EARLY NEURAL CREST SPECIFICATION, INDUCTION AND COMPETENCE

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Martín Leandro Basch

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To the memory of my grandmother

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ABSTRACT

The neural crest is a transient population of embryonic cells that originates at the border between the neural plate and the non-neural ectoderm. Near the time of neural tube closure, the neural crest go through an epithelial to mesenchymal transition and start an extensive migration throughout the embryo. During migration or shortly after they reached their final position, neural crest cells differentiate to form a wealth of derivatives. The mechanisms of migration and differentiation of neural crest have been vastly studied. Comparatively, much less is known about the embryological origins of the neural crest, and the nature of the interactions that generate them. To clarify the timing and nature of these inductive interactions, I examined the time of competence of the neural plate to become neural crest as well as the time of neural fold specification in chick embryos. The neural plate is competent to respond to inductive interactions with the non-neural ectoderm for a limited period, losing its responsive ability after stage 10. In contrast, non-neural ectoderm from numerous stages retains the potential to induce neural crest cells from competent neural plate. When I tested the ability of neural folds to produce neural crest, I found that folds derived from all rostrocaudal levels of the open neural plate of stage 10 embryos can generate neural crest when cultured in isolation. To further characterize the time of neural crest specification, I isolated regions of the epiblast from stages 3 and 4 embryos and identified a region that is already specified to adopt neural crest fates at the beginning of gastrulation. I describe the early expression pattern of the paired box transcription factor Pax-7, which correlates from stage 4+ onwards with the prospective neural crest forming region. Therefore, I propose that Pax-7 is the earliest neural crest marker described in chick. Furthermore, using a morpholino-based loss of function approach, I show that Pax-7 expression is required during neural crest development in chicks. Taken together, my results suggest that specification of the neural crest begins very early in development and it requires multiple and sustained signals and tissue intractions.

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Chapter 1:

Introduction to Neural Crest Induction

The neural crest is a transient population of cells that originates at the border between neural and non-neural ectoderm. Around the time of neural tube closure they undergo an epithelial to mesenchymal transition and migrate extensively throughout the embryo to give rise to a wide variety of derivatives. These include most of the craniofacial skeleton, cartilage, connective tissue, neurons and glia of the peripheral nervous system, all the pigment cells in the body and neuroendocrine cells of the adrenal medulla.

1.1 THE NEURAL CREST, A HISTORICAL PERSPECTIVE

Neural crest cells were first described in the avian embryo by His (His, 1868) as "band of particular material lying between the presumptive epidermis and the neural tube". In 1908, Brachet described similar observations in the amphibian embryos. In the 1920's Vogt mapped the prospective neural crest in Urodele embryos to a region in the gastrula between the neural plate and the prospective epidermis using vital dyes (Vogt, 1925). Still, in the 1930's researchers continued to debate whether the neural crest originated from the periphery of the neural plate, from the adjacent thin ectoderm, or from both. As Hörstadius points out, the real problem seemed to be: from which part of the ectoderm is the neural crest derived (Hörstadius, 1950)? In spite of all the progress made since those days, or perhaps because of it, it is almost embarrassing to admit that Hörstadius' question is still a valid one today. One of the first experiments to address induction of the neural crest was performed by

Raven and Kloos in 1945 (Raven and Kloos, 1945). These investigators showed that fragments of the archenteron roof (presumptive axial mesoderm) could induce neural tissue and neural crest when grafted to the blastocoel of a frog embryo, while fragments of the lateral archenteron (presumptive paraxial and lateral mesoderm) induced only neural crest (the implications of these findings will be discussed in a later section). Until the 1940's the main interest in the study of neural crest was its source of pigment cells and neural elements such as ganglia. Most of the research was carried on using amphibian embryos. By that time, studies by Horstadius and Sellman (1941, 1946) and de Beer (1947) moved the focus of attention towards the skeletogenic potential of the neural crest. In the 1960's researchers started to unveil the mechanisms of neural crest migration at the same time that chick embryos displaced amphibians as the model of choice (Weston, 1963; Johnston, 1966). More than a century after their initial description by His, Rosenquist fate-mapped neural crest precursors in the chick by performing isotopic grafts of tritiated thymidine labeled regions of the epiblast (Rosenquist, 1981). Similar to Vogt's findings, Rosenquist's studies place the neural crest precursors in a region of the epiblast between the prospective epidermis and the future neural plate.

1.2 THE NEURAL CREST TODAY

Over the past 20 years the study of neural crest has seen incredible advances due to the availability of new techniques and markers that allowed us

to characterize and begin to understand the molecular identity of these cells. The experimental approaches that have been used in the study of neural crest include a wide range of techniques, from classical embryological manipulations such as quail-chick interspecies grafts (Le Douarin and Jotereau, 1973), to large-scale genomic screens (Gammil and Bronner-Fraser, 2002).

In recent years, the neural crest has also been the focus of attention of emerging fields at the intersection of developmental biology and other disciplines. Here are just a few examples:

Neural crest as stem cells

The isolation of mammalian neural crest that have the capacity to self-renew and to give rise to differentiated progeny placed neural crests in the map of stem cell research (Stemple and Anderson, 1992). Since then, neural crest stem cells (NCSC) have been intensely studied as a model to understand the properties of stem cells and the cues and factors involved in maintaining their self-renewal ability and promoting cell fate decisions of their progeny (Shah and Anderson, 1997; Morrison et al., 1999).

