Instituto Juan March
de Estudios e Investigaciones

126 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Neural Prepatterning and Specification

Organized by

K. G. Storey and J. Modolell

D. J. Anderson
R. L. Cagan
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W. Chia
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Introduction

K. G. Storey and J. Modolell

Understanding the cellular and molecular mechanisms underlying neural specification is an important and fundamental challenge in Modern Biology. While much progress has recently been made in this area following the characterisation of neural inducing molecules, signalling pathways and transcription factors which mediate vertebrate neural specification, our understanding of the mechanisms underlying this process springs in large part from studies conducted in the fruit fly, *Drosophila melanogaster*. It is therefore important and informative to evaluate the similarities and differences manifest at both cellular and molecular levels during the generation of neural tissue in flies and vertebrates.

Recent advances in this field include the identification of multiple steps involved in defining vertebrate neural and neuronal precursors, some of which are homologous to steps identified in the fly. For instance, recent work in the fly has identified novel "pre-patterning" genes whose activity defines cell populations within which neural precursors can arise. Similar genes have now been identified in diverse vertebrates and it is currently being established whether these homologues also prefigure neural specification. Recent research in the fly also indicates that neural specification genes interact with the cell cycle machinery and the extent to which this is a universal mechanism that co-ordinates assignment of neural cell fate with patterns of cell proliferation is an important current issue. Topics of interest to be addressed at the workshop included: neural induction, neural pre-patterns, co-ordination of assignment of neural cell fate and patterns of cell proliferation, neuronal specification, evolutionary conservation of gene pathways and mechanisms.

The meeting brought together researchers investigating neural and neuronal precursor formation and activity in the fly and a variety of vertebrate embryos, including chick, frog, mouse and zebrafish. An initial emphasis was placed on understanding the role of the pre-patterning genes of the Irx family of homeo-domain transcription factors, but the meeting ranged more widely, addressing fundamental issues such as the evolution of proneural/*achaete scute* genes, early steps in vertebrate neural induction and the regulation of distinct spatial and temporal patterns of neuronal differentiation in both flies and vertebrates. Further talks addressed cell type specification within the nervous system particularly with respect to the glial cell lineage and the induction and differentiation of the vertebrate neural crest. Work on the specification and cell fate

choices of neuroblasts and their progeny in the fly were presented in the final session, which also addressed the involvement of cell cycle machinery in the generation of asymmetric divisions.

A number of issues were explored during these talks and subsequent discussions. An initial premise that "prepatterning" genes prefigure proneural gene expression in flies and vertebrates was addressed. This concept is well established in the fly and some instances were identified in vertebrates, e.g. the frog neural plate. Comparison of proneural gene function in flies and vertebrates further demonstrated conserved roles in cell type specification and cell identity as well as additionally regulation of cell cycle. Notch signalling in cell fate choice (neural vs glia) emerged as a current topic and new genes regulating this pathway were described in zebra-fish, frog and fly.

The entire Workshop cannot be summarised here. It must suffice to say that the forum created by the Instituto Juan March provided a unique opportunity for researchers working on common questions, but in a wide range of organisms and embryonic neural tissues, to identify new areas of research and to draw parallels and inspiration.

Kate Storey and Juan Modolell

Session 1: Neural prepatterning
Chair: José A. Campos-Ortega

The *iroquois* genes in *Drosophila* and vertebrates

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The *iroquois* (*iro*) genes, a paradigm for neural prepattern genes in *Drosophila*, were discovered in the course of a mutagenesis designed to identify genes that affected the pattern of the fly's bristles and other types of external sensory organs (Dambly-Chaudière and Leyns, 1992; Leyns et al., 1996). Some of the *iro* alleles obtained suppressed all the lateral bristles of the dorsal mesothorax (notum), an effect due to the failure of the proneural genes of the *achaete-scute* complex (AS-C) to be expressed. As a consequence, the sensory organ precursor cells were not formed. These results suggested that the *iro* locus might encode a factor(s) allowing expression of AS-C into the presumptive lateral notum. The subsequent molecular characterization of the *Drosophila iro* genes (Gómez-Skarmeta et al., 1996; McNeill et al., 1997) allowed the identification of homologues in *C. elegans* and several vertebrates, namely, *Xenopus*, mouse, zebra fish and chick (Bao et al., 1999; Bellefroid et al., 1998; Bosse et al., 2000; Bosse et al., 1997; Christoffels et al., 2000; Cohen et al., 2000; Funayama et al., 1999; Gómez-Skarmeta et al., 1998; Goriely et al., 1999; Peters et al., 2000; Tan et al., 1999). All Iro proteins share a characteristic homeodomain that has defined a new family of homeodomain proteins within the TALE class (Bürglin, 1997). In addition, the genomic organization of the *iro* genes is also apparently conserved. *Drosophila* has three *iro* genes, which together form the Iroquois complex. The individual genes have been named *araucan*, *caupolican* and *mirror* (Gómez-Skarmeta et al., 1996; McNeill et al., 1997). The Ara and Caup proteins are closely related while Mirr is more divergent. They appear to have partially redundant functions. Six *iro* genes (*m-Irx1-6*) have been identified in the mouse (Bosse et al., 2000; Bosse et al., 1997; Bruneau et al., 2001; Christoffels et al., 2000; Cohen et al., 2000; Peters et al., 2000). They are clustered in two groups of three genes each located in chromosomes 8 (*m-Irx1*, *m-Irx2* and *m-Irx4*) and 13 (*m-Irx3*, *m-Irx5* and *m-Irx6*) (Bosse et al., 2000; Peters et al., 2000). Six Irx genes have also been found in human databases and they are also grouped in two clusters of three genes each. Although only part of the *iro* functions in *Drosophila*, *Xenopus*, chick and mouse have been characterized, the emerging view is that the *iro* genes, both in the fly and vertebrates, are required at early stages of development to define large territories. Examples are the dorsal regions of the eye (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998; Cavodeassi, et al., 1999; Yang et al., 1999), head (Cavodeassi, et al., 2000; Pichaud and Casares, 2000) and mesothorax (Diez del Corral et al., 1998) of *Drosophila* and the neural ectoderm of *Xenopus* (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998, 2001). In some aspects they act like classical selector genes, but they display specific properties that place them into a category of their own. Later in development, and both in *Drosophila* and vertebrates, the *iro* genes function again to subdivide those territories into smaller domains and to help in their patterning. I will review our present understanding of the function of these genes during development.

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A common prepattern for the sensory bristles on the scutum of higher flies

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Many Brachyceran flies display species-specific bristle patterns on the notum. Bristles may be aligned into four rows on the scutum or may be present in a stereotyped arrangement. The four scutal rows are thought to represent an ancestral pattern from which the stereotyped arrangements were derived. The stereotyped patterns of *Drosophila* and *Ceratitis* result from a complex spatial expression of the proneural *achaete-scute* genes in a small cluster of cells at the site of each future bristle precursor. In *Drosophila* this has been shown to be dependent on an array of dispersed *cis*-regulatory elements present at the *achaete-scute* locus. The bristle rows present in *Calliphora* and *Phormia* arise from four stripes of expression of homologues of *scute* isolated from these two species. This suggests that a striped expression pattern may have preceded the evolution of discrete proneural clusters. This raises the question of whether the stripes are regulated by discrete *cis*-regulatory enhancer elements, and if so, do these bear any relation to the regulatory elements of *Drosophila*. To investigate this possibility we are cloning the *scute* complex from *Calliphora* with a view to identifying regulatory regions.

In the medial half of the notum of *Drosophila*, expression of *ac-sc* is regulated by the transcriptional activator Pannier. *pannier* is expressed in a single broad medial domain, but its activating potential is restricted to smaller domains by spatially-restricted negative co-factors such as U-shaped. Homologues of *pannier* have been isolated from *Ceratitis, Calliphora* and *Phormia*, and found to be expressed in a broad medial domain that is identical to that of *Drosophila pannier*. Within this domain, however, the bristle patterns of the four species differ considerably. This suggests that, although the function of Pannier in regulating *ac-sc* may have been conserved, expression of its co-factors may have changed throughout evolutionary time.

The Nematoceran flies display a number of ancestral features thought to have been present in the ancestor of the Brachycera. The thoracic bristles of most species of Nematocera are not arranged into reproducible patterns, and are generally randomly distributed. The Culicid mosquitos are amongst the exceptions to this rule and *Anopheles*, for example, display three rows of bristles on the scutum. We isolated homologues of *pannier* and *achaete-scute* from this species and found, surprisingly, that they are expressed in identical domains on the medial notum. This suggests that, in *Anopheles* too, AgPnr is an activator of *AgASH*, but that its function is not restricted by the presence of any co-factors. Furthermore, so far a single *ac-sc* homologue has been found in *Anopheles*. This may indicate that, unlike that of *Drosophila*, the regulatory region of *AgASH* may not be organised in a modular fashion with discrete regulatory enhancer elements. We are currently examining the regulatory region of *AgASH*. Interestingly bristle patterning is achieved differently in *Anopheles*. Bristles arise exclusively along the borders of four domains of expression of *Agpnr/AgASH* in the medial notum. Inside the expression domains sensory scales arise. This suggests special properties along the expression borders allowing cells to develop as bristle, rather than scale, precursors. The borders between *pannier*-expressing and non-expressing cells in *Drosophila* have been shown to display specific properties and be the site of a change in cell affinities similar to that

seen at compartment boundaries. This and other features have prompted the suggestion that *pannier* acts as a selector gene. Our results suggest that this selector gene function has been conserved. However, during the course of evolution, the nature of the regulatory interactions between Pannier and its target *achaete-scute* genes may have changed dramatically.

