Instituto Juan March
de Estudios e Investigaciones

65 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Signal Transduction in Neuronal Development and Recognition

Organized by

M. Barbacid and D. Pulido

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INTRODUCTION

M. Barbacid and D. Pulido

The development of the nervous system requires the proliferation of neuronal precursors, their migration to their respective locations, their differentiation into mature cells, and their survival since most nervous cells cannot be replaced. Moreover, the proper functioning of the nervous systems requires that neurons extend their axons according to a precise pattern so they reach their corresponding targets. Over the past several years, the molecular components responsible for neuronal survival and differentiation, as well as for axonal guidance are being defined with the discovery of new soluble or cell-attached factors, their cognate receptors, and their corresponding signal transduction pathways.

Neurons, the basic cellular components of the nervous system, control our autonomic, sensory and cognitive activities. Remarkably, neurons are only generated during early development. Since neurons are post mitotic cells, that is they cannot divide, our organism has to provide the means to maintain these cells alive and functional during our entire life span. Therefore, understanding the molecular mechanisms that control the generation of neurons from neuronal precursors during development as well as their long-term adulthood, is one of the major challenges in biomedical research today. Invariably, as our organism senesce, a certain population of neurons die and cannot be replaced. However under some pathological conditions such as Amyotrophic Lateral Sclerosis, Parkinson's and Alzheimer's disease, the process of neuronal degeneration becomes considerably accelerated resulting in significant loss of motor neurons and/or cognitive functions. Only when we can understand the cascade of events that led to premature neuronal cell death, we will be in a position to rationally prevent, or at least palliate, the pathological consequences of these neurodegenerative diseases.

During the last few years, we have witnessed several important developments in the field of signaling during neural development. For instance, several new neurotrophic factors have been discovered. Moreover, their receptors have been identified, thus making it possible to decipher the signal transduction pathways that mediate their neurotrophic properties. Mice defective in these neurotrophic molecules have also been generated by gene targeting approaches. These animals have provided the necessary tools to study the role of these molecules in neuronal differentiation and survival in vivo.

More recently, three families of proteins, the netrins, the Eph family of tyrosine protein kinase receptors and their cognate ligands (known as ephrins) have been implicated in axonal guidance and possibly in the establishment of topographic maps. The expression of ephrins and Eph receptors in complementary gradients during the development of the nervous systems suggests that these molecules may act as cues for axonal guidance. The generation of gene-targeted mice defective in some of these molecules should provide relevant information to determine the physiological role of these molecules in the development of the mammalian nervous system

The Juan March Workshop on "Signal Transduction in Neuronal Development and Recognition" held on April 21-23, 1997 provided a major opportunity to bring together an international group of prominent investigators working in these areas to survey and discuss the state of the art of signal transduction during neuronal development. Several topics were addressed at this meeting including neurotrophic factors and signal transduction, neuronal differentiation and survival and neural patterning during development. The second half of the meeting focused on a series of recently identified molecules involved in axonal guidance and recognition. Several talks were dedicated to the Eph receptors and their cognate ligands and their possible role as positional labels. The generation of mice lacking these molecules also revealed unexpected roles for these molecules during early development. The characterization of gene-targeted mice lacking netrins and their receptors illustrated the positive role that these novel class of molecules play in axonal guidance. Finally, the role of three other classes of molecules in axonal guidance processes, the semaphorins, certain tyrosine phosphatases and the Ras-like protein, Rac1, was illustrated by using elegant genetic experiments.

In summary, this Workshop provided the ideal forum to present the state of the art of two of the most exciting areas in neurobiology, the signaling mechanisms that control the growth and differentiation of neuronal cells and the identification of the molecular elements that guide axons to their physiological targets.

SESSION 1. NEUROTROPHIC FACTORS AND SIGNAL TRANSDUCTION

Chairman: David G. Wilkinson

Neurotrophin switching: Where does it stand?

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In vitro and *in vivo* studies suggest that certain populations of neurons switch their survival requirements from one neurotrophin to another during an early stage in their development. Although there is good evidence for neurotrophin switching in sensory neurons, the evidence for switching in sympathetic neurons is more controversial as is the identity of the factors that regulate responsiveness to particular neurotrophins.

The first clear evidence that certain populations of neurons switch their survival requirements from one set of neurotrophins to another during development came from *in vitro* studies of the embryonic mouse trigeminal ganglion neurons (Buchman and Davies, 1993). When these neurons are grown at low density at the stage when the earliest trigeminal axons are starting to grow to their peripheral targets, they do not survive longer than 24 hours unless BDNF or NT3 are present in the culture medium. Although NGF has a negligible effect on the survival of these early neurons, it supports an increasing proportion of the neurons in cultures set up at successively later stages. Concomitant with the acquisition of NGF survival dependence, responsiveness to BDNF and NT3 is rapidly lost in all neurons except a small subset. The number of dying neurons in the trigeminal ganglion of *trkB-/-* embryos (Pinon et al., 1996) and NT3-/- embryos (Wilkinson et al., 1996) is substantially increased during the early stages of the development when the neurons respond to BDNF and NT3 *in vitro*, whereas the increased neuronal death occurring in *trk*A-/- embryos peaks later in development (Pinon et al., 1996) at the stage when the neurons respond to NGF *in vitro*.

Unlike the onset of BDNF dependence, which is controlled by an intrinsic timing mechanism in early sensory neurons (Vogel and Davies, 1991, 1993), the switch to NGF dependence is triggered by signals that act on the neurons during the switchover period (Paul and Davies, 1995). The onset of neurotrophin dependence is associated with marked increases in the expression of the corresponding neurotrophin receptor tyrosine kinase (Ninkina et al., 1996; Wyatt and Davies, 1993), and the loss of BDNF dependence is associated with high levels of expression of non-catalytic trkB isoforms in neurons which act as negative suppressors of BDNF signalling (Ninkina et al., 1996). The dose responses of the neurons to BDNF and NT3 shift by several orders of magnitude to higher concentrations with age (Buj-Bello et al., 1994).

Accompanying the changing survival requirements of trigeminal neurons are matching changes in the expression of neurotrophins in their peripheral targets. BDNF and NT3 mRNAs are expressed in the peripheral trigeminal territory prior to the arrival of the earliest sensory axons (Buchman and Davies, 1993) and NGF mRNA and protein are expressed later with the arrival of sensory axons (Davies et al., 1987). The levels of BDNF and NT3 mRNAs are initially highest in the mesenchyme through which the axons grow to the periphery (Buchman and Davies, 1993; Wilkinson et al., 1996), whereas NGF mRNA is expressed predominantly in the target field epithelium (Davies et al., 1987).

Several *in vitro* and *in vivo* studies have led to the widely accepted view that NT3 is required for sympathetic neuroblast survival (Birren et al., 1993; Dechant et al., 1993; DiCicco-Bloom et al., 1993; ElShamy et al., 1996), induction of TrkA expression and the acquisition of NGF dependence (Verdi and Anderson, 1994; Verdi et al., 1996). It has also been proposed that depolarisation induces TrkA expression in sympathetic neuroblasts (Birren et al., 1992). However, we have previously shown that TrkA expression occurs normally in cultured sympathetic neuroblasts grown in defined medium without added NT3 or depolarising levels of KCl (Wyatt and Davies, 1995). In our most recent studies we have studied the development of the superior cervical sympathetic ganglion (SCG) of $NT3^{-/-}$ mouse embryos in detail to determine if NT3 plays any role in the early survival of sympathetic neuroblasts *in vivo*. We show that the number of neurons and the levels of trkA and p75 mRNAs in the superior cervical sympathetic ganglion (SCG) of $NT3^{-/-}$ mouse embryos increase normally up to E16, two days after SCG neurons start responding to NGF *in vitro*. At E18 and in the postnatal period, there are significant reductions in the number of SCG neurons and in the levels of trkA and p75 mRNAs. These results show that the neurotrophin survival requirements of SCG neurons do not switch from NT3 to NGF during development and that NT3 is not required for the expression of TrkA and p75 and the acquisition of NGF dependence. Rather, some sympathetic neurons have a late requirement for NT3 at the time when they also depend on NGF for survival. The expression of transcripts encoding catalytic TrkC is negligible at this stage, suggesting that NT3 acts mainly via TrkA. The demonstration that there are no statistically significant differences between the number of neurons in the SCG of $trkC^{-/-}$ and wild type mice throughout development (Fagan et al., 1996) likewise indicates that TrkC receptors are not required for sympathetic neuron survival.

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Neurotrophins: 2 receptors, 2 functions

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During the development of the vertebrate nervous system, many cells are eliminated by programmed cell death (PCD) When neurons contact their target cells, net cell numbers decrease in a variety of structures, and there is evidence to suggest that neuronal numbers are controlled by the limited availability of target-derived, secretory molecules belonging to the neurotrophin gene family. Four members have been identified in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5. All neurotrophins can prevent PCD by binding to and activating one of the 3 tyrosine kinase receptors of the *trk*-family, by mechanisms that remain to be worked out in detail[1].

All neurotrophins also bind to the neurotrophin receptor p75 ($p75^{NTR}$), a member of a gene family of non-catalytic receptors expressed in many cells and comprising more than 10 members, including both 2 TNF receptors and FAS. Recently[2], it has become apparent that NGF can mediate NF-κB activation in cultured Schwann cells following binding to $p75^{NTR}$.These cells do not express catalytic form of any of the *trk* receptors, and activation of NF-κB was only seen with NGF, and not with BDNF or NT-3. Furthermore in vivo, NGF induces PCD during normal development in CNS cells expressing $p75^{NTR}$ but not trkA[3].

NGF then controls cell numbers in opposite ways by interacting either with its tyrosine kinase receptor or with $p75^{NTR}$. Which cells deliver NGF to kill other cells and what are the mechanisms explaining the apparently unique ability of NGF to activate $p75^{NTR}$ are amongst the intriguing questions raised by these results.

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THE INSULIN/IGFs SYSTEM IN RETINAL NEUROGENESIS

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Insulin and the insulin-like growth factors I and II (IGFs) bind to and signal through two membrane tyrosine kinase receptors, the insulin receptor and the IGF-I receptor. Upon activation and autophosphorylation, these receptors phosphorylate endogenous substrates, such as IRS-1 and IRS-2, and engage in multiple signal transduction pathways. The cellular effects induced by these factors depend on the developmental stage of the target cell and can include stimulation of proliferation and differentiation, attenuation of apoptosis and metabolic regulation.

We have proposed that insulin (and/or its precursor proinsulin) and IGF-I have multiple, perhaps complementary, roles in early development and neurogenesis. The chick neuroretina provides a good system to analyze the expression of these factors, their receptors and cellular effects both in vivo and in organoculture during neurogenesis.

In the E4-E6 neuroretina, largely proliferative, there is predominant expression of preproinsulin mRNA, while preproIGF-I expression is lower and highly localized in the cilliary processes. In the E9-E15 neuroretina, largely differentiative, there is predominant expression of preproIGF-I mRNA. At stages of synaptic maturation in E17-E20, there is a second wave of preproproinsulin mRNA expression in neuroretina. Interestingly, between E6 and E15 the neuroretina lacks PC2, a necessary enzyme to convert proinsulin in insulin, while immunoactivity with the HPLC mobility of proinsulin is accumulated in the vitreous at higher concentration than IGF-I.

Characteristically, mRNA expression for both the insulin receptor and the IGF-I receptor is widespread in the developing retina and shows little temporal regulation. However, there is high regulation of receptors at the protein level. In E6 retina there are two types of receptors of the family. One type with high affinity and specificities typical of an IGF-I receptor, and an atypical high affinity -

low discriminating receptor with near equal affinity for insulin and IGF-I. This atypical receptor is not present in E12 neuroretina, where a population of more classic insulin receptors coexist with IGF-I receptors. The receptors in E6 are highly phosphorylated in the basal state although they respond to high concentrations of ligands with further autophosphorylation. The molecular basis of the atypical characteristis of E6 receptors are still under study.

The presence of this atypical receptor correlates with the observed biological actions of the factors. In organoculture of E5-E6 neuroretina insulin and IGF-I estimulate with near equal potency DNA and protein synthesis, as well as neuronal differentiation. Proinsulin is slightly less potent. Underlaying these effects on proliferation and differentiation may be a cell survival action of the peptides. Insulin indeed decreases the percent of apoptotic cells caused by deprivation of factors when the retina is placed in organotypic culture in defined medium. Studies on the cell cycle reveal that the major effect of insulin is an apparent shortening of the cell-cycle duration. However, this is concomitant with a decrease in the number of apoptotic cells, that may justify this apparent change in cell cycle period. Approaches using antisense oligonucleotides and ICE-protease inhibitors should clarify the receptors and mechanisms involved in these cellular processes in early neurogenesis.

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TRKA SIGNALLING IN CELL SURVIVAL.

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We have established a cell system to dissect the anti-apoptotic activity and pathways used by the TrkA/NGF receptor. In this model, Rat-1 fibroblasts express a conditional Myc-ER fusion protein which can be activated by the addition of oestradiol or 4-hydroxytamoxifen, leading to apoptosis in the absence of serum or survival factors. We have generated stable cell lines co-expressing wild type TrkA/NGF receptor and the Myc-MER fusion protein. Nanomolar concentrations of NGF can block Myc-induced apoptosis as efficiently as IGF-1, the classical survival factor in this system. This ability correlates with the level of TrkA expression and requires its kinase activity, since mutant TrkA receptors devoid of this activity fail to block Myc-induced apoptosis. Ligand activation of TrkA also blocks X-ray and UV-induced cell death in these cells.

