Instituto Juan March
de Estudios e Investigaciones

17

CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Cell Recognition During Neuronal Development

Organized by

C. S. Goodman and F. Jiménez

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INTRODUCTION

C. S. Goodman

Introduction: the aims of the workshop

Understanding how the nervous system becomes properly wired during development is one of the major unsolved problems in modern biology. Although there are still many unasnwered questions, the rate of progress over the past few years has been so impressive that the organizers thought a workshop on this topic would be timely and highly informative.

The participants in this workshop share a common interest in understanding the molecular mechanisms by which neuronal growth cones find their way towards, and ultimately recognize, their correct targets during development. Growth cones navigate over long distances and often through a series of complex choice points, appearing to follow signals on the surfaces of cells and in the extracellular environment. The aim of this meeting was to survey and discuss recent progress in uncovering the molecules and mechanisms that impart specificity to the developing nervous system and thus allow growth cones to recognize their correct pathways and targets. Over the past several years, an increasing range of cellular, molecular, and genetic techniques have been brought to bear on these problems. This workshop attempted to highlight recent discoveries in these areas, drawing from studies in diverse systems and organisms: from flies and worms to birds and mammals.

At the end of the last century, the great Spanish neuroanatomist Ramon y Cajal first coined the term "growth cone" to designate the expanded leading process of growing axons. Now, a century later, an influx of new techniques has brought significant progress in deciphering the mechanisms that guide growth cones along specific pathways and ultimately to their correct targets. Some of our participants study growth cone guidance in simple nervous systems such as the zebrafish; others focus on genetic analysis in the nematode and fruitfly. Still others work on the more

complex (but clinically relevant) nervous systems of mammals. Yet all share a common interest: trying to understand how neuronal growth cones find their targets.

Studies of specificity in neuronal recognition have their origins in the work of Sperry on the retinotectal system of goldfish and amphibians. Recent progress on the analysis of this classical system was the focus of one session in the workshop. Another session highlighted recent studies on the development of connections to and within the most important and complicated neural structure: the neocortex of mammals. Other sessions focused on molecular mechanisms, including guidance by repellant molecules, by diffusible chemoattractants, and by a multitude of cell surface and extracellular matrix (ECM) molecules around neurons, glia, and mesodermal cells.

In summary, the meeting focused on nine topics: (1) the mechanisms controlling pathway recognition; (2) the mechanisms controlling target recognition; (3) the mechanisms controlling specific cell migration; (4) the role of pioneer neurons; (5) the function of cell and substrate adhesion molecules; (6) the function of repulsive molecules in guidance and regeneration; (7) the function of target-derived chemoattractants; (8) the function of other types recognition molecules; and (9) the signal transduction mechanisms that control specific growth cone guidance.

MONDAY, May 31st

Growth Cone Guidance in the Zebrafish Central Nervous System

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The zebrafish embyro is quickly being established as an excellent vertebrate embryo for studying processes of development with a combination of genetic, molecular, and cellular techniques. One problem which is being fruitfully addressed is how growth cones navigate through embryos to find their targets. To do this growth cones distinguish appropriate pathways from inappropriate ones and know what direction they should extend on pathways. The clarity and simplicity of the zebrafish embryo combined with the availability of mutations and the ability to ablate and transplant specific cells has been particularly useful in elucidating the mechanisms that direct growth cones along their pathways.

Growth cones are guided by multiple guidance cues

The CNS of the early zebrafish embryo contains a simple scaffold of axonal tracts that are pioneered by the growth cones of specific classes of neurons in the spinal cord (Kuwada et al., 1990a; Kuwada et al., 1990b) and small clusters of neurons in the brain (Chitnis and Kuwada, 1990; Wilson et al., 1990). These early growth cones follow precise and cell-specific pathways to establish this scaffold of tracts. Growth cones that are projected later also follow stereotyped pathways by tracing out cell-specific routes within the early scaffold of axons.

Over the past several years, we have analyzed pathfinding by some of the earliest growth cones in the spinal cord and find that multiple, redundant cues act in concert at a single site to guide growth cones (Bernhardt et al., 1992a). The ventral midline of the spinal cord is the site of cell-specific turns made by some of the earliest spinal growth cones. At this site are two different classes of cells that growth cones may potentially interact with; the floor plate cells which occupy the ventral midline of the spinal cord and the notochord cells which are located just ventral to the floor plate cells. First, we showed that the floor plate serves as an intermediate target that provides multiple, cell-specific guidance cues to spinal growth cones (Bernhardt et al., 1992a; Bernhardt et al., 1992b). These growth cones dramatically increased their error rate at the ventral midline following laser ablation of floor plate cells in wildtype embryos and in *cyclops* mutants which are missing the floor plate cells (Hatta et al, 1991). Second, we demonstrated that notochord cells also provide guidance cues to the same growth cones (Greenspoon et al., 1993). Laser ablation of notochord cells in wildtype embryos increased errors at the ventral midline. Third, removal of both classes of cells by laser ablation of notochord cells in *cyclops* mutants gave higher error rates and more pronounced errors than elimination of either one alone.

We have also experimentally examined guidance of growth cones in the early zebrafish brain (Chitnis and Kuwada, 1991; Chitnis et al., 1992; Patel et al., submitted). Analysis of pathfinding by the growth cones of one particular cluster of brain neurons, the nucleus of the posterior commissure (nuc PC), demonstrated that these growth cones like their counterparts in the spinal cord were guided by redundant, extrinsic cues. Nuc PC growth cones extend to an intersection of axonal tracts and choose to extend posteriorly along one particular axonal tract although several other pathways are available at this

intersection. First, surgical elimination of this tract from the intersection significantly increased the proportion of errors made by nuc PC growth cones. However, many growth cones followed their normal pathway despite the absence of these axons. Second, we found that the scaffold of axonal tracts was perturbed in *cyclops* brains so that the axons nuc PC growth cones normally follow were eliminated. As with surgically manipulated wildtype embryos, nuc PC growth cones greatly increased their error rate at the intersection but many still followed their normal pathways in *cyclops* embryos. Both sets of results indicate that specific axons are an important source of guidance cues for nuc PC growth cones since elimination of the axons increased error rates. On the other hand, both sets of results suggest that additional nonaxonal cues must also act to guide these growth cones since many followed their normal pathway despite the absence of these axons. These experiments in the brain and spinal cord of zebrafish embryos along with experiments in other embryos suggest that multiple, redundant cues may be utilized to guide a wide variety of growth cones.

How do growth cones know what direction they should extend in the zebrafish embryo?

Our experiments demonstrated that intermediate targets and axonal tracts participate in guiding growth cones in the zebrafish embryo, but did not elucidate how these growth cones know what direction they should extend. Although microsurgical or genetic elimination of these guidance cues caused growth cones to follow aberrant pathways, they overwhelmingly extended in the correct direction even on aberrant pathways. This suggests that directional information that can guide growth cones is not limited to appropriate pathways. In order to determine the distribution of directionality cues for a set of growth cones, the highly accessible epiphysial neurons, were transplanted to ectopic sites in the brain; sites where their growth cones could have extended in any direction (Kanki et al., 1992). The growth cones of orthotopically transplanted donor neurons extended ventrally as they normally do, signifying that the transplant method does not damage the donor cells or the host environment. Similarly, the growth cones of donor neurons, presumably transplanted in random orientations to ectopic sites, overwhelmingly extended ventrally. These results demonstrate that directional cues that epiphysial growth cones can read are widely distributed in the embryonic brain. This in turn suggests that growth cones are guided by the action of two general classes of cues: spatially restricted cues that delineate specific pathways and widely distributed cues that provide directional information.

Are specific pathways delineated by axonal adhesion molecules?

Ultimately one would like to identify and determine the function of genes that may control growth cones. Although our understanding of molecules that may guide growth cones has increased greatly over the past several years with many studies demonstrating activities for molecules *in vitro*, relatively little is known about their role in developing embryos. This is particularly true of adhesion molecules that may delineate growth cone pathways in vertebrate embryos. As a first step toward *in vivo* analysis of such genes, we are investigating several cell surface molecules previously identified in other systems (Kanki and Kuwada, 1993). Amongst these are zebrafish genes that are similar to the chick DM-GRASP (Burns et al., 1991; Tanaka et al., 1991) and rat TAG-1 (Furley et al., 1990) genes. These genes are of interest to us, because their products promote neurite outgrowth *in vitro* and are expressed by developing axons and their substrates in patterns in the chick and rat embryos that suggest they influence pathfinding decisions. Given the recent advances made in the generation of transgenic zebrafish, we hope to be able to

determine the function of such genes by generating transgenic embryos in which their expression patterns are altered in meaningful ways.

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Adhesion Molecule and Cytoskeletal Mediated Aspects of Axonal Pathfinding in the Avian Limb. Lynn Landmesser, Ji-Cheng Tang, and Magdolna Szente. Dept. of Physiol. & Neurobiol. University of Connecticut, Storrs, CT.

The precise outgrowth of motor and sensory axons into the chick hindlimb occurs in several stages. First, as the many thousands of axons converge in the plexus region at the base of the limb, motor axons defasciculate and rearrange into target-specific fascicles, presumably due to growth cones interacting with extrinsic local cues and with cell surface molecules on other axons. Subsequently, subsets of motor axons diverge at precise points to project to their appropriate muscles. Since they are able to accomplish this even when they are surgically displaced and enter the limb from an abnormal position and in the company of foreign axons, it has been suggested that they are also capable of responding at some distance to target derived, possibly chemotactic, guidance cues. (See Landmesser, 1991 for review).

The importance of the initial rearrangement of axons at the base of the limb has recently been tested by interfering with this process by perturbing adhesion molecule function. Polysialic Acid (PSA), a carbohydrate moiety of NCAM has been shown to act as a negative modulator of cell-cell adhesive/signalling interactions, affecting both NCAM as well as L1 mediated interactions. Its onset of expression, just as axons defasciculated at the base of the limb suggested that it might play a role in this process. We have tested this idea by removing PSA at different stages of outgrowth via in-ovo injection of a PSA-specific endosialidase. When PSA was absent during the sorting out process in the plexus, a significant number of motoneurons made projection errors (Tang et al., 1992). In contrast removal after sorting out had occurred was without effect, indicating both that this process is important to accurate pathfinding and that the ability of motor axons to subsequently choose the correct muscle to project to, was not subject to PSA modulation. PSA removal in the plexus dramatically altered growth cone behavior resulting in more parallel trajectories with fewer turns.

Although little is known about the nature of the target derived guidance cues or the receptors that detect them, we have discovered a cytoskeletal alteration associated with growth cones detecting and responding to such cues. *In vivo*, Mab 5E10 recognizes an antigen which is expressed by growth cones from specific points, beginning slightly before they diverge to grow toward their own specific targets, and which has been identified as a specific phosphorylated epitope of the neurofilament protein NF-M (Landmesser and Swain, 1992). In cultured motor and DRG neurons two types of 5E10 expression can be distinguished. First, various stimuli including depolarization, Ca^{++} ionophore, and TPA produced a transient (lasting several hours) increase in 5E10 expression above the low background level characteristic of these neurons cultured alone. Second, a more intense and stable expression was induced when axons encountered a boundary; in this case expression was restricted to that portion of the neurite encountering the boundary and was maintained in the portion of the neurite that continued growth along the boundary after turning. This expression was not altered by subsequent treatment with PKC inhibitors such as staurosporine, but if treated with staurosporine just before reaching the boundary 5E10 expression was not induced, and the axons failed to turn. Associated with the boundary-induced 5E10 expression was a cytoskeletal alteration in which more of the 5E10 antigen became incorporated into the triton-insolubule cytoskeleton and in which the axons became more resistant the agents that cause axonal retraction. It is suggested that 5E10 expression and/or some associated cytoskeletal alteration is part of the mechanism by which growth cones respond to certain extrinsic guidance cues and alter their trajectories.

