Instituto Juan March
de Estudios e Investigaciones

114 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Genetic Factors that Control Cell Birth, Cell Allocation and Migration in the Developing Forebrain

Organized by

P. Rakic, E. Soriano and A. Álvarez-Buylla

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T. Curran
J. Frisen
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Instituto Juan March (Madrid)

INDEX

Instituto Juan March (Madrid)

Instituto Juan March (Madrid)

PAGE

Instituto Juan March (Madrid)

Instituto Juan March (Madrid)

Introduction

P. Rakic

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During mammalian evolution the cerebral cortex has undergone a thousand-fold increase in surface area and a remarkable elaboration of cytoarchitectonic areas subserving distinct functions. Execution of these functions range from sensory perception and motor control to cognition and volition, depending on the precise connections with sub-cortical structures within as well as between various areas. Before these connections are established, within each species, an appropriate number of neurons will be generated in the proliferative centers and allotment of postmitotic neurons transported to their proper laminar and areal positions. If these fundamental cellular events are not properly carried out, all subsequent steps, including the formation of connections are jeopardized, resulting in serious brain malformations.

The main goal of this meeting was to present and discuss the latest information on the control of cell production, allocation and migration in the forebrain and how this impacts formation of cortical areas. These early events set up the species-specific framework for subsequent formation of neuronal connections and synaptogenesis in the organ that is the major target of evolutionary expansion and the principle site of human mental capacity and skills. Recent advances in methods of molecular and cell biology have enabled identification of specific genes and signaling molecules that regulate production, differentiation and communication between heterogeneous classes of cells in the embryonic germinal zones of the telencephalon. These genes determine the number, identity and vulnerability of neurons before they arrive at their final destinations. However, it is also important to "climb back" from the whole animal to molecules and genes in order to develop rodent and possibly primate models of cortical dysgenesis that mimic specific genetic or acquired cortical disorders.

The participants of this meeting shared a common interest in the quest for how the proper number and mixing of progenitors dedicated to specific phenotypes and functions is achieved within the proliferative zones and primordial cortical plate. They are engaged in research at different levels of analysis, from anatomical to molecular and from *in vitro* to *in vivo* model systems with a goal of placing the identified genes and transcription factors within a mechanistic perspective.

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The meeting was subdivided into 4 topics that focus on the main events: 1) cell proliferation and elimination; 2) migration and settling; 3) allocation; and, 4) deficits of cell production and migration, including human congenital malformations. Since these themes are intimately related and overlapping, many of the participants contributed more than a single topic, which provided a rich environment for discussion.

Several basic principles as well as the large number of genes underlying early cortical development were discussed. Progress has been made in illuminating mechanisms and identifying genes that control the size of the cerebral cortex through regulation of proliferation and cell death. Specific cell types appear to be generated in distinct locations within the telencephalon. Thus, cells from the perventricular zones primarily migrate radially and generate the characteristic laminar structure of the cerebral cortex as well as the positional information for areal specification. On the other hand, tangential migration introduces a subclass of interneurons that are generated in the ganglionic eminence but populate the cerebral cortex; the distinguishing molecules between these classes of neurons are being identified. Several of the participants demonstrated the considerable degree of regionalization of the cerebral cortex before the arrival of thalamic afferents and suggested the existence of several genes and transcription factors that may be involved in the determination of areal specification. Although input from the thalamus appears to have little influence on the initial regionalization of the cortex, thalamocortical afferents are essential for its proper maturation. The meeting was concluded by discussing the implications of this research for understanding the mechanisms of evolutionary expansion of cortical size as well as pathogenesis of cortical disorders.

P. Rakic

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Session 1: Production and elimination (I)
Chair: Pat Levitt

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Stereotyped patterns of progenitor division and neuronal generation

Sally Temple

Development of the cerebral cortex is a four dimensional process, critically involving the element of time as well as the molding of different cell types into a final intricate form. How the generous variety of cortical cells is produced is a mystery made even more intriguing by the fact that this process has to be precisely scheduled to allow the normal cytoarchitecture to unfold. To understand how diverse cortical cell phenotypes are generated over time, we have focussed on characterizing the development of early progenitor cells that give rise to the dorsal telencephalon.

Removing early mouse embryonic cortical progenitor cells from the neuroepithelium and growing them in isolation has allowed us to focus on the fate of individual progenitor cells and to characterize their developmental potential. These clonal analyses indicate that the early cortical neuroepithelium contains diverse progenitor cell types, including a population of stem cells that make both neurons and glia. Diverse cortical cell types that arise in these clonal cultures bear close resemblance to *in vivo* phenotypes. Furthermore, they are produced on a similar time-course to *in vivo* cells. Hence much of the information for producing diverse cortical cell types on time during development resides within the incipient cortical progenitor cells and the cell-cell interactions that they create as they proliferate.

Continuous, long-term time-lapse recording of how cortical progenitor cells produce progeny over time, reveals that asymmetric cell division, (mitoses that produce two different daughter cells) plays a central role. Repeated asymmetric cell divisions occur when neurons are being generated, and asymmetric divisions are prominent when stem cells switch from making neurons to glia. We suggest that these asymmetric division patterns are critically involved in organizing the production of diverse cortical cell types over time.

In this regard, vertebrate and invertebrate neural development processes may be similar. Grasshopper, *Drosophila spp.* and *Caenorhabditis elegans* neural development also involve repeated asymmetric cell divisions, producing progenitor cell lineage trees that are remarkably similar to those we see in mouse cortex. We suggest that the conserved lineage trees result from conserved underlying mechanisms. To test this hypothesis we have begun to investigate the protein numb, which is important for generating asymmetric cell divisions and hence diverse cell fates in *Drosophila* neural development. Examining a vertebrate homologue of numb, we find that it can be asymmetrically segregated in cortical progenitor cells undergoing mitosis, and that unequal distribution of numb is linked to the generation of asymmetric cell divisions and diverse cortical cell fates.

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Epigenetic control of neurogenesis

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Cell type diversity in the CNS arises through complex interactions among extrinsic and intrinsic mechanisms. Extrinsic signals affect survival, proliferation, and specification of cell type, but intrinsic properties of progenitor cells determine whether and how these signals are received and interpreted at specific stages of development. CNS progenitors are heterogeneous and include multipotent stem cells and more restricted progenitor cells. Progenitor heterogeneity could be generated by cell-intrinsic mechanisms or cell-cell interactions. Our recent work provides evidence that a developmental change in multipotent stem cells in the cerebral cortex is controlled by antagonistic extrinsic signals.

In the cerebral cortex, multipotent stem cells have distinct properties at different stages of development. For example, at early embryonic stages (i.e., E10.5-E13 in mice and E12-E15 in rats), stem cells are mitotically responsive to FGF2 but not to EGF-family ligands, and they tend to generate more neuronal than glial progeny. In contrast, later embryonic and postnatal stem cells are mitotically responsive to EGF and to FGF2, and tend to generate more glial than neuronal progeny. Viral transduction of early progenitor cells with a retrovirus expressing wild-type EGF receptors revealed that a threshold number of receptors was required to elicit several responses to EGF among cortical progenitor cells, including proliferation as a multipotent stem cell. This threshold level of EGF receptors is normally achieved by a small population of progenitor cells at E15-16 (mice) or E16-17 (rats). Lineage tracing has demonstrated that EGF-responsive stem cells are descendants of early stem cells that do not divide in response to EGF. These findings suggest that multipotent stem cells change during development, from an EGF-unresponsive to an EGF-responsive state.

We have found that developmental changes in the EGF-responsiveness of stem cells occur on schedule in explant and aggregate cultures of early embryonic cortex, but not in monolayer cultures. This suggests that cell-cell signaling is involved in the development of EGF-responsive stem cells. To determine whether such signals act to promote or inhibit an increase in EGF receptor expression, we analyzed the development of stem cells in aggregate cultures made with a mixture of early and mid-embryonic cells. We found that early embryonic cortical cells delayed the developmental change in EGF receptor expression and responsiveness among the mid-embryonic cells. BMP4 could mimic the effect of early cortical cells, reversibly inhibiting the developmental change in EGF-responsiveness among stem cells. To determine whether endogenous BMP normally regulates this property of stem cells, we infected early embryonic progenitor cells in explants with a retrovirus transducing a dominant-negative form of BMPR1B. A greater proportion of progenitor cells infected with this virus at E12 (rat) or E10.5 (mouse) expressed high levels of EGF receptor 5-6 days post-infection compared to progenitor cells infected with a control virus. Moreover, infection with dnBMPR1B virus resulted in premature mitotic responsiveness to EGF, generating neurospheres that could differentiate into neurons and glia. This suggests that endogenous BMPs normally inhibit EGF receptor expression and mitotic responsiveness to EGF.

Instituto Juan March (Madrid)

We expected that the neurospheres generated in response to EGF prematurely would be derived from progenitor cells infected with dnBMPR1B virus. Surprising, the majority were derived from uninfected progenitors. This observation suggested that BMPs might normally suppress changes in EGF receptor signaling by a non-cell-autonomous mechanism. Such a mechanism was recently reported in the developing limb, where BMP was shown to negatively regulate the expression of several FGFs. Thus BMPs in the cortex might regulate EGF-responsiveness by inhibiting the expression of a signal that promotes responsiveness to EGF, and a good candidate for such a positive signal is FGF2. To test this idea, explants of early embryonic cortex were exposed to FGF2 (1-10 ng/ml) for 2-3 days. Exposure to high levels of FGF2 (10 ng/ml) resulted in premature mitotic responsiveness to EGF family ligands. BMP4 selectively antagonized the effects of FGF2 on EGF responsiveness, but did not inhibit proliferation stimulated by FGF2. FGF2 also antagonized the inhibitory effect of early cortical cells in mixed-age aggregate cultures.

These findings suggest that normal changes in EGF receptor expression and responsiveness among cortical stem cells are regulated by antagonistic interactions between positive and negative extrinsic signals. BMPs are a strong candidate for the negative signal, FGFs for the positive signal. Why is this change in stem cells important? Once a high level of EGF receptors are expressed, stem cells can respond to EGF-family ligands in several ways. In addition to stimulating their proliferation, EGF can alter their migration in a chemotactic manner, and higher concentrations of ligand can bias their fate, favoring astrocyte differentiation. Regulation of EGF-responsiveness therefore has profound consequences for the proliferation, migration, and differentiation of stem cells in the cerebral cortex.

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Characterization of neural stem cells in the adult CNS

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Neurons are continuously generated in certain regions of the adult mammalian brain. These neurons derive from multipotent, self-renewing neural stem cells. Such stem cells can be cultured from the walls of the ventricular system of the adult rodent and human brain. Under certain conditions, adult neural stem cells can generate a large number of different non-neural cell types. We have found, by *in vivo* labeling experiments, cell sorting and *in vitro* cultures, that ependymal cells have neural stem cell properties in the rodent. Ependymal cells divide rarely to give rise to subventricular zone progenitor cells which generate neuroblasts that migrate to the olfactory bulb. In response to a spinal cord injury, ependymal cells lining the central canal are induced to proliferate and generate migratory progeny which differentiate to astrocytes and contribute to scar formation. Further studies on the regulation of stem cell differentiation may allow the development of strategies to stimulate neurogenesis in the adult brain.

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Identification and regulation of stem cells for adult neurogenesis

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Neuronal birth, migration and differentiation occur constantly in the adult brain. The adult brain germinal layers provide unique advantage for identifying cell types important for neurogenesis and studying how this process is regulated. I will focus my discussion on the primary neuronal precursors in the adult brains of birds and mammals.

