In Vitro Detection of Cues that Guide Thalamocortical Development: A Role for Chondroitin Sulfate Proteoglycans

by

Daniel Eric Emerling

B.A., Biochemistry and Neurobiology
University of California, Berkeley, 1989

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Signature of Author:_____
Daniel Eric Emerling
Department of Brain and Cognitive Sciences
July 15, 1996

Certified by:_____
Arthur D. Lander
Associate Professor of Developmental and Cell Biology
Thesis Supervisor

Accepted by:_____
Gerald E. Schneider
Professor of Neuroscience
Chairman, Committee for Graduate Students

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ABSTRACT

In nervous system development, the growth cones of advancing axons are thought to navigate to their targets by recognizing cell-surface and extracellular matrix molecules that act as specific guidance cues. To identify and map cues that guide the growth of a particular axonal system, the thalamocortical afferents, an assay was devised to examine short-term interactions of dissociated embryonic thalamic cells with living, ~150 μm slices of developing mouse forebrain. Thalamic cells rapidly (<3 hours) and efficiently attached to and extended neurites on pre- and postnatal slices, but a broad zone throughout the neocortex was generally non-permissive for both thalamic cell attachment and the ingrowth of neurites. This zone coincided with the cortical plate at early stages (embryonic day 15), but later became restricted, in rostral-to-caudal fashion, to cortical laminae 2/3. In addition, neurites that extended on some layers were found to be significantly oriented in directions that coincide with the pathways that thalamic axons follow *in vivo*. These results imply that local adhesive cues and signals that affect process outgrowth are distributed among developing cortical laminae in a manner that could underlie much of the temporal and spatial patterning of thalamocortical innervation. To assess the role of chondroitin sulfate proteoglycans (CSPGs) in nervous system development, chondroitin sulfate was eliminated from these cocultures by adding a specific glycoeliminase to the culture medium. This treatment eliminated the layer-specific differences in thalamic cell attachment and neurite outgrowth that were observed when thalamic cells are plated onto embryonic day 16 cortex in untreated cocultures. Specifically, the cortical plate (a layer avoided by thalamic axons *in vivo*) is shown to possess an inhibitory activity (anti-adhesive, neurite repelling) and the intermediate zone and subplate (layers in which thalamic axons normally grow) are shown to possess a stimulatory activity (adhesive, neurite promoting). Biochemical analysis suggests that these opposing activities are not due to the presence of different CSPG species in different cortical layers. Rather, the data indicate that these activities may reflect the presence of differentially localized CS-binding molecules which, significantly, can also be competed away by soluble CS. This model provides a way to reconcile conflicting reports on the roles of CSPGs in neural development, and suggests a fundamental role for CSPGs in the organization of matrix-bound cues in the brain.

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CHAPTER I

INTRODUCTION

ABSTRACT

Nervous system function depends upon the proper connections being made between neurons. Although changes in synaptic contacts occur even in the adult, the initial development of neural circuitry happens during a phase of morphogenesis dominated by the proliferation and migration of neurons, the extension of neural processes, and the refinement of synaptic connections. Axons must extend via pathways that are often relatively long and convoluted to establish synapses with target tissues. Because the growth of processes and the creation of connections follow stereotyped patterns, it has long been suspected that they are regulated by specific mechanisms.

The goal of my thesis research has been two-fold: to characterize cues that guide the development of a particular axonal system, the thalamocortical afferents, and to assess the role of a major extracellular matrix constituent, chondroitin sulfate, in thalamocortical development. This chapter is intended to provide an overview concerning general mechanisms of axon guidance, the function of chondroitin sulfate proteoglycans in this process, and the cues that guide the development of thalamocortical axons, the model system on which I have focused.

OVERVIEW OF AXON DEVELOPMENT

Connecting Up The Brain

Compared to the proliferative and migratory events that occur during the development of other tissues, development in the nervous system would appear to present some special issues since axons are uniquely neural structures. Because they can extend far from the cell nucleus, the flow of information between the site of transcription and the distal tip of the axon can be significantly delayed compared to that which occurs between the nucleus and leading edge of a migrating cell. It is perhaps not surprising, then, that extending processes have motile structures, termed growth cones, at their tips that can migrate and navigate autonomously from the soma. Like migrating cells, growth cones have a dynamic cytoskeleton (O'Connor and Bentley, 1993; and reviewed by Solomon, 1992) and interact with their local environment to make pathway decisions (discussed below).

Understanding the cues that growth cones use to navigate and locate targets has been a long-standing goal of developmental neurobiology. Early models proposed by Ramon y Cajal suggested that axons might be guided by signals emanating from their targets. Only after eight decades did evidence exist that growth cones could be attracted by chemical gradients (Gundersen and Barrett, 1979). More recently, there has been growing interest and evidence that this mechanism does guide axons *in vivo*.

Hypotheses designed to address the precision of neural connections, such as Sperry's chemoaffinity model, invoked the idea that growth cones found their appropriate target cells by "differential chemical attraction" (Sperry, 1963). The proposal attempted to explain the point-to-point topography that exists in retino-tectal connections by mutual attraction between specific

retinal axons and chemical markers within the tectum. In this sense, axons extending in the tectum locate their exact targets by using substrate-bound cues along the growth cones' path, as opposed to following diffusible signals emanating from the target. This concept, that the substrate itself contains guidance information, has direct relevance to more recent hypotheses concerning axon development.

These attempts to explain the specificity of neural connections based on target-derived and substrate-bound cues, did not (nor could they based on knowledge at the time) account for the role that electrical activity plays in neural development. Extrinsic factors, such as information from the environment (sensory input or "experience") is required for the precise synaptic connections that constitute properly functioning sensory and motor systems. For example, the formation of the refined circuitry that constitutes ocular dominance columns and vibrissae-related barrels in sensory cortex can be irreversibly perturbed by eliminating sensory input or impairing electrical activity during critical periods of development (Hubel et al., 1977; Shatz and Stryker, 1978; Stryker and Harris, 1986; Woolsey and Wann, 1976; Belford and Killackey, 1980; Schlaggar et al., 1993).

Spontaneous electrical activity, which occurs before sense organs function, can also play an important developmental role. In utero blockade of electrical activity in the retina prevents the normal segregation of retinal axons that occurs among their target layers in the thalamus (Shatz and Stryker, 1988). Similarly, appropriate segregation of motor axons among muscle fibers in vertebrates is activity dependent, since electrical blockade prevents the loss of initial polyinnervation and exogenous electrical stimulation hastens it (reviewed by Fields and Nelson, 1992). Thus, in vertebrates and to a lesser degree in invertebrates (Budnik et al., 1990; Cash et al., 1992), electrical

activity is required for the development of precise axonal mapping and synaptic connectivity.

Despite this importance, studies of individual growth cones in developing insects have demonstrated that axons extend in a stereotyped manner prior to the onset of electrical activity (Bate, 1976; Goodman and Spitzer, 1981; Halpern et al., 1991). Furthermore, the pharmacological blockade of activity does not prevent motor (Liu and Westerfield, 1990; Dahm and Landmesser, 1991), retino-tectal (Meyer, 1983; Schmidt and Edwards, 1983), or thalamic (Herrmann and Shatz, 1995) axons from extending along stereotyped paths that lead them to their target tissues. These studies show that activity-independent factors account for the general pathfinding that allows axons to reach their targets. Thus, understanding the development of brain connectivity requires characterizing the pathways that developing axons follow (see section "Thalamocortical Development" below) and elucidating the mechanisms that guide them on these paths.

Attractive And Repulsive Cues Guide Growth Cones

Several studies have demonstrated that attractive cues help provide directional information to migrating growth cones. Target tissues or structures along the paths of extending axons, such as the ventral midline of the spinal cord, can secrete factors that attract growth cones at a distance *in vitro* (Lumsden and Davies, 1986; Tessier-Lavigne et al., 1988; Sato et al., 1994; Tamada et al., 1995). In these experiments, growth cones will migrate toward a point source from which a chemoattractant diffuses, indicating that diffusible factors can guide axons at a distance. Importantly, guidance cues can also be localized within the growth substrate. When the presumptive optic tract in Xenopus embryos is surgically removed and replaced in a different orientation,

retinal axons reorient with the rotated tissue when extending through it, indicating that a particular path is defined within the growth substrate (Harris, 1989). It is unlikely that axons already present within the rotated tissue provided such cues since, at the time of surgical manipulation, processes had not yet left the retina to create the optic tract

Nevertheless, axons and structures such as cell bodies do impart contact-mediated guidance cues. Proper development in certain systems requires the presence of "pioneer" axons which establish a pathway and subsequently act as guides, via fasciculation, for later growing processes. Growth cones of follower axons display aberrant pathfinding when fasciculation is impaired, such as in particular drosophila mutants (Grenningloh et al., 1991; Lin et al., 1994), or when neurons that project pioneer axons have been ablated (Raper et al., 1984; Bastiani et al., 1986). Contact-mediated guidance can also be provided by "guidepost" cells or transient targets along a growth cone's path (O'Conner et al., 1990). Ablation of such cells or structures will cause aberrant pathway decisions in developing grasshopper limbs (Bentley and Caudy, 1983) and nematodes (Walthall and Chalfie, 1988). Similarly in vertebrates, extending axons will deviate from their normal pathways after selective elimination of certain structures with which growth cones directly interact, such as the floor plate of the spinal cord in zebrafish (Greenspoon et al., 1995) and the subplate layer in the mammalian neocortex (Ghosh et al., 1993).

Although attractive cues clearly play a major role in axon development, it has recently become evident that repulsive interactions can also direct axon outgrowth. Neurites extending from ganglion cells of temporal retina will avoid membranes extracted from posterior optic tectum but will extend on membranes of anterior tectum (Walter et al., 1987; Cox et al., 1990), a

behavior consistent with the fact that axons from temporal retina project to anterior, but not posterior, tectum. Similarly, sensory neuron growth cones avoid the posterior scleratome *in vivo* (Tosney, 1988) and collapse *in vitro* upon contact with membranes of posterior, but not anterior, scleratome (Davies et al., 1990). Tissues that particular axons avoid, such as the septum by olfactory bulb axons (Pini, 1993) or the ventral spinal cord by certain motor axons (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995; Tamada et al., 1995), secrete factors that repel neurites from a distance *in vitro*. Thus, like attractive cues, repellent cues can involve local, contact-mediated interactions as well as diffusible signals.

Molecules That Guide Axons

Evidence that a particular molecule plays a role in a specific guidance event could come from *in vivo* manipulations (e.g. transgenics, surgery, pharmacology) or, less assuredly, from correlations of *in vitro* function and *in vivo* expression. In the latter case, it is necessary that the guidance event be well characterized since it must be modeled *in vitro*. *In vivo* studies of mutant nematodes indicate that UNC-6, a molecule homologous to laminin B2, acts in guiding commissural axons dorsally or ventrally depending upon the putative receptors expressed by growth cones (Hedgecock et al., 1990; Ishii et al., 1992). Mammalian homologues of UNC-6, netrin-1 and -2, are synthesized by cells in the ventral spinal cord and can diffuse from a source to attract axons from a distance *in vitro*. These data suggest that netrins likely act as chemoattractants for ventrally guided commissural axons (Kennedy et al., 1994; Serafini et al., 1994). Interestingly, netrin-1 may also act as a repellent to trochlear motor axons since these processes are repelled by netrins *in vitro* and extend dorsally, away from the ventral floorplate, *in vivo* (Collamarino and

Tessier-Lavigne, 1995). Thus, netrins may contribute to both attractive and repulsive cues.

Other molecules that repel neurites have also been characterized. In developing grasshopper, a member of the semaphorin/collapsin family likely acts to steer growth cones away from a stripe of epithelial cells that express the molecule in the limb bud (Kolodkin et al., 1992). A semaphorin may also prevent certain sensory axon projections from extending more ventrally within the dorsal spinal cord of the rat (Messersmith et al., 1995). These proposed functions are consistent with the fact that terminal axons arbors are inhibited from forming at sites where semaphorins are ectopically expressed in Drosophila (Matthes et al., 1995). Cell surface ligands for tyrosine kinases, RAGS and ELF-1 (Drescher et al., 1995; Cheng et al., 1995), are expressed in an anterior to posterior gradient in the tectum and at least RAGS has been shown to repel neurites *in vitro* (Drescher et al., 1995). Thus these molecules may underlie a repulsive cue which prevents temporal retinal axons from invading the posterior tectum. However, because RAGS also repels nasal retinal neurites, which do enter posterior tectum, other factors must also mediate retino-tectal specificity.

Guidance By The Growth Substrate

Besides being guided by secreted and substrate-bound factors that provide directional information, growth cone migration requires a surface or matrix that is permissive to outgrowth. Adhesive contacts between growth cones and the growth substratum are part of growth cone migration (Gundersen, 1988). Such contacts are mediated by transmembrane receptors (e.g. integrins, adhesion molecules) that attach to cytoskeletal components intracellularly and bind constituents of the growth substratum extracellularly

(reviewed by Reichardt and Tomaselli, 1991; Hynes and Lander, 1992). On a functional level, guidance effects of the chemoattractants and repellents mentioned above must modulate, probably via intracellular pathways, interactions between the cytoskeleton, receptors, and the substratum. Of course, growth cone migration can also be modulated by components of the growth substratum itself: these include molecules on other cell surfaces, such as cadherins and members of the immunoglobulin (Ig) superfamily (e.g. NCAM, L1, and F3/F11), or molecules of the extracellular matrix (ECM) such as laminin, fibronectin, tenascin, and thrombospondin. The possibility that such molecules mediate axon extension is supported by the fact that they all affect neurite outgrowth when presented as substrata *in vitro* (reviewed by Reichardt and Tomaselli, 1991; Hynes and Lander, 1992; Lander, 1989; Jessel, 1988; Takeichi, 1991; Hortsch and Goodman, 1991; Lander and Calof, 1993).

The ability of the substratum to guide growth cones was, for some time, believed to be mediated by differential adhesion. Although growth cones may choose a more adhesive substratum when given a choice between surfaces of different adhesiveness (Letourneau, 1975), they can also choose a less adhesive surface for migration (Gunderson, 1987; Calof and Lander, 1991). In some cases, growth cones will not even recognize differences in substrate adhesiveness (Gomez and Letourneau, 1994; Lemmon et al., 1992). Thus, it is clear that growth cones are not guided solely by differences in adhesion. However, the substrate must contain sites to which growth cones can make adhesive contacts and, in this sense, the growth substrate can provide guidance information just by delineating paths where suitable contacts can occur. Indeed, *in vivo* manipulations indicate that Ig family members and their glucoconjugates likely mediate selective fasciculation (Tang et al., 1992, 1994; Grenningloh et al., 1991; Lin et al., 1994; Pimenta et al., 1995) and

deccussation at the midline (Stoeckli and Landmesser, 1995) via their ability to promote specific interactions between growth-cones and the substrate.

Much of what is known about the function of ECM molecules in axon development is inferred from *in vitro* studies. When plated onto culture plastic, laminin, fibronectin, tenascin, thrombospondin, and numerous proteoglycans (PGs) all either promote or inhibit adhesion and outgrowth. Some do both, depending upon experimental conditions (e.g. the combination of molecules being plated, type of assay). All of these molecules interact with each other or with smaller factors within the ECM, and thus many of their functions in axon guidance may not be revealed when they are plated in purified form on plastic. Indeed, PGs affect neurite outgrowth *in vitro* and have also been shown to bind other ECM glycoproteins (laminin, fibronectin, tenascin, and thrombospondin), adhesion molecules and cell surface receptors, growth factors, and molecules that putatively guide axons such as netrins and semaphorins (Raper and Kapfhammer, 1990; Colamarino and Tessier-Lavigne, 1995; Litwak et al., 1995 and reviewed by Ruoslahti, 1989; Lander, 1994). As ECM constituents that interact with many other ECM components, PGs likely play an important role in substrate-dependent axon outgrowth.

Although PGs are among the most abundant of the known ECM proteins in brain (Herndon and Lander, 1990; Lander and Calof, 1993), there is little understood about their role in axon guidance *in vivo*. From studies of development in non-neural tissues, a good deal is understood about binding interactions between PGs, particularly heparan sulfate proteoglycans (HSPGs), and secreted molecules such as multi-domain ECM proteins (e.g. collagen, fibronectin) and growth factors (reviewed by Gallagher, 1989; Ruoslahti, 1989; Lander, 1993). Dissociation constants vary ($\mu M > Kd > nM$) and molecules can bind both the protein component of PGs and/or the carbohydrate portions

known as glycosaminoglycans (GAG). Applying this knowledge to studies of the developing brain, however, is difficult since most ECM proteins and HSPGs that have been studied extensively are not major constituents of brain ECM (reviewed by Lander and Calof, 1993). The fact that the expression of PGs and GAGs is highly developmentally regulated, together with evidence that neuronal adhesion and outgrowth can be directly influenced by both PGs and many PG- and GAG-binding molecules, would suggest that PGs and GAGs could play an important role in axonal development (see next section for references).

CHONDROITIN SULFATE IN THE DEVELOPING NERVOUS SYSTEM

As prominent PGs in brain ECM (Margolis et al., 1975b), CSPGs are believed to play some role in axonal development (Letourneau et al., 1994). The reasons for this belief stem from several lines of evidence including the patterns of expression of CS and CSPGs in the developing nervous system, the fact that other types of PGs (namely heparan sulfate proteoglycans) are functionally involved in various morphogenic processes, the evidence that CS and CSPGs bind and interact with other proteins that directly affect neuronal morphology, and the fact that CS and several CSPGs can themselves affect neurite outgrowth *in vitro*.

Overview Of Proteoglycan Structure

PGs are defined as any glycoprotein consisting of a core protein post-translationally modified with one or more O-linked, GAG polysaccharides. There are four general classes of GAG: hyaluronic acid (HA), keratan sulfate (KS), chondroitin sulfate/dermatan sulfate (CS/DS), and heparin/heparan sulfate (HS). All are linear polymers of disaccharide repeats. Different disaccharides characterize the different GAGs, but all disaccharides are composed of a hexuronic acid and an amino sugar, except for keratan sulfate which consists of hexuronic acid and galactose. All GAGs are covalently attached to proteins except for HA which exists purely as an unbound polysaccharide.

For CSPGs and HSPGs, GAG synthesis entails the serial addition of sugar residues to a linkage trisaccharide (xylose-galactose-galactose) that is attached to the core protein via a serine residue. Within the peptide sequence, this serine is usually, but not always, followed by glycine residues that together

form a GAG "attachment site." Not all such sites are necessarily glycanated (Bourdon et al., 1987). Keratan sulfate proteoglycans (KSPGs) are synthesized as either O- or N-linked polysaccharides (Stuhlsatz et al., 1989). HA is synthesized without a core into the extracellular space by a cell surface synthase (Prehm, 1989).

Once synthesized, HS, KS, and CS can be covalently modified. HS has the greatest potential for structural diversity (most sites per disaccharide that can be modified) because it can be N-deacetylated, N- and O-sulfated at various sites, and its glucuronic acid residues can be epimerized at C_5 to become iduronic acid. CS can be modified by 4- or 6-O-sulfation of N-acetylgalactosamine and 2-O sulfation of glucuronic acid. Epimerization of glucuronic acid into iduronic acid in CS creates DS sequences. CSPGs that have a high DS content are often referred to simply as DSPGs.

Expression Of Chondroitin Sulfate Proteoglycans In The Nervous System

In the developing rat, the total content of GAG in the brain (compared to DNA content) consistently increases before reaching adult levels. The relative content among the major GAGs also changes during this period. HA is the dominant species in the embryonic brain, constituting over 60% of total brain GAG (Oohira et al., 1986). Initially the relative HS content is greater than CS content, but by mid-gestation, this situation has reversed (Oohira et al., 1986). Around birth, HA content begins to decline, and between the second and third postnatal weeks, CS becomes the predominant GAG (≈50% total GAG; Oohira et al., 1986; Margolis et al., 1975a). In addition to these changes, the expression of specific CSPG core proteins is also developmentally regulated (Herndon and Lander, 1990; Oohira et al., 1994; Meyer-Puttlitz et al., 1995).

In relation to developing axon pathways, CS shows strong immunostaining within regions that axons actively avoid, regions that coincide with axon pathways, and regions where differential pathfinding occurs between different types of axons. The posterior scleratome acts as a barrier to spinal axons (Tosney and Oakley, 1990), contains an activity that repels neurites *in vitro* (Davies et al., 1990), and immunostains strongly for CS (Oakley and Tosney, 1991). Similarly, the epidermis in chick shows high levels of CS expression (Kitamura, 1987) and sensory axons avoid this tissue when extending within the underlying dermal layer (Hemming et al., 1994). In contrast, staining for CS is strong along the thalamic axon pathway in the developing cerebral cortex both prior to and during the arrival of thalamic axons (Bicknese et al., 1994; Miller et al., 1995). Similar staining for CS along developing axons tracts can be seen in the cerebellum (Flaccus et al., 1991) and tectum, however in the tectum, CS expression along the retinal afferents is probably due to the axons themselves (McAdams and McLoon, 1995). The dorsal midline of the spinal cord and optic tectum also stain for CS and are sites that many axons avoid but that commissural axons enter (Oakley and Tosney, 1991; Hoffman et al., 1996).

Thus, based on these varied correlations between axon guidance and CS expression, if CS has a role in axon development, it either interacts with other molecules that convey further information to growth cones (discussed further below), it has multiple functions, or its functions are context-dependent. Possible sources for functional diversity could be the many CSPG core proteins that have been identified in the developing nervous system (Herndon and Lander, 1990) or the variable structures that can occur within CS polysaccharides.

CSPG core proteins in nervous tissue: a diverse set

CSPG core proteins in the nervous system include glypiated proteins covalently attached to membrane lipids via glycosyl phosphatidylinositol (GPI) linkages as well as transmembrane and secreted proteins. Many of them have been shown to directly affect neuronal morphology *in vitro* (discussed further below).

Aggregating CSPGs

These CSPGs aggregate with the GAG hyaluronic acid (HA) due to link protein-like domains that bind HA. At least four members belong to a gene family that have many structures in common including an immunoglobulin-like domain, EGF-like repeats, a lectin-like domain, and complementary regulatory protein-like domain (Margolis and Margolis, 1994)

Brevican, the smallest member of the family yet cloned, can be both secreted (Yamada et al., 1994) and GPI-anchored (Seidenbecher et al., 1995). Although the protein is expressed in adult rat hippocampus and cerebellum (Seidenbecher et al., 1995), its expression in developing brain is yet to be reported.

Versican (Zimmerman et al., 1989; Perides et al., 1992; Crawford et al., 1993) and neurocan (Rauch et al., 1992) are secreted proteins expressed in nervous tissue. Several axon pathways immunostain for neurocan. In the subplate of the cerebral cortex, staining is coincident with CS expression and is strong both prior to and during the outgrowth of thalamic axons in this layer (Miller et al., 1995). Neurocan also stains intensely in the ganglion axon layer of the developing retina (Meyer-Puttlitz et al., 1996) and the fiber tracts of developing cerebellum (Flaccus et al., 1991). In contrast, versican immunostaining is strong in the posterior scleratome, a barrier to extending

motor and sensory axons (Landolt et al., 1995). Within the nervous system, expression of versican, or a proteolytic product of versican (GHAP), occurs mostly postnatally, showing widespread immunostaining on neurons in the adult (Bignami et al., 1993).

Aggrecan, the largest member of the family, is a main structural component of cartilage, and some evidence indicates that it may be expressed in nervous tissue (Yamagata et al., 1993). Several large CSPGs in the brain have biochemical characteristics similar to aggrecan (e.g. size and HA-binding properties). These include the secreted proteins pgT1 from rat brain (Iwata and Carlson, 1991; Iwata and Carlson, 1993; Iwata et al., 1993), S103L from developing chick brain (Krueger et al., 1992), and Cat-301 from cat brain (Zaremba et al., 1989; Fryer et al., 1992). Some of these CSPGs, such as S103L and Cat-301, contain little or no KS (Domowicz et al., 1995; Frer et al., 1992), whereas aggrecan bears KS as well as CS chains. Antibodies that recognize a peptide common to versican and aggrecan fail to react with pgT1 (Iwata and Carlson, 1993). Thus, the relation of these molecules to the aggrecan family is unclear.

S103L expression can be detected in embryonic chick brain, it reaches a maximum postnatally, and declines in the adult (Schwartz et al., 1993). The expression of pgT1 occurs throughout the adult brain, in both white matter and on neuronal surfaces, but expression during development has not been examined (Iwata and Carlson, 1993). The expression of the Cat-301 antigen on neurons in the thalamus and spinal cord is activity-dependent and correlates with the end of critical periods in synaptic refinement (Sur et al., 1988a; Kalb and Hockfield, 1994).

Receptor-type protein tyrosine phosphatases (RPTPs)

Members of this family can act as cell surface receptors that influence intracellular signaling since they contain a transmembrane domain, a pair of intracellular tyrosine phosphatase domains, and an extracellular domain, part of which is homologous to carbonic anhydrase. One member, RPTPβ, is a nervous system-specific CSPG expressed in developing cerebral cortex, dorsal midline of the spinal cord, and along nerve fibers (Canoll et al., 1993; Levy et al., 1993; Shitara et al., 1994). Phosphacan is a secreted CSPG and an apparent splice variant of RPTPβ, consisting of just the extracellular domain (Maurel et al., 1994). Phosphacan immunostains intensely along the dorsal midline and sensory and motor nerves in the developing rat spinal cord as well as in the subplate of developing cortex (Meyer-Puttlitz et al., 1996); however, another report concludes that there is no layer-specific immunostaining for phosphacan in cortex (Miller et al., 1995).

Decorin and small proteoglycans

This family includes two CS/DSPGs, decorin (Day et al., 1987; Scholzen et al., 1994) and biglycan (Fisher et al., 1989), as well as two PGs that contain the GAG keratan sulfate, fibromodulin and lumican (reviewed by Kresse et al., 1993). Members of this family contain leucine-rich repeats that are likely to be involved in binding with other proteins such as collagen (Kobe and Deisenhofer, 1994), with which members of this family colocalize in tissues outside the nervous system (Pringle and Dodd, 1990). At least decorin is expressed in the nervous system, being detected in the ventral midline of the developing spinal cord and adult pons in mice (Scholzen et al., 1994), schwann cells in the peripheral nervous system of adult rat (Hanemann et al., 1993),

and within the degenerative lesions seen in Alzheimer's diseased brain (Snow et al., 1992).

NG2

This transmembrane CSPG contains a large extracellular and a small cytoplasmic domain and does not have extensive homology with any other cloned molecules (Nishiyama et al., 1991). Its expression is developmentally regulated and widespread in nervous system including on O2A glial progenitors in developing optic nerve and cerebellum (Levine and Stallcup, 1987; Stallcup and Beasley, 1987; Levine and Card, 1987).

Neuroglycan C

This recently cloned CSPG is a nervous system-specific, transmembrane protein which also shows little homology to other known proteins. Both mRNA and immunoreactive material can be detected in neonatal and adult rat brain. Overall levels of protein expression are developmentally regulated and neonatal cerebral cortex immunostains for the protein (Watanabe et al., 1995).

Syndecan-1 and other syndecans

Although members of this four-member family (Bernfield et al., 1992) are typically considered HSPGs, at least syndecan-1 can also be a hybrid PG containing both CS and HS (Brauker et al., 1991). Messenger RNA for all of these transmembrane proteins can be detected in brain (Saunders et al., 1989; Carey et al., 1992; Kim et al., 1994), yet only syndecan-1, -3, and -4 have been shown to immunostain in nervous tissue (Corless et al., 1992; David et al., 1993; Baciu et al., 1993; Miller et al., 1995). In particular, syndecan-1 is expressed in developing cerebral cortex (Miller et al., 1995).

DSD-1PG

This large CS/DSPG is expressed on the surface of astrocytes and oligodendrocytes in developing mouse brain. It was identified with a monoclonal antibody that recognizes a CS/DS epitope that is apparently unique to this PG (Faissner et al., 1994). Structure of the core protein has not been characterized.

Astrochondrin

Three large CSPG core proteins with similar peptide maps are expressed by astrocytes of developing brain. These glycoproteins have in common, along with several cell adhesion molecules, a non-CS carbohydrate epitope, L2/HNK-1. The structure of the core proteins has yet to be deduced so its relation to other CSPGs is unclear. Immunostaining for astrochondrin in developing cerebellum shows an association of this CSPG with Bergmann glial process (Streit et al., 1990, 1993).

Amyloid precursor-like proteins (APLPs)

β-amyloid is a component of degenerative plaques found in Alzheimer's diseased brain and is derived from larger amyloid precursor proteins (APP) that belong to the extended APLP family. One member of this family, APLP-2, can be a CSPG if alternatively spliced to remove an exon that prevents GAG attachment (Thinakaran et al., 1995). APLP2 containing the exon is expressed in adult brain (Sandbrink et al., 1994), yet interestingly, transcripts lacking the exon (a putative CSPG) are selectively expressed in olfactory sensory neurons (Thinakaran et al., 1995), a neuronal population that continues to proliferate in the adult. Immunologically identified APLPs that are CSPGs have been

detected in the developing and adult rat brain and in cultured astrocytes (Oohira et al., 1995).

CD44

This cell surface receptor that binds hyaluronic acid is expressed in the developing nervous system (Vogel et al., 1992; Sretavan et al., 1994). A cell surface CSPG that is a likely splice variant of this protein has been identified (Faassen et al., 1992), but no reports address whether it is expressed in nervous tissue.

structure of CS: potential for diversity

Both the number and length of GAG chains attached to a core protein can vary. The aggregating CSPGs have multiple GAG attachment sites. Aggrecan can contain over 100 CS chains and 30 KS chains (Doege et al., 1987), whereas versican has approximately a dozen potential CS attachment sites (Zimmerman and Ruoslahti, 1989), neurocan 7 (Rauch et al., 1992), and brevican only 3 (Yamada et al., 1994). On the other hand some PGs, such as decorin (Chopra et al., 1985), have only one GAG attachment site.

The disaccharides that constitute different GAGs can affect the secondary structures of these molecules. For example, HA is rigid in aqueous solutions due to intramolecular hydrogen bonding that can occur because HA contains no covalent modifications (Scott, 1989). This fact is consistent with the notion that HA in the ECM occupies a great amount of hydrated space (Laurent, 1970) and may play a structural role in tissue development (Toole, 1976; and chapter 3). On the other hand, HS and DS are likely to be more flexible than HA due to the presence of iduronic acid which adds a degree of rotational freedom (Casu et al., 1988). Based on relations between primary

and secondary structure of these GAGs, one might expect CS to have flexibility somewhat between that of HA and HS or DS, since CS contains covalent modifications but does not contain iduronic acid.

Besides affecting secondary structure, the chemical composition of GAGs can also affect the functional diversity of GAG-protein interactions. HA binds some cell surface and secreted molecules (e.g. CD44, BEHAB, link protein, aggregating CSPGs), but there is little potential for functional diversity since HA is not modified and has no structural variability. HS, having the greatest potential for chemical variation, binds many more known proteins (Ruoslahti, 1989; Lander, 1994). In some cases, the negatively charged sulfate groups on HS may mediate some binding to positively charged amino acid side chains on GAG-binding proteins (Olson et al., 1991). Yet, there are several examples in which the specificity of GAG-protein binding requires particularly modified HS sequences and certain protein domains. These include the binding of HS to various members of the fibroblast growth factor family (Habuchi et al., 1992; Turnbull et al., 1992; Mach et al., 1993) and binding of a specifically modified HS pentasaccharide to antithrombin III (Atha et al., 1984; Lindahl et al., 1984; Atha et al., 1985).

Whether or not such specificity plays a role in the function of CS and GAG-binding proteins in nervous system development is unclear; however, two examples indicate that it is possible. Outside the nervous system, a particularly modified DS sequence specifically binds heparin cofactor II (Maimone and Tollefsen, 1990), demonstrating that covalent modifications of CS can dictate specificity in protein binding. Moreover, the nervous system specific CSPG, DSD-1PG, promotes *in vitro* neurite outgrowth in a manner dependent upon unique GAG sequences within the protein's CS chains. These sequences, most likely DS, are recognized by the DSD-1 monoclonal antibody

and can be selectively removed or retained using specific CS degrading enzymes. Removal of these epitopes or application of the antibody abolishes the neurite promoting properties of the entire PG (Faissner et al., 1994). Thus, it is conceivable that within the developing brain, structural variability of CS has functional consequences. These studies also raise the possibility that native CS structures, required for function, may be absent or lacking in commercial CS preparations.

Interactions Between Chondroitin Sulfate Proteoglycans And Other Proteins

Another possible explanation for why CS expression correlates both with barriers to axon outgrowth and pathways taken by developing axons is that CSPGs could interact with a functionally diverse set of molecules that modulate growth cone guidance. Indeed, some CSPGs have been shown to bind such proteins. For example, the cell surface adhesion and outgrowth promoting molecules NCAM and NgCAM (L1/NILE) specifically bind the CSPG neurocan (Kd ≈1 nM; Friedlander et al., 1994). If CS is digested from neurocan, binding is substantially reduced. It is not known if these interactions involve the HS binding domains known to be present in NCAM and NgCAM. Another cell surface protein that modulates cell adhesion, N-Acetylgalactosaminyl-phosphotransferase (NAcGalPTase, Bauer et al., 1992), binds an unidentified CSPG isolated from chick brain (Balsamo et al., 1995). In this case, the binding can occur whether CS chains have been digested or not.