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Axon guidance by chemoattractants in the developing vertebrate spinal cord

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To understand how the specific neural connections that underlie the functioning of the nervous system are generated during development, we have focused on the development of the vertebrate spinal cord, asking how spinal axons are guided to their target fields. This talk will focus on one form of axon guidance, axonal chemotropism. Although chemotactic mechanisms are central to the directed motion of a variety of cell types, and have long been suggested to guide axons to their targets, there has been little direct evidence for axonal chemotropism *in vivo*. Evidence for chemotropic axon guidance has, however, been obtained in embryological experiments in which explanted neurons confronted with their targets *in vitro* have been shown to grow toward those targets in a directed manner. We have, in this way, obtained evidence for the operation of chemotropic guidance mechanisms in the spinal cord. One set of spinal neurons, commissural neurons, extend axons to the brain via an intermediate target called the floor plate[1], located at the ventral midline of the spinal cord. We have shown that the floor plate secretes a diffusible factor that can selectively influence the direction of growth of commissural axons *in vitro*[2]. Consistent with a role in guidance *in vivo*, the factor is secreted only by floor plate cells and is an effective chemoattractant: it can diffuse considerable distances through the neural epithelium, setting up a concentration gradient capable of reorienting all commissural axons within its range of action[3].

To assess rigorously the role of the floor plate-derived chemoattractant in guiding commissural axons *in vivo*, and to determine how axons detect gradients of the attractant, it will be necessary to identify the attractant. Most of the talk will be devoted to describing our progress in this identification. In a search for an abundant source for biochemical purification, we discovered an activity in embryonic brain that is identical in its effects to the floor plate. In my laboratory at UCSF, we have purified the activity in embryonic brain extracts to homogeneity, using a six step purification, starting from 25,000 embryonic day (E) 10 chick brains. The activity in brain is due to two proteins of molecular weights 75 and 78kDa (p75 and p78), which each separately possess the activity observed for floor plate *in vitro*. Microsequencing of the proteins, and isolation of partial cDNAs encoding the proteins shows them to be novel and related. Importantly, although p78 was purified from E10 chick brain (i.e. long after commissural axons have grown to the floor plate), it is expressed in floor plate at E2.5, a time at which commissural axons are growing to the floor plate. This suggests that p78 contributes to the chemotropic effect of the floor plate. More detailed studies which are in progress will determine its precise contribution. We are also studying the expression of p75, as a first step towards assessing whether it too contributes to this effect. Finally, in the course of the purification, we discovered a third activity which has no effect on its own but which synergizes potently with p75 and p78 in promoting commissural axon outgrowth, and we have initiated the purification of the factor(s) responsible for this activity, which appears to reside in a protein(s).

Identification of the various proteins responsible for the chemotropic effect of the floor plate will enable us to manipulate their function *in vivo* and thus to determine their contribution to axon guidance during normal development.

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Cellular Recognition and the Development of Synaptic Connections in *Drosophila* Embryos. Haig Keshishian, Lansheng Wang, Akira Chiba, Te Ning Chang, Marc Halfon, and Jill Jarecki. Biology Department, Yale University, New Haven, CT 06511.

The neuromuscular connections of *Drosophila* are well-suited for studying synaptic function and development. Hypotheses about cell recognition can be tested in a simple array of 3 dozen glutamatergic motoneurons and 30 muscle fibers in each abdominal hemisegment. *Drosophila* muscle fibers are multiply innervated by individually identifiable motoneurons, each specialized by its morphology, cotransmitters, and connectivity. The initial wiring occurs during a period of about 3 hours during mid-to-late embryogenesis. Vital imaging of fillet cultures using laser confocal microscopy shows that the efferent growth cones sample multiple muscle fibers with rapidly moving filopodia. We have examined the role of intermediate cellular targets in guiding the axons to their destinations, tested with single cell lesions using laser microsurgery and by mutations (e.g. *pointed*) that disrupt trajectory choices by the nerve axons. Upon reaching their target muscle fibers, dye fills show that the growth cones rapidly differentiate into synapses whose morphology prefigure the larval junctions. Target mismatch, generated by laser microsurgery, mutations (*rhomboid, numb*) and heat shock (which duplicates specific fibers) was used to test the behavior of two identified motoneurons (RP3 & RP1) in response to alterations in the number and pattern of their synaptic partners. The experiments show that growth cones actively recognize the individual muscle fibers. However, synaptic connectivity is not hard-wired. When denied their preferred partner the embryonic motoneurons grow to the vicinity of their targets where they make ectopically placed but otherwise functional synapses on neighboring muscle fibers.

Drosophila motor endings show developmental plasticity. We denervated muscle fibers 7&6 by the laser ablation of motoneuron RP3. Denervation can also be achieved using mutations which disrupt RP motoneuron differentiation (e.g. *Toll*). The ablation of the native input leads to the appearance of physiologically functional foreign collaterals from neighboring synapses and nerves. The role of electrical activity in the refinement of synaptic contacts was also examined using both toxins (TTX or AR636, a glutamate receptor blocker) and by mutation (Na channel mutant *para*). Blocking nerve action potentials has two results: increased growth cone processes appear together with the appearance of collateral axon branches onto adjacent muscle fibers, reminiscent of those following muscle fiber denervation.

The mechanisms for cellular recognition during synaptogenesis remain to be identified. However, the embryonic motoneurons and muscle fibers are molecularly heterogeneous, expressing multiple cell surface glycoproteins known to mediate cellular adhesion events *in vitro*. We have examined the role of two glycoproteins (Fasciclins I and III) which are both expressed by the motoneurons RP1 and RP3, and by the muscle fiber targets of motoneuron RP3. Single null mutations for either protein have minimal effects. However, in double mutants the two motor axons defasciculate within the neuropilar ISN tract, and make aberrant collateral branches at trajectory changes. In the periphery both motoneurons arborized incorrectly over multiple ventral muscle fibers with aberrant processes observed in over a third of the dye-fills examined. Despite these errors in the embryos, later stages showed error correction: by the 3rd larval instar the double mutant synapses were indistinguishable from wild type.

The results show that several mechanisms are involved in synaptic connectivity: 1) Peripheral guidance steers motoneuron growth cones to the vicinity of their preferred targets. Axons grow to specific bodywall regions despite the absence of their synaptic targets. 2) The

selection of the correct target depends on the active recognition of individual muscle fibers. However, motoneurons can establish functional synapses with alternates if preferred targets are absent. 3) Both motoneurons and muscle fibers express an array of putative cell adhesion molecules. Loss of multiple adhesion proteins expressed by motoneurons and muscle fibers can disrupt targeting, when assayed in embryos. Finally, 4) denervation and activity-block show that the synapses are plastic and subject to activity-dependent refinement. The accessibility of these phenomena to experimental tests makes the *Drosophila* embryo valuable for analyzing problems of synaptic recognition.

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GENETIC ANALYSIS OF PATHWAY AND TARGET RECOGNITION DURING NEURONAL DEVELOPMENT IN DROSOPHILA

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Our studies are aimed at understanding the molecular mechanisms by which neuronal growth cones find their way toward, and ultimately recognize, their correct targets during development. A powerful approach to identify the key genes involved in a developmental process is to screen directly and systematically for mutations which perturb these events. One of the best examples is the pioneering studies of Nüsslein-Volhard and Wieschaus who over a decade ago undertook a near-saturation screen for mutations affecting segment number and polarity, scoring mutants by altered patterns of larval cuticle. The genes so identified revolutionized developmental biology. Modeled after these studies, in 1989 we designed a systematic screen to identify key genes that control CNS axon pathfinding. We collected mutants which affect embryonic CNS axon pathways as visualized with a MAb (BP102) that stains all CNS axon pathways. Based on the success of this screen, and the more recent availability of another antibody (MAb 1D4 against a specific form of fasciclin II) that allows motoneuron axons and growth cones to be visualized with remarkable clarity, in 1991 we began a second systematic mutant screen for mutations which affect the ability of motoneurons to find and recognize their correct target muscles. This second screen provides an even greater resolution of specificity than our initial screen, to the level of single identified growth cones and single identified synapses.

Growth cone guidance at the midline of the CNS. The midline of the CNS appears to provide both attractive and repulsive guidance cues; some growth cones are guided across the midline whereas others stay on their own side. We first described the midline glia, and then used genetic analysis and enhancer trap probes to characterize the identity and function of these and other midline cells. Based on this knowledge, we next performed a large-scale systematic screen for mutations that affect the development of CNS axon pathways, with particular focus on the midline. From a collection of hundreds of new mutations, we identified two new genes that play key roles during guidance at the midline. Mutations in the *commissureless* (*comm*) gene lead to an absence of nearly all CNS axon commissures such that growth cones that normally project across the midline instead now extend only on their own side. Mutations in the *roundabout* (*robo*) gene lead to the opposite misrouting such that some growth cones that normally extend only on their own side instead now project across the midline. The phenotypes of mutations in these two genes suggest that they encode key components or regulators of attractive and repulsive signaling systems at the midline. Embryos doubly mutant for *comm* and *robo* look identical to *robo* alone, suggesting that these two genes function in a common genetic pathway that controls guidance at the midline. We are presently cloning both genes.

Formation of specific connections between motoneurons and muscles. In Drosophila, the specificity of growth cones for target cells is most clearly studied in the ability of motoneuron growth cones to recognize specific muscle fibers. The body wall musculature is relatively simple and is arranged in a stereotyped array of 30 individually identified muscle fibers in each abdominal hemisegment. Most of these muscles are innervated by one or only a few of the 34 abdominal motoneurons, forming a highly stereotyped pattern of connections, as shown by detailed cellular analysis from the Whitington, Keshishian, Bate, and Goodman labs.

Using one of our MAbs (1D4) that selectively stains all motoneuron axons, growth cones, and synapses, in 1991 we initiated a large-scale systematic mutant screen with the goal of saturating the genome for mutations which affect motoneuron guidance and muscle target recognition. We recently completed a screen of the 2nd chromosome (40% of the genome); and are presently working on the 3rd chromosome (also 40%). Genes have been identified that perturb the projection and connectivity of specific subsets of motoneurons. Four new genes appear to control pathfinding and choice point navigation (*beaten-path*, *bypass*, *short stop*, and *stranded*). Mutations in two other genes (*walkabout* and *clueless*) prevent specific motoneurons from recognizing their correct synaptic partners from amongst the muscles they contact. The specificity of these mutant phenotypes for different aspects of pathfinding vs. targeting suggests that a hierarchy of molecular mechanisms controls the generation of neuromuscular specificity. We are presently cloning these six genes, and continuing to isolate additional mutations on the 3rd chromosome. Our goal is to identify all of the key genes involved in these events.

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Control of neurogenesis and migration in developing brain.