Neural crest and medicine

The DiGeorge syndrome, cleft palate and Hirschsprung disease are all neural crest derived birth defects. (Van de Putte et al., 2003; Goodman, 2003; Wilkie and Morris-Kay, 2001). Because these are some of the most common birth defects, it is not surprising that neural crest study has had a big impact on biomedical research.

Neural crest and evolution

Neural crest is a unique feature of the vertebrate embryos. Evolutionary biologists look at gene expression in the neural crest, and compare it to expression of homologous genes in invertebrate embryos. This type of analysis allows speculations about the changes in gene expression and regulation that must have taken place during the evolution of vertebrate ancestors, which allowed them to acquire all the specialized cell types that are derived from the neural crest (see Baker and Bronner-Fraser, 1998 for a review).

1.3 Induction of the Neural Crest

Near the time of neural tube closure neural crest cells go through an epithelial to mesenchymal transition and start an extensive migration throughout the embryo (Figure 2). During migration or shortly after they reached their final position, neural crest cells differentiate to form a wealth of derivatives. The mechanisms of migration and differentiation of neural crest have been vastly studied (Le Douarin, 1982; Le Douarin and Kalcheim, 1999). Comparatively, much less is known about the embryological origins of the neural crest, and the molecular nature of the interactions that generate them.

The description presented at the beginning of this chapter summarizes our current understanding of the neural crest. In the following sections, we will revisit this definition highlighting some of the gaps in the knowledge of neural

crest induction and how the work presented in the next chapters fits in the context of the data discussed here.

A cell or tissue is **specified** to adopt a certain fate if it does so in the absence of any further signals. Specification usually implies that the cell or tissue has already received the signal(s) that will instruct them to adopt a particular fate. A cell or tissue is **competent** to adopt a particular fate, if it does so in response to the right signal(s). A cell or tissue is **committed** to a particular fate when it maintains such fate even in the presence of signals that could challenge it.

1.3.1 Segregation of the ectodermal lineage: neural crest precursors

Individual neural crest cells can be identified as they begin to migrate away from the dorsal neural tube. Prior to migration these cells form part of a heterogeneous population of multipotent cells within the neural tube or the dorsal neural folds. Lineage analysis of the dorsal neural tube by single cell injection of fluorescent dextrans has shown that the progeny of the labeled cells contributed to neural tube and neural crest derivatives (Bronner-Fraser and Fraser, 1998; Collazo et al., 1993; Raible and Eisen, 1994; Serbedzija et al., 1994). Similar labeling experiments done on single cells of the neural folds prior to neural tube closure revealed that these younger cells have an even broader potential. Their progeny can contribute not only to the central nervous system and neural crest, but also to the epidermis (Selleck and Bronner-Fraser, 1995;

Figure 3). However, in some of the experiments described above, single cells were found that contributed to only one type of derivative, raising the possibility that some cells in the neural folds may have a more restricted fate potential. Another explanation for these results is that while these cells are still multipotent their progeny is biased toward one particular fate by signaling events within the neural folds. Taken together the data presented above suggest that neural crest cells fate is not determined until after the onset of migration. In fact, early migrating neural crest can form ventral neural tube derivatives when injected into the ventral side of a host neural tube (Ruffins et al., 1998), which implies that neural crest cells are not committed to their fate even after migration has started. The existence of such a heterogeneous population of cells that has the potential to generate neural crest poses an interesting challenge for the study of neural crest induction. As we will discuss next, a series of signaling events and tissue interactions take place very early in development to set up a domain in the ectoderm competent to generate neural crest. Thus, we can define neural crest induction as the process whereby ectodermal cells become specified as neural crest precursors. In other words, the step in which a group of cells in the ectoderm receive the signals that will instruct them to adopt a neural crest precursor fate. The expression of genes in this region of the embryo marks the localization of these precursors and not neural crest cells per se. In the following sections we will refer to these genes as early neural crest markers even though neural and neural crest fates do not segregate until the onset of neural crest migration (Figure 4).

1.3.2 Tissues and signals involved in neural crest induction

Signals from the mesoderm

One of the first experiments that addressed the issue of neural crest induction showed that portions of the archenteron roof of amphibian gastrula embryos had the capacity to induce neural tissue and neural crest when grafted into the blastocoel of a host embryo. If the tissue grafted was lateral archenteron only neural crest was induced in the host ectoderm. These experiments led to the proposal that a graded signal from the mesoderm was responsible for neural crest induction (Raven and Kloos, 1945). The ability of non-axial mesoderm to induce neural crest was confirmed later by recombination experiments *in vitro* both in amphibians (Marchant et al., 1998) and in chick embryos (Selleck and Bronner-Fraser, 1996). Amphibian embryos with surgically removed paraxial mesoderm failed to form normal neural crest derivatives, suggesting that signals from the mesoderm are required for neural crest induction (Bonstein et al., 1998). Furthermore, it has been shown that chick paraxial mesoderm can induce expression of Pax-3, an early marker of the neural plate border, when combined with either chick neural plate 'neuralized' *Xenopus* animal caps. (Bang et al., 1997). In addition, the induction of Pax-3 in these animal cap assays was prevented in the presence of a dominant negative Wnt-8, suggesting that Wnt signaling may mediate the inducing ability of paraxial mesoderm (Bang et al., 1999).