NGF-stimulated wild type TrkA receptors induce the activation of several signalling pathways in this model system, including the PI-3Kinase-AKT-S6Kinase, and the Ras-MAPK pathways, and activate PLCγ. The role of each of these pathways in mediating anti-apoptotic activity has been analysed by the use of specific enzyme inhibitors, as well as by mutation of TrkA aminoacid residues responsible for the binding and/or activation of specific signalling molecules. Rat-1-Myc-MER-derived cell lines expressing TrkA mutant receptors have been established and analysed for their ability to block Myc, X-ray and UV-induced apoptosis upon NGF treatment. The results of these studies will be presented and discussed in the light of current hypothesis on the survival pathways proposed for NGF/TrkA in other cell types.

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SESSION 2. NEURONAL DIFFERENTIATION AND SURVIVAL

Chairman: Martin Raff

ROLE OF MAMMALIAN POU DOMAIN TRANSCRIPTION FACTORS IN NEURAL DEVELOPMENT

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The POU domain family of transcription factors are characterized by a bipartite DNA binding domain containing the POU-specific domain, a highly conserved region of approximately 75 amino acids, tethered by a variable linker region to the POU-homeodomain, another 60 amino acid region of shared homology, related to the Drosophila homeodomain. Examination of the POU domain gene family provides an opportunity to explore the combination of shared features, functions and diversity of actions that are assumed by different members within a family of transcription factors. Following the initial discovery of the pituitary-specific Pit-1, the ubiquitously expressed, and B cell specific octamer binding proteins Oct-1 and Oct-2, and the C. Elegans unc-86 proteins, several new members in different species have been cloned, and grouped in six classes based on the amino acid sequence of their POU domains and conservation of the variable linker region (reviewed in Wegner et al., 1993; Ryan et al., 1996).

Based on their expression pattern in the developing and mature nervous system, the mammalian Class III factors Brn-1, Brn-2, Tst-1/Oct-6/SCIP and Class IV POU domain factors Brn-3.0 (Brn-3a), Brn3.1 (Brn-3c) and Brn3.2 (Brn-3b) have been hypothesized to play key roles in neural development. We and others have now generated mice harboring disruption of the genomic loci of Brn-2, Tst-1, Brn-3.0, Brn-3.1 and Brn-3.2, each exhibits a distinct phenotype in the nervous system, despite extensive temporal and spatial overlap in the expression patterns of genes within a class. Deletion of Brn-2 gene has a profound effect on the endocrine hypothalamus, where the paraventricular and supraoptic nuclei are markedly hypocellular, and do not produce corticotropin releasing hormone, oxytocin and vasopressin (Nakai et al., 1995; Schonemann et al., 1995). In Tst-1 null mice, peripheral myelination is affected, probably due to the disturbance of terminal differentiation of Schwan cells; only a small percentage of which are able to wrap the axons, despite an initial ensheatement of axons in a 1:1 ratio, that appears normal (Bermingham et al., 1996; Jaegle et al., 1996).

Disruption of Class IV POU domain transcription factors interferes with normal development of sensory and motor neurons, in accordance with the unique features of their spatial and temporal expression patterns. Brn-3.0 deletion causes defects in the brainstem nuclei resulting either from neuronal mismigration (inferior olive and compact formation of the nucleus ambiguus) or neuronal loss (red nucleus). In the peripheral nervous system, a severe cellular loss is observed in the trigeminal and upper cervical dorsal root ganglia accompanied by significant decreases in the expression of several neurotrophins and their receptors (McEvilly et al., 1996; Xiang et al., 1996). In contrast to Brn-3.0 mutant mice that die shortly after birth, Brn-3.1 and Brn-3.2 null mice survive to adulthood and appear healthy, with no noticeable behavioral defects in Brn-3.2 mutants. Brn-3.1 null mice however, develop severe balance problems, and later a hyperactive behavior characteristic of inner ear defects. Indeed, cochlear and vestibular hair cells fail to differentiate properly in Brn-3.1 mutant mice, causing secondary loss of the sensory ganglia and deafness (Erkman et al., 1996). The defect observed in mice harboring a targeted disruption of Brn-3.2 locus, is the loss of approximately 70% of retinal ganglion cells (Gan et al., 1996; Erkman et al., 1996). In Brn-3.2 (-/-) mice, these cells display abnormalities before cellular loss occurs, since Brn-3.0 expression is not observed at its normal time of onset, suggesting that they are not engaged in the correct differentiation program.

Despite their broad expression pattern early during neurogenesis, phenotypic manifestations of deletion of mammalian neural specific POU domain factors relates to relatively late events such as migration, neuronal survival, and terminal differentiation. Detailed analysis however, indicates that altered patterns of gene expression often precede the obvious morphological changes, suggesting, in many instances, that the POU domain transcription factors are indispensable from the onset of their expression. The lack of a discernible phenotype in many regions of the nervous system where these POU domain proteins are expressed, indicates that other members of the family can fulfill compesatory effects, and the study of combinatorial knockouts may reveal new roles during early embryogenesis.

Detailed analysis of the POU domain factor Pit-1 has provided insights into the molecular mechanisms of its initial activation and subsequent autoregulation, a characteristic of many POU domain factors. Genetic approaches have defined a paired-like homeodomain as a requirement for the asymmetrical cell division that generates the Pit-1 lineage. Both restricting and activating mechanisms dictate the cell-type specific expression of Pit-1 distal target genes in the distinct cell phenotypes that are regulated by Pit-1 (Sornson et al., 1996; DiMattia et al., 1997).

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Role of Eph-related receptor tyrosine kinases and their ligands in neural patterning

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Eph-related receptor tyrosine kinases and their ligands have been implicated in cell contact dependent interactions and are expressed in complex patterns during early vertebrate development. We have identified and analysed the function of members of these receptor and ligand families in the segmental patterning of the hindbrain and branchial neural crest. The Sek-1 receptor is expressed in rhombomeres r3 and r5 in the hindbrain (1,2), and we have shown that inhibition of receptor activation leads to the presence of cells with r3/r5 identity in r2/r4/r5 (3). Since ligands for this receptor - ELF2/Htk-L and Elk-L3 - are expressed in the complementary r2/r4/r6 (4,5), these data suggest that receptor-ligand interactions mediate a repulsion that restricts cell movement across rhombomere boundaries. In addition, we have shown that Sek-1 expression in r3/r5 is regulated by the transcription factor Krox-20, which provides a link between the processes of segmentation, segment identity and segmental restriction of cell movement. A further site of Sek-1 expression is in neural crest cells that migrate from r5 to the third branchial arch. This targeted migration is believed to be critical for craniofacial development, since before migration neural crest cells acquire a rostrocaudal identity that underlies their pattern of differentiation into cartilage. We find that inhibition of Sek-1 function in the Xenopus embryo leads to disruption of the targeted migration of third arch neural crest, with cells migrating rostral or caudal to their normal route (6). In addition, we find that ELF2/Htk-L is expressed in second arch neural crest, and that overexpression of this ligand also disrupts third arch crest migration. These data suggest that the complementary expression of Sek-1 and ELF2/Htk-L mediates repulsive interactions that guide neural crest cells to the correct target. Since in the retinotectal system, the complementary expression of members of these receptor and ligand families are involved in axon guidance by repulsive interactions, this indicates that similar mechanisms are involved in the pathfinding of neural crest cells and axons. In order to dissect the mechanism of cellular responses to receptor activation, it is essential to identify amino acid residues in the intracellular domain that mediate signal transduction. We will present the results of mapping residues required for Sek-1 function during neural patterning.

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Neuronal survival and axonal pathfinding defects in Trk and Eph receptor mutant mice

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The Trk family of RTKs (TrkA/B/C) are receptors for neurotrophins, a family of proteins that are important physiological regulators of the survival of specific peripheral neurons. Mice deficient in Trk RTKs demonstrate that essentially all sensory neurons require, in a modality-specific way, a single Trk receptor for survival. In the CNS, removal of a single Trk receptor does not result in major cell loss. Here, TrkB and TrkC receptors have redundant functions in promoting the survival of specific neurons, including hippocampal and cerebellar granule cells[1]. To study the downstream events triggered by activated TrkB receptors, the juxtamembrane docking site for the Shc adapter protein was mutated in the *trkB* gene. Mutant mice carrying this $trkB^{Shc}$ allele show severe balancing defects due to loss of vestibular neurons. These results indicate that coupling of Shc to the TrkB receptor is an essential pathway for the survival of at least a subset of TrkB-dependent sensory neurons.

The Eph family of RTKs and their ligands, the ephrins, have been implicated in cell movement and axonal pathfinding. To address the function of Eph receptors during mouse development, we generated mice deficient in EphB2 (Nuk) (collaboration with T. Pawson's lab) and EphB3 (Sek4) receptors. We show that the formation of the two major commissural axon tracts, corpus callosum and anterior commissure, that connect the two cerebral hemispheres, is critically dependent on B2 and B3 receptors[2,3]. Moreover, while mice singly deficient in either B2 or B3 are viable, most *ephB2/B3* double mutants die immediately after birth primarily due to a cleft palate. These results demonstrate essential and co-operative functions for B2 and B3 in establishing axon pathways in the developing brain and during the development of facial structures. Genetic evidence from a second *ephB2* allele (*nuk-lacZ*) suggests that transmembrane ligands may transduce signals in the developing embryo. We show that the cytoplasmic domain of the transmembrane (TM) ligand ephrin-B1 (Lerk2) becomes phosphorylated on tyrosine residues after contact with the EphB2 receptor ectodomain, suggesting that ephrin-B1 has receptor-like intrinsic signaling potential. Moreover, ephrin-B1 is an in vivo substrate for the platelet-derived growth factor (PDGF) receptor, suggesting crosstalk between ephrin-B1 signaling and signaling cascades activated by tyrosine kinases[4]. We propose that TM ligands of Eph receptors act not only as conventional RTK ligands, but also as receptor-like signaling molecules.

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NEUROTROPHINS AND THEIR RECEPTORS AS NEURAL ORGANIZERS IN HEARING AND BALANCE DURING DEVELOPMENT.

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The cochleo-vestibular nerve (cranial nerve VIII) primarily contains afferent fibres whose targets are the sensory epithelia of membranous labyrinth in the inner ear. Cochlear fibres commence in the organ of Corti, and vestibular fibres originate in the utricular and saccular maculae and the ampullary cristae of the semicircular canals. The somata of these afferent fibres is found in the cochlear (CG) and vestibular ganglia (VG). Most of the cochlear and vestibular neurons are bipolar. A peripheral process is contacting the hair cells in their respective sensory epithelia and a central process that projects in a topographical fashion to the cochlear and vestibular nuclei of the medulla. The inner ear also receives some efferent fibres from the centrally located efferent neurons that also use the cochleo-vestibular nerve to innervate the sensory epithelia (Spoendlin, 1988). Embryonic development of the vertebrate inner ear starts with the formation of the otic placode and then the otic vesicle. The otic vesicle subsequently grows and differentiates into the membranous labyrinth, which contains the cochlear (hearing) and vestibular (balance) sensory epithelia and the highly specific networks of afferent and efferent neuronal connections. In the developing CG and VG, neuronal death affects approximately to 20-30 % of the cellular population and that fits the pool of CG and VG neurones to the peripheral and central target fields (Aniko et al. 1983; Ard & Morest, 1984). On the other hand, distinct evidences suggest that cochlear and vestibular neurons require growth factors released from their natural targets to survive and differentiate (Ard et al., 1985).

As in other sensory systems, the Nerve Growth Factor (NGF) family of neurotrophins emerges as a candidate likely to be involved in guiding or maintaining otic innervation (Davies, 1986, 1994) . The NGF-family of factors includes the NGF itself, the Brain- Derived Neurotrophic Factor (BDNF) and Neurotrophins 3, 4 and 5 (NT3, NT4 and NT5), which are structurally homologous polypeptides sharing about 60% of aminoacid identity and membrane receptors, and biological effects (Hallbook et al., 1991; Berkemeier et al., 1991). The Trk family of protein tyrosine kinase receptors mediate the biological effects of the nerve growth factor (NGF) family of neurotrophins (Meakin and Shooter, 1992). The *trkA* and *trkC* gene encode high-affinity receptors for NGF (Kaplan et al., 1991; Klein et al., 1991a) and neurotrophin-3 (NT-3) (Lamballe et al., 1991), respectively. The product of the *trkB* gene serves as the receptor for two related neurotrophins, brain derived neurotrophic factor (BDNF) (Klein et al., 1991b) and neurotrophin 4 (NT-4) (Berkemeier et al., 1991; Klein et al., 1992) TrkB also binds NT-3, although the extent to which this occurs in vivo is controversial (Bothwell, M.,1995). Besides the Trks, all neurotrophins bind to another cell surface receptor, known as p75 or the low affinity NGF receptor (LNGFR) whose role may be to modulate Trk signaling (Meakin and Shooter, 1992,Davies et al., 1994; Hantzopoulos et al., 1994) .Over the past few years, in vitro studies, binding and in situ hybridization analysis and targeted gene disruptions have revealed that two neurotrophins (BDNF, NT-3) and two neurotrophin receptors (trkB, trkC) are essential to regulate the development of inner ear afferent innervation. Current research evidences that these neurotrophic factors are also involved in the trophic support of efferent innervation.