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The primary goal of our research is to identify the signals that regulate neurogenesis and migration in the developing mammalian brain. We have utilized two systems for the analysis of mechanisms of cell patterning in brain - the murine forebrain and cerebellar cortex. The developing forebrain has been useful as a model for the regionalisation of the brain and the mouse cerebellum has provided an experimental approach to the molecular control of the establishment of cell identity in brain. For the latter, we have focused on the granule neuron owing to the specific temporo-spatial pattern of differentiation of the granule neuron, arising in an external germinal layer (EGL) and migrating down the Bergmann glial fibers to establish the internal granule cell layer, and to the existence of the neurological mutant mouse *weaver*, an animal with phenotypic defects in granule cell differentiation and migration.

1. Dispersion of Neural Progenitors During the Development of the Forebrain

One of the early events in the establishment of regional diversity in brain is the subdivision of the forebrain into the cerebral cortex and underlying basal ganglia. This subdivision is of special interest, owing to the striking difference in cellular patterning in these two regions. Whereas the dorsal aspect of the telencephalon gives rise to the laminar, cortical regions of brain, the ventral aspect gives rise to nuclear, subcortical regions. To examine the cellular basis for the specification of these regions, we have visualized cell movement within the ventricular zones of the cortical and subcortical regions of the telencephalon. Over an 8-24h observation period, labeled cells moved extensively in the lateral plane of the cortical ventricular zone prior to migration along the radial glial fiber system. Cell dispersion was restricted, however, at the border between the cortical ventricular zone and the lateral ganglionic eminence, the subcortical ventricular zone. We suggest that this restriction of cell movements establishes a regional pattern of neurogenesis in the developing brain (Fishell et al, 1993).

2. Molecular Control of Granule Neuron Development

To examine the role of local cell interactions in the EGL on precursor cell proliferation, we purified precursor cells from the early postnatal mouse cerebellum, and measured DNA synthesis after dispersing the cells into a monolayer, reaggregating the cells or placing them in a three dimensional collagen matrix. These studies demonstrated that cell-cell contacts between EGL precursor cells stimulate cell proliferation and restrict cell fate toward a granule cell identity (Gao et al, 1991). To identify the signals that induce neural differentiation, we have taken a genetic approach, purifying EGL precursor cells from the cerebellar cortex of *weaver* mice and reaggregating them with wild-type cells. In vitro mixing experiments with dye-labeled mutant cells demonstrate that wild-type EGL precursor cells rescue the

weaver phenotype, suggesting that the *weaver* gene encodes an non autonomous signal for CNS neural determination (Gao and Hatten, 1992).

To study the molecular signals necessary for cerebellar granule cell migration in situ, precursor cells from the neurological mutant mouse weaver, an animal with phenotypic defects in migration, were implanted into the external germinal layer (EGL) of wild-type cerebellar cortex. In wild-type cerebellar cortex, labeled, weaver EGL precursor cells progressed through all stages of granule neuron differentiation, including the extension of parallel fibers, migration through the molecular and Purkinje cell layers, positioning in the internal granule cell layer and extension of dendrites. Thus, the weaver gene acts non autonomously in vivo, and local cell interactions may induce early steps in neuronal differentiation required for granule cell migration (Gao and Hatten, 1993).

To provide molecular markers for the stages of granule cell differentiation, we have constructed cDNA expression libraries from immature granule cells, purified from mouse cerebellum on postnatal days 3-5, and used novel antibody screening methods to identify cDNA clones that mark specific stages of granule neuron differentiation. 39 unique cDNA clones have currently been identified. Northern and in situ analysis of the pattern of expression of these cDNAs demonstrate a specific temporo-spatial pattern of expression, suggesting that there are at least four stages of granule cell differentiation (Kuhar et al, 1993).

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ROLE OF A TRANSIENT NEURAL SCAFFOLD IN CEREBRAL CORTICAL DEVELOPMENT.
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During the development of the mammalian cerebral cortex, axons from the lateral geniculate nucleus (LGN) must grow along appropriate pathways from thalamus to cortex, must select the visual cortex from other potential target areas, must invade layer 4, and finally must segregate according to eye of origin to form the system of ocular dominance columns. Experiments in cat suggest that subplate (SP) neurons may play important roles in facilitating these events. SP neurons are among the first postmitotic neurons of the cerebral cortex. Shortly thereafter, the axons of SP neurons are the first to grow from the cortex to the thalamus; thus SP axons pioneer cortical pathways later traversed by both the ascending thalamocortical axons and by the descending axons of the deep cortical layers.

The close proximity of thalamic axons and SP neurons during this early period suggests that interactions between them may be important for ensuing cortical development. To examine this possibility, SP neurons were deleted selectively by making local injections of kainic acid beneath visual cortex. As a consequence, LGN axons not only failed to initially accumulate and branch below visual cortex, but also subsequently failed to invade the visual cortex at later fetal and neonatal ages. In addition, the projection from layer 6 back to the LGN failed to form normally. Thus, SP neurons may be necessary for the processes of target selection and ingrowth in the formation of the reciprocal sets of connections between thalamus and cortex.

At slightly later ages, SP neurons send many axons into cortical layer 4 during the neonatal period when LGN axons have grown into layer 4 and are segregating into ocular dominance columns. To examine if SP neurons might be involved in segregation, they were deleted just before the onset of column formation. Subsequent examination of the pattern of the geniculocortical projection revealed that in the absence of SP neurons, LGN axons had failed to segregate, and instead remained diffusely distributed within layer 4.

These observations suggest that SP neurons are intimately involved in the formation of connections to and from the cerebral cortex throughout the course of development. Since SP neurons are present not only beneath visual cortex, but also throughout the mammalian neocortex, they may play multiple roles during cortical development, first by pioneering corticofugal pathways, and then by subserving a set of cellular interactions that are essential for the process of target selection and the formation of patterned sets of inputs to cortical layer 4. Moreover, since most SP neurons are eliminated by programmed cell death after birth, these interactions involve the construction and then dismantling of a transient neural scaffold within the forming cerebral cortex.

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Development of afferent and efferent cortical connections

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The mammalian cerebral cortex is divided into architectonically and functionally distinct areas which are further characterized by specific sets of connections, both with other cortical areas and with subcortical structures. Within each area of the cortex, however, there is a similar laminar organization of cortical inputs and outputs. To establish such orderly connections during development, growing axons have to find the area to which they project and, once there, they have to select the correct subset of target cells. The formation of cortical connections is further complicated by the fact that there is a temporal mismatch between the early development of subcortical structures and the late development of the cortex, where neurogenesis is protracted. How are then specific projections to and from the cortex established during development ? Studies made in intact animals have revealed the precise sequence of events which lead to the formation of the mature pattern of cortical connections. It has been found, for example, that in several species the input from the thalamus reaches the cortex even before their target cells have been generated. Thalamic afferents, however, do not enter the immature cortex. They are initially confined to the subplate zone beneath the immature cortical grey matter, and they invade the cortex only after their target layer is about to be formed. Recent in vitro approaches gave some cues on how this diffential growth of thalamic fibers is achieved and how cortical connections are specified during development. Studies listed below have shown that layer- and cell-specific afferent and efferent cortical connections are established in cortical slice cultures that are similar to those observed in vivo. The results of these experiments provide evidence for the existence of diffusible and membrane-bound guidance factors for specific sets of axons. Furthermore, they suggest that different molecules are involved that navigate axons towards their target, regulate target innervation and mediate target cell recognition.

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Specification of neocortical areas and thalamocortical projections.

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The mature neocortex is characterized by numerous anatomically and functionally distinct areas. We have been pursuing mechanisms which control the differentiation of cortical areas from a uniform appearing immature neocortex lacking the area-specific architectural and connectional distinctions seen in the adult. Our experiments have been directed at determining the extent to which cortical areas are specified to the neuroepithelium which gives rise to the neocortex and the extent to which agents extrinsic to the cortex govern the differentiation of cortical features.

One approach that we have taken to address this issue has been to heterotopically transplant pieces of fetal cortex to ascertain the competence of regions of the immature cortex to develop features ordinarily associated with other cortical areas. For example, the somatosensory cortex of rats is characterized by specialized functional groupings termed "barrels" - aggregates of layer 4 neurons innervated by clusters of ventrobasal thalamocortical afferents. Each barrel corresponds to a single specialized sensory hair on the body. The overall distribution of barrels, when viewed in the tangential plane of cortex, provides an anatomically demonstrable map of the body. We transplanted pieces of fetal visual cortex, which has continuous distributions of layer 4 neurons and thalamocortical afferents, into the somatosensory region neonatal rat pups and found not only that barrels form in visual cortex transplants, but the overall pattern of barrels can develop normally, spanning the host/transplant borders. In addition, complementary patterns of glycoconjugated molecules, suggested to be a prespecified template for barrel differentiation, also develop in the transplant. Other experiments which we have done demonstrate that the transplants develop the connections, both efferent and afferent, appropriate for their new area of cortex not for their origin. These findings show that different regions of the developing cortex have similar capacities to develop the unique features that distinguish cortical areas. We conclude that the neocortical neuroepithelium generates comparable populations of neurons and glia across its extent and when exposed to the same extrinsic influences, these cells will differentiate in comparable ways.

These findings indicate that thalamocortical afferents play a predominant and even primary role in cortical area differentiation. If so, the source of patterning information to somatosensory cortex will have the developmentally earliest demonstrable periphery-related patterning. Indeed, we find that ventrobasal thalamocortical afferents have a periphery-related pattern as early as the day before birth, and are distributed in a well-defined barrel pattern within 24 hours of birth - at least 2 days before any other barrel component yet identified. This temporal relationship is consistent with the notion that thalamocortical afferents are a primary agent controlling cortical differentiation.

This evidence, and that from many other groups, indicate that thalamocortical afferents influence the developmental differentiation of the functionally specialized, cytoarchitectonic areas of the neocortex. In this context, we are interested in defining mechanisms that control the development of thalamocortical projections. Thalamocortical axons have two distinct growth phases: (1) a targeting phase during which they extend within the subplate layer tangentially through the cortex to the appropriate area, and (2) a subsequent phase during which they extend superficially to invade the overlying cortical plate. In most systems studied in the mammalian brain, neurons project their axons more widely during development than at maturity. In these systems, development of functionally appropriate connectivity requires axon remodeling. However, we find that the development of connections between the principal sensory thalamic nuclei and their cortical targets appears to be specific from the outset. These findings suggest the operation of a highly effective marking system that controls the area-specific matching of developing thalamocortical connections.

Several lines of evidence suggest that the cortical subplate layer contains the information that allows thalamocortical axons to select their appropriate target areas. One way to lay down these cues would be for neuroepithelial cells to impart positional information to their progeny that form the preplate, from which the subplate is derived. Such a mechanism would require that the progeny of neighboring proliferative cells maintain some degree of neighbor relationships. However, several recent studies have shown considerable dispersion of cortical plate neurons and their progenitors. Through the use of time-lapse imaging of DiI-labeled cells in the neocortical neuroepithelium of fetal rat cortex maintained *in vitro*, we find that preplate neurons and their progenitors maintain spatial relationships during the period that the preplate is established. Therefore, positional information imparted to preplate cells around the time they are generated could later be effectively deployed in the subplate to control the targeting of thalamocortical axons.

