Instituto Juan March
de Estudios e Investigaciones

82

CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Wiring the Brain: Mechanisms that Control the Generation of Neural Specificity

Organized by

C. S. Goodman and R. Gallego

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Introduction

C. S. Goodman and R. Gallego

We met in Madrid in May of 1998 to discuss how the brain gets wired up. Specificity in nervous system wiring unfolds in a series of overlapping developmental steps. Neuronal growth cones navigate specific pathways and choice points. They find and recognize their correct targets and make initial patterns of stereotyped synaptic connections. The pattern and strength of these synapses is then refined, remodeled, and adjusted during development, a process that continues throughout life. Our Workshop reviewed progress in all of these areas, including the discovery of molecules, the elucidation of mechanisms, and the overall sophistication with which the field now approaches these questions.

A common coffee break topic was to marvel at just how far the field has come since we last met five years ago in Cuenca in 1993. Cuenca represented a transition from the cellular level of analysis of the 1980's as we ushered in the molecular genetic analysis of the 1990's. During the 1980's, specificity was defined, simple model systems explored, and a diversity of molecules and mechanisms implicated. It was a decade of clever in vivo and in vitro manipulations, from transplants and ablations to stripes and collagen gel assays. Elegant stories emerged concerning the development of motoneuron projections, retinotectal topography, other sensory projections, projections to and from the cortex, spinal cord wiring, and a variety of axon projections in flies and worms. We came to realize that repulsion is just as important as attraction, that guidance cues can work at long-range as well as short-range, and that guidance and connectivity are based on more than differential cell adhesion.

The either/or arguments of activity-independence vs. activity-dependence gave way to a perspective in which both mechanisms work in concert to construct the nervous system. Beautiful examples emerged showing just what activity does and does not normally do in patterning and refining connections. In short, during the 1980's, the dust settled on some of the old arguments in the field, and we got down to the hard work of trying to discover how nervous systems are actually constructed. As the 1990's approached, a variety of laboratories headed off in search of guidance and connectivity molecules, some using in vitro assays and biochemical approaches, while others took an in vivo genetic approach. Other laboratories used imaging and biophysical methods to further explore the mechanisms and roles of patterned activity.

What emerged at Cuenca in 1993 was that all of these approaches were beginning to pay off with great success. We heard about the discoveries of a variety of new IgCAMs and other cell adhesion molecules. Having heard for over a decade about what CAMs might be capable of doing, we finally started to hear what they actually do in the developing organism. We heard the further details of many different repulsion assays, and the characterization of a variety of activities and factors. The structure of the first growth cone collapse molecule -- Collapsin in chick -- was announced, and it was shown to be highly related to a recently described and otherwise novel guidance molecule -- Fasciclin IV in grasshopper. Related molecules were known in flies, and were just being discovered in humans. Within a few months, papers were published suggesting that both of these proteins were members of a broader family of molecules, called the Semaphorins, that were conserved across phylogeny. In the wind was the ongoing purification of the floor plate factor, which emerged one year later as the discovery of the Netrins, and their homology to UNC-6 in nematode. The genetic analysis in worms, with the interactions of *unc-6* with *unc-5* and *unc-40*, suggested candidate receptors for the Netrins in mammals. That too would emerge between Cuenca and Madrid. Topography was still in search of its first molecules -- the relevance of the Ephrins and Eph receptors in this process were still a

few years off. On the activity front, the first signs were starting to emerge that not all neurotrophins were functionally identical to NGF, and that some might do more than simply control cell survival.

What has happened since Cuenca? It was striking in Madrid in 1998 to see the ways in which our field has grown up, dug in, and begun to explore molecular mechanisms in a deeper and more biologically meaningful fashion. The field's assays and approaches have shifted towards more relevant ones. In the early 1990's, the dominant in vitro assays were based on simple neurite outgrowth; by 1998, many labs seemed to be using more relevant assays that provided choice and context, including both stripe and collagen gel matrix assays, and the use of transfected cells producing defined molecules. On the in vivo front, more people were using genetic or other molecular perturbations. Conditional genetic manipulations were just beginning to infiltrate our field.

In 1993 we knew that guidance could either be attractive or repulsive. By 1998 we had realized that most guidance decisions were not either one or the other, and were not based on a single molecule or mechanism, but rather that at most choice points, growth cones measure the relative balance of attractive and repulsive forces and respond accordingly. Names such as Netrins, Semaphorins, and Ephrins, and some of their receptors were household words that no longer needed introduction. At the same time, some new molecules emerged -- for example, the Slits and Robos. And intriguing transgenic results compelled us to consider that olfactory receptors led a double life in vertebrates, playing a role in guidance prior to their role in smell.

One theme of the meeting was the notion that changes in growth cone responsiveness are an important feature of the dynamic nature of guidance decisions. Growth cones in vivo can change their guidance receptors (either the levels or functions of these receptors) and thereby their responsiveness; in vitro, changes in second messenger systems and other associated proteins were shown to lead to dramatic changes in responsiveness. Some molecules that we thought functioned in repulsion can also function in attraction, and some that we thought were involved only in attraction can also function in repulsion. Detailed analysis in a number of well-known model systems, from the midline to motoneurons and from retinal to olfactory topography, showed us that guidance and targeting are not built with the principle one molecule/one choice or one molecule/one synapse. Knock-outs and knock-ins showed fascinating phenotypes, but rarely the simplest growth cone behaviors that would be predicted if single molecules controlled single guidance or connectivity decisions. Other systems, such as cortical and hippocampal connections, are just beginning to be explored, and appear to use many of these same molecules and principles.

In 1993, we heard about the first wave of new molecules, whereas in 1998 we heard about more members of those gene families, and some new gene families as well. The next frontier is integration and signal transduction, that is, dissecting how growth cones decipher and integrate all of the conflicting signals impinging on them, and translate them into meaningful guidance decisions. In 1998, a genetic analysis in Drosophila gave us our first glimpse of how guidance receptors might hook into local actin polymerization and depolymerization via adapter proteins (such as Dock) and small GTPases (such as cdc42). Five years from now, we'll no doubt have shed our innocence and joined the rest of cell biology in the morass of intersecting and intertwined signaling systems, hoping to understand how growth cones make decisions.

Synapse formation continues to be most elegantly dissected, in terms of cellular mechanisms and manipulations, at the neuromuscular junction. Although we know a great deal about the molecules that control receptor localization and gene expression, we are still in search of molecular mechanisms that control who wins and who loses during the process of synaptic refinement. One of the neurotrophins, BDNF, was shown to play an essential role in one form of synaptic plasticity in the adult, although it is still unknown how this and presumably other molecules control the developmental remodeling of synapses.

One principle that emerged in 1998 was the remarkable degree to which a variety of circuits generate their own bursting activity during development. First seen in the form of the retinal waves, and now extended to brain circuits throughout the visual pathway, such phenomena were shown for motoneurons in the spinal cord as well. Moreover, these patterns of electrical activity in the embryo have their own special biophysical and neurochemical properties that point to them being a unique and transient mechanism. We heard of the first successes using molecular methods to discover genes whose expression is turned on by activity. One of these activity-dependent genes, *MHC*, is particularly intriguing given how it functions in the immune system.

Interestingly, the temporal separation of activity-independent and activity-dependent mechanisms became further blurred. First the field learned that activity is likely to control synaptic growth and retraction by calling forth the same sorts of molecular mechanisms that function during activity-independent phases of development. Moreover, a beautiful example was described in which activity was shown to be necessary for an otherwise activity-independent process of axon defasciculation and selective refasciculation of motor axons.

Where will we be in 2003 when our community meets again for its 5 year progress report? Obviously, we'll have more molecules and a deeper understanding of mechanisms, particularly in terms of signal transduction, cytoskeletal changes, gene expression, and cell-cell interactions. We might even know (perhaps not in the full biblical sense, but nevertheless at some textbook level) how some guidance and connectivity decisions are made in the organism. But no doubt, we will see some additional transitions in our thinking and analysis, some predictable, and others not. For example, whereas the 1990's will have been the decade in which we embraced the remarkable conservation in gene structure and function across species, the next decade is likely to see us focus on the differences. The evolution of behavior must have its roots in specific changes in circuitry, and such differences in connectivity must have their basis in changes in the expression of guidance and connectivity molecules. Having learned that all organisms use the same molecules and mechanisms for wiring (and having exploited this conservation to learn how they function), the next challenge will be to discover how subtle differences in the expression of these molecules controls the evolution of brain and behavior.

Corey Goodman
Roberto Gallego

Session 1. Guidance (I)

Chair: Lynn T. Landmesser

MOLECULAR MECHANISMS OF COMMISSURAL AXON PATHFINDING

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The establishment of a functional nervous system crucially depends on the correct wiring of its individual parts. Thus, during embryonic development growing axons have to navigate through the preexisting tissue to find their way to and form stable contacts with their target cells. Many different families of molecules have been suggested to have a role as guidance cues for developing axons. The current hypothesis proposes that axons are guided by both long-range and short-range guidance cues which can be either attractive or repulsive (for a review see Tessier-Lavigne and Goodman, 1996). One of the best characterized model systems for growth cone guidance are the commissural axons in the embryonic chicken spinal cord. Commissural neurons are located in the dorsal half of the spinal cord, close to the dorsal root entry zone. They extend their axons ventro-medially toward the floor plate. After crossing the midline they turn rostrally into the longitudinal axis along the contralateral border of the floor plate.

Long-range as well as short-range guidance cues for commissural axons have been identified in both in vivo and in vitro studies (for a recent review see Stoeckli and Landmesser, 1998). It has been shown that the floor plate produces a long-range guidance cue, netrin-1, which is a chemoattractant for commissural axons (Kennedy et al., 1994; Serafini et al., 1994 and 1996). While netrin-1 is outlining the target for commissural axons, molecules of the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs) appear to serve as a track for the axons to reach their target. In an in vivo study, axonin-1 and NrCAM, two members of the of the Ig superfamily of CAMs, were shown to be important for the guidance of commissural growth cones across the midline (Stoeckli and Landmesser, 1995). If axonin-1 and NrCAM interactions were perturbed by injection of function-blocking antibodies or soluble axonin-1 into the central canal of the spinal cord in ovo, commissural axons turned into the longitudinal axis prematurely along the ipsilateral border of the floor plate.

A more detailed analysis of the mechanisms by which axonin-1 and NrCAM are guiding axons across the midline in vitro revealed a perturbation of growth cone/floor plate contacts in the absence of axonin-1 and NrCAM interactions (Stoeckli et al., 1997). Time lapse recordings of commissural growth cones contacting floor-plate cells in control cultures and in the presence of anti-axonin-1 or anti-NrCAM antibodies suggested that interactions of axonin-1 and NrCAM mask a collapse-inducing activity of the floor plate. Thus, a balance between positive and negative signals appears to determine the behavior of commissural axons at the midline. The same conclusions were drawn from studies in Drosophila, where a balance between Roundabout and Commissureless was found to be crucial for the correct formation of commissures in the central nerve cord (Kidd et al., 1998a and b).

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MECHANISMS OF AXON GUIDANCE IN THE HINDBRAIN

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The early pattern of the neuronal circuit in the vertebrate neural tube is composed of an orthogonal array of axonal tracts extending along circumferential and longitudinal axes. One major component that gives rise to the circumferentialy as well as longitudinally growing axons is commissural axons originating from the alar plate. Diffusible molecules secreted by the floor plate at the ventral midline of the neural tube appear to provide key signals for organizing neural patterning during development, by directing ventrally growing circumferential axons (1, 2, 3, 4). Moreover, chemotropic guidance by the floor plate, initially discovered in the spinal cord (4), has been extended to all axial levels where floor plate cells are present (2,3). At the molecular level, netrin-1 appears to be a key molecule in guiding ventrally directed axons (5, 6, 7).

The mechanisms that allow the growth of commissural axons across the floor plate has remained less clear. An intriguing possibility is that growth cones alter their responsiveness to a floor plate-derived chemoattractant when they encounter floor plate cells, enabling them to navigate beyond the floor plate. We tested this possibility using an in vitro preparation that reproduces the crossing of the midline floor plate by the cerebellar plate (CP) axons. When a strip of the rostral metencephalon that included the entire circumferential trajectory of CP axons was cultured alone in collagen gel, axons originating from the CP grew across the midline floor plate to extend contralaterally. A floor plate explant was then juxtaposed to the metencephalic strip on one side, and the behavior of CP axons was examined by implanting the fluorescent tracer DiI into the CP contralateral to the explant, to test whether or not CP axons, after they have crossed the floor plate, are attracted by an ectopic floor plate explant. Under such conditions,

CP axons which had crossed the midline floor plate did not show directed growth toward the ectopic floor plate explant (8).