In birds, new neurons are born in the ventricular zone (VZ) of the lateral ventricle and are incorporated throughout most of the telencephalon. The VZ of adult birds is composed of at least 2 classes of cells: ependymal cells and Type B cells. Ependymal cells, which can be identified by their ultrastructure and numerous long cilia, do not divide. In contrast, Type B cells, which have a single short cilium and an ultrastructure clearly different to that of ependymal cells, divide and function as the primary precursors for new neurons in adult birds.

The subventricular zone (SVZ) is a major site for neuronal birth in adult mammals. Neural stem cells can be isolated from the adult mammalian SVZ. Young neurons born in the SVZ of adult mice migrate as chains to the olfactory bulb where they replace granule and periglomerular neurons. Recent work in adult primates suggests that cells originating in the SVZ migrate into certain regions of neocortex. In many vertebrates an extensive network of pathways for chain migration exists throughout the SVZ. We have previously shown in mice that chains of migrating neuroblasts are ensheathed by slowly proliferating Type B cells, which have characteristics of astrocytes. Juxtaposed to the chains, are clusters of highly proliferative immature precursors called Typed C cells.

Are Type C cells the stem cells that give rise to the new neurons in the adult mammalian brain? Following antimitotic treatment with Ara-C, neuroblasts and Type C cells are eliminated, but some type B cells remain. Remarkably, the SVZ network rapidly regenerates. By 10 days, the migratory network is fully regenerated with the orientation and organization of chains resembling those of normal mice. These results indicated that Type C cells are not the primary precursors and allowed us to study the early stages of neurogenesis in the adult brain. Soon after Ara-C treatment, Type B cells divide and give rise subsequently to Type C cells and migrating neuroblasts. This suggests that during regeneration, the SVZ astrocytes act as the primary precursors. This is also the case in untreated mice: 1) SVZ Type B cells and not ependymal cells remain labeled with proliferation markers after long survivals in adult mice; 2) SVZ astrocytes specifically infected with a retrovirus give rise to new neurons in the olfactory bulb. Furthermore, SVZ astrocytes vitally labeled with an adenovirus give rise to cells that grow into multipotent neurospheres *in vitro*. Our results indicate that cells with features of astrocytes located in the SVZ of the adult mammalian brain act as neural precursors in both the normal and regenerating brain. Interestingly, SVZ astrocytes contain a single cilium similar to that seen in adult birds.

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The architecture that we have defined in the adult mammalian brain SVZ suggests that neurogenesis in this region may be regulated by the interaction of Type B cells with neighboring cells. I will present evidence using a novel *in vitro* system to study neurogenesis for the role of bone morphogenetic proteins (BMPs) and EPH receptors on the regulation of neural stem cell function. Finally, I will discuss the relevance of our findings to understand neuroepithelial development.

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Session 2: Production and elimination (II)
Chair: André M. Goffinet

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Orchestration of neuronal production, death and allocation in the cerebral cortex

P. Rakic

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The number and allocation of neurons in the given brain structure of each individual and species is determined by differential gene expression and the flow of intercellular information that underlies the logic of the system in which they participate. This is particularly evident in the cerebral cortex where specific cell classes are deployed into a radial and laminar arrays with a crystalline regularity that depends on the concerted production and migration of neurons from their place of origin to their final destination[1]. We have been studying these developmental events by analyzing cell production and by following their migration in transgenic and knockout mice, as well by testing the function of various candidate molecules *in vitro.*

We have identified several families of signaling and morphoregulatory molecules that are involved in control neuronal production by either modulation of the mitotic cycle (e.g., CamKII, GABA, Glutatamte, Notch)[2,3] or by regulating the rate of programmed cell death (e.g. Caspases 3/9, Jnks1/2)[4-7] . For example, we found that, mice lacking both copies of *Caspase 3 or 9* have an expanded pool of founder cells population generating supernumerary neurons that are deployed radially, resulting in enlarged cerebral surface with incipient convolutions[6]. We then examined the role of various antigens that may serve as mediators for neuron-glia interaction during migration of postmitotic cells (e.g. D4, NJPA1, GGF/neuregulins, integrins)[8-10]. In addition, we found that activation of specific ligand- and voltage-gated channel/receptors (e.g., NMDA-R2, N-type Ca receptor/channels) control the rate of neuronal movement[11,12]. We also found that cessation of cell movement requires both a specific signal, as well as a factor for dismantling neuron-glial cell junctional domains that are coupled with the cytoskeleton[13]. Lastly, we found that various transcription factors (e.g. Eph receptors, their ligands (ephrins), Tbr-1, Lhx-2, Emx-1, POU-domain containing genes) are expressed in distinct gradients and/or discrete domains in the large primate cerebral wall at early embryonic stages[14].

Together, these results suggest that intrinsic programs as well as communication between heterogeneous classes of cells may determine the species-specific size and primordial protomap that attract appropriate inputs for formation of synaptic connections which than can be validated through natural selection and individual experience[15].

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Instituto Juan March (Madrid)

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Functional radial units within the cortical ventricular zone

Arnold R. Kriegstein

At early developmental stages, the cortical ventricular zone consists of a mosaic of radially oriented, gap junction-coupled cell clusters. These clusters consist of precursor cells in G1, S, and G2 phases of the cell cycle, but do not appear to contain postmitotic neurons. Cells within these clusters uncouple through M phase and re-couple at G1 or S. A variety of manipulations that decrease gap junction conductance reversibly halt DNA synthesis. Coupling is thus required for cells to progress through at least S phase of the cell cycle. Indirect evidence based on observations of female mice expressing an X chromosome marker, suggests that coupled cell clusters may consist of clonally-related cells. Each cluster appears to behave as a discrete functional unit. Spread of intracellular signals, including calcium waves, is observed to be restricted to individual clusters of cells within the VZ. Moreover, coupled precursor cells express functional amino acid receptors for GABA and glutamate, and endogenous receptor activation appears to influence the transition of cycling cells from G1 to S phase. In addition to proliferating cells, each cluster also contains one or two radial glial cells. The coupling of precursor cells to radial glial fibers raises the possibility of intracellular signal propagation between VZ cells and postmigratory neurons in the cortical plate. Consistent with this hypothesis, stimulation of cells in the cortical plate can lead to increases in intracellular free calcium within individual VZ cell clusters. These proliferative units reflect a cellular organization within the ventricular zone that supports the radial unit hypothesis and indicates a potential compartmentalization of epigenetic influences at early stages of cortical development.

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Differential gene expression in the forebrain

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We only are beginning to examine the rules that govern differential gene expression in the brain. The forebrain, which in practice includes the midbrain, is special in the double sense that it receives mixed prechordal and chordal inductive influences and develops some of the most complex neural structures (thalamus, hypothalamus, telencephalon). Morphological analysis of a number of early gene expression patterns in the embryonic forebrain indicates the existence of expression boundaries distributed either longitudinally or transversally. Longitudinal gene domains bespeak of the hierarchy of gene effects related to dorsoventral (DV) patterning and reflect various equilibrium levels resulting from the competitive interaction of ventralizing and dorsalizing morphogens. Transversal gene patterns reflect anteroposterior (AP) patterning and, eventually, so-called segmentation effects. These processes are less well understood than DV processes, but may be compared also to diverse equilibrium states produced between competing cephalization and caudalization phenomena. The DV and AP patterning processes are less independent that is usually thought, given the frequent existence of point or regional singularities, where longitudinally expressed genes change into a transverse pattern, as occurs for instance at the isthmus or at the zona limitans intrathalamica, or appear with a different DV 'value' at a given AP level. This suggests that AP values affect DV patterning, leading to differential outcomes of the 'same' developmental process, and it is conceivable that DV values affect AP patterning (for instance, lack of interrhombomeric boundaries across the floor plate).

Later gene expression patterns in the forebrain are more varied in shape and position, although the diverse combinations strikingly adhere to the topology defined initially by the combined AP/DV patterning effects and therefore bespeaks of the participation of the early AP/DV boundary regions as local, short-range organizing centres. The interplay of a seemingly limited number of enhancer effects directing gene expression to specific loci increasingly calls our attention in the effort to better understand brain regionalization. Some of these late molecular codes seem to confer regional- or cell type-specific identities. Knowledge in this field clearly converges gradually with conventional morphological findings, insofar as phenotypes finally read out the diversity of superposed gene effects which cause individual neural structures. Cytoarchitectonic boundaries therefore are molecular boundaries whose genetic profile may be known or remain partially obscure.

Comparative neuromorphology increasingly can use gene expression patterns to distinguish developmentally homologous fields of the forebrain, as evidenced in recent studies on the telencephalic subpallium and pallium.

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A role for receptor protein tyrosine phosphatase alpha (RPTPα) in neuronal migration

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Receptor tyrosine phosphatase alpha (RPTPα) is intimately implicated in regulation of the activity of the tyrosine kinases SRC and FYN, and acts as a regulator of integrin signaling in fibroblasts[1]. The protein is itself a substrate for tyrosine phosphorylation, and is found associated in vivo with the adaptor protein Grb2, a regulator of Ras signaling. High levels of RPTPα expression are found in the nervous system, where it is found associated with the cell adhesion molecule contactin[2], and may mediate dephosphorylation of tyrosine phosphorylated Potassium channels[3].

To understand the physiological function of this PTP, we analyzed mice engineered to lack RPTPα expression. Histological analysis of RPTPα$^{-/-}$ animals revealed pleiotrophic developmental abnormalities in lamination of neocortex and hippocampus. The hippocampal stratum pyramidale was disorganized and contained reduced numbers of neuronal cell bodies. By contrast, an excess number of cell bodies were present in stratum oriens. Furthermore, use of BrdU birthdating revealed abnormal patterns of radial neuronal migration in RPTPα$^{-/-}$ cortex. Investigations are under way to identify how receptor tyrosine phosphatase α may tie into known signaling pathways involving tyrosine phosphorylated proteins critical to neuronal migration.

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Role of Sonic hedgehog and extracellular matrix glycoproteins in the production of cerebellar granule neurones

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The cerebellar cortex is formed from two distinct proliferative zones, a typical ventricular zone (VZ) and a displaced germinal zone, the external granule layer (EGL). Precursors of granule cells appear in the rhombic lip, just dorsal to the zone of Purkinje cell generation. Cells in the rhombic lip migrate up onto the surface of the anlage to constitute the EGL. After clonal expansion in the EGL, granule cells migrate through the field of differentiating Purkinje cells and set forth three layers- an outer molecular layer of granule cell axons and Purkinje cell dendrites, a layer of Purkinje cells, and an inner layer of granule cells. The signals involved in the subsequent steps of cerebellum development are being elucidated. The secreted protein Sonic hedgehog (SHH), within the EGL and from the Purkinje neurones, seems to act as a potent mitogenic signal to expand granule cell progenitors. In the search for signals involved in granule cells differentiation, in this study we have investigated the possible interaction between SHH and glycoproteins of the extracelular matrix. We show that Vitronectin (VN) is transiently expressed in the migrating Purkinje cells and thereafter in the internal part of the EGL, where granule cell precursors exit the cell cycle and commence migration. Furthermore, we show that in vitro, VN is able to partially antagonise the proliferative effect of SHH and to promote the differentiation of granule cell precursors. Based on these results we propose a model by which clonal expansion of the granule cell precursors is dependent on SHH mediated signals, whereas exit of the cell cycle and differentiation depends on SHH/VN mediated signals.

