layer I and all fire action potentials in bursts (Kasper, 1994a). This population differentiates anatomically and electrophysiologically from other layer V pyramidal cells in the VC during the third postnatal week and bursts of action potentials cannot be elicited from these neurons before P15 (Kasper et al, 1994b). This timing, along with the activity-dependent development of these cells' long axon projections, suggests that activity may play a role in the anatomical and electrophysiological differentiation of these cells as well.

Critical periods in the developing visual system were first recognized by Hubel and Wiesel, who saw that cortical cells with different eye preferences group into discrete clusters within layer IV of the primary visual cortex called ocular dominance columns (ODCs). Early disruptions of visual experience change the shape and distribution of these columns, but only during a "critical period" of development. Following early monocular deprivation labeled thalamic afferents representing the deprived eye narrow while the open-eye bands widen; physiological recordings tangential to the cortical surface reflect a similar shift in eye-preference (Hubel and Wiesel, 1962, 1969, 1972). In fact, critical periods have been described throughout the brain and in a wide range of systems and species including whisker-barrel map formation in rodents, chick imprinting, zebrafinch birdsong and even human language acquisition (Hensch, 2004). It would be interesting to see whether there is a "critical period" for the development of the corticocollicular projection as well. Delayed EO at P17-P18 appears to restart a program of exuberant growth and arborization within two days; by four days (P21-P22) appropriate TZs begin to form. Similarly, at this age the TZ is disrupted following four days of pattern-vision deprivation in animals with eyes re-closed on P17-P18. These delayed eye-opening and eye re-

closure experiments have not yet been carried out in the adult animal, however. Yoshii et al found that eye reclosure between P40 and P45 failed to alter PSD-95 levels in visual synapses (Yoshii et al, 2003). Consequently, if the absence of high levels of PSD-95 at visual synapses is required for synaptic branch retraction in the corticocollicular pathway, we predict that eye closure at this stage would have no effect on the maintenance of normal arbors. In addition, the observation of "critical periods" in so many systems involving activity-dependent development suggests that the plasticity of the corticocollicular projection might be lost at older ages. It would be very useful to define this time frame, not only to better understand the development of the sSC, but also because it might present a very useful model system in which to examine the molecular and physiological aspects of critical period closure.

Beyond it's implications for mechanisms of axon growth dynamics or for developmental plasticity, this work is exciting in the context of human vision. Human visual acuity improves dramatically over the first six months and postnatal anatomical and functional development takes place at all levels of the sensory pathway, from the retina to the cortex (Maurer and Lewis, 2001). Children with bilateral high-density cataracts are deprived of high contrast pattern vision and if these cataracts are not corrected within the first year of life visual acuity never completely recovers (Maurer et al, 2005). There is evidence that some functional vision can recover after correction as late as 12 years of age but spatial acuity and contrast sensitivity remain impaired (Ostrovsky et al, 2006).). In addition, these children suffer impairments of some higher levels of visual processing, including impaired face processing and the inability to distinguish global form or motion of objects (Maurer et al, 2005). Clearly a better understanding of plasticity in the visual system could help develop treatments to lead to better functional recovery of vision in

patients with such impairments. Furthermore, the impairments in higher level visual processing following recovery in these visually deprived children suggest the importance of understanding not only how sensory input develops, but also how central visual maps use experience to form appropriate functional connections to other brain areas.

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APPENDIX: METHODOLOGICAL CHALLENGES

Labeling the Corticocollicular Axons:

Tracing the corticocollicular projection over development proved a difficult technical problem. The projection, which is very long, originates in layer V pyramidal cells of the primary visual cortex, travels rostrally from the occipital cortex into the radiations and then caudally again along the dorsal diencephalon, entering the colliculus at its rostral pole (Sefton and Dreher, 1995).