There is also evidence that CSPGs bind ECM molecules. For example, phosphacan binds a member of the tenascin family (Kd ≈3 nM; Grumet et al., 1994), a set of multi-domain ECM molecules that have diverse effects on

neuronal adhesion and outgrowth under various conditions *in vitro* (Pesheva et al., 1989; Faissner and Kruse, 1990; and reviewed in Sage and Bornstein, 1991). Binding of other CSPGs to tenascin has been inferred by utilizing microspheres coated with covalently attached proteins. Microspheres coated with tenascin bind microspheres coated with neurocan and RPTPβ, but not with those coated with aggrecan (Grumet et al., 1994; Barnea et al., 1994; Hoffman and Edelman, 1987). Aggregation can be inhibited by antibodies against these molecules or soluble CSPGs. Utilizing GAG digesting enzymes, the authors of these reports conclude that CS is not involved in the binding of these PGs to tenascin. However, the same conclusion was reached using this technique to analyze the binding of neurocan to NCAM (Grumet et al., 1993), and it was subsequently revised when more rigorous biochemical analyses were used (mentioned above, Friedlander et al., 1994). Interestingly, a recombinant lectin domain of versican has been shown to bind carbohydrate residues on tenascin-R (Aspberg et al., 1995), suggesting that versican may bind this molecule in a manner that is CS-independent.

Thrombospondin, another ECM molecule that can affect adhesion and process outgrowth (Adams and Lawler, 1993; Taylor et al., 1993; and reviewed in Sage and Bornstein, 1991), can also bind CSPGs. Decorin binds thrombospondin in a solid phase binding assay under physiological salt concentrations (Kd ≈5nM), as does the isolated core protein. The CS chains from decorin bind Sepharose-bound thrombospondin (Winnemöller et al., 1992). Additional evidence also indicates that CS alone binds thrombospondin (Pancake et al., 1992; Holt et al., 1990; Herndon, 1996), but in most of these cases, HS is found to be a better ligand. Indeed, except for heparin cofactor II (mentioned above), most GAG-binding molecules bind HS better than CS/DS (Herndon, 1996). Yet, it is not clear how relevant this point is to brain

development given that CS is much more prevalent than HS during most of brain morphogenesis.

In addition to these interactions with molecules known to affect neurite outgrowth, CS also binds some proteins whose effects on neuronal morphology have not been studied. A novel protein that is the homologue of a drosophila cell cycle control protein binds both CS and other GAGs (Grammatikakis et al., 1995). In addition, interferon gamma binds a CSPG secreted by smooth muscle cells. Digestion of CS releases the interferon from ECM created by these cells *in vitro* (Camejo et al., 1995).

Functions Of Chondroitin Sulfate Proteoglycans

Although several CSPG core proteins have been cloned and a good deal is understood about CSPG synthesis, little is understood about the function of these molecules *in vivo*. There are no reports of transgenic animals and few of *in vivo* manipulations to provide insight about function. Most studies examining CSPG function in development have utilized *in vitro* techniques and have addressed the role of these molecules in adhesion and the motile behaviors of cells -- migration and process extension. However, a few studies have found that CSPGs can also act to promote neuronal survival *in vitro* (Nichol et al., 1994), mitigate the deleterious effects of excitotoxins (Okamoto et al., 1994), and play a role in the clustering of acetylcholine receptors during synaptogenesis (Mook-Jung and Gordon, 1995).

<u>CSPGs: adhesion and migration in non-neural tissues</u>

Studies using various non-neuronal cells have implicated CSPGs as mediators of cell adhesion and migration. The addition of exogenous CSPGs (aggregating type) inhibits cell migration on substrata composed of ECM

molecules such as laminin, fibronectin, vitronectin, elastin, and collagen (Perris et al., 1987). In some cases, exogenous CSPGs also inhibit cell attachment or adhesion to such substrata (Rich et al., 1981; Yamagata et al., 1989). In addition, enzymatic digestion of CS and HA in developing Xenopus allows migrating melanophores to enter a region they normally encounter but avoid (Tucker et al., 1986). For neural crest cells, the inhibition of migration requires the binding of CSPGs to the cell surface, as opposed to the substratum, and requires both CS chains, core protein (including HA-binding domain), and cell surface HA. These data suggest that CSPGs might inhibit migration by binding to cell surface HA and, in turn, inhibit interactions between HA and HA-binding proteins that mediate migration (Perris and Johansson, 1990).

However, the inhibitory effects of CSPGs are not always HA-dependent. Neural crest cells will avoid notocord explants in culture but will not find notocords inhibitory if cultures are treated with an enzyme that digests CS. Application of an enzyme that specifically digests HA produces no effect (Newgreen et al., 1986). Furthermore, CSPGs that do not bind HA, such as decorin, can also inhibit adhesion when added exogenously to cultures (Lewandowska et al., 1987; Bidanset et al., 1992). From these data, it can not be determined whether CSPGs act directly on neurons (i.e. bind specific receptors) or if CSPGs perturb the function of other molecules that directly affect adhesion and migration, possibly by competing with endogenous GAGs for GAG-binding sites on relevant proteins.

CSPGs as substrata for neurite outgrowth

Simplified *in vitro* models have been used by many researchers to assess the effects of CSPGs on neuronal morphology, adhesion, and process

outgrowth. Together, these studies, like those that examined *in vivo* expression, produced conflicting results from which no generalities about CSPG function in axon guidance can be cast. Many CSPGs, including neurocan (Friedlander et al., 1994), NG2 (Dou and Levine, 1994), and aggrecan (Snow et al., 1990a) inhibit neurite extension when plated in combination with molecules that are permissive for or promote outgrowth. Furthermore, CS alone was found to inhibit outgrowth when plated in combination with collagen (Verna et al., 1989) or synthetic polymers (Carbonetto et al., 1983). However, CS and CSPGs have also been found to promote outgrowth. When CS is plated with laminin (Fernaud-Espinosa et al., 1994) and poly-ornithine (Lafont et al., 1992), outgrowth is enhanced. DSD-1PG also promotes outgrowth when plated with poly-ornithine but only if its unique CS epitopes are left intact (Faissner et al., 1994). In contrast, the core proteins of CSPGs from neonatal rat brain can promote outgrowth in the absence of their CS chains (Iijima et al., 1991).

The relevance of these *in vitro* studies to CSPG function *in vivo* is not clear. Indeed, neurocan inhibits outgrowth *in vitro* but several axon pathways immunostain strongly for this core protein. These *in vitro* studies may produce results inconsistent with each other or with *in vivo* expression because they were designed under the assumption that CSPGs, as substrata, act directly on neurons. In many cases, particularly for the large CSPGs, inhibition may simply be due to steric hindrance of cell binding sites (cf. Lightner and Erickson, 1990). Furthermore, interpretation of data may be confounded by the fact that cells in culture secrete molecules that have autocrine effects on cell growth and morphology and can bind certain CSPGs (Yamaguchi et al., 1990). This may explain why phosphacan inhibits outgrowth when plated as a substratum only if cells are cultured at high, but not low, density (Maeda and

Noda, 1996). Thus, one possibility is that CSPGs produce various *in vitro* effects because they can interact with other molecules that directly bind and affect neurons (see chapter 3).

Nonetheless, one previously mentioned study has demonstrated that the effects of a particular CSPG are due to its binding a cell surface receptor. Addition of a chick brain CSPG to the medium of retinal cell cultures inhibits adhesion and outgrowth on cadherin substrata. Importantly, the authors demonstrated that the CSPG specifically binds, not the molecules of the substratum, but the cell surface NAcGalPTase that is involved in the inhibition (Balsamo et al., 1995).

CSPGs modulate neurite outgrowth in organotypic cultures

A few studies have assessed CSPG function in process outgrowth by perturbing CSPG expression in organotypic cultures. For example, the avoidance of epidermal explants by dorsal root ganglia neurites *in vitro* can be attenuated by adding to cultures β-xyloside, a competitive inhibitor of GAG attachment to core proteins (Fichard et al., 1991). Although β-xyloside preferentially inhibits CSPG synthesis, it does attenuate synthesis of HSPGs as well, and no controls were performed to address whether this latter activity caused the observed effects on neurite outgrowth. The results, however, are at least consistent with the notion that CSPGs are involved in the inhibition of process extension, as the authors of this study suggest.

A similar conclusion was drawn from a study in which endogenous CS was enzymatically digested within explants of developing retina using chondroitinase ABC. In particular, processes from ganglion cells that normally extend toward the optic disc showed aberrant growth into regions that contained CS prior to digestion and that they would normally not enter (Brittis

et al., 1992). Either cell migration and/or differentiation was also perturbed since neurons that differentiated during treatment were observed in aberrant locations. This study, however, did not eliminate the possibility that the effects were caused by the removal of endogenous HA, which is also digested by chondroitinase ABC. Interestingly, exposure of retinal explants to exogenous CS causes many of the same effects seen by digestion of endogenous CS (Brittis and Silver, 1994), putting into question hypotheses proposing that CS or CSPGs act directly on neurons in these morphogenic processes.

CSPGs and axon development: future directions

Given all the data implicating CSPGs in the regulation of process outgrowth, it is worthy to hypothesize that CSPGs have a role in axon development. However, reports addressing this issue have produced no unifying models, lack appropriate controls concerning the role of other GAGs and PGs in observed effects, or make assumptions about the exact mechanism of CS or CSPG action. As with other studies aimed at understanding molecules that guide axons (discussed above), assessing the role of CSPGs in axon development should be done within the context of well defined axon guidance events that are suspected of occurring *in vivo*. Thus, examining a system whose development is well characterized and correlates with CS expression would prove a useful model to address whether CSPGs play a role in axon guidance.

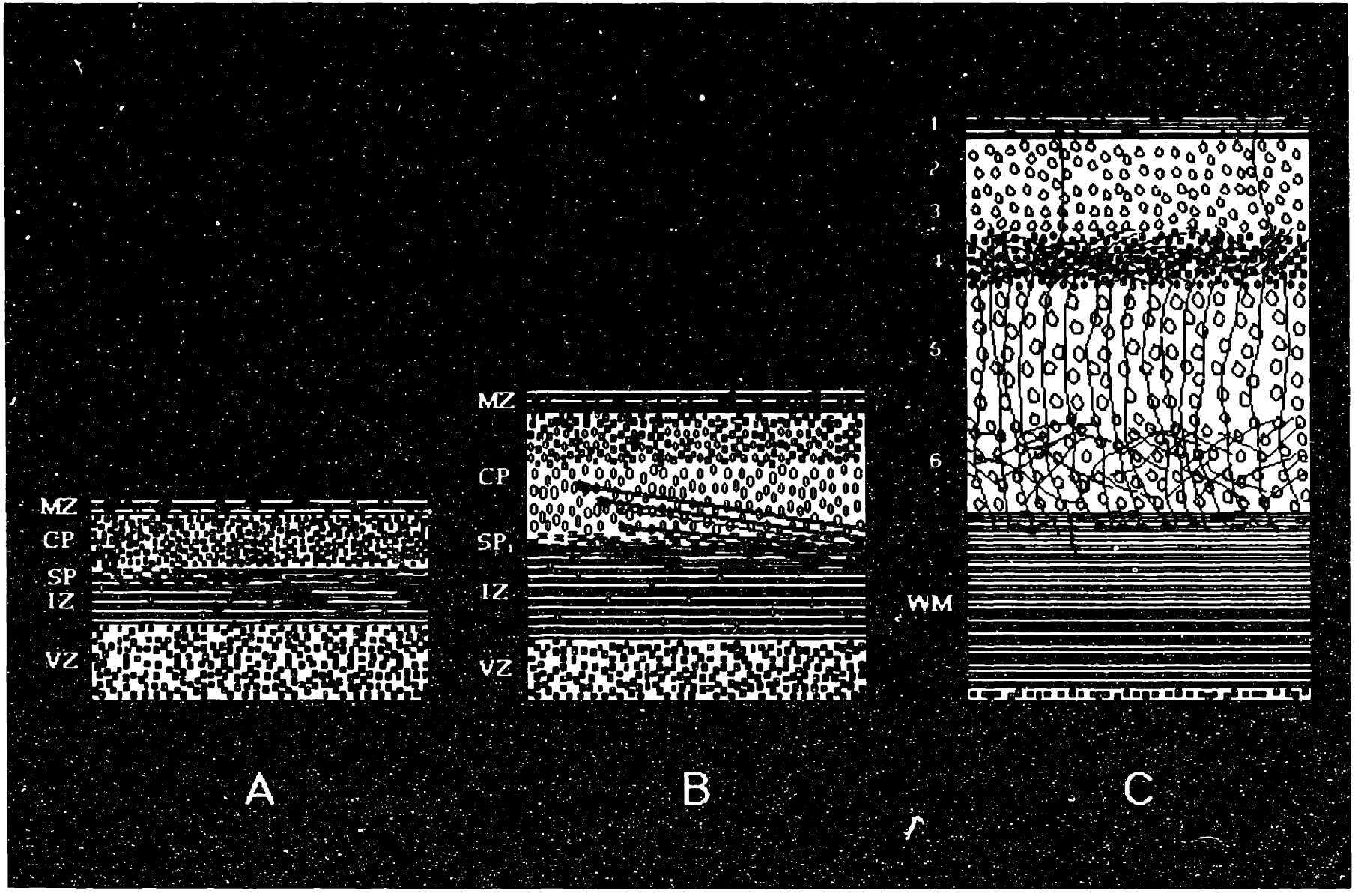
THALAMOCORTICAL DEVELOPMENT

In the adult mouse, the cerebral neocortex consists of six main layers (gray matter) that are distinguishable by cell density, neuronal morphology, and connectivity. Thalamic axons constitute the main input to the cerebral cortex and, in the adult, extend within the white matter before projecting toward the pial surface and into the numbered layers I-VI of the cortex (Fig 1c). All thalamic axons project to specific cortical layers (mostly IV and VI) along the radial axis of the cortical mantle and axons from particular thalamic nuclei project to specific cortical regions along the tangential axis (reviewed by Jones, 1985). Hence, during development, there must be mechanisms that provide for both laminar and regional specification of thalamocortical connections.

Lamination Of Cerebral Neocortex

Thalamic innervation of cortex occurs in concert with cortical lamination and efferent development, making these processes an important determinant of the environment that thalamic growth cones encounter. Tritiated thymidine birthdating has indicated that post-mitotic neurons are born in the ventricular neuroepithelium (ventricular zone) and migrate superficially, toward the pial surface. The oldest neurons originally form a layer (plexiform layer or preplate) between the pia and the neuroepithelium, but this layer is later bifurcated, into the subplate and marginal zone, by newly arrived neurons which form the cortical plate (Marin-Padilla, 1971, 1972; Kostovic and Molliver, 1974; Rickmann at al., 1977; Raedler and Raedler, 1978; Luskin and Shatz, 1985a; Bayer and Altman, 1991; Wood et al., 1992). Cortical layers II-VI differentiate in an inside-out manner as the deepest aspect

Figure 1. Pathway decisions made by developing thalamic axons. Layers of developing (A,B) and adult (C) cerebral cortex are indicated as at left of each panel. In the embryonic mouse (A), thalamic axons (red) extend mainly within the subplate (SP), but are also found in the intermediate zone (IZ) and ventricular zone (VZ). At this stage, thalamic growth cones encounter but do not enter the cortical plate (CP). Other axons extend within the marginal zone (MZ). As the cortical plate thickens due to post-mitotic neurons arriving from the ventricular zone (B), differentiation of numbered cortical layers occurs and thalamic axons change their course to enter the cortical plate. As development proceeds, layers 2-6 form out of the cortical plate, the marginal zone becomes layer 1, the subplate and intermediate zone become the white matter (WM), and the ventricular zone becomes the ependyma (C). Thalamic axons extend through developing layers 6 and 5, and most terminate in layer 4. A small subset of thalamic axons extend up to layer 1 (see text for references).


MZ
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VZ
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MZ
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B
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WM
C

of the cortical plate (Layer VI), adjacent to the subplate, differentiates first (Angevine, and Sidman, 1961; Hicks and D'Amato, 1968; Rakic, 1974; Luskin and Shatz, 1985b; Shimada and Langman, 1970). Younger neurons migrate through older layers, toward the marginal zone, via paths that cause both radial and tangential dispersion of neurons from their original birthplace (Walsh and Cepko, 1993; Tan and Breen, 1993; O'Rourke et al., 1992, 1995). Eventually, the ventricular zone becomes the ependyma, the marginal zone becomes layer I, and the subplate, along with the intermediate zone, becomes the white matter (Chun and Shatz, 1989; Woo et al., 1991).

The first axons to extend in the cortex are projected by the subplate neurons while they are still situated in the preplate, prior to its division by the cortical plate cells (Shatz and Rakic, 1981; McConnell at al., 1989; DeCarlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992; Bicknese et al., 1994). However, later born, layer 5 neurons project the first processes that reach the thalamus (Clasca et al., 1995). Within the cortex, efferents mostly extend within the intermediate zone between the subplate cell layer and the ventricular zone (Bicknese et al., 1994). The cortical efferents pioneer the main axon tract in the cortex (future white matter) as well as the internal capsule where they meet arriving thalamic afferents (see below).

Thalamic Axon Pathway Development - Descriptive Analyses

Thalamic growth cones enter the cerebral cortex via the internal capsule before their layer IV target cells are born (McConnell et al., 1989; Ghosh and Shatz, 1992a; Blakemore and Molnár, 1990; Reinoso and O'Leary, 1990; DeCarlos and O'Leary, 1992). Axons generally extend in the cerebral mantle between the subplate efferents and the cortical plate (Bicknese et al., 1994); however, some extending axons can be found within the intermediate and

ventricular zones (Reinoso and O'Leary, 1988; McConnell et al., 1989; Ghosh and Shatz, 1992a; Blakemore and Molnár, 1990; DeCarlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992).

Initially, thalamic growth cones migrate parallel with the laminae, encountering but not entering the cortical plate (Fig. 1a), where their future target cells will reside (Rakic, 1976; Lund and Mustari, 1977; Crandall and Caviness, 1984; Shatz and Luskin, 1986; Reinoso and O'Leary, 1988; Catalano et al., 1991; Kageyama and Robertson, 1993; Catalano et al., 1996). Thalamic axons extend collateral branches perpendicular to the layers within the subplate (Ghosh and Shatz, 1992a), and some of their growth cones probe the deepest aspect of the cortical plate (Naegle et al., 1988). In addition, thalamic axons form synaptic contacts with subplate cells (Herrmann et al., 1994). Initial studies found this period, during which thalamic axons are confined to the subplate, as relatively long in cats and primates and it was subsequently termed the "waiting period" (Rakic, 1977; Wise and Jones, 1978; Shatz and Luskin, 1986); however, it can be quite short in rodents (Catalano et al., 1991).

Once the cortical plate begins to differentiate, thalamic growth cones enter it (Fig. 1b), changing their course of migration toward the pial surface (Catalano et al., 1991; Blakemore and Molnár, 1990; DeCarlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992; Kageyama and Robertson, 1993). Primary and collateral branches extend into the cortical plate at angles both oblique and perpendicular to the cortical layers (Catalano et al., 1996). Growth cones pass through successively differentiating layers, but do not enter the most superficial, undifferentiated portion of the cortical plate (Catalano et al., 1991; Miller et al., 1993). Most thalamic axons terminate in layer IV and arborize in layers IV and VI (Fig. 1c), their main targets (Shatz and Luskin, 1986; Naegle et al., 1988; DeCarlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992; Miller et

al., 1993; Agmon et al., 1993; Kageyama and Robertson, 1993). A small subset of thalamic axons extend, unbranched, up to layer I (Shatz and Luskin, 1986; Naegle et al., 1988).

Projections of different thalamic nuclei to specific cortical regions correlates with different sensory modalities (e.g. lateral geniculate nucleus to visual cortex, medial geniculate nucleus to auditory cortex, ventral basal nucleus to somatosensory cortex, etc.). Axons accumulate within the subplate of appropriate cortical regions before invading the cortical plate (Lund and Mustari, 1977; Rakic, 1977; Shatz and Luskin, 1986; Wise and Jones, 1978; Catalano et al., 1991; Ghosh and Shatz, 1992a). Some reports indicate that axons (Reinoso and O'Leary, 1988) and collateral branches will extend into the subplate and deep cortical plate of "inappropriate" cortical areas (Ghosh and Shatz, 1992a; Naegle et al., 1988), however other reports claim that inappropriate projections do not enter the cortical plate (Crandall and Caviness, 1984; De Carlos and O'Leary, 1992). It is clear that extraneous collaterals are absent at the time when thalamic axons in layer IV begin displaying the region-specific arborizations that depend upon sensory modality -- such as ocular dominance columns in visual cortex (Hubel et al., 1977; LeVay et al., 1978, 1980) or barrels in somatosensory cortex (Killackey and Leshin, 1975; Wise and Jones, 1978; Erzurumlu and Jhaveri, 1990; Schlaggar and O'Leary, 1993, 1994). Interestingly, some region-specific axon patterning can be detected prior to innervation or even emergence of the final target, layer IV (Schlaggar and O'Leary, 1994).

Cues That Guide Thalamic Axons

Similar to the conclusions drawn from studies of other axon systems (outlined previously), the development of thalamocortical projections depends

upon both intrinsic and extrinsic factors. *In vitro,* thalamic explants project processes that find layer IV neurons in cortical tissue slices and form functioning connections with them (Yamamoto, et al., 1989, 1992; Bolz et al., 1992; Molnár and Blakemore, 1991), indicating that growth cones are able to locate target tissue in the absence of sensory input. Indeed, electrical activity is apparently not even necessary for pathfinding since, *in vivo,* thalamic axons still arrive at layer IV in the appropriate cortical regions after blockade of sodium action potentials. Under this treatment, however, axons do not form the expected arborizations within layer IV (Herrmann and Shatz, 1995) and blockade of sensory or electrical input from the eye prevents the segregation of thalamic arbors that creates ocular dominance columns (Sherman and Spear, 1982; Stryker and Harris, 1986). Thus, once thalamic axons have reached their target, extrinsic factors are required for the precise refinement of connections

For both early and late stages of thalamocortical development, subplate neurons play a necessary role in guidance and proper axon arborization. As mentioned above, the subplate constitutes the initial cortical pathway for most thalamic axons and thalamic growth cones interact extensively with subplate cells. Ablation of subplate cells by excitotoxins causes thalamic axons from the lateral and medial geniculate nuclei to extend past their appropriate cortical regions, and thus, fail to invade the cortical plate in the proper areas (Ghosh and Shatz, 1993). Ablation at later periods, after thalamic axons have already reached layer IV, prevents the formation of ocular dominance columns in visual cortex. At even later stages, just prior to arbor segregation into columns, ablation of subplate cells causes axons that are normally restricted to layer IV to extend into layers II-III (Ghosh and Shatz, 1994). Thus, the subplate provides cues necessary for thalamic axon pathfinding to appropriate cortical laminae and regions as well as for the subsequent layer- and region-

specific remodeling that ensues after synaptic connections with targets have been established.

The specification of thalamic axon outgrowth within and to particular layers is likely to be regulated by laminar-specific cues. Some have hypothesized that layer IV contains a "stop signal" for extending thalamic axons based on the fact that thalamic neurites will form connections with layer IV neurons in thalamocortical slice cocultures (Yamamoto, et al., 1989, 1992; Bolz et al., 1992; Molnár and Blakemore, 1991). However in these studies, growth cone migration was not monitored and thus it is unknown whether axons stopped extending specifically at layer IV or whether exuberant connections were selectively eliminated to leave connections to layer IV intact. Thus, from these studies it can only be concluded that target recognition cues exist within layer 4. Thalamic neurites will prefer growth on membranes extracted from the infragranular laminae (layers V-VI) as opposed to growth on other layers (Götz et al., 1992). Given that the axons must pass through layers V-VI to reach target layer IV, this result indicates that the relative permissiveness of cortical layers to process outgrowth may help define the thalamic pathway. However in these studies, it is not clear whether thalamic axon membranes within the preparation contribute to the preference (see below).

A similar study examining the stage of development when thalamic axons have not yet invaded the cortical plate also found laminar-specific outgrowth. *In vitro*, thalamic neurites avoid membranes extracted from the cortical plate (plus marginal zone) when they are given a choice for growth on membranes taken from whole cortex (Tuttle et al., 1995), suggesting that the cortical plate is a less preferred substratum than other layers. This may explain why the cortical plate is not invaded by thalamic axons when they

initially encounter it. Because thalamic axons intrinsic to cortical tissue will also be included in membrane preparations used in these studies, some of the factors that produce *in vitro* growth preferences may be produced by thalamic axons themselves. At the very least, this possibility indicates that homotypic interactions between early and late arriving thalamic axons could contribute to pathfinding. In any case, these experiments demonstrate that molecules which affect process outgrowth are expressed in a layer-specific manner and may contribute to the layer-specific development of thalamic axons. Further experiments are needed to establish the nature and detailed location of these factors (see chapters 2 and 3 for further discussion and more data).

Studies aimed at understanding the regional specification of thalamocortical connections have provided ample evidence that thalamic projections regulate region-specific cortical architecture (reviewed by Sur, 1993; O'Leary et al., 1994). Yet, less is understood about the mechanisms that regulate the region-specific projections of thalamic axons. It is tempting to hypothesize that thalamic axons are guided to appropriate cortical regions by gradients or the regionalized expression of guidance molecules, similar to models concerning the topographic specificity of retino-tectal projections. As of yet, there is little evidence for this proposal. Neurites from explants of lateral geniculate thalamus fail to discriminate between different regions of neocortex *in vitro* (Molnár and Blakemore, 1991). In contrast, thalamic axons normally destined for the limbic cortex will reroute to heterotopic transplants of this target tissue placed within neocortex (Barbe and Levitt, 1992). Thus, recognition molecules may play a role in specifying connections among neo- and paleocortex. However, for specification among neocortical regions, there is not enough data to even discriminate between different hypotheses, such as

those based on molecular recognition or the controlled timing of thalamic innervation.

CHAPTER II

Laminar Specific Attachment and Neurite Outgrowth of Thalamic Neurons on Cultured Slices of Developing Cerebral Neocortex

ABSTRACT

In nervous system development, the growth cones of advancing axons are thought to navigate to their targets by recognizing cell-surface and extracellular matrix molecules that act as specific guidance cues. To identify and map cues that guide the growth of a particular axonal system, the thalamocortical afferents, an assay was devised to examine short-term interactions of dissociated embryonic thalamic cells with living, ~150 μm slices of developing mouse forebrain. Thalamic cells rapidly (<3 hours) and efficiently attached to and extended neurites on pre- and postnatal slices, but a broad zone throughout the neocortex was generally non-permissive for both thalamic cell attachment and the ingrowth of neurites. This zone coincided with the cortical plate at early stages (embryonic day 15), but later became restricted, in rostral-to-caudal fashion, to cortical laminae 2/3. Thus, at each stage, thalamic cells *in vitro* avoided just that area that thalamic axons confront, but generally do not enter, *in vivo*. In addition, neurites that extended on some layers were found to be significantly oriented in directions that coincide with the pathways that thalamic axons follow *in vivo*. These results imply that local adhesive cues and signals that affect process outgrowth are distributed among developing cortical laminae in a manner that could underlie much of the temporal and spatial patterning of thalamocortical innervation.

INTRODUCTION

During brain development, the growth cones of advancing axons often travel relatively long distances to reach their targets. Evidently, this process involves specific decisions made by growth cones as they navigate (reviewed by Dodd and Jessell, 1988; Hynes and Lander, 1992). The formation of connections between the thalamus and the cerebral cortex is an attractive model for studying axonal development within the mammalian brain because the anatomy and timing of the growth of this pathway is relatively well characterized (reviewed by O'Leary and Koester, 1993).

In rodents, thalamic growth cones leave the thalamus and travel through the ganglionic eminence (anlage of the basal forebrain), to enter the developing cerebral cortex well before most of their target cells are born. The growth cones then travel mostly within the intermediate zone and subplate in a pathway that runs tangential to the cortical surface (the pia) and opposite in direction to the simultaneous growth of cortical efferents. Concurrently, the overlying cortical plate thickens as postmitotic neurons, including the target cells of thalamic axons, arrive from their birthplace in the ventricular zone. Thalamic growth cones project into the cell dense cortical plate only after the latter has begun to differentiate into distinguishable laminae (Lund and Mustari, 1977), eventually becoming cortical layers 2-6, which form in an "inside-out" fashion (reviewed by DeCarlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992; Kageyama and Robertson, 1993). After entering the cortical plate, thalamic growth cones migrate toward the pial surface, passing through the infragranular laminae (5/6), to the granular layer (4) or the lower portion of layer 3 where most axons terminate and arborize (Miller et al., 1993; Kageyama and Robertson, 1993; Agmon et al., 1993).

There is both *in vivo* and *in vitro* evidence suggesting that the pathway decisions made by thalamocortical afferents are controlled by cues residing in specific cortical layers. For example, Ghosh et al. (1990) have shown that ablating subplate neurons in developing animals prevents the innervation of the cortical plate by thalamic afferents. Studies of long term, thalamocortical co-cultures (Yamamoto et al., 1989, 1992; Molnár and Blakemore, 1991; Bolz et al., 1992; Götz et al., 1992) have demonstrated that axons extending from thalamic explants onto postnatal cortical slices generally stop growth in or near layer 4, the major target of these axons *in vivo*.

We wished to exploit the simplicity of the *in vitro* approach to look for some of the cues that guide thalamocortical afferents, especially those that might only be present transiently, and would therefore be difficult to identify in long-term explant co-cultures. Consequently, embryonic thalamic cells were dissociated, fluorescently labeled, and plated directly onto living, vibratome slices of mouse forebrain taken from different developmental stages. Cells were examined both for attachment and neurite outgrowth during one day in culture.

Within 1 hour of coculture, cells were observed to attach well to all embryonic (E15) cortical layers except for the cortical plate, which supported poor attachment. Neurite outgrowth was observed within 3 hours and, on some laminae, exhibited a significant tangential orientation. However, no neurites were observed to enter the cortical plate. As slices from older animals were used, the non-permissiveness of the cortical plate gradually changed, in a manner that correlated both spatially and temporally with ongoing events in thalamocortical development. These results indicate that lamina-specific signals that affect thalamic neuron behavior can be detected in as little as 3 hours *in vitro*. Moreover, they suggest that as simple a mechanism as local

differences in cell adhesion could play a major role in dictating the timing and location of thalamocortical innervation.

MATERIAL AND METHODS

All salts, sucrose, and glucose were purchased from Mallinckrodt. Tissue culture media, sodium pyruvate, penicillin, and streptomycin were purchased from Mediatech. All other reagents were purchased from Sigma unless otherwise indicated.

Dissection Of Tissue

Random-bred Swiss mice (CD-1, Charles River Laboratories) were naturally mated. Day of identification of vaginal plug was considered E0 and day of birth P0. For embryonic tissue, pregnant animals were killed by cervical dislocation and embryos removed. For postnatal tissue, animals were anesthetized by cooling on ice and brains were immediately removed. Brains were dissected in ice cold phosphate buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 7.83 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and prepared for sectioning (see below) or used to obtain tissue for dissociated cell preparations. In the latter case, further dissection of brains into regions (e.g. thalamus) was performed in ice cold PBS supplemented with 4% calf serum (HyClone Laboratories), 5.6 mg/ml glucose, 25 I.U./ml penicillin, 25 µg/ml streptomycin, and 0.001% phenol red, and the Atlas of the Prenatal Mouse Brain (Schambra et al., 1992) was used as a guide. The boundaries used in dissecting the thalamus were, in the rostrocaudal direction, the habenular recess and the dorsal diencephalic sulcus of the third ventricle, while in the dorsoventral direction the boundaries were the epithalamus and a point just dorsal to the ventral diencephalic (hypothalamic) sulcus. Careful attention was paid to the removal of tissue from the mammillary, preoptic, and tegmental nuclei as well of from the epi- and hypothalamus. Dissected tissue was immediately rinsed in multiple volumes of ice cold HHBSS: calcium-magnesium free, Hank's

Balanced Salt Solution (CMF-HBSS) supplemented with 10 mM HEPES, pH 7.2.