A recent study proposed that a member of the fibroblast growth factor (FGF) family, FGF-8, mediates the inductive effects of paraxial mesoderm on frog animal cap essays and that it is sufficient to induce expression of several neural crest markers (Monsoro-Burq et al., 2003). A requirement for FGF signaling in neural crest induction had been observed previously in an experiment where injection of a dominant negative FGF receptor prevented expression of neural crest markers (Mayor et. al, 1997). In a subsequent study it was shown that FGFs ability to induce neural crest in frog embryos was dependent on Wnt signaling (LaBonne and Bronner-Fraser, 1998). Monsoro-Burq et al. argue against a role of Wnt as the inducing signal from the paraxial mesoderm. They propose that treatment of the recombinants with dominant negative Wnts may have an effect on the inducing rather than on the responding tissue thus altering its inductive properties. They show that inhibition of the intracellular components of the Wnt pathway on the responding tissue does not prevent induction of the neural crest markers analyzed (Monsoro-Burq et al., 2003).

The involvement of Wnts and FGFs in neural crest induction is consistent with previous observations that this process requires posteriorizing signals, at least in amphibians (Villanueva et al., 2002). Interestingly, recombinants of Hensen's node and neuralized animal caps (ectodermal explants from frog blastula embryos that have been exposed to neural inducers) can induce expression of early border markers even in the absence of FGF, Wnt or retinoic acid signaling, suggesting that the node is also a source of a yet unidentified signal that has the capacity to induce neural crest (Bang et al., 1999).

Signals from the ectoderm

The localization of neural crest precursors at the border between neural plate and epidermis suggests a potential role for the interaction between these two tissues in the induction of neural crest. Rollhäuser-ter Horst grafting experiments in amphibians showed that gastrula ectoderm generated both neural and neural crest cells when grafted to the neural folds (Rollhäuser-ter Horst, 1979, 1980). The juxtaposition of these tissues in embryos of Axolotl, generated neural crest at the newly formed border (Moury and Jacobson, 1990). By grafting tissues from pigmented into albino host embryos, these researchers were able to observe that *de novo* neural crest originated from both the neural plate and the epidermis. Interestingly, while tissue from the neural plate tissue formed mostly melanocytes the epidermis tissue gave rise to spinal and cranial ganglia. The role of neural plate and epidermis interaction in the induction of neural crest was later confirmed *in vivo* in other organisms by similar tranplant experiments done in chick, fish and *Xenopus* embryos, which provided similar results (Selleck and Bronner-Fraser, 1995; Woo and Fraser, 1998; Mancilla and Mayor, 1996). *In vitro* co-cultures of epidermis and neural plate tissue from both chicken and frog embryos, proved that the interactions between these two tissues is sufficient to generate neural crest (Liem et al., 1995; Dickinson et al., 1995; Mancilla and Mayor, 1996). However, the competence of the neural plate to respond to signals from the ectoderm is lost by stage 10 HH (staging according to Hamburger and Hamilton, 1951, Fig.1) in chick embryos, suggesting that inductive interactions that lead to neural crest formation may be time limited in

the chick embryo (Basch et al., 2000; Chapter 3). *In vitro* experiments showed that addition of dorsalin-1 (dsl-1) to intermediate neural plate explants (INP, portions of the neural plate between the ventral midline and the neural folds) was sufficient to induce migratory neural crest (Basler et al., 1993). This tissue is considered naïve in the sense that it has not received signals to specify it as dorsal or ventral. Dsl-1 is a member of the transforming growth factor-β (TGF-β) family expressed on the dorsal neural tube. This observation suggested that other TGF-β family members expressed in the epidermis may also be involved in the induction of neural crest.

TGF-β family members

The firsts of such molecules to be identified were BMP-4 and BMP-7. Addition of these molecules to intermediate neural plate explants could substitute for the effects of the edpidermis, therefore they were proposed as the epidermal signal responsible for neural crest induction (Liem et al., 1995). At early stages of development (stages 4 and 5 HH), BMP-4 is expressed in the prospective epidermis of the chick epiblast and it is absent from the future neural plate. This expression is consistent with a role in neural crest induction. However, BMP-4 soaked beads implanted in the prospective neural plate at these stages cannot prevent neural fate (Streit et al., 1998). At later stages BMP-4 expression is downregulated in the epidermis adjacent to the closing neural folds and it is strongly expressed on the neural folds themselves. This expression pattern suggests a role for BMP-4 in the maintenance rather than the induction of neural crest. Consistent with this view, cells expressing Noggin, a BMP antagonist, can prevent expression of neural crest markers when injected in the

closing neural tube, but not when implanted next to the open neural folds, at a time when neural crest induction is still taking place (Selleck et al., 1998).

The analysis of phenotypes produced by mutations in different members of the BMP family and BMP antagonists suggests that these molecules are not required for proper neural crest formation, at least in mice. Embryos carrying a homozygous BMP-4 mutation usually die around gastrulation. However embryos that survive until neural fold stages do have some neural crest derivatives (Winnier et al., 1995). BMP-7 homozygous null mice present some craniofacial skeletal defects but they are more likely related to bone formation rather than neural crest (Dudley et al., 1995). In BMP-5 and BMP-7 double mutants neural crest cells are able to form and migrate normally. *In vitro* assays culturing neural tubes from these mice yielded neural crest that were indistinguishable from controls (Solloway and Robertson, 1999). Mice carrying homozygous mutations for the BMP antagonists Noggin (McMahon et al., 1998) or follistatin (Matzuk et al., 1995) do not exhibit defects in neural crest formation. While the normal expression pattern of BMPs could not account for the possibility of functional redundancy (see Streit et al., 1998) it is possible that in these mutants the expression of the other BMP genes is altered leading to ectopic function. However, we cannot rule out the possibility of other unidentified molecules (maybe other TGF-β family members) triggering BMP-like signals that could account for the effects described above.