Early steps of neural induction

Claudio D. Stern

In amphibians, neural induction has been hypothesized to occur by a "default" mechanism - ectoderm cells have a natural tendency to become neural, but this is prevented by intercellular signalling by BMPs. The organizer emits BMP antagonists, which release the neighbouring ectodermal cells of the prospective neural plate from this inhibition, allowing them to express their default, neural fate.

Recent data, however, suggests that the situation may be more complex than this. We have used the chick embryo to analyze neural induction. A screen for early response genes to signals from the organizer identified 15 genes (11 of them novel) all expressed before ectodermal cells become sensitive to BMP antagonists. Functional analysis revealed that many of these (but not all) are regulated by FGF8. Another molecular screen, for signals produced by the organizer, revealed 6 novel candidate signalling molecules expressed appropriately in the organizer.

Taken together, our results suggest that neural induction consists of a complex cascade of events, involving several signalling molecules, and where each signalling step stabilizes the previous ones.

The specification of prospective epidermal, neural crest and neural territories in early vertebrate embryos

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The ectoderm of early vertebrate embryos gives rise mainly to the epidermis and the central nervous system. Neural crest cells develop between these two territories, and give rise to diverse cell types such as neurons, muscle, or skeletal cells. We identified a novel chick gene, "GEM", which is expressed in the neural plate, the dorsal neural folds and in early migrating neural crest cells. Ectopic expression of GEM autonomously activated an expression profile typical for early neural crest cells. The GEM protein is localized in the cytoplasm, and nucleo-cytoplasmic shuttling is involved in the regulation of its subcellular distribution. An inhibition of nuclear transfer by anchoring the protein in the inner cytoplasma membrane via a N-terminal myristilic acid residue rendered it non-functional. GEM expression enhanced the emigration of cells from the dorsal neural tube *in vivo*, and triggered the emigration from ventral neural plate explants in tissue culture. An extended analysis employing BMP4, DLX5 and SLUG expression vectors allowed the positioning of GEM in a genetic network leading to early neural crest development.

The Wnt-activated Xiro-1 gene encodes a repressor that is essential for neural development and downregulates Bmp-4

José Luis Gómez-Skarmeta, Elisa de la Calle-Mustienes and Juan Modolell

Vertebrate neural tissue is generated by an inductive process. In *Xenopus* embryos, the Spemann organizer, which is located in the dorsal mesoderm, secretes neural inducers such as Noggin, Chordin and Follistatin that diffuse to the adjacent ectoderm and promote its neural development. In the last few years, it has become clear that neural induction is mediated by interference with BMP-4 signaling. The neural inducers bind to BMP-4, which is present in the ectoderm, and prevent its interaction with BMP-4 receptors, thus blocking signaling and allowing the acquisition of the default neural fate of the ectoderm. In the absence of neural inducers, BMP-4 can signal and promote the epidermal fate (reviewed in 1). Bmp-4 is initially expressed in the whole embryo. During gastrulation expression disappears from the dorsal side of the embryo, including the dorsal mesoderm and the prospective neural plate. Recently, it has been shown that neural inducers, while interfering with BMP-4 function, are unable to repress Bmp-4 expression in *Xenopus*. Instead, the repression seems to depend on Wnt signaling (2). In Xenopus three members of the Iroquis family of homeoproteins have been identified, Xiro1-3. Similarly to their *Drosophila* counterparts, they seem to control the expression of proneural genes such as Xash-3 and Xngnr-1. Thus, injection of Xiro mRNAs promotes ectopic expression of these proneural genes, an effect associated with an expansion of the neural plate, which occurs at least in part at the expense of the neural crests (3). In this work we report that Xiro-1 acts as a transcriptional repressor which is strictly required for neural development, even when BMP-4 signaling is impaired. Our data also indicate that there is a reciprocal inhibition between Xiro-1 and Bmp-4. Moreover, Xiro-1 is activated by Wnt signaling. Xiro-1 is probably a mediator of the repression of Bmp-4 by Wnt in the dorsal ectoderm.

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Molecular regulation of specification of *Xenopus* neuroectoderm and neural crest

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In *Xenopus* neural differentiation is initiated by neural inducers such as Chordin and Noggin, which inhibit BMP activities. Using differential screening, we have identified several genes acting downstream of neural inducers in *Xenopus* embryos. Among them are Zic-r1, Sox2, SoxD and a few other new genes, including secreted molecules. These genes are induced by BMP antagonists such as Chordin and suppressed by BMP4. Zic-r1, SoxD can induce neural differentiation of animal cap ectoderm when overexpressed. Sox2 does not have neuralizing activities while inhibition of Sox2 function by dominant-negative Sox2 results in suppression of neural differentiation. To address the question whether the genes involved in *Xenopus* neural induction play similar roles in the mouse system or not, we carried out studies using mouse epiblast explant and ES cells. I will discuss similarities and differences found in *Xenopus* and mouse neural differentiation.

I will also discuss about the role of FoxD3 on neural crest specification. Gain-of-function and loss-of-function studies have shown that FoxD3 is sufficient and essential for neural crest differentiation in *Xenopus* ectoderm. When FoxD3 function is reduced, prospective neural crest regions fail to express neural crest-specific genes such as Slug, and become positive for the neural plate marker Sox2. I will show detailed analyses on regulatory gene cascades in early neural crest determination.

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The SHH-GLI pathway and the control of dorsal brain development and tumorigenesis

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Elegant experiments by the groups of Jessell, McMahon and others have shown that the SHH-GLi pathway controls the development of the ventral neural tube. Specifically, ventral cell types such as floor plate cells and motor neurons depend on the activity of SHH for their differentiation.

We will present experiments that demonstrate that SHH signaling is required later on, at late embryonic and postnatal stages, for the growth of dorsal brain structures, namely, the cerebellum and cerebral cortex. SHH acts on stem/precursor cells as a mitogen. Our data also points to these populations of cells as possible sources of brain tumors after the inappropriate maintenance of the SHH-GLi pathway, giving rise to hyperplasia and tumor initiation. Alternatively, ectopic activation of this pathway in responsive cells may also initiate tumorigenesis. Together, our results indicate that the SHH-Gli pathway has been co-opted for CNS patterning and growth in an early-ventral/later-dorsal fashion, that regulation of progenitor cell proliferation is a basic function of the SHH-Gli pathway and that its deregulation leads to the formation of brain tumors.

Additional experiments beginning to address the targets of Gli proteins show that Wnts signals are regulated by and mediate Gli activity, providing a bases for an integration of pathways that regulate Gli function, such as Hedgheogs and FGFs with Wnt signaling.

Secondary organizers and brain patterning

Salvador Martínez

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Alicante

In the central nervous system of vertebrates distinct neural identities are acquired through progressive restriction of developmental potential of neuroepithalial domains under the influence of local environmental signals. Evidence for the localization of such morphogenetic signals at specific locations of the developing neural primordium has suggested the concept of "secondary organizer regions", which regulate one step further the identity and regional polarity of neighboring neuroepithelial areas. In recent years, the most studied secondary organizer is the isthmic organizer, which is localized at the hind-midbrain transition and controls the anterior hindbrain and midbrain regionalisation. *Otx2* and *Gbx2* expressions are fundamental for positioning the organizer and the stablishment of molecular interactions that induce *Fgf8* expression and then, stabilizes the autoregulative loop of *En1*, *Wnt1* and *Pax2* expressions (Wurst and Bally-Cuiff, 2001). Temporo-spatial patterns of such gene expressions are necessary for the correct development of the organizer that, by planar mechanism of induction, controls the normal development of the rostral hindbrain, from rh2, to midbrain-diencephalic boundary (Nakamura, 2001). *Fgf8* appears as the active diffusible molecule for the isthmic morphogenetic activity and has been suggested as the morphogenetic effector in other inductive actvities revelated in other neuroepithelial regions, which are ,therefore, considered candidates for rostral secondary organicers. Double *in situ* hibridisation for *Fgf8* and *Shh* expression patterns showed interesting spatial relations between the expression domains of these genes, actively involved in mophogenesys and regionalization of vertebrate neural tube (Martínez, 2001). *Shh* shows abrupt spatial changes in its expression pattern, which is systematically sifted dorsally where *Fgf8* is expressed: the isthmus (I), the zona limitans (ZLI) and at the rostral pole of the brain, the prsumtive commissural plate. It has been demonstrated an inductive activity in the isthmus (isthmic organizer) and in the rostral pole of the brain (Shimamura and Rubestein, 1997). The zona limitans is the region that appears now a suggestive area where a new organizer can display inductive and morphogenetic properties in diencephalic regionalization. This effect has been studied in our laboratory and seems to be related to a control of inductive opposite influences from the anterior pole of the brain and the isthmic organizer. In addition, recent experimental data demonstrate that an ectopic ZLI is induced at the ectopic border generated between prechordal and epichordal neuroepithelium, suggesting that, like the interaction between *Otx2* and *Gbx2* domains, prepatterning factors are underlying the specification and positioning the ZLI organizer.