Dissociation And Labeling Of Thalamic Cells

Thalami were incubated in HHBSS with trypsin (0.18 mg/ml) and DNAse (0.08 mg/ml) in a 15 ml polypropylene, conical tube for ≈10 minutes at 37°C with occasional, gentle mixing. The tissue suspension was then diluted with 1/3 volume of 37°C Complete Medium (CM): Dulbecco's modified Eagle's medium (DMEM: glutamine free, 4.5 g/L glucose) supplemented with 10 µg/ml transferrin, 5 mg/ml crystalline grade bovine serum albumin (BSA; ICN Biochemicals), 20 nM progesterone, 30 nM sodium selenite, 100 µM putrescine, 10 µg/ml bovine insulin, 1 mM sodium pyruvate, 50 I.U./ml penicillin, 50 µg/ml streptomycin, and 25 mM HEPES (pH 7.2). Soybean trypsin inhibitor (0.28 mg/ml) was added, the DNAse concentration was increased to 0.19 mg/ml, and the suspension was incubated for another 5 minutes at 37°C. Tissue was triturated gently ≈5X with a flame-polished pasteur pipette that was coated with a solution of 4% BSA in CMF-HBSS (brought to pH of 7.3 with 1 N NaOH). The suspension was brought to a final volume of ≈10 ml with HHBSS, undissociated tissue was allowed to settle (≈5 minutes), and the supernatant was transferred to a new tube. The cell suspension was centrifuged at 100 g for 10 minutes and the pellet gently resuspended in Labeling Medium which consisted of 30 µM Cell Tracker™ CMTMR (Molecular Probes, Eugene, OR) in CM; prepared, centrifuged to remove undissolved dye, and filter sterilized just before use. Cells were incubated at 37°C for 30-50 minutes. Two volumes of 37°C CM were added to the suspension along with DNAse (0.12 mg/ml) and incubation continued for 5-15 minutes. Several volumes of HHBSS were added and the suspension was underlayed with a 2 ml cushion of 4% BSA (crystalline

BSA in CMF-HBSS; brought to pH 7.3 with 1 N NaOH). Cells were then centrifuged at 150 g for 10 minutes. The pellet was resuspended in CM and cells were checked for viability using Trypan Blue. Routinely, >95% of cells excluded Trypan Blue. The cell suspension was diluted with CM to a concentration of ≈2.5 X 10^6 cells/ml .

Preparation And Fixation Of Living Slice Cultures

Brains were placed in 37°C, molten, low melting point agarose solution (Gibco BRL; 2% in PBS supplemented with 3 g/L glucose) which was immediately placed on ice to harden. Agarose blocks containing brains were cut to size (≈1.5 cm^3), glued to a vibratome tray (Technical Products International, Inc., St. Louis), immersed in ice cold PBS (supplemented with 0.45% glucose and 1 mM sodium pyruvate) and the tray was subsequently surrounded by an ice bath. Under the PBS bath, brains were vibratome-sectioned (150-200 µm thick) and slices were placed on nitrocellulose (0.45 µm; Sartorius, Bohemia, NY) filter disks (12 mm diameter) that had previously been sterilized, incubated in a sterile solution of concanavalin A (1.3 mg/ml in glutamine free DMEM) for 4-18 hours at 4°C, and rinsed several times in HHBSS before use. Disks with mounted slices were removed from the bath, placed on sterilized parafilm in plastic petri dishes, and 100 µl of ice cold CM was added, forming a bubble over the slices. Slices were kept on ice until all had been cut, medium was removed, 45 µl of fresh CM added, and then slices were moved to a 37°C, humidified incubator with a 8% CO_2 atmosphere. Cells were plated onto slices by carefully pipetting 45 µl of cells into the medium already on slices (≈10^5 cells/filter disk; ≈1000-2000 cells/mm^2). Cells were allowed to settle at 37°C and incubated for various periods. Cultures were transferred to a large volume of 37°C HHBSS and gently rinsed to remove unattached cells (for some

experiments, cells were visualized by fluorescence microscopy before the transfer and rinse procedure). Cultures were then added to warm (37°C) fixative: 4% paraformaldehyde (J.T. Baker, Phillipsburg, NJ) in 0.2 M sodium phosphate buffer (pH 7.5). After 15-30 minutes, the fixed tissue was rinsed in PBS, and counterstained (using 10 µg/ml bisbenzimide [Hoechst 33258] in PBS). Cultures were mounted on glass slides in a saturated sucrose solution containing 0.1% Sodium Azide (Fluka AG) and coverslipped.

Immunohistochemistry

Slice cultures were prepared, rinsed, and fixed as above except that fixation was limited to 18 minutes. Cultures were then washed several times in PBS and treated with 1% Triton X-100 in PBS for 7 minutes at room temperature. The following incubations were done at 4°C. Tissue was blocked (40% goat serum, 40% calf serum, 0.1% Triton X-100, 0.1% sodium azide in PBS; filtered) for 19 hours, washed (5% goat serum, 5% calf serum, 0.1% sodium azide in PBS; filtered) for 3 hours, and first antibody was added: CS-56 monoclonal mouse anti-chondroitin sulfate (Sigma), ascites fluid diluted in wash solution, 1:500. After 48 hours, cultures were washed several times over 20 hours, and then incubated with second antibody for 4 hours: FITC conjugated, goat anti-mouse IgM, µ specific (TAGO, Burlingame, CA), diluted 1:80 in wash solution. Cultures were washed several times, rinsed in PBS, counterstained with bisbenzamide, rinsed in 0.1 M $NaHCO_3$ (pH 9.2), and mounted in a saturated sucrose solution containing 0.1 M $NaHCO_3$ (pH 8.5) and 0.1% Sodium Azide.

For staining of dissociated cells the following methods were used: Cells were plated onto acid-washed glass coverslips (12 mm diameter) that had been treated with polylysine (100 µg/ml in PBS) overnight at 4°C, washed several times with PBS, incubated with concanavalin A (400 µg/ml in HHBSS) for 4

hours at 37°C, and rinsed several times in HHBSS. Plated cells were left for 3.5 hours at 37°C in a humidified incubator (8% CO_2 atmosphere), fixed for 22 minutes with 4% paraformaldehyde in 5% sucrose/PBS, and rinsed with PBS. For staining with antibodies to intracellular epitopes, cells were treated at this point with 0.5% Triton X-100 in PBS for 5 minutes at room temperature, and for all experiments cells were blocked with a solution of 5% goat serum, 5% calf serum, and 0.1% sodium azide in PBS. This solution was used for all subsequent washes and antibody dilutions. Anti-neurofilament (monoclonal antibody RT97, 1:666 dilution of ascites fluid [Wood and Anderton 1981]); anti-neuron specific β-tubulin (monoclonal antibody TUJ1, 1:200 dilution of concentration culture supernatant [Easter et al., 1993]), and anti-mouse NCAM (monoclonal antibody H28, 1:1 dilution of culture supernatant [Gennarini et al., 1984]) were applied at 4°C overnight. FITC- (Antibodies Inc., Davis, CA) and Rhodamine-conjugated (Kirkegaard & Perry Labs Inc., Gaithersburg, MD) secondary antibodies were used for visualization. Cell nuclei were counterstained with bisbenzamide, and coverslips mounted in 80% glycerol in PBS or 0.1 M $NaHCO_3$.

Preparation And Fixation Of Cryostat Section Cultures

Dissected brains were snap frozen in isopentane kept in a dry-ice/ethanol bath. Frozen brains were equilibrated to -20°C and mounted in OCT compound (Miles, Inc., Elkhart, IN). 20 µm slices were cut on a Reichert-Jung 2800 Frigocut-E cryostat. Slices were warmed onto ProbeOn Plus™ glass slides (FisherBiotech) and air dried. OCT was peeled off and a well was created (≈12 mm diameter) around the tissue slice with rubber cement (Dennison Stationary, Framingham, MA). Slices were then rehydrated with PBS, rinsed with CM, and 45 µl of fresh medium was added to the well. Slices were kept at

37°C for 30 minutes before 45 µl of cell suspension was added to the medium on each slice (≈10^5 cells/well), and cultures were incubated at 37°C for 3 hours. Unattached cells were gently rinsed off with 37°C HHBSS. The rubber cement was removed and warm (37°C) fixative was added for 10-20 minutes. After rinsing with PBS and counterstaining (10 µg/ml bisbenzimide in PBS), cultures were visualized by fluorescence microscopy.

Data Collection

The selection of fixed slice cultures for quantification was based solely on the integrity of slice anatomy, as visualized by bisbenzamide staining of cell nuclei. Cultures were chosen only if the laminar borders could be clearly discerned and the identity of individual laminae unambiguously determined. Borders were delineated by differences in the density of cell nuclei, and laminae were identified in some cases by specific landmarks, such as barrels within the granular layer of the P7 somatosensory cortex. Slices that had been folded or torn while being placed onto the nitrocellulose substrata, or had otherwise become distorted due to stretching or flattening, were not quantified. Importantly, slice selection was carried out under UV (bisbenzamide) fluorescence only. Since Cell Tracker™ CMTMR-fluorescence is not observed in this channel, it was possible to be confident that the selection of slices was carried out blind to patterns of thalamic cell attachment.

Measurements of cell attachment, neurite length, and neurite orientation were obtained from digitized images of fixed cultures. Images were collected using a Zeiss fluorescence microscope fitted with camera lucida optics that were set to view a video monitor. A video trace, that was superimposed over the fluorescence image of the culture within the microscope objectives, was drawn using the hardware and software of the Neuron Tracing System

(Eutectics Electronics Inc., Raleigh, NC). Cortical layers and their borders were identified and traced under UV fluorescence while labeled and attached thalamic cells were individually traced under rhodamine fluorescence. Using an automated, calibrated microscope stage and both fluorescence channels, a composite image of slice anatomy, attached cells, and neurite morphology was drawn to scale across several microscope fields.

Neurite data were collected from experiments using sagittal slices. Neurites were traced only if their growth cones and soma of origin could be clearly demarcated and if they were at least one soma diameter in length (≈10 μm). Since neurites often projected downward, into the slice tissue, they were traced in 3 dimensions (automated and calibrated control of the focus knob allowed for images to be traced through focal planes). Some cells had two neurites longer than a cell diameter; in these cases, both processes were traced. No neurites had branches which themselves met the length criterion.

Attachment Data Analysis

The surface area of and the number of cells on each layer were computed based on stored digital images, and attachment densities were calculated from these values. Distances from the cortical plate/subplate border of cells attached to the subplate and intermediate zone were calculated by the system software as the distance, within a plane parallel to the plane of the slice, between the center of each attached cell and the closest point on the cortical plate/subplate border to that cell.

Attachment data for different cortical layers were compared by a single factor analysis of variance (ANOV) to obtain reported P values. A Newman-Keuls test was used to verify the outlier values (Zar, 1974).

Neurite Data Analysis

Neurite lengths were calculated by the system software as the 3-D contour distance between a neurite's origin (point of connection to the soma) and its endpoint (center of the growth cone). Orientations for marginal zone, subplate/intermediate zone, and ventricular zone neurites were calculated with respect to the marginal zone/cortical plate, cortical plate/subplate, and intermediate zone/ventricular zone borders respectively. Values consisted of the angle between the reference border (or, if curved, a tangent to the border) closest to the neurite's origin and a line defined by the neurite's origin and its end point. Only coordinates along the rostral-caudal and pial-to-ventricular axes were used for this calculation. A neurite projecting rostrally, parallel with its reference border defined 0°. Projections directed toward the pia, perpendicular to the reference border, were assigned 90° and those directed toward the ventricle were assigned -90°.

For polar plots, neurites were separated according to their angle into twelve, 30° interval bins. Neurites that fell on the border between two bins were split with a value of 0.5 assigned to each bin. Each plot contains twelve points which consist of the angle of the *center* of the bin (q = -30°, 0°, 30, 60°, ...) and the number of neurites in that bin (r).

A chi-square analysis was used to test whether neurite data fit a uniform (random) circular distribution by comparing binned, neurite samples to a hypothetical sample consisting of the same number of neurites evenly distributed among all 12 bins (Zar, 1974).

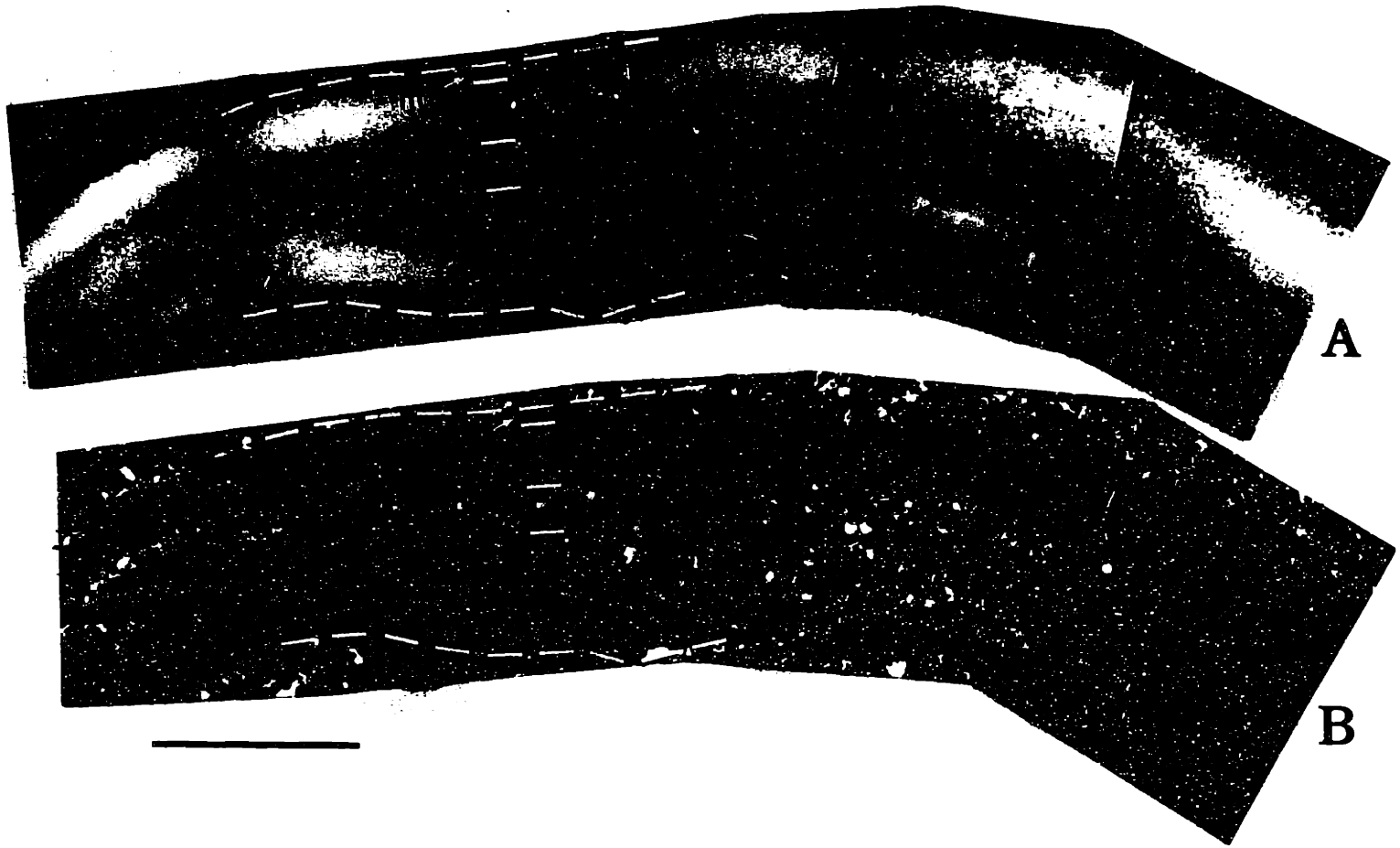
RESULTS

Region-Specific Attachment of Thalamic Cells to Living Slices of E15 Forebrain

To examine how thalamic neurons behave when confronted *in vitro* with different brain environments, embryonic day 14 or 15 (E14-15) mouse thalamic cells were dissociated, labeled with a vital fluorescent dye, washed into serum-free culture medium and allowed to settle onto freshly cut vibratome slices (150-200 μm) of developing mouse forebrain. After 3 hours at 37°C, the slices were gently rinsed to remove unattached cells and then fixed, counterstained with bisbenzimide (Hoechst 33258), and visualized by fluorescence microscopy (see Methods). To avoid the possibility of dye-transfer from thalamic cells to intrinsic cells of the slice, thalamic cells were labeled with a thiol-reactive fluorescent dye, CellTracker™ CMTMR, which diffuses into cells, becomes covalently coupled to intracellular components (e.g. glutathione), and is fixable by crosslinking agents. Immunocytochemical evaluation of dissociated thalamic cells prior to plating onto slices indicated that 91% (±3.3%, n=904) expressed neuron-specific class III β-tubulin and 70% (±3.5%, n=828) contained neurofilament protein. Thus, the vast majority of the cells were neurons. In addition, 83% (±4.0%, n=616) were immunoreactive for NCAM.

When thalamic cells were prepared and plated in this manner onto E15 forebrain slices, large differences were evident in the numbers of fluorescent cells that were found on different parts of the slice. The most striking differences were in the cerebral cortex, the anatomical layers of which could be identified by nuclear density (Fig. 1). Specifically, very few thalamic cells were found on the cortical plate, whereas numerous cells were found on each of the other cortical layers--the marginal, intermediate, and ventricular zones. On most subcortical regions (e.g. caudoputamen, globus pallidus, preoptic and

Figure 1. Laminar specific attachment of dissociated thalamic cells to slices of embryonic cortex. (A) Laminae of an E15 cortex can be seen under UV fluorescence as differences in nuclear density after a sagittal slice is cultured with thalamic cells (3 hours), fixed, and stained with bisbenzamide (see methods). Rostral is to the right, caudal to the left. (B) The same slice viewed under rhodamine optics shows the distribution of attached thalamic cells. Some attached cells are in clusters, which appear as larger, bright dots. The pial and ventricular edges of the cortex are demarcated by dotted white lines at the top and bottom of each figure. Lines are also positioned in the two photos to reference the same points in the two views of the slice. Very few thalamic cells attach to the cortical plate (CP) while more attach to the intermediate zone (IZ), marginal zone (MZ), and ventricular zone (VZ) as well as to the substratum off the slice (seen at the edges of the photos). Density of attached cells is greatest on the intermediate zone just subjacent to the cortical plate. Scale bar is 500μm.

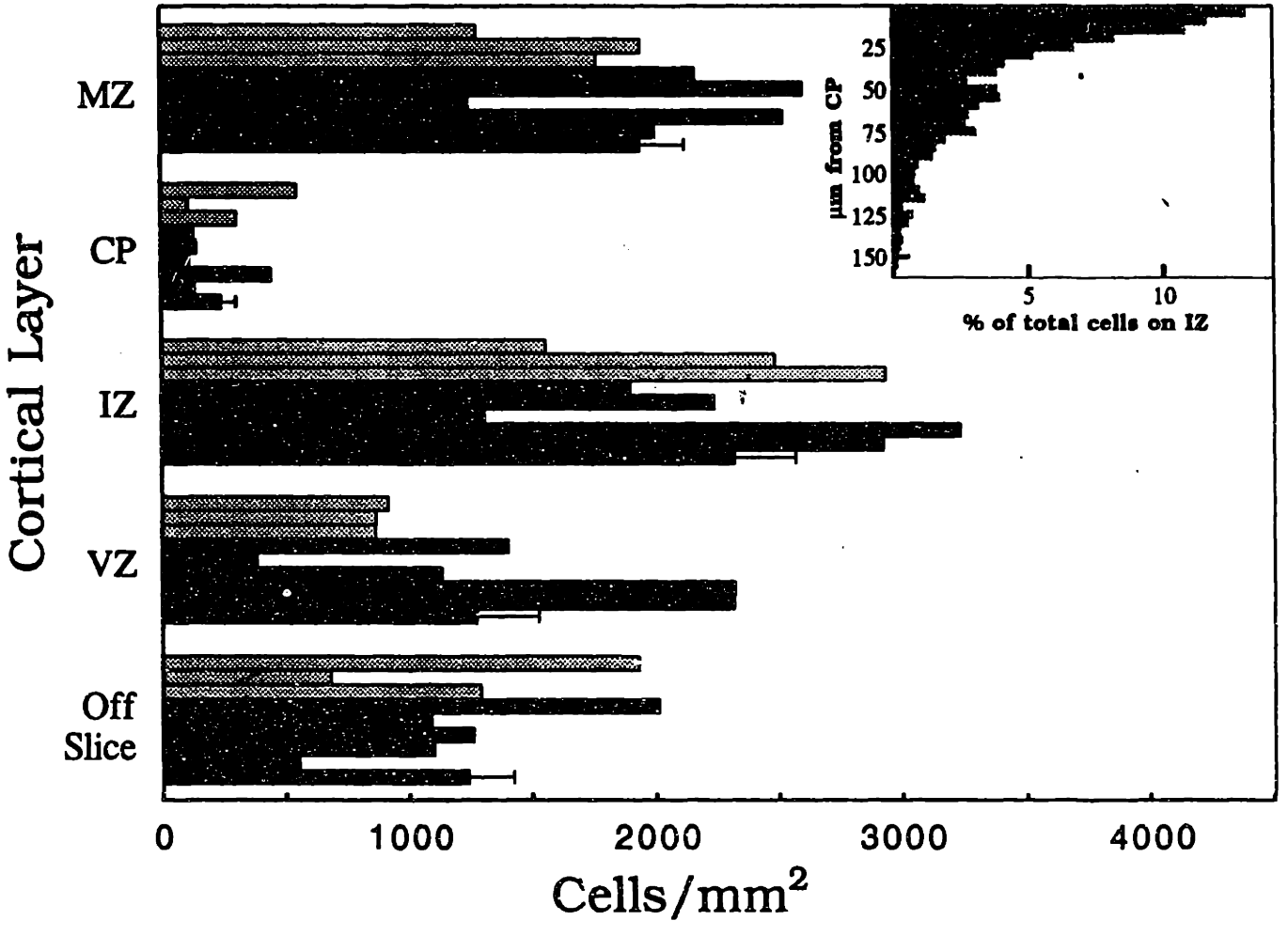

A
B

supraoptic areas, hypothalamus, thalamus) there was also a high density of thalamic cells, although on some (the anterior commissure, the claustrum, the amygdaloid area and olfactory nuclei of the ventral forebrain, and parts of the septum) few cells were seen (data not shown).

In some experiments, slices were examined just prior to rinsing and fixation. In these cases, thalamic cells appeared to be uniformly distributed over each slice. Thus, the differences in fluorescent cell density that were seen with rinsed, fixed slices apparently reflected region-specific cell attachment, rather than non-uniform plating of cells or migration of cells from some regions to others during the 3 hour culture period. In other experiments, thalamic cells were plated and cultured on forebrain slices for different periods of time before rinsing and fixation. Patterns of cell attachment similar to that in Fig. 1 were obtained whether rinsing and fixation were carried out as early as 1 hour, or as late as 24 hours, after plating (data not shown). We also found that the preincubation of slices for up to 5 hours at 37° C prior to the plating of cells produced the same pattern of cell attachment as seen in the previous experiments (data not shown).

To quantify thalamic cell attachment to the different layers of the E15 neocortex, measurements of the density of labeled cells on each layer were made for 8 slices, and are shown in Fig. 2. Mean thalamic cell attachment to the marginal, intermediate, and ventricular zones was in the range of 1300-2300 cells/mm^2 and was close to the density of cells that was initially plated onto the slices (~1000-2000 cells/mm^2; see methods). In contrast, thalamic cell attachment to the cortical plate was substantially lower (243 ± 60 cells/mm^2). This result was obtained regardless of whether coronal or sagittal forebrain slices were used (Fig. 2), and was qualitatively similar throughout the rostrocaudal and dorsoventral extent of the neocortex.

Figure 2. Densities of thalamic cells attached to embryonic cortical layers. The densities of cells attached to each of the major embryonic cortical layers -- the marginal zone (MZ), cortical plate (CP), intermediate zone (IZ), and ventricular zone (VZ) -- were determined for 3 coronal (light bars) and 5 sagittal (darker bars) slices. Black bars represent the means (± standard error) from the 8 samples. Data were collected from the portion of sagittal slices indicated by dotted lines in Fig. 1 or from the dorsolateral part of coronal slices because laminae could be unambiguously distinguished within these regions (total number of cells counted from the 8 slices: n_{mz}=1818, n_{cp}=316, n_{iz}=2001, n_{vz}=1647, $n_{off\ slice}$=2583). "Off Slice" data were collected from the culture substratum, adjacent to the pial surface. Mean values are significantly different among the four layers (Analysis of Variance test: $P<0.0005$). A Newman-Keuls analysis verified the value of the mean density on the cortical plate as a significant outlier ($\mu_{iz} = \mu_{mz} \neq \mu_{vz} \neq \mu_{cp}$). INSET shows that cells attached to the intermediate zone are not uniformly distributed on this layer. Cells that attached to the intermediate zone were binned according to their distance from the cortical plate border and the data, averaged from the 8 slices, were plotted as the proportion of total cells attached to this layer. On average, over half of the cells that attached to the intermediate zone are located within 25 μm of the cortical plate border.


Cortical Layer
MZ
CP
IZ
VZ
Off Slice
0
1000
2000
3000
4000
Cells/mm2
µm from CP
25
50
75
100
125
150
5
10
% of total cells on IZ

In photographs such as Fig. 1, the contrast in thalamic cell density between the cortical plate and intermediate zone is particularly noticeable because thalamic cells on the intermediate zone are especially concentrated near the border with the cortical plate (also see Fig. 3). This can also be illustrated by plotting cell density in the intermediate zone as a function of distance from the cortical plate (Fig. 2, inset). These data suggest that thalamic cell attachment is especially high in the subplate, the region of the intermediate zone subjacent to the cortical plate (Kostovic and Molliver, 1974; Luskin and Shatz, 1985a). Because the rodent subplate is only a few cell layers thick (Valverde et al., 1989; Bayer and Altman, 1990), its boundaries cannot be accurately determined in our thick slices by nuclear staining alone. In some experiments, the subplate was visualized by chondroitin sulfate immunohistochemistry (Sheppard et al., 1991), and was found to correspond to the part of the intermediate zone extending up to ~25 μm from the cortical plate border (data not shown), i.e., containing that region which supports the highest density of attached cells (Fig. 2, inset).

Neurite Outgrowth by Thalamic Neurons on Living Slices of E15 Neocortex

Even though thalamic cells were typically plated onto forebrain slices for only 3 hours, substantial neurite outgrowth occurred on all forebrain regions where thalamic cells attached, including the marginal, intermediate, and ventricular zones of the developing cortex (too few cells attached to the cortical plate to permit conclusions to be drawn about neurite outgrowth on this layer). There were no significant differences in the lengths of neurites on cortical layers (marginal zone: 16.1 μm [s.d.=5.7; n=85]; intermediate zone: 16.1 μm [s.d.=6.5; n=114]; ventricular zone 15.8 μm [s.d.=6.8; n=52]). Interestingly, the orientation of neurites on the intermediate zone appeared non-random. On

this layer, most neurites projected parallel to the cortical laminae (i.e. along the rostrocaudal axis), while few neurites projected radially (Fig. 3).

Fig. 4 shows the ranges of neurite angles (as measured with respect to nearby cortical layer borders; see Methods) that were observed for thalamic cells attached to marginal, intermediate and ventricular zones. The data confirm that a disproportionate number of intermediate zone neurites are rostrocaudally oriented ($P<0.001$), with no significant bias toward the rostral or caudal direction (52% and 48% of sample, respectively; n=114). In contrast, neurites extended by thalamic cells on the marginal and ventricular zones followed a much more uniform distribution, not significantly different from random (see Fig. 4, legend).

Since the subplate was included as part of the intermediate zone in the above analysis, an attempt was made to analyze subplate and non-subplate neurites separately. Neurites seen on the intermediate zone were therefore divided into two populations--those originating within one average neurite length (16.1 μm) of the cortical plate (probable subplate neurites) and those originating in the rest of the intermediate zone. As shown in Fig. 5, both sets of neurites exhibited orientations that were significantly biased toward the horizontal, i.e. along the rostrocaudal axis. Interestingly, among the putative subplate neurites (Fig. 5A), extremely few were oriented superficially (i.e., toward the cortical plate). Consistent with this observation, in no case were neurites observed that crossed from the subplate onto the cortical plate. Indeed, subplate neurites were occasionally seen that extended a short distance superficially, then turned sharply at the cortical plate border (not shown). In contrast, neurites were often seen that crossed the border between the intermediate and ventricular zones, without evidence of turning behaviors. Although these data raise the possibility that thalamic neurites actively avoid

Figure 3. Neurite extension on E15 cortex. (A) High magnification, UV fluorescence micrograph of the bisbenzamide stained cortex, from an experiment similar to that in Fig. 1, shows the cortical plate (CP), the cell poor intermediate zone (IZ), and the ventricular zone (VZ) as indicated at left. (B) The same field seen under rhodamine optics reveals attached thalamic cells and their neurites. Fluorescent cells (filled arrows) attached to the intermediate zone extend three neurites in directions parallel to the CP/IZ border. On the ventricular zone, neurites (open arrows) project both parallel to the cortical layers and along the radial axis (perpendicular to the lamina). Growth cones of those neurites projecting radially cannot be seen because the neurites extend into the slice and out of the plane of focus. Filled arrows and arrowheads in (A) point to nuclei whose corresponding cells are marked by the same labels in (B). These nuclei are out of focus because attached cells lie in a focal plane slightly above that of the cells of the slice. Scale bar is 10μm.

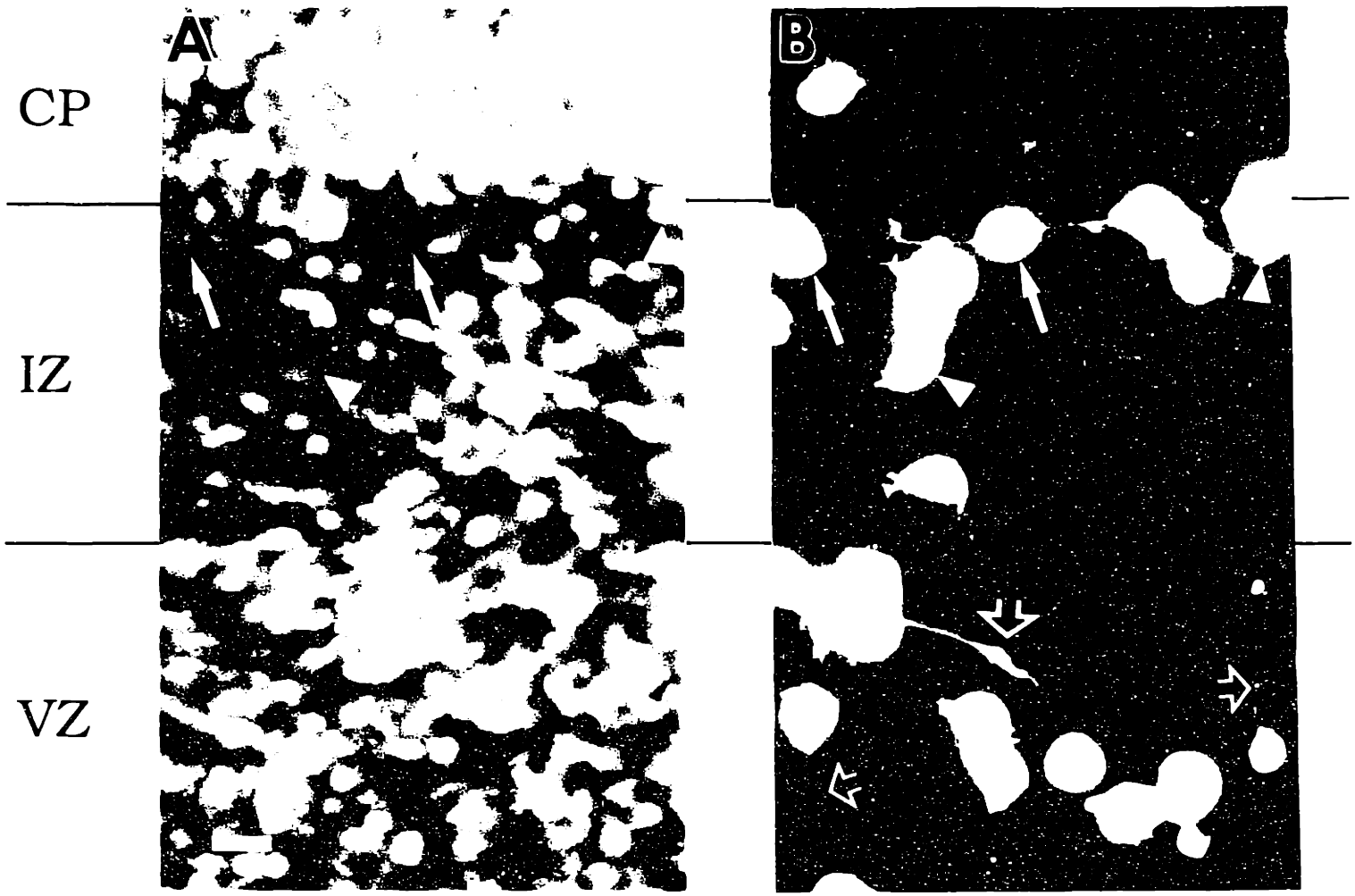
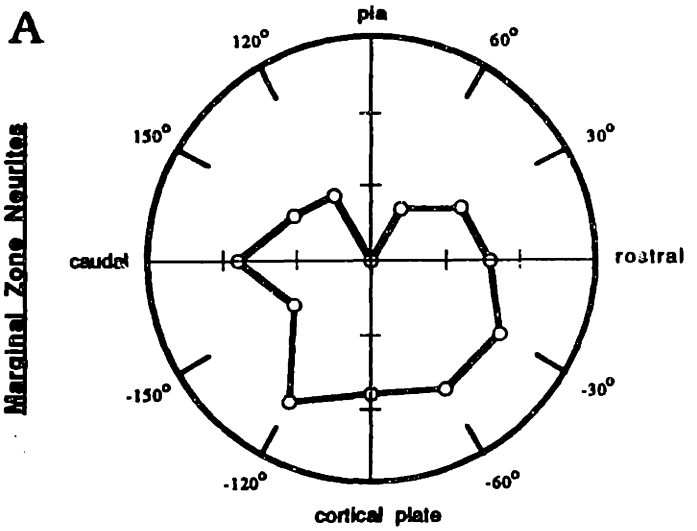
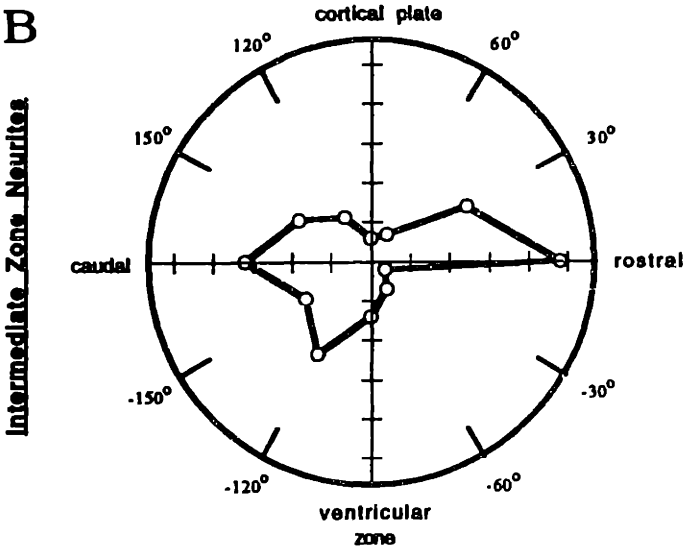

A
B
CP
IZ
VZ

Figure 4. Distribution of thalamic neurite angles on the different embryonic cortical layers . Polar plots show the angular distribution of neurites projected by thalamic cells on the marginal (A), intermediate (B), and ventricular (C) zones of E15 cortical slices. Neurite angles were calculated with respect to laminar borders and then segregated into twelve, 30° interval bins (see methods). Plotted points represent the center angle of each bin (q) and the number of neurites (r) in the sample with angles that are within that bin interval. Axis tick marks represent a count of 5 neurites in (A), 5 in (B), and 2 in (C). The shapes of the plots reflect the predominant directions in which neurites project on a given layer. Plot frames indicate the angle of each bin's center except for the bins centered on the axes, which are marked with cardinal directions based on cortical anatomy (90° and -90° represent projections toward the overlying and subjacent layers or tissue edges). Marginal (A) and ventricular (C) zone neurites project both rostrocaudally and radially (toward the pia and ventricle) and are not significantly different from a uniform circular distribution (chi-square to uniform sample: $P_{mz}>0.1$, $P_{vz}>0.5$). Intermediate zone neurites mostly project rostrocaudally and are different from a uniform distribution ($P_{iz}<0.001$). Data were taken from 4 sagittal slices (total number of neurites counted: $n_{mz}=85$, $n_{iz}=114$, $n_{vz}=52$).