Evidence for the requirement of epidermal BMP signaling in neural crest induction is more compelling in other vertebrates than in amniotes. Inhibition of BMP signaling by injection of a dominant negative BMP receptor, or the antagonists Noggin or chordin into the one cell frog embryo results in expression

of neural crest markers analyzed in explanted animal caps. (Marchant et al., 1998; LaBonne and Bronner-Fraser, 1998). The attenuation of BMP signaling elicits the expression of neural crest markers in a dose dependent fashion. The levels of BMP activity required to induce neural crest are intermediate between those required to specify ectoderm and neural plate. These findings led to the proposal of a model in which the different fates of the ectoderm derivatives are specified by a gradient of BMP activity (Marchant et al., 1998). Interestingly, overexpression of BMP-4 in *Xenopus* embryos is not sufficient to expand the expression domain of the neural crest marker slug, and while certain concentrations of chordin mRNA injection can induce expression of neural crest markers in animal caps, this expression was found to be weak compared to endogenous levels in the embryo. A much more robust induction occurred when inhibition of BMP signaling was accompanied by exposure to Wnts or FGFs. Taken together these data suggest that other signals are required in addition to BMPs in order to induce neural crest.

Genetic analysis of several mutations of the BMP signaling pathway identified in zebrafish embryos also suggests an important role in for these molecules in neural crest induction. *Swirl* (bmp2b), *snailhause* (bmp7) and *somitabun* (Smad5) mutants all display a great reduction in neural crest at trunk levels (Nguyen et al., 1998, 2000; Schmid et al., 2000). Interestingly, zebrafish bmp2b is functionally more similar to *Xenopus* BMP-4 than zebrafish bmp4 (Nikaido et al., 1997). The neural crest deficiencies observed in these mutants together with bmp2b and bmp7 expression patterns in the fish gastrulae are consistent with the BMP gradient model proposed for neural induction (Nguyen et al., 2000).

Wnt gene family members

Several lines of evidence suggest that members of the Wnt (wingless/INT) family of secreted glycoproteins can act as neural crest inducers (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Bang et al., 1999; García-Castro et al., 2002). Mice carrying a mutation in both Wnt-1 and Wnt-3a genes exhibit a significant reduction in the number of melanocytes and cranial and spinal sensory neurons as well as deficits in skeletal structures derived from cranial neural crest (Ikeya et al., 1997). However neural crest are induced in these animals, therefore it was perceived that these molecules were critical for the proliferation rather than for the initial formation of neural crest. Furthermore, it has been shown that neural crest arise *in vitro* in the absence of Wnt1 and Wnt3a (Dickinson et al., 1995).

These two Wnt family members also are strong inducers of neural crest markers when injected in neuralized animal caps. Overexpression of either Wnt-1 or Wnt-3a in whole embryos leads to an expansion in the neural crest domain and production of supernumerary neural crest cells (Saint-Jeannet et al., 1997). Because Wnt signaling can result in cell proliferation (Dickinson et al., 1994) the authors repeated the experiment blocking cell proliferation at gastrula stages, obtaining the same results. These data suggest a direct effect of Wnts on neural crest induction, perhaps at the expense of other ectodermal tissues. Similar experiments have shown that Wnt-7B and Wnt-8 can induce neural crest in ectodermal tissue that has been neuralized by noggin or chordin (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Bang et al., 1999).

Recent experiments in chick embryos have shown that Wnt signals are required for neural crest formation. Expression of the neural crest marker Slug was prevented by injecting COS cells expressing a dominant negative Wnt1 construct adjacent to the neural folds. Sufficiency was tested by experiments *in vitro*, where addition of wingless conditioned medium to intermediate neural plate explants generated migratory neural crest cells. In contrast to BMP-4, the generation of neural crest *in vitro* was achieved in a defined minimum medium lacking the cocktail of additives used in previous reports. Under these conditions, BMP-4 was unable to induce neural crest in the explants, suggesting that its effects might be the result of synergistic actions with other signaling molecules. In addition to be sufficient and required for neural crest induction, Wnt6 is expressed in the ectoderm. Taken together, these data suggest that Wnt is an epidermal inducer of neural crest (García-Castro et al., 2002). However, several other Wnts are expressed all around in the embryo including the neural folds themselves, making a simplified scenario impossible to sustain.

1.3.3 Neural crest induction: initiation versus maintenance

From the data presented above it is clear that interactions between the mesoderm or the epidermal ectoderm with the neural plate can generate neural crest. Members of the Wnt, bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGF) families have been shown to participate in the process of neural crest induction to different extents in different organisms. From

experiments in chick, we know that the induction of neural crest is a continuous process that can be disrupted at several points in time by manipulating some of these signaling pathways (Selleck et al., 1998). In frogs, analyses of neural crest induction are largely based on the expression of early neural crest markers, an event that is a consequence of the induction itself. Even though these markers are useful to interpret the role of different molecules in the induction process, their expression is too late to determine which are the initial events that lead to the specification of neural crest.

We can distinguish at least two steps in the process of neural crest induction. First, a region of the ectoderm has to receive instructive signals to become specified as neural crest precursors. Second, these neural crest precursors need to receive further signals that will allow them to maintain their identity in the developing embryo. Before we can understand completely how this induction process takes place and who are the molecular players involved in each step, we need to know when specification of neural crest occurs. None of the experiments described above allow us to determine accurately if the interactions or molecules studied participate in the initial induction or in the maintenance of the specified state of neural crest precursors.