After crossing the floor plate, commissural axons at all axial levels from the spinal cord to the mesencephalon abruptly change their growth direction to extend longitudinally (ref. 1, for example). The changes in growth cone responsiveness to guidance cues as described here might explain why commissural axons can grow along the longitudinal axis only on the contralateral side, despite the existence of identical ipsilateral cues. In addition, growth cone encounters with their intermediate targets might also cause sensitization of growing axons to subsequently encountered cues that guide them to their final destinations.

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Highways for axonal growth in the fore- and midbrain.

Kim Nguyen Ba-Charvet, Béatrice Llirbat, Stefania Guazzi, Edoardo Boncinelli, Ysander von Boxberg, Pierre Godement

1. Retinotectal projections.

In the retinotectal system, axons from both nasal and temporal retina invade the tectum from its anterior pole, but in the final map only temporal axons make connexions with anterior tectal cells, whereas nasal axons make connexions with posterior tectal cells. Several studies suggest that ligands of EPH kinase receptors (e.g., ELF1 and RAGS) exert a repulsive and differential influences on retinal fibers, from nasal and from temporal retina. Our in vitro and in vivo studies suggest that these two populations of fibers are also differentially funnelled as they grow through anterior aspects of the optic tectum. In vivo, growth cones of nasal fibers have mainly elongated morphologies in the anterior part of the embryonic mouse tectum, and much more elaborate morphologies posteriorly. In contrast, temporal fibers have mainly complex growth cones anteriorly, and collapsed growth cones posteriorly. These changes change appears to reflect a direct response of nasal fibers to their tectal environment, because they can be reproduced it in *in vitro* experiments: contact of nasal fibers with anterior tectal membranes leads to an increase in their growth rate, and to their adopting very elongated and filopodial morphologies. While temporal fibers are collapsed by posterior tectal membranes. We propose that selective funneling of nasal fibers through the anterior tectum may act to ensure that nasal fibers do not extensively pause to respond to other cues while in anterior tectum, and as an anti-branching effect and suggest that it represents a general guidance mechanism that acts to constrain the growth of subsets of fibers through select subregions in their target areas.

2. A potential role for the Otx2 homeoprotein in creating "highways" for axonal growth in the rostral brain.

Brain pattern formation starts with a subdivision of the neuroepithelium through site-specific expression of regulatory genes. It has been proposed that the boundaries between presumptive neuromeres may provide a scaffold for pioneer axon growth. In mouse forebrain, the transcription factor OTX2 is strongly expressed at several such boundaries. Combining dye tracing and staining for OTX2 protein, we show here that a number of early fibre tracts develop within stripes of OTX2 expression. To analyse a putative influence of OTX2 on the expression of molecules involved in neurite growth, we generated several clones of NIH3T3 cells stably expressing OTX2 protein at varying levels. As revealed by immunoblotting, *Otx2* transfection affects the expression of a variety of cell and substrate adhesion molecules, rendering the cells a favourable substrate in neurite outgrowth assays. Among the molecules upregulated with increasing levels of OTX2 are NCAM and DSD-1-PG, which also in situ colocalize with zones of OTX2 expression at boundaries. These data suggest that *Otx2* might be involved in defining "highways" for axon extension in the forebrain.

To Cross or Not to Cross: Genetic Analysis of Axon Guidance at the Midline

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Our goal is to understand the molecular mechanisms that control the wiring of the brain, that is, how neurons find and recognize their correct targets and make appropriate synaptic connections. We have focused our studies on the fruitfly Drosophila, a model system in which molecular genetic approaches can be applied to unraveling these mechanisms. For this talk, I will focus on the question: how do growth cones make decisions at choice points? We have focused these studies on guidance at the midline, in particular trying to understand why some growth cones cross the midline while others do not, and for those that do cross, why they never cross again. Our genetic screen for midline guidance mutants led to the identification of two key genes involved in short-range midline guidance: *commissureless* and *roundabout*. The *comm* gene encodes a transmembrane protein (Comm) expressed by midline cells; in its absence, axons approach the midline, but fail to cross it. Mutations in the *robo* gene lead to the opposite misrouting such that many growth cones that normally do not cross the midline, now do so; moreover, many growth cones that normally cross the midline only once, now freely recross the midline multiple times. The *robo* gene encodes a transmembrane protein of the Ig superfamily (Robo) that is expressed on growth cones and which functions as a repulsive guidance receptor. Comm functions as a key regulator of Robo expression. Overexpression of *comm* is dosage sensitive and leads to a phenotype identical to the *robo* loss-of-function: too many axons cross the midline. Comm controls Robo expression; increasing Comm leads to a reduction o Robo. The levels of Comm and Robo appear to be tightly regulated to assure that only certain growth cones cross the midline and that those growth cones that do cross never do so again. Thus, Robo functions as the key gatekeeper controlling which growth cones do or do not cross or recross the midline, and Comm functions as a key regulator of Robo expression. Growth cones expressing high levels of Robo do not cross the midline. We have discovered a family of Robo-like receptors in mammals, and have shown (in collaboration with Marc Tessier-Lavigne's lab) that they too are expressed by commissural neurons in the developing spinal cord.

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REGIONAL AND CELLULAR SPECIFICATION IN THE PROSENCEPHALIC ANLAGE OF THE NEURAL PLATE

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The anterior neural plate is the anlage of the prosencephalon. To study the process of prosencephalic regionalization we have developed a fate map of this area using quail-chick grafts. We have maped the topology of presumptive telencephalic areas (Rubenstein et al., 1998; Cobos et al. in preparation). During this study a tangential migration of cells in the marginal layer of the developing telencephalon was observed. These cells were generated in the neuroepithelium of the medial ganglionic eminence, and migrated dorsally into pallial domains. The characterization of these cells showed that oligodendrocytes and GABA-expressing neurons were present. Grafts of different telencephalic neuroepithelial areas did not show significative cell migration. These results are in agreement with recent data in mouse cortical development (Anderson et al.,1997.) and suggest the existence of ventricular compartments in the telencephalic neuropeithelium, where the regional and cellular identity of neural precursors are specified.

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Session 2. Guidance (II)

Chair: Corey S. Goodman

Role of Selective Fasciculation and Electrical Activity in Motor Axon Pathfinding. Lynn T. Landmesser, Victor F. Rafuse and Louise D. Milner, Dept. of Neurosciences, Case Western Reserve University School of Medicine, Cleveland, OH 44120

During motor axon outgrowth into the limb, we previously showed that motor axons regroup into target specific fascicles at the base of the limb, following a polysialic acid-mediated defasciculation process. We have recently found that within individual motoneuron pools that project to single muscles, neurons projecting to fast and slow myotubes exist as distinct subclasses. Although their somas are intermingled within the motoneuron pool, as their axons group into the target specific fascicles in the plexus region, the axons of fast and slow motoneurons segregate from one another into separate but adjacent fascicles, maintaining this segregation distally within the major nerve trunks. As the fast and slow axons approach their target muscles, they make divergent pathfinding decisions and project only into regions containing the appropriate type of myotube.

These observations indicate that the growth cones of fast and slow motoneurons must express different cell surface molecules that allow them to selectively fasciculate with like axons shortly after leaving the spinal cord. Additionally, fast and slow growth cones must also possess different combinations of receptors that allow them to distinguish fast and slow primary myotubes. A corollary is that fast and slow myotubes must also express molecularly distinct cell surface molecules. .Finally, in some pools, motoneurons projecting to slow and fast myotubes exhibit different electrical bursting patterns during spontaneous activity. Thus these motoneurons must also differ in intrinsic electrical properties and/or the manner in which they are connected to the interneurons that drive the characteristic bursting patterns. We are testing the ability of pure populations of such motoneurons, obtained by FACS sorting following retrograde labeling with DI-I, to respond to a variety of putative guidance cues in culture.

During the process of defasciculation and subsequent selective refasciculation described above during embryonic stages 23-25, we have found that motoneuron pools exhibit regularly spaced bursts of endogenous electrical activity. We have found that this activity is synaptically driven, and that this very early activity during axon outgrowth involved primarily cholinergic and GABAergic inputs. Although present, neither NMDA nor non NMDA glutamate receptors played a role in the endogenous electrical activity. This endogenous bursting activity could be blocked however, both in isolated cord preparations and in-ovo, by application of the GABA A agonist muscimol. Blocking of activity during the axonal sorting out period resulted in moderate but reproducible levels of pathfinding errors. Although we have not determined the cellular mechanisms responsible for such errors, one possibility is that the expression of some of the molecules involved in the axon sorting process in the plexus is

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Axon guidance of cranial motor neurons

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During development, cranial motor neurons extend their axons along distinct pathways into the periphery. Neurons may be classified as branchiomotor (BM), visceral motor (VM) or somatic motor (SM) depending on their position, axonal trajectory and synaptic target. For example, BM and VM neurons extend axons dorsally to leave the hindbrain via large dorsal exit points, whilst SM axons exit the neuroepithelium ventrally in small groups. BM and VM axons subsequently grow in association with sensory ganglia, whereupon BM axons navigate towards the muscles of the branchial arches. We have investigated the possible role of diffusible chemorepellents or chemoattractants in cranial motor axon pathfinding in rat embryos, by co-culturing explants containing cranial motor neurons with pathway tissues in collagen gels. Previous experiments showed that the floor plate exerts a chemorepellent influence on motor axons (Guthrie and Pini, 1995), deflecting them from the midline during the early part of their course. These effects may be mediated by the chemorepellents netrin-1 and semaphorin D; differential sensitivity of motor neuron subpopulations to these molecules may underlie segregation of ventral and dorsal axonal pathways (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarría et al., 1997). Oculomotor neurons were sensitive neither to netrin-1 nor to semaphorin D (Varela-Echavarría et al., 1997).

Recently we have examined the idea that other pathway tissues might provide diffusible cues for cranial motor axons. We have found that explants of dorsal neural tube or of the hindbrain roof plate were chemorepellent, reflecting a role for these structures in excluding motor axons, whilst explants of cranial sensory ganglia were weakly chemoattractant. The most striking effects observed in our experiments concerned explants of branchial arch mesenchyme, which were strongly growth-promoting and chemoattractant for cranial motor axons. All subpopulations of cranial motor neurons displayed chemoattraction to arch tissue, including those that normally innervate targets other than the branchial arches, such as trochlear and oculomotor neurons which supply extra-ocular muscles. Chemoattraction could also be elicited by purified Hepatocyte Growth Factor (HGF) loaded on beads and used in the co-cultures. Furthermore, anti-HGF antibodies largely blocked the chemoattractant effect of the branchial arch, suggesting that HGF may be the relevant arch-derived activity *in vivo*. *HGF* was found to be expressed by myogenic cells in the branchial arches and other regions of the head innervated by cranial motor neurons, whilst the HGF receptor *Met* was expressed by all subpopulations of cranial motor neurons with the exception of the oculomotor nucleus.

Myogenic cell populations in the branchial arches and elsewhere thus act to guide motor axons by chemoattraction, at least partially due to the production of Hepatocyte Growth Factor. Chemoattraction of oculomotor axons to the branchial arch was not blocked to a significant degree by anti-HGF antibodies, consistent with the lack of Met expression by oculomotor neurons and implying a separate mechanism for navigation of these axons to their targets. In addition, a component of the branchial arch chemoattraction on all other cranial motor neuron populations appeared to persist in the presence of anti-HGF antibodies, pointing to the existence of HGF-independent chemoattraction for motor axons in the head.

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ATTRACTIVE ASPECTS OF REPULSIVE PROTEINS: SEMAPHORINS CAN ACT AS BIFUNCTIONAL AXONAL GUIDANCE SIGNALS

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The precise navigation of axons is essential for the establishment of the complex pattern of neuronal connections in the vertebrate nervous system. Proteins encoded by the semaphorin gene family have been shown to act as growth cone guidance signals in vertebrates and invertebrates. To date 16 different semaphorins have been identified in vertebrates. They fall into five distinct classes that can be distiguished by the presence of class-specific carboxyterminal domains (1-3).