Several studies have successfully labeled long projections in rodents with horseradish peroxidase (HRP) alone or conjugated to wheat germ agglutinin (WGA-HRP; Thong and Dreher, 1986; Harvey and Worthington, 1990; Ramirez et al,1990; Tan and Harvey, 1997), biotinylated dextranamine (BDA; Garcia del Cano et al, 2002; Reiner et al, 2000), *Phaseolus vilgaris* leucoagglutinin (PHA-L; Garcia del Cano et al, 1997; Ling et al 1997), the B fragment of cholera toxin (CTB; Ling et al 1997) and Lipophilic carbocyanine dyes such as DiI (Koester and O'Leary 1993; Khachab and Bruce, 1999). In addition, several studies have used fluorescently labeled latex microspheres to examine the developmental time course of this anatomical projection (Aarnoutse et al, 1995; Rumberger et al 1998).

Although this wide range of tracing techniques has been used on the corticocollicular projection, each presented technical problems. The HRP-label is frequently incomplete and fills

cell somas and primary dendrites far better than axons (Kobbert et al, 2000). It has been used successfully as a *retrograde* tracer of the corticocollicular projection (Thong and Dreher, 1986) but even conjugated to WGA it does little more than show the presence of cortical axons in the colliculus, after lesions for example (Tan and Harvey, 1997). Latex microspheres are also transported retrogradely and so, while useful in studying the gross time course of development of topography, cannot be used to look at changing axon projections. Tracings using plant lectins and toxins (BDA, PHA-L, CTB) clearly show the presence of axons in the colliculus. In some cases, for example Garcia del Cano et al.'s 1997 study in which rabbit corticocollicular fibers were labeled with PHA-L, cortical axons in the colliculus were clear and traceable (Garcia del Cano et al, 1997). In our hands, however, none of the plant lectins or toxins yielded labeling consistent enough to trace axon projections (Table 1).

Unlike plant lectins and toxins, DiI regularly produces beautiful axon staining in prenatal and neonatal animals when applied to fixed tissue and allowed to diffuse for several months (Koester and O'Leary 1993; Khachab and Bruce, 1999). In fixed tissue lipophilic dyes diffuse laterally through membranes and, because of changes in membrane properties and an increase in myelination, diffusion rates slow in older animals (Kobbert et al, 2000; Vercelli et al, 2000). We found this to be the case, as in our hands this technique yielded beautifully filled axons, but only at P3 and younger. At older ages, fixed tissue incubated with DiI for up to a year showed filled axons that extended forward to the radiations but no farther. We found that new formulations intended to increase the diffusion rate (*FAST* DiI, Molecular Probes) did not increase the labeling of this long tract.

DiI can be used *in vivo* as well, however. Lipophilic dyes diffuse in the plasma membrane but *in vivo* they are rapidly internalized by endocytosis, incorporated into vesicles and actively transported both anterogradely and retrogradely (Kobbert et al, 2000; Vercelli et al, 2000). In this case, no transfer occurs between cells (a concern in the fixed condition) and the dye remains viable for long periods of time (Kobbert et al, 2000). The disadvantage is that the clear membranous labeling seen in fixed tissue is quickly replaced with granular cytoplasmic cluster-like labeling (Vercelli et al, 2000). Of all the labeling techniques we tried, *in vivo* DiI application was the only one that produced traceable labeled axons at all ages. A further technical problem was presented by the delivery of the DiI. DiI in a solution of DMSO injected slowly into the VC through micropipettes yielded little to no labeling; only when loaded onto thin gelfoam strands, which allowed slow release of DiI into the tissue, was good labeling obtained (Table 1).

Tracing the Labeled Corticocollicular Axons:

Once corticocollicular axons were successfully labeled it became clear that both developmental and EO-dependent changes take place during the second and third post-natal weeks. The DiI-loaded-gelfoam method of tracing is remarkably consistent but it remains impossible to control precisely the injection size. Moreover, injections regularly label several hundred axons in the SC and because the rat brain undergoes noticeable changes in size during the developmental time period we wished to study, we could not ensure we labeled the exact region of topographic space in every injection. Thus rather than compare whole SCs with one another we sought to trace individual axons in the sSC.