1) Neurotrophic factors and their receptors follow specific patterns of expression in the developing ear. Cranial sensory neurons have been especially useful for studying the specificity and time-course of the survival-promoting effects of neurotrophins.TrkB, TrkC and LNGFR are expressed in the cochlear and vestibular ganglion before and during innervation of their target fields, whereas TrkA is expressed earlier and at a low level (Emfors et al., 1992; Pirvola et al., 1994; Schecterson and Bothwell, 1994; Vazquez et al., 1994). Further Trk analysis revealed a apparent preference for different

neuronal types such as TrkB for vestibular and TrkC/TrkA for cochlear. On the other hand, BDNF, NT-3 and NGF but not NT-4 is detected in the sensory epithelia (Pirvola et al., 1992, 1994; Schecterson and Bothwell, 1994, Bernd et al., 1994). BDNF and NT-3 show comparable temporal, but a different spatial pattern of expression. BDNF is found in the hair cells of all inner ear sensory epithelia (except in the semicircular canals) through development and continues to be expressed in decreased amounts during neonatal life and adulthood. While initially expressed in cochlear and vestibular hair cells, NT-3 becomes progressively restricted to cochlear inner hair cells.

2) BDNF and NT-3 show specific biological effects on cochlear and vestibular neurons. In explant cultures, exogenous BDNF and NT-4 promote neurite outgrowth from the cochlear and vestibular ganglion neurons, while NT-3 is mainly effective on cochlear neurons (Avila et al., 1993; Pirvola et al., 1994; Vazquez et al., 1994). In dissociated neuron cultures, BDNF and NT-3 promote survival of both cochlear and vestibular neurons (Davies et al., 1986; Avila et al., 1993; Pirvola et al., 1994, Vazquez et al., 1994). Results on the effectiveness of NGF in bioessays are controversial (Lefebvre et al., 1990, 1991; Pirvola et al., 1992, 1994). Based on the spatiotemporal expression patterns and the biological effects, BDNF and NT-3, rather than NGF and NT-4, are responsible for the survival and neurite outgrowth of cochlear and vestibular neurons, acting via their high-affinity receptors TrkB and TrkC, respectively.

3) The phenotypes of germline-targeted mutant mice lacking functional neurotrophins and their receptors define the specific neuron populations that are dependent on neurotrophin action during development. Consistent with the NT-3 expression pattern and in vitro experiments, NT-3 (-/-) mutant mice show reductions in the cochlear and vestibular ganglion (Fariñas et al., 1994). BDNF (-/-) mutant mice have severe deficiencies in coordination and balance, suggesting abnormalities in functions of the vestibular system (Ernfors et al., 1994a, Jones et al., 1994). Vestibular ganglia are drastically depleted of neurons and the vestibular fibres fail to innervate the sensory epithelia in postnatal animals. With the known expression pattern of BDNF and its effects in vitro systems, the results suggest that most of the vestibular ganglion cells require BDNF for survival. In contrast, the cochlear ganglion, the cochlea and the innervation of inner and outer hair cells seem unaffected in BDNF (-/-) mice (Ernfors et al., 1994a). The development of the vestibular and auditory system in mice mutant for the TrkB and TrkC receptors (Klein et al., 1993) has been analyzed. Most *trkB* (-/-) homozygous mutants die at postnatal day 1 (P1) with neuronal deficiencies in the central (facial motor nucleus and spinal cord) and peripheral nervous system. The results reveal further abnormalities in the part of the peripheral nervous system that is responsible for the innervation of the inner ear. Vestibular nerve fibres and nerve fibres contacting the outer hair cells of the cochlea initially reach their targets, but fail to maintain the innervation with their respective sensory epithelia. TrkB receptors therefore seem to be dispensable for establishing innervation of ganglion cells with their peripheral targets in *trkB* mutants, but are essential for the maintenance of functional nerve terminals in the sensory epithelia. Mice carrying an inactive form of *trkC* have been also analyzed for inner ear defects (Klein et al., 1994b, Schimmang et al., 1995), showing a major loss (<50%) of cochlear neurons but minor losses in vestibular neurons (Bianchi et al., 1996). Cochlear innervation pattern in this *trkC* mutant showed a complete loss of innervation in inner hair cells.

Data obtained in double *trkB/trkC* null-mutant mice proved that the absence of TrkB and TrkC leads to a further reduction of vestibular and cochlear neurons and a complete loss of all afferent and efferent innervation within the inner ear sensory epithelia (Minichiello et al.,1995).

4) Potential uses of neurotrophins as therapeutic agents for hearing disorders. Recently it was shown that neurotrophins are capable to reverse an experimental neuropathy of the cochleovestibular nerve induced by ototoxins, proving a neuroprotective action of BDNF and NT-3 (Zheng & Gao 1996).

As it is apparent from the above revised data, neurotrophins play a critical role in embryonic organization and stabilization of inner ear innervation during development. Consistently in the adult cochlear and vestibular ganglia they may also play a role in maintaining neurons. Finally neurotrophins are candidate factors as therapeutic agents for hearing disorders.

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Mammalian Numb homologues and their roles in mouse asymmetric cell divisions and cortical neurogenesis

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During mammalian cortical neurogenesis, a cortical progenitor cell may divide asymmetrically to generate two distinct daughter cells, a post-mitotic neuron and another progenitor cell. During asymmetric divisions of *Drosophila* neural precursor cells, differential segregation of the cell-intrinsic Numb protein (d-Numb) is necessary for their daughters to adopt distinct fates. d-Numb functions by biasing the cell-cell interaction mediated by Notch, a transmembrane receptor, possibly via direct physical interaction with the intracellular domain of Notch. To uncover molecular mechanisms that allow daughters of cortical progenitor cells to adopt distinct fates, we isolated the mammalian homologues of *d-numb*, mouse *numb* (*m-numb*) and *numblike* (*nbl*).

m-Numb and Nbl are both highly homologous to d-Numb, especially in the phosphotyrosine binding (PTB) domain. However, when expressed in *Drosophila*, only m-Numb is localized asymmetrically to one half of the cell membrane in dividing neural precursors and segregated primarily to one daughter cell, just like d-Numb. Nbl is symmetrically distributed in the cytoplasm and segregated to both daughter cells. Consequently, in *d-numb* loss of function mutant embryos, expression of m-Numb allows neural precursor cells to divide asymmetrically, whereas expression of Nbl allows daughter cells of a neural precursor to both adopt the fate of the cell that normally inherits d-Numb.

In developing mouse neocortex, Nbl is only expressed in postmitotic neurons in the cortical plate, whereas m-Numb and Notch1, a mouse homologue of Notch, are also expressed in progenitors within the ventricular zone. In dividing cortical progenitors at the ventricular surface, m-Numb is asymmetrically localized to the apical membrane while Notch1 is distributed around the entire membrane. As a result, while Notch1 is always segregated to both daughter cells, m-Numb may be differentially segregated to only one daughter cell. Furthermore, m-Numb and Nbl can both physically interact with the Notch1 intracellular domain.

We propose that an evolutionarily conserved interplay between cell-intrinsic mechanisms (executed by *m-numb* and *numblike*) and cell-extrinsic mechanisms (mediated by *Notch1*) may be involved in both progenitor cell proliferation and neuronal differentiation during mammalian cortical neurogenesis. We have generated *m-numb* and *nbl* mutant mice through gene targeting and are currently analyzing their phenotypes in asymmetric cell divisions and neurogenesis.

SESSION 3. NEURONAL DEVELOPMENT

Chairman: Yves-Alain Barde

CONTROLS ON CELL NUMBERS DURING NEURAL DEVELOPMENT

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The size of an organism or of an organ such as the brain largely depends on the number of cells it contains(1). How is the number of cells determined? We have been addressing this problem in one of the simplest parts of the CNS, the optic nerve, and have focused on how the final number of oligodendrocytes in the rodent optic nerve is determined(2).

Oligodendrocytes are nondividing cells that develop from dividing precursor cells that migrate into the optic nerve early in development. Their final number depends on how many precursor cells migrate in, how many times each precursor cell divides before it stops and differentiates, and how much cell death occurs normally in this cell lineage. We still do not know how many precursor cells migrate into the developing nerve, but we have learned something about how their proliferation and survival are controlled.

At least 50% of the oligodendrocytes produced each day in the developing optic nerve undergo programmed cell death, apparently in a competition for limiting amounts of survival factors provided by axons (3): if the axons are made to degenerate, most of the oligodendrocytes die(4); if the number of axons is experimentally increased, few oligodendrocytes die and their numbers automatically increase to match the number of axons(5).

The proliferation of oligodendrocyte precursor cells is stimulated mainly by PDGF. But even in saturating amounts of PDGF, the precursor cells divide a limited number of times before they stop and differentiate(6). This limitation reflects the operation in the precursor cell of an intrinsic "clock"(7,8), which consists of at least two components: (1) a counting mechanism that measures times and (2) an effector mechanism that stops the cell cycle when time is reached(9). I shall present evidence that the effector mechanism depends on thyroid hormone(9) and that an accumulation of the cyclin-dependent kinase inhibitor p27 may be part of both the counting and effector mechanisms(10,11).

It seems very likely that the mechanisms that control the numbers of neurons in the nervous system will prove to be very similar to those that control the number of oligodendrocytes in the optic nerve.

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COUPLING SIGNALLING TO TRANSCRIPTION: CREB AND CREM AS TARGETS OF THE NGF-RAS-RSK2 PATHWAY

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The events occuring in the nucleus in response to activation of intracellular pathways determine, through the modulation of gene expression, whether the cell will differentiate, proliferate or die. Thus, a fundamental stride has been the discovery that many transcription factors constitute final targets of specific transduction pathways. Many distinct kinases have been shown to directly or indirectly modulate the activity of various nuclear factors. For example, activity of transcription factor AP-1 maybe increased by inducing c-*fos* gene transcription, a process mediated by the ERK-1 and -2 mitogen-activated protein (MAP) kinases, which directly phosphorylate the transcription factor Elk-1/TCF, which then binds to the c-*fos* serum response element. Alternatively, AP-1 activity maybe be enhanced by direct phosphorylation of Jun by a different type of MAPKs, the stress-activated protein kinases (JNK/SAPK). Transcription factor ATF-2, a dimerization partner of Jun, is also a target of the JNK kinase. Interestingly, ATF-2 was first cloned as member of the ATF/CREB family of transcription factors and was shown to bind to cAMP-responsive elements (CREs). The ATF/CREB family includes several members, of which only the CREB, CREM and ATF-1 gene products have been shown to be directly phosphoryalted by the cAMP-dependent protein kinase A. Cross-talk between the mitogenic signalling pathways and the cAMP-responsive transcription has been established, which reinforces the notion of converging signalling within the PKA and PKC pathways in the cytoplasm and in the nucleus.

An interesting example of signalling cross-talk in the nucleus involves the pathway coupled to the Nerve Growth Factor (NGF) receptor, Trk, which results in the activation of several kinases. Trk is a receptor tyrosine kinase which, once activated, stimulates the activity of the small GTP-binding protein Ras. Activation of Ras triggers the MAPK pathway, which includes the MAP kinase kinase (MEK) and the ribosomal S6 kinase pp90rsk. Interestingly, constitutively activated expression of MAPK and MEK is sufficient to induce neurite outgrowth in PC12 cells indicating a direct role of this pathway in eliciting the changes in gene expression required for the neuronal differentiation program. Although MAPK and MEK have not been shown to directly phosphorylate CREB, the use of cells expressing a dominant-interfering Ras mutant has revealed the involvement of this pathway for CREB phosphorylation upon NGF-induction. Indeed, it has been proposed the involvement of a CREB-kinase which could have characteristics similar to pp90rsk. Interestingly, pp90rsk is likely to be responsible for CREB phosphorylation in human melanocytes, while the other member of the RSK family, p70s6k, also possesses CREB phosphorylation activity. Thus, two different signalling pathways may converge to modulate gene expression via the same transcriptional regulator, CREB.

The CREM gene, because of its modular structure, also generates antagonists of cAMP-induced transcription. In particular, an alternative promoter which lies within an intron near the 3' end of the CREM gene, directs the transcription of a truncated product, termed ICER (Inducible cAMP Early Repressor). The ICER open reading frame corresponds to the C-terminal segment of the gene and generates a protein that, compared with the previously described CREM isoforms consists of only the bZip DNA binding-dimerization domain. The bZip domain directs specific ICER binding to a consensus CRE element. ICER is able to heterodimerize with CREB and thus to function as a dominant repressor of cAMP-induced transcription.

The expression of ICER was first described in the pineal gland where it is the subject of a dramatic circadian pattern of expression. Additional evidence implicates dynamic ICER expression as a general feature of neuroendocrine systems. The key feature of ICER is its inducibility. This makes ICER the only CRE-binding protein

whose function is physiologically regulated by alteration in its cellular concentration.