The secreted class III semaphorins are synthesized as proproteins which are proteolytically cleaved by a furin-like proprotein convertase (4). Proteolytic processing of SemD at several highly conserved dibasic sequences generates two isoforms of 65 kDa and 95 kDa which differ in their repulsive activities. SemD is secreted as a homodimer that is linked by one intermolecular disulfide bridge (5). This dimerization is necessary but not sufficient for SemD to display its repulsive activity. While SemD(95k) forms dimers, the removal of the carboxyterminal domain by proteolytic cleavage results in the dissociation of SemD homodimers to monomeric SemD(65k).

In explant cultures, SemA, D and E act as chemorepellents on specific populations of axons (4). While SemD repels a wide range of sensory and sympathetic axons, the effects of other semaphorins such as SemA and E are restricted to sympathetic axons of a specifc developmental stage.

Their expression in mesodermal structures of the developing mouse embryo such as the somites and the branchial arches suggest that one function of the secreted semaphorins is to restrict the choice of pathways accessable to sensory axons during the innervation of their peripheral targets.

Semaphorins and their receptors display highly dynamic expression patterns in the developing CNS. In order to test their role in cortical development the response of cortical axons to gradients of secreted semaphorins was analyzed. In contrast to the repulsive effects of SemD, SemE had an attractive effect on cortical axons. Thus, like netrin-1, at least one semaphorin can act as a bifunctional signal and has both attractive and repulsive properties.

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The Molecular Biology of Smell

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Mammals possess an olfactory system of inordinate discriminatory power. How is the diversity and specificity of olfactory perception accomplished? Analysis of the spatial patterns of expression of odorant receptor genes suggest a logic for olfactory perception in which neurons expressing a given receptor, and therefore responsive to a given odor, project to precisely defined loci in the olfactory bulb creating a topographic map of receptor activation. The identity of an odorant stimulus will therefore be encoded in distinct spatial patterns of activity in the brain. Support for this model derives from recent genetic experiments that permit the visualization of the individual axons from sensory neurons expressing a given receptor as they course through the olfactory epithelium into the olfactory bulb. These experiments reveal that neurons expressing a given receptor project to only two topographically-fixed loci in the mouse olfactory bulb such that the pattern of convergence is absolute and invariant. The bulb, therefore, provides a spatial map that identifies which of the numerous receptors have been activated such that the quality of an olfactory stimulus would be encoded by specific combinations of glomeruli activated by a given odorant. How do neurons expressing a given receptor know which target to project to in the olfactory bulb? In recent experiments, we have used gene targeting to introduce either deletions or substitutions into specific odorant receptor genes, and have visualized the effect of these mutations on the precision of axon targeting. These data support a model in which the olfactory receptor plays an instructive role in axon targeting as one of a complement of guidance receptors involved in the generation of a precise topographic map.

Signal transduction mechanisms affecting chemorepellent effects of collapsin-1/Sema III

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Collapsin-1/Semaphorin III/D (Sema III) has been implicated as a diffusible axonal chemorepellent in patterning the central and peripheral projections of sensory afferents from developing dorsal root ganglia. To begin to elucidate the mechanisms that account for the repulsive actions of Sema III, we have used expression cloning to search for Sema III binding proteins from a cDNA library derived from E14 rat DRG cells. We have shown that a type I transmembrane protein, neuropilin-1, can bind Sema III with high affinity, and antibodies against the ectodomain of neuropilin-1 can block the ability of Sema III to repel sensory axons and to induce collapse of their growth cones (He & Tessier-Lavigne, Cell 90, 739-751, 1997). Subsequently we also identified a neuropilin-1 related protein, neuropilin-2 whose expression in developing axons is largely nonoverlapping with that of neuropilin-1. Unlike neuropilin-1, which has similar affinity for Sema III, Sema E, and Sema IV, neuropilin-1 shows high affinity to Sema E and Sema IV, but not Sema III (Chen et al., Neuron 19, 547-559, 1997). Similar results have been reported by Kolodkin et al (Cell 90, 753-762, 1997). The differences between neuropilins in sites of expression and affinity of ligand binding might contribute to the specificity of action of semaphorins on different classes of axons during development.

The high conservation in the short cytoplasmic domains of neuropilin-1 from different species stimulated us to search for its binding proteins. Using a yeast two hybrid screening strategy, we identified a novel neuropilin association protein (NAP) which can bind to the cytoplasmic domains of both neuropilin-1 and neuropilin-2. The sites of expression of NAP in developing neurons overlaps with that of neuropilins. We are currently examining the functional relevance of NAP in Sema III-mediated responses.

Sema III-induced repulsive effects have also been demonstrated in a *Xenopus* spinal neuron culture assay. We have shown that microscopic gradients of Sema III trigger a repulsive turning response by the growth cones of *Xenopus* spinal neurons. Interestingly, the repulsive effects of Sema III on *Xenopus* growth cones can be converted to attraction by pharmacological activation of the cGMP-dependent signaling pathway. Similarly, the collapse-inducing effect of Sema III of rat sensory growth cones can be inhibited by activation of the cGMP pathway. These results point to a key role of cyclic nucleotides in regulating growth cone behavior, and suggest potential approaches to alleviate the action of repulsive factors that inhibit nerve regeneration *in vivo*.

CHARACTERISATION OF THE ROLE OF COMMISSURELESS DURING AXON GUIDANCE IN THE *DROSOPHILA* CNS

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comm is required for the formation of almost all the commissures in the *Drosophila* embryonic CNS. It encodes a novel transmembrane protein that is expressed by glia at the ventral midline of the CNS at the time of commissure formation. As commissural axons contact these glia and cross the midline of the CNS, Comm protein is apparently transferred onto commissural axons. The intracellular portion of Comm is essential for function since a truncated form of the protein lacking this region lacks all function.

To investigate further the role of Comm during axon guidance we have used the intracellular region of Comm in a yeast two-hybrid screen to identify potential downstream components of a Comm signalling pathway. The Comm intracellular region was fused to the GAL4 DNA-binding domain and used to screen *Drosophila* embryonic cDNAs fused to the VP16 activation domain. From 15 x 10^6 transformants 41 positives were isolated, as detected by the expression of two reporter genes. Isolation of plasmid DNA has revealed that a large subset of these clones encode a protein containing two WW domains. The WW domain is an approximately 40 amino acid domain that has been implicated in protein-protein interactions through binding to a proline consensus sequence XPPXY. In addition, mutation of either a WW domain or the proline consensus sequence that it recognises has been implicated in a number of human diseases. From the homology of our WW domains to a known vertebrate protein we suggest that Comm's role in axon guidance may be via the regulation of protein degradation.

Session 3. Targeting (I)

Chair: Jeff Lichtman

DEVELOPMENT OF THE OLFACTORY SYSTEM: EARLY FIBER PROJECTIONS AND CELL MIGRATION

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Primary olfactory fibers originate from the first neurons found in the olfactory pathway, the olfactory receptor neurons (ORN). These neurons are localized outside the brain, in the olfactory epithelium (OE) of the nasal cavity, and project their axonal processes to the glomerular layer of the olfactory bulbs (OB), where they make sinaptic connections with periglomerular, tufted and mitral cells[1, 7, 9]. The processes of these four types of neuron form anatomically specialized synaptic structures called olfactory glomeruli. During early embryonic development, before the anatomical formation of the OB, ORN originate in the olfactory placode. The olfactory placode develops as a local thickening of the epithelial tissue in the rostral tip of the embryonic head[6, 14]. The folding and invagination of this structure gives rise to the olfactory pit[15] and later to the OE, which contains ORN, and sustentacular and basal cells. Soon after their generation, ORN send neurites toward the rostral pole of the telencephalic vesicle (TV), the location of the presumtive OB. The ORN are bipolar neurons with two neuronal processes: a short peripheral process and an unmyelinated long process. The short peripheral process is directed toward the surface of the mucosa, where it ends in an expanded olfactory knob covered by cilia. The long unmyelinated central process penetrates the lamina propia of the OE accompanied by specialized glial cell processes (ensheathing cells)[5, 12], that project through the cribriform plate to the ipsilateral OB. From the very early stages of development, a fibrillar and cellular continuity persists between the olfactory placode and the central nervous system[2]. Thus, some cells originating in the olfactory placode leave this structure and migrate toward the TV. Among these cells there are two populations that are not associated with olfactory functions. One, the luteinizing hormone-releasing hormone (LHRH)-secreting cells that migrate from the medial site of the olfactory placode and enter the TV via the nervus terminalis to reach their targets in the hypothalamus and septum[11, 13]. The second, a novel population of cells described by our group that may be implicated in neocortical development[3]. Two other population of cells are of olfactory nature, the olfactory marker protein (OMP)-immunoreactive cells and the ensheathing cells. OMP is a protein restricted to mature olfactory neurons and, although some OMP-immunoreactive neurons migrate from the OE to the ventrolateral aspect of the OB, usually they are anchored in the OE[13]. Ensheathing cells are presumptive glial cells that guide the olfactory fibers to the OB. These cells enter the telencephalon superficially, contributing to the formation of the olfactory glomeruli[10, 12].

The aim of this communication is to describe the development of primary olfactory axons and the possible migration patterns of cells derived from the olfactory placode, olfactory pit or OE. To answer these questions, we performed acetylcholinesterase (AChE) histochemistry, immunocytochemistry to visualize the distribution of β-tubulin type III (TuJ1 antibody) and the growth-associated protein 43 (GAP43 antibody), *in vivo* and *in vitro* labelling with carbocyanine fluorescent tracers, and *in utero* injections of retroviral vectors containing the gene encoding for Green Fluorescent Protein (GFP). As a result of our experiments the incorporation of migrating cells from the olfactory placode or OE into the brain was demonstrated and different mechanisms of migration put forward[4]: 1) Dorsally migrating neurons which travel following a path not associated with any fiber and which interact with the neocortical preplate layer. During their migration these cells develop processes; 2) OMP-immunoreactive neurons which navigated through the area occupied by the olfactory fibers without associating with bundles of fibers and without emiting processes. Only when they arrived to the rostro-ventral part of the TV/OB did these cells emit fibers; 3) LHRH-producing neurons are generated in the medial olfactory placode

and migrated medially associated with the nervus terminalis to enter the TV ventro-medially. These neurons emit processes into fibers bundles aiding their migration; 4) Some ORN in the OE project long fibers which enter the TV neuroepithelium. Once these fibers have reached their destination, these cells leave the OE and migrate toward the TV, shortening their long process. A fifth mechanism of migration is present, taken by the glial ensheathing cells that guide the olfactory fibers to their target. Finally, we have used the mouse carrying a mutation in the Pax-6 gene to study whether the nasal olfactory structures intervene in the formation of central olfactory structures. This mutant, as well as lacking eyes and nasal cavity, is reported to lack olfactory epithelium and OB. However, we have found an ovoid cellular structure localized in the rostral part of the brain, and some cells in this structure project axons toward the piriform cortex forming a presumptive lateral olfactory tract. We conclude that the referred structure is an OB, which fails to develop because the mutation in the Pax-6 gene affects the formation of nasal structures. As such, fibers of the ORNs are necessary for the protrusion and layered formation of the OB, but these inputs are not necessary for the establishment of the central olfactory projections[5].

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DEVELOPMENT OF NEURAL CONNECTIONS IN THE HIPPOCAMPUS.

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The main afferent connections to the hippocampus are the entorhino-hippocampal pathway and the commissural/associational pathway. In the adult hippocampus these connections are organized in a layer-specific fashion, in which the entorhinal afferents terminate in the outer layers (stratum lacunosum-moleculare and outer molecular layer) and the commissural/associational connections innervate the lower layers (stratum radiatum and oriens and inner molecular layer). Studies in developing mouse embryos have shown that this pattern of connections is formed in a highly stereotyped way. Thus entorhinal axons grow in one direction towards the hippocampus about E14, and soon after (E16) they invade their specific layer, the stratum lacunosum-moleculare. Commissural axons grow dorsally along the fimbria from E15 onwards and innervate the appropriate target layers in the contralateral hippocampus.