A
Marginal Zone Neurites
pia
120°
60°
150°
30°
caudal
rostral
-150°
-30°
-120°
-60°
cortical plate


B
Intermediate Zone Neurites
cortical plate
120°
60°
150°
30°
caudal
rostral
-150°
-30°
-120°
-60°
ventricular
zone

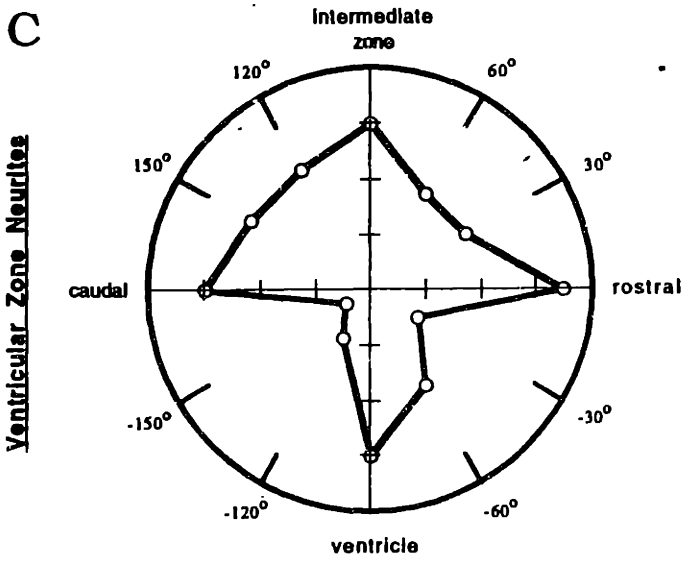
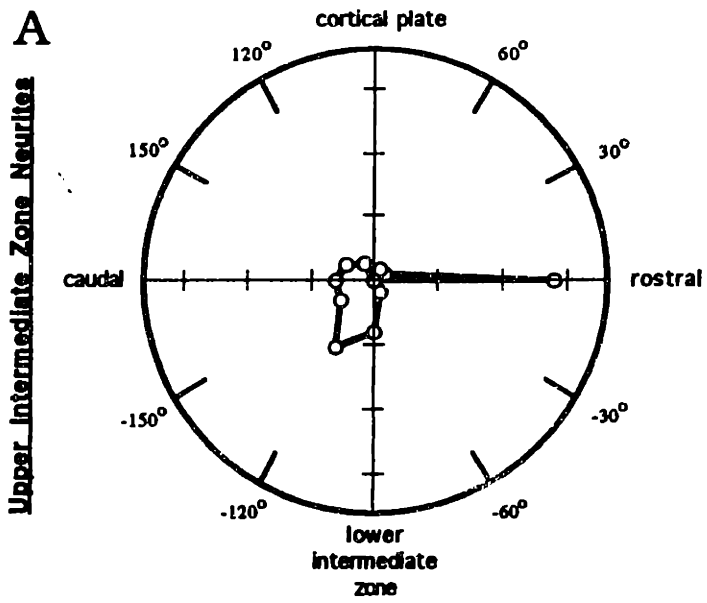
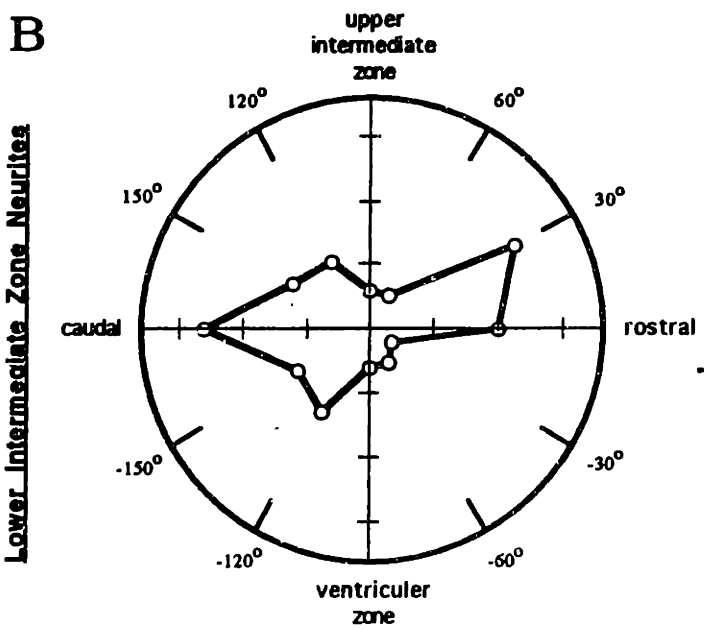

C
Ventricular Zone Neurites
intermediate
zone
120°
60°
150°
30°
caudal
rostral
-150°
-30°
-120°
-60°
ventricle

Figure 5. Neurites on the E15 intermediate zone and subplate do not cross onto the cortical plate. The intermediate zone neurite sample shown in Fig. 4B was subdivided into two samples and replotted using the same graphing procedure. (A) The upper sample consists of those intermediate zone neurites (n=37) that originate within 16 μm (one mean neurite length) of the cortical plate. This sample approximates the neurites that are generated on the subplate region of the embryonic cortex. (B) The lower sample represents the rest of the intermediate zone neurites (n=77). Both samples are rostrocaudally oriented (parallel to the cortical lamina) and are significantly different from uniform circular distributions ($P_{upper}<0.001$, $P_{lower}<0.01$). Although both samples contain some radially directed projections (along vertical axis), there are almost no neurites on the upper intermediate zone that project superficially, toward the cortical plate. Neurites projecting on the lower intermediate zone are not biased toward either direction along the radial (vertical) axis. Axis tick marks represent a count of 5 neurites.


A
Upper Intermediate Zone Neurites
cortical plate
120°
60°
150°
30°
caudal
rostral
-150°
-30°
-120°
-60°
lower
intermediate
zone

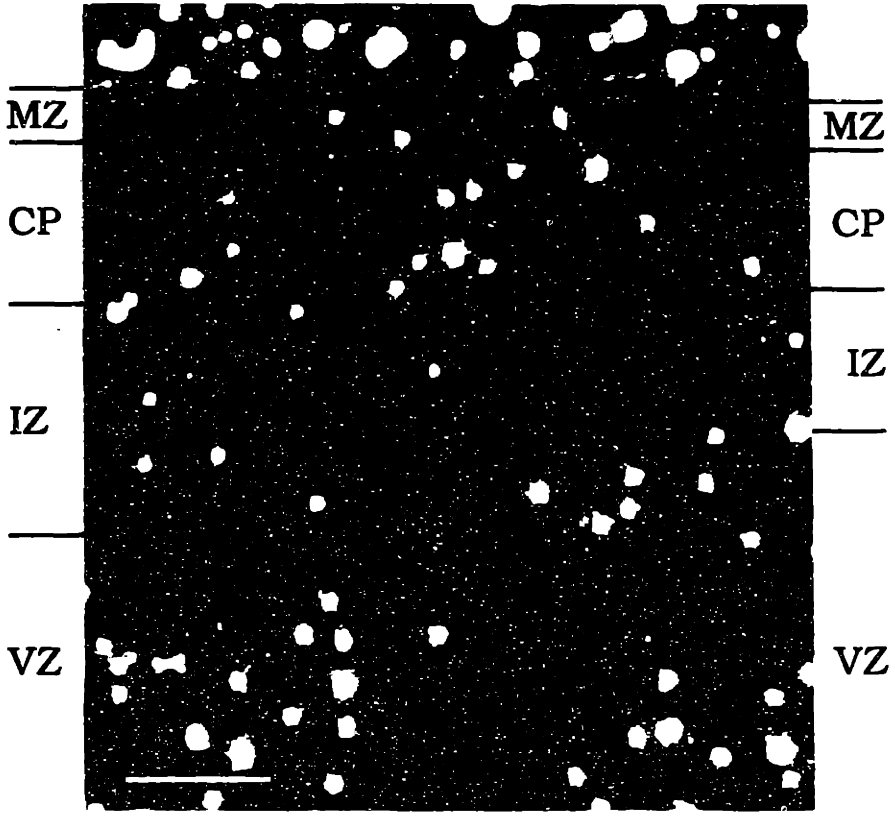

B
Lower Intermediate Zone Neurites
upper
intermediate
zone
120°
60°
150°
30°
caudal
rostral
-150°
-30°
-120°
-60°
ventriculer
zone

the cortical plate, time lapse cinematographic observations will be required to establish such a conclusion.

Behavior of Thalamic Cells on Frozen Sections of E15 Forebrain

Others have found that neurons can exhibit region-selective attachment and/or neurite outgrowth on frozen (cryostat) sections of brain, nerve, or muscle (Carbonetto et al., 1987; Sandrock and Matthew, 1987; Covault et al., 1987; Watanabe and Murakami, 1989; Savio and Schwab, 1989; Crutcher, 1989; Geisert, 1991; Tuttle and Matthew, 1991). To determine whether this method would reveal thalamic cell behaviors similar to those observed using living slices, E15 thalamic cells were prepared and labeled as before, but plated onto air-dried 20 µm cryostat sections of fresh-frozen E15 forebrain. After 3 hours at 37°C, non-attached cells were rinsed off and cultures were fixed and counterstained. Compared to previous experiments with living sections, the absolute density of attached cells was much lower (Fig. 6). Moreover, attachment appeared to be fairly uniform across all cortical layers. In addition, little neurite outgrowth from cells attached to the cryostat sections was seen. The fact that frozen sections could not substitute for living slices in revealing cortical layer-specific behaviors of thalamic neurons raises the possibility that those behaviors require living cortical cells. However when vibratome slices were lightly
fixed with formaldehyde before thalamic neurons were plated onto them, thalamic cells displayed layer-specific attachment much like that which was seen when they were plated onto living slices (data not shown). Thus, it appears that cortical cell viability is not required to obtain cortical layer-specific thalamic cell attachment. It seems more likely that critical molecules

Figure 6. Cryostat sections of E15 forebrain support only a low density of thalamic cell attachment which is uniform across embryonic cortical layers. Both the laminar structure of the E15 cortex and attached thalamic cells can be visualized in this photo taken under both phase contrast transmittance and rhodamine fluorescence microscopy. The larger, fluorescent dots are clusters of 2-4 cells. Cells can be seen to attach to all embryonic cortical layers, i.e. the marginal zone (MZ), cortical plate (CP), intermediate zone (IZ), and ventricular zone (VZ). At the top of the photo, adjacent to the marginal zone, fluorescently labeled cells can be seen which are attached to the substratum of the slide. The photo is taken from the lateral cortex of a coronal section. Ventral is to the left, dorsal to the right. Markings on the two sides of the photo are of different scales due to the thinning of the telencephalon that occurs along the ventrodorsal axis of coronal sections. Scale bar is 100μm.


MZ
CP
IZ
VZ
MZ
CP
IZ
VZ

or structures that are preserved in vibratome sections are lost or disrupted in frozen sections.

Developmental Changes in the Attachment and Neurite Outgrowth Activities of Neocortex.

At E15, the cerebral cortex is at a relatively early stage in its development. To determine whether the laminar-specific differences in the behavior of embryonic thalamic neurons change as the cortex matures, embryonic (E14-15) thalamic neurons, prepared as before, were plated onto living slices cut from older brains. Postnatal days 1 and 7 (P1 and P7) were chosen to represent intermediate and late stages in cortical histogenesis (reviewed by Bayer and Altman, 1991) and thalamocortical innervation (reviewed by Erzurumlu and Jhaveri, 1992; Kageyama and Robertson, 1993).

Fig. 7 shows the results of a typical experiment involving a sagittal slice from a P1 animal. At the caudal end of the slice, the pattern of thalamic cell attachment is similar to that observed with E15 slices: Cells attached to the marginal zone (i.e., cortical layer I) and the intermediate zone (i.e., the developing white matter), but not to the cortical plate, which at this age consists of developing layers 2-6 (the ventricular zone is not indicated in Fig. 7 because at this age it has become too thin to distinguish clearly). Moving toward the rostral end of the slice, however, one sees increasing numbers of labeled cells attached to the cortical plate, especially the deeper regions of the cortical plate. Since the maturation of the neocortex is known to follow a rostral-to-caudal gradient (Smart, 1983; Bayer and Altman, 1991), these results suggest that the cortical plate may become permissive for thalamic cell attachment as the cortex matures.

Figure 7. Thalamic cell attachment to P1 cortex. (A) Laminae of a P1 cortex can be seen under UV fluorescence after a sagittal slice is cultured with thalamic cells (3 hours), fixed, and stained with bisbenzamide. By this age, the cortical plate (CP) has matured and some differentiated laminae can be distinguished within it. (B) The same slice viewed under rhodamine optics shows the distribution of fluorescently labeled and attached thalamic cells. Many cells attached to the developing white matter (WM, formerly the intermediate zone) and layer 1 ("1", formerly the marginal zone). Cells also attach to the cortical plate (not seen on E15 sections), but in a non-uniform manner. The rostral portion (left) supports the greatest level of attachment while the caudal portion (right) is almost devoid of fluorescent cells. Dotted white lines indicate the edges of the cortex (pia at the top) and reference the same points of the slice in both photos. Scale bar is 500μm.


A
B

In support of this idea, when E14-15 thalamic cells were plated onto P7 sagittal slices, cell attachment to the cortical plate was observed throughout the rostrocaudal extent of the slices (Fig. 8). As was seen on E15 and P1 slices, thalamic cells also attached well to cortical layer I and the developing white matter. Unlike what was observed using E15 slices, labeled cells on the developing white matter (former intermediate zone) were not concentrated near the cortical plate border (site of the embryonic subplate).

The data on thalamic cell attachment to P7 slices are presented quantitatively in Fig. 9. When these data are compared with Fig. 2, the most striking difference is the increase in the level of thalamic cell attachment to the P7 cortical plate relative to other cortical layers. Closer inspection of the sections revealed, however, that it is not the entire P7 cortical plate that supports thalamic cell attachment, but only its deeper layers. Using nuclear staining alone, it was possible to divide the P7 cortical plate into supragranular (laminae 2/3), granular (lamina 4) and infragranular (laminae 5/6) layers, and to measure cell attachment separately for each layer. As shown in Fig. 9 (inset), thalamic cell attachment to the P7 cortical plate was largely restricted to the granular and infragranular layers (laminae 4-6).

As with E15 slices, thalamic cells plated onto P7 slices were observed to extend neurites on all cortical layers to which they attached (including, in this case the cortical plate). Although measurements of neurite angles were not made, no obvious orientation of neurites on any layer was observed. Significantly, neurites were found that extended between the developing white matter and layer 6 of the cortical plate, without evidence of turning at this border (data not shown).

Figure 8. Thalamic cell attachment to P7 cortex. (A) Laminae of a P7 cortex can be seen under UV fluorescence after a sagittal slice is cultured with thalamic cells (3 hours), fixed, and stained with bisbenzamide. The cell-dense, barrel walls of the somatosensory cortex can be seen and used to identify the granular layer 4 (Woolsey and Van Der Loos, 1970; Rice and Van Der Loos, 1977). Rostral is to the left, caudal to the right. (B) The same slice viewed under rhodamine optics shows the distribution of fluorescently labeled and attached thalamic cells. As in the E15 and P1 experiments, cells attached well to layer 1 ("1", formerly the marginal zone) and the white matter (WM, formerly the intermediate zone). Attachment to the cortical plate (laminae "2-6") can be seen throughout the rostrocaudal extent of the slice, however it is limited to layers 4-6, i.e. the granular ("4") and infragranular ("5/6") layers. Attachment to the supragranular layers ("2/3") is relatively low. Dotted white lines indicate the edges of the cortex (pia at the top) and reference the same points of the slice in both photos. Scale bar is 1mm.

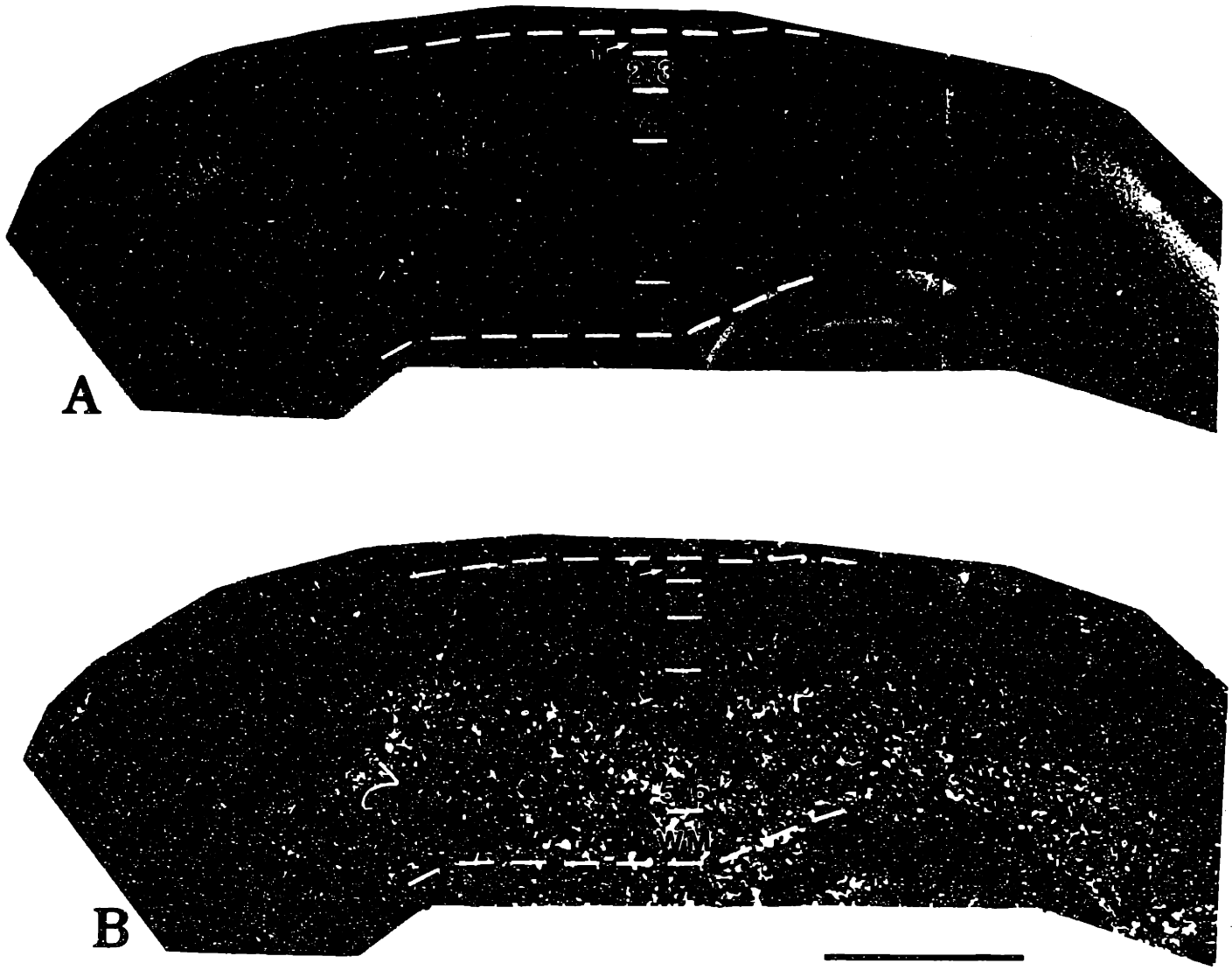
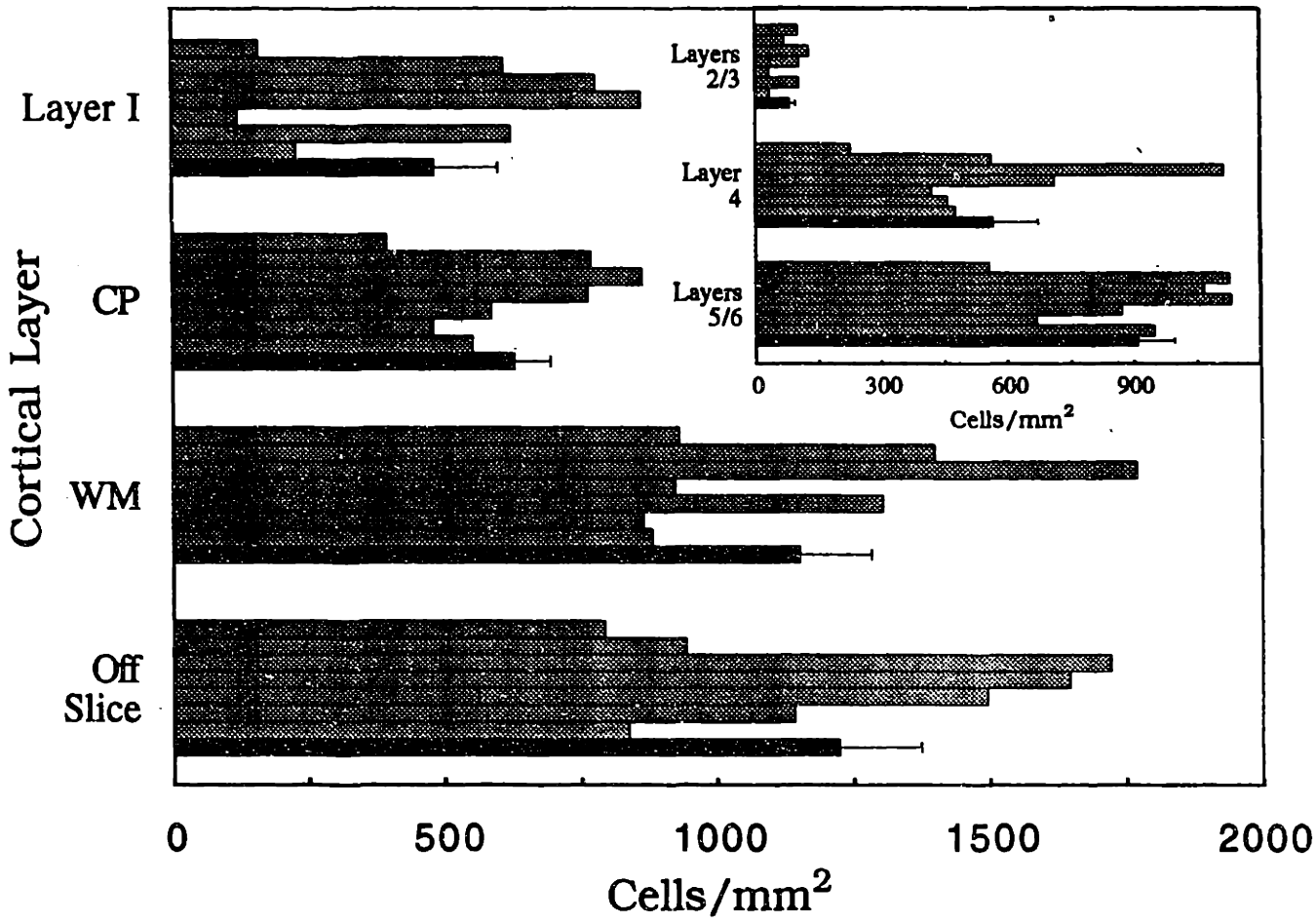

A
B

Figure 9. Densities of thalamic cells attached to postnatal (P7) cortical layers. The densities of cells attached to Layer 1, the cortical plate (CP, laminae 2-6), and the white matter (WM) were determined from 7 sagittal slices (light bars). Black bars represent the mean (± standard error) from the 7 samples. Data were collected from the presumed somatosensory cortex, the extent of which is indicated by the dotted lines in Fig. 8, because the laminae could be unambiguously distinguished within this region (total number of cells counted from the 7 slices: n_1=891, n_{2-3}=330, n_4=1379, n_{5-6}=7042, n_{wm}=2400, $n_{off\ slice}$=1874). "Off Slice" data were collected from the culture substratum adjacent to the pial surface. INSET shows the density of cells attached to the laminae within the cortical plate. Cell density on supragranular layers (2/3) was much lower than on granular (4) and infragranular (5/6) layers (analysis of variance test: $P<0.0005$). A Newman-Keuls analysis verified the mean density on laminae 2/3 as an outlier ($\mu_{5/6} \neq \mu_4 \neq \mu_{2/3}$).


Layer I
CP
WM
Off Slice
Cortical Layer
Layers 2/3
Layer 4
Layers 5/6
0
300
600
900
Cells/mm2
0
500
1000
1500
2000
Cells/mm2

DISCUSSION

These experiments demonstrate that living, but not cryostat, sections of cerebral cortex support selective attachment of and neurite outgrowth from embryonic thalamic neurons in a manner that depends upon the developmental state of the cortex. Embryonic cortical plate and postnatal supragranular layers act as *in vitro* substrata which, compared to all other cortical laminae, support little neuronal attachment. Neurites of cells attached to the embryonic subplate and intermediate zone project mostly parallel to the cortical layers and neurites on the subplate do not cross onto the cortical plate. These results can be obtained in serum-free medium, and are apparent after very short periods in culture (3 hours).

The Correlations Between *in vitro* Behavior of Thalamic Neurons and *in vivo* Development

Both the patterns and timing of thalamic cell attachment and neurite outgrowth in this *in vitro* assay correlate well with *in vivo* patterns and timing of thalamocortical development: At each developmental stage examined, thalamic cells failed to attach well to, and failed to extend neurites onto, essentially those territories that thalamic axons encounter, but avoid, *in vivo*.

For example, at the earliest stage examined in this study (mouse E15, which is roughly equivalent to rat E17), tangentially-oriented thalamic axons normally occur abundantly in the cortical subplate, but are absent from the adjacent cortical plate. Thalamic axons are also present in the deeper intermediate zone, and are occasionally seen in the ventricular zone, (Crandall and Caviness, 1984; Reinoso and O'Leary, 1988; Catalano et al., 1991; Erzurumlu and Jhaveri, 1992; Kageyama and Robertson, 1993). *In vitro*, embryonic thalamic cells seeded onto E15 cortical slices attached especially

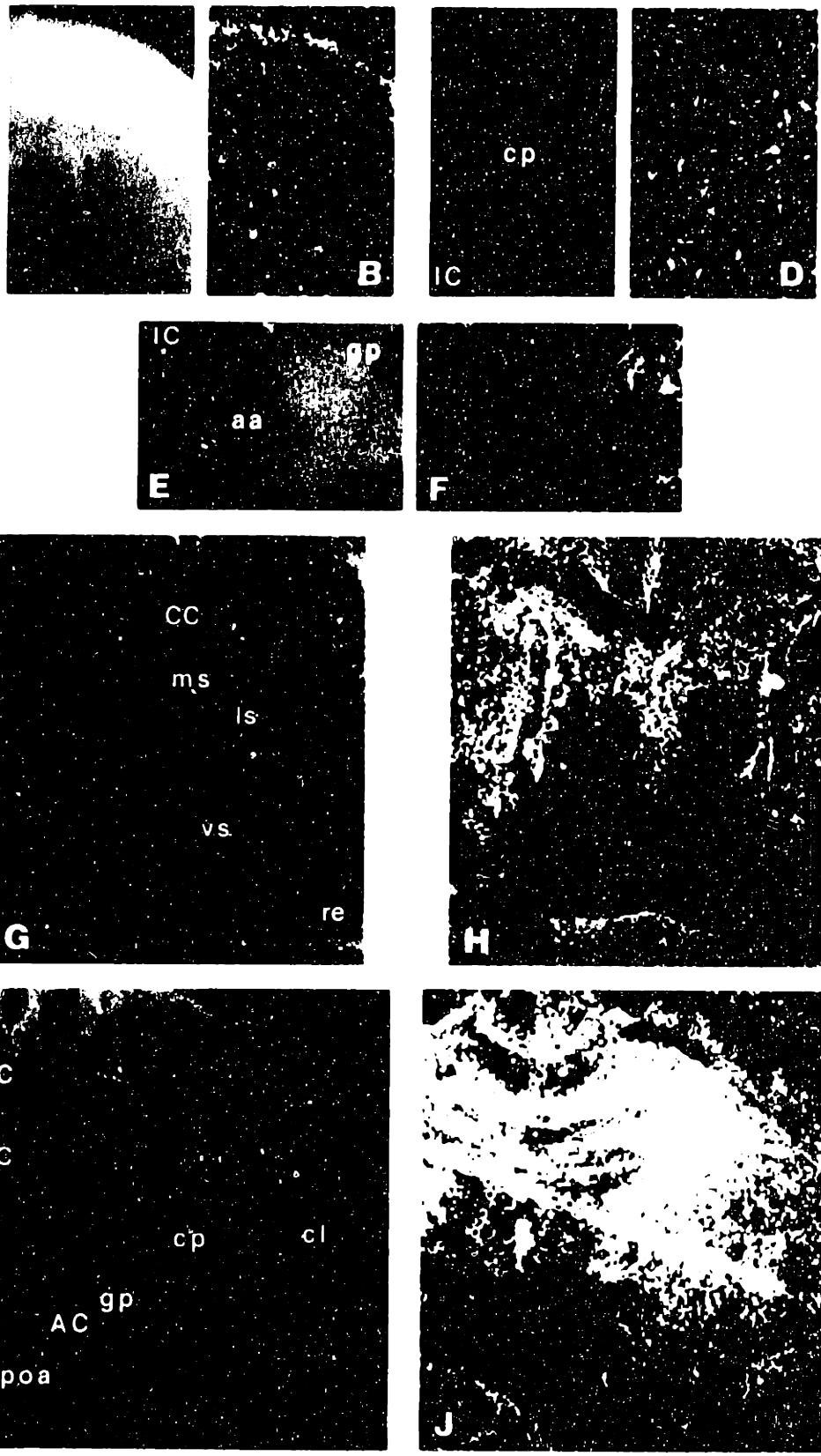
well to the superficial intermediate zone (the presumptive subplate), as well as throughout the intermediate zone and, to a lesser extent the ventricular zone, but they attached very poorly to the cortical plate (Figs 1, 2). Neurites of thalamic cells that attached to the intermediate zone (including the subplate) tended to be oriented tangentially and, like thalamocortical axons *in vivo*, failed to enter the cortical plate (Figs 3, 4B, 5).