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Pathways and mechanisms of tangential neuronal migration in the developing mammalian forebrain

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Recent studies suggest that neurons born in the developing ventral forebrain migrate long distances perpendicular to radial glial and many of these cells reach the developing neocortex. This form of tangential migration, however, has not been demonstrated *in vivo*, and the sites of origin, pathways of migration and final destination of these neurons in the postnatal brain is not fully understood. Using the ultrasound guided transplantation we mapped in utero migratory pathways of cells born in the medial and lateral ganglionic eminences (MGE and LGE) directly in 13.5 day old mouse embryos. We demonstrate that MGE and LGE cells migrate along different routes to populate distinct regions in the developing brain. We show that LGE cells migrate ventrally and anteriorly to colonize the developing striatum, olfactory tubercle, nucleus accumbens and olfactory bulb. In contrast, we show that the MGE is a major source of neurons migrating dorsally and invading the developing neocortex. MGE cells migrate tangentially through the neocortical subventricular zone and differentiate into a transient neuronal population in the marginal zone and into a stable population of interneurons in the cortical plate. We propose that MGE cells are guided towards the neocortex by a selectively permissive environment and chemotactic gradients.

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Session 3: Migration and settling
Chair: Dennis D.M. O'Leary

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Regulation of neuronal migration in the developing CNS

Li-Huei Tsai

Programmed cell migration plays a pivotal role in the histogenesis of the mammalian central nervous system. In particular, the cytoarchitecture of the cerebral cortex and cerebellum relies on distinct modes of migration of multiple cell types in different developmental stages. A small protein ser/thr kinase Cdk5 in conjunction with its regulatory partners, p35 and p39, plays an indispensable role in these processes. We and others showed that in the absence of Cdk5, neuronal migration is impaired resulting in cell positioning defects in the cerebral cortex, hippocampus, cerebellum and other hind brain structures. Cdk5 may function in neuronal migration at least in part, through its impact on cytoskeletal dynamics and neuronal adhesion. In light of the similar defects displayed by the Cdk5 or p35 deficient mice and reeler/scrambler, we employed genetic and biochemical analyses to determine if Cdk5 interacts with the *reelin/disabled* pathway. We have evidence indicating that Cdk5 can be regulated by the reelin signaling pathway, and conversely, *disabled* can be modulated by Cdk5. These observations suggest that Cdk5 cooperates with the reelin/disabled pathway to regulate neuronal migration during CNS development. In addition to Cdk5 and reelin signaling cascades, the type I lissencephaly gene product, Lis1 also regulates neuronal migration. We found that Lis1 interacts with cytoplasmic dynein and upregulates dynein function. Furthermore, we identified a Lis1 interacting protein Nudel (NudE-like) that also interacts with cytoplasmic dynein. Interestingly, Nudel is an *in vivo* substrate of Cdk5 and phosphorylation of Nudel by Cdk5 changes its subcellular distribution. Together, our observations indicate crosstalk between Cdk5 and Lis1 pathways in regulation of neuronal migration and suggest that Cdk5 activity may regulate cytoplasmic dynein.

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Molecular basis of cell migration

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We are interested in molecular mechanisms guiding the direction of neuronal migration. We have shown that a guidance cue for axons functions similarly in guiding neuronal migration. The guidance cue that we are currently focusing on is a secreted protein named Slit, which binds to a transmembrane receptor Roundabout. The concentration gradient of the Slit protein is essential for its function as a repulsive cue for both axons and neurons.

We have asked the question whether neuronal guidance cues can function on no-neuronal cells. We will present evidence that the same molecule can also function outside the nervous system. These findings suggest that mechanisms involved in regulating cell migration are conserved in most, if not all, cell types.

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Morphological aspects of defective brain patterning in Pax-6 mutant mice

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Pax genes are members of a large superfamily of developmental control genes that encode transcription factors. Failure of expression of these regulatory genes produce severe brain alterations and influence the developmental pattern of connectivity. In the mouse, homozygous mutant embryos for Pax-6, one of the members of this gene family, and known as *Small eye (Sey)*, lacks nose and eyes, and their brains become dominated by a broad spectrum of neuronal migration disorders and several malformations (Walther and Gruss, 1991; Schmahl et al., 1993; Stoykova et al., 1996; López-Mascaraque et al., 1998; Valverde et al., 2000; Jiménez et al., 200).

In this study we have used mice carrying the mutation designated as *Sey* ^{Neu}, which has a similar phenotype to *Small eye (Sey)*, to describe, in homozygous animals, the presence of a neural structure in the rostral pole of the telencephalic vesicle resembling the olfactory bulb (OB) of wild-type animals, combining several morphological approaches cell markers and axonal tracers. Among other brain alterations, we also found that the mammillary bodies apparently are not affected in the mutant mice; the mammillotegmental bundle had normal development, but the mammillothalamic tract was missing.

In mutant mice, the prospective OB, that we have named *Olfactory Bulb Like Structure* (OBLS), consists of two well-differentiated areas, a large one called A1 measuring about 500 µm, and a smaller one approximately 100 µm in diameter. A distinctive OBLS formation was first observed at embryonic day 12 (E12). The identification, morphological description and projections of the cells forming the regions A1 and A2 were examined by injecting DiI in the ventrolateral surface (a region corresponding to the prospective lateral olfactory tract) of fixed mutant mouse brains at different developmental ages. Retrogradely labelled cells were found throughout the entire A1 area, clearly delimiting this region. Some cells resembled immature mitral neurons.

To further investigate cellular distribution in the presumptive OB (OBLS), and to determine whether cell generation in the OBLS corresponds to that found in the control OB, we performed a series of birthdating experiments analyzing the incorporation of BrdU following short and long post-injection survival times. We also examined different molecules that are known to be expressed in the OB of control mice (Jiménez et al, 2000). These experiments provided indirect evidence suggesting that area A1 of the mutant OBLS correspond to a partially developed OB, while the A2 would correspond to the AOB.

In mutant embryos, the nasal placode never develops, therefore olfactory nerves are absent and the OB, as we know it, simply fails to develop (Hogan et al., 1986; De Carlos et al. 1996). However, the presence of an OBLS and the persistence of projections in the prospective lateral olfactory tract in the absence of olfactory neuron signals suggest that the initial development of the central olfactory tract projections does not depend on the olfactory epithelium induction. Recent studies show the existence of early-generated neurons designated as *lot cells* that specifically mark the future site of the lateral olfactory tract. These cells are generated from the neocortex following a tangential migration stream toward the lateral olfactory tract position (Tomioka et al., 2000). We consider that these cells, which have been suggested to act as guidepost for mitral cell axons, are also present in mutant mice in view of the persistence of projecting axons from the OBLS in the prospective lateral olfactory tract.

The Pax-6 deficiency in mice has been reported to cause, among other malformations, an abnormal diencephalic morphology with an enlarged third ventricle and loss of the dien-/mesen-

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cephalon border (Warren and Price, 1997), pathfinding defects of some of the major axonal tracts (Kawano et al., 1999), and abnormal differentiation of both dorsal and ventral thalamus (Stoykova et al., 1996). All these studies confirmed that expression of *Pax-6* is necessary not only for specification of the distinct brain territories but also for the establishment of proper neuronal connections within and across the different prosomeric boundaries. The mammillary bodies, which are derived from the mammillary area or the basal zone of prosomere 4, lie outside the expression domain of *Pax-6*. However, the mammillothalamic tract - collateral branch of the principal mammillary tract - seems to originate within an ill-described basal region at the base of the *zona limitans*, where *Pax-6* is expressed intensely. In this study we found that the mammillotegmental bundle appeared normal in the mutant, but the mammillothalamic tract was lacking entirely (Valverde et al., 2000). We have extended these observations to the study of the development of the mammillothalamic tract in wild type mice and correlate the absence of this collateral tract with alterations found in this area in the mutant, suggesting a possible relation of the Pax-6 expression domain.

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Role of Cajal Retzius cells

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Cajal Retzius (CR) cells are transient, pioneer neurons located in the developing layer I of the neocortex and hippocampus. Although they were discovered more than a century ago, their functions have been controversial until recently. We have started to address the functions of CR cells using several paradigms. Experimental ablation of CR cells in the developing layer I of newborn mice leads to malpositioning of cortical migrating neurons destined to upper layers II-III. This indicates that CR cells are essential for proper cortical migration throughout corticogenesis, even long after the split of the preplate. Studies with glial markers indicate that this alteration is mediated by abnormal transformation of radial glial cells. Further, transplantation experiments in the developing cerebellum suggest that CR cells exert an attractive influence on migrating granule cells and that they induce the re-expression of radial glia markers in the adult Bergman glia.

Expression studies in the developing neocortex indicate that Reelin is expressed from middle stages of corticogenesis in layer V, in addition to CR cells in layer I. This pattern of expression is regulated by thyroid hormone in a positive way. Lastly, analyses of transgenic mice overexpressing BDNF indicate that this leads to alternate patches of CR cells and non-CR cells in layer I, which creates a mosaic-like structure in the neocortex. However, CR cells appear to express normal levels of Reelin. *In vitro* studies of recombinant Reelin and blocking reagents against Reelin receptors are underway to characterize the effects of Reelin on migrating neurons.

Secondly, in the developing hippocampus ablation studies have shown that CR cells are essential for the ingrowth of hippocampal afferents. Recent data obtained using ectopic CR cell transplantation suggest that this function is mediated by chemoattraction and that CR cells are also essential for the regeneration of axotomized hippocampal connections.

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Session 4: Allocation
Chair: Christopher A. Walsh

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The role of hepatocyte growth factor/scatter factor in the developing telencephalon

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Cortical interneurons arise from the proliferative zone of the ventral telencephalon, the ganglionic eminence. Recent studies from other laboratories have demonstrated the migration of postmitotic, GABAergic neurons around the cortical-striatal sulcus into the developing neocortex, where initially they are restricted to the cortical marginal and intermediate zones. The molecular cues that guide this migration are unknown, although repulsive signals from the midline (slit) initially may direct the cells laterally. The spatial patterns of migration and settling patterns of the interneurons reflect our recent discovery of complementary expression patterns of hepatocyte growth factor/scatter factor (HGF/SF) and its receptor, C-Met in the forebrain. HGF/SF can serve as a motogen, both to induce cell movement away from a source and prevent cell movement into regions of high HGF/SF concentration. We will discuss experiments that document the biological scatter activity of HGF/SF in the developing telencephalon and show that addition of HGF/SF perturbs cell migration from the ganglionic eminence in organotypic slice cultures. We also will describe our most recent findings of altered cortical interneuron development in a knockout mouse that exhibits reduced HGF/SF and C-met activity. This work was supported by NIMH grant MH455507 to PL and NIMH grant F32 MH12651 to EMP.

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Genetic control of cortical parcellation

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Dorsoventral regional specification within the telencephalon is controlled by transcription factors that respond to signals produced by patterning centers located along the midline of the rostral neural tube. Mutations in these transcription factors in mice have been an efficient method to study this process. I will describe the effects of mutating the Nkx2.1, Gsh2, Dlx1/2, Pax6 and Lef1 transcription factors in patterning ventral, intermediate and dorsal telencephalic structures. A hypothesis that is derived from interpretation of these studies is that regional specification is tightly linked with cell type specification. Thus, ventral-most specification appears to produce cholinergic neurons, intermediate specification produces GABAergic neurons, and dorsal specification produces glutaminergic neurons. Regions that don't produce a particular cell type, get these neurons through their tangential migration. For instance, there is evidence that the cerebral cortex doesn't produce GABAergic neurons; rather these neurons migrate to the cerebral cortex from the basal telencephalon where they are specified.