Early axonal trajectories are influenced by diffusible signals. To study the possible involvement of long-range diffusible signals in the patterning of hippocampal projections we co-cultured embryonic explants in collagen gels. We showed that hippocampal axons are repelled when confronted by entorhinal or neocortical explants, and that entorhinal axons are also repelled by the neocortex. These results indicate that repellent diffusible signals prevent hippocampal axons from invading the entorhinal cortex and direct them instead to the midline of the telencephalon. Conversely, entorhinal axons are repelled by the adjacent neocortex and enter the hippocampus. Expression studies and experiments with COS-transfected cells suggest that the secreted semaphorins Sema III and Sema IV mediate these responses via receptors of the neuropilin family. Similar experiments with cells producing Netrin 1 indicate that the expression of this factor in the fimbria exerts an attractive function for axons from the hippocampus and entorhinal cortex.

Pioneer neurons are involved in the formation of layer-specific connections. Studies *in vivo* show that, when they reach their target, developing hippocampal afferents overlap various populations of early-generated neurons that are arranged in distinct layers, and form transient synaptic contacts with them. One such population is formed by Cajal-Retzius (CR) cells, which are transient targets for entorhinal axons. Studies of organotypic slice cultures show that ablation of CR cells prevents the ingrowth of entorhinal axons in the hippocampus. Conversely, the ectopic location of CR cells disturbs the layer-specific targeting of entorhinal axons. These results indicate that distinct sets of pioneer neurons arranged in a layer-specific fashion may guide developing afferents to specific layers.

Reelin - an extracellular protein expressed by Cajal-Retzius cells - contributes to the development of the entorhino-hippocampal pathway. Studies *in vitro* using the CR-50 blocking antibody and analysis of the reeler phenotype - defective in Reelin - indicate that this protein is not essential for the ingrowth of entorhinohippocampal axons. However, both in *vivo* and *in vitro* these afferents show numerous target errors as well as decreased axonal arborizations. Some of these axonal abnormalities are recovered at late postnatal stages. In contrast, commissural afferents are targeted correctly to the appropriate layer. These findings

are supported by ongoing studies on the direct effects of Reelin on developing axons, in which addition of recombinant protein decreases axonal outgrowth and increases axonal branching of entorhinal axons. These findings indicate that Reelin modulates the targeting and growth of entorhinal afferents.

Neurotrophins contribute to axonal branching and synaptogenesis. Analyses of the phenotype of trkB (-/-) and trkC (-/-) mutant mice indicate that neurotrophins are not essential for the ingrowth or the layer-specific targeting of hippocampal afferents. Similar findings are reported in double mutant trkB/trkC (-/-) mice. However, at late postnatal stages single axons display less elaborate axonal arbors in these mutant mice. In addition, electron microscopic analyses show that trkB (-/-) mice have decreased densities of axonal boutons as well as decreased densities of synaptic vesicles and absence of vesicle clustering near active zones. These fine structural abnormalities correlate with selective reduced expression of v-SNARE and t-SNARE proteins, responsible for calcium-mediated neurotransmitter release. These findings indicate that neurotrophins contribute to the regulation of numbers of synaptic inputs and synaptic machinery.

Taken together, these findings show that the development of hippocampal connections is a multifactorial process in which different signals, acting at sequential stages, provide the cues necessary for the ingrowth, layer-specific targeting and elaboration of specific hippocampal connections.

HOW DO CORTICAL WIRING MOLECULES SPECIFY CORTICAL CONNECTIONS ?

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Local circuits in the cerebral cortex are based on the precise arrangement of axon collaterals of pyramidal neurons which form precise connections between distinct cortical layers (Bolz et al., 1996). During cortical development, these connections are highly specified from the initial outgrowth of collateral branches. Our previous work provided evidence for positional cues confined to individual layers that induce and/or prevent the formation of axon collaterals in specific populations of cortical neurons and restrict their growth to individual laminae (Castellani and Bolz, 1997). However, very little is known about the molecular nature of the signals that influence the growth, targeting and arbor formation of cortical neurons. We have been examining several candidate molecules which in have been implicated in axonal guidance and which are exhibit specific laminar expression patterns at developmental stages when local connections are being formed in the cortex. Our results indicate that these molecules have a wide and complex range of actions, and that an individual molecule can exert highly selective effects on cortical axons. The effects are cell-type specific, and some molecules regulate selectively axonal guidance, or axonal branching or axonal elongation, whereas others influence any combination thereof. For example, ephrin-A5, a ligand for Eph receptor tyrosine kinases, is a repellent axonal guidance signal ("RAGS") for a population of fibers that avoid the cortical layer expressing ephrin-A5. However, in contrast to its established role as a repulsive axonal guidance signal, ephrin-A5 specifically mediates sprouting of those cortical axons that target the ephrin-A5 expressing cortical layer in vivo (Castellani et al., 1998). Results obtained with semaphorins, which have been previously described as mediators of repulsive growth cone guidance ("collapsin"), have indicated that at least one semaphorin acts as an attractive guidance signal (Bagnard et al., 1997). These results illustrate that a given molecule might be either "attractive" or "repellent". Moreover, the same study demonstrated that the effects of semaphorins depend on the spatial distribution of these molecules: effects were different when cortical growth cones encountered a gradient, a patterned distribution or a uniform concentrations of semaphorins. These results indicate that, in the developing cortex, it is not possible to classify molecules as attractive versus repulsive factors, or as "guidance signals", "branching signals" etc. This is why we propose to call such molecules collectively "wring molecules": although they can function in alternative ways, they serve as signals for assembling the intricate network of neuronal connections (Bolz and Castellani, 1997).

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Genetic analysis of axon outgrowth and guidance in C. elegans

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The simple nervous system of C. elegans with its stereotypic axon patterns provides an opportunity to examine the pathways that control neuronal development at single-cell resolution. We have been studying the development of the chemosensory neurons, a group of neurons that are part of the animal's central nervous system.

A screen for mutants with defects in the development in chemosensory axons led to the identification of the immunoglobulin superfamily member SAX-3, which is similar to the Drosophila Robo protein. sax-3 mutants have defects in the positioning of the nerve ring, the major neuropil of the C. elegans central nervous system. sax-3 is also important for guidance of axons to the midline and for preventing ectopic midline crossing by axons; the latter function is analogous to the role of Robo at the Drosophila midline.

While SAX-3 determines the final position of the nerve ring, other genes help guide chemosensory axons to this position. The chemosensory axons grow ventrally before they reach the nerve ring, and two redundant groups of genes mediate this first guidance event. This guidance event and nerve ring positioning are superficially independent of one another.

The chemosensory neurons are born in the embryo and reach their mature morphology before the animal hatches. As the animal grows, the axons grow while the neurons maintain their functions. Activity of the sensory neurons is required for neuronal growth to proceed normally. Mutations that disrupt sensory activity lead to characteristic axon sprouting events. The same defects can be produced by blocking the access of sensory neurons to odorants, by disrupting sensory transduction, or by inhibiting electrical activity. Sensory activity contributes to the maintenance of normal axon structure even into the adult stage. These results reveal an unexpected role for activity-dependent processes in axon growth in the nematode nervous system. It is striking that the effects of activity are independent of initial guidance and targeting events.

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REG PROTEINS IN NEURAL DEVELOPMENT AND REGENERATION

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We have recently characterised a novel neuron-glia signalling protein, Reg-2/Reg-IIIβ, which is a component of a neuron-glia signalling pathway involved in motor neuron regeneration (Livesey et al., 1997). Reg-2 mRNA and protein are expressed by developing and regenerating mammalian motor and sensory neurons and the protein is transported along regrowing axons. A necessary step in successful regeneration is axon-stimulated Schwann cell proliferation and we have shown that Reg-2 is a potent Schwann cell mitogen *in vitro*. Inhibition of Reg-2 signalling *in vivo*, by administration of an anti-Reg-2 polyclonal antiserum which blocks the mitogenic efects of Reg-2 in culture, significantly retards the regeneration of Reg-2-containing axons. During development, Reg-2 production by motor and sensory neurons is regulated by contact with peripheral targets. Strong candidates for the peripheral factors regulating Reg-2 production are cytokines of the LIF/CNTF family, as there are two interleukin-6 response elements (IL-6REs; STAT-binding elements) in the promoter region of the rat Reg-2 gene (Dusetti et al., 1995). These cytokines are well-characterised as peripherally-derived neurotrophic factors for developing motor and sensory neurons and all signal through receptor complexes containing the LIF receptor (LIFR) to activate the intracellular Jak-STAT signalling pathway. We have found that Reg-2 is not expressed in developing motor or sensory neurons of mice carrying a targeted disruption of the LIFR gene, a common component of the receptor complexes for all of the LIF/CNTF family (Li et al., 1995), indicating that LIFR activation is necessary for Reg-2 expression. Reg-2 is one of six closely related proteins, all expressed in the adult pancreas and gastrointestinal tract. One of the family, Reg-1, has been reported to be expressed in human developing and Alzheimer's disease-affected cerebral cortex (Ozturk et al., 1989). In the developing rat embryo, Reg-1 protein is expressed in pyramidal neurons in the cerebral cortex and area CA1 of the hippocampus and in a subpopulation of neurons in the olfactory bulb. A detailed study of the function of this protein in the developing nervous system is currently being carried out.

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The C. *elegans mig-1* gene acts to guide longitudinal cell migrations and encodes a *frizzled*-related transmembrane protein

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A number of long-range cell migrations during C. *elegans* development occur along the anteroposterior body axis. For example, the M mesoblast migrates from the head to the posterior body region and the HSN neuroblasts migrate from the tail to the midbody. Mutations in the gene *mig-1* cause these cells to terminate their migrations prematurely, suggesting that *mig-1* functions to guide them to their normal final destinations.

mig-1 also specifies certain cell fates. In wild type animals, the descendants of the QL neuroblast migrate posteriorly, whereas the descendants of the QR neuroblast migrate anteriorly. In *mig-1* mutants, the QL descendants migrate anteriorly like the QR descendants, suggesting that *mig-1* determines the fates of the QL descendants. Interestingly, the *mig-1* cell migration and cell fate defects are region-specific, occurring in the posterior half of the animal.

We determined that *mig-1* and *cfz-1*, which encodes a *frizzled*-related transmembrane protein, are the same gene. Like other *frizzled*-related genes, *mig-1/cfz-1* encodes a protein with an amino-terminal signal sequence, a conserved cysteine-rich extracellular domain and seven membrane-spanning domains. *frizzled*-related proteins can act as *wingless* or *wnt* receptors, suggesting that MIG-1 is also a *wnt* receptor and that *wnt* signaling functions in the guidance of long-range longitudinal cell migrations in C. *elegans*.

The migration defects conferred by *mig-1* mutations vary in severity and penetrance. At least two *mig-1* alleles are null mutations by molecular criteria, suggesting that multiple guidance processes influence these migrations.

We constructed a *mig-1::gfp* translational fusion to examine the expression pattern of *mig-1*. *mig-1::gfp* is membrane localized and first expressed in embryonic epidermal cells during the period that M and the HSNs undergo their migrations. *mig-1::gfp* expression was also been observed in some neurons (including the HSNs, QR, and QL), body wall muscle and M. Thus, *mig-1::gfp* appears to be expressed in the affected migratory cells and in the surrounding epidermal tissue. We are currently investigating where *mig-1* function is needed for normal development.

Session 4. Targeting (II)

Chair: Carla J. Shatz

Axon targeting and turning in the developing visual system of Xenopus

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Our research has focused on two of multiple steering decisions that retinal axons must make to correctly navigate the embryonic optic pathway: 1) exit from the retina, and 2) entry into the target, the optic tectum. Recent evidence suggests that netrin-1, whose expression is localized to the optic nerve head within the developing vertebrate retina (Sretevan et al, 1997; de la Torre et al, 1997), may entice axons to leave the retinal surface by attracting them into the optic nerve head, and thus out of the retina. Growth cone turning assays in culture demonstrate that a localized source of netrin-1 acts as a chemoattractive signal that elicits chemoattractive turning. This chemoattraction is mediated via the netrin-1 receptor, DCC, since function blocking antibodies abolish attractive turning (de la Torre et al, 1997). Interestingly, this attractive turning response can be converted to repulsive turning in the presence of soluble laminin or a specific laminin peptide, YIGSR. Experiments in which levels of intracellular cAMP were altered indicate that the laminin-induced switching involves cytosolic cAMP. Our findings suggest that external factors in the extracellular matrix in vivo may play an important role in modulating the responsiveness of growth cones to specific guidance cues.