1.3.4 Initial steps of neural crest induction: what do we really know?

The experiments described in Chapter 2 are the first attempt to look at the initial steps in neural crest specification in chick embryos. Our results suggest that neural crest in avian embryos is already specified by the start of gastrulation.

Even though our findings suggest that neural crest is specified in the chick embryo before the generation of paraxial mesoderm, precursors for this tissue are present in the epiblast adjacent to the neural plate and thus could be the source of some planar signaling. The hypoblast and the emerging definitive endoderm could also be a source of inductive signals acting on the overlying ectoderm.

In fish and frog, a model has been proposed in which a gradient of BMP patterns the ectoderm and specifies epidermal, neural plate and neural crest fates during neural induction (Mayor et al., 1996; LaBonne and Bronner-Fraser, 1999; Aybar and Mayor, 2002). Because the formation of neural crest is tightly associated both temporally and spatially with the formation the neural plate, we might gain some insight into the initial steps of neural crest induction by analyzing the molecular events that lead to neural induction and the establishment of the neural plate border.

1.3.5 Another look at neural induction

Neural Induction in *Xenopus*

In 1924 Hilde Mangold, a graduate student in Hans Spemann's laboratory, performed a classic experiment in the history of developmental biology that opened the doors to the study of neural induction. By grafting the dorsal lip of the blastopore (the dorsal most mesoderm) from pigmented newts into the ventral side of albino host embryos, Mangold and Spemann showed that this

tissue, now known as Spemann's organizer, could induce the formation of a secondary axis. The difference in pigmentation between donor and host embryos and histological analysis showed that the mesodermal structures in the newly formed axis were derived from the donor tissue but the bulk of the neural tissue was derived from the host (Spemann and Mangold, 1924). The implication of these results was that a signal or signals from the organizer could respecify the fate of the ventral tissue that would normally give rise to epidermis and redirect it towards a neural fate.

The "default model" of neural induction

The first insight on the nature of these signals awaited until the late 80's, when it was shown that dissociation of epidermal cells from *Xenopus* animal caps induced neuronal differentiation (Grunz and Tacke, 1989; Godsave and Slack, 1989; Sato and Sargent, 1989). The interpretation of this result led to the proposal of the "default model" where neural is the default state of the ectoderm but is inhibited by a signal that becomes diluted in the dissociation experiments.

Injection of a dominant negative receptor for activin, a BMP related molecule, inhibited the formation of mesoderm but induced the formation of ectopic neural tissue in *Xenopus* embryos (Hemmati Brivanlou and Melton, 1994). In addition, exposure of dissociated animal cap cells to BMP-4 restored their epidermal fate (Suzuki et al., 1997). This restoration to epidermis was prevented if the embryos had been previously injected with the activin dominant negative receptor (Wilson and Hemmati-Brivanlou, 1995). In addition, activated effectors of the BMP pathway also induced epidermis in dissociated cells (Mahoney et al., 1997; Suzuki et al., 1997). BMP-4 is expressed throughout the ectoderm in

Xenopus at the beginning of gastrulation and subsequently disappears from the neural plate (Fainsod et al., 1994; Schmidt et al., 1995). Taken together these data indicate that BMP-4 can act as a neural inhibitor as proposed in the default model.

During this same time period, the search for neural inducers from the organizer continued. Several secreted molecules with diverse structures were identified. Noggin, chordin, follistatin, Cerberus, Gremlin and Xnr3 are expressed in or close to the organizer at late blstula or aearly gastrula stages and all of them generate ectopic anterior neural tissue or an expansion of the neural plate when overexpressed. In addition to being neural inducers, all these molecules had the ability to antagonize BMP signaling either by direct binding to BMPs or by competing with them for receptor binding (Xnr3) (Lamb et al., 1993; Hemmati Brivanlou et al., 1994; Sasai et al., 1995; Bouwmeester et al., 1996; Hsu et al., 1998; Hansen et al., 1997). Because the newly induced neural tissue was anterior in character, it was believed that some of these cells were subsequently caudalized by other signals from the organizer (Nieuwkoop et al., 1952). Among the signals proposed for this caudalizing activity are retinoids, FGFs and Wnt3A (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991; Isaacs et al., 1992; Cox and Hemmati Brivalnou, 1995; Lamb and Harland, 1995; Launay et al., 1996; McGrew et al., 1995, 1997) These data suggest a simple model for neural induction in *Xenopus* where BMP antagonists secreted by the organizer suppress the inhibition of neural fate mediated by BMP-4 on the prospective neural plate. According to this model neural induction would take place during gastrulation by signals from the organizer at the dorsal lip of the blastopore.

Challenging the default model

Accumulating evidence however, suggests that this may not be the complete story. A recent study has shown that neural tissue can form even in the absence of mesoderm, suggesting that the organizer is not required for neural induction. In this study, formation of mesoderm was prevented by injection of a truncated form of Cerberus, which antagonizes nodal-related genes necessary for mesoderm specification. Interestingly, expression of the BMP antagonists chordin, Noggin, follistatin and Cerberus was detected before the onset of gastrulation and their expression was shown to be dependent on β-catenin, a downstream effector of the Wnt pathway (Wessely et al., 2001). Two other studies show restricted expression of neural markers in prospective neural tissue, suggesting that neural fate is specified at late blastula stages, before the formation of the organizer (Kroll et al., 1998; Gamse and Sive, 2001). In addition to BMP antagosnists, Wnt signals have also been proposed as neural inducers. Injection of Wnt8 and β-catenin can induce neural tissue in *Xenopus* animal caps and neural induction is blocked after inhibition of Wnt signaling (Baker et al., 1999). This study also provides evidence that Wnts are responsible for repressing expression of BMP-4 in the neural plate of early gastrulae. The authors suggest that Wnt signals may sensitize the dorsal side of the embryo to respond to neural inducing molecules from the organizer (Baker et al., 1999). Several studies have proposed a role for FGFs in neural induction, although this remains an issue of debate. This argument is based on the observation that exposure of animal caps to bFGF can induce expression on neural markers without formation of mesoderm (Lamb and Harland, 1995), and injection of a dominant negative FGF