ICER inducibility is achieved via the unique organization of its regulatory region. In contrast to the CREM P1 promoter which generates all the other CREM isoforms, and which is GC-rich and not inducible, the intronic P2 promoter has a normal A-T and G-C content and is strongly inducible by cAMP. It contains two pairs of closely-spaced CRE elements organized in tandem. In all analyses conducted to date, it has been shown that ICER expression is not inducible by phorbol esters, dexamethasone and growth factors. The restriction of ICER inducibility to the cAMP-dependent signalling pathway had suggested a well-defined, non-promiscuous function of this powerful repressor.

We have found that ICER is inducible in PC12 pheochromocytoma cells upon a differentiating treatment with NGF. This result shows that CREM might have a key function in nuclear cross-talk mechanisms of signal transduction. Indeed, we show that a CRE-binding repressor is activated by a cAMP-independent pathway. Importantly, we show that induction of ICER expression by NGF occurs via promoter elements which have been shown to direct cAMP-inducibility. This is achieved via the NGF-mediated phosphorylation of CREB. As for CREB phosphorylation, also ICER inducibility requires an intact Ras-dependent signalling pathway. Physiologically, an important aspect of this work consists in the activation of a powerful repressor of the cAMP-responsive pathway by NGF, whose transduction signalling is cAMP-independent.

The CREB-kinase responsive to NGF and EGF is RSK2. Three proteins constitute the p90rsk activity, RSK1, RSK2 and RSK3. In collaboration with A. Hanauer and J. L. Mandel we have found that the X-linked Coffin-Lowry syndrome (CLS) is due to a defect in the RSK2 kinase. Cells derived from CLS patients have non-functional RSK2 and show no phosphorylation of CREB in response to EGF. In these cells EGR responsiveness of the c-*fos* gene is impaired.

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GENETIC DISSECTION OF SIGNALING PATHWAYS IN DROSOPHILA

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Cell fate specification in the developing eye of *Drosophila* is dependent on inductive signals that are mediated by receptor tyrosine kinases such as Sevenless (Sev). Screens for mutations that dominantly enhance or suppress signaling from the Sev receptor led to the identification of mutations in genes coding for components of the conserved Ras/MAP kinase pathway. *daughter of sevenless* (*dos*) encodes an adaptor protein that provides binding sites for multiple SH2-containing proteins including Drk, the SHP-2 homologue Csw, and PI-3 kinase (1). Dominant negative mutations in the gene coding for the heat shock protein 83 (Hsp83) were identified as suppressors of activated Raf (2,3). This provided evidence that the interaction between the Raf kinase and Hsp83/90 is essential for Raf function. While the Ras/MAP kinase pathway is used at multiple steps during development, genetic analysis of the parallel Jun kinase (JNK) also called Stress-Activated Protein (SAP) kinase pathway suggests a much more restricted requirement during development. Mutations in *basket* (*bsk*) encoding DJNK and *hemipterous* (*hep*) encoding DJNKK specifically block dorsal closure during embryogenesis but do not interfere with proliferation or cell fate specification in the imaginal discs (4,5). Dorsal closure is a process by which lateral epithelial cell sheaths stretch to fuse at the dorsal midline of the embryo. We show that activation of the DJNK pathway results in the activation of *dpp* expression, which encodes a TGF-β homologue, in the dorsal row of cells. The local activation of *dpp* is controlled by two antagonistic transcription factors, DJun and the ETS repressor Aop/Yan. Like DJNK DJun is not required for cell proliferation or cell fate specification in the developing eye. These results indicate that the JNK pathway is conserved in *Drosophila* and that it plays a restricted role during development.

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In Vitro Analysis of Axon Guidance and Axon Targeting in the Vertebrate CNS

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A nervous system is composed of a network of synaptic connections among excitable cells. This network comes into being during development as axons extend from presynaptic neurons and grow often long distances to reach their correct postsynaptic partners. A growth cone at the tip of an extending axon is guided along a specific trajectory by cues present in its immediate environment. Using these cues, a growth cone is guided to a target region, where it has the task of identifying correct targets and initiating synaptogenesis with them. We employ an *in vitro* approach to the identification and characterization of the molecules mediating both axon guidance and target recognition in the developing vertebrate central nervous system.

Analysis of growth cone guidance in vitro

Chemoattractants were first proposed as axon guidance cues over one hundred years ago by Ramón y Cajal. We have used an *in vitro* system to identify the first known endogenous chemoattractants for developing axons, the netrins. Using the outgrowth of commissural axons from explants of embryonic spinal cord as an assay, we have purified to apparent homogeneity from embryonic chick brain two outgrowth-promoting proteins, named "netrin-1" and "netrin-2" (1). The netrins are chemoattractants *in vitro* for commissural axons that project circumferentially to the ventral midline of the spinal cord during development (2). We were also able to identify another component, Netrin Synergizing Activity, that dramatically enhances netrin activity under certain conditions. Cloning of cDNAs encoding the netrins showed that these proteins are roughly 50% identical to the predicted product of the *unc-6* gene of *C. elegans*, which when mutated leads to defects in circumferentially extending axons in the nematode, thus suggesting similar functions for these proteins in organisms separated by over 600 million years of evolution. Netrins are bifunctional guidance cues *in vitro*, displaying both chemoattractant and chemorepellent activities on growth cones (3). Netrins have also been described in *Drosophila*, where they seem to have a role in axon targeting in the periphery as well as a guidance role at the midline (4).

Although the *in vitro* activities and expression patterns of the netrins strongly suggested that they were required for chemotropic axon guidance *in vivo*, this evidence has only come recently through our analysis of mutant mice deficient in netrin-1 (5). We have found that the commissural axons that are attracted to a source of netrin-1 *in vitro* are misrouted in the absence of netrin-1 *in vivo*. Thus, netrin-1 is required for axon guidance *in vivo*, and the defects observed are consistent with the protein acting as a chemoattractant. Furthermore, axonal systems in the brain, such as the hippocampal commissure and corpus callosum, require netrin-1 for proper formation.

Analysis of target recognition in vitro

Synaptic specificity and partner selection, occurring in the end phase of axon guidance, have also been addressed *in vitro*, where specific pre- and postsynaptic neurons can be placed together in isolation. Studies using invertebrate and vertebrate neurons indicate that growth cones often change selectively upon target contact. One change often observed is the arrest of presynaptic growth cone extension upon contact with postsynaptic targets (6-8).

We are employing two systems displaying this growth arrest phenomenon to study target recognition between neurons *in vitro*. The first has been described by Baird, Hatten, Mason and colleagues (8, 9). If explants of neonatal murine basilar pons (which provides a substantial portion of the mossy fiber afferent system to the cerebellum) are plated onto a laminin-coated surface, many mossy fiber axons extend in excess of 500 μm from the explant over 48 hours. If, however, the explants are plated onto a laminin-coated surface onto which purified postnatal granule cells (their correct postsynaptic target) have been plated, a four- to five-fold reduction in neurite lengths is observed. This growth arrest requires growth cone-target contact, and growth arrest is maintained for at least 16 hours.

We are also employing a second system to study target recognition *in vitro*. During the development of the spinal cord, the NT-3 responsive axons of proprioceptive dorsal root ganglion (DRG) neurons enter the spinal cord dorsally and project ventrally, where they synapse with motoneurons. The NGF-responsive axons of other DRG neurons also enter dorsally but synapse in the dorsal aspect of the spinal cord with sensory relay neurons. We have very recently been able to demonstrate that purified motoneurons will arrest the growth of NT-3 responsive axons, but not NGF-responsive axons. We hope that this assay, together with the assay described above, will allow us to identify and characterize molecules mediating target recognition during the development of the vertebrate central nervous system.

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Vertebrate homologues of *C. elegans* UNC-5 are candidate netrin receptors

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In the developing nervous system, migrating cells and axons are guided to their targets by cues in the extracellular environment. The netrins are a family of phylogenetically-conserved guidance cues that can function as diffusible attractants and repellents for different classes of cells and axons. Recent studies in vertebrates, insects and nematodes have implicated members of the DCC subfamily of the immunoglobulin (Ig) superfamily as receptors involved in migrations toward netrin sources. The mechanisms that direct migrations away from netrin sources (presumed repulsions) are less well understood. In *Caenorhabditis elegans*, the transmembrane protein UNC-5 has been implicated in these responses, as loss of *unc-5* function causes defects in these migrations, and ectopic expression of *unc-5* in some neurons can redirect their axons away from a netrin source. Whether UNC-5 is a netrin receptor or simply an accessory to such a receptor has not, however, been defined. We report the identification of two vertebrate homologues of UNC-5, which, with UNC-5, define a novel subfamily of the Ig superfamily, and whose mRNAs show prominent expression in various classes of differentiating neurons. We provide evidence that these UNC-5 homologues are netrin-binding proteins, supporting the hypothesis that UNC-5 and its relatives are netrin receptors.

SESSION 4. NEURONAL RECOGNITION (I)

Chairman: John B. Thomas

EPH LIGANDS AND RECEPTORS AS POSITIONAL LABELS IN NEURAL MAP DEVELOPMENT

John G. Flanagan

Topographic maps, which maintain the spatial order of neurons in the order of their axonal connections, are found throughout the nervous system. The visual projection from the retina to the tectum has for decades been a leading model to study topographic mapping. Based mainly on studies of this system, Sperry first proposed in the chemoaffinity theory that such maps could develop in response to complementary position-specific labels in gradients across the projecting and target fields. However, the molecular identification of these labels has long remained an elusive goal. Eph family ligands and receptors are now implicated as topographic mapping labels.

The Eph receptors are by far the largest known family of receptor tyrosine kinases, with at least 14 members so far identified in vertebrates. Remarkably, however, all were initially identified as orphan receptors without known ligands. ELF-1 (Eph ligand family-1) was cloned by methods we developed to characterize orphan receptor ligands, using a soluble receptor probe with an alkaline phosphatase tag to determine ligand expression patterns directly in the embryo, and then using this spatial information to construct and screen a cDNA expression library (1). Since then, we have identified two additional ligands, ELF-2 (2) and ELF-3, and at least 8 Eph family ligands have now been found.

In the chick retinotectal system at the time of mapping, we find that ELF-1 is expressed in the tectum and its receptor Mek4 in the projecting retinal ganglion cells. Both are in gradients along matching axes that map to one another. In addition, a functional test of binding activities, using alkaline phosphatase (AP) fusions of ELF-1 and Mek4, showed that each can detect a matching gradient of binding sites in the reciprocal field, providing direct evidence for the gradient complementarity that would be predicted from the chemoaffinity theory (3). We have also tested the effects of ELF-1 on retinal axon behavior. ELF-1 acts as a repellent axon guidance factor in vitro. In vivo, when the tectal ELF-1 pattern is modified by retroviral overexpression, retinal axons avoid ectopic ELF-1 patches and map to abnormally anterior positions. All these effects were seen on axons from temporal but not nasal retina, indicating ELF-1 could determine nasal versus temporal retinotectal specificity, and providing the first demonstration of a cell recognition molecule with topographically specific effects on neural map development (4). Current experiments are further testing the roles of Eph ligands and receptors in retinotectal mapping, and in other aspects of nervous system development.

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Functional characterization of Eph-related receptor tyrosine kinases and their ligands during development of the chicken retinotectal system

Dieter Dütting, Bruno Monschau, Claus Kremoser, Susanne Lang, Claudia Handwerker, Friedrich Bonhoeffer and Uwe Drescher

Results from an in vitro guidance test, the stripe assay, indicated the presence of a repulsive axon guidance activity for temporal retinal axons in the posterior part of the vertebrate optic tectum. RAGS and ELF-1 are glycosylphosphatidyl-anchored ligands for the Eck subfamily of Eph related receptor tyrosine kinases, which are expressed in overlapping gradients in the posterior part of the tectum. When recombinantly expressed, both proteins can act as axon guidance molecules for retinal ganglion cell axons in the stripe assay. Membrane attachment of these ligands is necessary for exerting this activity (for review see 1).

Data will be presented showing that soluble forms of RAGS and ELF-1 at nanomolar concentrations can block completely the endogenous repulsive activity of the posterior tectum seen in vitro. Similar results were obtained with the soluble extracellular domain of Cek4, which is a receptor for ELF-1 and RAGS, but not with the corresponding domain of Cek10, which is a receptor for the transmembrane class of Eph-ligands. These competitive inhibition experiments show, that the endogenous repulsive axon guidance activity, which shows up in the stripe assay, is mediated by glycosylphosphatidyl-anchored ligands for the Eph-related receptor tyrosine kinases.

In vivo, retrovirally driven ectopic overexpression of RAGS in the anterior tectum leads to projection errors of both temporal and nasal retinal ganglion cell axons.

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NEURONAL DEFECTS IN MICE LACKING EPHA8 (EEK) TYROSINE PROTEIN KINASES RECEPTORS AND ITS COGNATE LIGAND EPHRIN-A5 (AL-1/RAGS).