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Patterning the fly retina

Ross L. Cagan

By overlaying simple patterns of molecules and cell interactions, a sheet of cells is transformed into a complex epithelium. This includes cell proliferation, fate selection, and cell death.. My laboratory uses the *Drosophila* retina to study the mechanisms that generate patterned arrays of cell types in a developing epithelium. We have focused on two steps: the emergence of the first cell fate into an organized array, and the use of cell death to refine this initially loose pattern into a highly precise, functioning retina.

Establishing the first cell fate: How can the first cell fate emerge from an initially homogeneous, naïve epithelium? In the mature larval retina, the pioneering R8 photoreceptor neuron emerges in a square array within the 'morphogenetic furrow'. We have demonstrated a central role for regulators of the *Drosophila* EGF Receptor (dEGFR) in establishing domains of high dEGFR activity. These 'R8 equivalence group' domains, found within the larger 'proneural clusters', establish a stepwise pattern of R8-competent regions within which R8s emerge. We have recently identified a transmembrane regulator of dEGFR which is required to properly space regions of high *vs.* low dEGFR activity.

Notch, Scabrous and boundary formation: dEGFR activity is further refined by the secreted factor Scabrous. Loss of *scabrous* activity (or its overexpression) leads to poor proneural cluster resolution and ectopic R8s within each proneural cluster field. Our *in vivo* and tissue culture results support a direct interaction between Scabrous and the Notch signaling receptor, at a site distinct from the binding domains of the Notch ligands Delta and Serrate. This association stabilizes Notch protein at the surface, preventing the normal turnover associated with Delta. Overall, our data suggest a model in which Scabrous binds to Notch and inhibits Delta-mediated *Notch* activity within the proneural clusters; by restricting its expression, Scabrous can set boundaries of *Notch* activity within the neuroepithelium, a view supported by mosaic analysis. Thus, Scabrous represents a new class of 'modifying ligand' that acts to pattern *Notch* activity.

Programmed cell death: The selective use of programmed cell death provides an addition layer of precision to emerging epithelia. Nearly every tissue makes extensive use of programmed cell death to sculpt their final pattern. For this process to succeed, unneeded cells must be removed in a precisely-regulated manner and, just as importantly, other cells must remain untouched. Although we have learned an increasing amount about the downstream signals that determine life *vs.* death in a cell, little is understood of the upstream signals that tip the balance in one direction or the other.

In the developing retina, programmed cell death is used to bring the ommatidia into a tight hexagonal array. Approximately one-third of the interommatidial cells are removed. We have used laser ablation and high resolution 'movies' to explore the spatial aspects of the signaling that leads to selective cell death. We have combined this with a large-scale genetic screen and organ culturing to identify many of the factors that regulate this process. Our evidence indicates that spatially-precise regulation of the EGF receptor, p38, and Notch signaling pathways is critical. We have also identified several new and perhaps surprising players in this cell death game, and are now working to understand their role in the spatially precise culling of interommatidial cells.

The role of geminin in vertebrate neural cell fate determination

J. Bernard, J. Bainter and K. Kroll

Washington University School of Medicine

The tremendous complexity of the vertebrate nervous system arises through a series of cell-cell signaling events occurring during embryonic development. The first such signaling event is neural induction, signaling from dorsal mesoderm to adjacent ectoderm. The early response of ectoderm to this neural inducing signal is responsible for specifying neural cell fate and defining neural plate boundaries; the molecular basis of this ectodermal response remains largely unknown.

Here, we have used a functional cloning screen in *Xenopus* embryos to isolate a number of new molecules involved in formation and patterning of the neural plate. One of these molecules, which we call geminin, plays an essential role in the earliest aspects of neural cell fate determination. Geminin is essential for neurogenesis and its expression at the onset of gastrulation provides perhaps the earliest defining characteristic of the future neural territory. Misexpression of geminin outside of the nervous system leads to ectopic neurogenesis, while interfering with geminin's function causes failure of neural plate formation, leading to development of those cells as epidermis. Our current studies focus on: (1) identifying pathways regulating geminin's expression in the forming neural plate of the early embryo (2) Characterizing geminin's activities in mammalian neural cell fate determination and in later aspects of neurogenesis and (3) Testing geminin's biochemical role in the embryo. Geminin's expression and activities offer us a unique point of entry into the earliest regulatory pathways controlling neurogenesis.

Neural patterning along the antero-posterior axis in vertebrate embryos

Nobue Itasaki

During vertebrate embryogenesis, neural tissue acquires positional identities along the antero-posterior (A-P) axis that can be defined by combinatorial expression of various molecules. At the level of rhombomeres and the spinal cord, Hox gene clusters play a central role for regional specification. There is a considerable degree of plasticity in the neural patterning along the A-P axis during embryogenesis, and strong signalling activity from the somites is supplied to the neural tube (Itasaki et al., 1996). We are currently pursuing the nature of the somite activity by testing of known candidate molecules and by non-biased screening of a somite library.

Retinoic acid (RA) and fibroblast growth factor (FGF) are well known molecules as transforming neural tissue to more posterior character when applied to embryos. We found in chick neurula stage embryos that these molecules are effective for the posterior transformation only at limited levels of the A-P axis. This result was unexpected because Hox clusters show spatial linearity and are organised in a sequential manner. This shared responsibility for organising the entire axis will be discussed.

From the systematic screening of a somite-derived library using *Xenopus* animal cap explants, a couple of clones were isolated so far, both of which are related to wnt pathway. We found that a number of wnt pathway components can alter the neural patterns both in animal cap and in vivo, and effect of β-catenin is not cell-non-autonomous, suggesting another signalling pathway is involved in the effective action of the pathway.

Patterning the neural crest in the ectoderm of *Xenopus*

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The neural crest cell is a migratory embryonic cell population that forms at the border between the neural plate and the future epidermis. This border of the neural plate, or neural plate border, correspond to the neural fold. The neural fold surround the entire neural plate but only the lateral and posterior portion of the fold give rise to the neural crest cells, while the anterior neural fold differentiate as forebrain. We will analyze some aspects concerning the specification of the neural crest at the lateral-posterior border of the neural plate. We have shown that an interaction between neural plate and epidermis is enough to induce neural crest cells, and currently this seems the most accepted model for neural crest induction. However, there are several results in amphibian and zebrafish embryos that suggest a model based on a BMP gradient for neural crest specification. We have shown that levels of BMP signaling intermediate to those that specify neural plate and epidermis play a role in establishing neural plate border fates, including neural crest. Our recent results show that in order to induce neural crest properly a posteriorizing signal coming from the posterior mesoderm pattern the neural plate border as anterior neural fold (forebrain) and posterior neural fold (neural crest). The molecular nature of this posteriorizing signal seems to be the same as that for the posteriorization of the neural plate: FGF, *Wnt-8* and retinoic acid. Finally, we should try to reconcile the BMP gradient model for the induction of the neural crest with the model based on the interaction between neural plate and epidermis.

The Snail gene family and the evolution of the neural crest

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Functional analysis of the Snail gene family during embryonic development showed that *Slug* was needed in the chick embryo for the delamination of the neural crest and the mesoderm (Nieto et al., 1994) and for the induction and migration of the neural crest in *Xenopus* (Mancilla and Mayor, 1996; LaBonne and Bronner-Fraser, 2000). At the cellular level, its function in mesoderm and neural crest delamination is mediated by the triggering of epithelial-mesenchymal transitions (EMT) that allows an epithelial cell to separate from its neighbours and migrate to populate different regions within the organism. The generation of a *Slug* null mutant in mice by homologous recombination (Jiang et al., 1998) indicated that, however, it is not required for mesoderm or neural crest development in mice. This unexpected result and the simultaneous description of a very striking interchange in the expression patterns of the two family members (*Slug* and *Snail*) between chicken and mouse embryos led to the suggestion that *Snail* rather than *Slug* could be the gene involved in EMT in the mouse (Sefton et al., 1998). We have recently confirmed that *Snail* triggers EMT in mammalian cells, acting as a repressor of the epithelial phenotype (Cano et al., 2000).

This raises the question of whether Snail and Slug can be functionally equivalent when ectopically expressed at the appropriate sites both intra- and interspecies. I will discuss a series of overexpression experiments that we have carried out to directly address this possibility.

Another important question is when this unusual interchange in the expression patterns occurred in evolution. This has led us to carry out a careful analysis of the gene family from an evolutionary point of view that involves the analysis of different vertebrates and the study of the phylogenetic relationship between the family members. In addition to learning about the change in expression patterns, this analysis is very useful since the neural crest is believed to have been crucial in the formation of the "new head" of vertebrates (Gans and Northcutt, 1983). Interestingly enough, although the neural crest is considered as a vertebrate character, non-vertebrate chordates such as ascidian and *amphioxus* embryos show expression of *snail* homologues in the dorsal neural tube, precisely the region where the neural crest is formed in vertebrates. I will present data on the expression of Snail and Slug in fish, turtle, lizard, avian and mammalian embryos and discuss the potential ancestral and derived functions of the gene family in evolution.