What governs target entry? We have found that experimental pertubations of fibroblast growth factor receptor (FGFR) signaling in growing retinal axons in vivo cause errors in target recognition. For example, when FGFRs are activated by exogenous FGF-2 or FGF-2-binding heparan sulfates, or when it is blocked by expression of a dominant negative protein, retinal axons fail to enter the tectum and instead skirt around its perimeter (McFarlane et al, 1995, 1997; Walz et al, 1997). Our findings support a model of target recognition in which a key element is a change in levels of FGFR signaling in retinal growth cones when they reach the tectal border triggered, for example, by a drop/elevation in ligand concentration or a change in ligand type or modulatory factor (Holt and Harris, 1998). Such a signaling change could alter the responsiveness of retinal growth cones causing a repulsive signal in the tectum to become an attractive one, thus permitting target entry.

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Growth Cone Guidance in the Drosophila Visual System

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We have been studying the mechanisms regulating the formation of specific patterns of neuronal connections in the Drosophila visual system. The compound eye of the fly contains some 750 simple eyes called ommatidia. Each ommatidium contains 8 photoreceptor neurons (so-called R1-R8 neurons). These neurons can be classified into three groups based on synaptic specificity. The R1-R6 neurons form connections in the first optic ganglion, the lamina, whereas the R7 and R8 axons project through this region and make specific connections in two different layers of the second optic ganglion, the medulla. In addition to ganglion target specificity, photoreceptor neurons elaborate a topographic map.

To begin to dissect the histological mechanisms regulating R-cell axon guidance and target recognition, we carried out a screen for mutations disrupting R-cell connections. A set of mutations were identified that affected various aspects of axon guidance including outgrowth, pathfinding and target recognition. Our studies on one of these, called *dreadlocks (dock)*, provided a starting point for dissecting signal transduction mechanisms within the growth cone (1). Dock encodes an SH3/SH2-domain containing protein regulating aspects of pathfinding and target recognition. The finding that the mammalian ortholog of Dock, Nck, interacts with Eph and c-Met receptors suggests that Dock/Nck may play an evolutionarily conserved role in regulating signalling in the growth cone. Phenotypic studies will be presented arguing that Dock functions in many, but not all, neurons to control aspects of guidance and targeting. *dock* mutants exhibit distinct phenotypes in a subclass of motoneurons and in the projections of photoreceptor neurons in the larval eye (i.e. Bolwig's organ). Progress in understanding the molecular mechanisms by which Dock functions in the growth cone to link extracellular signals to changes in cytoskeletal organization will be discussed.

The presence of an SH2 domain in Dock led us to pursue a candidate gene approach to identify genes regulating phosphotyrosine signaling in R-cell growth cones. A receptor tyrosine phosphatase, DPTP69D, is required for the termination of R1-R6 growth cones in the lamina; in *dptp69d* mutants R1-R6 axons remain fasciculated with R8/R7 and project into the medulla. Studies indicated that though DPTP69D is required for R1-R6 growth cones for their termination in the lamina, misexpression of DPTP69D in R8/R7 is not sufficient to re-specify their connections. These data suggest that DPTP69D functions in the context of other guidance molecules to regulate R1-R6 termination in the lamina. Mutational analysis of the role of DTPTP69D in motoneurons by Zinn and colleagues (2) and our analysis in Bolwig's organ reveals that 69D functions differently in the context of different growth cones.

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AXON GUIDANCE, *IN VIVO* AND *IN VITRO* EXPERIMENTS

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Generally it is believed that the Eph family exerts its function in neuronal patterning by the complementary expression of receptors and ligands. Here we show that in the retinotectal projection Ephrin-A ligands are co-localized with their EphA receptors on nasal, but not temporal retinal ganglion cells at the time of ingrowth into the tectum. Moreover, Eph receptors and ligands are expressed surrounding the optic fibre tract towards their target area, the optic tectum. Retrovirally driven overexpression of Ephrin-A5 in the retina leads to the inability of temporal axons to respond to tectal guidance cues in vitro concommittant with a change in the phosphorylation pattern of EphA receptors. In vivo, retinal axons show servere pathfinding errors and a disturbed mapping on the optic tectum. These data suggest a role of Ephrin-A ligands in the modulation of the activity of co-expressed EphA receptors.

Mechanisms and molecules controlling retinal axon guidance, branching, and topographic mapping in the developing brain. Dennis D.M. O'Leary, Janet E. Braisted, Seung Choi, Todd McLaughlin and Paul A. Yates. Molecular Neurobiology Lab, The Salk Institute, La Jolla, CA 92037 USA

Our objective is to define mechanisms and molecules involved in the development of orderly spatial arrays of axonal connections in the brain, using as a model the topographic mapping of retinal axons onto the chick optic tectum or its rodent homologue, the superior colliculus (SC). The Eph receptor tyrosine kinase, EphA3 (Mek4), and its ligands, ephrin-A2 (ELF-1) and ephrin-A5 (AL-1/RAGS), are candidates for controlling topographic specificity of retinal connections. F. Bonhoeffer, J. Flanagan and their colleagues have shown in developing chicks that EphA3 and its ligands are expressed in complementary gradients, with EphA3 highest in temporal retina (maps to rostral tectum) and lowest in nasal retina (maps to caudal tectum), and ephrin-A2 and ephrin-A5 present in an increasing rostral to caudal gradient in tectum. In collaboration with John Flanagan and colleagues (Nakamoto et al., 1996), we have shown using the membrane stripe assay that ephrin-A2 specifically repels temporal axons. In addition, temporal axons avoid ectopic patches of ephrin-A2 overexpression created by infection with recombinant retrovirus. Thus, ephrin-A2 could determine temporal versus nasal retinotectal specificity.

In collaboration with Jonas Frisen and Mariano Barbacid (Frisen, Yates, et al., 1998), we have addressed the requirement of ephrin-A5 for retinal axon guidance by analyzing ephrin-A5 null mice created by targeted gene deletion. We find that in mice ephrin-A5 has a graded high caudal to low rostral expression across the SC, whereas ephrin-A2 is expressed at high levels in mid SC and lower or non-detectable levels in more rostral and caudal SC. Ephrin-A5 (-/-) mice have normal anterior-posterior patterning of the midbrain, as indicated by the expression patterns of ephrin-A2 and the engrailed genes, En-1 and En-2. However, in the SC, retinal axons establish permanent projections to topographically incorrect sites that correlate with locations of low expression of ephrin-A2. Thus, ephrin-A5 is required for the proper topographic mapping of retinal axons. In addition, in ephrin-A5 (-/-) mice retinal axons transiently overshoot the SC and extend into the inferior colliculus (IC). This defect is consistent with a high level of ephrin-A5 expression in the IC, and our finding that the level of ephrin-A5 in the IC inhibits retinal axon growth in vitro. Thus, ephrin-A5 may also serve to restrict retinal axons to the SC.

Additional findings suggest that ephrin-A2 and -A5 act by regulating rostral-caudal specificity in axon branching and arborization. Although ephrin-A2 and ephrin-A5 are expressed in graded or restricted patterns in the tectum/SC when retinal axons first arrive, they fail to guide or restrict growing retinal axons to topographically correct sites. Our in vivo work indicates that the development of topographically specific connections occurs principally through the formation of collateral branches which go on to establish arbors. In vitro studies show that guidance molecules can control the collateral branching of retinal axons in a topographically specific manner. These data, and modeling of the extension and topographic specific branching of retinal axons, make predictions on the patterned distributions of receptors and ligands required to generate topographic specificity.

Complementing the proposed roles of ephrin-A2 and -A5 in regulating topographic specificity of retinal axon branching through a repellent mechanism, we have been investigating molecules that might promote retinal axon branching. BDNF is an attractive candidate: retinal ganglion cells are known to express TrkB, the high-affinity receptor for the neurotrophin BDNF. In addition, BDNF is expressed in the tectum, and has been shown by S. Fraser and colleagues to regulate the morphology of retinotectal axonal arbors. Our ongoing work indicates that BDNF presented in vitro to localized sites along retinal axons in either a soluble or substrate-attached form can induce branch-like structures. Further experiments indicate that this effect is specific and appears to act through a Trk-specific mechanism. These findings support the hypothesis that BDNF directly regulates retinal axon branching in the optic tectum.

Finally, our expression analyses done with Hai Wang and David Anderson also suggest a role in retinotectal axon patterning for ephrin-B1 and ephrin-B2, ligands for the B subfamily of Eph receptors (Braisted et al., 1997). Ephrin-B1 is expressed in a high dorsal to low medial gradient in developing chick tectum, complementing the high ventral to low dorsal graded expression of its receptor, EphB2, by retinal ganglion cells. Ephrin-B2 is distributed uniformly in the tectum but selectively in the deep retinorecipient layers. These patterns implicate ephrin-B1 in retinal axon mapping along the dorsal-ventral tectal axis, and ephrin-B2 in the development of layer-specific projections. Supported in part by NEI R01 EY 07025.

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Emx2 in the developing cerebral cortex

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Emx1 and Emx2 are two vertebrate homeobox genes related to the fruit fly gene *empty spiracles* (*ems*). They are expressed in the developing rostral brain of mouse embryos, in a region including the presumptive cerebral cortex and the olfactory bulbs. In the developing cerebral cortex, Emx1 is expressed in most neuroblasts and neurons at all stages of development, whereas Emx2 expression is restricted to proliferating neuroblasts of the so-called ventricular zone and is undetectable in post-mitotic neurons. It is conceivable to hypothesise that Emx2 plays a role in the control of proliferation of cortical neuroblasts or in the regulation of their subsequent migration.

Mutations in the human EMX2 gene have been reported in sporadic cases of schizencephaly. This rare developmental disorder is characterised by a full-thickness cleft within the cerebral hemispheres. Large portions of the cerebral hemispheres may be absent and replaced by cerebro-spinal fluid. The presence of Emx2 mutations in cases of schizencephaly lends support to the hypothesis of a requirement of Emx2 gene products for the correct formation of the human cerebral cortex.

The implication of Emx2 gene products in the control of proliferation and/or migration of cortical neurons is confirmed by the analysis of Emx2 null mutant mice. Their brain displays several abnormalities including a general reduction of the cortex, both in extension and thickness. The dentate gyrus is missing, the hippocampus proper and the medial limbic cortex are greatly reduced in size and olfactory bulbs are disorganised.

We are currently analysing the detailed distributions of the EMX2 homeoprotein using a polyclonal antibody. In the pallial neuroepithelium EMX2 is expressed in a graded way in cell nuclei along the rostro-caudal axis with a caudal maximum as expected, but in addition to that some anti-EMX2 immunoreactivity is also detectable in Cajal-Retzius cells. This suggests for the EMX2 homeoprotein a more direct role in the migration of cortical neurons.

SEMAPHORINS III AND IV REPEL HIPPOCAMPAL AXONS VIA TWO DISTINCT RECEPTORS.

Chédotal Alain[1], Jose A. Del Rio[4], Zhigang He[2], Fernando de Castro[1], Monica Ruiz[4], Hang Chen[2],Victor Borrell[4], Frederic Ezan[1], Corey S. Goodman[3], Marc Tessier-Lavigne[2], Constantino Sotelo[1] and Eduardo Soriano[4].

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In the developing central nervous system (CNS), axonal growth cones are known to be guided, at a distance by a variety of diffusible molecules. Some of these secreted factors can attract the axons (chemoattraction) whereas others repel them (chemorepulsion). To date, semaphorins constitute the largest family of chemorepulsive molecules and many of them are expressed in the CNS. Secreted semaphorins have recently been shown to bind receptors of the neuropilin family.

Our goal in these experiments was to determine whether chemorepulsion and semaphorins might play a role in axon guidance in the hippocampus, a structure whose connections are highly organized. Using collagen gel co-cultures taken from E16-E17 mouse embryos, we have shown that axons growing out of explants from CA1 and CA3 can be selectively repelled by entorhinal cortex explants. We also found that two secreted semaphorins, Sema III and SemaIV, as well as their receptors neuropilin 1 and 2 are expressed in diverse components of the hippocampal formation at the time the connections are being formed. We showed in vitro that Sema III and SemaIV, expressed in COS cells, strongly repel CA1 and CA3 axons. Entorhinal axons were only repelled by Sema III. Finally, we found that an antibody directed against neuropilin-1, when applied to our cultures, could block the repulsive action of Sema III, but not Sema IV.