Once arriving at the correct area, developing thalamocortical axons wait in the subplate before invading the cortical plate. We have used the membrane stripe assay to address whether changes in membrane-associated molecules expressed by cortical plate cells regulate the ingrowth of thalamocortical axons. Membranes prepared from late embryonic cortical plate before thalamocortical ingrowth, and neonatal cortical plate soon after ingrowth begins, were attached to a substrate in alternating 90 μm wide lanes. When given this choice, embryonic thalamic axons prefer to grow on the lanes of neonatal membranes. This finding suggests that the end of the waiting period is not due to a change in the responsiveness of thalamocortical axons to existing molecules; rather, changes in membrane-associated molecules may signal the end of the waiting period and promote thalamocortical ingrowth into the cortical plate.

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TUESDAY, June 1st

MYELIN-ASSOCIATED NEURITE GROWTH INHIBITORS INFLUENCE DEVELOPMENT OF FIBER TRACTS, AND IMPEDE REGENERATION

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Oligodendrocytes and CNS myelin contain an activity which leads to arrest of neurite growth in culture. This activity was identified as two membrane protein fractions of molecular weights 35 and 250 kD (NI-35/250) (for review: Schwab et al., Ann. Rev. Neurosci. 16: 565-595, 1993). Partial amino acid sequencing resulted in novel sequences. These oligodendrocyte membrane proteins inactivate growth cone movement upon contact (Bandtlow et al., J. Neurosci. 10: 3837-3838, 1990). A large increase in intracellular calcium is an obligatory step of the signal cascade triggered by the purified inhibitory proteins in DRG growth cones (Bandtlow et al., Science 259: 80-83, 1993). - Monoclonal antibodies (IN-1) neutralizing the inhibitory activity of NI-35/250 have been generated and characterized in several in vitro systems (Caroni and Schwab, Neuron 1: 85-96, 1988). Application of the antibody IN-1 into the CSF of spinal cord lesioned rats led to successful regeneration and elongation of cortico-spinal tract (CST) fibers over 5 - 20 mm (in 2 - 4 weeks) (Schnell and Schwab, Nature 343: 269-272, 1990). In contrast, control antibodies only permitted local sprouting not exceeding 0.5 - 1 mm. Identical results were obtained in rats X-irradiated at birth, thus eliminating oligodendrocytes and myelin (Savio and Schwab, Proc.Natl.Acad.Sci. USA 87: 4130-4133, 1990). A similar, highly significant regeneration response was obtained in the hippocampus (septohippocampal fibers) (Cadelli and Schwab, Europ. J. Neurosci. 3: 825-832, 1991) and in the optic nerve. - During development, oligodendrocyte differentiation occurs in a tract- and region-specific manner in the CNS. We studied the effect of oligodendrocyte elimination or of the antibody IN-1 on CST development (Schwab and Schnell, J. Neurosci. 11: 709-722, 1991). In both cases branching of CST fibers occurred into the sensory tracts, a phenomenon never observed under normal conditions. These results attribute a guidance function ("guard rails") to NI-35/250 for the developing CST. - In the developing hamster visual system oligodendrocytes appear in the stratum opticum of the superior colliculus (SC) soon after the optic fibers have grown in. If one SC is removed, the target-deprived optic fibers sprout to the other side, but grow only very superficially and never enter the stratum opticum. In presence of IN-1 this pattern is reversed to a normal neuroanatomical innervation (Kapfhammer et al., J. Neurosci. 12: 2112-2119, 1992). In this case NI-35/250 restrict the access of regenerating fibers to particular tectal layers. - Results in the oligodendrocyte-free rat optic nerve (P15) indicate that axons sprout along the nerve in the absence of oligodendrocytes, indicating a stabilizing function of these inhibitory molecules in the adult CNS.

unc-44 Encodes Ankyrins Required for Neuronal Development in *Caenorhabditis elegans*

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In neurons and other cells, a meshwork of actin and spectrin filaments underlies the plasma membrane. By tethering integral membrane proteins, these filaments control the static shape of cells and establish stable membrane domains with specialized functions. During division and migration, they respond to signals from the mitotic apparatus, or cell surface receptors, with directed cellular protrusion. Several families of bifunctional adaptors (e.g., ankyrins, ezrin/talin/band 4.1) link cytoskeletal filaments directly to integral membrane proteins. Ankyrins, in particular, have been shown to bind spectrin filaments to a variety of membrane proteins including the Na+/K+ ATPase, the anion exchanger, the amiloride-sensitive Na+/H+ antiporter, CD44 (H-CAM), and neurofascin-like glycoproteins (Davis et al., 1993. JCB, 121:121). Evidently, ankyrins and other adaptors couple specific cell surface receptors and cytoskeleton during neuronal differentiation.

The *unc-44* gene is required for axonal outgrowth in sensory, motor and interneurons throughout the *C. elegans* nervous system (Hedgecock et al., 1985. Dev. Biol 111:158; McIntire et al., 1992. Neuron 8:307). Few, if any, neuronal types are unaffected. In a homozygous viable mutant, *unc-44 (e362)*, neurons have several short, abnormally branched axons. Both pioneering and fasciculative phases of axonal outgrowth are poorly directed. However, neuronal cell lineages and neuroblast migrations, as well as non-neural tissues, are normal in this mutant.

To identify the gene product, *unc-44* was transposon-tagged and cloned. The gene spans about 30 kb and expresses multiple mRNAs (3.2, 5.1, 6.0, 6.9 and 14 kb). From genomic and cDNA sequencing, these messages encode a variety of ankyrin isoforms. Like brain and erythrocyte ankyrins, UNC-44 isoforms have four structural domains, i.e., repeat, linker, spectrin-binding, and regulatory. The repeat domain comprises twenty-three tandem copies of an ancient protein module called the *ankyrin* motif. This domain is over 50% identical in UNC-44 and human ankyrins. Specific repeats have been implicated as binding sites for particular membrane proteins. The acidic linker is poorly conserved in length and sequence, but the rest of the spectrin-binding domain is highly conserved. Finally, the regulatory domains have diverged greatly. In size and sequence, two UNC-44 isoforms resemble erythrocyte and brain ankyrin 2. From Southern and Northern analysis of *unc-44* mutants, the 14 kb mRNA may encode a novel neuron-specific isoform responsible for axonal defects in *unc-44* mutants.

The ankyrins illustrate two important problems for the cortical cytoskeleton. How can diverse, evidently unrelated, membrane proteins be linked to a common filament meshwork using just a handful of distinct adaptors? Apparently, each membrane protein has evolved binding sites *ad hoc* for available adaptors. How can a specific membrane protein (e.g., Na+K+ ATPase) be localize to distinct membrane domains in different cell types? Presumably, different ankyrin isoforms define different membrane domains.

unc-44 encodes several distinct proteins each with multiple domains. This structural complexity was not anticipated from the original genetic analyses. Therefore we have begun to isolate new alleles in non-complementation screens to learn whether *unc-44* has additional functions in neurons or other cells, and whether its various functions are genetically separable.

Cell adhesion and neural development in *Drosophila*: Analysis of *neurotactin*

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Neurotactin is a *Drosophila* transmembrane protein that was initially characterized by means of monoclonal antibodies against embryonic tissue (1-3). It is found mainly in the nervous system, and also in some mesodermal derivatives and in imaginal discs, during the stages of cell proliferation and differentiation. Like many cell adhesion molecules, Neurotactin accumulates in parts of the cell membrane where Neurotactin-expressing cells contact each other. In addition, *Drosophila* S2 cells, transfected with a *neurotactin* cDNA, do not aggregate but are able to bind to a subpopulation of embryonic cells (4). This suggests that Neurotactin functions as a heterophilic cell adhesion molecule.

Sequencing of *neurotactin* cDNAs (2, 3) indicates that the deduced protein sequence contains a large cytoplasmic domain rich in charged residues, a single transmembrane domain, and an extracellular domain homologous to serin-esterases, although it lacks the active site serine required for esterase activity. This similarity is found at two different levels. First, there is a strong sequence homology within a region of about two hundred amino acids. The second level of homology is shown by the conservation of the relative positions of six cysteines involved in the formation of intramolecular disulfide bonds. The extracellular domain also contains three copies of the tripeptide leucine-arginine-glutamate, a motif that forms the primary sequence of the adhesive site of vertebrate s-laminin.

Cell adhesion molecules are one of the most important types of guidance cues used during axon outgrowth. This conclusion is mainly based on the results of antibody perturbation experiments carried out *in vitro*. It would be desirable, however, to know whether the elimination of an adhesion molecule may affect axon outgrowth *in vivo*, in an otherwise intact organism. Hence the need for the genetic analysis. The first analyses of mutations in several cell adhesion genes, performed mainly in *Drosophila* (see 5 for a review), but also in vertebrates (6), indicate that mutation in a single gene has little or no effect on neural development. The interpretation of these results is that there is redundancy in the molecular mechanisms of axon growth or that different mechanisms are acting in concert (7).

To study the role of *neurotactin* during development, we have undertaken its genetic analysis. Using an immunoscreening protocol we have isolated one mutation in the gene (nrt^1) that produces a truncated protein, lacking the most distal portion of the extracellular domain. nrt^1 homozygotes, or heterozygotes over a chromosomal deletion of the gen, are semiviable

and display discrete but clear defects in the axon patterning within the CNS. This result demonstrates a role for Neurotactin in axon outgrowth. However, the mutant phenotype is not as strong as it could be expected from the generalized expression of *neurotactin* in the CNS. This is in agreement with the hypothesis on the concerted or redundant function of cell adhesion molecules. To test this hypothesis, we have studied the phenotypic interactions between the nrt^1 allele and mutations in other cell adhesion genes.

Neuroglian is a cell adhesion protein, member of the immunoglobulin superfamily, that mediates cell adhesion in a homophilic manner. *neuroglian*, like *neurotactin*, is widely express in the CNS (8). Mutations in *neuroglian* do not affect the gross morphology of the CNS. Double mutants for *neurotactin* and *neuroglian* display a synergistic phenotype, affecting both viability and CNS morphology. This result, in line with a previous study on the genetic interaction between *fasI* and *abl* in *Drosophila* (9), strongly supports the above mentioned hypothesis.

It has been reported that *amalgam*, that encodes a protein of the immunoglobulin superfamily, is transcribed in the epidermis (10), although the protein is secreted and found in other tissues, with a pattern of accumulation that strongly resembles that of Neurotactin (11). This suggests that both proteins interact physically. Indeed, we have found that, in ntc^1 embryos, *amalgam* is transcribed normally, but the protein does not accumulate in the CNS. This result strongly suggests that Neurotactin is a physiological receptor for Amalgam, although the function of this interaction is currently unknown.

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ADHESIVE INTERACTIONS THAT REGULATE AXON GROWTH IN THE EMBRYONIC VISUAL SYSTEM. Louis F. Reichardt, Blaise Bossy, Ivan de Curtis, Michael DeFreitas, Karla M. Neugebauer, Barbara Varnum-Finney, Kristine Venstrom, and David Sretavan. Department of Physiology and Howard Hughes Medical Institute, UCSf School of Medicine, U426, San Francisco CA 94143-0724

In past work, this laboratory has identified several cell adhesion molecules and extracellular matrix constituents recognized by neurons in the retina, using cell adhesion and neurite outgrowth assays. Cell surface molecules of potential importance in mediating interactions with astroglia include the cell adhesion molecules L1, NCAM, and N-cadherin. Extracellular matrix proteins include laminin, thrombospondin, tenascin, and vitronectin. Integrin-class receptors have been identified for each of the extracellular matrix constituents. These include the first β_1-class receptors identified for tenascin and thrombospondin.