receptor can prevent neuralization of animal caps by exposure to Noggin or chordin (Launay et al., 1996; Sasai et al., 1996). These results are in conflict with other studies in which expression of a dominant-negative FGF receptor prevents the acquisition of posterior neural fates but not neural induction (Holowacz and Sokol, 1999). Furthermore, neural induction does not appear to be suppressed in transgenic *Xenopus* embryos that express a dominant negative FGF receptor (Kroll and Amaya, 1996).

Taken together, these data suggest that in addition to BMP antagonists secreted by the organizer, other signals might be required for neural induction and that the timing of neural specification may precede the formation of the organizer.

Neural induction in other vertebrates

Challenging the default model even further: is the organizer necessary for neural induction?

Transplantation experiments have identified functional equivalents of Spemann's organizer in other vertebrate embryos. Hensen's node in chicks, the embryonic shield of zebrafish and the mouse node, all can induce ectopic neuraxis when grafted into host embryos (Waddington, 1932; Gallera, 1971; Beddington, 1994; Oppenheimer, 1936; Storey et al., 1992; Shih and Fraser, 1996). However, the involvement of the organizer in neural induction has been questioned in all these organisms. Complete removal of the embryonic shield in zebrafish embryos cannot prevent neural induction although it disrupts

anteroposterior patterning, as revealed by analysis of of midbrain and hindbrain markers (Shih and Fraser, 1996; Saude et al., 2000). Similarly, mouse embryos mutant for the HNF3 gene fail to form node or notochord and do not express Noggin nor chordin but still form neural tissue (Ang et al., 1994). Embryos with mutations in both Noggin and chordin genes display a reduction in anterior neural tissue but neural induction still occurs (Bachiller et al., 2000). In chick embryos, grafts of chordin expressing cells in regions of the epiblast that are competent to form neural tissue failed to induce expression of general neural markers. However, these grafts were able to maintain the expression of neural markers after the competent tissue had been exposed to a grafted node for five hours (Streit et al., 1998). Taken together these results suggest that signals from the organizer are not required or sufficient to induce neural tissue but instead could play a role in the maintenance of induction.

Coming to terms with the default model: variations on BMP inhibition

The nature and timing of the signaling events required for neural induction in the chick came from evidence gathered in the recent years. Removal of the endoderm and the adjacent hypoblast of chick early gastrulae showed that vertical signals from these tissues are required for the formation of the neural plate. In the absence of these tissues, the expression of epidermal markers was expanded covering the area of the tissue removed (Pera and Kessel, 1999). Expression of FGF 8 is consistent with a role as a neural inducer from the endoderm/hypoblast (Streit et al., 2000). Indeed, evidence from two groups suggests that FGF signaling is necessary albeit not sufficient for neural induction. Two novel genes, ERNI and Churchill (ChCh) were identified in a screen for

early responses to neural inducing signals from Hensen's node. Surprisingly, the expression of ERNI begins before the formation of the primitive streak. Ectopic expression of ERNI by grafts of Hensen's node into the area opaca can be mimicked by FGF 8 coated beads and prevented by the presence of an FGF inhibitor. Even though FGF 8 can induce expression of the early neural markers ERNI and Sox3, it failed to induce the later neural markers Sox2 and chordin by itself (Streit et al., 2000). The second gene reported from this screen, Churchill, is also expressed in response to FGF but slightly later than ERNI. ChCh is a zinc finger transcription factor that was shown to play multiple roles in development. Because it can induce the expression of Sip1, a cofactor of the BMP downstream effector Smad1, the authors propose that it plays a role in sensitizing the epiblast to BMP antagonists after the initiation of neural induction (Sheng et al., 2003). Further evidence for the role of FGF in neural induction came from *in vitro* experiments testing the specification state of very early embryos. Medial epiblast explants (explants taken from the prospective neural plate) from st. 3 HH (definitive streak) chick embryos grown in culture express anterior neural markers after 12 hours of incubation. This observation suggests the specification of anterior neural fates occurs before gastrulation (Muhr et al., 1997). To further analyze the timing of neural specification in a later study, the researchers analyzed the state of specification of two regions of the epiblast in stages VIII, IX and XII (according to Eyal-Giladi and Kochav, EG&K) embryos. By the time the egg is laid, the embryo is already stage X EG&K. Explants from lateral or medial regions of the epiblast were grown in culture for 40 hours and later analyzed for expression of ectodermal or neural markers. Lateral explants from all stages expressed epidermal markers. In contrast, medial explants taken from stages IX