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Session 2: Neural determination
Chair: Claudio D. Stern

Neural crest stem cells

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Neural crest stem cells are self-renewing, multipotent neural stem cells that can give rise to multiple peripheral neuron cell types, glia (Schwann cells) and smooth muscle cells. Cells with similar properties can be prospectively isolated from uncultured neural tissue by fluorescence activated cell sorting using specific cell surface antibody markers. These cells self-renew *in vivo* as well as *in vitro*. I will discuss current studies of the control of cell fate decisions in these stem cells by extracellular signals and by transcription factors.

Patterning neurogenesis in the zebrafish embryo

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Our goal is to identify cellular, molecular and genetic mechanisms that determine how neurons are made in the appropriate number and location in the neural plate. In order to identify such mechanisms we have identified zebrafish mutants with an aberrant pattern of early neurons. Analysis of these mutants is beginning to define mechanisms that are essential for patterning early neurogenesis in the zebrafish embryo.

The *headless* (*hdl*) mutant [1] was identified by its expanded domain of trigeminal neurons. The aberrant pattern of trigeminal neurons in this mutant is due to posteriorization of the anterior neurectoderm, where the relatively caudal midbrain-hindbrain boundary (MHB) domain is expanded at the cost of rostral domains that form the eyes, forebrain and most of the midbrain. Ectopic activation of Wnt/Wingless signaling during early gastrulation has previously been shown to have similar effects on anterior neural development development suggesting that there is loss of a mechanism that inhibits Wnt signaling in *hdl* mutants. Genetic mapping of the mutation revealed that *hdl* encodes a member of the Tcf/Lef family, Tcf3, a transcription factor that acts as a repressor of Wnt target genes through its association with co-repressors, or as an activator through its association with beta-catenin. Our results suggest that basal repression of Wnt targets by Tcf3 during early gastrulation is essential for expression of genes responsible for determining anterior neural fate. Previous studies have suggested a critical role for secreted Wnt antagonists as head inducers. Our studies suggest that these Wnt antagonists act as head inducers by maintaining basal repression of Wnt target genes provided by Tcf3. In the absence of this repression the Wnt antagonists are ineffective at inducing the most anterior neural tissues. Furthermore, our studies suggest that Tcf3 helps establish a gradient of Wnt signaling that is essential for proper specification of neural fates in the anterior neurectoderm.

The neurogenic mutant, *mind bomb* (*mib*) [2,3], is characterized by an over-production of early neurons. Functional analysis suggests these mutants have a defect in the Notch signaling pathway. Previous studies have shown that Notch signaling mediates lateral inhibition and normally limits the number of neurons produced in the embryo. We showed that activation of Notch signaling reduces the neurogenic phenotype and ectopic expression of genes that inhibit Notch signaling mimic the *mib* phenotype. By positional cloning we have recently identified *mib* as a novel gene that is widely expressed at low levels in the embryo. We are now investigating how this novel protein contributes to Notch signaling.

Together these studies illustrate how the analysis of zebrafish mutants has provided important insights into molecular mechanisms that determine how neurons are made in the correct number and location in the nervous system.

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Proneural genes control the proliferation of cortical progenitors

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The generation of the complex cytoarchitecture of the mammalian cerebral cortex depends on a precise schedule of neuronal and glial differentiation. Currently, the molecular mechanisms regulating the stereotyped patterns of cortical progenitor cell division and differentiation are poorly understood. We have begun to examine the role of the proneural genes Neurogenin 1 (Ngn1) and Ngn2, which encode basic-helix-loop-helix transcription factors, in regulating the proliferation and differentiation of cortical progenitors. Mutant analyses have revealed distinct requirements for these two genes during corticogenesis. In Ngn1 mutant embryos, cortical progenitors undergo a premature burst of proliferation and neuronal differentiation. In contrast, Ngn2 mutant progenitors exhibit a reduced rate of proliferation that is accompanied by defects in the expression of key cell cycle regulators, suggesting that proneural genes may directly control cell cycle progression. Strikingly, defects in the proliferative properties of single Ngn1 and Ngn2 mutant cortical progenitors do not lead to gross defects in the laminar organisation of the cerebral cortex at birth. In contrast, both the radial glia scaffold and cortical layering are disrupted in Ngn1;Ngn2 double mutants. We are currently examining whether this is a consequence of the altered developmental potential of mutant progenitors. In conclusion, we suggest that the Ngns are integral components of the regulatory cascades that control the timing and pattern of cortical progenitor cell divisions.

Cell type specification in the developing spinal cord of the zebrafish

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We are interested in understanding how the composition of the cell lineages in the developing spinal cord of the zebrafish is regulated. We follow a combination of three different approaches to this problem, results of which will be subject of my presentation. The first approach is clonal analysis, which involves either labelling individual cells within the neural plate by intracellular injection of fluorescein-dextran or transplanting cells that express GFP, and analysing the development and composition of the clones generated by them. We find that the cell lineages have stereotypic, region-specific composition. Intracellular injection of rhodamine-dextran followed by repeated injections of BrdU indicates that progenitor cells divide asymmetrically with a stem cell-like pattern, giving rise to daughter cells that differentiate either as neurones or as glia cells, and daughter cells that continue to divide. In the second approach, transgenic embryos expressing GFP fused to either histone 2A or a tau protein are used to investigate various aspects of the pattern of mitotic divisions in the developing spinal cord *in vivo*. Particular attention is given to the orientation of the mitotic spindle in the ventricular zone and the behaviour of the daughter cells. We find that the mitotic spindle is always oriented parallel to the ventricular surface, irrespective of whether the daughter cells differentiate or continue to divide.

In the developing spinal cord, specification of progenitor cells and of the different cell types within lineages is mediated by diffusing morphogens and by direct cell-to-cell interactions. In the third approach, we use the Gal4-UAS technique for directed gene expression to analyse molecular genetic aspects of cell lineage development. We are currently studying the role played by Sonic hedgehog and Notch signalling in the specification of progenitor cells and of the various cell types. Zebrafish from activator strains, which express Gal4 in particular regions of the neural plate driven by specific promoters, are crossed to individuals of effector strains carrying *UAS:Shh* and *UAS:notch1a-intra* transgenes, and the effects on spinal cord development in the progeny are studied. Results from this work will be discussed.

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Spatial and temporal control of neurogenesis in *Xenopus* embryos

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The neuroectoderm forms early in the development of the embryo and gives rise to neurons and glia that populate the nervous system. Differentiation within the embryonic neuroectoderm takes place in a spatial and temporal order. This allows the nervous system to grow since some progenitor cells divide longer before they differentiate and is important for generating the correct cell type diversity in the nervous system. I will present two mechanisms by which neuronal differentiation is controlled in space and time during embryogenesis.

Over the last few years several transcription factors that either suppress or promote neuronal differentiation have been identified (e.g. [1], [2], [3]). These factors have a restricted spatial distribution on the neural ectoderm. How is their expression controlled? *XBF-1* is a winged helix transcription factor that is expressed in the embryonic forebrain. Analysing the activity of *XBF-1* we have found that it controls the expression of both differentiation-promoting and differentiation-inhibiting factors. Thus, *XBF-1* is a bifunctional transcription factor, acting either as a suppressor or an activator of neuronal differentiation. Whether the neuronal differentiation-inhibiting or -promoting activity predominates depends on whether *XBF-1* is expressed at a high or low concentration, respectively. Based on these findings, we propose that one mechanism of spatial and temporal is based on the localised expression of transcription factors, such as *XBF-1*. Like *XBF-1*, such factors could position neurogenesis by virtue of their dual activity as activators or suppressors of differentiation in a dose dependent manner [4]. Recently, we have found that *XBF-1* also affects the proliferation of neuroectodermal cells by controlling the transcription of a cell cycle inhibitor, thereby co-ordinating cell division and neural cell fate specification in the ectoderm [5].

However, some parts of the neuroectoderm do not differentiate in response to a low dose of *XBF-1*, suggesting the existence of additional mechanisms of spatiotemporal control. In the second part of my talk, I will present evidence for another mechanism, which may regulate the generation of primary (early) versus secondary (late) neurons. At the neural plate stage, the *Xenopus* neuroectoderm consists of a superficial and a deep layer of cells. In a fate mapping study of the neural plate, the deep layer cells were found to differentiate early in development while the superficial layer cells remained undifferentiated as late as the tadpole stage [6]. We have found that these superficial cells do differentiate at a later stage and is therefore justified to consider them as precursors of secondary neurons. Furthermore, primary and secondary precursors can be mapped to different layers of the ectoderm as early as at the gastrula stage [7].

We have found that these two cell populations have intrinsic differences in their capacity for early neuronal differentiation in response to a variety of inducing factors, including the endogenous neuronal inducer [8]. We have also found that these two layers are the result of early oriented cell divisions [9]. Such divisions may result in the unequal inheritance of factors that influence the competence of the cells for neuronal differentiation.

We propose that this may represent an additional mechanism to control neurogenesis and I will present strategies to identify molecular differences between the two layers at the time when these are generated.