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Regulation of neocortical arealization and thalamocortical axon pathfinding

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The specification and differentiation of neocortical areas are controlled by intrinsic genetic programs and extrinsic influences such as thalamocortical afferents (TCAs). To identify genes differentially expressed across the developing neocortex, and potentially involved in neocortical arealization, we carried out a differential display PCR screen using RNAs from motor and visual areas of E16 rat (Liu et al., 2000). Several differentially expressed clones were identified and will be described. To address the interplay between intrinsic and extrinsic mechanisms, we analyzed the requirement of TCAs in establishing areal patterns of gene expression in embryonic mouse neocortex. Several cadherins and regulatory genes exhibit normal expression patterns in Mash-1 mutants which fail to develop a TCA projection. Thus TCAs are not required for the establishment of these expression patterns suggesting that their regulation is intrinsic to neocortex (Nakagawa et al., 1999). Further studies indicate that these intrinsic patterns of gene expression, as well as the process of arealization, are regulated by the transcription factors Emx2 and Pax6, which are expressed in countergradients in the neocortical ventricular zone. Our evidence suggests that areas are disproportionately altered in Emx2 and Pax6 mutants in opposing manners, and that Emx2 and Pax6 cooperate to regulate arealization and confer area identity to cortical cells (Bishop et al., 2000). Data consistent with this interpretation has also been obtained using recombinant adenovirus to overexpress Emx2 (A. Leingartner, L. Richards, D. O'Leary, unpub. obs.). Finally, since TCAs are critical for neocortical differentiation, we have studied TCA pathfinding. Our findings implicate several spatially and functionally distinct guidance cues, including netrin-1, slit-1 and slit-2, and specific cell domains in the forebrain, in TCA pathfinding (Tuttle et al. 1999; Braisted et al., 1999, 2000a,b).

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Acquisition of areal identity : a study using expression of H-2Z1 a somatosensory area specific mouse transgene

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The H-2Z1 mouse lacZ transgene is expressed postnatally in layer IV neurons of the somatosensory area. *In vivo*, H-2Z1 expression coincides with invasion of the cortical plate by thalamic afferents. We therefore investigated the role of thalamic innervation in the onset of H-2Z1 expression. For this purpose, we examined the pattern of H-2Z1 expression in perinatal cortical explant, in reeler mutant and MaoA deficient mice, or in animals which had received neonatal lesions affecting the somatosensory cortex or the thalamocortical projection. We found that, around birth, a switch occurs in the control of H-2Z1 expression: whereas H-2Z1 expression developped autonomously *in vitro* in embryonic parietal cortex explants in the absence of thalamic fibers, a transient requirement for a thalamic axon derived signal was observed around birth. This property has interesting implications for the plasticity of cortical areas in development and evolution. We have used H-2Z1 embryos in heterotopic transplantation experiments both *in vivo* and *in vitro* to investigate the chronology of determination of areal identity. From the onset of neurogenesis (E11.5), the cerebral cortex may be subdivided in domains fated to express or not the somatosensory area specific transgene. Determination occured one day later by E12,5. Thus, whereas region specific cell-cell interactions produce neurons which are competent to express area specific traits, their actual expression *in vivo* depends on late thalamocortical interactions. This may allow a precise alignment of the domain expressing "somatosensory cortex properties" (i.e. H-2Z1 positive) with the thalamocortical projection field. This plasticity also opens the possibility to form new cortical domains during development or evolution.

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The Dab-1 gene: Role in the Reelin signaling pathway and effects on Tau phosphorylation

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The extracellular protein Reln controls neuronal migrations in parts of the cortex, hippocampus and cerebellum. In vivo, absence of Reln correlates with up-regulation of the docking protein, Dab1, and decreased Dab1 tyrosine phosphorylation. Loss of the Reln receptor proteins apoER2 and VLDLR results in a Reln-like phenotype accompanied by increased Dab1 protein expression. Complete loss of Dab1, however, recapitulates the Reln phenotype. To determine if Dab1 tyrosine phosphorylation affects Dab1 protein expression and positioning of embryonic neurons, we have identified Dab1 tyrosine phosphorylation sites. We then generated mice in which the Dab1 protein has all the potential tyrosine phosphorylation sites mutated. This mutant protein is not tyrosine phosphorylated during brain development and is not upregulated to the extent observed in the reln or the apoER2 and VLDLR receptor mutants. Animals expressing the non-phosphorylated Dab1 protein have a phenotype similar to the dab1 -null mutant. We conclude that Dab1 is downregulated by the Reln signal in neurons in the absence of tyrosine phosphorylation. Dab1 tyrosine phosphorylation sites and not downregulation of Dab1 protein are required for Reln signaling. In addition, we have examined Tau phosphorylation state in brains of homozygous dab1 mice. We find that Tau phosphorylation is increased by the Dab1 mutation, dependent on genetic background. Tau hyperphosphorylation appears to correlate with early death. Strain backgrounds with low Tau phosphorylation show normal lifespan, despite exhibiting the developmental defects associated with Dab1 deficiency. This suggests that Dab1 tyrosine phosphorylation directly or indirectly inhibits Tau phosphorylation, dependent on other genetic or epigenetic effects.

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Session 5: Defects of migration
Chair: John L.R. Rubenstein

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Reelin and early cortical development

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Reelin, the extracellular glycoprotein defective in reeler mice, plays a key role in cortical development. In the embryonic cortex, Reelin is secreted by neurons in the marginal zone, including Cajal-Retzius cells, and governs the architectonic organization of the cortical plate [1]. Full-length Reelin is about 400 kDa and composed of an N-terminal region with weak similarity to F-spondin, followed by a segment without specific feature, and then a succession of eight repeats of about 350 residues centered on an EGF motif; the protein ends with a short basic stretch [2]. In the tissue, most of the protein is cleaved at two sites by a Zn^{++}-dependent proteinase activity. The two cleavage sites are located approximately 150-180 kDa and 300-310 kDa from the N-terminus. Experiments with various inhibitors suggest that the enzyme responsible for Reelin processing does not belong to the matrix metalloproteinase family. Thus far, this enzyme could not be identified. The functional implications of processing are unknown; however, it has been detected in all species examined thus far, including fishes, reptiles and mammals, and is thus evolutionarily conserved.

Reelin acts via two receptors of the lipoprotein receptor family, namely VLDLR and ApoER2, and binds specifically to their extracellular portion [3, 4]. Several Reelin constructs are being tested for their ability to bind to receptors, using co-immunoprecipitation experiments with Fc-tagged receptor constructs (gift of J. Herz). Recombinant proteins up to the second repeat are apparently not implicated in receptor binding. Constructions containing Reelin repeats 7 and 8 bind very weakly. By contrast, constructions that contain repeats 3-8 bind very well, whereas deletion of repeats 3 and 4 reduce binding drastically. Although more data are required, these preliminary results suggest that the central region of reelin, repeats 3-6, is particularly important in receptor binding. The Reelin signal is transmitted in the cell via the adapter Disabled1 (Dab1) [5-7], and tyrosine phosphorylation of Dab1 is stimulated when Reelin is added to neurons in vitro [8]. It will be important to correlate receptor binding with the Dab1 phosphorylation assay. Thus far, other partners of the intracellular cascade have not been unequivocally identified, although a link between lipoprotein receptors, Dab1 and tau phosphorylation suggests that the cytoskeleton is probably implicated.

Despite considerable progress, a lot of uncertainties remain regarding the action of Reelin on cortical neurons. Studies of reelin and Dab1 expression in the developing cortex in several species are compatible with the hypothesis, first proposed by A. Pearlman, that Reelin may provide a stop signal to migrating neurons. This hypothesis is mostly based on the observation that Reelin-positive cells are commonly surrounded by profuse extracellular space, as if target neurons were prevented from migrating in a Reelin rich environment. By using the 3D collagen assay, we have tested a putative action of Reelin on growing cortical axons. When reeler cortical explants were tested in the presence of reelin-transfected cells (source of full-length Reelin) or of normal explants (source of processed Reelin), no effect of Reelin could be detected, suggesting that the action of Reelin occurs mostly at the level of dendrites and/or cell bodies. Neuron migration requires at least the elongation of the leading tip, the engagement of the nucleus in the cytoplasmic furrow - a process referred to as nucleokinesis - and the retraction of the trailing process. Reelin might stop migrating neurons

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by regulating nucleokinesis [9]. Such a mechanism would explain that Reelin does not interfere with growth cone elongation, but prevents cell bodies from invading areas where the extracellular milieu contains Reelin. Arguments, admittedly indirect, for this view, are that several of the genes involved in neuron migration disorders, particularly Lis1 and Doublecortin, seem to act also on nucleokinesis. Another possibility to explain actions of Reelin is that it promotes adhesion among cortical plate cells. Thus far, however, these hypotheses on the cell biological actions of Reelin remain hard to test until a reliable in vitro system is developed to study not only radial migration but also early cortical plate formation.

Another set of questions concerns the regulation of reelin expression. Comparative studies show that the amplification of reelin mRNA and protein levels is a key factor in cortical development in mammals as compared to other species. That this amplification of reelin expression in marginal zones was necessary to allow the increase of the cortex during evolution, particularly in primates, is evident from observations of reeler mice, and even more so from the observation that cortical foliation is drastically reduced in humans with defective reelin, (as shown by C. Walsh). Studies of the reelin promoter region will probably require the analysis of very large introns in the 5' part of the gene, that are likely to contain regulatory regions. The promoter and the first introns of reelin have been cloned as a PAC and will be characterized by transgenesis. Thus far, a few reports suggest that the factors Emx2, Pax6 and p73 may influence reelin expression, but the mechanisms remain to be demonstrated. For example, p73 KO mice have decreased levels of reelin but their cortical plate develops almost normally. A more direct effect of Tbr1/CASK on the reelin promoter has been suggested but similarly needs to be better defined. Cotransfection with these factors and reelin promoter constructs are under way to test the role of these factors.

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Role of the Reelin pathway in the control of brain development

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The formation of the mammalian nervous system requires a choreographed series of cellular interactions that involve dramatic reorganization and cell migration during specific time periods. These processes are defective in several strains of ataxic mice. The mouse mutants *reeler*, *scrambler*, and *yotari* provide unique opportunities for deciphering the complex molecular mechanisms underlying vertebrate brain development. In these mice, neuronal migration is disrupted throughout the brain, resulting in disorganization of many laminar structures. The *reeler* gene, *reelin*, encodes a novel extracellular protein that is secreted by pioneer neurons in various structures of the developing brain. The gene disrupted in *scrambler* and *yotari*, *disabled-1* (*dab1*), encodes an intracellular adapter-like protein similar to the *Drosophila disabled* gene product. Dab1 protein, but not mRNA, is present at a higher level in the ectopically placed neurons in *reeler*. This suggests that Dab1 accumulates in the absence of a Reelin-evoked signal. Dab1 is phosphorylated on tyrosine after treatment of cells with Reelin. It is believed to function as an adaptor molecule in a signaling pathway that is required for the regulation of neuronal positioning during development. Mice lacking the very low density lipoprotein receptor (VLDLR) and the apolipoprotein receptor 2 (ApoER2) also exhibit a *reeler*-like phenotype. We found that Reelin binds directly to lipoprotein receptors on the cell surface. Binding is inhibited in the presence of apoE3 and apoE4 but not in the presence of apoE2. These results imply that Reelin is a ligand for lipoprotein receptors. Dab1 interacts with a motif (NPxY) that is present in the cytoplasmic domain of lipoprotein receptors and the amyloid precursor gene family (APP). These findings suggest that the Reelin pathway, which controls cell positioning in the developing brain, may have additional functions that impact on pathophysiological processes associated with neurodegeneration. We are addressing the role of Reelin in controlling cell migration by ectopic expression in transgenic mice. The results of these studies challenge simple interpretations of effects of Reelin on cell positioning during development. We have also found that the function of the Reelin pathway is not restricted to cell positioning as there is a defect in retinal circuit formation in the mutant mice.