and XII but not VIII expressed all neural markers analyzed. No expression of mesodermal markers was detected in these explants as analyzed by RT-PCR. This surprising result indicates that neural specification can take place in utero shortly after st VIII (Wilson et al., 2000). Addition of BMPs to the prospective neural explants prevented the expression of neural markers and ectodermal markers were detected instead. When this experiment was repeated on stage 4HH explants, the prospective neural cells maintained expression of neural markers. This result is consistent with other reports that gastrula stages neural plate is refractory to the effects of BMP (Streit et al., 1998). Suppresion of FGF signaling prevented the acquisition of neural fates in the medial explants unless it was accompanied by inhibition of BMP signaling. Futhermore, RT-PCR analysis showed that FGF downregulates expression of BMP-4 and –7 in medial explants (Wilson et al., 2000). Using a similar experimental design, another study showed that Wnt3a and Wnt8 are normally expressed in lateral explants at stages X-XIII EK&G. Inhibition of Wnt signaling in lateral explants promotes specification of neural cells unless accompanied by inhibition of FGF signaling. In medial explants addition of both FGF and Wnts results in expression of epidermal marker. These data suggest a model in which FGF represses BMP signaling in medial epiblast, thus promoting neural fates. In lateral explants Wnt represses FGF signaling thus allowing BMP expression and acquisition of epidermal specification (Wilson et al., 2001).

Collectively, all the experiments discussed above suggest that specification of neural fates is a very early event and imply a role for FGF in early neural induction. This function of FGF, however, is tightly associated with its ability to attenuate BMP expression. The intimate association of the FGF, Wnt

and BMP signaling pathways results in epidermal and neural fates. Interestingly, the specification of the neural fate seems to be the result of BMP antagonism albeit at a transcriptional and not at a post-translational level (See Wilson and Edlund, 2001; Stern, 2002 for a review).

1.3.6 A trip to the neural plate border

Where is the border?

In light of recent evidence it seems evident, at least in chicken embryos, that intrinsic differences between prospective epidermal and neural cells exist very early in development even before the egg is laid (Wilson et al., 2000). Work from *Xenopus* also seems to indicate some degree of prepaterning in the ectoderm before the proposed time for neural induction (Kroll et al., 1998; Gamse and Sive, 2001). However, most of what we know about the formation of the border comes from studies done at later stages, mainly due to the lack of early molecular markers and the fact that a visible thickened neural plate does not exist before gastrulation (García-Martínez et al., 1993 and references therein). The location of the border region between neural and non-neural tissue has been established by two types of experiments, fate mapping studies of the prospective neural plate at early stages and the analysis of the early expression patterns of genes that are considered to be either neural or general non-neural markers. Among the genes that label neural plate are Sox2 and Sox 3, Otx2, Gsx, Six3 Gbx2, Ganf, Lmx1, Frzb1 and Plato (Rex et al., 1997 a, b; Lawson et al., 2000; Bally-Cuif et al., 1995;

Lemaire et al., 1997; Bovolenta et al., 1998; Shamin and Mason, 1998; Knoetgen et al., 1999; Yuan and Schoenwolf, 1999 Baranski et al., 2000). The genes that label non-neural ectoderm include Gata2 and 3, BMP-4 and BMP-7, Dlx5, Crescent, Smad6 (Sheng and Stern, 1999; Liem et al., 1995; Ferrari et al., 1995; Pera and Kessel, 1999; Pfeffer et al., 1997; Yamada et al., 1999). However, the expression of BMP-4 and its targets Dlx-5 and Msx-1 is later confined to a more medial region of the non-neural ectoderm surrounding the neural plate, therefore they are also considered border markers (Streit et al., 1998; McLarren et al., 2003; Tríbulo et al., 2003; Pera and Kessel, 1999). Several fate map studies have analyzed the prospective neural plate in stages 3 and 4 chick embryos by several methods including fluorescence labeling, interspecies grafts between quail and chick embryos, homotopic labeled grafts or a combination thereof (Rosenquist 1981; García-Martínez et al., 1993; López-Sánchez et al., 2001; Fernández-Garré et al., 2002). The evidence gathered from these studies conflicts with some of the assumptions made by analyzing gene expression patterns. For example, the expression patterns of Sox2 and Otx2 are much broader than the limits of the prospective neural plate, suggesting that these genes also label some non-neural ectoderm. The most medial limit of Dlx-5 expression was found at a considerable distance from the prospective neural plate at stage 4 HH suggesting that this marker does not label the border region of the non-neural ectoderm (Fernández-Garré et al., 2002). Thus, establishing the precise location of the neural plate border will require further studies, similar to the ones described above, combining fate mapping techniques with a detailed and exhaustive analysis of gene expression.

How is the border established?

Two groups have recently proposed a model for the establishment of the border between neural and non-neural ectoderm. According to this model, the border is set up by signals from the organizer and the endoderm acting together to establish a domain of BMP-4 expression on the non-neural ectoderm at the border with the neural plate (Streit and Stern, 1999; Pera and Kessel, 1999). The evidence for this model comes from several experiments. Grafts of Hensen's node into the area opaca of host embryos not only can induce neural tissue, but also expression of border markers surrounding it. Beads coated with FGF-4 (expressed both by the Henses's node and the hypoblast) can induce expression of msx-1 a transcription factor that is both upstream and downstream of BMP-4. Removal of the hypoblast underlying the neural plate shifts the expression of border markers. Finally, BMP-4 or BMP antagonists secreted by the node can affect the position of the border but not the fate of neural or non-neural ectoderm. Data from experiments in frogs, also suggest that manipulations of BMP signaling modify the position of the border by either expanding or reducing the size of the neural plate (LaBonne and Bronner-Fraser, 1998).