In functional studies, different classes of neurons have been shown in many instances to utilize different integrins to interact with individual extracellular matrix constituents. In the neuroretina, a major receptor for laminin has been shown to be the integrin $\alpha_6\beta_1$. Two isoforms of the α subunit of this receptor, derived by alternative splicing, are present with distinct localization patterns in the embryonic retina. During development, there are dramatic changes in the interactions of subpopulations of retinal neurons with laminin. These appear to be caused by both changes in expression of the integrin α_6 gene in retinal ganglion cells and by changes in the functional activity of the $\alpha_6\beta_1$ integrin on the surface of other classes of retinal neurons.

In order to examine axonal outgrowth within the native embryonic cellular environment, the movements of growth cones or retinal ganglion cells in the optic stalk, optic chiasm, and optic tract have been imaged using time-lapse videomicroscopy. Several integrins, cell adhesion molecules and extracellular matrix proteins are strongly expressed by either cellular substrates or axons in this pathway. Molecular perturbation experiments examining growth cones *in vivo* indicate that at least some of the molecules important in mediating growth cone movements *in vitro* are also important *in vivo*.

Imaging experiments also suggest that interactions between retinal ganglion cell growth cones and cells in the optic chiasm determine whether individual growth cones progress into the ipsilateral or contralateral optic tracts. The optic chiasm before the arrival of retinal axons includes a population of antigenically recognizable cells that appear to be an early differentiating population of neurons. Ablations of these early chiasm cells result in dramatic perturbations in axonal guidance, suggesting that interactions between embryonic retinal axons and these chiasm cells are required for normal development of the primary visual pathways.

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ROLES OF A CELL SURFACE PROTEIN A5 IN DEVELOPING NERVOUS SYSTEMS

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Cellular interaction is the one of essential mechanisms in axonal growth and guidance, and target recognition, resu ing in stereotyped neuronal connections. Recent works have been focus on the identification of molecules relevant to neuronal cell interaction. Here, we reporte the involvement of a cell surface-associated protein designated A5 in axonal fasciculation and guidance in Xenopus frog and mouse.

The A5 is a protein with apparent molecular weight of 140 kd which has originally been identified in Xenopus tadpole nervous tissues, by means of a hybridoma technique (1). The A5 is a novel class I membrane protein containing two different internal repeats homologous to the domain III of complement components C1r and C1s, and to the coagulation factors V and VIII (2). Molecular cloning has revealed the existence of the mouse homologues of the Xenopus A5. In Xenopus the A5 is expressed in both the pre-and post-synaptic elements in the visual and general somatic sensory systems, and have been proposed to be involved in the target recognition (1, 3, 4).

While, another lines of evidences indicate that the A5 may play roles in axonal fasciculation and guidance, other than the target recognition. First, in Xenopus, olfactory receptors are divided into several subclasses which differentially express the A5 and an other membrane protein named B2, and their fiber

pathways are sorted out depending on their cell surface constituents. Second, the A5 promotes neurite outgrowth for the A5-expressing neurons but not for the A5-negative ones *in vitro* (5). Third, in mouse, the A5 is transiently expressed in the growing PNS fibers (spinal and some of the cranial nerve fibers), and also, in the mesenchymal cells surrounding these nerve fibers. To clarify the function of the A5 *in vivo*, we have undertaken to produce A5-expressing mouse embryonic stem cells, and to obtain chymeric mice in which the A5 is over expressed. In these mice the fasciculation and pathways for the spinal nerve and the cranial nerves (such as the vagus nerve) was partially but not seriously disorganized.

These findings suggest that the A5 mediates interactions between growing axons, and also, between axons and their surrounding cells. The balance of these two cellular interactions seems to be important for fiber fasciculation and axonal growth, at least in the mouse PNS.

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PROTEOGLYCANS AS MODULATORS OF NEURITE OUTGROWTH.

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During development and regeneration of the mammalian central nervous system process outgrowth is modulated by molecules which favour or inhibit neurite extension (Dodd and Jessell, 1988; Kapfhammer and Schawb, 1992). Recent *in vitro* studies have also shown that there exist molecules that can exert both growth promoting and inhibiting activities depending on their exact molecular composition and how they are presented to neurons. Sulfated proteoglycans represent a class of complex macromolecules that might have this capability (Sheppard et al.,, 1991; Britts et al., 1992; Snow and Letourneau, 1992).

We have isolated a detergent soluble proteoglycan complex associated with plasma membranes of adult rat hippocampal tissue, injured by a kainic acid injection in the lateral ventricle. This proteoglycan prevented neurite initiation of hippocampal and thalamic neurons when it was presented as a surface bound molecule. When added in a soluble form, it bound to neuronal cell bodies and growth cones, causing their growth arrest and ultimately their collapse. The inhibiting complex had a molecular weight of 160-220 kDa, with a main core protein of 48 kDa, containing glycosaminoglycans (GAG) of the heparan sulphate (HS) and chondroitin sulphate (CS) type. Digestion with both heparinase and chondroitinase ABC abolished inhibition, implying that both sugar moieties were actively involved in the inhibitory activity (Bovolenta et al.,1992, 1993).

In contrast, when added to the culture medium, soluble chondroitin sulfate type C, isolated from shark cartilage, promoted axonal vs dendritic growth from thalamic neurons but not from hippocampal neurons. The effect on thalamic neurons was concentration dependent and was mediated by the selective binding of CS to the neuronal surface and its subsequent internalization. On hippocampal neurons, CS bound to the plasma membrane but was not internalized. This effect was specific for this type of glycosaminoglycan, since chondroitin sulfate type B (dermatan sulfate, DS), isolated from bovine mucosa, did not have the same activity.

These results indicate that proteoglycans can modulate neuronal process outgrowth positively or negatively. In particular, the GAG component of these class of molecules can differentially modulate neurite outgrowth, depending on their biochemical composition and on the type of neurons they bind to.

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Repulsive cues in axonal guidance
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Our laboratory is studying how the growing axons of neurons navigate accurately through the developing nervous system. How do axons choose to extend on one pathway as opposed to another? It now appears that in many instances molecules that repulse growing axons play an important role in directing axon extension. In addition, repulsive guidance cues may prevent damaged axons from regenerating in the mature central nervous system. Our aim is to identify some of these repulsive cues.

We have recently identified, cloned, sequenced, and expressed a 100 kDa chick brain glycoprotein, collapsin, that induces the morphological collapse and paralysis of the growing tips of axons in culture. Native collapsin is highly potent, inducing paralysis at a concentration around 10 pM. Biologically active, recombinant collapsin is secreted into the medium of COS-7 cells transfected with collapsin cDNA. The recombinant activity is far more effective in causing the paralysis of DRG axons than retinal ganglion cell axons. The N-terminal half of collapsin shares a significant homology with that of fasciclin IV, a protein in the grasshopper that is implicated in axon guidance. The C-terminal half contains a single immunoglobulin-like domain and an additional highly basic region. We propose that collapsin serves as a ligand that can guide axon extension by a repulsive, motility inhibiting mechanism.

We have also begun to characterize a second paralytic activity that is expressed on peripheral axons and that affects the extension of retinal ganglion cell axons. This activity is neutralized by general serine proteinase inhibitors and by PPACK, an irreversible inhibitor of a sub-class of trypsin-like serine proteinases. A similar PPACK sensitive activity can be recovered from adrenal gland membranes. We hope to use adrenal membranes as the starting material for the biochemical enrichment of this new repulsive molecule.

The molecular characterization of these repulsive guidance cues is the first step in understanding their roles in development and regeneration.

WEDNESDAY, June 2nd

P84: a novel member of the immunoglobulin superfamily expressed by the floor plate promotes the adhesion of spinal and sensory neurons

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The floor plate is a transient epithelial cell group located at the ventral midline of the neural tube and has been implicated in the organization of the developing spinal cord. Signals from the floor plate appear to contribute to the determination of cell fates in the ventral neural tube and to guide axons to and across the ventral midline. In the rat, a subset of commissural neurons which differentiate in the dorsal spinal cord can be identified by their expression of the axonal glycoprotein TAG-1 (Dodd et al. 1988). Axons of these neurons grow ventrally in the transverse plane in part under the control of a chemoattractant released by the floor plate (Tessier-Lavigne et al. 1988). Commissural axons cross the midline at the floor plate, then turn orthogonally, joining the contralateral ventrolateral funiculus and grow rostrally in the longitudinal plane of the spinal cord. The floor plate may have a role in the guidance of commissural axons through the midline and into the ventrolateral funiculus. Commissural growth cones that contact the floor plate exhibit a more complex morphology than those in the dorsal spinal cord (Bovolenta and Dodd, 1990). In a mouse mutant, Danforth's Short Tail, caudal regions of the spinal cord form without a floor plate. In such embryos commissural axons follow aberrant pathways, failing to join the ventrolateral funiculus and continuing to grow in the transverse plane into the contralateral dorsal spinal cord (Bovolenta and Dodd, 1991). The change in trajectory of commissural axons at the floor plate could be mediated, in part, by molecules that are selectively expressed on the surface of floor plate cells. We have therefore begun to analyze cell surface glycoproteins that are specifically expressed by the floor plate as commissural axons encounter these cells. Here we describe the cloning and *in vitro* characterization of one such protein, P84.

In a screen for antibodies directed against rat floor plate surface components we identified one monoclonal antibody, 6G3, which recognizes a surface antigen expressed selectively by floor plate cells from E11 - E15. With increasing developmental age, the expression of this antigen broadens and postnatally it is expressed throughout the grey matter of the spinal cord. Affinity purification of the antigen has identified a protein of 84 kDa. The immunohistochemical and biochemical characterization of the 6G3 antigen indicates that it is closely related to the mouse P84 protein (Chuang and Lagenaur, 1990).

To characterize the P84 protein in more detail we determined the N-terminal amino acid sequence of the affinity-purified protein and used PCR to obtain a probe that we used to clone the corresponding cDNA. The deduced amino acid sequence of P84 encodes a protein with a molecular weight of about 50 kDa. P84 contains 13 potential N-glycosylation sites which probably account for the difference in apparent molecular weight between the native protein and that predicted by the cDNA clone. Analysis of the sequence of P84 shows that it is a member of the immunoglobulin superfamily with one amino terminal V domain and two carboxy terminal C domains. In addition, the cloned cDNA predicts a transmembrane domain and a short cytoplasmic domain. P84 can therefore be classed with neural cell adhesion proteins which consist solely of Ig domains.

In preliminary studies to analyze the properties of P84 *in vitro* we immobilized the protein onto a nitrocellulose substrate and performed cell binding studies. Neuronal and non-neuronal cells from embryonic dorsal root ganglia and dorsal spinal cord bind to P84 *in vitro*. In addition, dorsal root ganglion neurons extend neurites on a substrate of P84. Transfection of the cDNA coding for P84 in drosophila S2 cells did not reveal a homophilic binding activity of P84. The observed cell binding to P84 *in vitro* is likely therefore to be mediated by a receptor distinct from P84.

The expression pattern, structural and functional properties of P84 raise the possibility that it mediates cellular interactions between commissural neurons and floor plate cells. Further *in vitro* studies are required to reveal the nature of such interactions and the involvement of P84 in the guidance of axons by the floor plate.