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Eph8A/Eek is a member of the Eph family of tyrosine protein kinase receptors that binds with similar nanomolar affinity at least three members of the GPI-linked subfamily of Eph ligands, ephrin-A2/Elf-1, ephrin-A3/Ehk1-L and ephrin-A5/AL-1. To determine the role of the Eph8A/Eek receptor *in vivo*, we have disrupted the *eph8A/eek* locus in ES cells by inserting in frame a *lac*Z reporter gene. Mice homozygous for this mutation do not show any overt anatomical or behavioral phenotype. Eph8A/Eek expression (as determined by X-gal staining) is restricted to a very small subpopulation of neurons located in the rostral part of the superior colliculus and to discrete regions of the hindbrain, the dorsal part of the rostral spinal cord and the naso-lacrimal groove. Axonal tracing in these homozygous mice indicates that axons from a subpopulation of superior colliculus neurons failed to reach their targets located in the contralateral inferior colliculus. More importantly, these mutant mice display an aberrant ipsilateral axonal tract to the cervical spinal cord. Retrograde labeling experiments revealed that these projections originate in superior colliculus neurons that normally express Eph8A/Eek receptors. Although the developmental origin of these abnormal projections remains to be established, our results raise the possibility that they represent misguided commisural axons that fail to identify appropriate positional cues due to the absence of Eph8A/Eek receptors.

Recently, we have generated mice defective in ephrin-A5/AL-1, a cognate ligand for the EphA subclass of receptors and a repulsive axonal guidance factor. Most of the ephrin-A5/AL-1 (–/–) mice survive without obvious anatomical and/or behavioral defects. However, about 20% of these mutant animals are born with developmental deficiencies of variable penetrance in the dorsal midline of the head. The milder defects consist of a hematoma or a small aperture in the dorsal midline of the cranium with a slight protrusion of brain tissue. The most severely affected mice have a completely open cranium with cleft nose and palate, anencephaly and absence of pituitary gland and trigeminal ganglia. Analysis of ephrin-A5/AL-1 (–/–) embryos revealed that these defects are caused by incomplete closure of the rostral neuropore. Interestingly, ephrin-A5/AL-1, along with its cognate receptor EphA7/Ehk3, are transiently expressed at the edges of the forebrain neural folds during neurulation, thus suggesting that at this developmental stage (E8.5-E9) ephrin-A5/AL-1 may play a role in promoting cell-cell interactions. Characterization of the retino-tectal projection in ephrin-A5/AL-1 (–/–) mutant mice is currently been carried out in collaboration with Dr. D. O'Leary's group.

RETINAL AXON GUIDANCE: MECHANISMS AND MOLECULES. Dennis D.M. O'Leary, Molecular Neurobiology Lab, The Salk Institute, La Jolla, CA 92037 USA

Most neural connections in the brain are topographically organized such that one neuronal population maps precisely onto another. The retinotectal projection has been a model system for studying the mechanisms that control the development of these maps. This talk will consider work from our lab and others on the behavior of retinal ganglion cell axons during map development and the genes and molecules which may control their behavior.

The development of retinotopic maps is widely believed to be controlled by axonal guidance molecules present in a regional or graded distribution in the superior colliculus (SC) / tectum. Work done in the past few years has demonstrated that putative guidance molecules are present in the tectum as developing retinal axons enter it, but that these molecules fail to guide or restrict the growth of retinal axons to their topographically correct sites, and that the formation of appropriate connections is developed principally through the formation of collateral branches which go on to form arbors. In vitro studies have shown that guidance molecules can control the collateral branching of retinal axons in a topographically specific manner. These findings suggest that a prominent role of guidance molecules is to regulate the topographic formation of collateral branches which go on to develop ordered synaptic connections. Ligands of the eph subfamily of receptor tyrosine kinases have been implicated as guidance molecules. One of these, Elf1, has been found to specifically inhibit or repel the growth of temporal retinal axons.

Another line of investigation has suggested that the engrailed genes, which encode homeobox transcription factors, control retinal map development by regulating the expression of guidance molecules through a genetic cascade. Previous transplantation studies have shown that the pattern of engrailed expression correlates with the polarity of the retinotectal map. Work using recombinant retroviruses has directly implicated engrailed as a regulator of retinal mapping in the tectum.

The Semaphorins: A Family of Axonal Guidance Molecules

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During development axons navigate with remarkable specificity to their peripheral and central targets. Proteins encoded by the semaphorin gene family have been proposed to act as growth cone guidance signals in vertebrates and invertebrates. To identify candidate molecules involved in axonal pathfinding during mouse embryogenesis we have isolated cDNAs encoding seven new members of the semaphorin gene family (semA - G). These can be grouped into three classes by comparison of semaphorin domain sequences and the presence of class-specific carboxyterminal domains (1, 2). The secreted class III semaphorins are synthesized as proproteins which are proteolytically cleaved in the secretory pathway by a furin-like convertase. proSemD is activated upon processing at one of several highly conserved dibasic cleavage sites. The murine semaphorin genes are differentially expressed in mesoderm and neuroectoderm before and during the time when axons select their pathways in the embryo (E9 - E15). In explant cultures recombinant Sem D and Sem E convert a matrix permissive for axonal growth into one that is inhibitory for neurites of peripheral ganglia. Whereas all sensory neurites are responsive to Sem D early in murine development (E12.5), proprioceptive neurons selectively lose their sensitivity towards semD at the time when they begin to extend collaterals into the spinal cord (E14.5). Thus, semaphorins may pattern the innervation of target areas by providing local signals that specify territories inaccessible for subsets of axons (3).

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NOVEL EXPRESSION GRADIENTS OF EPH-LIKE RECEPTOR TYROSINE KINASES IN THE DEVELOPING CHICK RETINA

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Classical experiments led Sperry to propose that correct retinotectal mapping is achieved by the detection of spatial gradients within the tectum by receptors present in the projecting retinal ganglion cell axons. Recently, much attention has been paid to the role that members of the Eph-like family of receptor tyrosine kinases (RTK's) and their corresponding ligands play in the establishment of the retinotectal map. Complementary gradients of receptors and ligands in the retina and tectum, have previously been described in both the dorsoventral and nasotemporal (anteroposterior) axes. Two ligands, RAGS and Elf1 (expressed in posterior to anterior gradients within the tectum) show repulsive activity towards temporal axons (Drescher et al, 1995; Nakamoto et al, 1996), fulfilling the criteria proposed by Sperry required to encode retinotectal topography.

We have been interested in the role played by RTK's in the development of the chick retina and have identified a large number of RTK's expressed during retinal development including several members of the Eph-like family of kinases. We have deteceted a novel centroperipheral gradient of expression for one member of this family of receptors, Cek9, suggesting that retinotectal projections may involve coordinate mapping along each of the three body axes. In relation to this, we have found matching gradients of two cytoplasmic kinases, compatible with their putative involvement in the intracellular signalling pathways used by these receptors in the retina. Furthermore, we demonstrate a dorsal to ventral expression gradient for Cek11, an Eck-like receptor, the Eph subclass previously suggested to specify positional information along the nasotemporal axis.

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SESSION 5. NEURONAL RECOGNITION (II)

Chairman: Dennis D.M. O'Leary

Receptor tyrosine kinases and axonal guidance in *Drosophila*

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Developing neurons are capable of selecting specific pathways which lead them to their appropriate target areas. Upon arrival within their target areas, similar recognition events occur as neurons select their correct target cells. A variety of molecular mechanisms are thought to mediate these cell-specific choices, including cell adhesion, repulsion and chemotropism. However, apart from a few molecules which have been shown genetically to be involved, the mechanisms underlying neuronal pathway and target selection are largely unknown. We have taken a genetic approach in *Drosophila* to isolate and study genes that control neuronal guidance and target recognition.

To facilitate our studies of pathway selection and target recognition, we created an axon-targeted fusion reporter, *tau-lacZ*, that efficiently labels the axons and dendrites of expressing neurons (Callahan and Thomas, 1994). The *tau-lacZ* reporter has allowed us for the first time to identify and determine the relationships among many neurons within the CNS. We used a *tau-lacZ* P element transposon in an enhancer trap screen to isolate genes expressed in subsets of embryonic neurons that project along common pathways. The underlying assumption in this approach is that such genes might be involved in the recognition events mediating the specific pathway choices. The *derailed* (*drl*) gene was identified in this enhancer trap screen (Callahan et al., 1995). *drl* is expressed in a subset of interneurons, the majority of which cross the midline in the anterior commissure and choose a common pathway within the longitudinal connective. By fasciculating with their homologs in adjacent segments, the *drl* neurons form distinct axon bundles that run the length of the CNS. In *drl* mutants, the *drl* neurons project abnormally within the CNS, often crossing the midline in the wrong commissure and extending axons along inappropriate pathways within the connectives. The result of these pathfinding defects is that the distinct *drl* axon bundles are obliterated. However, not all pathfinding behaviors of the *drl* neurons are disrupted in *drl* mutants. For example, they still cross the midline (albeit some in the wrong commissure) and extend anteriorly within the connective, indicating that these more general aspects of pathfinding, in contrast to the more specific aspects of pathway selection and fasciculation, are mediated by a different mechanism.

drl encodes a member of the receptor tyrosine kinase (RTK) family, displaying the greatest similarity to mammalian Ryk. Drl and Ryk define a distinct RTK subfamily, as they both share novel extracellular domains and unusual amino acid substitutions within their catalytic domains. Drl protein is localized to the growth cones and axons of the *drl* neurons as they cross the midline within the anterior commissure, but not on the axon segments within the connective after the neurons have turned anteriorly. Thus, Drl probably does not physically participate in the adhesion and fasciculation of *drl* neurons within the connective. Instead, our results suggest the existence of multiple, discrete steps in pathway selection with Drl playing a key controlling role in the initial recognition event. We envision that once activated by its ligand, Drl controls pathway selection by

modulating the function of specific cell adhesion molecules that mediate proper axon fasciculation.

Drl is also expressed on a subset of developing muscles and neighboring epidermal cells during the period of muscle attachment site selection. Normally, muscles extend growth cones along the epidermis and form attachments with specifically positioned epidermal cells. In *drl* mutants, the *drl* muscles often fail to insert at these locations, although the attachment cells are present and appear unaffected (Callahan et al., 1996). This result shows that in addition to its role in axon pathway selection within the nervous system, *drl* participates in a recognition mechanism underlying muscle attachment site selection, and further suggests that both neurons and muscles use similar mechanisms to recognize their paths or targets.

There is mounting evidence from studies in vertebrates that the Eph RTK subfamily, like Drl, is involved in neuronal pathfinding (e.g., Henkemeyer et al., 1996). In collaboration with Mike McKeown we have cloned a *Drosophila* member of the Eph subfamily. This *Drosophila* RTK shares significant sequence homology to Cek/Mek subfamily members within both the kinase and extracellular domains and thus we have named it Dek. *In situ* hybridization of *dek* probes to embryos reveals a pattern of expression restricted to the CNS during periods of axon outgrowth and pathfinding. We have raised antibodies to Dek and found that the protein is expressed on the growth cones and axons of differentiating neurons within the CNS. We have mapped the gene and are currently isolating mutations to elucidate its possible role in axon guidance.

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Henkemeyer M., Orioli, D., Henderson, J.T., Saxton, T.M., Roder, J., Pawson, T. and Klein, R. (1996). Nuk controls pathfinding of commissural axons in the mammalian central nervous system. Cell 86, 35-46.

Title: **Ariadne and the neural development in Drosophila**

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Ariadne is a novel protein encoded in the HL-V transcription unit of the haplo-lethal region in the Shaker gene complex (Ferrús et al., 1990). Two major structural features are evident from the deduced amino acid sequence: a Zn^{2+} binding domain of the RING finger type and a long Cys-rich region towards the carboxy terminus. We have sequenced four lethal mutant alleles of *ari* and localized two of them to key amino acids of these two motives. The remaining two alleles are severe hypomorphs located outside of the open reading frame.

The existence of mutations in the RING finger and the Cys-rich region allows a functional dissection of these structural motives. Using germ line mosaics of these mutations we find that the RING motif is essential for oogenesis while the Cys region is not. Later in development, however, at the time of metamorphosis when the central nervous system undergoes a major reorganization and the adult projection pathways are established, both structural motives are required since all mutations yield the same neural phenotype. In the four alleles, the lethal phase coincides with the mid-pupal period and the CNS of the pharate adults exhibits many aberrant projections in all neural centers. Interestingly, the sensory projections in these pharate adults or in somatic mutant mosaics appears to be normal. This is evidence in support for a role of ARI in the axonal projection specifically in the CNS versus the PNS. A more direct evidence for the role of ARI in pathfinding is provided by the phenotype observed in a migratory population of optic glial cells. These cells show an aberrant migration in the mutant.

Although ARI is a novel protein, data from various genome sequence projects indicate that conserved homologs exist in yeast, nematods and humans, albeit with unknown function.

Ref: Ferrús et al. (1990) Genetics 125, 383-398.

NEURONAL RECOGNITION AND SIGNAL TRANSDUCTION BY PROTEIN TYROSINE KINASES IN DROSOPHILA

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

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

We are interested in understand the signaling processes generated by neuronal recognition molecules in particular those generated by protein tyrosine kinases and phosphatases. Using different approaches we have isolated several genes coding for protein kinases exclusively or mainly expressed in the nervous system. These proteins are directly (receptors) or indirectly (cytoplasmic proteins) involved in the generation of intracellular signaling events mediated by phosphorylation. Some of these molecules include two tyrosine kinase receptors (D*trk* and D*ret*) and a cytoplasmic Ser/Thr kinase (Dgcn2).