At P1 *in vivo*, thalamocortical axons from the subplate have begun to invade the cortical plate in at least parts of the cortex (Agmon et al., 1993). *In vitro*, thalamic cells attached to much the same areas of the P1 cortex as observed with E15-E16 slices, except that, at this stage, the cortical plate in the rostral, but not caudal, cortex also supported thalamic cell attachment (Fig. 7). This rostral to caudal gradient of attachment to the P1 cortical plate *in vitro* parallels the general rostral to caudal gradient by which cortical histogenesis (Smart, 1983; Bayer and Altman, 1991) and innervation of the cortical plate (cf. Reinoso and O'Leary, 1988) occur *in vivo*.

Finally, by P7 *in vivo*, thalamocortical axons have invaded the cortical plate throughout the rostrocaudal extent of the cortex, with most axons terminating in layer 4 and the deepest aspect of layer 3 (Naegele et al., 1988; Miller et al., 1993; Kageyama and Robertson, 1993; Agmon et al., 1993). *In vitro*, thalamic cells also attached to and extended neurites on the cortical plate throughout the rostrocaudal extent of the cortex, but attachment and neurite outgrowth within the cortical plate was limited to layers 4-6 (Figs 8, 9), i.e. the layers through which thalamic axons grow *in vivo*.

At each of these stages, thalamic cells *in vitro* also attached to and extended neurites on the marginal zone (layer I) and on particular non-cortical regions which thalamic axons do not normally enter, such as the postnatal hippocampal commissure and the medial septum (Figs. 1, 2, 8, 9, and 10).

Figure 10. Embryonic thalamic neurons attach to various subcortical regions. Photos show cocultures of embryonic thalamic cells plated onto sagittal sections of E16 (A-F) or coronal sections of P1 (G-J) forebrain. Photos represent slices cut from different regions along the mediolateral or rostrocaudal axes so as to include different subcortical structures. The first panel in each pair of photos shows slice anatomy (A,C,E,G,I) as revealed by UV fluorescence after cocultures were stained with the nuclear marker bisbenzimide. The second panel shows attached thalamic cells (B,D,F,H,J). Cells attach well to all regions of embryonic thalamus (A,B), the caudate-putamen (C,D), and globus pallidus (E,F), however cells avoid the amygdaloid area that borders the internal capsule and basal ganglia in the ventral forebrain (E,F). On P1 slices, cells attach well to the striatum (globus pallidus and caudate-putamen) as well as to the hippocampal commissure and medial septum (G-J). Cells also display some attachment to the preoptic areas of the ventral forebrain. Many regions, however, support almost no attachment -- such as the lateral and ventral regions of septum, the corpus collosum, the claustrum, and the anterior commissure. White lines extending diagonally across panels G-J are produced by a dye within the nitrocellulose substratum that autofluoresces. Abbreviations: *t*, thalamus; *cp*, caudate-putamen; *IC*, internal capsule; *gp*, globus pallidus; *aa*, amygdaloid area; *CC*, corpus callosum; *s*, septum; *ms*, medial septum; *ls*, lateral septum; *vs*, ventral septum; *re*, rhinencephalon; *HC*, hippocampal commissure; *AC*, anterior commissure; *cl*, claustrum; *poa*, preoptic area.


B
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Thus, territories not occupied by thalamic axons *in vivo* can be permissive for thalamic cell attachment and neurite outgrowth *in vitro*.

Given that thalamic cells attach to and extend neurites on regions that thalamic axons do not enter or encounter, it would appear that these cells are able to recognize cues that may be pertinent to the development of other migrating cells or axons. Recognition of cues meant for other axons has often been seen when neurons are transplanted into environments that they do not normally encounter. For example, after grafting or rerouting into tissues that they do not normally encounter, retinal axons will reproducibly follow particular paths or terminate at targets appropriate for other axons (Katz and Lasek, 1978, 1979; Sur et al., 1988b; Zwimpfer et al., 1992; Roe et al., 1993). Thus, it should not be surprising that thalamic cells are also able to attach to and extend neurites on regions which do not lie directly on the path of thalamic axons *in vivo* -- such as the embryonic marginal zone, the hippocampal commissure, and the medial septum (Fig. 10G-J). Based on the idea that thalamic cells may recognize non-thalamic guidance cues, one may also expect some non-thalamic cells to recognize cues that guide thalamic axons (see below for further discussion and references)

Although no quantitative analyses were made of thalamic cell behavior on subcortical regions, qualitative assessments indicate some correlations between *in vitro* attachment to subcortical regions and *in vivo* pathway development. In particular, cells attach well to the embryonic thalamus (Fig. 10A-B) and lateral ganglionic eminence (presumptive caudate-putamen; Fig. 10C-D), regions through which thalamic axons extend to reach the cortex. At the embryonic stage, cells avoid the ventral forebrain (Fig. 10E-F) which may represent a region that thalamic axons encounter but do not enter when they extend from the thalamus to the caudate-putamen.

Indeed, there are two other regions that thalamic cells avoid *in vitro* that are also avoided by thalamic axons *in vivo*: At least at postnatal stages, neither the ventral and lateral septum nor the corpus collosum support cell attachment (Fig. 10G-J). The lateral septum borders the striatum and may be encountered by thalamic axons as they extend to and through the caudate-putamen. The corpus collosum is contiguous with the white matter of the cerebral cortex, but thalamic axons do not cross the corpus collosum even though the do extend in the white matter. It would be interesting to assess whether thalamic cell avoidance of these regions represents developmentally relevant, inhibitory phenomena. In any case, data presented here do establish that at least some territories that mark boundaries of thalamocortical axon growth *in vivo* (i.e. the entire cortical plate at early stages; layers 2 and 3 at later stages) were consistently non-permissive for thalamic cell attachment and neurite outgrowth *in vitro*.

Are Thalamic Cells Detecting Axon Guidance Cues?

It is striking that regions of cortex that thalamic axons confront, but into which they do not normally grow, are readily detected in culture as regions to which dissociated thalamic cells do not attach. This observation raises the possibility that the adhesive behavior of neuronal cell bodies can, in some cases at least, reveal the locations of axonal guidance cues. Such a view has also been suggested by investigators studying the developing chick retinotectal system (Barbera et al., 1973; Gottlieb et al., 1976).

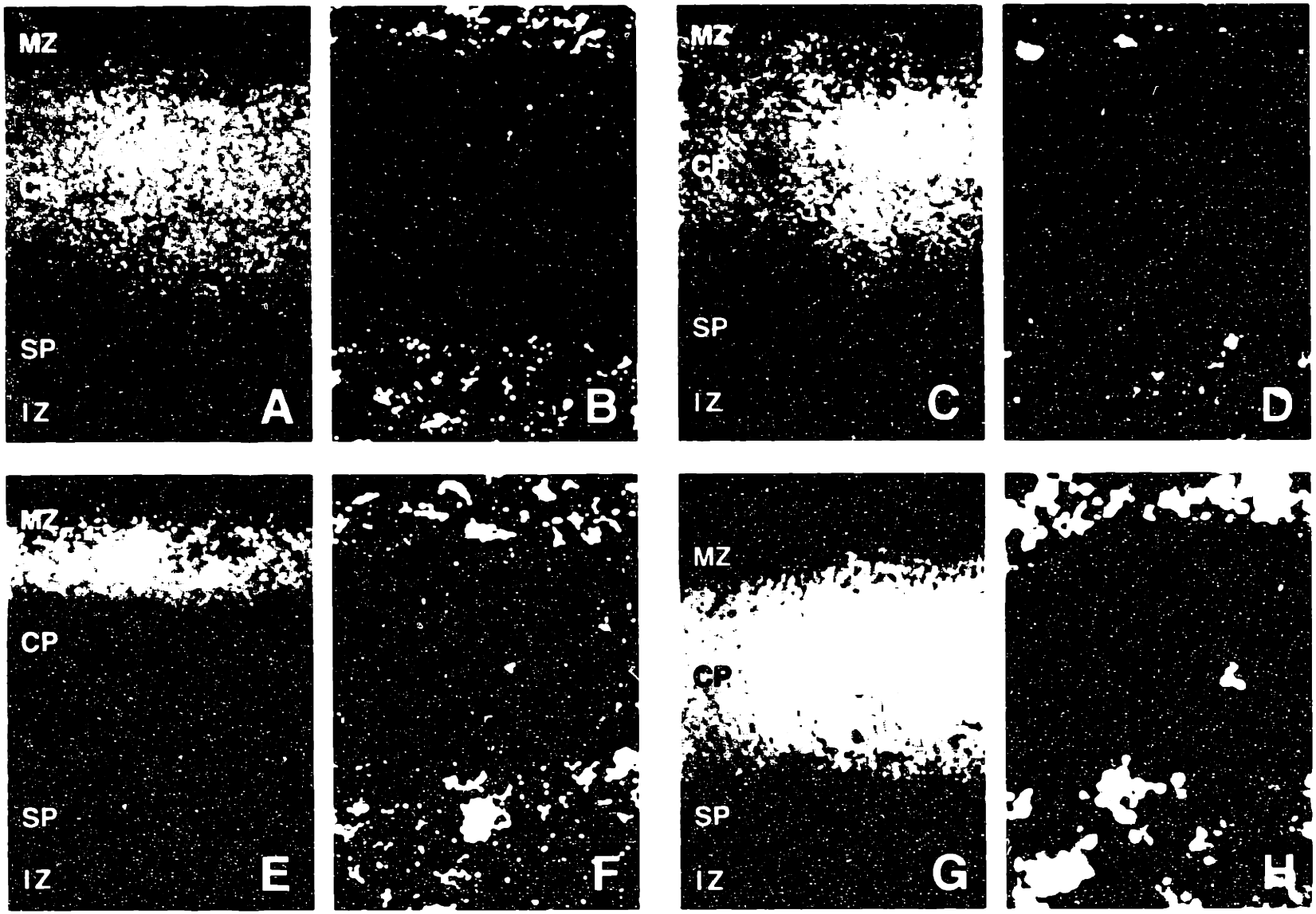
It is interesting in this regard to compare the conclusions of the present study with observations by other investigators on the *in vitro* growth of thalamic axons on substrata of isolated cortical membranes: Götz et al. (1992) found that embryonic (E16), rat cortical membranes were relatively non-

permissive for axon outgrowth, but postnatal (P7) membranes were not. More recently, Tuttle et al. (1995) showed that the change in the outgrowth-promoting properties of cortical membranes--from non-permissive at embryonic stages, to permissive at postnatal stages--could also be observed with membranes prepared from only the superficial cortex (i.e. the cortical plate and marginal zone--of which the cortical plate is the major component).

Thus, both these and the present study point to the cortical plate as the site of transient non-permissiveness, both for neuronal attachment and axon growth. It is not unreasonable to suppose that the cause is the same for both the lack of adhesion and the lack of axon growth, especially given that many of the molecules that promote axon growth also mediate cell adhesion (cf. Lander, 1989; Hynes and Lander, 1992). Indeed, while there are certainly cases in which the guidance of neurons by their substratum is not controlled by differential adhesion (cf. Gundersen, 1987; Calof and Lander, 1991; Lemmon et al., 1992), the development of the thalamocortical system may be a case where adhesion does play a dominant role.

One issue not discussed above is whether the non-permissiveness of the cortical plate (and subsequently layers 2/3) is a property that only thalamic neurons can recognize, or whether other classes of neurons might be able to detect the same cues. Although extensive studies have not been carried out, preliminary experiments support the latter view. To date, dissociated cells from E15 cerebral cortex, E14 ganglionic eminence, and P4 cerebellum have been labeled and plated onto E15 forebrain slices, and all showed markedly poorer attachment to the cortical plate than to other layers (Fig. 11; for cerebellar cells, the difference between attachment to the cortical plate and to other layers was apparently not as great as for thalamic cells, but was still noticeable). These results suggest that the non-adhesive properties of the

Figure 11. Non-thalamic neurons and non-neuronal cells also avoid the cortical plate. High-magnification photographs display cocultures in which E15 cerebral cortex (A,B), E14 ganglionic eminence (C,D), P4 cerebellum (E,F), and 3T3 fibroblasts (G,H) were dissociated and plated onto sagittal slices of E16 mouse forebrain. The first panel in each pair of photos shows cortical layers (A,C,E,G) as revealed by UV fluorescence after cocultures were stained with the nuclear marker bisbenzimide. Regions corresponding to the marginal zone (MZ), cortical plate (CP), subplate (SP), and intermediate zone (IZ) are indicated. The second panel in each pair (B,D,F,H) shows attached cells. In all cases, the density of cells attached to the marginal zone and subplate/intermediate zone is greater than the density of attachment to the cortical plate.


MZ
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cortical plate are quite general, and even extend to cell types that never contact the cortex. Such results are perhaps not surprising, given that many axon guidance cues are known to act on cells that normally never see those cues. Examples include the fact that most classes of neurons extend neurites in response to laminin, even though laminin is present in only a few parts of the developing brain (reviewed by Lander and Calof, 1993); that retinal axons will regenerate through peripheral nerve grafts and terminate specifically within structures that are not normal targets (Zwimpfer et al., 1992; Carter et al., 1994); and that even fibroblasts--which are not found in the central nervous system--respond to the motility-inhibiting effects of the central myelin-derived inhibitor of neurite outgrowth (Caroni and Schwab, 1988).

What Molecules are Thalamic Neurons Detecting *in vitro*?

The general non-permissiveness of the embryonic cortical plate and postnatal layers 2/3 for cell attachment and neurite outgrowth *in vitro* suggests that these regions either lack molecules that promote neuron adhesion and outgrowth, or possess molecules that inhibit adhesion and outgrowth. Although the data presented here cannot distinguish between these possibilities, at least one major mediator of neural cell adhesion--NCAM--is clearly expressed in the embryonic cortical plate and postnatal supragranular layers (Fushiki and Schachner, 1986; Chung et al., 1991; and unpublished observations). In addition, no dramatic differences have been seen in the relative isoform composition or polysialic acid content of the NCAM in these, versus other layers, of the developing cortex (Chung et al., 1991; and unpublished observations).

Considering that NCAM can act as a homophilic adhesion molecule and that the majority of plated thalamic cells plated in the present study were

NCAM-immunoreactive, the possibility that the embryonic cortical plate and postnatal layers 2/3 contain molecules that inhibit cell adhesion and axon growth is worth considering seriously. Recently, several molecules, particularly certain components of the extracellular matrix, have been claimed to have "anti-adhesive" (reviewed by Sage and Bornstein, 1991) and "neurite-repulsive" (e.g. Snow et al., 1990a; Faissner and Kruse, 1990) properties. Indeed, in the retina, the gradual central-to-peripheral disappearance of one such molecule has been suggested to play a crucial role in guiding axons towards the optic fissure (Brittis et al., 1992).

Whatever the molecules are that account for the different adhesive and neurite outgrowth-promoting properties of different cortical layers, it should not be assumed that their source is the intrinsic cells of the cortex. Thalamic axons themselves, growing within certain cortical layers, could contribute some of the cues that thalamic cells detect when they are plated onto cortical slices (present study) or cortical membranes (Götz et al., 1992; Tuttle et al., 1993). For example, some or all of the attachment of thalamic cells to the embryonic subplate and intermediate zone (Figs 1, 2), the tangential orientation of neurites growing on those zones (Figs 3, 4B, 5), and the thalamic cell attachment to postnatal layers 4-6 (Figs 8, 9), could reflect the binding of thalamic neurons to thalamic axon membranes. Such homotypic interactions could play just as important a role in guiding the majority of thalamic axons *in vivo* as interactions of thalamic axons with intrinsic cortical cells. The assay described in the present study, performed following the selective ablation of appropriate cells or fibers *in vivo* (cf. Ghosh et al., 1990), should prove useful in determining the sources of the cues that are responsible for the layer-specific behavior of thalamic cells and their axons.

Overall, the results presented in this paper suggest that molecules that influence neuronal cell adhesion may, through their patterns and timing of expression, provide important cues for the growth of thalamic axons along the radial axis of the cortex. The continued use of rapid *in vitro* assays, such as the one described here, should aid in determining whether and how known adhesion molecules participate in this process.

Acknowledgments

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CHAPTER III

Inhibitors and Promoters of Thalamic Neuron Adhesion and Outgrowth in Embryonic Neocortex: Functional Association with Chondroitin Sulfate

ABSTRACT

To investigate the roles of chondroitin sulfate proteoglycans (CSPGs) in nervous system development, a tissue culture assay was exploited in which mouse thalamic neurons are plated onto living slices of embryonic cerebral cortex. In this assay, dramatic differences are seen in the ability of particular cortical layers to support thalamic cell attachment and neurite outgrowth, and these differences correlate spatially and temporally with patterns of thalamocortical innervation (Emerling and Lander, 1994, Development 120: 2811-2822). Here we show that these layer-specific differences can be eliminated from the embryonic day 16 cortex by enzymatic removal of chondroitin sulfate (CS). Specifically, the cortical plate (a layer avoided by thalamic axons *in vivo*) is shown to possess an inhibitory activity (anti-adhesive, neurite repelling) and the intermediate zone and subplate (layers in which thalamic axons normally grow) are shown to possess a stimulatory activity (adhesive, neurite promoting). Both activities are chondroitinase-sensitive. Biochemical analysis suggests that these opposing activities are not due to the presence of different CSPG species in different cortical layers. Rather, the data indicate that these activities may reflect the presence of differentially localized CS-binding molecules which, significantly, can also be competed away by soluble CS. This model provides a way to reconcile conflicting reports on the roles of CSPGs in neural development, and suggests a fundamental role for CSPGs in the organization of matrix-bound cues in the brain.

INTRODUCTION

Extracellular matrix (ECM) molecules that have dramatic effects on neuronal adhesion, shape, migration, and neurite outgrowth are expressed in many regions of the developing nervous system, and are thought to play crucial roles in neural development (reviewed by Sanes, 1989; Lander and Calof, 1993; Letourneau et al., 1994). Although its organization is not well understood, the ECM of the vertebrate central nervous system is unique in its relative lack of fibril- and polymer-forming matrix molecules (collagens, elastin, laminins, etc.) and relative abundance of chondroitin sulfate proteoglycans (CSPGs) and hyaluronic acid (Margolis et al., 1975a; and reviewed by Toole, 1976; Lander and Calof, 1993).

CSPGs are a heterogeneous set of proteins that are substituted with O-linked glycosaminoglycans of the chondroitin sulfate (CS) class. A linear polymer of repeating dissacharides, CS is itself heterogeneous due to variations in polymer length, degree of sulfation, and degree of epimerization (the latter change producing carbohydrate structures known as dermatan sulfate). During development, strong immunostaining for CS often localizes to territories that are thought to act as barriers to migrating neurons or extending axons. These include the posterior sclerotome (Perris et al., 1991; Oakley and Tosney, 1991), the dorsal midline of the spinal cord and optic tectum (Oakley and Tosney, 1991; Hoffman-Kim, D., 1996), regions of the developing retina (Snow et al., 1991; Brittis et al., 1992), and the epidermis and basal lamina of innervated skin (Kitamura, 1987; Hemming et al., 1994). *In vitro*, CSPGs (Perris and Johansson, 1987; Snow et al., 1990a; Friedlander et al., 1994; Dou and Levine, 1994; Maeda and Noda, 1996), the isolated core proteins of CSPGs (Oohira et al., 1991; Dou and Levine, 1994; Maeda and Noda, 1996), and/or CS by itself (Carbonetto et al., 1983; Verna et al., 1989) have been shown to inhibit cell

migration or neurite outgrowth on defined, growth-promoting substrata. Data obtained using organotypic cultures have also suggested that inhibition of CSPG expression can result in neuronal migration or outgrowth into previously avoided territories (Newgreen et al., 1986; Fichard et al., 1991; Brittis et al., 1992).

Despite these findings, it is clear that regions of tissue that strongly express CS do not exclude the entry of all axons (Yaginuma and Oppenheim, 1991; Oakley and Tosney, 1991), and in some cases CS immunostaining coincides with developing axon pathways (Flaccus et al., 1991; Sheppard et al., 1991; Bicknese et al., 1994; McAdams and McLoon, 1995). Paralleling these findings have been several *in vitro* studies suggesting that CSPGs (Streit et al., 1993; Faissner et al., 1994), CS (Lafont et al., 1992; Fernaud-Espinosa et al., 1994), and isolated core proteins (Iijma et al., 1991) can promote rather than inhibit neurite outgrowth.

To better understand what role, if any, CSPGs play in axonal targeting, we chose to study the thalamocortical system. When thalamic axons enter the cerebral cortex, they elongate within the subplate, a layer that immunostains strongly for CS (Bicknese et al., 1994; Miller et al., 1995). Although they encounter and even probe the overlying cortical plate, thalamic growth cones initially do not penetrate that layer (Lund and Mustari, et al., 1977; Crandall and Caviness, 1986; Catalano et al., 1996). Eventually, once appropriate target neurons have appeared within the cortical plate, thalamic axons extend to those target cells. Coincident with axon invasion of the cortical plate, staining for CS in that layer increases (Miller et al., 1995; Tuttle et al., 1995). Thus, the thalamocortical system provides examples of territories that axons choose, territories that axons avoid, and a changing landscape of CS expression over the course of development.

Recently, we obtained evidence that cues to which thalamic axons respond *in vivo* can be rapidly detected in cultured vibratome slices of cerebral cortex (Emerling and Lander, 1994): Within three hours of plating dissociated embryonic thalamic neurons onto living cortical slices, strong layer-specific attachment and neurite outgrowth by the plated neurons becomes apparent. On cortical slices from several developmental stages, thalamic cells attach well and extend neurites into layers in which thalamic axons normally grow (such as the embryonic subplate), and attach poorly and fail to extend neurites into layers that thalamic cells encounter but normally avoid (such as the embryonic cortical plate, and postnatal layers II-III).

In the present study, we demonstrate that, in the embryonic cortex, at least two types of cues--permissive and repulsive--are responsible for the laminar specific behaviors of thalamic cells. We further show that both types of cues are functionally associated with cortical CS, i.e. they require CS either for their biological activity or for their localization within the cortex.

METHODS

Preparation of slice cultures

All materials, recipes, and experimental protocols used in preparing slice cocultures are as described previously (Emerling and Lander, 1994) except as noted below.

Briefly, thalami were dissected from CD-1 mouse embryos, dissociated, and labeled with Cell Tracker™ (Molecular Probes, Eugene, OR), fluorescent dye as previously described. For pretreatment with chondroitinase, dissociated cells were gently agitated for 90 minutes at 37° in complete medium (CM: glutamine free Dulbecco's modified Eagle's medium with serum-free supplements) containing carrier (5 mg/ml bovine serum albumin [BSA, crystalline grade, ICN] in calcium-magnesium free, Hank's Balanced Salt Solution [MediaTech] supplemented with 10 mM Hepes, pH 7.2 [HHBSS]), CM containing carrier and 6 µg/ml cycloheximide (from 12 mg/ml stock in ethanol; Sigma), or CM containing cycloheximide plus chondroitinase ABC (Seikagaku USA; reconstituted with 5 mg/ml BSA in HHBSS). Cells were removed from these additives and/or dye by centrifugation into a cushion of 4 % BSA in HHBSS and diluted to 1.8 X 10^6 cells/ml before plating onto slices.

Forebrains were dissected, embedded in low melting point agarose, sliced with a vibratome to 160 µm thick in a bath of ice-cold phosphate buffered saline (PBS with supplements, see previous), and placed on sterile, pretreated, nitrocellulose filter disks (12 mm diameter; 2-4 slices/disks). Disks with mounted slices were kept under a 100 µl bubble of CM on ice until all slices were cut. For treatment with enzymes or other additives, culture medium was then replaced with 90 µl of either fresh CM (carrier) or fresh CM containing one or more of the following: chondroitinase ABC (Seikagaku USA) reconstituted

with CM or 5 mg/ml BSA in HHBSS; streptomyces hyaluronidase (Seikagaku USA) reconstituted with CM; cycloheximide (see above); a mixture of CS isomers [4:1 ratio of CS A from bovine trachea and CS B from bovine mucosa (Sigma)] reconstituted to 500 μg/ml total with CM. The CS mixture, which contained some of chondroitin 4-, chondroitin 6-, and dermatan sulfate, was chosen so that the final concentrations of each of the different CS isomers were, respectively, ≈30-fold, ≈10-fold, and ≈10-fold greater than the dissociation constant (K_d = 487nM) for CS (from bovine trachea) and the CS-binding protein thrombospondin-1 (Herndon, 1996). Slices were then kept in a 37°C, humidified incubator with an 8% CO_2 atmosphere for 4 hours before being rinsed successively in three volumes (≈14 ml) of HHBSS. Subsequently, 90 μl of CM containing labeled thalamic cells (8 X 10^4 cells/disk) and, if applicable, fresh additives were applied to slices. Cocultures were then placed back in the incubator for 3 hours, rinsed to remove non-attached cells, fixed, counterstained with bisbenzamide, and mounted on slides as previously described.

Staining of cultures slices for CS and hyaluronic acid

Forebrain slices were incubated for 4 hours in CM with or without enzymes, rinsed, and fixed (25 minutes) as above, followed by several washes with PBS. The following incubations were done in a dark, humidified chamber:

For CS-staining, all rinses and dilutions were done with a blocking solution of 1% fish gelatin (Sigma) in PBS with 0.04 % sodium azide. Tissue was blocked for 45 minutes at room temperature, and then left overnight at 4°C in the presence of blocking solution or antibody (CS-56 monoclonal mouse anti-CS (Sigma) ascites fluid diluted 1:500 in block). Slices were subsequently rinsed (4 X 10 minutes) and gently agitated for 4 hours in secondary antibody

[Cy3-conjugated, goat anti-mouse IgM, μ chain specific (Jackson Immunoresearch) diluted 1:200 in blocking solution] at room temperature. After rinsing (5 X 10 minutes), slices were counterstained (10μg/ml bisbenzamide in PBS) and mounted in saturated sucrose containing 0.1% azide and an anti-photobleaching agent (Prolong™ [Molecular Probes], component A).

For hyaluronic acid staining, all rinses and dilutions were done with 3% BSA (Sigma) in TBS (0.1 M Tris-HCl, pH 8, 150mM NaCl). Tissue was blocked (3% BSA, 0.3% Triton X-100 [Pierce] in TBS with 0.1% azide] for 2 hours at room temperature, rinsed (4 X 10 minutes), and then left overnight at 4°C in the presence of rinse solution or 1 μg/ml biotinylated hyaluronic acid -binding protein (courtesy of Bryan Toole). Slices were subsequently rinsed (4 X 10 minutes) and gently agitated for 2 hours at room temperature in 1 μg/ml texas red-conjugated avidin (Zymed). After rinsing (5 X 10 minutes), slices were counterstained and mounted as above.

Data collection and analysis

Fixed cocultures were selected for quantification, as before, based solely on whether cortical, laminar anatomy was preserved as detected under visualization of cell nuclei by bisbenzamide staining. Measurements of cell attachment were obtained as previously described. Briefly, cocultures were visualized using a fluorescence microscope fitted with camera lucida optics set to view a video monitor on which digitized traces were drawn using the hardware and software of the Neuron Tracing System (Eutectics Electronic Inc., Raleigh, NC). Switching between bisbenzamide and rhodamine filters allowed for one trace to be made for each coculture that included both the borders of cortical layers and attached thalamic cells. The area of each layer and the

number of attached cells were extracted from these computer traces. For cocultures in which exogenous CS was used, cell attachment measurements were determined from images collected with a CCD camera and NIH image software. Images taken under bisbenzamide fluorescence revealed the cortical layers as differences in nuclear density which were traced to determine area. Traces were then overlaid on an image of the same slice taken under rhodamine optics showing the attached thalamic cells which were subsequently counted.

Neurites were examined using fluorescence microscopy by switching between bisbenzamide- and rhodamine-filters to reveal cortical layers and neurites of attached thalamic cells, respectively. Neurites were only scored if their entire length, from origin at the soma to the growth cone, could be visualized. A calibrated reticule was used to determine if a given neurite's origin was within 25μm of the border between the subplate and cortical plate.

Photos of CS-immunofluorescence were taken using a standard fluorescence microscope, however a confocal microscope was used to quantify levels of immunofluorescence. The gain and black levels on the microscope photomultiplier were set so that all pixels in images of CS-56 stained slices had values below saturation (maximum pixel intensity of 255) and so that all pixels in images of immunostained control slices (secondary antibody alone) had values of above zero. For all slices examined, gain and black levels were then left at these settings, and images were collected from three separate fields in each of the cortical plate and the subplate/intermediate zone. Fields extended from the marginal zone border to the subplate border for the cortical plate images and from the cortical plate border to the ventricular zone border for subplate/intermediate zone images. A maximum projection image was created from a z-series of images (12 X 4.5 μm optical sections) that began at the focal plane of the tissue surface and ended at a plane 54 μm deep to the

surface. From this image, immunofluorescence levels were defined as the area under the pixel histogram ("pixel total") for each summed image divided by the area of the field itself ("μm^2") and, for each slice and layer, represent the mean of the three values derived from the three separate fields examined. The mean values for each layer of 4 slices are reported.

Attachment and immunofluorescence data were compared by a single factor analysis of variance (ANOV) or a two-tailed t test, as appropriate, to obtain reported P values. A Newman-Keuls test was used to verify outlier values. For neurite data, a chi-squared test was used in which actual data were compared with the values expected should chondroitinase have no effect on neurite crossing.

Microdissection and proteoglycan analysis

Slices of embryonic forebrain (200μm) were prepared as above and then further dissected under ice-cold dissection medium (50 mM Tris-HCl [Fisher] pH 8.0$^{4°C}$, 150 mM NaCl [Mallinckrodt], 1 mM EDTA [Mallinckrodt]) using tungsten (Ted Pella) microknives that were frequently sharpened by electrolysis in 10% KOH and rinsed in PBS (REF). Cortical layers within slices could be discerned under a dissecting microscope using oblique-transillumination (Tuttle et al., 1995). To ensure that the cortical plate sample did not contain tissue from other layers, cuts were made within this layer so that the marginal zone and subplate/intermediate zone samples contained small amounts of cortical plate tissue. Tissue from different layers was collected into 3.5 ml of ice cold homogenization buffer (HB: 50 mM Tris-HCl pH 8.0$^{4°C}$, 150 mM NaCl, 1 mM EDTA, 1μg/ml Pepstatin [Sigma], 1 mM PMSF [Sigma], 250 μg/ml NEM [Sigma], 1% CHAPS [Boehringer Mannheim]) and homogenized at 4°C in a teflon-on-glass homogenizer (Thomas Scientific) with pestle rotation (setting

50) provided by a T-line laboratory stirrer (Talboy's Engineering, Emerson, N.J.). Homogenates were then centrifuged (423,500 x g for 40 minutes) at 4°C. Supernatants were collected, snap frozen in a dry ice/ethanol bath, and stored at -80°C.

PGs were then purified by anion exchange chromatography based on procedures of Herndon and Lander (1990). All steps were carried out at 4°C. Extracts from several dissections were pooled (58 slices total) for each sample layer [total protein by amido black binding (Schaffner and Weissman, 1973): marginal zone, ≈34 µg; cortical plate, ≈60 µg µg/ml; subplate/intermediate zone, ≈ 54 µg; ventricular zone, ≈ 64 µg]. DEAE Spectra/Gel™ M beads (Spectrum), washed in HB, were then added to each of the 4 samples (≈2µl packed beads/µg total protein; ≈40 ml total sample volumes) which were gently agitated overnight. Bead slurries were transferred to separate Poly-Prep columns (BIO-RAD) through which the following buffers were passed (rate of ≈7ml/hour): 20 ml of 50 mM Tris-HCl pH 8.0$^{4°C}$, 150 mM NaCl, 0.5% CHAPS, 10 ml of 50 mM Tris-HCl pH 8.0$^{4°C}$, 250 mM NaCl, 0.5% CHAPS, 10 ml of 50 mM Tris-HCl pH 8.0$^{4°C}$, 250 mM NaCl, 0.5% CHAPS, 6M Urea (Anachemia), 10 ml of 50 mM sodium formate (EM Science) pH 3.5, 200 mM NaCl, 6 M Urea, 0.5% CHAPS, 8 ml of 100 mM Tris-HCl pH 8.0$^{4°C}$, 0.5% CHAPS, and 7 ml of 50 mM Tris-HCl pH 8.0$^{4°C}$, 0.5% CHAPS. Beads were left in the final buffer overnight and, then, washed with 8 ml of 50 mM Tris-HCl pH 8.0$^{4°C}$,150 mM NaCl to remove detergent.

Bound material was radioiodinated using chloramine T as in Herndon and Lander (1990). After washing with 50 mM Tris-HCl pH 8.0$^{4°C}$, 150 mM NaCl, 0.5% CHAPS, radioiodinated material was eluted with 50 mM Tris-HCl pH 8.0$^{4°C}$, 750 mM NaCl, 0.5% CHAPS. Samples were brought to ≈300 µg/ml BSA (crystalline, ICN). Trichloracetic acid-preceptible, radioactive counts were

determined and samples were snap frozen in a dry ice/ethanol bath, and stored at -80°C.