These results show that chemorepulsion plays a role in axonal guidance in the hippocampus, and that secreted semaphorins may be responsible for this action. Our results also demonstrate that the same axons can be repelled by two distinct semaphorins via two different receptors, one of which involves neuropilin-1. This also suggests that the receptor mediating the Sema IV repulsive effect might involve neuropilin-2.

Session 5. Developmental plasticity

Chair: Roberto Gallego

Synaptic Competition at the Neuromuscular Junction

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Mammalian muscle fibers undergo a major change in the pattern of innervation during early postnatal life. This phenomenon, which is known as "synapse elimination" has interesting parallels to alterations in connectivity in other parts of the developing nervous system including autonomic ganglia, the cerebellum, and the visual system. In muscle, competition between axons seems to be the driving force behind the changes in synaptic connectivity. At birth, each muscle fiber is innervated by multiple motor axons that converge at a neuromuscular junction. Although all the axons are appropriate in the sense they come from the correct motorneuron pool, within several weeks (~2 weeks in rodents) each neuromuscular junction loses input from all axons but one. The loss of connections is the result of extensive axonal branch retraction on the part of each motorneuron. Each neuron disconnects with more than half the muscle fibers it contacted earlier. My laboratory has focussed on attempting to understand this process by studying individual neuromuscular junctions as they undergo this competitive loss of multiple innervation. Using a variety of physiological and anatomical techniques, we have found that the interaxonal competition is a stepwise process. Axons converging at a neuromuscular junction begin with approximately equal territory and synaptic strength but become gradually different as one axon comes to occupy more territory and have greater quantal content than other axons. Initially the inputs are highly intermingled at each junction but as synapse elimination progresses we have recently found that they segregate. Synaptic endings nearest competitor synapses are withdrawn first. The withdrawal is due to a local atrophy of individual branchlets within the junction. As the presynaptic axonal changes take place, we have found a correspondingly rapid disassembly of the postsynaptic apparatus. We find that sites of elimination ultimately loose all signs of pre- or postsynaptic specializations. Thus, the withdrawal of an axon is not due to the invasion and innervation of its territory by another input. Rather, synaptic competition is due to competition at a distance with the susceptibility for loss being greatest for competing branches that are in close proximity. Previous experiments in which we focally blocked neuromuscular transmission at parts of junctions have suggested to us that neurotransmitter receptor activation by one axon initiates the disassembly of synaptic sites that are not active at the same time. These results indicate that the postsynaptic cell is the likely intermediary in the competition between axons. In trying to understand this mechanism of loss we have posited that there needs to be three different kinds of signals: 1) a local protection signal that prevents active postsynaptic sites from disassembling themselves, 2) a punishment signal that decrements rapidly in space and time that disassembles the postsynaptic sites associated with other axonal inputs, and finally 3) a retrogradely acting signal that causes nerve terminals to atrophy and withdraw from postsynaptic sites that are themselves disappearing. Recent work with muscle fibers whose protein synthesis has been blocked and transgenic animals overexpressing various growth factors suggests that the atrophy of axonal branches is due to the absence of a retrogradely acting factor that is continually required for the ongoing maintenance of synaptic endings.

FORM FROM FUNCTION: NEURAL ACTIVITY SCULPTS CONNECTIVITY IN VISUAL SYSTEM DEVELOPMENT.

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Connections in the adult central nervous system are highly precise. In the visual system, ganglion cells in each retina confine their axonal connections with target neurons in the LGN to adjacent, non-overlapping eye-specific layers. During development, however, layers are not present. Inputs from the two eyes are intermixed initially, and the adult layered pattern emerges as connections from the two eyes remodel. Remodeling requires ganglion cell signaling, since blocking action potentials prevents formation of the layers. These action potentials are endogenously generated in utero long before rods and cones are present: the ganglion cells fire spontaneously and synchronously, generating "waves" of activity that sweep across domains of the retina. Calcium imaging and microelectrode recordings show that an early synaptic network of ganglion cells and cholinergic interneurons is required to produce these waves of activity. The periodic activity, in turn, is relayed synaptically from retina to LGN. Physiological recordings indicate that the strength of this synapse can be altered in an LTP-like way. Moreover, the spontaneous activity present in the retinogeniculate pathway is required for the regulation of gene expression by LGN neurons, as revealed by differential display comparing expression between control and activity-blocked LGN. These observations indicate that an early neural circuit within the retina generates discrete spatiotemporal patterns of activity which are then relayed across central synapses. Thus, long before visual experience, nerve cell function is essential for specific activity-dependent gene expression and for the initial structural remodeling that leads ultimately to the adult precision of visual system connectivity. Since spontaneous neural activity is present elsewhere in the developing CNS, this activity-dependent process is likely used throughout the nervous system to refine early connections into their precise adult patterns. NSF IBN 93-19539, March of Dimes, and NIMH 48108.

IN VIVO PATTERNS OF SPONTANEOUS ACTIVITY IN THE DEVELOPING VISUAL THALAMUS: IMPLICATIONS FOR THE DEVELOPMENT OF CORTICAL FUNCTIONAL ARCHITECTURE

Lawrence C. Katz, Justin C. Crowley

It is a widely held belief that understanding the patterning of functional architecture in the mammalian visual cortex, in particular the pattern of ocular dominance columns, will serve as a sort of "Rosetta stone" for understanding the basic rules by which circuits in the mammalian brain are formed and altered by experience. Ocular dominance columns consist of interdigitating stripes of thalamic terminations, each carrying information originating from a single eye. Based on the pioneering work of Hubel and Wiesel, it is abundantly clear that the organization of this pattern can be dramatically altered by visual experience. As these alterations require neuronal activity, a large body of research has emerged implicating patterned neuronal activity and activity-dependent plasticity processes, such as LTP, in the formation and plasticity of these structures. However, in recent years it is becoming increasingly clear that the forces guiding plastic rearrangements of ocular dominance columns and those guiding their initial formation need not be identical. For example, in 1996, Hocking and Horton, following an earlier work by Rakic (1976) showed that newborn monkeys have completely developed ocular dominance columns. Thus, whatever patterning forces cause the formation of columns, they must exist before the onset of visual experience.

Since columns are present at birth, it follows that spontaneous activity in the developing visual pathway must be important. Work by Shatz and colleagues (L.C. Katz and C.J. Shatz, Science 274: 1133-1138) has shown that the developing retina produces rhythmic waves of spontaneous activity, even at very early times in development. These waves have been implicated in segregating the eye-specific layers in the lateral geniculate nucleus (M. Weliky & L.C Katz, Nature 386: 680-683). Similar patterns could also be important subsequently, in development for patterning of ocular dominance columns.

Remarkably little is known however, about the patterns of spontaneous activity in awake behaving animals during the period during which ocular dominance may be constructed. To address this, we developed a multi-electrode recording technique to record from eye and response-type specific layers in the lateral geniculate nucleus of ferrets during the time that ocular dominance columns may be first forming (PND 24-28).

Several clear findings emerged from these experiments. First, retinal wave-like activity is clearly transmitted to the thalamus *in vivo*. We observed periodic bursts of activity in thalamic neurons lasting for several seconds and occurring at approximately every 20 seconds. Several properties of these waves were consistent with the actions predicted by models of independent retinal oscillators. For example, the correlations within a given eye-specific layer were high. However, there were also substantial correlations between the two eye-specific layers, which should be relatively rare if the two eyes were acting as two independent oscillators. These binocular correlations require feedback from the visual cortex, as cortical ablation, eliminating the between-eye correlations. Furthermore, if both optic nerves were cut, correlated bursts of activity soon returned and these were extremely

correlated between the two eye-specific layers, implying that a thalamocortical loop is capable of sustaining patterned activity.

Interestingly, the two eyes were not equivalent in their ability to drive the thalamus. Cutting the ipsilateral optic nerve had virtually no effect on the patterns of thalamic activity, whereas cutting the contralateral nerve effectively silenced the thalamus. This suggests that input from the contralateral eye is much stronger physiologically at these early times in development, consistent with recent findings by others. We next inquired whether it was possible for geniculocortical axons to segregate into ocular dominance columns in the complete absence of the two retinas. Animals were binocularly enuculeated at P15, just after thalamocortical axons reached layer 4. At P70, after the critical period for ocular dominance columns in ferrets, layer-specific injections of biocytin were made under visual control into the thalamus.

Remarkably, in these animals segregated geniculocortical axons were clearly segregated into stripes that were indistinguishable from normal control animals. This finding was buttressed by retrograde transport studies, which showed that small injections of retrograde tracers into cortex led to labeling in one-specific layer or the other in enucleated animals.

Thus, independent of the presence of the two retinas and any activity they generate, the thalamocortical axons can segregate into eye-specific layers. We propose that the standard theories of the activity-dependent competitive interactions based on classical Hebbian mechanisms and LTP are not adequate to explain the mechanisms driving the initial formation of ocular dominance columns. While it is possible that subtle patterns of correlation within the oscillating thalamocortical loop could utilize such mechanisms, specific molecular markers present in the cortex may be used to determine the pattern of eye-specific terminations in layer 4. In this context, activity may act as a permissive force to allow axons to decode these signals. Thus neurons may need to be active in order to properly organize their terminal arbors, but the precise pattern or correlational structure of activity is of little consequence.

Long-Term Changes in Synaptic Efficacy: Neurotrophins and Synaptic Specificity

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Long-term potentiation (LTP) has been proposed as an important mechanism underlying various forms of learning, synaptic plasticity, and circuit reorganization. LTP is generally thought to be induced by concurrent pre- and postsynaptic activity leading to elevated calcium levels in the postsynaptic cell. How the subsequent synaptic enhancement is achieved and later maintained is much less clear, but it is very likely that at least part of the expression of synaptic enhancement is presynaptic, i.e. caused by enhanced transmitter release. The fact that the induction of LTP is postsynaptic whereas part of its expression is presynaptic calls for a retrograde messenger being involved in the process of LTP expression.

I will present evidence for brain-derived neurotrophic factor (BDNF) being a potential candidate for such a retrograde messenger. LTP is severely compromised in BDNF-knockout animals[1] and long-lasting LTP (L-LTP; t>3h) is even completely abolished[2]. It can be rescued by locally infecting cells with an adenovirus vector containing the BDNF gene[3], indicating that the BDNF protein is required at the time of LTP-induction. BDNF might act instructively as a retrograde messenger, but a permissive action also has to be considered. Since it is well known that BDNF can influence the morphology of neurons[4], it is attractive to speculate that this molecule might provide a link between functional and morphological aspects of synaptic plasticity which almost always go hand in hand.

I will furthermore present results showing that LTP is not confined to single synapses. If synapses are enhanced by simultaneous pre- and postsynaptic stimulation, neighboring synapses participate in this enhancement[5]. These results are in line with a diffusible retrograde messenger being responsible for expression of synaptic enhancement but they also suggest that the strict concept of a Hebbian synapse has to be modified to encompass the notion of enhancement spreading several tens of microns from the site of initiation. If one believes that the Hebbian mechanism is the basis for information storage in the brain these results indicate that for information storage - not necessarily for information processing - groups of synapses rather than single synapses are the basic computational unit.

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POSTERS

Target recognition and axon growth arrest are two independent processes in *Drosophila* mechanoreceptors.

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Single neuron changes in connectivity have been analyzed in somatic mosaics of *Drosophila gigas* mutant after horseradish peroxidase retrograde backfillings. This lethal mutant was isolated on the basis of its enlarged cell phenotype, including the nucleus. Mosaics obtained after exposure to X-rays show somatic spots of mutant cells that are normal in morphology but two to three times larger than wild types. Mutant *gigas* mechanoreceptors project their axons, over a genetically normal substrate, to the regular targets maintaining their stereotyped profile. However, they extend additional projections into novel areas of the central nervous system. Boutons are observed in the normal target as well as along the new extended projection. The additional targets in other neuromeres still correspond to mechanosensory domains. Under electron microscopy analysis, the new invading projections do not seem to elicit gliotic reaction nor to cause necrosis. The functional consequences elicited by these mutant mechanoreceptors were assayed using the grooming reflex behavior. It is observed that mosaic flies carrying a single *gigas* mechanoreceptor produce normal as well as modified, but still context-coherent, grooming reflex responses in agreement with the new projection targets reached. These experiments show that growth cone arrest and target recognition can be considered as two separable mechanisms, at least in this sensory system. Also, these observations underly the autonomous role of the growth cone during the formation of this sensory map.