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Cellular and molecular mechanisms underlying the progressive generation of the spinal cord

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The chick spinal cord is generated over an extended period of time in a head to tail sequence such that there is a spatial as well as temporal separation of the events of neurogenesis. Cells that give rise to spinal cord reside in the caudal neural plate, a unique region of the neural plate which regresses alongside the primitive streak to the caudal end of the embryo. As cells leave the caudal neural plate they form the neural tube (Brown and Storey, 2000) where the first neurons are born adjacent to the first formed somites.

Here we review key steps underlying the generation of the spinal cord from its primordium, the caudal neural plate. Cells leaving this region down regulate caudal neural plate specific genes, such as the proneural gene homologue *cash4* (Henrique et al., 1997) and begin to express genes characteristic of the differentiating neural tube, *Irx-1, Pax6* and the marker of differentiating neurons *NeuroM*. We demonstrate an inverse relationship between the expression of caudal neural plate genes, which are maintained by underlying presomitic mesoderm and FGF signalling, and neuronal differentiation, which is repressed by such signals and accelerated by somitic mesoderm. Strikingly, we find that somites can act by attenuating FGF signalling in the caudal neural plate. However, while blocking the FGF pathway up-regulates some genes characteristic of the differentiating neural tube, such as *Pax6* (see (Bertrand et al., 2000) this is not sufficient to elicit neuronal differentiation. These findings identify the caudal neural plate as a unique region in which neuronal differentiation is repressed and suggest that an initial step in neurogenesis is the removal of FGF signalling. Further signals provided by the somites are however required to promote neuronal differentiation. We discuss these results in the form of a model in which the changing signalling properties of the paraxial mesoderm regulate the onset of neuronal differentiation in the spinal cord.

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Proneural gene function during *Drosophila* neural specification

Andrew Jarman

Development of sensory neurons of the *Drosophila* PNS has provided a particularly important model for neurogenesis, not least as a paradigm for the function of bHLH proteins. The best known proneural genes are those encoded by *atonal* and the *achaete-scute* complex (AS-C). These genes have much in common: they are required for neural precursor fate, are expressed in ectodermal proneural clusters, their proteins share a related bHLH domain (~40% identical), and all appear to activate certain neural-specific target genes. In each case, lateral inhibition refines neural fate within proneural clusters.

In addition to these common features, there are major differences in the function of proneural genes. They are required for distinct subsets of neural precursors, and so contribute to the specification of neuronal subtype identity as well as to general neural fate (Jarman and Ahmed, 1998). We believe that the proneural genes are actively involved in the processes and mechanisms that translate these conceptual identities into biological differences between sensory neurons. Different neuronal fates must be specified by the selective regulation of different downstream targets (Jarman and Jan, 1995). I am particularly interested in this subtype specificity, both in terms of the target genes that must be differentially activated and the developmental and molecular mechanisms by which the proneural proteins achieve this regulation.

One possible difference between the proneural genes may be in the differential activation of EGFR-mediated neural recruitment by *atonal*, leading to *atonal*-dependent sensory neurons (chordotonal organs) forming dense clusters (zur Lage and Jarman, 1999) (although the recent discovery of EGFR-mediated lateral cooperation complicates this, Culi et al., 2001). Ongoing work is aimed at defining the molecular basis of how *atonal* regulates EGFR signalling and how this signalling results in neural recruitment.

We recently described *amos* as a putative proneural gene for some classes olfactory sensilla (Goulding et al., 2000). Since this report, we have succeeded in isolating several mutations in *amos*, preliminary characterisation of which suggests that there is plenty to learn about the development of these classes of sensilla.

We have used an *atonal*-induced 'rough' eye phenotype (White and Jarman, 2000) as the basis for an F1 genetic modifier screen. One of the modifier mutations isolated defines a new gene required for refinement of neural fate in proneural clusters and we are investigating how it fits in with Notch signalling.

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Regulation of the Notch pathway during neurogenesis

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During animal development, the Notch family of receptors plays an instrumental role in regulating the pattern of neurogenesis, both during the process of lateral inhibition, and potentially by prepatterning proneural domains. To analysis the role of Notch in regulating neurogenesis in *Xenopus* embryos, we have studied two genes that regulate Notch signaling. One gene, called *Nrarp*, encodes a small protein containing two ankyrin repeats and is upregulated in *Xenopus* embryos by Notch mediated transcription. Overexpression of *Nrarp* in embryos blocks Notch signaling and inhibits the upregulation of other Notch target genes. Nrarp forms a ternary complex with the intracellular domain (ICD) of XNotch1 and the CSL protein XSu(H), and promotes the loss of ICD. By downregulating ICD levels, Nrarp could function as a negative feedback regulator of Notch signaling that attenuates Notch-mediated transcription. The other gene is the *Xenopus* homolog of *neuralized*, which encodes a RING finger domain protein with unknown function in the Notch pathway. Neuralized is expressed in *Xenopus* embryos in a pattern reminiscent of *Drosophila neuralized*, and when overexpressed produces neurogenic phenotypes. The neuralized protein fulfills the biochemical criteria for an ubiquitin ligase, and interacts physically with the Notch ligand, XDelta1. We propose the Neuralized negatively regulates XDelta1 activity during neurogenesis via ubiquitination, and that negative regulation is essential for maintaining proper levels of ligand required for Notch signaling.

Session 3: Neural specification and identity
Chair: David J. Anderson

Specification and differentiation of the *Drosophila* CNS midline glial cells

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Any complex nervous system is made up by two major cell types: neuronal and glial cells types. Whereas neurons have the task to send out long processes to form the intricate neuronal network, which collects and integrates information, glial cells have numerous functions ranging from important functions during the development of the complex neuronal network to electrical insulation of neurons.

We have used the embryonic *Drosophila* CNS as a model system to understand the molecular logic orchestrating the development and function of glial cells. About 65 glial cells are found in each abdominal neuromere of the embryonic ventral cord. To date the lineage relationships of all glial cells – as well as the lineage relations of the neurons - are known. Some glial cells are associated with neuronal cell bodies, however, most are found in close association with neuronal axon tracts. The major axon tracts in the embryonic CNS of *Drosophila* are organized in a simple, ladder like pattern. Each neuromere contains two commissures that connect the two contra-lateral sides and two longitudinal connectives connecting the different neuromeres along the anterior-posterior axis. The formation of the commissural tracts, in which all contra-lateral projecting neurons send their axons across the CNS midline, occurs in close association with the CNS midline glial cells. In the absence of midline glial cells a characteristic CNS phenotype develops.

To unravel the genes controlling midline glia development in the embryonic ventral nerve cord, we conducted a large scale saturating EMS mutagenesis. Genetic and phenotypic analyses showed that the midline cell lineages are specified by the combined action of *single minded* and several segment polarity genes. Subsequent differentiation of the midline glial cells requires more than 20 different gene functions. Analyses of cell autonomously acting genes regulating glial cell polarity (*klötzchen*) as well as non cell autonomously acting genes influencing glial differentiation will be discussed (*kette, schmalspur*).

Neural stem cells sequentially express transcription factors which specify the temporal identity of their neuronal progeny

Chris Q. Doe

Stem cells often generate distinct cell types in a specific order, but the intrinsic or extrinsic cues regulating temporal cell fate specification remain mysterious. I will show that most *Drosophila* neural stem cells sequentially express the transcription factors Hunchback, Krüppel, Pdm, Castor, and that their neuronal progeny maintain the transcription factor profile present at their birth. Hunchback is necessary and sufficient for the first-born cell fate in all neuroblast lineages assayed, independent of whether the first-born cell will be a motoneuron, interneuron, or glia. Similarly, Krüppel is necessary and sufficient for second-born cell fate, independent of whether the second-born cell will be a motoneuron, interneuron, or glia. We propose that Hunchback and Krüppel control early-born temporal identity in neural stem cell lineages.

Cell cycle, cell fate, asymmetric cell divisions and the generation of neuronal diversity

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Asymmetric cell divisions can be mediated by the preferential segregation of intrinsic cell fate determinants into one of two sibling daughters. For this to occur the orientation of mitotic spindle and the localisation of the determinants need to be coordinated. In *Drosophila* neural progenitors divisions the mediation and coordination of these processes (along with RNA localisation and some aspects of sibling daughter cell size asymmetry) require several proteins, including Bazooka (Baz), Inscuteable (Insc) and Partner of Inscuteable (Pins), which localise as an apical cortical complex starting at interphase. Here I will: 1) briefly summarise our present knowledge on the formation and function of this apical complex; 2) present results from experiments which shed some light on the question of why the localisation of the various asymmetrically localised proteins shows cell cycle dependence; 3) summarise findings which provide some initial genetic evidence supporting the existence of a cryptic mechanism which can partially compensate for the absence a functional apical complex.

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Neural identity in the sense organs of fly and fish

N.Gompel, S.Layalle, A.Ghysen and C.Dambly-Chaudière

Our aim is to understand the rules that govern specificity during neural development. We first approached this question in the sensory system of the fly. More recently, we have extended our analysis to the development of the lateral line of the fish, a sensory system that presents the same qualities of accessibility, definition and reproducibility as in *Drosophila*.