For gel electrophoretic analysis, proteoglycan core proteins were diluted 4-fold in digestion buffer (50 mM Tris-HCl pH $7.1^{37°C}$, 15 mM phosphoric acid [Fisher Scientific], 1 mM EDTA, 1µg/ml Pepstatin, 1 mM PMSF, 250 µg/ml NEM, and ≈400 µg/ml BSA) and either chondroitinase ABC (0.05 U/ml), a combination of chondroitinase and heparitinase (4 µg/ml of enzyme prepared from flavobacterium heparinum by hydroxyapatite chromatography as described by Linker and Hovingh [1972]; see Herndon and Lander, 1990), or no enzyme at 37°C for 3 hours. Digested samples were then analyzed by SDS-PAGE (Laemmli, 1970) under reducing conditions. Each lane was loaded with the equivalent of ≈10,000 counts/minute of labeled material. Dried gels were autoradiographed using HyperFilm™-MP (Amersham) at -80°C.

RESULTS

Treatment of embryonic forebrain slices with chondroitinase ABC alters laminar-specific attachment of thalamic neurons to cortical tissue

When plated onto living slices of pre- and postnatal mouse forebrain, embryonic thalamic neurons display laminar-specific attachment and neurite outgrowth that spatially and temporally correlate with events in thalamocortical development (Emerling and Lander, 1994). Layers of the embryonic cortex that thalamic axons enter *in vivo* (i.e. the subplate and intermediate zone) support the attachment of thalamic neurons *in vitro*, as do regions that thalamic axons do not normally enter (i.e. the marginal and ventricular zones). In contrast, neurons attach poorly to the embryonic cortical plate, a layer that marks a boundary to thalamic axon growth *in vivo*. Furthermore, neurites extending *in vitro* from cells attached to the embryonic subplate and intermediate zone orient parallel with the cortical plate/subplate boundary and fail to enter the cortical plate (thalamic axons cross readily between other layers). When thalamic neurons are plated onto postnatal slices--a time when thalamic axons have invaded the lower layers of the former cortical plate (cortical layers IV-VI)--they attach well to those layers that thalamic axons have invaded, but continue to attach poorly to higher (supragranular) cortical plate-derived layers (layers II-III).

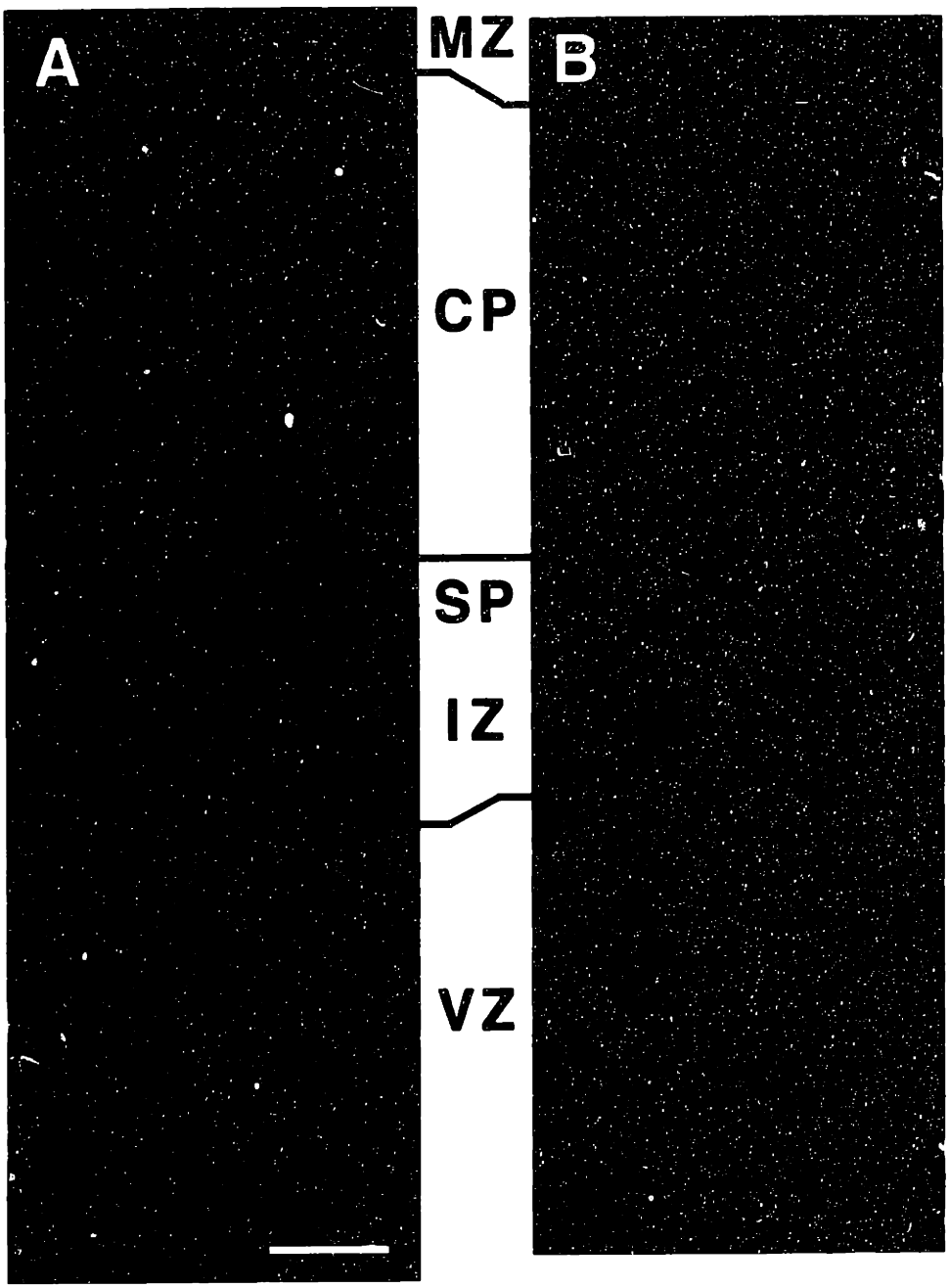
To examine whether CSPGs play a role in the laminar specific behavior of thalamic neurons plated on cortical tissue, thalamic neurons and forebrain slices were cocultured in the presence of chondroitinase ABC, an endoglycoeliminase that degrades all forms of CS (including those containing dermatan sulfate sequences). Briefly, embryonic day 14 (E14) mouse thalamic cells were dissociated, labeled with a fluorescent vital dye, and allowed to settle on freshly cut vibratome slices (160 μm thick) of mouse E16 forebrain that had

been exposed to medium containing either 1 U/ml chondroitinase or carrier alone (complete, serum-free medium) for 4 hours at 37° C. After 3 hours of culture in the continued presence of either chondroitinase or carrier, slices were rinsed to remove unattached cells, fixed, counterstained with bisbenzamide, and visualized by fluorescence microscopy.

In cultures not exposed to the enzyme, thalamic neurons attached in the expected non-uniform manner: they bound efficiently to the subplate, the intermediate zone, and the marginal zone, somewhat less to the ventricular zone, and were almost absent from the cortical plate (Figure 1a). In contrast, when chondroitinase had been included in the culture medium, attachment to the cortical plate was markedly enhanced and attachment to the subplate and intermediate zone was diminished. There was little, if any, change in attachment to the marginal and ventricular zones (Figure 1b). As in previous experiments, levels of thalamic cell attachment to each of the cortical layers were similar throughout the rostrocaudal extent of the neocortex in both treated and untreated cocultures and the non-uniform, layer-specific distribution was noticeable only after cocultures had been rinsed to remove non-attached cells (i.e. effects were due neither to cell migration nor the differential settling of cells; data not shown).

In the above experiment, chondroitinase would have had the opportunity to digest CS both within the forebrain slice and on dissociated thalamic cells. Experiments were next carried out to determine which site of action was responsible for the observed changes in cell attachment. One set of cultures was prepared using embryonic forebrain slices that had been pretreated with chondroitinase, washed extensively to remove the enzyme, and cocultured with untreated thalamic cells at 37° C for 3 hours. A parallel set of cultures was prepared using chondroitinase pre-treated thalamic cells and untreated

Figure 1. Effect of chondroitinase on the attachment of dissociated thalamic cells to embryonic day 16 cerebral neocortex. Sagittal forebrain sections were exposed to complete tissue culture medium containing (A) carrier (complete serum-free medium) or (B) 1 U/ml chondroitinase for 4 hours at 37°C. Subsequently, dissociated E14 thalamic neurons, labeled with a fluorescent vital dye, were plated onto the sections in the continued presence of either carrier or chondroitinase. After 3 hours, slices were rinsed, fixed, and counterstained with bisbenzamide (see methods). The photographs, which are of corresponding regions of dorsomedial neocortex, show the nuclei of endogenous cortical cells, in blue, and attached embryonic thalamic cells in red. Laminae can be discerned as differences in nuclear density. In the absence of chondroitinase, few cells attach to the cortical plate (CP) while more attach to the subplate/intermediate zone (SP/IZ), marginal zone (MZ), and ventricular zone (VZ). The addition of enzyme to the culture medium causes both more cells to attach to the cortical plate and fewer cells to attach to the subplate/intermediate zone. Scale bar, 50 μm.


A
MZ
B
CP
SP
IZ
VZ

forebrain slices. In both sets of cultures, cycloheximide, an inhibitor of protein synthesis, was included both during chondroitinase digestion and afterward (a total of 5-7 hours) to block the re-synthesis and replenishment of CSPGs. Control experiments were also performed in which cycloheximide alone was added. Cultures were rinsed, fixed, and counterstained as before, and the densities of labeled cells attached to each of the cortical layers was determined for several cultures. As in previous analyses, the subplate and intermediate zone data were combined because the border between these two layers could not be accurately determined (Emerling and Lander, 1994).

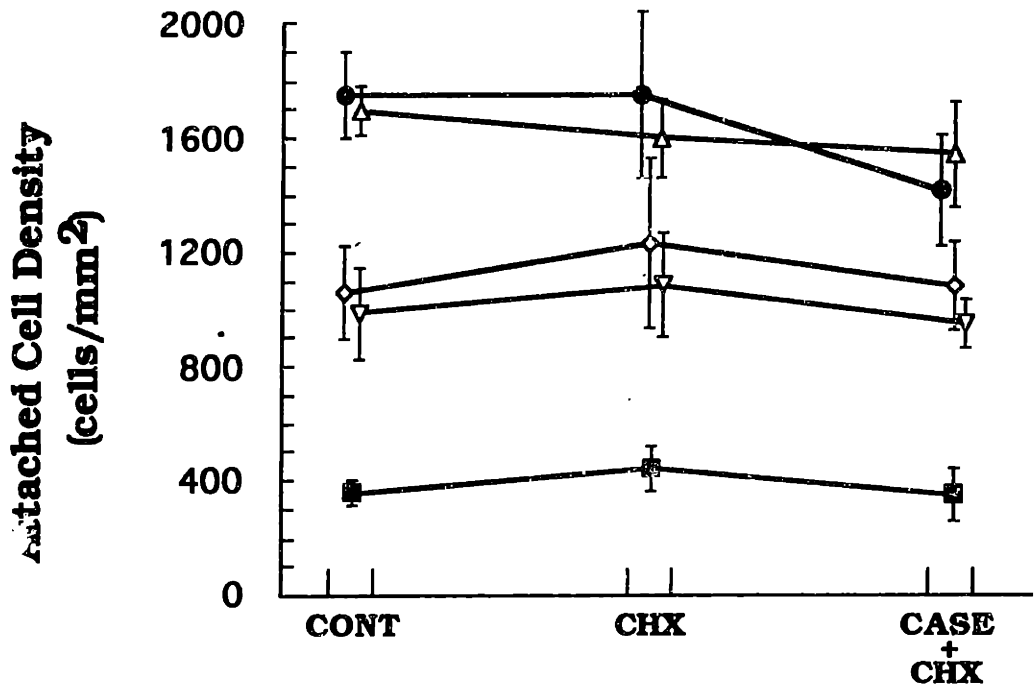
Measurements from cultures that were neither treated with cycloheximide nor chondroitinase (Fig 2a-b; "CONT") quantify the layer-specific differences seen in Figure 1a and agree with previous results. In particular, thalamic neurons attached much less efficiently to the embryonic cortical plate than to other cortical layers. Chondroitinase treatment of thalamic neurons (but not forebrain slices) had no noticeable effect on these results (Figure 2a). In contrast, when forebrain slices (but not thalamic neurons) were treated with chondroitinase, two changes were observed (Figure 2b): a significant increase in thalamic cell attachment to the cortical plate *and* a decrease in attachment to the subplate/intermediate zone. Attachment to other layers was unaffected. In neither case did cycloheximide treatment alone have any detectable effect in these assays. The data imply that chondroitinase acts on cortical tissue to effect changes in thalamic cell attachment to cortical layers.

The effects of chondroitinase correlate with the removal of chondroitin sulfate

As shown in Figure 3, the effects of chondroitinase on thalamic cell attachment are dose dependent. Both the increase in cell attachment to the cortical plate and the decrease in attachment to the subplate/intermediate

Figure 2. Densities of cells attached to embryonic cortical layers after limiting chondroitinase treatment to either thalamic cells or forebrain slices alone. Dissociated embryonic thalamic neurons (A), or E16 forebrain slices (B), were treated with carrier (balanced salt solution, CONT), carrier plus cycloheximide (6 μg/ml, CHX), or 1 U/ml chondroitinase ABC plus cycloheximide (CASE+CHX) before being extensively rinsed and added to cocultures with untreated slices or cells, respectively (see methods). For both (A) and (B), coculture medium contained either carrier (for CONT) or cycloheximide (for CHX and CASE+CHX) but did not contain chondroitinase (see methods). Mean values (± standard error) of densities of cells attached to each of the cortical layers -- the marginal zone (Δ), the cortical plate (■), the subplate/intermediate zone (●), and the ventricular zone (∇) -- were calculated from 8 (A), or 7 (B), slices. 'Off slice' data (◇) were collected from the culture substratum adjacent to the pial surface. When thalamic cells alone were pre-treated (A), neither cycloheximide nor chondroitinase significantly altered cell attachment to any layer (analysis of variance: $P_{\text{all layers}} > 0.2$). Selective treatment of forebrain slices with chondroitinase (B), however, did produce noticeable effects on the densities of attachment to the cortical plate ($P_{cp} < 0.001$) and the subplate/intermediate zone ($P_{sp/iz} < 0.005$) while not significantly affecting attachment to other layers ($P_{mz} > 0.2$, $P_{vz} > 0.2$, $P_{\text{off slice}} > 0.2$). Mean attachment to the cortical plate increased about 4-fold whereas attachment to the subplate/intermediate zone decreased by approximately 50%. A Newman-Keuls analysis verified that both effects were caused by the combined chondroitinase/cycloheximide treatment (CASE+CHX) and not by cycloheximide alone (CHX): $\mu_{cont,cp} = \mu_{chx,cp} \neq \mu_{case+chx,cp}$ and $\mu_{cont,sp/iz} = \mu_{chx,sp/iz} \neq \mu_{case+chx,sp/iz}$.

A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1600
1200
800
400
0
Attached Cell Density (cells/mm2)
CONT
CHX
CASE + CHX
Cell Treatment Prior to Coculture

B

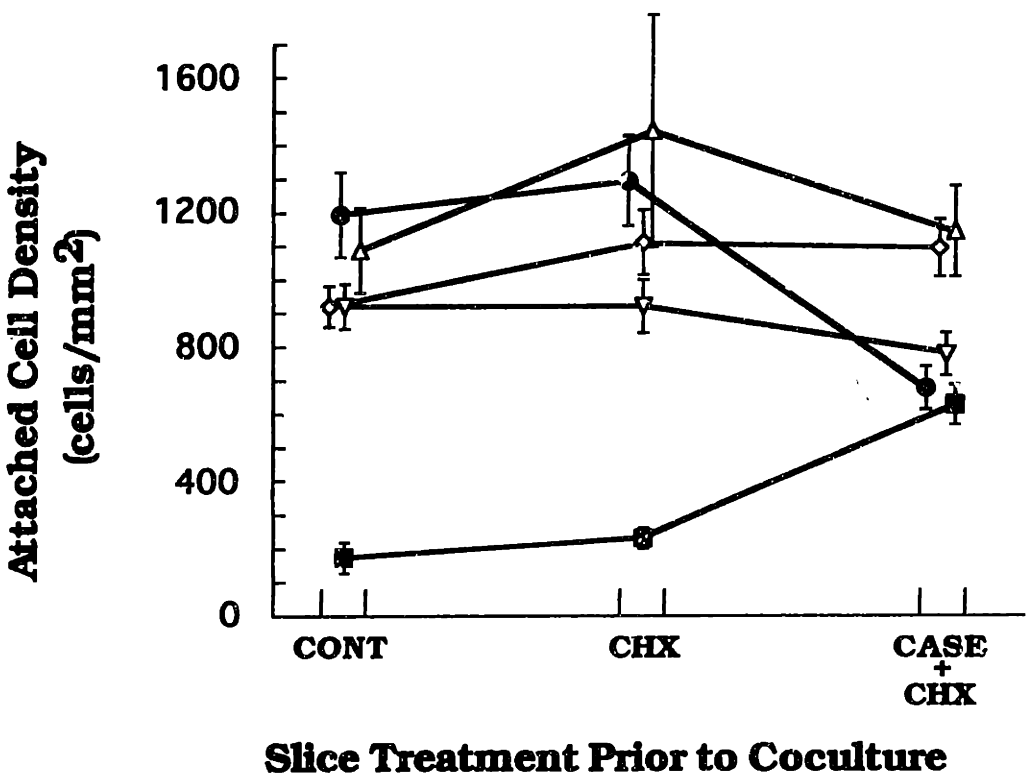
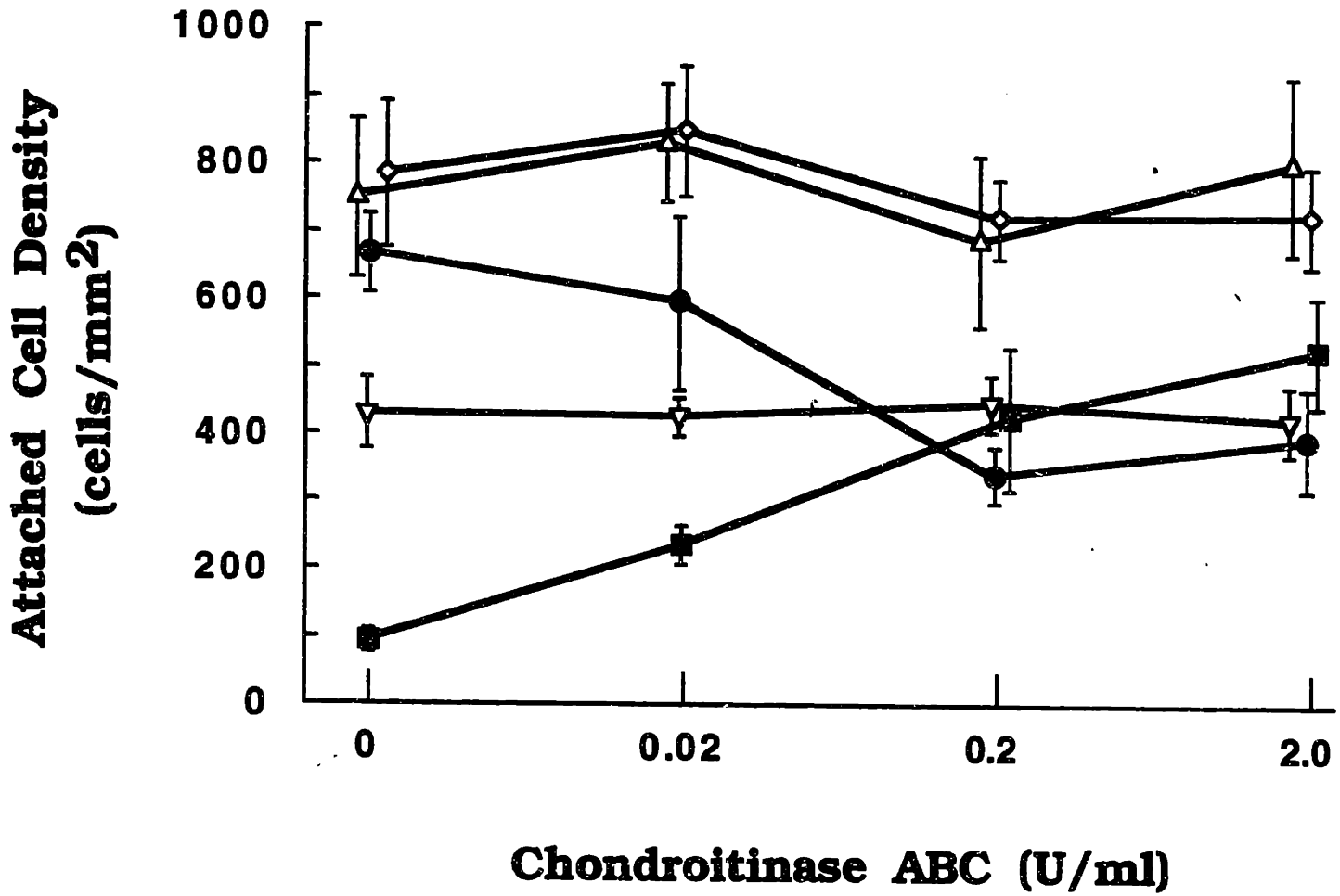

1600
1200
800
400
0
Attached Cell Density (cells/mm2)
CONT
CHX
CASE + CHX
Slice Treatment Prior to Coculture

Figure 3. Effects of chondroitinase ABC dose on thalamic cell attachment to embryonic cortex. E16 forebrain slices were exposed to carrier (complete medium) or various concentrations of chondroitinase for 4 hours at 37°C, followed by coculture with embryonic thalamic neurons in the continued presence of chondroitinase (at the same concentration). After 3 hours, slices were rinsed, fixed, and counterstained. Densities of cells attached to each of the cortical layers -- the marginal zone (Δ), the cortical plate (■), the subplate/intermediate zone (●), and the ventricular zone (∇) -- as well as to the culture substratum ('off slice', ◇), were determined from 8 cocultures for each dose. Mean values (± standard error) are indicated and demonstrate the effect of dose on attachment to the cortical plate and the subplate/intermediate zone, but show no effect on attachment to other layers (analysis of variance: $P_{cp} < 0.001$, $P_{sp/iz} < 0.03$, $P_{mz} > 0.2$, $P_{vz} > 0.2$, $P_{off\ slice} > 0.2$). Mean density of attachment to the cortical plate increased ≈5-fold (X_0=92, $X_{2.0}$=522 cell/mm^2) whereas attachment to the subplate/intermediate zone decreased about 50% (X_0=666, $X_{2.0}$=393 cell/mm^2).


Attached Cell Density
(cells/mm2)
1000
800
600
400
200
0
0
0.02
0.2
2.0
Chondroitinase ABC (U/ml)

zone were noticeable at 0.02 U/ml of enzyme (a concentration within the range used to digest CS from trace amounts of proteoglycans in solution; cf. Herndon and Lander, 1990), and both were saturable between 0.2 and 2.0 U/ml. Saturating effects corresponded to an approximate 5-fold increase in mean cell attachment to the cortical plate and roughly a 50% decrease in attachment to the subplate/intermediate zone.

To test whether the dose-dependence of these changes in cell attachment correlates with the removal of CS from the cortical slice (which should be the case if those effects are due to the CS lyase activity of the enzyme), embryonic forebrain slices were digested exactly as in the coculture experiments, and immunostained using CS-56, an antibody that recognizes both the 4- and 6-sulfated forms of CS (Avnur and Geiger, 1984). Immunofluorescence was quantified from confocal images. An example of an immunostained slice that was not treated with chondroitinase is shown in Figure 4. CS immunoreactivity corresponds well with that seen at this stage *in vivo*, namely that the marginal zone and subplate regions stain more densely than the intermediate and ventricular zones, whereas the cortical plate stains faintly (Sheppard et al., 1991; Tuttle et al., 1995; Miller et al., 1995; unpublished observations). Figure 5 shows the effect of chondroitinase concentration on CS immunoreactivity in the cortical plate and subplate/intermediate zone of cultured slices. For both regions, there was a noticeable decrease after exposure to 0.02 U/ml chondroitinase and a significant decrease across the whole dose curve. Overall, the concentration range over which CS-immunoreactivity is removed from the slice closely parallels the range over which alterations in cell attachment occur.

Although these data are consistent with the idea that digestion of CS causes the observed changes in cell attachment, they can not eliminate the

Figure 4. Immunolocalization of chondroitin sulfate in the dorsomedial cortex of a cultured, E16 sagittal slice. Forebrain slices were cultured at 37°C for 4 hours, fixed, incubated with the CS-56 antibody followed by a Cy3-conjugated secondary, and then counterstained with bisbenzamide. (A) Cortical laminae can be seen under bisbenzamide fluorescence as differences in nuclear density. (B) The same slice viewed under rhodamine optics shows laminar-dependent immunostaining for chondroitin sulfate. Staining is strongest in the subplate (SP) and marginal zone (MZ). However. weak staining can also be detected in the intermediate zone (IZ), ventricular zone (VZ), and the cortical plate (CP). Staining with the secondary antibody alone produced no visible signal above tissue autofluorescence (data not shown). Scale bar, 50 μm.

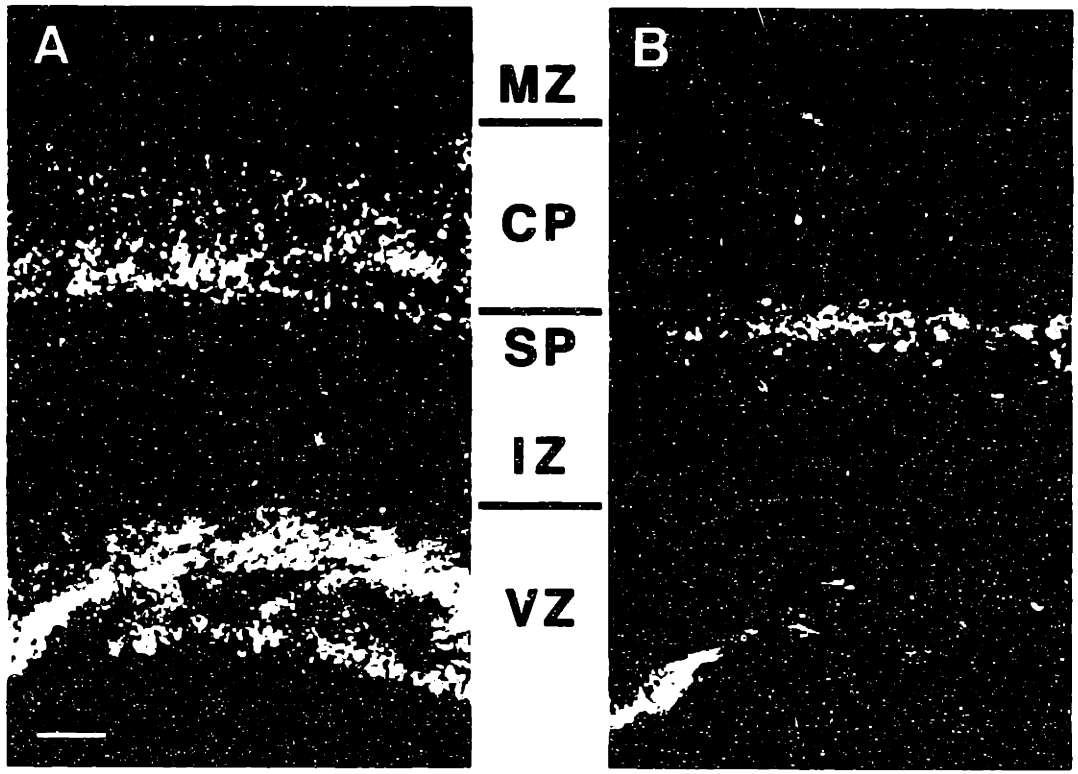
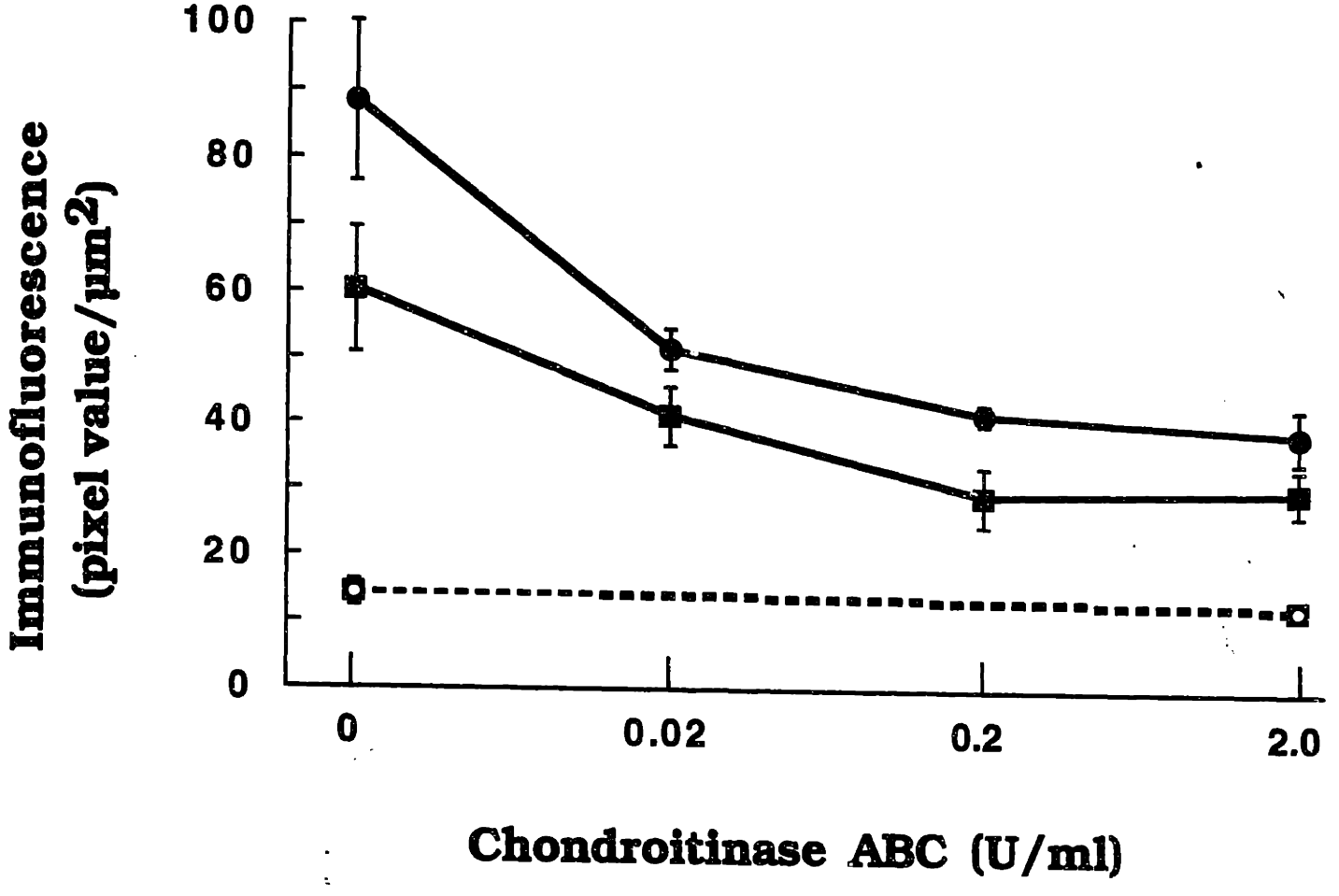

A
MZ
CP
SP
IZ
VZ
B

Figure 5. Dose-dependent removal of chondroitin sulfate immunoreactivity by chondroitinase ABC. E16 forebrain slices were cultured in the presence of carrier (complete medium) or various concentrations of chondroitinase for 4 hours at 37°C, fixed, incubated with the CS-56 antibody followed by a Cy3-conjugated secondary, and counterstained with bisbenzamide. Digitized images of the cerebral neocortex were collected by confocal microscopy from each immunostained slice. Fluorescence levels, obtained from these images, were defined as the area under the pixel histogram divided by the area of the corresponding region within the image. Data points from the cortical plate (■) and the subplate/intermediate zone (●) represent the mean values (± standard error) for 4 slices and demonstrate that chondroitinase removes a substantial fraction of the CS-immunoreactivity (analysis of variance: $P_{cp} < 0.02$, $P_{sp/iz} < 0.001$). Effects are noticeable at 0.02 U/ml chondroitinase and level-off between 0.2 and 2.0 U/ml. To control for the possibility that chondroitinase affects tissue autofluorescence or non-specific binding of the secondary antibody, untreated (n=4) and chondroitinase treated (2 U/ml; n=4) slices were incubated exclusively with secondary antibody and immunofluorescence levels were determined for the cortical plate (□) and the subplate/intermediate zone (○). Data points are mean values (± standard error), and were not altered by chondroitinase treatment (t test: $P_{cp} > 0.4$, $P_{sp/iz} > 0.5$). Differences between "background" fluorescence (dotted line) and fluorescence levels at saturating concentrations of chondroitinase presumably represent undigested CS which is inaccessible to the lyase (e.g. CS "stubs" left at protein attachment sites, intracellular CS) or non-specific, primary antibody binding.