Role of NMDA and AMPA receptors in regulating giant depolarizing potentials (GDPs) in developing hippocampus

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Giant depolarizing potentials (GDPs) observed in the hippocampus during the first postnatal week are synchronized network oscillations generated by the interplay between GABA and glutamate. At this stage of development GABA, acting through $GABA_A$ receptors, is depolarizing and hence allows calcium entry into the cell through activation of voltage-dependent calcium channels. This divalent cation plays an essential role in synapse formation.

The aim of this study was to determine the source of the glutamatergic drive and to evaluate the role of ionotropic glutamate receptors in GDP generation. To this purpose, single and dual whole cell recordings have been performed from CA3 pyramidal cells in hippocampal slices perfused with artificial cerebrospinal fluid at 34 °C. Rats (3-6 days old) were decapitated under urethane (10%) anaesthesia, and hippocampal slices were prepared using a standard procedure. Patch pipettes (resistance 4-6 MΩ) were filled with (in mM): KCl or KF 140; $MgCl_2$ 1; NaCl 1; EGTA 1; HEPES 5; K_2ATP 2; (pH=7.3). The intracellular solution containing KF was used to block $GABA_A$ receptor-mediated responses. With KF the responses to exogenous application of 100 μM GABA (in TTX 1μM) were blocked, while those to AMPA (5 μM) or NMDA (10 μM) were still present. In KF GDPs could still be recorded and were synchronous with those detected simultaneously with KCl-filled electrodes. However, their amplitude was smaller as shown by the slope obtained by the plot of GDP amplitude versus membrane potential (slope values with KF and KCl were -0.26 ± 0.03, n=11 and -0.77 ±0.06, n=6 respectively, mean ± SEM). This suggests that also in pyramidal cells as in interneurons (Khazipov et al. 1997) the block of $GABA_A$ receptors with KF reveals a glutamatergic component of the GDP. Similar results were found in pair recordings obtained from minislices containing only a small portion of the CA3 area, indicating that the glutamatergic component may derive from a local network. In order to identify which type of ionotropic glutamatergic receptor is involved in GDP generation, experiments were performed in the presence of selective antagonists for these receptors. GYKI 52466 (100 μM) or CNQX (40 μM), selective antagonists of AMPA and kainate receptors, reversibly blocked GDPs, while the NMDA receptor antagonists CPP (30 μM) or D-AP5 (50 μM) did not abolish them. However, in the presence of GYKI (100 μM) or CNQX (40 μM), GDPs could be still induced by application of GABA (20 μM). When GABA, at the same concentration, was applied in the presence of both NMDA and non-NMDA receptor antagonists, it failed to induce GDPs.

These results suggest that generation of GDPs normally requires non-NMDA receptors activation, while NMDA receptors are not essential. However, when non-NMDA receptors are blocked, the NMDA activation is essential for GDPs induction by GABA

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CLONING OF VERTEBRATE HOMOLOGUES OF THE IMMUNOGLOBULIN SUPERFAMILY MEMBER ROUNDABOUT (ROBO) AND CHARACTERIZATION OF THEIR ROLE IN AXON GUIDANCE AT THE CNS MIDLINE

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An important aspect of nervous system wiring is that information from the two sides of the body is integrated by axons that project to and cross the midline, forming axon commissures. Embryological experiments in vertebrates and genetic manipulations in invertebrates have suggested that growth cones sense specific cues at the midline which influence their decision to cross or not to cross. The specific midline cues and the precise molecular mechanisms mediating this choice are not well understood. The Drosophila mutant *roundabout* (*robo*) is defective in this process of commissure formation. In robo mutants, axons that normally would never cross the midline, now cross and axons that would normally cross only once, now cross multiple times, suggesting that robo is part of a signaling system which prevents inappropriate crossing. To determine the mechanisms that regulate pathfinding at the vertebrate midline, we cloned and characterizing vertebrate homologues of components of this robo signaling system. We have identified two rat homologues of Drosophila *robo*, *rRobo1* and *rRobo2*, both of which are expressed in the rat spinal cord at the time of commissural axon crossing of the ventral midline. *rRobo1* mRNA is expressed in the dorsal spinal cord, in a pattern apparently corresponding to the cell bodies of commissural neurons, as well as in a subpopulation of ventral cells in the region of the developing motor column. *rRobo2* is also expressed in the region of the developing motor column, but in a distinct pattern from that of *rRobo1*. In contrast, *rRobo2* mRNA is expressed in the dorsal root ganglia, where *rRobo1* is not expressed, and is expressed in the dorsal spinal cord in a much more restricted pattern than is *rRobo1*. Therefore, like that of its Drosophila homologue, *rRobo1* and *rRobo2* mRNAs appear to be expressed by both neurons with crossing axons and neurons with non-crossing axons, suggesting that Robo may in vertebrates play a role similar to that of Robo in Drosophila. To confirm whether Robo protein is indeed expressed on commissural axons and whether it is localized in a manner consistent with its postulated role as a receptor preventing inappropriate crossing, we have generated antibodies against rRobo1 and rRobo2 and are characterizing Robo expression at the time of midline crossing. We are also pursuing a number of molecular, cell biological and embryological approaches to gain insight into Robo's potential role in directing guidance at the midline: (1) We are actively seeking the identification of candidate Robo ligands, which may act as a midline repellent for commissural axons. (2) We are developing *in vitro* assays for midline crossing. Such assays should not only gain us valuable insight into mechanisms of Robo function but will also be a valuable tool for identifying other elements of the Robo signaling pathway. (3) To determine Robo's function in vivo, Andy Plump is "knocking-out" the mouse homologues of rRobo1 and rRobo2. (4) In collaboration with Corey Goodman's lab, we are beginning to examine potential signaling effectors downstream of Robo.

Observations of Rapid Translocation of Signalling Receptors *in vivo*: Time-lapse Imaging in *C. elegans* sensory neurons

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The regulation of the distribution of signalling receptors on neurons is a problem ripe for study. With the recent discovery of many receptors for axon guidance cues such as netrin receptors, robo/sax-3, and Eph receptors, it is now possible to ask how the localization of these receptors is dynamically regulated by the neuron during development. Changes in localization of signalling receptors may govern how neurons make decisions such as growth cone turning, interstitial branch formation, arbor elaboration or pruning, and synapse stabilization or elimination. Fascinating examples of how receptor distribution influences developmental decisions include acetylcholine receptor dynamics at the NMJ during synapse elimination, stabilization by FasII on both sides of the synapse, and the complementary changes in derailed and robo protein expression in axons upon midline crossing. What are the factors that determine receptor concentration and location? Guideposts, targets, and local changes in activity or calcium are likely to play key roles, but the links between these influences and receptor expression or localization are largely a mystery. Spatially and temporally controlled endocytosis or secretion of vesicles containing receptors may play a role in receptor regulation, and thereby in neuron development and plasticity.

We have been studying transport and localization of seven transmembrane domain receptors in chemosensory neurons in *C. elegans*. Previously we identified a novel protein that is required for proper localization of a subset of odorant receptors. Recently we have been studying the rapid transport of receptors in neurons within the living worm. This is possible through the use of functional GFP-tagged receptors, and rapid time-lapse imaging using a high-resolution CCD camera and Metamorph software. We have found that receptors can be translocated in the dendrite at very high speeds, up to microns per second. Receptors appear to be transported in vesicles and tubules of heterogenous sizes, and travel at a variety of speeds, both anterogradely and retrogradely. This process is dependent on ATP, and could be mediated by microtubule motors, although existing motor mutations do not disrupt this movement. Since little is known about how receptors are targeted to and transported in dendrites, the ability to couple genetics with *in vivo* observation of transport dynamics could add greatly to the field.

The surprisingly rapid rates of translocation of these receptors suggest that changes in patterns of receptor distribution on neurons can take place within seconds. It is possible that the mechanisms used in *C. elegans* dendrites will also be used in mammalian dendrites for regulating the distribution of receptors that then effect structural changes.

Axons of the Ocellar Sensory System of Drosophila show discrete requirements for specific CAMs and the FGFr function during guidance.

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The development of sensory nerves in the adult dorsal head of Drosophila offers a simple model system to study the mechanisms of growth cone guidance. Early during the metamorphosis of the fly, two different types of neurons differentiate side by side in the terrritory that will become the apical part of the adult head: pioneer neurons of the ocelli (OP) and mechanoreceptor neurons of the bristles (MN). These two types of neurons initially select either of two alternative substrates for axon extension. OP axons extend in the Extracellular Matrix (ECM) without adhering to the underlaying epithelium while MN axons grow in association with the epithelium[1]. These two substrates guide these axons to different targets in the brain. Before reaching them, OP axons cross from the ECM surrounding the head capsule to the ECM covering the dorsal side of the brain while MN axons continue following the epidermal head contour up to the region of the antenae, where they leave this surface and cross to the brain.

Different Cell Adhesion Molecule (CAM) and Growth Factor Receptor (GFR) mutants reveal discrete functions during the different steps of development and guidance of these neurons and their axons. Laminin A (LamA) is required in the ECM for OP axons to select it as their substrate for extension. In mutant conditions for LamA, OP axons project in the epidermal layer where they stop growth[1]. In a collaborative work with the labs of C. Goodman and R. Murphey, we have found that Laminin B1 (LamB1) and B2 (LamB2) are required by MN axons to both project in the epidermis and follow here their correct projection pathway. Interstingly, triple LamA/LamB1/LamB2 mutants reveal a similar requirement for LamA, not previously uncovered by the single mutant.

Neurotactin (Nrt) is a CAM molecule especifically expressed by OP neurons. Nrt is required in the OP axons to mantain a tight fasciculation. In the absence of Nrt, isolated axons or small bundles can select the epidermis for extension where they eventually stop growth, suggesting that fasciculation of many axons may be a way to ensure precision during guidance. Conversely, ectopic expression of Nrt in MN axons allows them to recognize and select the OP axons as a substrate for extension. Ectopic expression of Nrt in the epidermal cells cause OP axons to project in the epidermis where they again stop growth. Obviously, the growth inhibitory influence of the epidermis over the OP axons is a specific characteristic of these neurons, since MN axons use the epidermis as their normal substrate for extension[2].Another CAM, Neuroglian (Nrg) is expressed by OP and MN neurons, as well as by the epithelium. Nrg seems to be required by MN axons to attach to the epidermis, since Nrg mutants show bristle axons leaving the epidermal layer soon after their birth.

Finally, we are studying the requirements for GFRs during development of OP and MN neurons. While the Epidermal Growth Factor receptor (EGFr) is required for the initial determination of both types of neurons, the Fibroblastic Growth Factor receptor (FGFr) seems to be specifically required for axon guidance. Thus, loss of function conditions of the FGFr generated using the GAL4 system show prominent phenotypes in both OP and MN axon projections. In both cases, axons are unable to make appropriate decisions at critical choice points. OP axons sometimes fail to project in the ECM but most frequent is a failure to cross from the head's ECM to the brain. In addition, most MN axons are unable to leave the epidermis and cross to the brain at the antennal choice point. Other aspects of axon development like fasciculation and growth do not seem to be affected at all under our experimental conditions. Therefore, and in line with current ideas derivated from experiments in vertebrate cell culture systems and transgenic mice, GFRs seem to play a crucial role in integrating and transducing signals triggered by CAMs during growth cone guidance in Drosophila.