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Neural recognition molecules and signal transduction

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The transduction of cell surface recognition to consecutive signals in the cell interior appears to be instrumental for a neural cell and its growth cone to decide which interactions to engage in within its cellular and acellular environment. The cellular responses towards the cues that impinge on the cell surface depend on the integration of multiple signals that result from the activation of cell recognition molecules (1). The neural adhesion molecules L1 and NCAM activate distinct intracellular signals depending on the cell type expressing them (2). Interaction of neural cells with the oligodendrocyte-derived J1/janusin extracellular matrix molecule leads to the repulsion of neurons and adhesion of astrocytes with time of interaction (3, 4). Repulsion of neurons and their growth cones is mediated by the immunoglobulin superfamily recognition molecule F3/11 (5). Functional association of recognition molecules with ion pumps, such as the Na/K-ATPase (6, 7), and ion channels (8) exemplify the various routes that recognition molecules may use to exert their influence on the ionic milieu. L1 and NCAM influence the tyrosine kinase $pp60^{c\text{-}src}$ in growth cone membranes and affect the phosphorylation of tubulin, thus influencing the cytoskeleton (9). The linkage of the high molecular weight form of NCAM, NCAM 180 to the cytoskeleton endows this particular isoform with the ability to accumulate at and stabilize cell contacts between neural cells (10, 11, 12). The association of NCAM 180 with postsynaptic densities favours this molecule as a candidate for the stabilization of synaptic contacts (13). Neural recognition molecules may also influence the expression of other recognition molecules (14). These diverse functional associations of neural recognition molecules with the cell interior may not only be important during development, but also for the maintenance and plasticity of the adult nervous system.

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SIGNAL TRANSDUCTION BY PROTEIN TYROSINE KINASES AND PHOSPHATASES DURING *DROSOPHILA* NEURAL DEVELOPMENT.

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The functioning of the nervous system depends on the actions of highly stereotyped patterns of neuronal circuits. These circuits function correctly because the neuronal components are appropriately interconnected. An important problem in neurobiology is to understand how these precise patterns of synaptic connections is established during embryonic development.

Three major classes of molecules are though to provide the local signals that influence the specificity of axon projection in the developing embryo: a) integral membrane proteins on the surface of cells encountered by the growth cone; b) molecules associated with the extracellular matrix that surround neural cells, and c) diffusible factors, some of them known as neurotrophic factors. The capacity of growth cones to respond to this group of molecules implies that they expresses a complex array of cell surface receptors that can transduce the extracellular signals into specific local changes in the cytoskeleton of the developing axon.

Although we are beginning to identify some of the molecules responsible for neuronal recognition processes, little is know however, about the intracellular signalling events induced by the specific interactions between them. Research over the past two years has provided some evidences suggesting that phosphorylation/dephosphorylation, mainly in tyrosine residues, could be a general mechanism directly associated to these events.

We are interested in understand the signalling processes generated by neuronal recognition molecules, in particular those generated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). To this end, and using different approaches we have isolated several genes coding for PTKs and PTPs exclusively or mainly expressed in the embryonic nervous system. These proteins are directly (neuronal receptors) or indirectly (cytoplasmic proteins) involved in the generation of intracellular signalling events mediated by protein phosphorylation. Some of these molecules include:

- Two PTK receptors (D*trk* and D*ret*-like) expressed in developing axons. D*trk* shows some similarities with the mammalian *trk* family of neurotrophin receptors, and promotes calcium independent homophilic cell adhesion.
- Two PTPs, including a receptor-type (DPTP10D) and a cytoplasmic-type (DCPTP62A). Both proteins are specifically expressed in developing axons. In the case of DCPTP62A we suspect that it correspond to the product of the *roughoid* locus.
- A Ser/Thr kinase (DHCI) that is specifically expressed in a restricted group of cells of the midline (two cells per neuromer). This protein shows similarities to the product of the yeast GCN2 and is able to phosphorylate in vitro the *Drosophila* and rabbit eIF2 initiation factor.
- Finally, a putative neurotrophic factor (*ama*) that may work as a diffusible and membrane bound signalling (trophic) molecule.

Through a combination of biochemical, cell-culture and genetic analysis, we are currently investigating the putative roles of these proteins in modulating neuronal recognitions processes.

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Cell signalling events associated with cell adhesion molecule function

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Members of three well characterised Cell Adhesion Molecules (CAM) families are involved in a variety of recognition events during the development of nerve and muscle. These are the immunoglobulin superfamily, the cadherins and the integrins. A variety of perturbation strategies using mostly antibodies have implicated CAMs in processes such as axonal growth towards target tissue. The protein called NCAM is relatively unique as a CAM because it exists in a number of different isoforms generated through alternative splicing of a single copy gene. Additionally post translational modifications involving glycosylation in particular a unique form of carbohydrate called polysialic acid lead to additional diversity. However some general rules regarding classes of isoform expression are emerging. Neurons express exclusively transmembrane isoforms of 180 and 140 kD while cells such as glia and myotubes express mainly GPI-anchored N-CAM isoforms of 120 - 125 kD. One explanation for the strict patterns of expression may be cell signalling events. It is possible that the transmembrane NCAM isoforms could activate second messenger pathways following homophilic (self) binding. In contrast the GPI-anchored NCAM can bind but may not be involved in cell signalling. Other alternatively spliced variants such as the VASE exon in the 4th immunoglobulin domain may modulate the avidity or signalling capacity of NCAM isoforms. We have attempted to explore these hypotheses via gene transfer approaches. Full length cDNA clones are available for all NCAM isoforms of interest and these have been placed in different cellular backgrounds. These are NCAM negative 3T3 cells and NCAM expressing PC12 neuronal cells. The results to date show that neurons can respond to different NCAM isoforms (both transmembrane and GPI anchored) when expressed in the 3T3 monolayer. However isoforms expressing the VASE exon are inhibitory for growth although the mechanism of this effect are not known. In a second system PC12 neuronal cells were transfected with transmembrane or GPI-linked NCAM. We found that NCAM requires a cytoplasmic domain in order to transduce a homophilic binding signal to a neurite growth response. GPI-linked NCAM acted as a "dominant-negative" variant and was inhibitory to growth. The results strongly suggest that transmembrane NCAM isoforms are involved in signalling. A signalling pathway has been recently described by us. This involves the activation of a pertussis-toxin sensitive G-protein downstream of CAM action and the influx of calcium via L- and N- type calcium channels.

Tyrosine phosphorylation is also important in CAM mediated neurite outgrowth. A number of tyrosine kinase inhibitors have been tested for their ability to inhibit neurite outgrowth over parental 3T3 monolayers which we show to be largely dependent on neuronal integrin receptor function, as compared to neurite outgrowth stimulated by different CAMs. Whereas genistein (100μM) lavendustin A (20μM) and tyrphostins 34 and 213 (both at 150 μM) specifically inhibited the response stimulated by all three CAMs. CAM stimualted neurite outgrowth, the erbstatin analogue (10-15μg/ml) and tyrphostins 23 and 25 (both at 150 μM) can be accounted for by a G-protein

dependent activation of neuronal calcium channels; experiments with agents that directly activate this pathway localised the erbstatin analogue site of action upstream of both the G-protein and the calcium channels, whereas tyrphostins have sites of action downstreaam from calcium channel activation. The activation of some tyrosine kinases requires their dephosphorylation by a tyrosine phosphatase. These data suggest that increased tyrosine phosphorylation is an important step upstream of calcium channel activation in the second messenger pathway underlying the neurite outgrowth reponse stimulated by a variety of CAMs

An emerging theme in CAM research is that these CAMs can be viewed as recognition molecules that are involved in cell signalling events and that the ability of a NCAM to function is not directly related to adhesive interactions.

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SUMMARY AND CONCLUSIONS

C. S. Goodman

Summary and conclusions from the workshop

Everyone who attended agreed that the workshop was an enormous success. Scientists from around the world, including Spain, United States, England, France, Germany, Switzerland, and Japan, came together for a three day workshop to discuss the mechanisms of growth cone guidance and target recognition, and left with a sense of excitement about the rapid progress and recent breakthroughs in this field. Below is a summary of some of the research discussed at the meeting.

Guidance at the midline. Evidence is starting to accumulate to suggest that the developing midline of the CNS in both vertebrates and invertebrates provides both attractive and repulsive guidance cues. At the optic chiasm in the mammalian brain, the ventral floor plate of the vertebrate neural tube, and the midline of the developing Drosophila CNS, some growth cones cross the midline whereas others do not. One model to explain this behavior in all three cases is to propose the differential responsiveness of different growth cones to attractive vs. repulsive cues at the midline.

John Kuwada described experiments from his laboratory showing that in the developing neural tube of the zebrafish embryo, some growth cones project ventrally towards and then across the floor plate, whereas others extend towards the floor plate, but then abruptly turn away and remain on their own side. When the floor plate cells are removed by either the *cyclops* mutation or by laser ablation, these growth cones often take aberrant pathways near the midline, either failing to cross or abnormally crossing the midline. Kuwada also described studies from his laboratory on the formation of early axon pathways in the developing brain of the zebrafish.

J. Walter, a postdoctoral fellow in Tom Jessell's lab, described a number of new molecules that are specifically expressed at the floor plate of the developing neural tube, including two extracellular matrix (ECM) molecules (F-spondin and S-laminin) and several different cell adhesion molecules (p84, SC1/DM1/BEN, NCAM-PSA, and several cadherins). Walter focused his talk on the structure and expression

of p84, a member of the immunoglobulin (ig) superfamily that can induce neurite outgrowth from certain neurons in vitro, and yet lead to adhesion but no outgrowth of other neurons. Walter and colleagues are currently investigating the function of this new floor plate cell surface (and potential guidance) molecule.

In mammals, the optic chiasm, a specialized midline structure at the intersection of the two optic nerves, appears to provide attractive signals for those axons that project contralaterally, and repulsive signals for those axons that project ipsilaterally; in contrast to lower vertebrates where all retinal axons project across the midline, this combination of crossed and uncrossed projections in mammals is the basis for binocular vision. The two subpopulations of retinal ganglion axons grow together, intermix throughout the optic nerve, and sort out only at or near the midline chiasm; the growth cones of nasal retinal axons cross the chiasm.

Louis Reichardt discussed recent experiments from his laboratory that are beginning to determine the cellular and molecular mechanisms of guidance at the optic chiasm. Video microscopy of retinal axons in organ culture shows that growth cones of the non-crossing (temporal retinal) axons grow towards the midline, then encounter a substrate that induces growth cone collapse, and finally turn sharply away from the midline and into the ipsilateral optic tract. Reichardt's group has identified a population of special midline neurons that pioneer the first axon pathways from the optic chiasm to the brain. When these neurons are eliminated, the axons of the retinal ganglion cells extend towards the chiasm, but then stop and stall at the chiasm without crossing it or going any further. He also described the expression of CD44 at the midline, and proposed that CD44 might play a role in guidance at the midline.

Guidance at the midline by a diffusible, chemoattractants. At the turn of the century, Ramon y Cajal first proposed that growth cones might be guided by gradients of soluble diffusing signals emanating from their targets. The first compelling example that growth cones (from sensory neurons) can respond to a gradient of a soluble

molecule (in this case, nerve growth factoror NGF) was reported in 1979. Although the physiological relevance of this study remains unclear, it nevertheless fueled a resurgence of interest in chemotropism and what followed were several elegant tissue culture experiments which showed that target tissues taken from embryos at relevant stages could attract the appropriate growth cones from a distance.