Immunofluorescence
(pixel value/μm2)
100
80
60
40
20
0
0
0.02
0.2
2.0
Chondroitinase ABC (U/ml)

possibility that the effects are produced by another enzymatic activity of chondroitinase, namely the ability to degrade the glycosaminoglycan hyaluronic acid (Yamagata et al., 1968), which is abundantly present in the developing brain (Margolis et al., 1975a; Toole, 1976). To test this possibility, a lyase specific for hyaluronic acid (streptomyces hyaluronidase) was added to cocultures to determine whether it mimicked the effects of chondroitinase on cell attachment. As shown in Figure 6, hyaluronidase used at a variety of concentrations had no significant effect on thalamic cell attachment to any layer. Staining performed using a biotin-conjugated hyaluronic acid binding protein verified that most of the hyaluronic acid was removed at the concentrations of hyaluronidase used (data not shown). Experiments using even greater concentrations of the enzyme (500 TRU/ml) caused forebrain slices to disintegrate (data not shown), an effect never seen with chondroitinase.

Chondroitinase treatment alters the relative permissiveness of subplate and cortical plate tissue for thalamic neurite outgrowth.

In previous work (Emerling and Lander, 1994), we proposed that layer-specific differences in the attachment of thalamic neurons to embryonic cortex reflected the presence of localized axon guidance cues. This idea was supported by the fact that layer specific differences were also observed in the behavior of the neurites extended by attached thalamic neurons. In particular, neurites that originated on the subplate/intermediate zone rarely crossed into the cortical plate, even when they originated within one cell diameter of the border between the two layers.

In the present study, neurite outgrowth near the cortical plate border was examined after chondroitinase treatment. Figure 7a-b shows an example of a typical neurite from an attached thalamic cell in a control culture (no

Figure 6. Effect of streptomyces hyaluronidase on thalamic cell attachment to embryonic cortex. E16 forebrain slices were exposed to carrier (complete medium) or hyaluronidase for 4 hours at 37°C, before coculture with embryonic thalamic neurons in the continued presence of enzyme. After 3 hours, slices were rinsed, fixed, and counterstained. Densities of cells attached to each of the cortical layers -- the marginal zone (Δ), the cortical plate (■), the subplate/intermediate zone (●), and the ventricular zone (∇) -- as well as from the culture substratum ('off slice', ◇), were determined from 8 cocultures for each dose. Mean values (± standard error) are indicated and fail to demonstrate an effect of any dose on attachment to any layer (analysis of variance: $P_{\text{all layers}} > 0.2$).

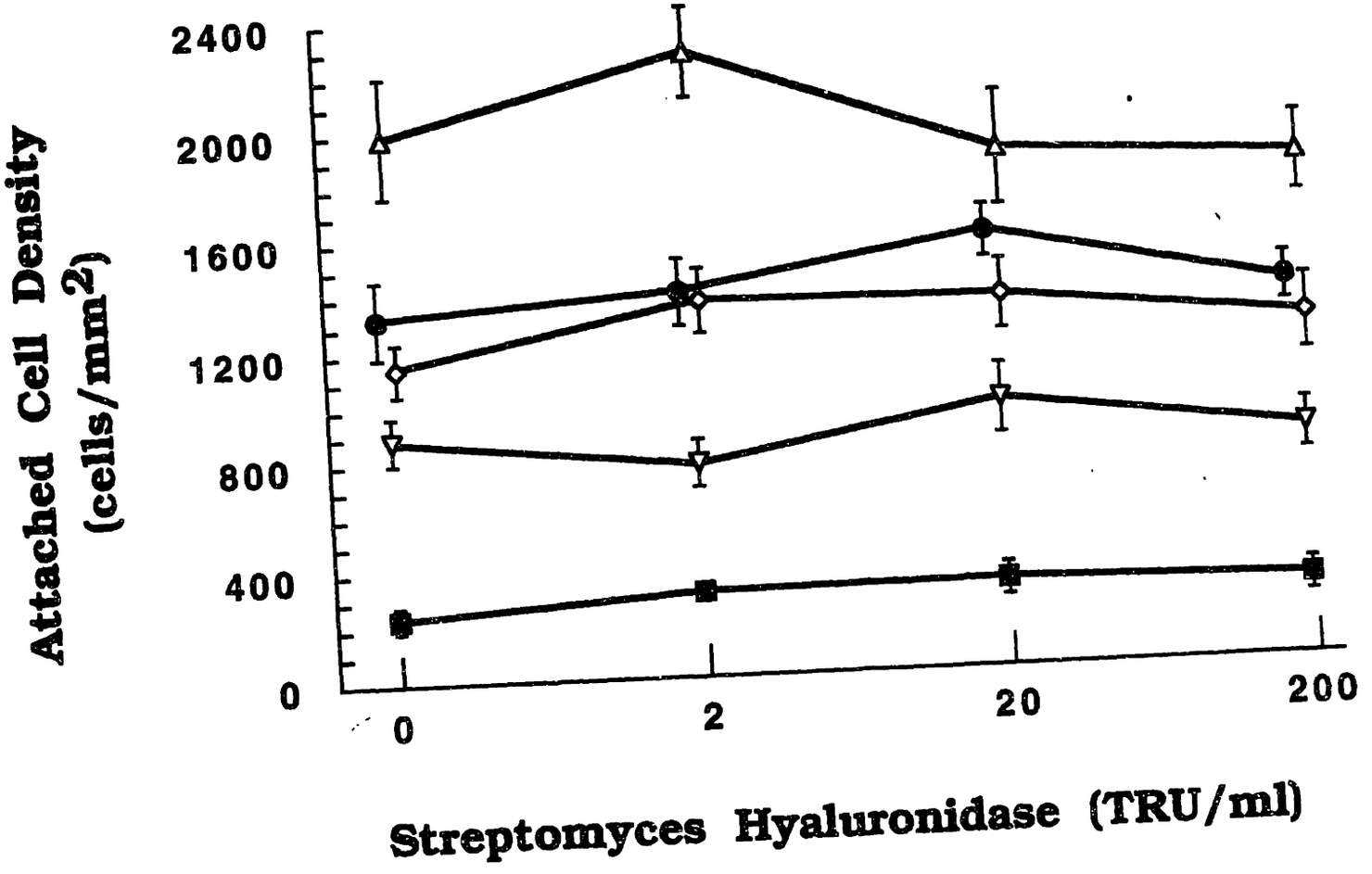
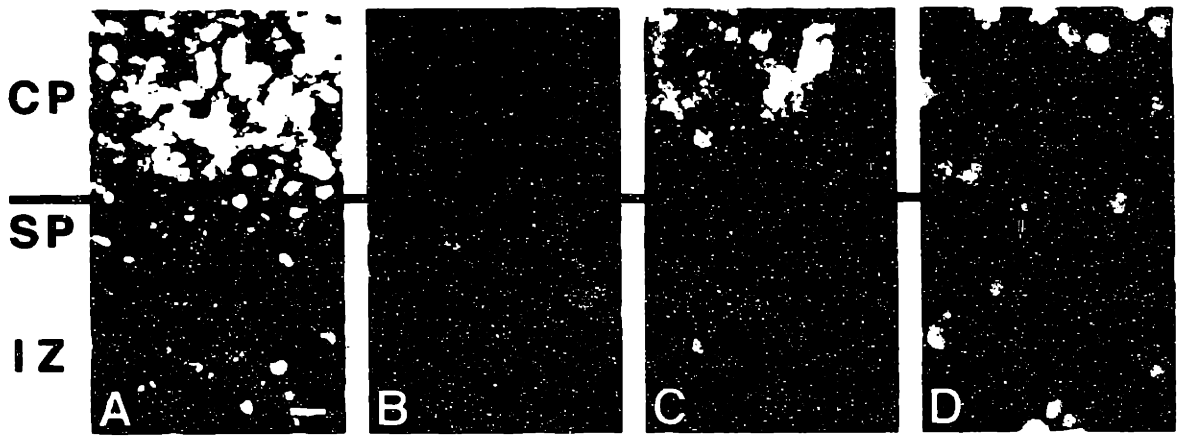

Attached Cell Density
(cells/mm2)
2400
2000
1600
1200
800
400
0
0
2
20
200
Streptomyces Hyaluronidase (TRU/ml)

Figure 7. Thalamic neurite extension on the cortical plate and subplate in cocultures treated with chondroitinase ABC. High-magnification, fluorescence micrographs show the cortical plate (CP), subplate (SP), and intermediate zone (IZ) of bisbenzamide-stained, E16 cortices from cocultures treated with carrier (complete medium, A), or 1 U/ml chondroitinase (C) as in Figure 1. The same slices viewed under rhodamine optics reveal the attached, embryonic thalamic cells and their neurites (B, D). In untreated cocultures (A, B), neurites that extend on the subplate/intermediate zone tend to orient parallel with the cortical layers. Neurites that cross from the subplate onto the cortical plate are extremely rare, as are neurites that originate on the cortical plate (Emerling and Lander, 1994). However, when chondroitinase is added to the medium (C, D), neurite outgrowth on the cortical plate is enhanced and processes that originate on the subplate often cross onto the cortical plate. Scale bar, 10 μm


CP
SP
IZ
A
B
C
D

enzyme); the neurite runs parallel to the laminar boundary and fails to enter the cortical plate. In contrast, in the presence of chondroitinase (1 U/ml), neurites that clearly cross from the subplate to the cortical plate are readily seen (Fig. 7 c-d). Also visible in Figure 7c-d are neurites that originate from the numerous cells that attach to the cortical plate as a result of chondroitinase treatment.

Neurite behaviors were quantified by scoring all neurites that originated within 25 μm of the border between the cortical plate and subplate as either having "crossed" or "not-crossed" this border (Table 1; see Methods). The proportion of all neurites that cross the border was significantly increased by the enzyme treatment. A similar increase is seen if one restricts the analysis to neurites that originated on the subplate and crossed onto the cortical plate. It was not possible to accurately determine whether there was also an increase in the proportion of neurites originating on the cortical plate that crossed onto the subplate, since so few cells attach to the cortical plate in the absence of chondroitinase that the number of neurites originating there under control conditions is extremely low (Table 1). Treatment of similar cultures with streptomyces hyaluronidase produced no noticeable effects on neurite behavior (data not shown),
consistent with the interpretation that the effects of chondroitinase on neurite outgrowth are due to the CS-lyase activity of this enzyme.

Chondroitin sulfate may act indirectly, by immobilizing other molecules in the ECM

The results outlined above indicate that at least two activities in the embryonic cortex that influence thalamic neurons are functionally associated with CS: one is localized to the subplate/intermediate zone and promotes

		Crossed Border	Failed to Cross Border	% Crossed
All Neurites Originating Within 25μm of Subplate-Cortical Plate Boundary	Control	6	203	3%
	Chondroitinase	80	219	27%
Neurites Originating On Cortical Plate	Control	5	3	*
	Chondroitinase	38	148	20%
Neurites Originating On Subplate	Control	1	200	0.5%
	Chondroitinase	42	71	37%

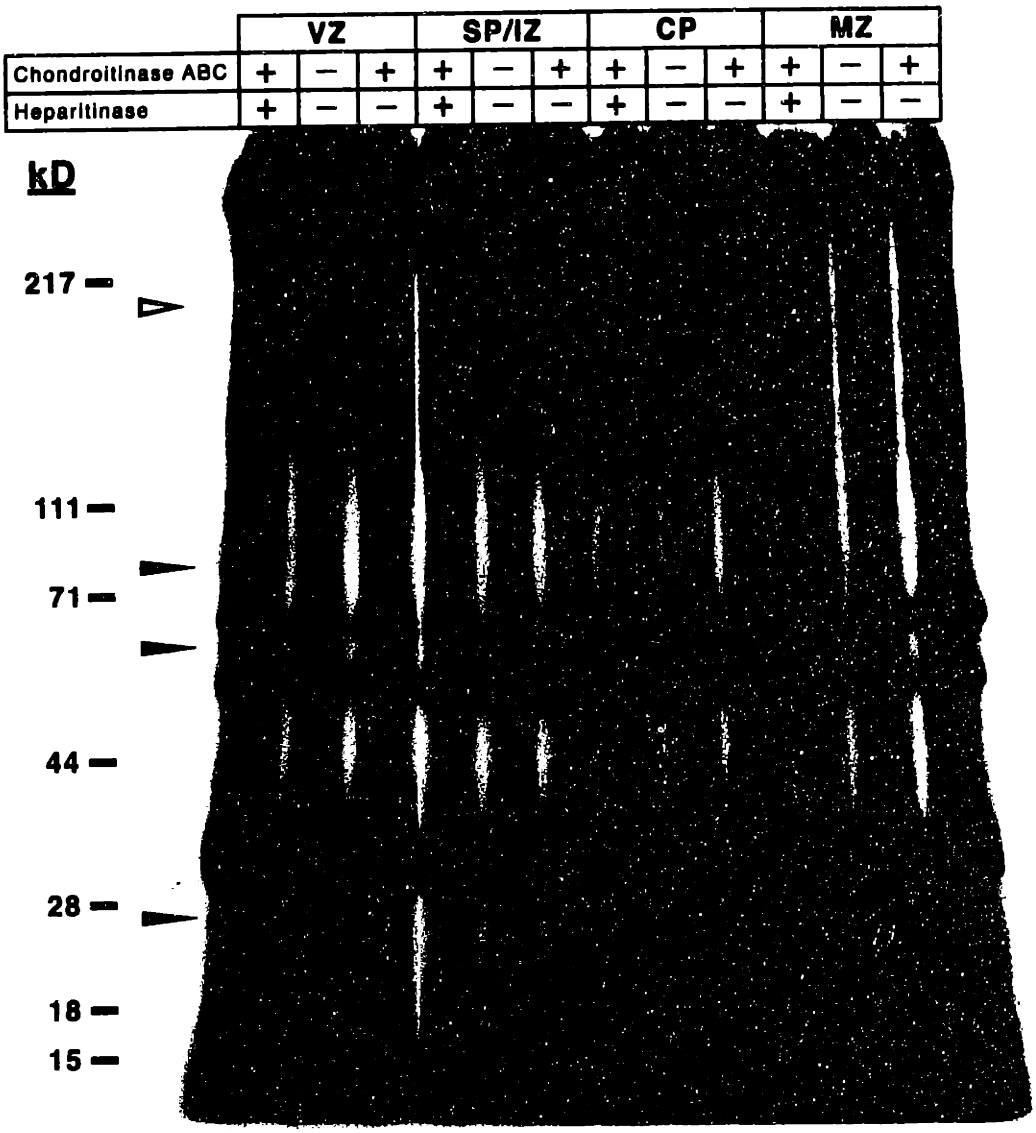
Table 1. Frequency of neurites that cross the border between the subplate and cortical plate in control and chondroitinase treated cocultures. Cocultures of embryonic thalamic cells and E16 forebrain slices were exposed to carrier (complete medium, "control") or 1 U/ml chondroitinase as in Figure 1. All neurites that originated within 25μm of either side of the subplate/cortical plate border were scored from 14 control and 24 treated cocultures. A neurite was defined as having crossed the subplate/cortical plate border if at least half of the neurite's length was on the opposite side of the border from the neurite's point of origin. All other processes were labeled as having not-crossed (see Methods). The data (top) indicate that the frequency of overall neurite crossings increases with enzyme treatment ($P < 0.001$, chi-squared analysis). A similar analysis of just those processes that originate on the subplate (bottom) shows that they cross onto the cortical plate much more often in chondroitinase treated cocultures ($P < 0.001$). Too few neurites originated on the cortical plate under control conditions to make statistical conclusions about the effects of enzyme on these processes (asterisk).

thalamic neuron attachment; one is localized to the cortical plate and inhibits thalamic neuron attachment. One or both of these activities renders the cortical plate non-permissive for the entry of axons from the underlying subplate.

If CSPGs are directly responsible for mediating these two opposing activities, it is reasonable to assume that different CSPGs would have to be involved in the different layers. Experiments were therefore undertaken to look for CSPGs in the E16 cortex that are present in the cortical plate but not in the subplate/intermediate zone, or present in the subplate/intermediate zone but not in the cortical plate. Proteoglycans were directly isolated from laminae that had been individually micro-dissected from slices of E16 cortex. Tissue was homogenized in the presence of detergent, and proteoglycans were enriched by ion exchange chromatography and radioiodinated (Herndon and Lander, 1990). Analysis of PGs was accomplished by comparing core protein banding patterns before and after treatment with glycosaminoglycan lyases (see Methods). As shown in Figure 8, the core proteins of several heparan sulfate-containing proteoglycans, as well as some co-purifying non-proteoglycans, were found to be expressed either exclusively or predominantly in certain layers. However, no CSPG (or mixed heparan sulfate/CSPG) core proteins were found exclusively or predominantly in the cortical plate or subplate/intermediate zone.

Although there may be differences in CSPG structure and/or expression that could not be detected by this analysis (see discussion), these data raise the possibility that CS-dependent activities of the cortical plate and the subplate/intermediate zone may not reside in layer-specific CSPGs themselves, but rather in layer-specific molecules that are associated with CSPGs. The idea that some biologically active proteins are held within the ECM through

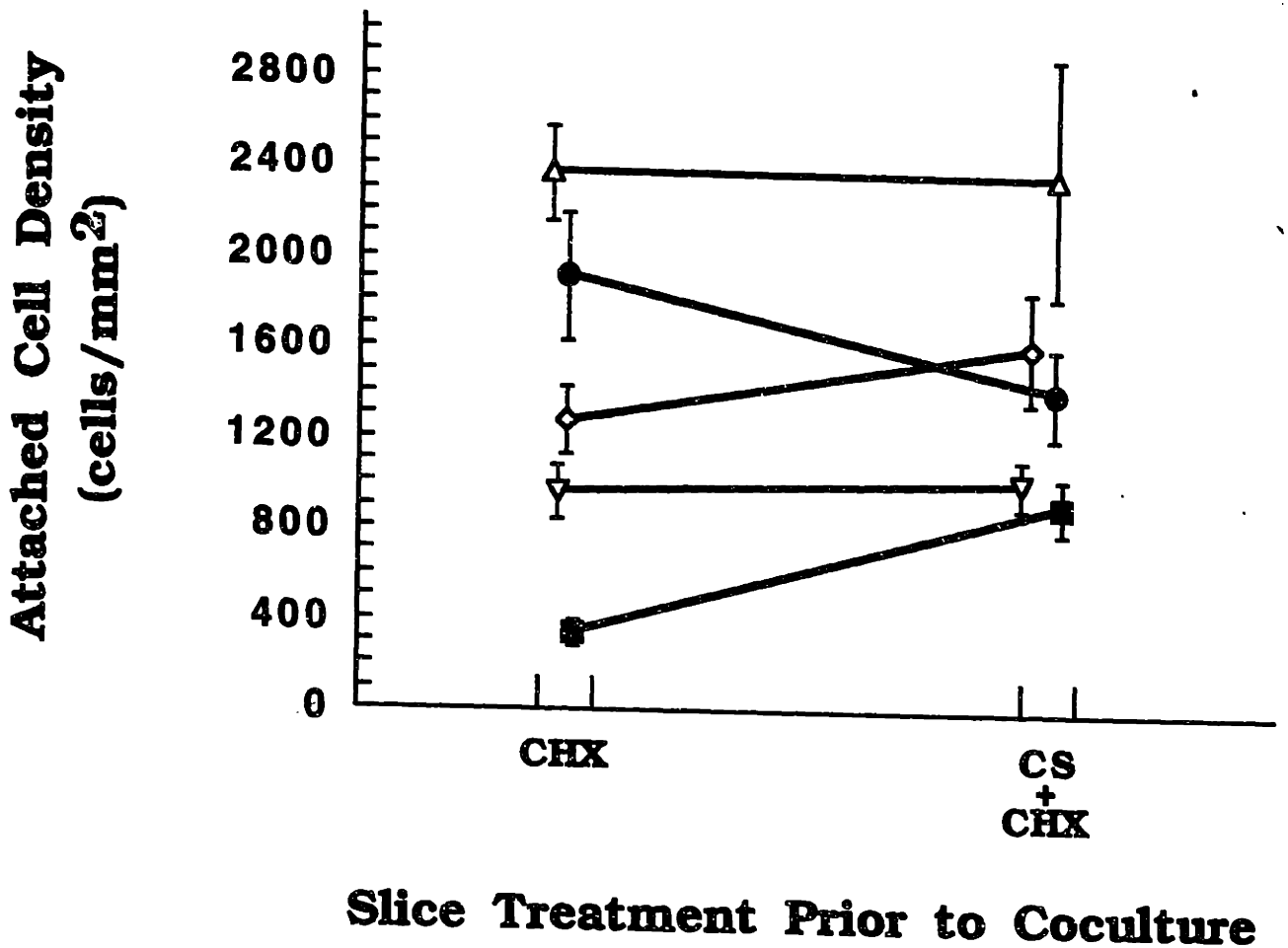
Figure 8. SDS-PAGE of PGs isolated from different layers of the embryonic neocortex. Laminae from E16 cortices were microdissected and homogenized in the presence of CHAPS. PGs were subsequently purified by ion-exchange chromatography and radioiodinated (see methods). Samples from the marginal zone (MZ), cortical plate (CP), subplate + intermediate zone (SP/IZ), and ventricular zone (VZ) were treated with chondroitinase ABC, chondroitinase ABC plus heparatinase, or left untreated prior to being separated by SDS-PAGE under reducing conditions. Bands that appear only after treatment with lyases represent the core proteins of PGs expressed in the respective layers. Some PG core proteins (filled arrowheads) show layer-dependent expression such as the ≈26kD (presumptive M16; Herndon and Lander, 1990) and ≈88kD (presumptive syndecan-1; Ivins and Lander, unpublished data) HS or HS/CS hybrid cores expressed in the VZ and MZ and the ≈67 kD (presumptive glypican; Litwack et al., 1994) HS or HS/CS hybrid core expressed in the VZ. One ≈206 kD non-PG (open arrowhead) is expressed exclusively in the SP/IZ, while another is expressed exclusively in the VZ (≈170 kD, not indicated). No CSPG or HS/CS hybrid cores are exclusively expressed in the CP or SP/IZ. Several PG cores, however, are expressed in all layers: CSPGs at ≈ 137kD, ≈154 kD, ≈168 kD, ≈188 kD, and at least three with apparent weights above 217kD; HS or HS/CS hybrid PGs at ≈79 kD and ≈118 kD.


VZ
SP/IZ
CP
MZ
Chondroitinase ABC
Heparitinase
+ − + + − + + − + + − +
+ − − + − − + − − + − −
kD
217
111
71
44
28
18
15

associations with glycosaminoglycans is well precedented (Roberts et al., 1988; Moscatelli, 1992), and many proteins that bind glycosaminoglycans, including CS, are known to influence cell adhesion and neurite outgrowth (see Discussion). According to this view, removal of CS from cortical slices alters thalamic neuron attachment and outgrowth by causing the release of CS-bound factors. If this model is correct, one should also be able to release the same CS-bound factors by adding an excess of soluble CS, to displace them. The data shown in Figure 9 are consistent with this predication.

In this experiment, forebrain slices were pretreated with exogenous CS (mixed isomers) and rinsed prior to coculture. Cycloheximide was included both during pretreatment and during coculture to prevent re-synthesis both of CSPGs and CS-binding proteins. CS pretreatment increased the level of attachment to the cortical plate, reproducing the effect of chondroitinase on this layer. Attachment to the subplate/intermediate zone also decreased after this treatment, however, experimental variability was such that statistical significance at the 5% level could not be established ($P = 0.16$). Attachment to other layers and to the substratum adjacent to the slice remained essentially unchanged. Overall, the data are consistent with the interpretation that the removal of CS-binding proteins from the cortical plate, and possibly from the subplate/intermediate zone, could account for the marked effects of chondroitinase on the ability of these layers to support cell attachment.

Figure 9. Densities of cells attached to embryonic cortical layers after pretreatment of forebrain slices with CS. E16 forebrain slices were treated with carrier (complete medium) plus cycloheximide (CHX, 6 μg/ml) or CS (500 μg/ml) plus cycloheximide (CS+CHX) before being extensively rinsed and cocultured with untreated cells and cycloheximide-containing medium (see methods). Mean values (± standard error) of densities of cells attached to each of the cortical layers -- the marginal zone (△), the cortical plate (■), the subplate/intermediate zone (●), and the ventricular zone (▽) -- were calculated from 10 cocultures. 'Off slice' data (◇) were collected from the culture substratum adjacent to the pial surface. Cycloheximide alone had no effect on layer-specific attachment (see Fig. 2). Cells attached well to the marginal zone, subplate/intermediate zone, and ventricular zone, but poorly to the cortical plate (analysis of variance: $P < 0.0005$; Newman-Keuls: $\mu_{chx,mz} = \mu_{chx,sp/iz} \neq \mu_{chx,vz} \neq \mu_{chx,cp}$). Pre-treatment of slices with CS caused an approximate 3-fold increase in attachment to the cortical plate (t test: $P_{cp} < 0.001$). Mean density of attachment to the subplate/intermediate zone decreased, although the data were not sufficient to establish statistical significance with 95% confidence ($P_{sp/iz} = 0.16$). Attachment to other layers was not affected ($P_{mz} > 0.5$; $P_{vz} > 0.5$; $P_{off\ slice} > 0.2$). CS pre-treatment caused levels of attachment on the cortical plate to be comparable to those seen on other layers (analysis of variance: $P < 0.01$; Newman-Keuls: $\mu_{cs+chx,mz} \neq \mu_{cs+chx,sp/iz} = \mu_{cs+chx,vz} = \mu_{cs+chx,cp}$).


Attached Cell Density
(cells/mm²)
2800
2400
2000
1600
1200
800
400
0
CHX
CS
+
CHX
Slice Treatment Prior to Coculture

DISCUSSION

The results presented here demonstrate that treatment of embryonic cerebral cortex with chondroitinase ABC dramatically alters the laminar-specific behaviors seen when thalamic neurons are plated upon it. Enzyme treatment caused the embryonic cortical plate, normally a poor substratum for cell attachment and neurite outgrowth, to support attachment and outgrowth at levels comparable to those seen on other layers. Simultaneously, the subplate and intermediate zone, normally excellent substrata for attachment and outgrowth, come to support only half the level of attachment seen under control conditions (Fig. 2, 3). Paralleling these changes in cell attachment were changes in neurite outgrowth, such that thalamic neurites crossed frequently between the subplate and the cortical plate, a phenomenon rarely seen in cultures not treated with enzyme (Fig. 7, Table 1). Analysis of proteoglycan expression in different cortical layers failed to reveal any CSPGs with appropriate laminar-specific expression to account for these chondroitinase-sensitive cell behaviors (Fig. 8). However, similarities between the effects of treatment with chondroitinase and treatment with exogenous CS (Fig. 9) suggested that CS-bound molecules might be responsible for the layer-specific activities of the cortex.

Adhesive and Repulsive Cues in the Developing Cortex

In a previous study (Emerling and Lander, 1994), we argued that the laminar specific differences in attachment and neurite outgrowth that are seen when thalamic neurons are plated onto cortical slices are likely to reflect axon guidance cues within the developing cortex. In that study we were unable to resolve whether the poor attachment of thalamic cells to the embryonic cortical plate results from a lack of promoters of adhesion or the presence of inhibitors

of adhesion. We were also unable to address whether any of the cues detected by thalamic neurons in cortical slices were intrinsic to the cortical tissue, or associated with thalamic axons already present within the slice.

The present study resolves both those issues. The fact that treatment with chondroitinase (or CS) renders the cortical plate a good substratum both for attachment and neurite ingrowth indicates that one or more inhibitory (anti-adhesive, neurite-repellent) factors is found in the embryonic cortical plate. Similarly, the data also point to the presence of a stimulatory (adhesive) activity in the subplate/intermediate zone. Since the cortical plate does not contain endogenous thalamic axons (at this stage), the activity localized to this layer must be intrinsic to the cortex.

Taken together with our earlier results, these data strongly suggest that the developing cortical plate produces at least one neurite-repellent molecule at a time when thalamic axons normally come into contact with the cortical plate, but fail to enter it. Since, at later (postnatal) stages when thalamic axons do invade the cortical plate, the *in vitro* non-permissiveness of the lower cortical plate (layers IV-VI) disappears (Emerling and Lander, 1994), it is likely that the expression of this molecule is down-regulated in those layers. Although it is tempting to speculate that the remaining non-permissiveness of layers (II-III) derived from the cortical plate is due to the persistence of this molecule, we suspect this is not the case, since treatment of postnatal day 7 forebrain slices with chondroitinase ABC fails to have any effect on the non-permissiveness of the supragranular layers (unpublished observations).

The idea that the embryonic cortical plate is inherently non-permissive for thalamic axon growth, and becomes much more permissive postnatally is also suggested by the recent studies of Tuttle and colleagues (1995). These investigators allowed thalamic neurites to choose between substrata composed

of membranes isolated from postnatal or embryonic cortical plate. Although the embryonic cortical plate was a distinctly less preferred substratum for growth, the experiments failed to detect the presence in it of inhibitors of neurite growth. Significantly, the membranes used by Tuttle and colleagues were shown to be lacking in CSPGs (and, presumably, CSPG-binding proteins as well), so it is unlikely that the inhibitory activity detected in the present study would have been seen by them. Together, the data suggest that cues of different types, stimulatory and inhibitory, CS-dependent and CS-independent, act in concert to regulate the timing of thalamic axon invasion of the cortical plate.

Activities associated with chondroitin sulfate may reflect the functions of proteins bound to proteoglycans

Numerous authors have argued that CS and CSPGs are inhibitory to axon growth (Verna et al., 1989; Snow et al., 1990a; Snow et al., 1990b; Snow et al., 1991; Fichard et al., 1991; Friedlander et al., 1994; Dou and Levine, 1994; Maeda and Noda, 1996). Others, using different assays, have argued that these molecules are growth-promoting (Lafont et al., 1992; Streit et al., 1993; Faissner et al., 1994; Fernaud-Espinosa, 1994). The present study demonstrates that, within a single assay, CSPGs can be implicated in both kinds of effects. Interestingly, little correlation exists between levels of overall CS expression and either stimulatory or inhibitory activity. The embryonic cortical plate, for instance, is the site of very weak CS-immunoreactivity, but very potent CS-dependent inhibition of attachment and neurite outgrowth. Both the subplate and marginal zone stain strongly for CS, but only the former was found to possess CS-dependent attachment activity.

These findings imply that, to understand the origins of the CS-dependent activities described in the present study, it is necessary to look beyond the bulk properties of CS. There are two possible explanations for the opposing CS-dependent activities in the cortical plate and the subplate/intermediate zone: These layers could contain CSPGs that have inherently different biological activities, or they could owe their differences to the activities of proteins that are not CSPGs, but whose functions are CS-dependent. The simplest example of the latter type of molecule would be a CS-binding protein that is released from the cortical slice when its interactions with CS are disrupted.

Although our analysis of proteoglycans isolated from different layers of the embryonic cortex failed to identify any CSPGs that were conspicuously present in, or absent from, the cortical plate or the subplate/intermediate zone, these negative results do not constitute proof that no such CSPGs are present. The conditions used for tissue extraction are known to solubilize most, but not all, of the CS in the brain (Margolis et al., 1975b; Iwata and Carlson, 1993). In addition, it is theoretically possible that some CSPG core proteins were missed because they did not label well with ^{125}I; they were insufficiently sulfated to bind well to DEAE-cellulose; or they were simply too rare to be noticed. Indeed, others have reported that immunoreactivity for the CSPG neurocan is more intense in the subplate and marginal zone than in the cortical plate and ventricular zone (Oohira et al., 1994; Miller et al., 1995; Meyer-Puttlitz et al., 1996) and cortical immunoreactivity for the CSPG phosphacan may (Meyer-Puttlitz et al., 1996) or may not (Miller et al., 1995) show laminar specificity. Whether immunoreactivity proves to be a reliable indicator of the level of expression of these CSPGs remains to be seen, especially since the mRNA for both of these core proteins appears to localize

mainly to layers different from those that immunostain strongly for them (Engel et al., 1996).

Even if there are no layer-specific CSPG core proteins in the developing cortex, there still exists the possibility that differences in CS structure could assign different biological activities to common core proteins (cf. Faissner et al., 1994). Yet despite these caveats, the results of one experiment in the present study independently suggests that CSPGs themselves are not directly responsible for the layer-specific adhesive and repulsive activities of the cortex. The fact that both the removal of CS (chondroitinase treatment) and the addition of excess soluble CS have the same effects on the cortical plate (and possibly on the subplate/intermediate zone as well) strongly suggests that both treatments work by disrupting CS-mediated binding of molecules that need not themselves be proteoglycans. There are intriguing parallels between these data and those of Brittis and Silver, who found that treatment of retinal explants with either chondroitinase (Brittis et al., 1992) or soluble CS (Brittis and Silver, 1994) causes ganglion cell processes to extend into a region (the ventricular surface) they would normally not enter. It may be the case that factors that affect axon growth in many regions of the nervous system are bound to endogenous CS.