D*trk* codes for a novel type of neural cell adhesion molecule with some homology to the TRK family of neurorotrophin receptors. Trangenic lines expressing mutant forms of this receptor shows clear defects in the axon patterning within the central nervous system (CNS), suggesting a role for D*trk* in axon outgrowth. In the case of D*ret*, this gene codes for a receptor with high homology to the mammalian RET proto-oncogene. Recently, several papers have show that mutations in the human RET gene are associated with several diseases, including several cancer syndromes and a neurodegenerative disease. Interestingly, many of these mutations occurred within codons specifying residues that are conserved between the human and *Drosophila* Ret proteins, suggesting a high degree of functional conservation between both receptors. Dgcn2 codes for a Ser/Thr kinase that at the end of the embryogenesis is specifically expressed in a restricted group of neurons of the CNS (two pairs by neuromer). This gene is the homologue of the yeast GCN2 locus and encodes a protein that is able to phosphorylate the *Drosophila* eIF2α initiation factor.

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

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The GTPase Rac1 and the Transmembrane Tyrosine Phosphatase DLAR Cooperate to Control Motor Axon Guidance in Drosophila. Nancy Kaufmann, Zak Wills, Daneiila Scalice and David Van Vactor. Department of Cell Biology and Program in Neuroscience, Harvard Medical School, Boston, MA 02115, U.S.A.

The stereotyped pattern of neuromuscular connections in the Drosophila embryo provides an excellent context in which to identify and understand the function of axon guidance molecules at single cell resolution (1). Our recent studies have demonstrated that specific motor axons require the receptor-like protein tyrosine phosphatase (RPTPase) DLAR to correctly enter the appropriate muscle target domain (2). DLAR is a member of a large class of RPTPases, many of which are expressed in the developing nervous systems of vertebrates and invertebrates alike. Despite the tremendous significance of tyrosine phosphorylation in regulating a variety of cellular behaviors, the molecules that participate in RPTPase signaling pathways are poorly understood. In an effort to better define the DLAR signaling mechanism, we have begun to search for candidates that may transduce guidance information in the context of a very specific axon guidance choice.

It has been known for many years that the directional specificity of axon outgrowth depends upon cytoskeletal dynamics at the leading edge of the growth cone. From studies in a variety of systems, it seems likely that actin is a major effector system downstream of many axon guidance signals. The GTPases Rac, Rho and Cdc42, are key regulators of actin structures and motility behavior. Here we demonstrate that neuronal expression of a dominant negative Drac1 shows the same phenotype as loss of DLAR. This phenotype is also reproduced consistently by low doses of Cytochalasin D, which blocks actin assembly. These observations show that Rac is necessary for normal axon guidance, and suggest that Rac mediates signals to the actin cytoskeleton from cell surface molecules including DLAR. However, when DLAR is eliminated by null mutations, Drac1 still shows a requirement for target entry. This indicates that Rac must respond to other extracellular cues, and suggests a model where Rac is an integrator of multiple sources of guidance information.

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POSTERS

Calmodulin is involved in membrane depolarization-mediated survival of motoneuron by PI-3K and Ras/MAPK independent pathways

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During embryonic development of the vertebrate nervous system, approximately one half of all the neurons produced die as a result of a process known as natural or programmed cell death. In motoneurons (MTNs) of the lumbar spinal cord of chick embryos, this process takes place between embryonic days 6 and 10. It is now clear that specific target-derived neurotrophic factors have a decisive role in this mechanism. There is growing evidence that other non-target-derived factors may also be involved in the regulation of neuronal survival. Bioelectric activity plays a crucial role in both the differentiation and survival of nerve cells. Several populations of neurons can be maintained in culture in the absence of neurotrophic factors when their plasma membrane is chronically depolarized by an increased K^+ concentration in the culture medium.

We have analyzed the effect of elevated extracellular K^+ on survival of primarily cultured MTNs from chick spinal cord and we explore some of the molecular mechanisms involved. We find that the elevation of extracellular K^+ concentration promotes the in vitro survival of chick spinal cord motoneurons deprived of any neurotrophic support. This treatment induces chronic depolarization of the neuronal plasma membrane, which activates L-type voltage-dependent Ca^{2+} channels, resulting in Ca^{2+} influx and elevation of the cytosolic free Ca^{2+} concentration. Pharmacological reduction of intracellular free Ca^{2+} or withdrawal of extracellular Ca^{2+} caused motoneuron death. The intracellular Ca^{2+} response to membrane depolarization developed as an initial peak followed by a sustained increase in intracellular Ca^{2+} concentration. Exposure of motoneurons to BDNF, but not depolarization, caused activation of the tyrosine kinase activity of Trk, suggesting that depolarization causes its effect without Trk activation. Both neurotrophin and depolarizing treatments caused tyrosine phosphorylation of MAPK. The calmodulin antagonist W13 inhibited the survival-promoting effect, induced by membrane depolarization, but not the tyrosine phosphorylation of the MAPK. Moreover, the PI-3K inhibitor worthmannin did not supress the survival promoting effect of K^+ treatement. These results suggest that calmodulin is involved in calcium-mediated cell survival of motoneurons through the activation of PI-3K and Ras/MAPK independent pathways.

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ODORANT RECEPTORS AND THEIR LOCALIZATION

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Recent discoveries in signal transduction in many systems have shown that spatial determinants play a critical role in signal transduction. At cell-cell junctions during development, at the neuromuscular junction, and at neuronal synapses, signal transduction components including receptors and channels are often clustered together. Membrane domains specialized for signal transduction keep signaling machinery in close proximity so that signal transduction is rapid and efficient. Little is understood about how signaling molecules are targeted to and maintained at these plasma membrane specializations. We have been studying two genes involved in odorant receptor localization.

The chemosensory system of *C. elegans* has provided a useful model for neuronal signal transduction and development. This worm can detect hundreds of chemicals. Through the use of powerful behavioral genetic screens, our laboratory has identified many signaling components involved in odorant recognition. Among these are over two hundred putative odorant receptors, a G protein, a cyclic nucleotide-gated channel, and a calcium channel. All of these components are localized to the cilia of chemosensory neurons, membrane structures at the tips of the dendrites specialized for reception of chemicals. How is this limited localization of signaling components accomplished within a cell?

The genes *odr-4* and *odr-8* seem to be involved in this process. In *odr-4* and *odr-8* mutants a subset of odorant receptors are not localized properly to the cilia. One of the mis-localized receptors is the putative receptor for the odorant ligand diacetyl, a chemical with a butter-like smell. Mutants in which this receptor is not localized to the cilia are not able to sense diacetyl. Hence, localization to the cilia is critical for olfactory transduction to occur.

odr-4 was cloned positionally and encodes a novel protein of 445 amino acids with a transmembrane domain at the C-terminus and no signal peptide. The gene is expressed in chemosensory neurons, and we are currently trying to determine its specific role in receptor localization.

IMPLICATION OF DCC ("*DELETED IN COLORECTAL CANCER*") GENE IN EPITHELIAL DIFFERENTIATION.

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In 70% of colorectal tumors analyzed, Fearon *(1)* demonstrated the presence of a loss of heterozygosity (LOH) in the locus 18q21.3. In this region, was identified by positional cloning a tumor suppressor gene, called DCC for "Deleted in Colorectal Cancer", of 1.4Mb, codifying for a transmembrane protein of the Ig-CAM superfamily. Recent data indicate that the DCC protein is implicated in the morphogenesis of the nervous system during embryonic development, functioning as a receptor for netrin - 1, a diffusible factor controlling axon guidance *(2)*. On the other hand, Hedrick *(3)* evidenced a possible relation between DCC expression and mucosecretory differentiation in colonic epithelium. However, the mechanism through which it would act still remains to be elucidated. To answer this question, we decided: a) to analyze the mechanisms of DCC inactivation in colon cancer; b) to characterize its function in epithelial differentiation by identifying its ligands and intracellular substrates.

First, in order to determine if DCC was specifically implicated in the differentiation of mucosecretor cells, we analyzed DCC expression by RT-PCR in normal colon as well as in a panel of 22 cell lines derived from colorectal carcinomas, with different capacities of differentiation in culture. This study has been realized using primers specific for extra- and intra-cellular domains of DCC. In parallel, the expression of different mucin genes (MUC2, 3 , 4, 5ac, 5b, 6) was examined by northern blot. Results were confirmed, at protein level, on the cell lines analyzed as well as in normal colon mucosa, by immunohistochemistry with antibodies directed against the DCC protein and colonic mucins, and by alcian blue staining to detect mucus secretion. The results were the following ones: 1) DCC expression was detected in the majority of cell lines (16/22), independently of the phenotype; 2) Some of the cell lines were expressing only one of the two domains of the protein (5/22); 3) only one cell line did not present any detectable level of DCC; 4) We did not observe any specific correlation between DCC and mucin genes expression in the studied cell lines, neither did we find any specific colocalization between mucus secretion and DCC in normal colon epithelium.

Secondly, we developed a working model in culture consisting of HT-29 cells (undifferentiated cell line with barely detectable levels of DCC expression) transfected with the DCC cDNA, inserted in the eucaryotic expression vector pCMVneo. We selected several clones expressing the DCC protein at the membrane and observed that they still grow as a multilayer of unpolarized cells as shown by semithin sections. Western blot and immunofluorescence staining analysis of the HT-29/DCC transfectants did not detect expression of molecular markers for intestinal epithelial differentiation such as mucins, DPPIV or sucrase-isomaltase.

In conclusion, in addition to the general mechanism of DCC inactivation in colon cancer by allelic loss, our preliminary results suggest that, in some cases (25%), the remaining allele can be affected by alterations leading to the synthesis of a truncated form of its mRNA, either as a result of aberrant splicing or differential use of exons. Regarding to its function in the differentiation process, the lack of specific correlation observed between DCC expression and the mucosecretory pathway suggests that, first, DCC may not be directly implicated in differentiation; second, that the expression of a truncated form of DCC (as occurs in tumors) may function as a dominant negative in terms of competition for interacting molecules; and, third, that the limiting factor would then be the expression of its ligand(s) or substrate(s). Given the recent results obtained in the nervous system, we are currently investigating the possibility that one of the DCC ligands in the epithelium of the colon could be either a secreted diffusible factor netrin-like or a component of the extracellular matrix, both at the epitelio/mesenchymal interphase.

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Eph family receptors and their ligands can be divided into two major specificity subclasses that form embryonic boundaries

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The Eph family of receptor tyrosine kinases has 13 distinct members, and eight ligands for these receptors have been described to date. Emerging functional evidence indicates that Eph family ligands elicit responses from neurons and their precursors that are quite different from those seen in response to classical neurotrophic and survival factors such as the neurotrophins. Eph family ligands may function in regulating axonal bundling, as signals involved in patterning axonal projections, and in the formation or in refinement of the sharp boundaries seen between segments in the developing hindbrain, somites and elsewhere. In nonneuronal cells such as endothelial cells, Eph ligands seem to be able to regulate cell migration. We find that in both cell binding and receptor activation studies receptors of the Eph family and their ligands can be divided into just two major specificity subclasses. Moreover, whole embryo binding experiments show that all receptors of a particular subclass identify similar ligand patterns in the embryo, while all ligands of a particular subclass detect similar receptor profiles. Interestingly, these patterns reveal multiple boundaries in the embryo--in the developing brain and spinal cord, limb buds, pharyngeal arches, somites, and elsewhere--where ligands of a particular subclass interface with their corresponding receptors. These results are consistent with roles for Eph family members as axon guidance cues, and also implicate the Eph family in the formation or refinement of boundaries in multiple embryonic structures. The surprising lack of binding diversity in this large family raises major issues concerning shared activities and redundancy for members of this family, and also demand that future functional analyses simultaneously consider all members of a particular subclass as potentially equivalent mediators of particular processes. The data also present a context in which to interpret emerging functional data for particular members of this large family.

In vivo Cooperation of the Neuropoietic Cytokine LIF and the Multifunctional Growth Factor IGF-I

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Leukemia inhibitory factor (LIF), also known as Cholinergic differentiation factor, is a neuropoietic cytokine that acts through a heterodimeric receptor composed of the LIFR subunit and the gp130, signal transducing component. The dimeric receptor associates with several cytoplasmic JAK kinases. Interestingly, some of the JAKs are able to phosphorylate IRS1 and IRS2, the main substrates of the Insulin and insulin-like growth factor I (IGF-I) receptors, both tyrosine kinases. Thus, there is convergence of these two signalling systems. The factors, LIF and IGF-I are both expressed and modulated during development in the nervous system and other tissues. There is increasing evidence that both proteins, depending on the cellular context, are able to regulate proliferation, differentiation, survival and gene expression in the central and peripheral nervous system.