Several years ago, in vitro experiments from **Mark Tessier-Lavigne** and his colleagues showed that the floor plate, the specialized ventral midline cells of the vertebrate neural tube, provides chemotropic guidance signals for the growth cones of commissural interneurons. Experimental manipulations from a number of groups suggest that these neurons are guided by chemotropism in the developing embryo. At the workshop, Tessier-Lavigne described his group's recent efforts at characterizing the midline chemoattractant. They have now purified, microsequenced, and cloned the genes encloding two proteins purified from the chick embryo brain, called p75 and p78, which are novel, distinct, but related secreted proteins. They represent two members of a new family of diffusible guidance molecules. Tessier-Lavigne showed that one of these new molecules, p78, is expressed specifically by the floor plate of the neural tube in the early chick embryo.

Generation of neuromuscular specificity. The ability of motoneuron growth cones to find and recognize their correct muscles has been a model system for studies on the mechanisms of pathway and target recognition in a wide range of organisms, including the chick (the subject of Lynn Landmesser's talk) and the Drosophila (the subject of Haig Keshishian's and Corey Goodman's talks) embryos. In both of these organisms, motoneuron growth cones make stereotyped pathway choices as they extend towards the regions where their appropriate target muscles arise.

Lynn Landmesser described studies from her laboratory that show the events and mechanisms that guide chick hindlimb motoneurons to their muscle targets. As these motoneurons exit the neural tube, motor axons destined to innervate different

muscles are intermingled within the spinal nerves. Upon reaching the base of the limb bud, they pause and sort out at the plexus region. It is within the plexus that specificity first unfolds, as motor axons sort out into motoneuron pool-specific groups, and make their first motoneuron-specific directional decisions in the form of dorsal vs. ventral and anterior vs. posterior pathway choices. The motor axons exit each plexus in dorsal vs. ventral trunks which, as they enter the limb, make their next motoneuron-specific decisions as they further diverge into individual muscle nerves.

Landmesser described how changes in cell adhesion can influence the ability of motoneuron axons to sort out in the plexus. Cell-cell interactions can be modulated by the polysialic acid (PSA) moiety associated with the neural cell adhesion molecule NCAM. The higher the levels of PSA, the lower the axon-axon adhesion mediated by either NCAM or L1 (another neural CAM). During normal development, when motor axons reach the plexus region, the tightly fasciculated axons defasciculate and begin to sort out. In this region, the levels of PSA go up dramatically on motoneuron growth cones. When PSA is enzymatically removed during the period of axonal sorting out in the plexus region, an increase in the number of projection errors is observed. These results suggest that the PSA-mediated decrease in axon adhesion helps facilitate the ability of motoneuron growth cones to respond to specific guidance cues in the plexus and sort out into their appropriate nerves; the molecular nature of these specific guidance cues remains unknown.

Just as in chick, so too in insect embryos, identified motoneuron growth cones extend toward and innervate the appropriate target muscles in a highly specific fashion. **Haig Keshishian** described studies from his laboratory that show that in the Drosophila embryo, the body wall musculature is relatively simple and is arranged in a stereotyped array of 30 individually identified muscle fibers in each abdominal hemisegment. Most if not all of these 30 muscles are innervated by one or only a few motoneurons, and the patterns of innervation are highly specific. To test the specificity

of motoneurons for specific muscles, Keshishian and his colleagues have conducted a series of experiments that either duplicate or delete specific muscles. These experiments argue for a high degree of specificity in the ability of motoneurons to recognize particular muscles, and suggest that different genes act in specific target regions to generate these precise patterns of connectivity.

How can these genes be identified? The most powerful approach to identify molecules involved in pathfinding and target recognition is to directly screen for mutants which perturb these events. **Corey Goodman** described a large-scale systematic mutant screen in his laboratory with the goal of saturating the genome for mutations which affect neuromuscular specificity. Goodman reported on five genes required for specific aspects of pathway (*beaten path*, *stranded*, and *short stop*) and target (*walkabout* and *clueless*) recognition. The different classes of mutant phenotypes suggest that neural specificity in this system is controlled by a hierarchy of molecular mechanisms: motoneurons are guided towards the correct region of mesoderm, in many cases navigating a series of choice points along the way; they then display an affinity for a particular domain of neighboring muscles; and finally, they recognize their specific muscle target from within this domain. He revealed that one of these genes, *stranded*, encodes profilin, a protein that binds both actin and PIP_2. Goodman speculated that profilin might regulate a specific signal transduction pathway that controls specific events of growth cone guidance, in this case, the choice of motoneuron growth cones to enter their specific muscle target domains.

Development of the neocortex and cerebellar cortex. Four talks at the workshop (Mary Beth Hatten, Carla Shatz, Jürgen Bolz, and Denis O'Leary) focused on the development of two different areas of the mammalian brain: the neocortex and the cerebellar cortex.

Mary Beth Hatten described her studies on the control of cell migration (of cerebellar granule cells) by radial glia cells. She described recent experiments on

weaver mutant mice in which granule cell migrations do not take place. By using mosaic experiments in vitro, she and her colleagues were able to show that the granule cells require wild type *weaver* function for their normal migration. In the absence of *weaver*, these neurons express a number of general neural cell adhesion molecules (e.g., NCAM and L1) but do not express other more specific markers (e.g., TAG-1). She then showed that *weaver* neurons can migrate on radial glia cells in vitro if they are incubated with wild type neurons, an inductive interaction that requires cell contact. She concluded that *weaver* encodes a non-autonomous signal (or regulator of a signal) for the proper differentation of the granule neurons.

Carla Shatz focused her talk on the role of a transient population of neurons, called subplate neurons, in the initial formation of connections between the thalamus and the cortex. In the developing visual system, axons from the lateral geniculate nucleus (LGN), one region of the thalamus, project to the visual cortex, one region of the cortex. The growth cones of LGN axons are faced with the job of navigating long distances to visual cortex, and en route they must make a number of important decisions: They initially decide to grow up into the cerebral hemispheres (rather than down into the spinal cord), they bypass many inappropriate cortical areas en route to recognize and grow into the appropriate region of cortex (visual cortex), they recognize the appropriate layer of cortical cells (layer 4), they project in a topographic map, and finally they segregate to form ocular dominance columns. While the molecular mechanisms that control these decisions are unknown, it is clear that a special transient cell type plays an essential role. These are the subplate neurons which are among the first postmitotic neurons generated in the telencephalon. Subplate neurons are present throughout neocortex and visual subplate axons pioneer the pathway from visual cortex towards the LGN; they normally disappear by cell death during neonatal life. Shatz described how ablation of these neurons during the period when LGN axons have just arrived below visual cortex prevents LGN axons from entering cortex;

moreover, ablating them later on, once LGN axons have grown into layer 4, prevents ocular dominance columns from forming.

Jürgen Bolz described his studies using the in vitro slice culture system aimed at elucidating the mechanisms of target recognition in the developing cortex. He showed that any thalamic nucleus, when placed on either side of any region of the cortex, would establish connections with the appropriate layer and cell type in the cortex. He also described the reverse experiments in which he studied the projections of cortical axons into thalamic or superior colliculus explants in culture. He showed that layer 5 neurons in the cortex project to the superior colliculus, whereas layer 6 neurons project to thalamic nuclei. Finally, he described his most recent assays in vitro in which he is beginning to determine the nature of the attractive and repulsive guidance cues that control the way in which thalamic axons grow into specific regions of the cortex.

Denis O'Leary discussed his laboratory's studies on the mechanisms that control the projection and organization of axons from the cortex to other regions of the CNS, and from the thalamus to the cortex. For example, sensory axons innervating whiskers from the face in mammals normally project thru the brainstem and thalamus to the barrel fields in the S1 region of neocortex. O'Leary reported that although tenasin, an ECM molecule with interesting functions and expression, is expressed at the boundaries around the barrel fields, the thalamic afferents sort into barrels 2-3 days before the onset of tenasin expression. O'Leary asked the question: can other cortical areas segregate into barrel fields if giventhe appropriate thalamic input? The answer is "yes". Even visual cortex, when transplanted into the region of somatosensory cortex, was able to form barrel fields and to then induce rings of tenasin. Thus, thalamocortical inputs can control the cytoarchitecture of the cortex.

O'Leary also presented further analysis of the projections from layer 5 neurons in the cortex to subcortical areas, and from thalamic regions to layer 4 neurons of the

cortex. For the descending projections from the cortex, he described a common, early pattern followed by selective pruning (selective axon elimination) of individual projections. For the ascending projections to the cortex, he described that at no time during development did he see any targeting errors, both in terms of area specific targeting and the specific invasion of specific targets.

The precise mechanisms that control the projections of thalamic axons to specific regions and cell types in the cortex is not yet well understood. Nevertheless, the workshop had three of the world's experts in this active area of research -- Shatz, Bolz, and O'Leary -- all in the same session, which led to considerable questions and discussions of alternative models and speculative interpretations that lasted well into the evening. All of the workshop's participants were treated to some lively exchange of views amongst these three speakers which gave all of us a good idea of just what is presently known about the postional specification of projections to and from the cortex, and what areas are still unclear but under intense experimental investigation.

Guidance by repulsive or inhibitory molecules. One of the most important discoveries in the field of growth cone guidance over the past decade is the observation that growth cones respond not only to attractive cues, but also to contact-mediated repulsive signals. The workshop featured three talks by scientists who work on molecules that provide repulsive or inhibitory guidance signals -- Paola Bovolenta, Jonathan Raper, and Martin Schwab.

Paola Bovolenta described studies that she and her colleagues have performed in an attempt to better understand why CNS axons do not regeneration after injury. She reported on experiments in the hippocampus after injury (induced by kainic acid) that suggests that gliotic tissue is inhibitory to neurite growth. The inhibitory factor appears to a proteoglycan of around 160-200 kD in molecular weight. The active component appears to be the carbohydrate (or GAG) chains. She described experiments now in progress aimed at determining where this inhibitory

factor comes from, how it functions, and to further characterize its sequence and biochemical conponents.

Jonathan Raper talked about studies that he and his colleagues began in the mid-1980's in which they showed that in vitro, retinal growth cones avoid sympathetic axons, and vice versa. Not only do growth cones of one cell type avoid the axons of the other, but on contact (at least, using an in vitro assay), the growth cones collapse: that is, filopodia and lamellipodia retract and the growth cone contracts. Moreover, sprinkling membrane vesicles from the second cell type onto the surface of the growth cones causes dramatic collapse.

At the Cuenca workshop, Raper announced that he and his colleagues had purified their growth cone collapse protein from several thousand chick brains, microsequenced fragments of it, and used this sequence information to clone the gene encoding it. They put the gene back into COS cells, and used the expressed (in this case, secreted) protein to confirm that the cloned gene encoded a protein which indeed had the expected growth cone collapsing activity. Raper showed that the chick protein, called Collapsin, is significantly related to the published grasshopper Fasciclin IV sequence (from the Goodman laboratory). Raper reported that the overall structure of the amino acid sequence of Collapsin was: signal sequence, small unique region, 400 amino acid Fas IV domain, small unique region, single immunoglobulin domain (C2 type, ~75 amino acids), small unique region, and finally ending in a small region of positively charged amino acids. The structure suggested to him that the protein would be secreted, and indeed, when transfected into COS cells, it was secreted into the medium. Raper he did not know that the Goodman laboratory had cloned several additional Fas IV-related genes in *Drosophila*, but with Goodman at the workshop, the relationship of these proteins was discussed. Raper's report on Collapsin, and its relationship to the Fas IV-related genes in insects, led to lively discussion and

speculation about the evolutionary conservation of this family of related genes that appears to encode repulsive guidance molecules.