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Instituto Juan March (Madrid)

Lessons from the cerebellar system : The neurophilic migration of precerebellar neurons

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The vanguard ideas of Cajal (1892) of chemotactic mechanisms regulating the orientation of axon growth cones have now become a reality: developing axons are guided by a variety of molecules that can be diffusible (long-range cues) or bound to a substrate (short-range cues), and exert either attractive or repulsive effects (Tessier-Lavigne and Goodman, 1996). Are similar kinds of diffusible signals also implied in the guidance of neuronal migration ? My presentation will provide some evidence showing that neurophilic migration (Rakic, 1990) is also guided by long-range cues and, more importantly, that at least one of these cues, netrin-1, is common to both processes. Thus, the main difference between axon guidance and neuronal migration is the process of nucleokinesis, that only occurs in the latter.

Precerebellar neurons follow circumferential migratory routes. The basilar pontine (BPN), external cuneatus (ECN), lateral reticular (LRN) and inferior olivary (ION) nuclei all migrate from their ventricular neuroepithelium (the lower rhombic lip), along the external aspect of the brainstem, to their final locations (Bourrat and Sotelo, 1990). However, while the cell bodies -specified by the cell nucleus moving through its respective leading process- of the LRN and ECN neurons cross the midline, those of the BPN and ION neurons move up to the floor plate, but stop before crossing the midline. These anatomical results prompted us (Bourrat and Sotelo, 1990) to propose a double role for the floor plate in the migration of precerebellar neurons. First, it would attract the migrating neurons towards the midline. Second, it would be a decision-making region ("stop" or "go") specifying whether the projection of these neurons will be crossed or uncrossed.

Study of the phenotype of the olivocerebellar system in mice with inactivation of the netrin-1 gene. The floor plate is an important source of netrin 1, one of the first discovered chemotactic molecules in axon guidance. We found that the patterns of expression of netrin 1 and its receptors (DCC, neogenin and members of Unc5 family) in wild-type mouse embryos were consistent with a role of netrins in directing the migration of precerebellar neurons. We studied mice deficient in netrin-1 production, and found that up to P0 (these mice die at birth) the number of BPN and, particularly, ION neurons was remarkably decreased. In the ION, the normal lamination failed to develop and the surviving neurons established an abnormal projection with the cerebellum : the projection was ipsilateral instead of being contralateral as in normal mice. These results establish a requirement for netrin-1 in the migration of precerebellar neurons. Netrin-1 may function as a specific guidance cue for the initial steps of migration, and later as a stop signal for ION neurons. The results also establish a requirement for netrin-1, directly or indirectly, for the survival of precerebellar neurons (Bloch-Gallego et al., 1999).

Netrin1 acts as an attractive cue for precerebellar neurons. Using explants of the lower rhombic lip dissected from E12 to E14 wild-type mouse embryos, we have directly analyzed netrin-1 function in the migration of precerebellar neurons. The explants were grown for 24-72 hours in collagen, adjacent to control or netrin-1 transfected EBNA-293 cells. We showed that netrin-1 exerts a strong chemoattractive effect on migrating precerebellar neurons. Netrin-1 promotes the exit of postmitotic neurons from their ventricular zone, and the movement of their cell nuclei through the leading processes. In the absence of netrin, only few neurons migrated in isolation, whereas in the presence of netrin, they migrated as clusters, in association with one another, forming thick fascicles of neurites, typical of the neurophilic way of migration. Thus, molecular mechanisms considered as primarily involved in axon guidance appear also to steer neuronal cell migration (Alcantara et al. 2000).

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Functional analysis of genes involved in Lissencephaly

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Keywords: lissencephaly, mouse model, brain development, cytoskeleton, WD repeat protein. Formation of the brain structure in human is a complex process that occurs during several months of prenatal development. One of the most striking features of the human brain is characteristic convolutions. These convolutions are lacking in a severe human brain malformation known as lissencephaly (lissos = smooth, encephaly = brain). About one in 30,000 life births are affected with this disease. Lissencephaly patients have a reduced life expectancy and a severe mental retardation. So far, two genes have been found to be mutated in lissencephaly; LIS1 located on chromosome 17 and Doublecortin and X-linked gene.

LIS1

LIS1 is a WD repeat protein, belonging to a big family of proteins with this protein motif, including the β-subunit of G proteins. LIS1 was found to be a subunit of an intracellular enzyme PAF-AH (platelet-activating-factor acetylhydrolase). The LIS1 subunit does not have any catalytic activity and its regulatory role is not known yet. In addition, we have discovered that LIS1 interacts with tubulin and microtubules. LIS1 affects parameters of dynamic instability of the microtubules. We hypothesize that this property is of importance during migration of neurons in the developing brain. The interaction with microtubules is conserved during evolution. The LIS1 protein is highly conserved among different species: in human, mouse, bovine, and chicken, the amino acid sequence identity levels exceeds 99%. In *Aspergillus nidulans* (a fungus) the *nudF* gene shares homology to *LIS1* (42% identity). *NudF* is required for microtubule-dependent nuclear migration in this organism. One mode of regulating protein interactions is via phosphorylation, and indeed LIS1 is a phospho-protein and its phosphorylation is regulated during development. The LIS1 locus is very dosage sensitive. We have demonstrated that all truncations and point mutations result in a protein that is not properly folded. In order to study the function of LIS1 during development we are using the mouse model. We have mutated the endogenous Lis1 gene. Mice that are homozygote to this mutation die during early embryogenesis. Heterozygote mice exhibit a phenotype in the developing brain that can explain some of the lissencephaly features. One of the abnormalities observed was retardation of innervation of the cortex by thalamocortical fibers. The neurons of the mutant mice migrated slower, confirming the general belief that lissencephaly is a defect in neuronal migration.

Doublecortin

X-linked lissencephaly is a severe brain malformation affecting males. Recently it has been demonstrated that the *doublecortin* gene, is implicated in this disorder. Doublecortin was found to bind to the microtubule cytoskeleton. *In vitro* and *in vivo* assays demonstrate that Doublecortin stabilizes microtubules and causes bundling. The results obtained in this study with Doublecortin and previous work with LIS1 emphasize the central role of regulation of microtubule dynamics and stability during neuronal morphogenesis. An autosomal human

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gene, KIAA0369, with a high level of similarity to doublecortin has been cloned from human adult brain. This gene product contains a kinase domain in addition to a doublecortin-like domain. Three cDNA products of this gene were cloned: one, doublecortin-like kinase, the second containing only the doublecortin-like region, and the third containing only the kinase domain with no calcium calmodulin binding domain, a homolog of the previously cloned rat CPG16 gene. We studied doublecortin-like kinase expression and conclude that Doublecortin-like kinase (DCLK) is expressed in multiple regions of embryonic brain including the developing cerebral cortex. Ectopic expression of results in colocalization with microtubules, and phosphorylated DCLK copurifies with microtubules during assembly from embryonic brain extract. We demonstrated that DCLK is a microtubule-associated active protein kinase expressed in growth cones of postmitotic neurons.

LIS1 and DCX

Mutations in either LIS1 or DCX are the most common cause for type I lissencephaly. We detected that LIS1 and DCX interact physically both *in vitro*, and *in vivo*. Epitope-tagged DCX transiently expressed in COS cells can be co-immunoprecipitated with endogenous LIS1. Furthermore, endogenous DCX could be co-immunoprecipitated with endogenous LIS1 in embryonic brain extracts, demonstrating an *in vivo* association. The two protein products also colocalize in transfected cells and in primary neuronal cells. Using fragments of both LIS1 and DCX the domains of interaction were mapped. LIS1 and DCX interact with tubulin and microtubules. Our results suggest that addition of DCX and LIS1 to tubulin enhances polymerization in an additive fashion. We conclude that LIS1 and DCX cross talk is important to microtubule function in the developing cerebral cortex.

Objectives and Future Directions

Our general objective is to understand corticogenesis through analysis of genes involved in human neuronal migration disorders. Our Future directions include; (1) Developmental functions elucidation by generation of mouse models for Doublecortin and Doublecortin-like kinase. (2) Identification of additional functions of LIS1, Doublecortin and Doublecortin-like proteins by isolation of interacting proteins. (3) Dissecting signaling pathways using DNA microarrays and tissue culture models.

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Making up your mind: genes that regulate human cerebral cortical development

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Although reeler is one of the best known murine neurological mutants, its relevance for human disease has been uncertain. We identified a human autosomal recessive brain malformation, lissencephaly (which means "smooth brain") with cerebellar hypoplasia (LCH) with anatomical abnormalities analogous to those of the reeler mouse. LCH shows lissencephaly (usually a sign of abnormal neuronal migration in the cortex), and severe anomalies of cerebellum, hippocampus and brainstem. LCH maps to 7q22 and is associated with human RELN gene mutations that disrupt splicing, resulting in low or undetectable amounts of Reelin protein.

Although Reelin encodes a large extracellular matrix protein, its mode of action has been uncertain. Recent work shows that Reelin acts as a stop signal for migrating neurons, causing them to dissociate from radial glial cells. Moreover, Reelin binds to $\alpha 3\beta 1$ integrin, protocadherins, and LDL superfamily receptors, but its antimigratory effect appears to depend upon $\alpha 3$ integrin. Therefore, Reelin appears to have direct anti-adhesive effects, as well as activating downstream signaling cascades.

The finding that human Reelin mutations cause lissencephaly suggests that the Reelin pathway might ultimately interact with other human lissencephaly genes, such as LIS1 and DCX. Although LIS1 and DCX are microtubule-associated proteins, their precise cellular mechanisms of action are unknown. While DCX binds $\alpha\beta$ tubulin directly, Lis1's interaction with tubulin is less clear. We identified a Lis1 interacting protein encoded by a mouse homolog of *NudE*, a nuclear distribution gene in *A. nidulans* and a multicopy suppressor of the *LIS1* homolog. mNudE is located in the centrosome/microtubule organizing center (MTOC), and interacts with several centrosomal proteins including pericentrin and γ-tubulin. Overexpression of mNudE dissociates γ-tubulin from the centrosome and disrupts the normal interphase microtubule network. Missense *LIS1* mutations that cause lissencephaly interfere with Lis1-mNudE binding. Misexpression of the Lis1 binding domain of mNudE in *Xenopus* embryos causes defects in the architecture and lamination of the CNS. Our results suggest that Lis1-mNudE interactions regulate microtubules through dynamic reorganization of the MTOC, and that Lis1-mNudE interactions are essential for normal neuronal migration.

Finally, many other human developmental disorders of the cortex exist but the responsible genes are not yet known. Further genetic analysis of these disorders is likely to enlighten us further about how our brains developed, both in terms of ontogeny and phylogeny. Supported by the NIH and the March of Dimes.

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POSTERS

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Altered migration of cortical gabaergic neurons in mice that overexpress BDNF

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Neurotrophic factors have recently been implicated in neuronal migration. Application of NT4 and BDNF to embryonic cortex produces heterotopies in the marginal zone (Brunstrom et al., 1997), and in transgenic mice, ectopic overexpression of BDNF prior to the onset of endogenous expression down-regulates reelin and produces the disorganization of Cajal-Retzius cells and aberrant cortical lamination (Ringstedt et al., 1998). Here we analyze the neuronal migration in the neocortex of the transgenic mice that overexpress BDNF under the nestin promoter. At E18 the subplate and marginal zone were in the appropriate locations. Levels of reelin expression were normal in Cajal-Retzius cells but decreased in GABAergic cells. GABAergic neurons generated in the ganglionic eminence migrate tangentially trhough the subventricular zone and then radially to reach their appropriate cortical layer (Anderson et al., 1997; Tamamaky et al., 1997). In the nestin-BDNF mice we found that GABAergic neurons reached the neocortex but then failed to migrate radially, accumulating at the level of the subplate and deep cortical plate, where they overexpressed calbindin and glutamic acid decarboxylase. The few GABAergic cells that entered the cortical plate formed columns below heterotopyc clusters of GABAergic cells in the marginal zone. In the nestin-BDNF transgenic mice the preplate spliced into marginal zone and subplate in spite of reduced reelin expression, suggesting that migration of early-born cortical neurons is largely normal. Taken together, these results indicate that BDNF induced malformation differs from reeler mutation. We propose that BDNF overexpression affects GABAergic neurons, mainly their maturation and radial migration. We are now examining the effect of BDNF on the guidance mechanisms involved in GABAergic cell migration and in the maintenance of the radial glial scaffold.