To explore whether there is any cross-regulation or cooperative effects of LIF and IGF-I in the nervous system, we have chosen initially to study a model of peripheral nervous sytem injury/regeneration in normal and the LIF-null mutant mice. After sciatic nerve crush in normal mice, both LIF and IGF-I mRNA are induced. Markers of Schwann cells, such as Glial Fibrillary Acidic Protein (GFAP), are also increased. In the LIF-null mutant mice, 1 day postcrush, the GFAP$^+$ cells are much less abundant in the crushed side, compared to the normal mice. One day postcrush, the levels of IGF-I mRNA are low both in wild type and mutant mice but the IGF-I$^+$ cells, possibly macrophages, are less abundant in the crushed side of the LIF-null mutant mice. At later times (3-4 days) during sciatic nerve regeneration, IGF-I mRNA is increased several fold in the crushed side of both normal and LIF-null mutant mice and a few large cells, possibly mast cells, are strongly stained for IGF-I. These cells are invading the endoneurium of the crushed side, earlier and in larger numbers in the LIF-null mutant mice than in normal mice. Thus, IGF-I that has a role in myelinization and astrocyte proliferation in the CNS and acts as neurotrophic factor in the peripheral nervous system, may contribute to compensate the absence of LIF in the regeneration of the lesioned sciatic nerve of mice lacking LIF. A possible approach to study functional interactions between LIF and IGF-I *in vivo* is the generation of double null mutant mice for these factors.

CALCIUM TRANSDUCTION SIGNAL IN NON SECRETORY CELLS

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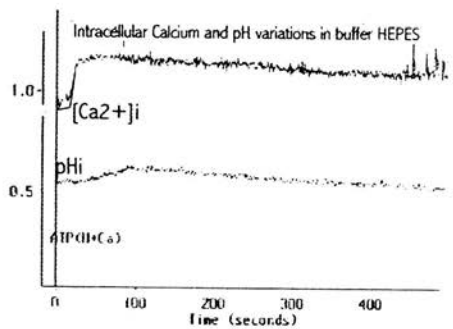
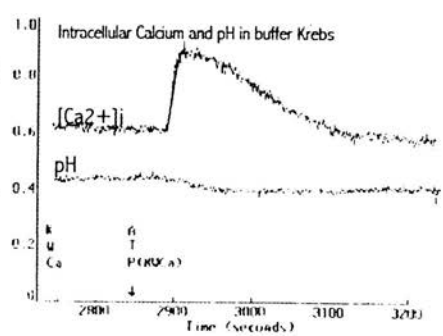
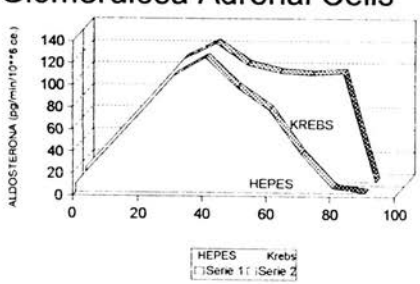
We have strong evidence that the pHi and [Ca2+]i signal transduction mechanisms are interelated.
We measured intracellular calcium in different conditions that modifye the management of calcium. Using Hepes buffer versus Krebs with CO2 we got different patterns of calcium signals very well related with pHi changes measured by BCECF-AM.
We got the same pattern by using either Aequorin a photoprotein derived from Aequoriae Forskolea and introduced inside the cells by reversible permeabilization and by using Fura 2-AM that goes through the membrane and is deesterified inside the cells. Fig. 1 and 2.
These effects affect different functions as we have documented for the second phase secretion of aldosterone by the glomerulosa cells and for the second phase secretion of insulin by the pancreatic islets Figs. 3 and 4.

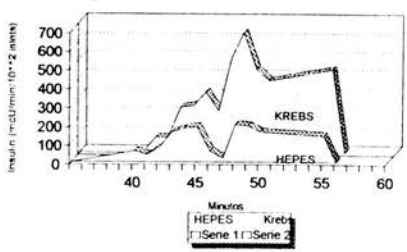




Aldosterone Secretion by Glomerulosa Adrenal Cells



Insulin Secretion in buffer KREBS and HEPES



REGULATION OF THE BRAIN-SPECIFIC GENE RC3/NEUROGRANIN BY THYROID HORMONE IN VIVO AND IN CULTURED NEURONAL CELLS

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RC3/neurogranin is a neuron-specific protein present in the soma and dendritic spines of telencephalic neurons. It is a PKC substrate that binds calmodulin and is likely to be involved in synaptic plasticity. We have recently shown that thyroid hormone controls in vivo the expression of the RC3 mRNA. The goal of this work was to study the mode of action of T3 on RC3 expression using rats and neuronal cultures.

RC3 expression was studied by in situ hybridization in rat brains at postnatal day 22. In agreement with previous studies RC3 expression was decreased by hypothyroidism in layer 6 of cerebral cortex, retrosplenial cortex, dentate gyrus and antero-lateral caudate. A single injection of T3 restored RC3 mRNA levels in these regions. The T3 action was at the transcriptional level, as shown by run-on assays using nuclei isolated from the caudate. However, in other regions the RC3 expression was not affected by thyroidal manipulations. To check the possibility that this affected regions express a specific T3 receptor isoform or that the unaffected regions express abundantly the $\alpha 2$ variant receptor, we studied the colocalization of T3 receptors and RC3 mRNA in normal, hypothyroid and hypothyroid T3 treated rats. We obtained no evidence for a specific expression of a receptor isoform in a subset of RC3 expressing neurons, and T3 increased RC3 mRNA in cells that express the $\alpha 2$ variant receptor.

To check whether the action of T3 is direct on the RC3 gene, we studied regulation of RC3 expression in the GT1-7 neuronal cell line. These cells express functional T3 receptors, as shown by T3 binding experiments (7000 sites per nucleus) and by activation of a reporter gene containing a T3 responsive element. Under basal culture conditions the cells express low levels of RC3 mRNA, but increase 10-20 fold 24 hours after addition of 150 nM T3. The effect of T3 was already observed at 6 hours and was not blocked by cycloheximide, suggesting a direct action of T3, without involvement of protein synthesis. The half life of the RC3 mRNA in these cells was 20 hours, and was not changed by T3. However, T3 failed to activate the RC3 promoter region in constructs containing up to 3 kbp of flanking sequence.

Conclusion: T3 regulates expression of the RC3 gene directly at the transcriptional level. The DNA sequence mediating the T3 response remains to be found. RC3 could be a component of a subset of genes which are insensitive to the inhibitory effects of the $\alpha 2$ variant on thyroid hormone action in the brain.

BIRECTIONAL SIGNALLING THROUGH THE EPH RECEPTOR NUK AND ITS TRANSMEMBRANE LIGANDS

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Receptor tyrosine kinases of the Eph class have been implicated in the control of axon guidance and fasciculation, in regulating cell migration, and in defining compartments in the developing embryo. Efficient activation of Eph receptors generally requires that their ligands be anchored to the cell surface, either through a transmembrane (TM) region or a glycosyl phosphatidylinositol (GPI) group. These observations have suggested that Eph receptors can transduce signals initiated by direct cell-cell interactions. Nuk belongs to a subclass of Eph receptors that bind specifically to TM Eph receptor ligands. Genetic analysis of *Nuk* has revealed a physiological requirement for this receptor for correct pathfinding of specific anterior commissure (AC) axons in the mouse brain. Surprisingly, however, a truncated Nuk polypeptide containing the extracellular and TM domains, but lacking the kinase domain supports normal AC formation in some genetic backgrounds. Nuk is primarily expressed in cells immediately ventral to the AC, while TM-ligands are expressed in the commissural axons, thus raising the possibility that the TM-ligands might themselves possess a signalling function, which is activated by binding of the Nuk extracellular domain. Consistent with this suggestion, the three known TM-ligands have a highly conserved cytoplasmic region with multiple potential sites for tyrosine phosphorylation.

We have demonstrated that challenging cells expressing Elk-L or Htk-L with the clustered ectodomain of Nuk induces phosphorylation of the ligands on tyrosine, a process which can be mimicked both in vitro and in vivo by an activated Src tyrosine kinase. These results suggest that the TM-ligands are associated with a tyrosine kinase, and are inducibly phosphorylated upon binding Nuk, in a fashion reminiscent of cytokine receptors. We have substituted tyrosine residues in the conserved C-terminal tail of Elk-L with phenylalanine in order to map these phosphorylation sites. Co-culturing of cells expressing a TM-ligand with cells expressing Nuk leads to tyrosine phosphorylation of both the ligand and Nuk. Eph receptors and their TM-ligands can therefore mediate bi-directional cell signalling. Furthermore, we show that TM-ligands, as well as Nuk, are phosphorylated on tyrosine in mouse embryos, indicating that this is a physiological process. Eph family tyrosine kinases may thus be viewed not only as receptors, but also as ligands for the activation of transmembrane proteins such as Elk-L, Htk-L and presumably the recently cloned Elk-L3.

A Role for the mDab1 Adaptor Protein in Neural Development

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We have recently reported the identification of a mouse homolog of the Drosophila Disabled (*dab*) gene, *mdab1*, and show it encodes a family of alternatively spliced products that encode adaptor molecules functioning in neural development. We find that mDab1 is expressed in certain neuronal and hematopoietic cell lines, and is localized to the growing nerves of embryonic mice. The p80 and p120 isoforms of mDab1 are tyrosine phosphorylated when the nervous system is undergoing dramatic expansion early in embryogenesis. However, when nerve tracts are established, mDab1 lacks detectable phosphotyrosine. Tyrosine phosphorylated mDab1 associates with the SH2 domains of Src, Fyn and Abl. mDab1 contains an aminoterminal phosphotyrosine binding (PTB) like domain which we now show binds to the cytoplasmic portion of the amyloid precursor protein.

To investigate the role of mDab1 in neurogenesis we have generated a loss of function allele of *mdab1*. This mutant allele, *mdab1-1*, encodes the p120 but not the p80 isoform of the mDab1 protein. Mice which are homozygous for *mdab1-1* are born, and appear outwardly normal for the first 10 days post partum. These mice are noticeably ataxic by P10, display a tremor and frequently fall over. The cerebella of these mice are dysplastic and share many characteristics with the cerebella of *reeler* mice, that fail to produce the extracellular matrix protein reelin. Like the *reeler* mice the *mdab1* mutants have defects in cortical and hippocampal development as well. The developmental role for signals which are conveyed by mDab1 and how this may involve reelin will be discussed.

The Role of the Agrin Binding Protein, α-Dystrogylcan, in MuSK Signal Transduction and Acetylcholine Receptor Clustering. Christian Jacobson, Federica Montenaro, Michael Lindenbaum, Salvatore Carbonetto and Michael Ferns. Centre for Neuroscience Research/McGill University, 1650 Cedar Ave., Montreal, Quebec, H3G 1A4, Canada.

The interaction of agrin with the Muscle Specific tyrosine kinase (MuSK) initiates the formation and differentiation of the neuromuscular synapse. Although MuSK appears to be the signalling receptor responsible for these events, there is evidence that agrin may require a co-receptor for binding to MuSK. Here, we show that the agrin binding protein α-dystroglycan (α-DG) and heparin sulfate proteoglycans (HSPGs) in general are not required for agrin stimulated MuSK autophosphorylation or the downstream phosphorylation of the acetylcholine receptor (AChR) β-subunit. Using several approaches, including assaying the activity of truncated agrin fragments with drastically reduced binding to α-DG, and analysis of cell lines with reduced α-DG expression we conclude that α-DG plays a minimal role in the initial stages of agrin induced AChR clustering. Nevertheless, α-DG deficient muscle cells have dramatically reduced levels of agrin induced AChR clusters relative to wild type. Taken together these results argue that AChR clustering is bi-phasic and that α-DG, though minimally involved in initiation of aggregation, may play a significant role in the consolidation and maintence of acetylcholine clusters.

Chromophore Assisted Laser Inactivation of Proteins During Growth Cone Motility and Axon Guidance

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The major paradigm for understanding axon guidance is the chemoaffinity hypothesis by Roger Sperry that states that neurons generate unique axon morphologies in response to extracellular cues. The field of axon guidance has been limited to defining extracellular cues and there have been few studies on the intracellular proteins of the growth cone that respond to those cues because of technical difficulties. We seek to understand how the growth cone acts as a sensory and motile machine to translate signals encountered in the environment to directed motility and guidance. This involves the reception of extracellular cues, signal tranduction and the regulation of the cytoskeleton and its associated motors. To address this question, we developed chromophore assisted laser inactivation (CALI), that can inactivate any protein of interest at specific times and locations in the embryo or cell to ascribe functional roles to that protein in complex cellular processes. This is done by microinjecting a malachite green-labeled non-blocking antibody that binds to the protein of interest. Laser excitation of the dye results in selective free radical damage to the bound protein with a high degree of spatial and temporal resolution. CALI has led to the determination of the functional roles of 10 intracellular proteins in neuronal growth cone motility and guidance decisions. These include cell adhesion molecules, signal transduction molecules, cytoskeletal-associated proteins and molecular motors. Establishing the functions of these proteins provides the first opportunity to think about molecular mechanisms for growth cone motility and axon guidance. We have shown that these proteins act independently on 2 discrete steps of growth cone behavior: filopodial motility and neurite extension.