In *Drosophila*, we pursue the characterization of the gene *poxn*, which controls all aspects of the development of chemosensory organs including the chemosensory specific lineage and the connectivity of the sensory neurons. In order to get more information about how *poxn* controls these two processes, we made a substractive library of genes expressed in the legs of $poxn^{+}$ but not of $poxn^{-}$ pupae (in collaboration with A.Giangrande and G.Ragone). The resulting library yielded 80 candidates. 40 have been sequenced, 36 of which are homologous to known genes while 4 are novel genes. *in situ* hybridisation on embryos are in progress.

In zebrafish, we have recently shown that the projection of the mechanosensory lateral line is somatotopically organized, with posteriormost neuromasts projecting dorsalmost in the hindbrain. Since both sensory cells and neurons originate from an ectodermal placode, they might be sibling cells and therefore share some common marker that might allow mutual recognition later on. We examine the lineage relationships between sensory neurons and sensory cells, and show that there is no strict relationship between the two cell types.

We are now trying to identify what drives the correspondance between the position of a sense organ, and the position where its innervating neuron will arborize in the brain. We examined the development of the sensory neurons during embryogenesis, and discovered that central projections form much before the neurons innervate their peripheral targets. We observed that morphological differences among the sensory neurons can be detected much before the corresponding neuromasts are laid down. We show that these differences are correlated to the position of the neuromasts that the neurons will eventually innervate, suggesting that the determining factor in somatotopy lies not in the position of the sense organ, but in the neurons themselves, or in their central projections.

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Fate choices and asymmetric cell divisions in fly neural stem cells

Angela Giangrande

One of the most challenging issues in biology is to understand the mechanisms governing the establishment of cell diversity during development. The complex information processing capacity of the nervous system relies on the ability of stem cells to produce all the classes of neurons and glial cells using developmental pathways that are genetically determined. These pathways require a combination of extrinsic and/or intrinsic signals that progressively restrict the potential of stem cells and allow the generation of different cell identities. One of the first decisions that must be taken is whether to become a neuron or a glial cell. Due to the simplicity of its nervous system, *Drosophila melanogaster* represents an ideal genetic model system to analyze the molecular and cellular bases of this fate choice. In addition, all the neural lineages have been traced with respect to the progeny issued from each precursor. Furthermore, previous analyses have allowed the identification of each stem cell on the basis of their position and profile of gene expression. Finally, the determinant responsible for the choice between the neuronal and glial fate has already been identified: the gliogenic factor Glial cell deficient/Glial cell missing (Glide/Gcm).

glide/gcm mutations result in embryonic lethality due to the lack of glial cells (1,2,3). *glide/gcm* codes for a novel type of transcription factor that positively autoregulates (4,5,6,7) and is located close to a related gene called *glide2* (8). Strikingly, the expression of both genes is necessary and sufficient to promote the glial fate *in vivo*, indicating the key role of these factors in glial differentiation (1,2,9,10,11,12). Moreover, glial differentiation is triggered at the expense of neurons, since lack of Glide/gcm converts glial cells into neurons. The identification of the glial fate determinant constitutes a real breakthrough in our understanding of how cell diversity is established in the nervous system.

The mechanism by which the fate choice is induced is asymmetric distribution and inheritance of *glide/gcm* RNA (10,11,13). By looking at a specific lineage, we have shown that two steps are required, asymmetric distribution of *glide/gcm* RNA in the neuroglioblast (NGB) and maintenance of *glide/gcm* expression in the cell that inherits most transcripts, the glioblast (13). The latter step requires the Prospero transcription factor. We have also found that RNA asymmetry is established progressively and that it only become apparent at NGB metaphase. In addition, we have found that *glide/gcm* RNA displays a different subcellular localization compared to that of other fate determinants of the nervous system. Finally, the overall mode of division of the NGB is different from other asymmetric divisions with respect to mitotic apparatus and orientation of division.

In order to determine the molecular and cellular bases of the fate choice between neurons and glia, we are now using *in vivo* and *in vitro* approaches to understand how *glide/gcm* expression and RNA asymmetry are regulated.

We are also analyzing the differentiation of peripheral glial cells, which display features similar to those of oligodendrocytes. These cells originate from sensory organ lineages that express *glide/gcm* (14,15,12). *glide/gcm* is not required in the asymmetric division that generates the glial precursor (GP), but is necessary in the GP to activate the glial program. The differentiation of peripheral glial cells depends on fixed cues and on cell cell

interactions: Notch represses *glide/gcm* and thereby gliogenesis (16). Once the GP has differentiated, it stops expressing *glide/gcm* and starts moving and proliferating. The profiles of migration and division are very variable, suggesting that the late steps of gliogenesis do not depend on lineage decisions. We are now using several approaches to determine the cues underlying these processes.

The results of these analyses will help us understanding how neural precursors are able to produce glia and neurons and, more in general, how cell diversity can be generated during development.

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POSTERS

Xenopus Enhancer of Zeste (XEZ); an anteriorly restricted polycomb gene with a role in neural patterning

M.W.Barnett, R.A.Seville, S.Nijjar, R.W.Old and E.A.Jones

We have identified the *Xenopus* homologue of *Drosophila* Enhancer of Zeste using a differential display strategy designed to identify genes involved in early anterior neural differentiation. XEZ codes for a protein of 748 amino acids that is very highly conserved in evolution and is 96% identical to both human and mouse EZ(H)2. In common with most other *Xenopus* Pc-G genes and unlike mammalian Pc-G genes, XEZ is anteriorly restricted. Zygotic expression of XEZ commences during gastrulation, much earlier than other anteriorly localized Pc-G genes; expression is restricted to the anterior neural plate and is confined later to the forebrain, eyes and branchial arches. XEZ is induced in animal caps overexpressing noggin; up-regulation of XEZ therefore represents a response to inhibition of BMP signalling in ectodermal cells. We show that the midbrain/hindbrain junction marker En-2, and hindbrain marker Krox-20, are target genes of XEZ and that XEZ functions to repress these anteroposterior marker genes. Conversely, XEZ does not repress the forebrain marker Otx-2. XEZ overexpression results in a greatly thickened floor of the forebrain. These results implicate an important role for XEZ in the patterning of the nervous system.

Functional and structural analysis of the murine homeobox gene Irx2

May-Britt Becker

Irx2 belongs to the Iroquois gene family which is classified as a subgroup of the TALE homeobox superfamily (Bürglin, 1997). The murine family contain so far six members and all of them show a very high degree of sequence identity in the homeodomain and in the "iro-box" (Bosse et al, 1997 and 2000; Cohen et al, 2000). In D. melanogaster the genes of the Iroquois complex (IRO-C) are mediators of the neuronal regulation cascade and are implicated in the development of the peripheral nervous system. By functional analysis we would like to know if the neurogenic pathway is conserved between fruitfly and mice. The Irx genes are the possible missing link in the regulatory cascade among the BMP, Gli and Mash genes. Here we are presenting the expression analysis of Irx2 and its loss of function experiment. The expression pattern of Irx2 is very specific concerning time and space parameters. The expression starts in rhombomere four in the phase of neurulation. In the developping CNS Irx2 is present in the regulative areas between fore- and midbrain and around the mid-hindbrain-border. Outside of the nervous system Irx2 activity is present in the early lung, the limbs and the forming cartilage of the vertebral column. The functional analysis of this gene is done by replacing the DNA binding domain - the homeobox - with an lacZ-Neo-construct. In respect to the region specific begin of Irx2 expression without overlap to other members of the family we exspect morphological changes in the mutant hindbrain.

Requirement for neural crest in the development of epibranchial ganglia

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The cranial sensory ganglia have a dual embryonic origin, arising from both the neural crest and neurogenic placodes. In this way they differ greatly from those of the trunk which are derived exclusively from neural crest. Neurogenic placodes are specialized regions of embryonic ectoderm within which neurons are born. We have previously shown that the neurogenic placodes which contribute to the epibranchial series of ganglia (the geniculate, petrosal and nodose) arise independently of the neural crest. The epibranchial placodes are induced in the ectoderm by the pharyngeal endoderm, in an interaction which requires the signaling molecule BMP-7 (Begbie et al., 1999).

Here I will present data which suggest that although the neural crest is not required for the induction of the epibranchial placodes, it is necessary for post-inductive events. We have examined the migration of placodal cells as they leave the placode and move towards the site of ganglion formation. Both the migration of the cells, and the subsequent innervation of the hindbrain by the post-mitotic neurons are perturbed by the absence of crest, while maturation and ganglion formation seem unaffected.

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Functional analysis of Sox group E genes during gliagenesis

Yi-Chuan Cheng, Ching-Jung Lee, and Paul J. Scotting

At least 30 Sox (SRY-box related) genes have been identified in different species to date and divided into seven groups, designated A-G. Members of the same group usually share >80% amino acid identity within the HMG domains. Sox group E comprises Sox8, Sox9, and Sox10. Mutations in SOX9 cause campomelic dysplasia and autosomal XY sex reversal, mutations in SOX10 cause Waardenburg-Shah syndrome and Yemenite deaf-blind hypopigmentation syndrome, and recently SOX8 had been suggested a candidate gene for contributing mental retardation phenotype in ATR-16.