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REELIN AND DAB1, TWO COMPLEMENTARY MOLECULES THAT GOVERN MOUSE BRAIN DEVELOPMENT

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Reelin is an extracellular matrix protein specifically expressed during brain development. In mice, reelin deficiency results in the reeler phenotype, characterized by anomalies of neuronal cell patterns in the cerebral cortex, cerebellum, hippocampus and several other structures in the CNS. In the cortex, reelin is secreted by pioneer neurons such as Cajal-Retzius cells, as well as in cerebellar external granule cells and reticular neurons in the brainstem. Surprisingly, most reelin–producing neurons are not affected by the reeler trait. The reeler phenotype is also produced by mutations of the disabled1 gene (mDab1), a putative adaptor of non receptor tyrosine kinases homologous to the drosophila disabled gene product. Reelin expression is normal in animals with these mutations, including scrambler, yotari and mdab1-1 knock out. mDab1 is expressed in neurons of the cortical plate, in Purkinje cells, neurons of the inferior olivary complex, etc... all well-known targets of the reeler gene. Expression of mDab1 appears better correlated to the reeler phenotype than that of reelin. There is thus some complementarity between reelin and mDab1 expression, suggesting that reelin acts in a juxtacrine fashion on target neurons, the response of which requires the mDab1 gene product. Reelin is a large, more than 388 kDa glycoprotein and immunohistochemical studies suggest that it does not diffuse over appreciable distances from producing cells. Furthermore, binding studies on tissue sections using several fragments of reelin as ligands did not reveal any significant specific signal. The mechanism by which target neurons sense and respond to the presence of reelin thus remains to be explained further.

Ephrin-B class ligands in the patterning of retinal projections in the developing chick tectum

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Several Eph receptor tyrosine kinase receptors are expressed in the chick retina during the development of the topographic retinotectal map. EphA3, an EphA class receptor, is expressed in a high temporal to low nasal gradient in the retina. Two ligands for EphA3, ephrin-A2 and ephrin-A5, are expressed in high caudal to low rostral gradients in the developing tectum and have been functionally implicated in the development of the retinotectal projection along the rostral-caudal axis of the tectum by specifically repulsing retinal ganglion cell axons expressing high levels of EphA3 from regions of the tectum expressing high levels of ephrin-A class ligands. At least one other Eph receptor, the EphB class receptor EphB2, is distributed in a gradient in the chick retina and is present on retinal ganglion cell axons during the development of the retinotectal projection. However, EphB2 is expressed orthogonally to EphA3 in the retina, being present in a high ventral to low dorsal gradient. A high affinity ligand for EphB2, ephrin-B1, is distributed in a high medial to low lateral gradient in the tectum during the time of initial retinotectal mapping and is found on the endfeet and along the processes of radial glial cells. These corresponding distributions of EphB2 and ephrin-B1 are suggestive of a role in retinotectal mapping along the medial-lateral axis. However, distinct from the EphA3 - ephrin-A class ligand interactions along the rostral-caudal axis, along the medial-lateral axis, regions of retina expressing high levels of EphB2 map to regions of tectum expressing high levels of ephrin-B1, suggesting the novel possibility of an attractive interaction between Eph receptor and ephrin. To date, no molecule has been directly implicated in mapping along the medial-lateral axis of the tectum. A recombinant replication competent avian retrovirus has been constructed and is being used to misexpress ephrin-B1 in the developing chick tectum to assess its role in the mapping of the dorsal-ventral axis of the retina onto the medial-lateral axis of the tectum.

A second ephrin-B class ligand is also expressed in the developing tectum. At the time of retinal ganglion cell axon arborization, ephrin-B2 mRNA and protein are localized to deeper retinorecipient tectal laminae. Although the distribution of ephrin-B2 in the tectum is not appropriate for influencing topography, the timing and localization of expression suggests that ephrin-B2 may play a role in the laminar patterning of retinal axon terminations. Ephrin-B2 may act to specifically attract those retinal axons which arborize within ephrin-B2 expressing laminae, or ephrin-B2 may act to specifically repel those retinal axons which arborize in more superficial tectal laminae. A recombinant replication competent avian retrovirus has been constructed and is being used to misexpress ephrin-B2 in the embryonic chick tectum to assess the function of ephrin-B2 in determining the laminar distribution of retinal ganglion cell arborizations.

Kerry L. Tucker

"Optical Imaging of Mouse Brain using Green Fluorescent Protein"

One of the major problems in the study of central nervous system (CNS) development is that it is difficult to image large numbers of dendrites or axons in a living tissue. While excellent imaging techniques exist, typically they allow sampling of few cells (e.g. the intracellular injections of fluorescent dyes) or require fixed tissue (e.g. DiI or tracers detected through immunocytochemical techniques). We are attempting to specifically label neurons and their subcellular compartments in the CNS of the mouse, utilizing the genes encoding the green fluorescent protein (GFP) from *Aequoria victoria* and its mutant derivatives. Live analysis *in situ* and in organotypic slice cultures prepared from mouse brain will be performed with the recently-developed 2-photon laser microscope, a technique which produces both unparalleled spatial resolution and reduced cell toxicity.

Expression of GFP will be effected by targeting its cDNA to the tau gene, a microtubule-binding protein localized in the axons of neurons in both the CNS and PNS. This will confer abundant neuron-specific expression upon the GFP cDNA from the endogenous promoter of the tau gene. The tau gene has been previously inactivated through gene targeting (Harada *et al.*, *Nature* 369: 488-91), and the resultant homozygous mutant mice are viable, fertile, and show no obvious morphological defects in CNS structure. Thus, the expression of GFP from the targeted tau locus and the concomitant loss of tau expression from this allele should allow for the analysis of a normal CNS. At the time of abstract submission, embryonic stem cells have been transfected with a targeting construct and are being analyzed for the targeting of tau and the expression of GFP.

As the ubiquitous expression of GFP in the CNS may present problems in resolving individual neurons, an inducible system of GFP expression is also being designed by placing a transcription-terminating "STOP" cassette upstream of the GFP cDNA. This STOP cassette is flanked by loxP sites, so that expression of the GFP cassette can be induced by the application of Cre recombinase, either through transgenic mice expressing Cre or through the localized application of Cre-expressing adenoviruses. Altering the applied titer of such adenoviruses should allow for the expression of GFP in only a fraction of neurons in a given tissue, thus allowing individual neurons to be visualized against a background of non-expressing cells. Another parameter to be considered is the targeting of the expressed GFP to axons or dendrites by coupling to the tau or MAP2 proteins, respectively.

We are particularly interested in the effects of neurotrophins upon patterns of visual cortex innervation. Recent research has uncovered a role for neurotrophins in the activity-dependent growth and connectivity of neurons in the CNS. For example, neurotrophins have been found to regulate ocular dominance column formation and dendritic growth in the visual cortex of cats and ferrets. Mice bearing a targeted GFP cDNA will be crossed to mice deficient for the neurotrophins Brain-Derived Neurotrophic Factor, Neurotrophin-3, and Nerve Growth Factor. Axonal and dendrite arborizations will be the quantifiable parameters to be examined first in neurons, but other activity-dependent morphological changes will be considered.

VOLTAGE-GATED SODIUM, CALCIUM AND POTASSIUM CHANNELS IN DEVELOPING RAT SPINAL MOTONEURONES: PHARMACOLOGICAL AND MOLECULAR CHARACTERIZATION

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The regulated expression of voltage gated ionic channels plays an important role in the changes that take place in action potential duration (Ribera, 1996) and repetitive firing properties (Viana et al., 1995) observed in developing neurones. Spontaneous elevations in intracellular Ca^{2+} are also necessary for the developmental of distinct neurotransmitter phenotypes (Gu and Spitzer, 1995). We have used cultures of embryonic rat spinal motoneurones and the patch-clamp technique to investigate the expression of different Na^+, Ca^{2+} and K^+ channels, and their pharmacological sensitivity.

Embryonic (E14) rat spinal motoneurones, maintained in culture for 1-4 days, were able to fire repetitive trains of actions potentials upon depolarizing current injections. Action potentials were completely abolished in the presence of the Na^+-channel toxin TTX. Furthermore, the duration of the action potential was highly sensitive to the application of K^+-channel blockers TEA and 4-AP.

In the presence of Na^+ and Ca^{2+} channel blockers, 1-s duration depolarizing pulses evoked outward K^+ currents with transient (IK_A) and sustained (IK_D) components, with distinct kinetic and pharmacological properties. IK_D activated around -30 mV, was blocked by TEA (IC_{50} = 2 mM), 4-AP and quinine. IK_D was also blocked by the Ca^{2+} channel blocker mibefradil, but this effect did not depend on intracellular Ca^{2+} elevations. A small fraction of IK_D was also blocked by charybdotoxin and α-dendrotoxin. IK_A activated at more positive potentials (~ -55 mV), inactivated rapidly and almost completely, and showed pronounced steady-state inactivation ($V_{1/2}$ ~ -75 mV). IK_A was insensitive to TEA, α-dendrotoxin and mibefradil but blocked by quinine and 4-AP. Unlike previously cloned Kv 1.4 channels, the amplitude and inactivation characteristics of IK_A were little affected by removal of external K^+ and oxidizing reagents. Using RT-PCR, we investigated the expression of K^+ channel mRNAs. We could amplify messengers coding for the Kv1.4, Kv4.2, and Kv 4.3 K^+ channels.

Ca^{2+} currents were recorded in the presence of Na^+ and K^+ channel blockers (Cs^+ and TEA). From -90 mV, depolarizations to -30 mV revealed a small (< 40 pA) inward current that inactivated rapidly (τ ~ 20 ms) and more completely. This current was preferentially blocked by amiloride and nickel. All these features are characteristic of T-type Ca^{2+} channels. A much larger, sustained inward current was evoked upon steps to 0 mV. The sustained current was partially blocked by nifedipine (L-type current, 15%), ω-conotoxin GVIA (N-type current, 45%) and ω-agatoxin IVA (P/Q-type current, 20%). A residual component of inward current (R-type) was blocked by Cd^{2+}.

In conclusion, our results suggest the presence of multiple pharmacological and molecular components of K^+ and Ca^{2+} channels in cultured embryonic rat motoneurones. These diversity is similar to observations made in postnatal rat motoneurones studied in slices and suggests an early specification of ion channel phenotypes in spinal rat motoneurones.

THE CONSERVED SAX-3/ROBO IMMUNOGLOBULIN SUPERFAMILY MEMBER DIRECTS AXON GUIDANCE AND CELL MIGRATION IN *C. ELEGANS*

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Mutations in the *sax-3* gene lead to widespread misrouting of axons throughout the *C. elegans* nervous system. *sax-3* encodes a predicted transmembrane protein with immunoglobulin-like domains and fibronectin type-3 repeats that is closely related to *Drosophila* Robo (Zallen *et al.*, 1998, Kidd *et al.*, 1998). In one model, SAX-3/Robo may function as a receptor in the growth cone for choosing among available substrates in the enviroment.

sax-3 mutants exhibit defects in the guidance of pioneer axons that travel independently, as well as axons that travel together in axon bundles. For example, *sax-3* mutants are defective in the guidance of many axons that travel in the nerve ring, the largest axon bundle in *C. elegans*. To determine where *sax-3* functions to guide nerve ring axons, we examined *sax-3* mosaic animals. Mosaic anlaysis demonstrates that SAX-3/Robo can function non-autonomously in the nerve ring. In *sax-3* mosaic animals, neighboring axons have similar phenotypes regardless of whether they express *sax-3*, suggesting that axon guidance in the nerve ring is directed by a subset of SAX-3-expressing cells. These results are consistent with SAX-3/Robo acting in pioneer neurons, or alternatively as a ligand for a nonautonomous cell interaction. Experiments are in progress to distinguish between these models.

In addition to axon guidance defects, *sax-3* mutants also exhibit defects in cell migration, suggesting that these distinct cell biological processes can utilize some of the same molecular components. In *sax-3* mutants, the CAN neuron fails to complete its posteriorly-directed cell migration. In contrast, overexpression of SAX-3 causes CAN to migrate past its normal destination. These results suggest an instructive role for SAX-3/Robo in guiding CAN migration. Similarly, SAX-3/Robo overexpression and loss of function can have reciprocal effects on axon trajectories.

The guidance information mediated by SAX-3/Robo acts in concert with other guidance systems in *C. elegans*. For example, some axons that are affected by mutations in *sax-3* are also similarly affected by mutations that disrupt signaling mediated by netrin or integrin pathways. We are currently investigating *in vivo* interactions between *sax-3* and other guidance genes, to understand how growing axons integrate guidance information from multiple sources to achieve their precise trajectories.

LIST OF INVITED SPEAKERS

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