Initially, to trace axons in the sSC confocal z-stacks were opened in ImageJ (NIH), converted to black-on-white images and compressed into a single 2D projection of the 3D stack. These 2D projections were then imported into Adobe Photoshop (Adobe Systems) where adjacent sections were manually aligned to assemble a complete montage of the A-P length of the labeled sSC (Chapter 2, Supplementary Fig. 1A). Reconstructions of the arborization were made in Photoshop by zooming to a highly pixilated view and manually filling adjacent darkened pixels on a separate Photoshop layer, using the paintbrush tool (Chapter 2, Supplementary Fig. 1B). This produced very detailed reconstructions but unfortunately, because the sections were flattened, we could not ensure they contained single axons. In fact, these flattened image reconstructions contain many more branches and a much denser area of arborization than the Neurolucida reconstructions subsequently made from the same SC or an SC prepared in the exact same manner. Even though this method fails to produce single axon reconstructions, however, it is still informative as the development of a focal, dense locus of arborization in the retinotopically appropriate region of the SC indicates that relative topographic order is achieved in the corticocollicular projections.

Quantification of the Labeled Corticocollicular Axons:

Quantification of changes observed in the labeled corticocollicular axons posed another challenge. The reconstruction and analysis software, Neurolucida, allowed us to trace single axons. Frequently, however, axons ran at a slight angle to the section (Chapter 2, Supplementary Fig 3), making a reconstruction of even an incomplete axon arbor impossible. We could not find sufficient numbers of axons that ran the whole length of the SC to perform a statistical analysis.

While a single axon could not be reconstructed from these angled sections, however, it was clear from looking at the EO P15-19 samples that topography was maintained: axons at the anterior end of the SC were smooth and ran parallel to one another; axons at the posterior end of the SC branched, terminated and arborized in a haphazard manner. Thus we devised a "grid sampling" procedure to sample the tissue for analysis. A 40x40μm grid overlay was placed atop 100μm-deep image stacks, and aligned to cover the A-P length of the sSC (Chapter 2, Supplementary Fig 3). This yielded 160mm^3 volumes of tissue for tracing and analysis. All processes in every fourth volume along the A-P axis of the sSC were traced in Neurolucida and analyzed in Neurolucida Explorer. The numbers of branch points the numbers of normal endings were the parameters that most captured the robust changes seen over development and between the EO and EC conditions. For statistical analysis these were normalized to the number of traced branches in a grid sample to control for variation in injection size/location. Other parameters, such as tortuosity, branch order and ending type appeared promising initially, but yielded no statistically significant results.

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Tracer:	Delivery System:	Resulting label:
HRP-FITC	Solution; pressure injection	Cell bodies in VC, no axons in SC
HRP-FITC	Solution; pressure injection	Cell bodies in VC; no axons in SC
HRP-FIT C	Crystals	Cell bodies in VC; no axons in SC
CTB	Iontophoresis	Background only
CTB	Pressure injection	Cell bodies in VC; no axons in SC
CTB	Coated insect pins	Cell bodies in VC; no axons in SC
CTB	Coated gelfoam	Cell bodies in VC; no axons in SC
BDA	Pressure injection	Cell bodies at injection site only; faint axons in SC
BDA	Coated insect pins	Cell bodies at injection site only; no axons in SC
GFP-Adenovirus	Pressure injection	Cell bodies in VC; no axons in SC
DiI	Pressure injection	Cell bodies at injection site only; no axons in SC
CM-DiI	Pressure injection	Background Only
CM-DiI	Paste application	Grainy cell bodies in VC; no axons in SC
DiI	Crystals	Cell bodies in VC; inconsistent axons in SC
DiI	Coated insect pins	Cell bodies in VC; inconsistent axons in SC
DiI	Coated gelfoam	Cell bodies in VC; consistent axons in SC*
DiI; *FAST* DiI	Post-fix application (crystals, pins or gelfoam)	Very well filled cells at injection site; well filled axons <P2

Table 1. Tracers and delivery systems tested and resulting label in VC (visual cortex) and SC (superior colliculus).