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Characterization of a new gene involved in neural migration during the development of the mouse forebrain

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The functionality of the adult nervous system requires that different subset of neurons migrate from the germinal zones till their definitive localization and that the axons recognize specific targets to establish the appropriate pattern of connectivity. In the last years it has become apparent that the molecules involved in these processes are well conserved through evolution. C.elegans unc-53- is a not yet fully characterized new gene involved in some A-P migratory processes in the worm, i.e controlling migration in several subsets of neurons and myoblasts as well as the excretory canals. UNC-53 is a multidomain protein that plays a role as regulator of 'directional' cell motility, by transducing receptor tyrosine-kinase signals into recruitment of F-actin and microtubuli in the nematode and in mammalian cells (1). A human gene family of at least three members has been cloned but their role in vertebrate development has not been investigated yet (2,3). For this reason we have started a new project isolating and characterizing the murine counterparts of this human family. Preliminary experiments showed that a mouse EST with high identity to human gene 1 displayed a very restricted neural expression. Using this EST as an starting probe we have cloned 135 positive clones in several screens of a mouse brain cDNA library which reconstructed a cDNA of more than 9 kb with an ORF of 5667 bp. We are currently investigating if there is a further 5' extension in the whole transcript. Northern blot analysis at very high stringency revealed a complex transcriptional pattern with 14, 12.5 and 11 kb as major mRNA species. The encoded protein of, at least, 203.8 kDa shows very high identity with the encoded by the human gene 1 (3) and displays the typical features of the family. The spatial-temporal expression pattern of m-unc-53-1 has been analyzed in great detail by ish techniques. This gene has very restricted expression to SN. The onset is seen already in E10 and the maximal expression is achieved in E18-P0 and P5. The expression in juveniles and adults is very low. We have dissected the expression in cortex, hypocampus, cerebellum, thalamus and hypothalamus, olfactory bulb, and others. The cells expressing m-unc-53-1are mainly post-mitotic neurons which are no longer migrating. These would argue for a more generic role of this gene in neuritic maturation. Preliminary experiments of knocking down the gene expression using an antisense approach in dissociates of cortical tissue at E17 stage showed a marked neuritic collapse compared with the sense treatments. But in addition, we have also seen a striking expression in some circunferencial and longitudinal migratory streams like the pontine migration and, at lower level, the rostral migratory stream form the olfactory bulb. The expression in these cells could argue for a role of m-unc-53-1in the directional guidance of these cells along their migration in the same way it has been described in C.elegans.

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REELIN IMMUNOREACTIVITY IN THE CEREBRAL CORTEX OF EMBRYONIC AND POSTNATAL FERRETS.

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Reelin, the protein mutated in reeler mice, mediates neural migration and axonal branching in the developing cerebral cortex. We have analyzed reelin immunoreactivity (Reln-ir) in the cortex of fetal (E23, E25, E28, E31, E34, E40) and postnatal (E44, E46, E54, E61) ferrets. Serial sections were obtained from paraffin-embedded or frozen brains fixed in 4% paraformaldehyde. Sections were assayed with a monoclonal antibody (#142) against the F-spondin similarity domain of Reln (a gift from Dr. André Goffinet, Namur, Belgium). Some sections were counterstained with cresyl violet for cytoarchitectonic identification. In all ages analyzed, the marginal zone (MZ) shows the heaviest and most consistent Reln-ir. This labeling involves both cellular profiles, including somata and proximal dendritic segments, as well as pericellular clumps of dense immunoprecipitate around some of these cells. In addition, a fainter, homogeneous staining covers the whole width of the marginal zone but abruptly disappears at the MZ-cortical plate border. The hippocampo-septal region nad the lateral olfactory palecortex are the first to show Reln-ir cells (E23), and consistently contain the largest numbers of these cells. Labeled MZ cells are first detected in the neocortical pallium between E28 and E31, and, by E34, they are present throughout the hemisphere. Labeling appears first in the ventromedial and ventrolateral aspects of the hemisphere, subsequently extending to more dorsal zones. At younger ages, most Reln-ir cells seem to be intimately associated to the pia, to the extent that, at the points where the pia is artifactually detached from the cortex in our sections, most Reln-ir cells remain attached to it. After E46, additional Reln-ir neuronal profiles appear in layers III-V. This is the first report of the spatio-temporal distribution of reelin in the cortex of developing carnivores.

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Proliferation and cell cycle exit in the retina are mediated in part by the cyclin-dependent kinase inhibitors $p19^{INK4d}$ and $p27^{KIP1}$

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The development of the CNS requires the proliferation and the subsequent differentiation of multipotential progenitor cells in a highly coordinated manner. The signals that trigger cells to leave the cell cycle and differentiate at the appropriate time are mediated in part by cyclin-dependent kinase inhibitors (CKI). mRNA expression of $p19^{INK4d}$ and $p27^{KIP1}$ in the cerebral cortex and retina suggests that the function of these genes may be conserved throughout the CNS. We hypothesized that loss of both gene functions might alter retinal development due to continued cell proliferation.

Retina derived from $p19^{INK4d}$-null mice appeared normal whereas retina from crosses of $p19^{INK4d}$-null with $p27^{KIP1}$-null mice exhibited dysplasia. Cells displaced in the outer segment layer were positive for the Muller glia marker CRALBP. Double-null mice pulse labeled with bromodeoxyuridine (BrdU), showed BrdU positive cells in the outer nuclear layer (ONL), inner nuclear layer (INL) and a few cells in the ganglion cell layer (GCL). This labeling was not observed in either of the single-null or wt animals. The cell proliferation appeared to be compensated by apoptosis. We are currently investigating whether the BrdU positive cells are displaced progenitors or if these are differentiated cell types that are re-entering the cell cycle. Preliminary evidence from double labeling with BrdU and neurofilaments protein suggested that differentiated cells are re-entering the cell cycle. These data suggest that $p19^{INK4d}$ and $p27^{KIP1}$ may be important for withdrawal from the cell cycle, differentiation and/or maintaining retinal cells in their post-mitotic state.

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Migration of oligodendroglial progenitors is guided by secreted semaphorins and netrins in the embryonic optic nerve

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Oligodendrocytes are the myelin forming cells of the central nervous system. We have previously shown that, in the embryonic brain of mouse and birds, the ventricular cells expressing plp/dm-20 (the gene encoding proteolipid protein and its isoform DM-20) define the restricted territories of the germinative neuroepithelium from where oligodendrocyte progenitor cells (OPC) originate. To follow the fate and migration of these plp/dm-20 precursors, we have generated several mouse transgenic lines expressing reporter genes (eitherlacZ or GFP), under the control of regulatory sequences of plp/dm-20 gene. The aim of the present study was to investigate whether molecular cues governing axonal outgrowth, may also be involved in OPC migration. We took advantage of plp transgenic lines and used the optic nerve as an experimental model to analyze the effect of candidate molecules on glial progenitors migration. We first demonstrate that cells exiting optic nerve explants from E16 mouse transgenic embryo all expressed the transgene, and were immunolabeled with A2B5 and AN2 (equivallent to NG2 proteoglycan) mAbs, two established markers of OPC. Optic nerve explants were then cocultured with aggregates of COS cells transfected with putative chemotactic factors. We show that among the secreted semaphorins, netrins and slits tested, only Semaphorin3B was chemorepellent for the optic nerve OPC whilst Semaphorin3F and Netrin-1 were chemoattractant. The expression pattern of these molecules and of their receptors in the optic tract and its related structures were consistent with their involvement in the migration of OPC.

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PHYSIOLOGICAL NITRIC OXIDE SYNTHESIS CONTROLS PROLIFERATION OF NEURAL PRECURSORS IN THE ADULT MOUSE SUBVENTRICULAR ZONE-OLFACTORY BULB NEUROGENIC SYSTEM

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The capacity to generate new neurons persists well into adulthood in the mouse subventricular zone (SVZ). Neuroblasts formed in this region migrate toward the olfactory bulb (OB), where they differentiate as granular and periglomerular interneurons (1). Immunohistochemical detection of the nitric oxide (NO) synthesizing enzyme NOS I, revealed two possible sources of NO in relation with the SVZ-OB system. First, well differentiated nitrergic neurons were located in the limits of the SVZ, with long varicose processes extended within the area of neurogenesis. Second, in the olfactory bulb, some of the newly-formed periglomerular neurons, which were identified by PSA-NCAM staining, expressed NOS I (2).

To analyze the possible effects of NO on neuronal precursors, a long-lasting reduction of cerebral NOS activity was produced in adult mice by administration of the specific inhibitor L-NAME (90 mg/kg/day, i.p. for 7 days). Control and treated animals were injected with bromo-deoxyuridine (BrdU; three doses of 200 mg/kg, i.p., separated by 2.5-h) and perfused 2.5 h after the last injection, to identify cells which underwent mitosis during this time interval. NOS inhibition produced significant increases in the number of mitotic cells in the SVZ (157 ± 10 %), rostral migratory stream (130 ± 5 %) and periglomerular area of the OB (168 ± 10 %).

To investigate the mechanisms of action of NO in neural precursors, cells obtained from the SVZ of postnatal mice were either maintained in primary cultures or grown as neurospheres, in the presence of epidermal growth factor (EGF). After three days, the primary cultures contained cells which proliferated in vitro and expressed either glial or neuronal phenotypes. Many cells in these cultures expressed NOS I. Addition of L-NAME (100 μM) significantly increased precursor cell proliferation, although the number of cells expressing neuronal or glial markers was unchanged. Conversely, the NO donor DETA-NO (10 μM) reduced cell proliferation to levels similar to those measured in the absence of EGF. Western blot analysis of neurosphere-derived cells showed that NO donors inhibited the autophosphorylation of the EGF receptor (EGFR) produced by EGF.

The results indicate that endogenous NO participates in the control of postnatal and adult neurogenesis by reducing precursor cell proliferation. This effect is due, at least in part, to an impairment in the transmission of the proliferation signal initiated by EGF.

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Cell types responsible for adult neurogenesis in lizards

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Previous studies have shown that several areas of the telencephalon in lizards undergo neurogenesis even in fully-grown, adult animals. The areas with adult neurogenesis include the olfactory bulbs (main and accessory), the cerebral cortex, anterior dorsal ventricular ridge, striatum, and nucleus sphericus. Where sufficient data are available, proliferating cells seem to be largely restricted to the walls of the lateral ventricles from where they migrate to their final destination to differentiate. In the present study, we used serial ultrathin sections to reconstruct the composition and three-dimensional organization of the ventricular wall in lizards. In addition we used [3H]-thymidine autoradiography to identify cell types responsible for adult neurogenesis. Two main cell types were identified in the ependymal wall in contact with the ventricle: Type I cells and type II cells. Both cell types were ultrastructurally similar, but type I cells had a characteristic single cilium whereas type II cells were multiciliated cells resembling the mammalian ependymal cells. One hour after a single injection of [3H]-thymidine, all labeled cells corresponded to the single cilium type. This suggests that type I cells are primary neuronal precursors for adult neurogenesis in lizards. We discuss these results in the context of recent findings on adult neurogenesis in birds.