We have focused on understanding how different proteins act in growth cone motility by studying the proteins, talin, vinculin, and the actin-based motors, myosin I and V. Our work has provided evidence that a cytoskeletal associated protein, talin, couples filopodial motility to actin dynamics. Talin simultaneously binds F-actin and the integrin receptor that in turn binds to the extracellular matrix, and the local inactivation of talin in the growth cone causes filopodia to transiently stall. In contrast, vinculin (which binds to talin) appears to have no role in motility but is required for filopodial structure. A key point of controversy has been whether filopodial movement is powered by the retrograde flow of F-actin or by actin-based motors. We have shown that myosin V is specifically required for filopodial extension and this is the first direct evidence that an actin-based motor acts in growth cone motility. Myosin I acts in the opposing direction to hold the periphery of the growth cone in check and the local balance of forces between these myosins may dictate where filopodia will emerge. These experiments address fundamental biophysical processes of how proteins work together to determine the shape and subsequent movement of cells.

We are also addressing how signal transduction regulates neurite outgrowth and guidance. An important signal in these processes is the localized change of intracellular calcium. The downstream effectors that mediate calcium-induced responses were not previously known. The loss of calcineurin, a calcium dependent serine phosphatase, within a subregion of the growth cone results in localized filopodial retraction and growth cone turning. These findings provide evidence that calcineurin can act to transduce localized rises in internal calcium to direct growth cone motility. We are extending this work by inactivating putative upstream and downstream components that may interact with calcineurin in the growth cone to begin to elucidate how this protein could modulate motility. Our future work will be dedicated to understanding molecular pathways of how extracellular cues elicit localized signal transduction changes to direct growth cone motility.

Stefan Jungbluth, Georgy Koentges, and Andrew Lumsden:

Coordination of early neural tube development by BDNF/trkB

Neurotrophins signal through members of the trk family of tyrosine kinase receptors and are known to regulate several neuronal properties. Although initially characterized by their ability to prevent naturally occuring cell death of subsets of neurons during development, neurotrophins can also regulate the proliferation and differentiation of precursor cells. We have found a novel involvement of neurotrophins in early dorso-ventral patterning of the neural tube. We demonstrate that a functional trkB receptor is expressed by motor neuron progenitors in the ventral neural tube and that treatment of explants with the trkB ligand Brain-Derived Neurotrophic Factor (BDNF) leads to a significant increase in the number of motor neurons. The only *BDNF* expression detectable at this stage is by a subset of ventrally projecting interneurons in the dorsal neural tube; ablating this region in vivo leads to a reduction of motor neuron numbers that can be prevented by treatment with recombinant BDNF. We propose that BDNF produced by dorsal interneurons stimulates proliferation and/or differentiation of motor neuron progenitors after anterograde axonal transport and release in proximity to the trkB-expressing motor neuron precursors, thereby coordinating development between dorsal and ventral regions of the neural tube.

EPH RECEPTOR TYROSINE KINASES ARE OVEREXPRESSED IN ADULT HIPPOCAMPUS AFTER INJURY.

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The molecular mechanisms underlying the processes implicated in response to injury in the Central Nervous System (CNS) are incompletely understood. Many cell activation system may be involved, some of them previously used during development and others specific of type of insults.

Receptor tyrosine kinases (RTK) and their ligands play key roles throughout life. The Eph family of RTKs, named for its first described member, are developmentally regulated, with temporally and spatially restricted expression patterns. They and their ligands have been implicated in processes of neural pathfinding. However we do not know anything about their role in the adult brain.

We have used a model of CNS lesion in rat, in which intraventricular injection of kainic acid was performed by using stereotaxic surgery. This produces neuronal death at CA3 and glial activation in the hippocampus. Three days post-injury, ipsilateral hippocampi of three rats were dissected and used to extract total RNA.

Degenerated primers described by Lai and Lemke, 1991, in the catalytic domain of RTK, with high degree of conservation for all the families of protein tyrosine kinases were used for Reverse Transcription-PCR of pooled RNA extracted from injured hippocampi. The amplified products were ligated to the pGEM-T vector (Promega) and cloned. A total of one hundred clones were examined and inserts sequenced. We obtained four clones containing inserts which belong to the Eph family of RTK. Two of these inserts were Sek and the other two were Tyro 5 and Ehk1.

We examined the expression in hippocampus of these members of Eph family by *in situ* hybridization. Preliminar results confirm their presence in all fields of hippocampus. After lesion they appear to be up-regulated in CA1. This qualitative evaluation of the *in situ* examination is currently being confirmed by Northern analysis.

The exact significance of these results is currently unknown because the lack of information of Eph RTK in the adult brain. Mice deficient in Nuk (Tyro 5 homologue in mice) and Sek4 appear to exhibit defects in pathfinding of anterior commissure axons and corpus callosum, respectively. Both hippocampi are interconnected through the anterior commissure, so these axons belong to paths affected in these mice. The function of these Eph RTK both in normal adult brain and in injury deserves further attention.

MUTATIONS AFFECTING CONSERVED AMINOACID RESIDUES WITHIN THE SECOND IMMUNOGLOBULIN-LIKE DOMAIN OF TRKA PROMOTE RECEPTOR DIMERIZATION AND ACTIVATION.

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NGF initiates its biological effects by promoting the dimerization and activation of the tyrosine kinase receptor TrkA. The extracellular region of the TrkA receptor is distinguished by several structural features, including leucine-rich repeats, cysteine-rich clusters and two immunoglobulin-like domains. We have previously identified the two immunoglobulin domains as those involved in NGF binding (Perez *et al.*, 1995) although the specific sequences required for ligand binding and dimerization have not been determined.

Mutations that promote ligand-independent dimerization or catalytic activation of TrkA receptor have been described; partial deletion of the second Immunoglobulin-like domain or point mutation of a cysteine to a serine in this domain can cause activation of the TrkA tyrosine kinase domain (Coulier *et al.*, 1990). To explore the mechanism of dimerization of the TrkA receptor we have performed several point mutations in both immunoglobulin domains. Those mutations change aminoacid residues conserved in all the family of Trk receptors as well as in other immunoglobulin domains and are probably involved in the maintenance of the immunoglobulin structure.

We have performed NGF binding assays with those mutant receptors stably transfected into 293 cells in order to see how the ligand binding was affected. Analysis of the receptor activation and transforming activity was done in transfected Rat-1 cells by determining growth and colony formation in soft agar plates and by measuring ^{3}H-Thymidine incorporation in the absence of serum.

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A KINASE-DEAD MUTANT OF p70^{S6K} INDUCES EPITHELIAL-MESENCHYMAL TRANSITION LIKE ACTIVATED RAS IN RESPONSE TO TGFβ IN MOUSE MAMMARY EPITHELIAL CELLS.

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We have previously shown, that mammary epithelial cells (EpH4) cells undergo growth arrest and apoptosis in response to TGFβ. In contrast, *v-Ha-Ras*-transformed EpH4 cells (EpRas cells) undergo epithelial/fibroblastoid conversion in collagen matrices in the presence of TGFβ, which is characterised by a change from a polarised, epithelial morphology to a spindle shaped morphology, by acquiring the expression of mesenchymal marker proteins and by showing invasive behaviour in *in vitro* assays (Oft *et al*, 1996). EpRas cells form tumors in nude mice quite rapidly, acquire fibroblastoid features, start to express TGFβ themselves and grow invasively (Oft *et al*, 1996). These data suggested that an activated Ras pathway protects epithelial cells against TGFβ-induced apoptosis and rather induces in synergy with TGFβ epithelial/fibroblastoid conversion that results in invasive growth of tumor cells. Activation of the Ras pathway and increased levels of TGFβ have in fact been correlated with certain human tumors and in addition, highly invasive spindle cell tumors have been shown histologically to originate from epithelial cells. Therefore, the synergy of TGFβ and activated Ras might well explain the onset of invasive growth of tumor cells *in vivo*.

The Ras pathway is a mitogen-induced signal transduction pathway, that transmits external growth factor signal via *erk*-encoded MAP kinases and p90^{rsk} into the cell nucleus. We wanted to know whether a distinct growth-factor induced pathway, namely the p70^{S6k} pathway can also protect EpH4 cells against apoptosis and induce epithelial/fibroblastoid conversion in mammary epithelial cells in response to TGFβ.

p70^{S6k} activation has been shown to be essential for G_1 progression of fibroblasts (Lane *et al*, 1993) and lymphocytes (Chung *et al*, 1992) and is involved in control of translation initiation of certain mRNAs in response to growth factor stimulation of cells (rev. by Brown and Schreiber, 1996). Activation of p70^{S6k} has been shown to be independent of active p21^{ras} (Ming *et al*, 1994), although a cross-talk of the two pathways might take place at the level of PI 3-K (Rodriguez-Viciana *et al*, 1994).

We have created several phosphorylation mutants of p70^{S6k} (Edelmann *et al*, 1996), expressed them stably in EpH4 cells (EpS6k1-6) and examined the response of the cells to TGFβ. Interestingly, a mutant version of p70^{S6k} that is catalytically inactive,

protected the cells (EpS6k4) against TGFβ-induced apoptosis and rather caused epithelial/mesenchymal transition in response to TGFβ. In my poster, I describe the characterisation of the mutant $p70^{S6k}$ enzymes that we have created and the mutant phenotype of the EpS6k cells.

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DISSECTING NEUROTROPHIN SIGNALING IN COCHLEAR NEURONS BY HSV-1 MEDIATED GENE TRANSFER

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Defective Herpes Simplex Virus Type 1 (HSV-1) vectors, or amplicons, permit transfer and functional studies of recombinant genes in postmitotic neurons. We have used this system to examine neurotrophin-mediated signaling in avian cochlear neurons at a developmental stage (HH E35), when these neurons show a well-characterized biological response to brain derived neurotrophic factor (BDNF). Dissociated cultures of cochlear neurons were transduced with an amplicon expressing human BDNF (pHSVbdnf) and analysed after 48 hours in culture. Expression of BDNF produced neuritogenesis in 60% of cochlear neurons, comparable with the maximal effect obtained using recombinant BDNF. Surprisingly, pHSVbdnf infected neurons revealed a glia-contact independent growth of neurites, whereas untransfected cultures treated with exogenous BDNF showed a strict association between neurons and glia cells. Transfer of conditioned medium from cultures transduced with pHSVbdnf to control cultures resulted in association of neurons and their extensions with glial cells. Therefore, expression of pHSVbdnf in cochlear neurons apparently results in a glia-independent mode of differentiation using an autocrine mechanism. In contrast in cultures treated with recombinant BDNF glia cells appear to act directly or indirectly as mediators of neurotrophin signaling.

In a different study we examined the potential of changing the neurotrophin responsiveness of avian cochlear neurons, which only respond to BDNF and NT-3, but not NGF. We therefore transduced neurons with an amplicon expressing the high-affinity receptor for NGF, TrkA (pHSVtrkA). Upon treatment with NGF, neurons infected with pHSVtrkA survived and differentiated reflecting their capacity respond to this factor. Interestingly, the extending fibres were short and ramified unlike the long bipolar fibres produced by BDNF or NT-3. Therefore, although cochlear neurons can be rendered NGF-responsive, they appear to respond in a morphologically distinct manner from other neurotrophins. The various phenotypes may reflect differences between the signaling pathways of individual neurotrophin-Trk receptor pairs inside these neurons.

DUAL ROLE OF INSULIN-LIKE GROWTH FACTOR-I IN INNER EAR DEVELOPMENT

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ABSTRACT

We have investigated the cellular mechanisms involved in the growth promoting effects of insulin-like growth factor-I (IGF-I) in the otic vesicle. The results suggest that IGF-I stimulated growth of the otic vesicle is associated with the induction of the expression of the nuclear proto-oncogene c-*jun*. IGF-I promoted the hydrolysis of a membrane glycosyl-phosphatidylinositol, which was characterised as the endogenous precursor for IPG. Both IPG and its synthetic analogue 6-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-D-*myo*-inositol-1,2-cyclic phosphate were able to mimic the effects of IGF-I on Jun expression. Anti-IPG antibodies blocked the effects of IGF-I, which were rescued by the addition of IPG or its analogue. These results along with previous work suggest that there is a release of IPG upon IGF-I binding to membrane receptors which, in turn, induces the expression of c-*fos* and c-*jun*. IPG release seems to be necessary to achieve full response to IGF-I in the stimulation of cell growth during embryonic development of the chicken inner ear.

In situ hybridisation analysis of c-*fos* and c-*jun* in the otic vesicle revealed that expression domains overlapped but were not identical. c-*fos* was broadly distributed in the otic vesicle, whereas c-*jun* was restricted to the dorsal region of the otic vesicle.

Effects of IGF-I were also studied on cochleo-vestibular ganglion neurones. In organotypic explant cultures of 70-72 h, IGF-I stimulated neurite outgrowth and expression of the neuron-specific antigen G4. This effect was neither mimicked by IPG nor blocked by anti-IPG antibodies.

Taken together, these results indicate that IGF-I displays multiple roles during development of the inner ear. Differential activation of signalling mechanisms may account for specific effects. The sequence involving the hydrolysis of membrane glycolipids and the expression of c-*jun* and c-*fos* proto-oncogenes is part of the mechanism that activates cell division. However, neurite outgrowth and expression of neuronal differentiation markers appear to be independent of generation of IPG.

List of Invited Speakers

Workshop on

SIGNAL TRANSDUCTION IN NEURONAL DEVELOPMENT AND RECOGNITION

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SIGNAL TRANSDUCTION IN NEURONAL DEVELOPMENT AND RECOGNITION

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