Raper also described his laboratory's work in progress aimed at purifying another growth cone collapsing activity found in both membranes from PC12 cells and adrenal medullary cells. This factor appears to be related to thrombin, to have serine protease activity, but to not be thrombin itself.

Repulsive factors may prevent regeneration. Axons in the CNS of humans, unlike axons in the peripheral nervous system, do not show significant abilities to regenerate after injury. In contrast, axons in the CNS of lower vertebrates, such as fish and amphibians, are capable of regenerating. In the early 1980's, many scientists thought that this lack of regeneration in mammals and particularly in humans resulted from the absence of the appropriate growth-promoting factor(s), which may in part be true. But with the discovery of growth cone repulsion, many workers shifted their attention from the absence of an attractive factor, to the potential presence of a repulsive factor in preventing regeneration.

During the 1980's, numerous reports were published showing that mammalian CNS axons will extend for long distances in peripheral nerves, but as soon as their growth cones contact the CNS, they often stop. At the workshop, **Martin Schwab** described his laboratory's efforts to further characterize this form of inhibition using in vitro as well as in vivo assays. Schwab showed that CNS growth cones are inhibited by contact with oligodendrocytes, a major glial cell type in the brain and spinal cord that forms the myelin wrappings around axons. Interestingly, growth cones from fish or amphibian neurons are also inhibited by mammalian oligodendrocytes, but neither they nor mammalian growth cones are inhibited by fish or amphibian oligodendrocytes. This suggests that during evolution, mammalian glial cells began expressing a growth cone inhibitor that already existed in lower vertebrates, but was used in some other way.

Schwab described his most recent studies on his oligodendrocyte growth inhibitor that he has speculated might be a major impediment to regeneration. The concept is that in order to restrict adult plasticity in the CNS to the grey matter (in other words, to local changes in synaptic strength, rather than long distance growth), a mechanism has evolved to prevent sprouting in the white matter - the myelinated axon tracts. By having a growth inhibitor in the myelin, boundaries are established that separate tracts, stop collateral sprouting, and rather give axons access to grow and retract in their appropriate layers and territories without sprouting into inappropriate regions. Naturally, although very good for controlling CNS adult plasticity, such a growth inhibitor would cause severe problems during regeneration.

Schwab previously generated MAbs against oligodendrocyte membrane proteins, and discovered one MAb (IN-1) that neutralizes the inhibitory effect of these glial cells. Schwab and his colleagues have shown that the IN-1 MAb, when applied to the site of a spinal cord lesion in a rodent model, will allow some modest amount of regeneration, whereas in its absence, no regeneration is observed. Schwab reported on his efforts to purify his inhibitory protein and clone the gene encoding it. The inhibitory protein appears to be a complex of two proteins, one with a molecular weight of 215 kilodaltons (kD) and the other at 45 kD. Schwab calls this oligodendrocyte protein a "neurite growth inhibitor" (NGI). Schwab reported that he had now microsequenced several fragments of the 45 kD protein, and was proceeding with the cloning.

Schwab has done some experiments on the signal transduction systems that are activated by his myelin-specific growth inhibitor. He reported that calcium influx is the key signal. Moreover, experiments suggest that the inhibitor binds to an unknown receptor, that stimulates a G protein, and that leads (via the PIP_2 and IP3 pathway) to the release of calcium from internal stores (these are caffeine-sensitive internal stores, and the process is specifically blocked by dantrolene).

Several years ago, Schwab published experiments which showed that when the IN-1 MAb was added to the site of a spinal cord lesion in mammals, that some corticospinal axons grew across the lesion area and extended for a modest distance. At the workshop, he reported that although not many axons extended, and those that did extend did not grow very far, he now can do much better. The simultaneous addition of NT3 (one of the neurotrophins) greatly enhances this regenerative growth in the presence of MAb IN-1, whereas NT-3 alone only affects local sprouting. BDNF and NGF, in this particular spinal cord assay, did not show the synergistic effects with MAb IN-1 as did NT3. In summary, adding the appropriate trophic factor enhances spinal cord regeneration, even if the oligodendrocyte inhibitor is blocked.

Generation of retinotectal specificity. In the 1950's, Roger Sperry and his colleagues investigated the specificity by which retinal ganglion cell axons regenerate into the optic tectum in amphibians. He discovered that retinal axons reinnervated the optic tectum with remarkable precision in that neighboring retinal ganglion cells from a particular location in the retina connect to neighboring target neurons in a predictable location in the tectum. In this way, nasal retina projects to posterior tectum, temporal retina to anterior tectum, dorsal retina to ventral tectum, and so on. Based on his experimental analysis of this system, in 1963 Sperry proposed the chemoaffinity hypothesis. Since that time, much work has been aimed at trying to uncover the nature of the recognition molecules that guide retinal growth cones.

Hajime Fujisawa reported on his studies, beginning in amphibians and now in mice, aimed at uncovering the molecular mechanisms that control the specificity of retinal projections. Fujisawa reported on the further characterization of the A5 cell surface protein which is expressed on the superficial layers of the optic tectum, the termination sites of the retinal ganglion cells in lower vertebrates. The A5 protein has an interesting sequence, containing a signal sequence, regions with homology to complement component, coaggulation factors, unique sequences, a transmembrane

domain, and a short cytoplasmic domain. In amphibians, A5 is expressed both pre- and postsynaptically during the development of the visual system. In vitro, A5 can promote retinal neurite outgrowth. Fujisawa presented the hypothesis that A5 might be involved in target recognition. To test this hypothesis, Fujisawa cloned the A5 gene in the mouse, and generating mice in which the protein is both over (or ectopic) expressed, and eliminated by a knock-out mutation. He reported that over-expression of A5 leads to a varietyof abnormalities in the CNS, PNS, and blood vessels.

Genetic analysis of growth cone guidance. Five speakers -- Ed Hedgecock, Haig Keshishian, Corey Goodman, Fernando Jiménez, and Diego Pullido -- reported on the use of genetic analysis in both the fruitfly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* to study the mechanisms and molecules that control growth cone guidance and target recognition. Four of the five presentations are mentioned elsewhere in this report.

Ed Hedgecock described two different but related projects from his laboratory concerning the genetic analysis of growth cone motility and guidance. In the first, he described mutations in *unc-44* which produce lots of variable defects in neurons giving rise to abnormal short axons and ectopic axons. The *unc-44* gene encodes ankyrin, and Hedgecock speculated on the role that ankyrin might play in stabilizing the domains of expression of certain transmembrane proteins.

He also described the continuing studies from his group and his collaborators on the molecular genetic analysis of the *unc-6* and *unc-5* genes which encode, respectively, a laminin-related (but smaller) ECM molecule, and a transmembrane protein that is likely to function as the UNC-6 receptor. The most interesting new result comes from Joe Culloti's group who have ectopically expressed UNC-5 (using a cell-specific promoter) in a group of neurons that normally do not express it. When these neurons, which normally extend ventrally and anteriorly, now express UNC-5, they

grow dorsally. This altered growth cone behavior requires wild type UNC-6, thus further confirming that UNC-5 is indeed the receptor for UNC-6.

Cell adhesion molecules and signal transduction mechanisms. Over the past decade, an increasing number of cell adhesion molecules (CAMs) have been cloned and characterized, many of which are expressed in the developing nervous system. Many of these neural CAMs in both vertebrates and invertebrates are members of two different gene families, the immunoglobulin (Ig) superfamily and the cadherin family, although other gene families exist as well.

Fernando Jiménez described the studies from his group using genetic analysis to study the function of a novel CAM, called neurotactin, in *Drosophila*. Neurotactin defines a new family of CAMs containing a serine esterase-like domain. Neurotactin can not function as a homophilic CAM in vitro, but rather appears to function in a heterophilic fashion using an unknown partner. Jiménez reported that knock-out mutations in the *neurotactin* gene are partially viable and show only very subtle mutant phenotypes. However, in double mutant combination of null alleles in both *neurotactin* and *neuroglian* (a CAM of the Ig family that is highly related to vertebrate L1), Jiménez reported that 100% of the embryos show an abnormal CNS phenotype with disrupted or absent axon pathways. He also described in vitro binding assays which show that amalgam, a secreted protein, appears to be a ligand for neurotactin.

Melitta Schachner reported on the phenotypes of mouse knock-out mutations in two different CAMs: P_0, a glial CAM with a single Ig domain; and AMOG, the β2 homologue to the β1 subunit of Na/K-ATPase. The first clear phenotype in the P_0 knock-out becomes apparent at the end of the 3rd week postnatal. The mice show motor problems and tremors. They have either very little myelin, or disorganized myelin. Schachner reported that in the absence of P_0, the levels of other glial CAMs, such as myelin associated glycoprotein (MAG), are altered. AMOG mutants, on the

other hand, die abruptly. Certain brain areas have large holes, the ventricles are enlarged, and there are holes in blood vessels. Interestingly, although AMOG was isolated based on a putative cell migration function during cerebellar development, the AMOG mutant shows no abnormality in the cerebellum.

Recent experiments have begun to shift attention from considering the function of CAMs simply in terms of their adhesivity and instead to studying the growth-promoting and signalling functions of these and other molecules. At the workshop, **Frank Walsh** described the signal tranduction events that mediate the ability of NCAM, a homophilic CAM, to promote neurite outgrowth. The extracellular domain of NCAM consists of 5 Ig domains and two fibronectin type III domains. The protein comes in multiple forms, either attached to the membrane by a phospholipid anchor (NCAM 120), or having a transmembrane domain and either a short (NCAM 140) or long (NCAM 180) cytoplasmic domain. Walsh reported that as the ligand to stimulate neurite outgrowth, any form of NCAM will do, so long as it does not contain a special 10 amino acid extracellular variable alternatively spliced exon, called the VASE exon. On the neuronal receptor side, NCAM 120 will not do, but rather neurite outgrowth requires the 140 form without the VASE exon, or the 180 form.

Walsh went on to show that the cytoplasmic domain triggers a signal transduction mechanism that leads to activation of calcium influx. This signal transduction cascade is blocked by Pertussis toxin, indicating that G proteins mediate this event. He concluded by showing recent results that suggest that both protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) are involved in transducing the signal from NCAM to G proteins.

Diego Pullido reported on the diversity of PTKs and PTPs of both the transmembrane and cytoplasmic varieties that are expressed in the axons of developing neurons in the *Drosophila* embryo. One of the receptor PTKs, called Dtrk, has an extracellular domain consisting of Ig domains that look like many other CAMs;

in vitro experiments using S2 cells show that Dtrk can function as a homophilic CAM. He then gave a progress report on work in progress aimed at using genetic analysis to determine the function of a number of different PTKs and PTPs in developing axons.

List of Invited Speakers

Workshop on

CELL RECOGNITION DURING NEURONAL DEVELOPMENT

List of Invited Speakers

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Workshop on

CELL RECOGNITION DURING NEURONAL DEVELOPMENT

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