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Development of neurotrophic responsivity in avian spinal MNs: Intrinsic and extrinsic influences

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In the developing nervous system, many neuronal populations undergo histogenetic cell death during the period of target innervation, resulting in the loss of 50% of the neurons produced. While extracellular target-derived signalling has been amply demonstrated to regulate the survival of embryonic spinal MNs, the competence of subpopulations of these MNs to respond to specific factors appears to be defined earlier. We are testing the hypothesis that such differential responsivity is part of a general program of cell fate specification. For example, we are interested if MNs which settle in discrete regions of the lumbar lateral motor column (LMC) and exhibit defined transcription factor identitites also display different patterns of trophic receptor expression. Our preliminary studies have identified specific motor pools within the lumbar LMC which express different components of the GDNF receptor system. In particular, we are studying the expression of these factors in motor pools which innervate muscles of common embryonic origin, twitch type and function. The response of these pools to an array of trophic factors is currently being examined *in vivo* and *in vitro*. Finally, we are investigating the onset and regulation of trophic receptor expression within MNs, as well as of trophic factor expression within MN target areas.

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Doublecortin (DCX) and DCAMKL1 are expressed during neuronal migration in the developing avian nervous system

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The development of functional layers in the brain involves spatially and temporally regulated gene expression. Many genes expressed in the course of development can be used as markers for specific processes such as neuronal migration. One such gene is doublecortin (Dcx), which encodes a microtubule- associated protein expressed during migration. In humans, mutations in Dcx are known to cause disorders of neuronal migration such as lissencephaly and subcortical laminar heterotopia ("double cortex"), resulting in epilepsy and mental retardation. In other species such as the chick, our group has previously shown that a homolog of Dcx exists and that avian Dcx mRNA is similarly expressed in migrating neurons, suggesting a common role for this gene in neurogenesis (Hannan et al. (1999) J.Neurosci.Res. 55: 650). The current study aims to extend this analysis to consider protein expression in the chick, looking at both DCX and the product of a related gene known as DCAMKL1. DCAMKL1 (also referred to as KIAA0369) has homology to Dcx and to protein kinases, suggesting a role for this gene in linking kinase activity to the cytoskeleton. Polyclonal antibodies were raised for this study against human DCX and DCAMKL1 peptides and used to detect specific proteins of 40 and 85 kDa, respectively, in both human and chick brain samples. Immunohistochemistry showed that DCX and DCAMKL1 proteins are expressed in the developing chick brain (embryonic days 8-18) in layered structures such as the cortex, optic tectum and cerebellum. In the chick forebrain and tectum, DCX and DCAMKL1 are initially located in the ventricular zone and shift with migration to the post-proliferative neuronal population. Both proteins were also readily detected in afferent tracts of the developing tectum and in the Purkinje cells and molecular layer of the cerebellum. Similar expression patterns have been reported previously in mammalian species (Mizuguchi et al. (1999) Am. J. Pathol. 155: 1713). DCX and DCAMKL1 are expressed in identical regions of the avian brain, suggesting that these proteins are co-expressed and function together in migrating neurons.

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TAG-1 MEDIATES THE MIGRATION OF CORTICAL GABAERGIC NEURONS FROM THE GANGLIONIC EMINENCE ALONG THE AXONS OF THE CORTICOFUGAL FIBER SYSTEM

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The unravelling of the mechanisms that give rise to the excitatory pyramidal and the inhibitory nonpyramidal neurons is fundamental to our understanding of cortical development. Both cell types are derived from distinct proliferative zones. Pyramidal neurons are generated in the cortical ventricular zone and migrate along the processes of radial glia to reach their positions in the cortical plate (CP). Nonpyramidal cells, the GABAergic interneurons of the cortex, originate in the ganglionic eminence (GE) of the ventral telencephalon, and follow tangential migratory paths to the developing cortex.
The mechanisms that guide tangentially migrating neurons from the GE to the cortex are unknown. We followed the migration of such neurons by making small placements of DII crystals in the medial GE of cultured slices prepared from brains of rat embryos (E14-E16). We found that labelled cells first appeared in the marginal zone and later in the lower intermediate zone and CP. They appeared to follow distinct pathways to the cortex in close association with the axonal bundles of the developing corticofugal fiber system. The order of appearance of these cells in the different layers of the developing cortex coincided with the temporal pattern of expression of the neuronal adhesion molecule TAG-1 in the corticofugal axonal system. Moreover, a significant reduction in tangentially migrating neurons and in the population of cortical GABAergic cells was observed in slice cultures incubated in the presence of TAG-1 blocking antibody. These results suggest that TAG-1 mediates the tangential migration of cortical GABAergic neurons that arise in the GE along the axons of the corticofugal projection system.
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The evolution of cortical development. An hypothesis based on the role of the reelin signaling pathway

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Comparative studies of reelin and Dab1 expression during cortical development in embryonic turtle (chelonian), lizard (squamate), chick, mouse and man reveal interesting correlations with different neuronal cell patterns. In all species, reelin is produced by neurons in cortical marginal zones (MZ) in the medial and dorsal cortical areas (homologous, respectively, to mammalian hippocampus and neocortex), whereas Dab1, an adapter that mediates the response to reelin, is synthesized by cortical plate (CP) neurons. This pattern is thought to reflect the situation in stem amniotes and to define an homology. Mammals are characterized by i) the presence of a laminated, radially organized CP and ii) maturation of the CP from inside to outside. These two features are reelin-dependent and related to an enormous amplification of reelin production in the MZ as compared to other amniotes. Lizards also develop an elaborate CP, but CP maturation proceeds from outside to inside as in other reptiles and birds. In lizards, the high level of CP architectonic development is accompanied by the development of an additional layer of reelin production in subcortex. These observations suggest that the reelin signaling pathway has been used in the evolution of CP architectonics in at least two lineages leading to mammals and squamates, presumably reflecting an homoplasy. Our data point to the importance of architectonic patterns as a key parameter of cortical evolution, in addition to more widely appreciated features such as neuron numbers and differentiation, and hodological organization.

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Origin and molecular specification of striatal interneurons

Oscar Marín

The striatum, the largest component of the basal ganglia, contains projection neurons and interneurons. Whereas there is considerable agreement that the lateral ganglionic eminence (LGE) is the origin of striatal projection neurons [1,2], less is known about the origin of striatal interneurons. Using focal injections of retrovirus into the ventral telencephalon in vitro, we demonstrate that most striatal interneurons tangentially migrate from the medial ganglionic eminence (MGE) or the adjacent preoptic/anterior entopeduncular areas (POa/AEP) and express the NKX2.1 homeodomain protein. While the majority of striatal interneurons (cholinergic, calretinin+ and parvalbumin+) maintain the expression of NKX2.1 into adulthood, most of the interneurons expressing somatostatin (SOM), neuropeptide Y (NPY) and neural nitric oxide synthase (NOS) appear to downregulate the expression of NKX2.1 as they exit the neuroepithelium. Analysis of striatal development in mice lacking Nkx2.1 suggests that this gene is required for the specification of nearly all striatal interneurons. Similar analysis of mice lacking the Mash1 bHLH or both the Dlx1 and Dlx2 homeodomain transcription factors demonstrate that these genes are required for the differentiation of striatal interneurons. Mash1 mutants primarily have a reduction in early born striatal interneurons, while Dlx1/2 mutants primarily have reduced numbers of late born striatal interneurons. We also present evidence implicating the Lhx6 and Lhx7 LIM-homeobox genes in the development of distinct interneuron subtypes. Finally, we hypothesize that within the MGE, radially migrating cells generally become projection neurons, whereas tangentially migrating cells largely form interneurons of the striatum and cerebral cortex.

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Doublecortin expression in human embryonic and fetal neocortex

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The gene *doublecortin* (DCX) is required, along with other genes, for proper cortical migration and differentiation. We studied its expression in developing human neocortex from 5 to 40 gestation weeks (GW). At 5 GW, DCX partially colocalized with Reelin in the earliest cells of the preplate, suggesting that DCX may play a role in this early migration. The first few DCX-immunoreactive cells in the ventricular zone appeared at 7 GW in a DCX-negative neuroepithelium, and steadily increased in number from 8 GW to midgestation. We distinguished two opposite patterns of DCX-expression. In the cortical plate, moderate expression was found in distal radial processes presumably of pyramidal cells. In the deeper compartments of the cortical wall (ventricular, subventricular and intermediate zones), DCX was strongly positive in mostly non-radially oriented mono- or bipolar neurons, which assumed a more vertical orientation upon entering the subplate. In addition, DCX was expressed by cells in the subpial granular layer and by Cajal-Retzius cells. During the 13 - 17 GW period, DCX in ventricular, subventricular and intermediate zones was extensively colocalized with calretinin, a marker of nonpyramidal neurons, suggesting a nonpyramidal phenotype of DCX-expressing cells in these compartments. By contrast, LIS-1 seemed to stain virtually all migrating neurons. DCX-negative interspaces were most prominent in intermediate zone, and occupied by clusters of radially-oriented LIS1-immunoreactive cells, further indicating a distinct migration pattern of DCX-positive neurons. A 19 GW-old Miller-Dieker case was compared to the normal, age-matched cortex. DCX was present in all compartments of the cortical wall, but in extremely low numbers, suggesting that LIS-1 deficiency adversely affects migration and differentiation of DCX-expressing neurons.

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Developmental changes in neuronal domains from mouse visual cortex

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Spontaneous correlated activity is causally related to circuit reorganizations during the development of the visual system. Neuronal domains are a type of spontaneous activity present in slices of developing cortex. They consist of groups of neighboring neurons that increase their calcium concentration in synchrony (Science 257:665, 1992). These neuronal coactivations are thought to be mediated by gap junctions. Although their ulterior fate and potential role in cortical development remains unknown, their columnar shapes suggest that domains might be related to radial units and/or adult functional circuits. In previous studies in rats and mice, neuronal domains could only be studied until P7, because older animals were not successfully loaded with calcium indicators. We have recently developed new loading procedures that permit adequate fura-2 AM loading up to P23 in mice. This enables us now to study neuronal domains in the second postnatal week, period during which gap junctional coupling among most neurons disappears (Neuron 10:103, 1993). Using small temperature drops to activate domains, we characterize their development in mouse visual cortex. After P8, we encounter a progressive decrease in the number of domains and their areas. Thus, at P4 we find 11.2 domains per slice, with an average area of 1736.2±1204.4 mm2 (mean±SD; n=158), whereas at P10, we observe 3 domains per slice with an area of 1177.3±695.7 mm2 (n=9), and at P14, 0.8 domains per slice with an area of 558.4±231 mm2 (n=5). This time course parallels the previously described decrease in gap junctional coupling and suggests that domains disappear during the second postnatal week. We are testing whether neurons within a domain become synaptically interconnected. Supported by NATO and the NEI.

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Development of specific cellular phenotypes in the two compartments of the mammalian striatum

Eleonora Fusco y Rosario Moratalla

The molecular mechanisms that lead to striosome/matrix pattern formation in the developing striatum are likely to involve two diferent programs working coordinately: one intrinsic determined geneticaly, and another afferent-dependent that incorporates extrinsic information. Selective expression of regulatory genes, such as those encoding transcription factors, could be critical for establishing the pattern formation events that lead to groups of neurons to form morphological and functional units in the mammalian brain.