We have recently cloned chick Sox8 and chick Sox10 homologs and, together with the previously identified chick Sox9, analyzed their expression in detail in the developing nervous system. The transcripts of these genes were all detected in the early neural crest cells and macroglia of the cerebellum, although there was a striking degree of variation in their expression in different cell populations. These genes were also expressed in several glial derived tumours and cerebellar tumours, therefore providing useful potential prognosis marker. In addition, in ovo electroporation has been performed on chick embryos in order to study the biological functions of this highly conserved subgroup in developing nervous system.

The BMP/CHORDIN antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo

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We have investigated the role of the Bone Morphogenetic Protein (BMP) pathway during neural tissue formation in the ascidian embryo. The orthologue of the BMP antagonist, chordin, was isolated from the ascidian Halocynthia roretzi. While both the expression pattern and overexpression phenotype of chordin and BMPb, the dpp-subclass BMP, did not suggest a role for these factors in neural induction, the BMP/CHORDIN antagonism affected neural tissue patterning. BMPb induced ectopic sensory pigment cells in the other brain lineages and suppressed pressure organ formation. Reciprocally, chordin suppressed pigment cell formation and induced extra pressure organ. We show that pigment cell formation occurs in three steps. (1) During cleavage stages ectodermal cells are neuralized by a vegetal signal that can be substituted by bFGF. (2) At the early gastrula stage, BMPb secreted from the lateral nerve cord blastomeres induces the a8.25 neuralized blastomeres to adopt a pigment cell fate. (3) At the tailbud stage, among the pigment cell precursors, BMPb induces the anterior type of pigment cell, the otolith, while CHORDIN suppresses BMP activity and allows ocellus differentiation posteriorly.

Key words: neural induction, neural patterning, sensory pigment cell, BMP, CHORDIN, bFGF, ascidian.

Progression of neurogenesis is regulated by paraxial mesoderm in the chick spinal cord

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Coordination of neural and somitic development is essential for the ordered outgrowth of neurons from the spinal cord, however, little is known about how this is achieved. In most vertebrates neurogenesis takes place in a head to tail sequence within the spinal cord and occurs in parallel with the differentiation of underlying paraxial mesoderm into somitic tissue. We address the possibility that the changing signalling properties of the paraxial mesoderm regulate the progression of neurogenesis in the chick prospective spinal cord. Our approach is to compare the production of neurons in explants of this early neural plate in the presence or absence of specific mesodermal tissues. We find that neurons, identified by the expression of NeuroM, a gene related to the *Drosophila* proneural gene atonal, differentiate earlier in the presence of somitic tissue. On the other hand, removal of presomitic mesoderm in vivo leads to an expansion of the domain of NeuroM expression indicating that this mesodermal tissue normally represses the differentiation of neurons. We further identify fibroblast growth factor (FGF) as a repressor of neuronal differentiation. However, we find that although somites act to attenuate FGF signals in the spinal cord, inhibition of FGF signalling alone is not sufficient to trigger the formation of neurons, indicating that somites interact with multiple pathways during this process. We propose a model in which somitic and presomitic signals act during normal development to control the timing of neurogenesis, a key step of which is the regulation of fibroblast growth factor signalling.

Molecular analysis of the induction and positioning of the midbrain-hindbrain boundary in *Xenopus*

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During development, the emergence of domains with specific gene expression patterns within the neural tissue define groups of cells with different abilities and fates. An example of this kind of processes is the establishment of the midbrain-hindbrain boundary (MHB). It has been shown in chicken that this tissue has organizer properties (1) and that this ability is due to the expression of fgf8 (2,3). The use of transgenic and knockout techniques in mouse have shown that positioning of this region depends on the expression of Otx2 and Gbx2, perhaps due to inhibitory activities, they have over each other (4,5). Thus the interaction of large territories of gene expression in the embryo define the position of a new emergent domain which has the capacity of inducing changes in surrounding cells. We have studied the genetic interactions leading, not only to the positioning but also the induction of the MHB in *Xenopus*. We have demonstrated that, as in mouse, Otx2 and Gbx2 have a crucial role in the positioning of this region. These genes participate as transcriptional repressors having cross inhibitory activities as in the mouse. More importantly, the contact of explanted tissues expressing these genes is sufficient to induce the expression of fgf8 and, moreover, this induction is dependent on FGF. Furthermore, we have observed that Xiro regulates, as a repressor, the expression of Gbx2. Therefore, Xiro is an uncharacterised upstream component of the mechanism that permits the correct MHB positioning.

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Neural patterning around the vertebrate embryonic midbrain-hindbrain boundary

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Segmentation is an important mechanism involved in patterning the neural tube which allows to control the spatial organization along the anteroposterior axis of the neural tube. It has been particularly well-characterized for the embryonic hindbrain region which is transiently subdivided into segments called rhombomeres. Under the influence of genes expressed in segment-specific patterns, the generation of distinct and regionally specific structures from each rhombomere is achieved with the almost complete absence of cell mixing between neighbouring rhombomeres.

We have now examined patterning mechanisms at the midbrain-hindbrain boundary (MHB) where *Otx2*-expressing midbrain cells abut *Gbx2*-expressing hindbrain cells. This sharp line of demarcation between the two expression domains suggests that this interface would be a compartment boundary, with no intermixing of cells, but this has not previously been examined.

We have used short-term re-aggregation assays to compare the adhesive properties of cells derived from midbrain and anterior hindbrain, and cell labelling *in vivo* directly to monitor cell behaviour at the midbrain/hindbrain boundary. Interestingly, our data demonstrate that --- in contrast to the hindbrain rhombomeres --- differential adhesion does not seem to operate between the midbrain and anterior hindbrain and that --- at least to a certain extent --- cells move between the two territories. In contrast to the inter-rhombomeric boundaries, the MHB, therefore, does not represent a compartment boundary *sensu strictu*. We conclude that these two subdivisions are not separated and maintained by cell lineage restriction but by cells maintaining labile fates, such that cells change their *Otx2/Gbx2* expression profile as they move from midbrain into hindbrain and *vice versa*.

Patterning of anterior neural plate occurs and is maintained in cripto-/- embryos, despite the absence of the node

Giovanna Liguori

Cripto is the original member of the EGF-CFC family of proteins, which play key roles during vertebrate embryonic development. Adult cripto+/- heterozygous mice were apparently healthy and fertile, while, cripto null homozygotes died during gestation, between 8.5 and 9.5 days of development. cripto-/- embryos do not form the primitive streak and node, thus lacking all embryonic posterior structures and are mostly constituted by anterior neuroectoderm. The intriguing phenotypic characteristics of cripto null mutants (absence of the node and mislocalization of the anterior visceral endoderm) made them very useful to gain insight into the complex interactions that give rise to the anterior neural plate development. A two-step model has been proposed, identifying an initial induction of the anterior neural identities, mediated by AVE, and the subsequent maintenance and reinforcement of these identities by the node-derived anterior mesendoderm. The ectopic distribution of anterior neuroectoderm in cripto mutants, in the absence of the node and corresponding to the AVE mislocalization, confirms this model, clearly identifying in the AVE, and not in the node, the ability to induce the anterior neural plate specification. Moreover and more interestingly, the finding of a correct polarity inside the anterior neuroectoderm, which mostly constitutes the extremely degenerated cripto mutant embryos, might indicate that the node is not essential even for the early maintenance of the rostral neural plate. Thus, the AVE might be sufficient to maintain the anterior neural identities, at least until early somite stage.

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Changes in cSox3 gene expression during patterning of the epibranchial placodes and subsequent neurogenesis

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Until recently, studies of the neurogenic placodes has been impeded by a lack of early markers. Over the past few years however, several genes encoding transcription factors have been shown to be expressed early during placodal neurogenesis. We have identified cSox3, a member of the Sox HMG family of transcription factors, as a gene expressed in placodal ectoderm prior to the onset of neurogenesis. We have used in vivo experimental strategies to investigate the relationship between changes in cSox3 expression and the early stages of epibranchial placode development. Fate mapping and in ovo electroporation have been used to determine the relationship between cSox3 expression and forming placodes and the role of cSox3 in the transition from surface epithelium to migrating neuronal precursor. We will describe how the expression of cSox3 relates to markers of neurogenesis such as the neurogenins, and how we have used cSox3 to demonstrate the patterning of the surface ectoderm which gives rise to the placodes.