This idea is particularly exciting because at least two families of ECM proteins--the tenascins and the thrombospondins--are known to bind CS or CSPGs, to be expressed in the developing brain, and to sometimes have anti-adhesive and/or neurite repellent effects in tissue culture (Chiquet and Fambrough, 1984; Hoffman and Edelman, 1987; Grumet et al., 1994; Barnea et al., 1994; Winnemöller et al., 1992; Pancake et al., 1992; Kruse et al., 1985; O'Shea and Dixit, 1988; Pesheva et al., 1989; Faissner and Kruse, 1990; Taylor et al., 1993; and reviewed in Sage and Bornstein, 1991). Tenascin-C, in

particular, is often localized to regions of the CNS that act as boundaries to cell migration and neurite outgrowth (reviewed by Faissner and Steindler, 1995), and it is known to be present in the embryonic cortical plate (Tuttle et al., 1995). In addition to these ECM molecules, two other classes of secreted molecules that can provide repulsive cues to some types of axons are known to bind glycosaminoglycans: semaphorins (collapsins) and netrins (Raper and Kapfhammer, 1990; Colamarino and Tessier-Lavigne, 1995; Jon Ivins, unpublished data). At least one semaphorin is expressed in mouse embryonic cortex (Inagaki et al., 1995). However, only netrin-1 has so far been shown to bind specifically to CSPGs (Litwack et al., 1995).

Do chondroitin sulfate proteoglycans "organize" the brain extracellular matrix?

Is it merely coincidental that disruption of CS eliminates both stimulatory and inhibitory properties of cortical tissue, essentially eliminating the ability of cortical layers to selectively support thalamic attachment and restrict thalamic neurite growth? One explanation why a single treatment can cause opposing effects may be that CSPGs play a general role in organizing the brain ECM, such that interference with the interactions of CS causes the removal or inactivation of a large number of ECM-associated molecules. A similar role has been proposed for heparan sulfate proteoglycans in other types of ECMs, especially with regard to the sequestration of polypeptide growth factors (Roberts et al., 1988; Moscatelli, 1992; Klagsbrun, 1992). In the brain, however, CS is much more abundant than heparan sulfate (Margolis et al., 1975a), and most of the heparan sulfate proteoglycans found in the brain so far are cell-surface, rather than ECM, molecules (Lander, 1993).

The glycosaminoglycan hyaluronic acid is also very abundant in brain and likely plays a major role in organizing brain ECM (Margolis et al., 1975a; Toole, 1976; Delpech et al., 1989; Bignami et al., 1993). However, experiments described here suggest an interesting functional difference between it and CSPGs: Treatment of cortical slices with high levels of hyaluronidase caused overt disintegration of the tissue, whereas chondroitinase had no discernible effect on tissue integrity. These results suggest a model in which hyaluronic acid plays a structural role in brain ECM, analogous perhaps to that of collagens in other ECMs. CSPGs might then associate with the matrix via hyaluronic acid (many CSPGs, including several in brain, bind hyaluronic acid via their core proteins [Zimmermann et al., 1989; Rauch et al., 1992; Iwata, et al., 1993; Yamada et al., 1994]). Finally, other molecules that interact directly with cells would become incorporated into the ECM via interactions with the CS chains of CSPGs. It will be interesting to test the predications of this model, especially as they relate to the mechanisms of localization of numerous glycosaminoglycan-binding factors that are thought to influence neuronal proliferation, survival, cell migration and axon guidance (Parr et al., 1993; Lander and Calof, 1993; Eckenstein, 1994; Shepherd et al., 1996).

Acknowledgements

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CHAPTER IV

CONCLUSION

The data presented in the previous chapters demonstrate that short-term cocultures of dissociated neurons and tissue slices can be effectively used to characterize the location and nature of potential axon guidance cues. Application of this technique to the study of the developing thalamocortical system has indicated that neural cells will display rapid, laminar-specific behaviors *in vitro* that correlate with the laminar-specific development of these systems (chapter 2, see also appendix). For thalamocortical cocultures, this correlation was utilized as a means to assay for activities and molecules that may contribute to thalamic axon guidance.

Because the examination of cell attachment within thalamocortical cocultures was used as a means to search for potential axon guidance cues, it is important to discuss what relation cell attachment has to process outgrowth. Like growth cone and cell migration, attachment is a process that involves interactions between the cytoskeleton, the cell surface, and the substratum. Both cell motility and cell attachment require the regulation of cell surface-substratum contacts (reviewed by Hynes and Lander, 1992; Mitchison and Kirschner, 1988). Little of the strength of cell adhesion is mediated by the passive binding of molecules on the cell surface to molecules within the substratum. Instead, most of the forces that provide for attachment are mediated by contacts that develop over time and involve changes in cytoskeletal organization and cell morphology (McClay and Ettensohn, 1987; Lotz et al., 1989), just as occurs in cell and growth cone migration. Thus, some of the same factors that control cell-substrate contacts during growth cone and cell migration may also affect contacts that manifest cell attachment. This idea is supported by the fact that molecules that mediate attachment are expressed both on the cell body surface and on growth cones (van den Pol and Kim, 1993; reviewed by Rutishauser, 1985) and by the fact that that membrane

constituents added to the growth cone during axon extension flow back to the cell body (Dai and Sheetz, 1995). Furthermore, many molecules that mediate cell attachment also promote growth cone migration (reviewed by Jessell, 1988; Lander, 1989). Indeed, a protein isolated from myelin based on its ability to inhibit the attachment and spreading of fibroblasts is believed to be the activity responsible for the inhibiting effects of myelin on axon outgrowth (Caroni and Schwab, 1988).

The data presented in the previous chapters strongly support the notion that factors that affect cell attachment and those that mediate process outgrowth can, at least sometimes, be one and the same: The behavior of thalamic cell bodies on cortical layers *in vitro* correlated with spatiotemporal patterns of thalamocortical development, suggesting that common mechanisms mediate cell attachment and *in vivo* axon guidance. Furthermore, patterns of cell attachment also correlated with patterns of neurite outgrowth in all cases where processes were examined *in vitro*. Thus, the data collected using thalamocortical cocultures support the notion that cell attachment and axon extension share common molecules and mechanisms.

Besides providing a rapid means to elucidate factors that potentially guide axons, in the case of the experiments described in chapter 3, cell attachment also provided information beyond that which could have been provided by data on neurite outgrowth alone. For example, the effects of chondroitinase on the frequency of neurites crossing from the subplate onto the cortical plate could have been caused by an enzyme-induced decrease in the permissiveness of the subplate, an increase in the permissiveness of the cortical plate, or both. The attachment data provided evidence that the substrate properties of both the subplate and the cortical plate were altered by chondroitinase. Thus, it appears that the preferential attachment of thalamic

cell bodies on cortical tissue indeed provides a useful means of elucidating factors that mediate the laminar-specific process outgrowth which is also observed in these cocultures.

Other studies have also demonstrated that thalamic neurites show laminar-specific outgrowth preferences using substrata composed of extracts from different cortical layers (Götz et al., 1992; Tuttle et al., 1995). However, neither of these studies eliminate the possibility that these preferences are caused by thalamic axon membranes included in the preparations. The same holds true for other slice coculture studies (Yamamoto et al., 1989; 1992; Bolz et al., 1992; Molnár and Blakemore, 1991). In addition, the reports on membrane extracts do not localize the exact layer in which suspected cues exist: one study shows neurites prefer membranes of postnatal layers 5 and 6 combined (Götz et al., 1992), the other shows neurites avoid membranes of embryonic cortical plate and marginal zone combined (Tuttle et al., 1995). The data presented in chapter 3 demonstrate that the cortical plate contains an inhibitory activity, and that a permissive activity exists in the subplate/intermediate zone. Although the activity in the subplate/intermediate zone may derive from thalamic axons, the inhibitory activity in the cortical plate can not be of thalamic origin, since afferents have not entered the cortical plate at the stage examined. This activity is, thus, the first example of a suspected thalamic axon guidance cue that must be of cortical origin. It is also the first evidence that inhibitory cues may contribute to thalamocortical development.

Assessing whether the activities within the cortical plate and subplate/intermediate zone *actually* guide thalamic axons requires an *in vivo* approach. Because the molecule(s) that underlie these activities are not known, the use of transgenics or function blocking antibodies is not yet

possible. However, the fact that these activities are chondroitinase sensitive means that the *in utero* application of this enzyme could address the issue. This experiment could be performed by placing a polymer matrix (e.g. Elvax) containing chondroitinase onto the cortical surface of mice embryos at a stage prior to thalamic axon invasion of the cortical plate (before ≈E16). If processes are found to prematurely invade the cortical plate after this procedure, it would provide strong evidence that thalamic axons initially extend in the subplate and do not enter the cortical plate due to one or both of the CS-dependent activities localized to these regions.

Of course, it would also be desirable to isolate the molecules which underlie the activities discovered *in vitro*. It is possible to take a biochemical approach, particularly if a simplified assay could be devised to screen fractions for the activities. Examining neurite outgrowth in cultures of dissociated thalamic neurons could be such an assay. The permissive activity in the subplate/intermediate zone might be expected to increase outgrowth compared to controls whereas the putative inhibitor in the cortical plate might decrease outgrowth or cause growth cone collapse, such as other outgrowth inhibitors like semaphorins (Luo et al., 1993) and RAGS (Drescher et al., 1995) do *in vitro*. If the molecules that underlie the activities are released from the slice upon chondroitinase or exogenous CS treatments -- as is proposed for the inhibitory activity of cortical plate (chapter 3) -- then the putative factor(s) should be recoverable from the medium of treated slices. It would, thus, be worth testing whether such a "releasate" has an activity that attenuates neurite outgrowth. If there is an inhibitory activity within the media, it would not only verify the usefulness of a simplified assay, it would also demonstrate that CS does serve to localize the inhibitory molecule within the tissue, as is hypothesized.

Indeed, the data presented in chapter 3 raise interesting issues concerning the function of CS in nervous system development. Contrary to other proposals that CSPGs are directly inhibitory to process outgrowth (reviewed by Silver, 1994; Letourneau et al., 1994), I found that CS contributes to both inhibitory and stimulatory phenomena. It would also appear that, at least for the inhibitory phenomena, CS acts in concert with other factors since both digestion of CS and addition of exogenous CS cause the loss of inhibitory activity. Therefore, preliminary data concerning this issue would suggest that CS correlates both with regions that axons enter and regions they avoid (chapter 1) because it plays a role in localizing or restricting the distribution of other molecules that affect axon outgrowth.

It's conceivable that secreted guidance molecules need to bind ECM constituents to prevent their diffusion into regions where their activity would be deleterious to proper development. For example, if the cortical plate contains a secreted inhibitor of outgrowth, what would prevent this putative inhibitor from diffusing into the adjacent subplate where thalamic axons appropriately extend? Binding to localized ECM constituents could mitigate such diffusion and allow for specific regionalization of secreted guidance factors.

A modified version of this model would incorporate the concentration of CS as an important parameter which modulates the accessibility of putative GAG-binding factors to growth cones. This model, based on hypotheses of PG-growth factor interactions in other tissues (reviewed by Ruoslahti and Yamaguchi 1991; Moscatelli, 1992), would suggest that CS acts to sequester guidance factors from growth cones when the concentration of GAG is high. At low GAG concentrations, factors would become more accessible to cells. Applying this model to the role of CS in axon guidance would require

information about the local concentrations of CS in regions that growth cones encounter and the binding constants for CS and putative guidance factors, information which is not known. Furthermore, the kinetics of fibroblast growth factor binding to heparan sulfate suggests that sequestration of growth factors does not necessarily occur even in the presence of high heparan sulfate concentrations in the ECM (Moscatelli, 1992).

Interactions between CS and guidance factors could also be regulated by specificity in GAG-guidance factor interactions. As mentioned in chapter 1, there are a couple of examples in which specific CS sequences have functional significance. The assay described in previous chapters provides a means to assess whether specific CS sequences play a role in the chondroitinase sensitive phenomena described. Specific glycoeliminases exist which selectively digest different forms of CS. For example, chondroitinase ACII is an exoglycoeliminase that digests only chondroitin 4-sulfate and chondroitin 6-sulfate but not DS. Thus, if this enzyme does not produce the same results as chondroitinase ABC, it would indicate that the phenomena are DS dependent and may involve specificity like that seen with DSD-1PG, or DS and heparin cofactor II (chapter 1). Because exogenous CS produces some of the same effects as chondroitinase, addition of different exogenous CS isomers may provide another means of assessing whether specific GAG sequences are involved in laminar specific behaviors.

The data presented in this thesis provide evidence that CS could play a role in specific thalamocortical guidance cues. The assay used to obtain these data can be further used to address the nature of the role of CS. At the least, the data raise questions about current models in which CS acts directly on neurons as an inhibitor, or promoter for that matter, of axon outgrowth. As

more guidance molecules become characterized, and tools become available for studying already characterized ones, it will be interesting to see whether they interact with CSPGs and if such interactions have functional significance *in vivo*.

APPENDIX

Laminar-Specific Attachment of Retinal and Tectal Cells on Slices of Optic Tectum: A Qualitative Study

INTRODUCTION

The coculture technique described in the previous two chapters was designed to examine the role of ECM constituents as axon guidance cues. Although many studies have addressed axon development utilizing other *in vitro* methods, these techniques can pose problems for those interested in studying the ECM. Both dissociated cell cultures (Baird et al., 1992) and artificial matrices, such as collagen gels (Placzek et al., 1990), disrupt or exclude the endogenous ECM, and substrata composed of membrane extracts can be deficient in ECM components (Tuttle et al., 1995).

The ECM is generally preserved in slice cocultures, however the typical design for these types of assays, two tissue slices juxtaposed so that processes can extend between them (Gawäiler, 1981, 1988), presents other problems. For example, in cocultures of thalamic and cortical slices, thalamic neurites reach their target layer 4 but do so via paths that are not followed by thalamic axons *in vivo* (Yamamoto et al., 1989; 1992; Bolz et al., 1992; Molnár and Blakemore, 1991). Thus, analyzing cues located along the endogenous pathway using these cultures can be difficult. Furthermore, because coculture periods are long (several days), temporally regulated cues may be disrupted (Bolz et al., 1993) and biochemical perturbations can be difficult to perform.

The assay I utilized in my thesis research was designed to address these issues. Tissue slices were used to preserve the endogenous ECM within the substratum, neurons were plated directly onto slices so that they would immediately interact with regions suspected of containing guidance cues, and only behaviors that occurred within a short period were examined. This technique proved useful for collecting data on thalamocortical development (previous chapters). Yet, preliminary experiments were also performed to assess the usefulness of this technique using chick retinal and tectal tissue.

This chapter outlines the qualitative data that was collected from these experiments in which retinotectal development was analyzed.

Lamination of axons within the tectum

The laminar development of chick retinotectal projections has been well characterized (reviewed by Yamagata et al., 1995). Retinal axons first extend within the tectum along the superficial surface (stratum opticum, SO), beneath the pia. At approximately post-fertilization day 10 (d10), the axons project collateral branches and change their course of migration to enter deeper layers. Axons terminate and arborize in the relatively cell-dense superficial sublaminae, A-F, of the stratum griseum et fibrosum superficiale (SGFS). Axons do not enter the cell-dense sublamina G of the SGFS and this layer demarcates the deepest extent of the retinal axons within the tectum. Cells within the stratum griseum centrale (SGC) project tectofugal axons within this layer and the subjacent stratum album centrale (SAC). These two layers are relatively cell sparse and are located near the ventricular surface.

Key to tectal layers

SO	stratum opticum - beneath pia, retinal afferent tract
SGFS	stratum griseum et fibrosum superficiale - sublaminae A-F constitute the superficial portion of this layer and are target layers for retinal afferents
SGC	stratum griseum centrale - contains tectofugal projection neurons and efferents
SAC	stratum album centrale - adjacent to ventricular surface, tectal efferent tract

METHODS

All methods for preparation, fixation, and mounting of cocultures were performed as indicated in chapter 2, except as noted below

Dissection Of Tissue

Retinas were dissected from post-fertilization day 7 chicks by dissecting the eyes, removing the vitreous, and separating the retina from the pigment epithelium. Tectal tissue used for dissociated cell preparations consisted of the entire tectum. Tectal tissue slices were obtained by embedding whole chick brains in low melting-point agarose and selectively slicing the tecta.

DiI labeling of axons in tectum

DiI was mixed with rubber cement (40µl fast DiI [5mg/ml in methanol] to ≈1 ml of rubber cement). Small (≈5mm length) steel insect pins were coated with the wet mixture which was allowed to air dry. Pins were placed onto paraformaldehyde-fixed slices of tectum in an orientation perpendicular to the tectal surface and crossing all tectal layers. After ≈16 hours at 37°C, pins were removed. Slices were then counterstained with bisbenzamide, mounted on slides, and visualized by fluorescence microscopy.

RESULTS

Detection of tectal anatomy

Laminae of the tectum could be discerned as differences in cell density which were detected using the nuclear dye bisbenzamide. Reports that describe the anatomy of developing tectum were used as guides (LaVail and Cowan, 1971; Yamagata et al., 1995). DiI was used to label axon layers within the tectum as a means of verifying that the assignment of laminae based on bisbenzamide staining was correct (see Fig. 1 for details).

Retinal cells display laminar-dependent attachment on tectal slices

To determine whether retinal cells respond differentially to various regions of tectum, d7 retina were dissociated and plated onto ≈200μm sagittal sections of tectum. After a 3 hour coculture period, retinal cells plated onto tectal slices were uniformly distributed across the entire culture substratum (data not shown). Only after rinsing and fixation was a non-uniform distribution of cells noticeable (Fig. 2). These results indicate that cells did not migrate, but that they preferentially attached to certain regions. Few attached retinal cells projected neurites within the 3 hour coculture period.

Retinal cells attached to tectal slices within two bands parallel with the tectal surface. Cells attached to the superficial layers of tectum that are innervated by retinal axons (SO and superficial SGFS) and also attached to cell-sparse and cell dense layers corresponding sublaminae H and J, respectively, of the SGFS. In addition, some cells attached to the SGC and SAC, layers that contain axons and neurons that project tectofugally. Interestingly, cells did not attach to a region that corresponds to the cell dense sublamina G of the SGFS and the superficial aspect of the cell-sparse

Figure 1. Localization of axon pathways in the d10 tectum. Fluorescence micrograph shows the laminae of d10 tectum after a 200 μm, vibratome-cut slice has been stained with the nuclear marker bisbenzamide (left panel). The same slice viewed under rhodamine optics (right panel) shows processes within the slice stained with DiI (see methods). The pial (superficial) surface is at the top of both photos. Labeled axons can be seen in the most superficial and most deep (ventricular) layers of the tectum. Axons near the pial surface extend in the SO and the superficial aspect of the SGFS (sublaminae A-F). These regions correspond to a thin and cell-sparse layer at the pial surface as well as the superficial aspect of a cell dense layer. Axons near the ventricular surface are located within the SGC and SAC which correspond to a relatively cell sparse layer. Axons are not seen in the middle cell-sparse or cell dense layers (putative sublaminae H and J of the SGFS) nor within the deepest aspect of the superficial cell-dense layer (putative sublaminae G)

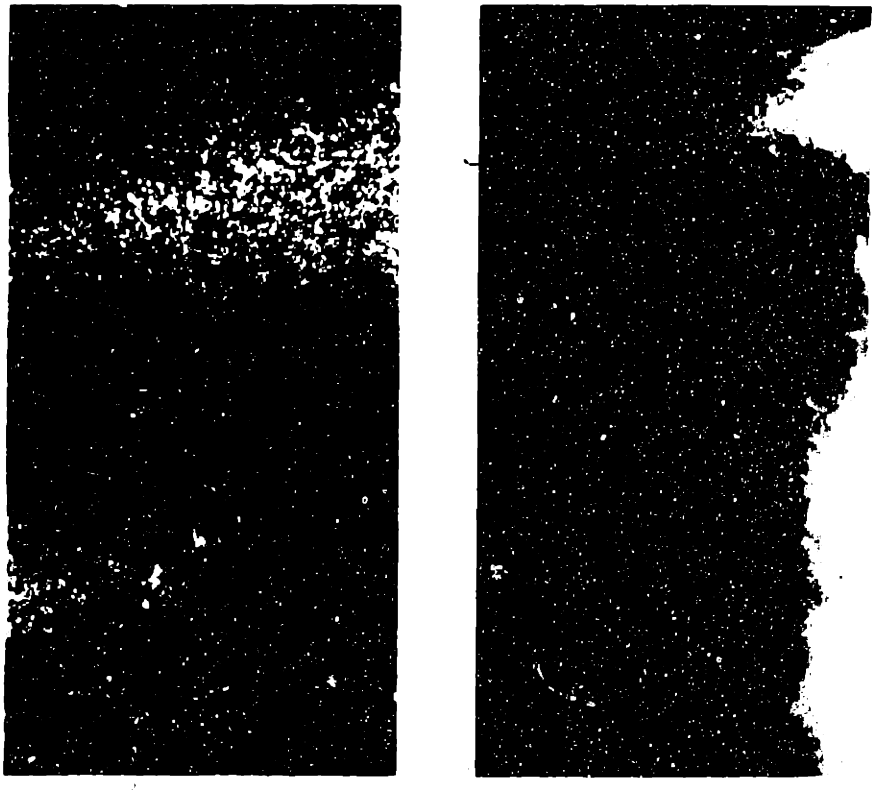
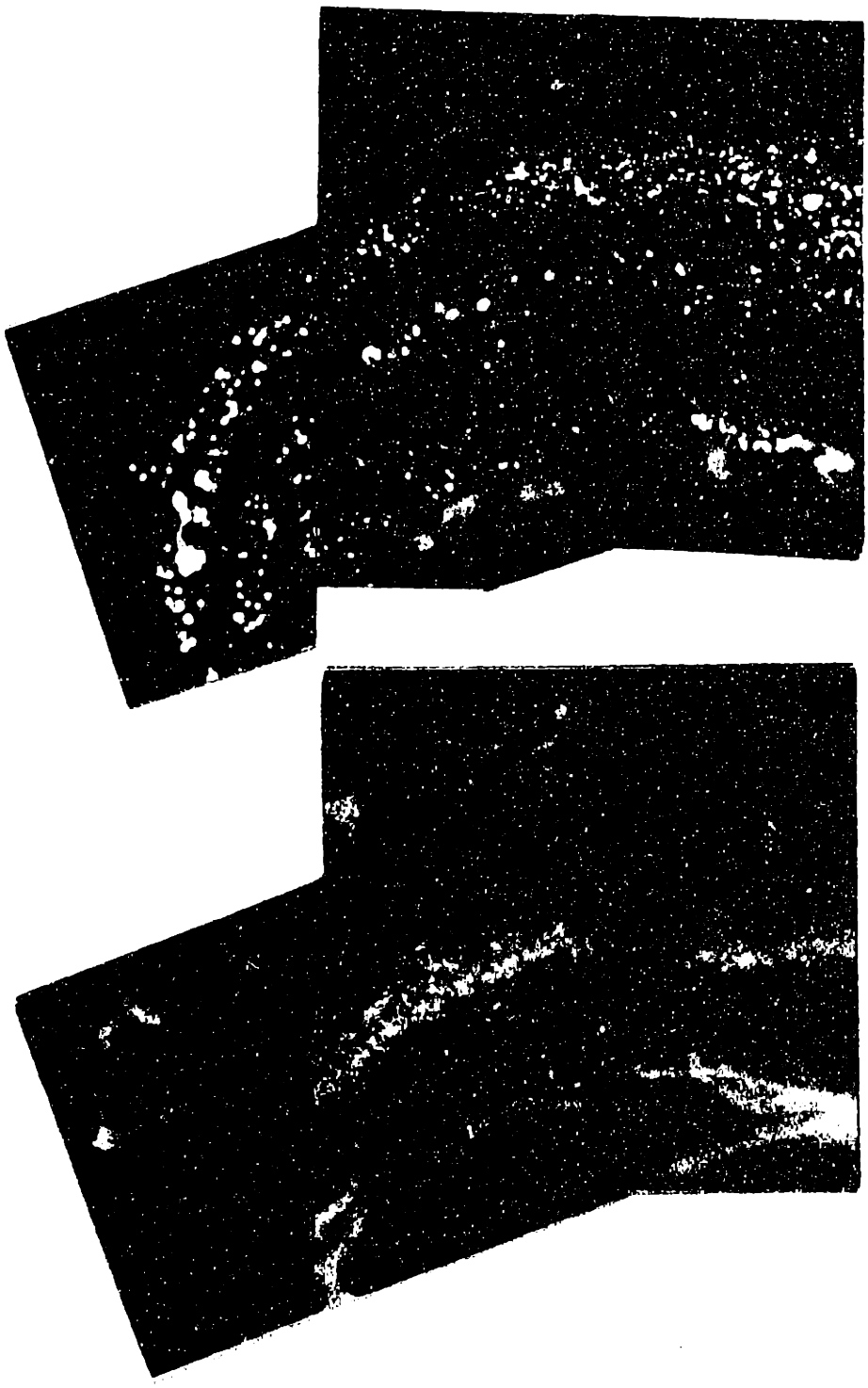


Figure 2. Attachment of d7 retinal cells to slices of d10 tectum. Fluorescence micrograph shows the laminae of d10 tectum after a cultured slice has been stained with the nuclear marker bisbenzamide (left panel). The same slice viewed under rhodamine optics shows the attached retinal cells (right panel). Dorsal is at the top and the pial surface extends along the right side and top in each photo. Cells attached well to the most superficial aspect of tectum including the cell-sparse retinal fiber tract (SO) and layers beneath the tract which are innervated by retinal axons (sublaminae A-F of SGFS). A second band of retinal cells attached to the deep aspect of a cell-sparse layer and the superficial aspect of a cell-dense layer which correspond to sublaminae H and J of the SGFS. Cells did not attach to the cell-dense layer that corresponds to sublamina G of the SGFS. Cells did attach to sublamina I of the SGFS (most cell-dense band within the slice) and to the SGC and SAC, layers near the ventricular surface that contain tectofugal projecting neurons and axons.

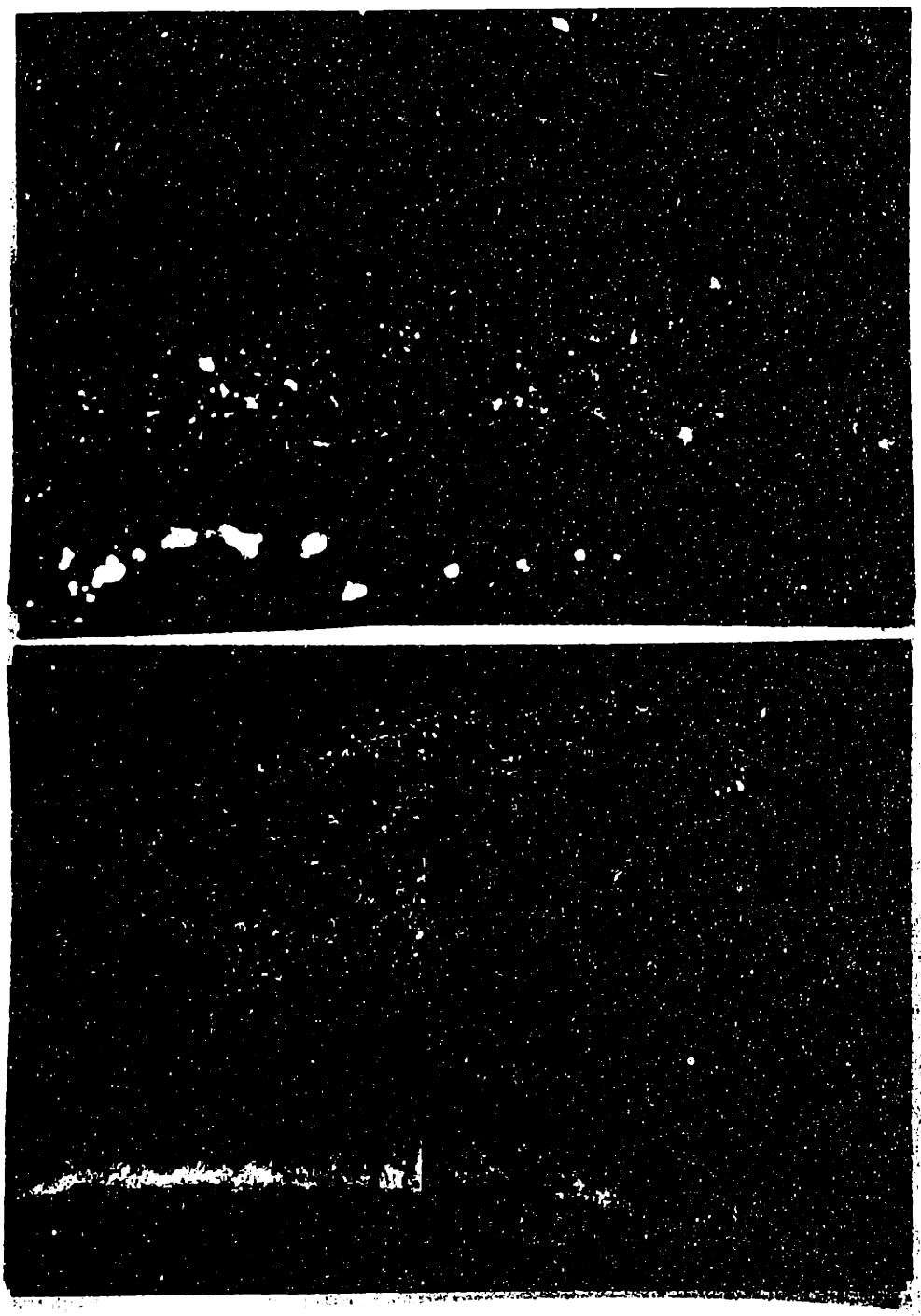


sublamina H, regions that the retinal axons encounter but through which they do not extend (see Fig 2 for details).

Tectal cells attach to tectal laminae in a pattern different from that of retinal cells

When d7 tectal cells were plated onto d10 tectum following the same procedure outlined above, a non-uniform pattern of cell attachment was revealed only after cocultures had been rinsed and fixed (Fig. 3). As with retinal cells, few attached tectal cells projected neurites. Tectal cells attached almost exclusively to regions that contain neurons and axons that project out of the tectum, the SGC and the SAC.

Figure 3. Attachment of d7 tectal cells to slices of d10 tectum. Fluorescence micrograph shows the laminae of d10 tectum after a cultured slice has been stained with bisbenzamide (left panel). Dorsal is at the top and the pial surface extends along the right side in each photo. The same slice viewed under rhodamine optics shows the attached tectal cells (right panel). Cells attached to the SGC and the SAC. A few cells may have attached to the deepest aspect of the SGFS, sublamina J, however because the cell density of this layer is not very different from the SGC, it can not be distinguished by the counterstain used in these experiments.



DISCUSSION

The results presented here indicate that tectal and retinal cells attach to tectal tissue in different patterns. Because these data were not quantified to assess the consistency of the described results, interpretations regarding their relevance to development would be preliminary. Nonetheless, similar patterns of attachment were seen across several cocultures and some interesting interpretations can be made with respect to the laminar specificity of retinotectal development.

The fact that retinal and tectal cells attached in different patterns would seem to indicate that they are recognizing different cues within tectal tissue. Retinal and tectal cells attached *in vitro* to the layers through which their respective axons project *in vivo*. Thus, cells *in vitro* may be recognizing cues that restrict axons to their proper layers *in vivo*. However, it is also possible that laminar specific attachment is mediated by homophilic interactions between plated cells and endogenous cells or axons within the slice. Indeed this effect could explain all of the attachment of tectal cells since they apparently attached only to layers which contain tectal axons and projection neurons. Interestingly, no tectal cells attached to any lamina superficial to the SGC and SAC, even though tectal cells plated onto slices contained cells from all tectal layers -- since whole tecta were dissociated. Thus, homophilic adhesion of tectal cells to tectal slices is either restricted to particular types of tectal cells or is not the only mechanism that accounts for the laminar specific attachment.

It is interesting to note that retinal cells attached to most layers of the tectum but particularly avoided sublaminae G of the SGFS, the layer that delineates the deepest extent of retinal axons in the tectum. Given the data concerning the thalamocortical system, it's intriguing to consider regions

which do not support attachment *in vitro* and that are encountered but not entered by axons *in vivo* (e.g. embryonic cortical plate) as containing an inhibitory activity (Chapter 3). Indeed, using cocultures of retinal and tectal slices, others have found that retinal neurites will extend on all layers of tectum, but that sublaminae G-J of the SGFS are substantially worse at supporting outgrowth than other layers (Yamagata and Sanes, 1995). Thus, it is possible that sublamina G of the SGFS acts as a barrier to retinal axons and prevents them from entering deeper layers of the tectum.

SUMMARY

At the least, the results presented here demonstrate the applicability of the technique described in this thesis to the study of non-thalamocortical neural systems. The results also indicate that different types of neurons can respond differently to the same tissue-slice substratum, an effect that was not observed with thalamocortical cocultures (chapter 2, discussion). Quantitative analyses like those described in previous chapters would be required before experiments could be carried out to elucidate the mechanisms behind the laminar specific behaviors of retinal and tectal cells plated on tectal slices. Nonetheless, the interpretations of these preliminary data imply that inhibitory cues may play a role in retinotectal development just as they likely play a role in thalamocortical development (chapter 3).

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