In the experiments described here, we found that transcription factors of two immediate-early gene (IEG) classes, Fos and Ziff, have selective and temporal spatial patterns of expression that could define the developmental phases of compartment formation in the striatum. We traced the onset and developmental expression of Fos and Ziff proteins in the striatum of mouse embryos. We found that these two proteins show a strict and selective expression in parallel with the developing striosomal compartment. First, there was a ventrolateral phase (E14—E16) in which Fos- and Ziff-positive nuclei and future striosomal neurons were localized at the ventrolateral edge of the striatum; following that, there was a dispersed phase (E16—E17), in which the IEGs were dispersed throughout the striatum. IEG expression in the last prenatal days was increasingly defining clusters of neurons corresponding to future mature striosomes. This clustering phase extended through the first postnatal week, by the end of which IEG expression lost comparment constraints and diversified in gene-specific patterns. Fos becomes downregulated while Ziff expression is increased to include the matrix. Even though it is not clear which aspects of the differentiation procces Fos/Ziff may regulate, these results suggest that constitutive IEG expression could be part of the gene regulation necessary for the development of the striatum. Funded by FIS grant 98/1368 and by CAM 8.5/43.1/99.

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Involvement of neural BHLH genes in the commitment of multipotential cortical progenitors to neuronal versus glial fate

Marta Nieto

A main issue in neuronal development is to understand how neuronal and glial fate decissions are made during differentiation of neural stem cells. The bHLH genes *Mash1* and Neurogenins are involved in the determination of neuronal lineages. We observed premature and ectopic expression of astrocyte markers in the subventricular zone of dorsal telencephalon of *Ngn2;Mash1* double mutant mice. To characterize the molecular mechanisms responsible for this phenotype, we analyzed the development of neuronal progenitors at a single cell level. Progenitors were studied based on their expression of a LacZ gene reporter knocked in the *Ngn2* locus. Dissociated cells from the cerebral cortex were labeled with the fluorescence galactosidase substrate FDG, FACS sorted and cultivated at clonal density on a layer of embryonic rat cortical cells. Results from wild-type mice showed that *Ngn2*LacZ positive (FDG positive) cells contained neuronal and astrocyte restricted progenitors, while cultures of the *Ngn2* negative (FDG negative) progenitors contain a subpopulation of bipotential clones with a higher proliferation rate. Progenitors from *Ngn2* mutant mice showed only a slight reduction in the size of neuronal restricted clones, indicating functional compensation by *Ngn1* and *Mash1* proneural genes. Interestingly, cortical precursors from *Ngn2;Mash1* double mutant mice change their neuronal fate to generate mainly larger astrocytic clones and showed a reduction in both the number and size of neuronal clones. These results demonstrate a neuronal versus glial determination role for bHLH proneural genes in the mouse telencephalon and show that in the absence of these genes, neuronal progenitors adopt a glial fate rather than remain in an undifferentiated state. Thus, bHLH proneural genes are required for the generation of neurons and glial at appropriated time and cell numbers.

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Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1

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The central control of motor output depends critically on the function of local circuit interneurons that are generated in the ventral spinal cord. Ventral horn interneurons are assembled into local neural circuits whose primary function is to regulate the pattern of motor neuron discharge in response to incoming sensory information. The cell bodies of many of these distinct subclasses of ventral interneurons are segregated into distinct domains within the ventral spinal cord. Classical physiological and anatomical studies of ventral interneurons have defined several neuronal subclasses that differ in their neurotransmitter phenotype, cell body position and initial axonal trajectory. The developmental mechanisms that establish these diverse aspects of spinal interneuron identity and function, however, remain poorly understood.

We have examined the role of the homeodomain protein Dbx1, a protein restricted to a subset of ventral neural progenitor cells, in establishing the distinct developmental features of two major classes of ventral interneurons, V0 (Evx1/2) and V1 (En1) neurons. Our genetic studies show that Dbx1 regulates the transcription factor profile, neurotransmitter phenotype, intraspinal migratory path and axonal trajectory of V0 neurons - features that distinguish them from an adjacent set of V1 neurons. Misexpression of Dbx1 within the chick neural tube results in an expansion in the domain of generation of V0 neurons, and a repression of V1 neuron generation. Conversely, inactivation of the mouse *Dbx1* gene blocks the generation of V0 neurons. In *Dbx1* mutants, progenitor cells present in the former p0 domain now give rise to interneurons that exhibit molecular features of V1 neurons, as assessed by the profiles of transcription factor and neurotransmitter expression, cell body migration and settling position, and certain aspects of axonal trajectory. These findings provide direct evidence that the spatially-restricted expression of a single progenitor homeodomain protein coordinates several distinct phenotypic features that contribute to interneuron diversity and function within the developing spinal cord.

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Restricted developmental expression of SFRP-2 and WNT-7b at the corticostriatal sulcus is regulated by pax-6

Samuel Pleasure

The wnt-signaling pathway is implicated in a wide range of developmental processes (Moon, Brown et al. 1997; Lee and Jessell 1999). I am interested in the functions of both wnts and wnt-inhibitors in patterning, cell fate and migration events in the developing forebrain. One family of wnt-inhibitors is the soluble frizzled related protein (SFRP) family (Rattner, Hsieh et al. 1997). We have found that SFRP-2 and wnt-7b are expressed in a dynamic fashion in the corticostriatal sulcus (CSS), a developmentally important structure at the neocortical-striatal border that regulates migration of neurons from the ganglionic eminences to the neocortex (Stoykova, Götz et al. 1997; Chapouton, Gartner et al. 1999). From embryonic day 10.5 (E10.5) to E17.5, SFRP-2 expression is concentrated in a region at the CSS between the lateral ganglionic eminence (LGE) and the cortical ventricular zone. The expression domain of SFRP-2 is complementary to dlx-2 expression ventral to the CSS and overlaps the region of pax-6 expression at the CSS. Wnt-7b is also concentrated at the CSS but is expressed in the LGE as well. From E10.5 to E17.5, SFRP-2 expression is also concentrated at the zona limitans interthalamica (ZLI), a boundary region between dorsal thalamus and ventral thalamus. Previous work has shown that pax-6 mutant mice have a loss of boundary function of the CSS, increased migration of cells from the striatal compartment into the cortex, disrupted radial glial fascicles at CSS, and disruption of the ZLI neocortex (Grindley, Hargett et al. 1997; Stoykova, Götz et al. 1997; Götz, Stoykova et al. 1998; Chapouton, Gartner et al. 1999). Because of these phenotypes, we analyzed the expression of SFRP-2 and wnt-7b at the CSS and ZLI in this mutant. SFRP-2 expression is dramatically reduced in the telencephalon of pax-6 -/- mice, and concentrated SFRP-2 and wnt-7b expression are absent from the CSS. The coordinate regulation of SFRP-2 and wnt-7b was also seen in the ventral spinal cord at E10.5. Interestingly, wnt-7b expression in the ZLI, cortical plate and cortical hem is preserved, which suggests that an alternate mechanism regulates wnt-7b expression in these areas. These data provide evidence that pax-6 regulates SFRP-2 and wnt-7b expression in the CSS and ZLI, and that components of the wnt-signaling pathway are likely to be involved in the normal formation of these two boundaries in the developing forebrain.

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Presenilin-1 and Notch-1 in neural development

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Mutations in *Presenilin-1* (*PS1*) are a major cause of familial Alzheimer's disease. Our previous analysis of *PS1-/-* mice showed that PS1 is required for murine neural development. The *PS1-/-* embryonic brain displays a reduction in the neural progenitor population, disrupted cerebral laminar architecture, and extensive neuronal loss in specific brain regions, including Cajal-Retzius neurons in the marginal zone. Here we show that lack of PS1 leads to premature differentiation of neural progenitor cells, indicating a role for PS1 in a cell fate decision between postmitotic neurons and neural progenitor cells. Neural proliferation and apoptotic cell death during neurogenesis are unaltered in *PS1-/-* mice, suggesting that the reduction in the neural progenitor cells observed in the *PS1-/-* brain is due to premature differentiation of progenitor cells, rather than to increased apoptotic cell death or decreased cell proliferation. In the ventricular zone of *PS1-/-* mice, expression of the Notch1 downstream effector gene *Hes5* is reduced and expression of the Notch1 ligand *Dll1* is elevated, whereas expression of *Notch1* is unchanged. These results provide direct evidence that PS1 controls neuronal differentiation in association with the down-regulation of Notch signalling during neurogenesis. We are in the process of investigating the role of PS1 in cortical layer formation and the cause of the extensive cell loss in the *PS1-/-* brain by analyzing our newly generated conditional *PS1* KO mice, in which PS1 function is eliminated in neural progenitor cells or postmitotic neurons. The results of the analysis will be presented.

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Differentiation of cortical astrocytes y cAMP-dependent signaling

Mario Vallejo

During development of the CNS, the phenotypic fate of proliferating neural precursors is determined in response to signals delivered to the cell membrane by neurotrophic factors. Two major pathways, those dependent on CNTF and BMPs, respectively, have been implicated in astroglial differentiation (1, 2). We have found that stimulation of the cAMP-dependent pathway by 8Br-cAMP (1mM) or forskolin (10 mM) results in astrocyte differentiation of rat E17 cortical precursor cells maintained in primary cultures in vitro (3). Differentiation was evidenced by morphological changes, stimulation of GFAP expression, downregulation of nestin expression, and decreased proliferation. We looked for ligands that could act via G-protein coupled receptors to trigger the differentiative response. We found that treatment of cortical precursors with PACAP (10-100 nM), but not with the related peptide VIP, resulted in astrocytic differentiation. Short term exposure of the cells to PACAP was sufficient to trigger the response. RT-PCR experiments confirmed the presence of PACAP type I receptors on cortical precursor cells, and revealed that upon treatment with PACAP an splicing event occurs such that the isoform containing the "hop" cassette is downregulated. We also determined that treatment of primary cortical precursors with PACAP results in a dose-dependent increase in the synthesis of intracellular cAMP. Our results suggest that PACAP may act as an instructive signal for astrocyte differentiation during cortical development.

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CNS development in mice lacking insulin-like growth factor-I (IGF-I) and leukemia inhibitory factor (LIF)

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IGF-I and LIF are pleitropic proteins that support the proliferation, survival and differentiation of a variety of cell types in culture. Signaling pathways activated by IGF-I and LIF may converge through the interactions of IRSs and STAT proteins, and the expression of IGF-I is regulated by LIF in a model of sciatic nerve crush. Single IGF-I or LIF knockout (KO) mice display alterations in the mature CNS and PNS. To study whether IGF-I and LIF cooperate during development, IGF-I and LIF heterozygous KO mice (provided by A. Efstratiadis and C.L. Stewart) were intercrossed. Mouse embryos (E18.5) lacking IGF-I ($LIF^{+/+}/IGF\text{-}I^{-/-}$) were 34% smaller than the control mice, and 59% of them died before weaning. In contrast, $LIF^{-/-}/IGF\text{-}I^{+/+}$ KO mice were nearly normal in weight and only 10% of them died before weaning. Double homozygous KO ($LIF^{-/-}/IGF\text{-}I^{-/-}$) were 47% smaller than controls and 100% of them died at birth. In H and E-stained sections from the nervous system of E18.5 embryos, the gross anatomy of regions such as the spinal cord, cerebellum, cortex, hippocampus, retina, and olfactory epithelium appeared normal in these various KO mice. Immunostaining with antibodies against calbindin, calretinin, or GFAP confirmed normal presence of cerebellar Purkinje neurons, olfactory receptor neurons and hippocampal and spinal cord astrocytes. A moderate deficit in the number of motoneurons in the trigeminal nucleus was found in the $LIF^{-/-}/IGF\text{-}I^{-/-}$ KO mice. Strikingly, the olfactory bulb of the $LIF^{+/+}/IGF\text{-}I^{-/-}$ KO presented a 70% decrease in the number of mitral neurons and an apparent disorganization of the granular and periglomerular layers. Similar results were obtained in the double $LIF^{-/-}/IGF\text{-}I^{-/-}$ KO mice. Altogether, these data suggest that IGF-I and LIF may collaborate to promote general growth and vital functions during development but only IGF-I is required for the survival and/or differentiation of olfactory mitral neurons.

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