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Distinct sites of origin of oligodendrocytes and somatic motoneurons in the chick spinal cord: oligodendrocytes arise from Nkx2.2-expressing progenitors by a Shh-dependent mechanism

Cathy Soula

In the vertebrate spinal cord, oligodendrocytes arise from the ventral part of the neuroepithelium, a region also known to generate somatic motoneurons. The emergence of oligodendrocytes, like that of motoneurons, depends on an inductive signal mediated by Sonic hedgehog. We have defined the precise timing of oligodendrocyte progenitor specification in the cervico-brachial spinal cord of the chick embryo. We show that ventral neuroepithelial explants, isolated at various development stages, are unable to generate oligodendrocytes in culture until E5 but become able to do so in an autonomous way from E5.5. This indicates that the induction of oligodendrocyte precursors is a late event that occurs between E5 and E5.5, precisely at the time when the ventral neuroepithelium stops producing somatic motoneurons. Analysis of the spatial origin of oligodendrocyte progenitors, evidenced by their expression of O4 or PDGFR indicate that they always lie within the most ventral Nkx2.2-expressing domain of the neuroepithelium, and not in the adjacent domain characterized by Pax6 expression from which somatic motoneurons emerge. We then confirm that Shh is necessary between E5 and E5.5 to specify oligodendrocyte precursors but is no longer required beyond this stage to maintain ongoing oligodendrocyte production. Furthermore, Shh is sufficient to induce oligodendrocyte formation from ventral neuroepithelial explants dissected at E5. Newly induced oligodendrocytes expressed Nkx2.2 but not Pax6, correlating with the *in vivo* observation. Altogether, our results show that, in the chick spinal cord, oligodendrocytes originate from Nkx2.2-expressing progenitors in response to Shh and rule out the hypothesis that somatic motoneurons and oligodendrocytes could arise from a common Pax6-expressing progenitor.

The *retina aberrant in pattern (rap)* gene encodes the *Drosophila* Fizzy related protein (Fzr) and regulates cell cycle exit and neural pattern formation in the developing eye

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The development of the *Drosophila* compound eye offers an excellent system for studying the mechanisms that regulate neural pattern formation. In the developing eye, neural patterning and differentiation are linked to the precise spatio-temporal regulation of mitotic cell cycles. In the developing eye imaginal disc, neural patterning begins as a wave of morphogenesis marked by the morphogenetic furrow (MF). Cells anterior to the MF are unpatterned while cells posterior to the MF differentiate into photoreceptor clusters. During the morphogenesis of the eye imaginal disc, two domains of mitosis are seen, the first wave of mitosis is anterior to the MF, and the second mitotic domain is seen immediately posterior to the MF. Previous studies have shown these mitotic events are strictly regulated by a stereotypical spatial and temporal distribution of the key cell cycle regulators such as cyclins and cyclin dependent kinases. Our previous studies have shown that, *retina aberrant in pattern (rap)* gene is required for retinal pattern formation and the differentiation of a subset of photoreceptor neurons (Karpilow et al., 1989; 1996). Our recent molecular and genetic studies show that *rap* encodes the cell cycle regulatory protein, Fizzy related (Fzr). Fzr has been shown to be involved in the regulation of the levels of Cyclin B in the developing embryo (Sigrist and Lehner, 1997). In *rap* mutant eye imaginal discs, the pattern of mitosis is abnormal and neuronal precursors undergo additional mitotic cycles. Confocal image analysis of the mitotic patterns in *rap* mutant eye imaginal discs shows that, loss of *rap* function results in the persistence and abnormal distribution of Cyclin B, leading to the initiation of additional mitotic cycles. The abnormal mitosis is aborted and the neuronal precursors are eliminated by programmed cell death (Apoptosis). *In situ* hybridization experiments show that rap (fzr) mRNA is expressed at the anterior edge of the MF and in the posterior regions of the disc. Confocal microscopic immunolocalization experiments show that Rap (Fzr) is expressed in the MF and in the post-mitotic regions posterior to the MF. Targeted ectopic expression of Rap leads to premature cell cycle exit and cell fate changes in the retinal precursor cells. These studies suggest that Rap (Fzr) plays a critical role in the mechanisms that coordinate cell cycle exit with neural differentiation and pattern formation (supported by the NIH-RCMI and SCORE grants).

Induction and patterning of telencephalon in *Xenopus laevis*

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Forebrain patterning is controlled by several regulatory genes expressed in specific regions of the anterior CNS. The *emx1* homeobox gene is almost exclusively expressed in the dorsal telencephalon. We have studied the mechanisms of *Xemx1* induction within the anterior neural plate compared to other neural specific genes, such as pan-neural genes (*nrp-1*), general anterior neural plate genes (*Xotx2*), retinal specific genes (*Xrx1*), telencephalic specific genes (*XBF-1*) and ventral forebrain specific genes (*Xvax1b*) by means of specification assays, and of molecular marker analysis of different combinations of inducing and responding tissues as well as of animal caps treated with secreted neural inducing/patterning molecules.

Autonomous activation of *Xotx2*, *Xrx1*, *XBF-1* and *Xvax1b* suggest that the presumptive forebrain may be already specified and considerably patterned by midgastrula stage. However, proper regulation of *Xemx1* expression in the prospective neural plate is only achieved by late gastrula. Neither the prechordal mesoendoderm, nor the anterior notochord are sufficient for dorsal forebrain induction in naive ectoderm, which requires the action of both tissues. Besides, patterning signals from the anterior dorsal endoderm are important for the proper activation of *Xemx1* in the presumptive neural plate.

We found that secreted BMP antagonists, though able to trigger an anterior neural fate, are not sufficient to set up full regionalization within the induced tissue, and to activate several telencephalic genes such as *eomesodermin*, *Xemx1*, *Xnkx2.1*. However, when they are combined with FGFs, strong activation of the ventral marker *Xnkx2.1* occurs, while the dorsal marker *eomesodermin* is also activated, though to a lesser extent. Little, if any, activation of *Xemx1*is observed, suggesting that more complex signalling is required for the full specification of the dorsal telencephalon.

Dorsoventral patterning within the *Drosophila* CNS: identification of transcriptional targets of the homeodomain proteins Ind and Vnd

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The insect CNS initially develops from a bilaterally symmetric neuroectoderm on the ventral surface of the embryo. The earliest sign of neural dorsoventral patterning is the expression of three homeobox genes in the neuroectoderm -- *ventral nervous system defective* (*vnd*), *intermediate neuroblasts defective* (*ind*), and *muscle segment homeobox* (*msh*) – which are expressed in ventral, intermediate, and dorsal columns of neuroectoderm, respectively. The expression of these genes is subsequently inherited by the neuroblasts that form in each column. The use of these homeobox genes to pattern the dorsoventral axis of the CNS appears to have been evolutionarily conserved. In vertebrates homologous genes are also in dorsoventral columns of the developing CNS: *Nkx* genes (homologous to *vnd*) are expressed ventrally, *Gsh1/2* genes (homologus to *ind*) are expressed in intermediate regions, and *Msx* genes (homologous to *msh*) are expressed in the dorsal CNS. To gain a greater understanding of the function of these genes we have been using microarrays to identify transcriptional targets of *vnd* and *ind*. To do this we have isolated RNA from embryos of the following genotypes (1) control embryos, (2) embryos overexpressing *ind* and (3) embryos overexpressing *vnd*. We chose this approach over a loss of function approach because we can get virtually complete transformation of the CNS into intermediate or ventral cell fates following misexpression of *ind* and *vnd*, respectively. In addition we are testing a regulatory relationship between *ind* and a putative target, *eyeless (ey)*, the *Drosophila* Pax6 orthologue. *ey* is expressed in a subset of intermediate column cells within the embryonic CNS; the onset of *ey* expression follows that of *ind* and results in repression of *ind* leading to mutually exclusive domains of *ey* and *ind* in the intermediate column. Misexpression data shows that *ind* activates *ey* but *ey* represses *ind*. The function of *ind* and *ey* in the intermediate column will be discussed.

- Irx1 -
Structural and functional analysis of a murine *Iroquois* homeobox gene

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In *D. melanogaster* genes are composing a network, which determine neural cell lineage. Among them the genes of the *Iroquois* complex *(IRO-C)* are playing a crucial role in regulating the function of this regulatory cascade. Members of the *D. melanogaster Iroquois* homeobox gene family are implicated in the development of peripheral nervous system and the regionalization of wing and eye.

Increasing evidence emphasizes that the function of the neurogenic network is similar in fly and in vertebrates. By functional analysis we would like to know if the neurogenic pathway is conserved between fruitfly and mice and of course if the *Irx* genes are the missing link in the regulatory cascade including the *BMP, Gli* and *Mash* genes. Four mouse *Iroquois* homeobox *(Irx)* genes, named *Irx1*, *Irx2*, *Irx3* and *Irx5*, have been identified in our lab (Bosse et al., 1997).

Here we are presenting the structural and functional analysis of one member - *Irx1*.

The murine *Irx1* belongs to the homeobox gene family and all of the *Iroquois* genes contain a typical *Iroquois* box at the 3´ prime end. *Irx1* shows a very specific expression pattern during mouse development starting during the early steps of murine gastrulation. In the functional analysis of this gene we replaced the functional domain - the homeobox - with an *IRES-TAU-lacZ-loxPNeoloxP*-construct to produce a null mutant. For the generating of the mutants we used the described construct containing the *Neo* gene and further we removed the *Neo* gene by a *Cre*-recombination event in the ES cells.

In the *Irx1* mutant mice the development stops before E7.0. Embryos null for *Irx1* are not able to fulfill the gastrulation and are stopping during the process of forming mesoderm structures. *Irx1* is a mayor player in the early steps of murine gastrulation. If this gene is affected no further development will take place (paper in preparation). In future we will analyze the genetic relations of *Irx1* to genes of the prospective regulative cascade like for example *Wnt3a*, *BMPR* and *BMP4*, which all three are also crucial players in the early development and are members in the potential regulative cascade.

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