Dlx-3 and Dlx-5, two other downstream targets of BMP-4, play an important role in positioning the border. These transcription factors are normally expressed in the non-neural ectoderm abutting the border with the neural plate. Gain and loss of function experiments in frogs have shown that these transcription factors can repress or expand the neural plate respectively. Overexpression of Dlx-3 causes an expansion of non-neural ectoderm at the expense of neural plate. Surprisingly, this non-neural ectoderm does not express

epidermal markers. The opposite result was observed after injection of a Dlx dominant-negative (that targets both Dlx-3 and –5). However, expression of border markers is shifted but not affected (Woda et al., 2003). A similar observation was made by electroporation of a tagged Dlx-5 construct in chick embryos. Overexpression of this construct in the neural plate inhibited the expression of neural markers and upregulated the expression of the border markers Msx1, BMP-4 and Six4. Interestingly, the expression of these markers was noticed not only in cells that expressed the construct but also in neighboring cells, suggesting that the effect of Dlx-5 is not cell autonomous. Consistent with the observation made in frogs, ectopic expression of Dlx-5 was not sufficient to induce the expression of epidermal markers (McLarren et al., 2003).

What marks the border?

The molecular identity of the neural plate border can be defined by the combinatorial analysis of genes expressed in this region of the ectoderm. As mentioned above, the expression of several neural and non-neural 'specific' markers overlaps at the border of the neural plate. In addition several genes are expressed exclusively at the border and their sustained expression in the neural folds makes them useful markers for prospective neural crest. Table 1 is modified from a recent review in which the authors provide a comprehensive list and description of these neural, non-neural and border specific genes (Gammill and Bonner-Fraser, 2003). We will briefly refer to a selected subset of genes

expressed at the border because of their historical or functional significance in neural crest specification.

- Snail/Slug: This family of zinc finger transcription factors was first identified in frogs by homology with the Drosophila gene Slug (Sargent and Bennet, 1990; Nieto et al., 1994) and like their fly homolog, these genes act as transcriptional repressors (Gray et al., 1994; LaBonne and Bronner-Fraser, 2000). At least one copy of Snail and Slug has been described in mouse, chick and frogs, while zebrafish seems to carry two copies of Snail. Slug and Snail are expressed in premigratory and/or migratory neural crest of mouse, chick, frog and fish and have been considered among the earliest markers for neural crest (Nieto et al., 1992, 1994; Hammerschmidt and Nusslein Volhard, 1993; Essex et al., 1993; Thisse et al., 1993, 1995; Mayor et al., 1995; Sefton et al., 1998; Jiang et al., 1998). Functional studies in both chick and frog have shown that overexpression of these genes results in an expansion of the neural crest forming region, while inhibition of their function blocks neural crest specification and migration (LaBonne and Bronner-Fraser, 1998, 2000; del Barrio and Nieto, 2002; Aybar et al., 2003). However, mice carrying a homozygous null mutation for Slug do not seem to display an obvious neural crest phenotype (Jiang et al., 1998) although this may be due to a functional redundancy with Snail. The precise function or functions of Slug and Snail in neural crest specification is yet to be determined. However, studies in tumor transformations and cardiac development have shown that Slug can mediate epithelial to mesenchymal transitions by downregulation of adhesion molecules

(Romano and Runyan, 1999; Conacci-Sorrel et al., 2003), which is a role that has been proposed for Slug in neural crest (Nieto et al., 1994).

- Msx-1 and Msx-2: These homeobox genes are transcriptional repressors expressed at the border of the neural plate in mouse, chick and frog embryos (Davidson, 1995; Shimeld et al., 1996; Catron and Wang, 1996). Msxb and Msxc in zebrafish have a similar pattern of expression (Ekker et al., 1997). In chick embryos, expression of these genes at stage 4 is epidermal but progressively becomes restricted to the border (Streit et al., 1998). Gain or loss of function experiments in frog result in ectopic expression or inhibition of neural crest markers respectively, suggesting that these genes play an important role in neural crest specification (Tríbulo et al., 2003). Accordingly, Msx-1 null mice exhibit a loss of neural crest derivatives in the face (Satokata and Maas, 1994). Msx –1 and Msx-2 are direct downstream targets of the BMP and Wnt signaling pathways (Suzuki et al., 1997; Hu et al., 2001; Tríbulo et al., 2003).
- Sox 9 and Sox 10: These genes contain a high mobility group (HMG) domain and are transcriptional activators (Rehberg et al., 2002; Chiang et al., 2001). Expression of Sox9 and Sox10 is highly specific to premigratory and/or migratory neural crest in mouse, chick, frogs and fish embryos (Mori-Akiyama et al., 2003; Britsch et al., 2001; Cheng et al., 2000; Cheung and Briscoe, 2003; Honoré et al., 2003; Spokony et al., 2002; Dutton et al., 2001; Chiang et al., 2001). Loss of function studies using morpholino antisense oligonucleotides in *Xenopus* have shown that both Sox9 and Sox 10 are required for specification of the neural crest (Spokony et al., 2002; Honoré et al., 2003), while *in vivo* and *in vitro* overexpression studies in

chick suggest that sox 9 is sufficient to induce neural crest markers in competent ectoderm (Cheung and Briscoe, 2003). In addition, Sox10 can inhibit neuronal differentiation and maintain multipotency of neural crest stem cells (Kim et al., 2003). However, mutations of Sox 10 in mouse and zebrafish embryos do not prevent specification of neural crest but instead neural crest cells fail to migrate and/or differentiate and undergo premature apoptosis (Dutton et al., 2001; Mollaaghababa et al., 2003). The Waardenburg-Shah syndrome, which is related to defects in neural crest derivatives, has been mapped to several mutations of the human Sox 10 gene (Pingault et al., 1998).

- Pax-3 and Pax-7: these two genes are members of the paired box family of transcription factors. According to the genomic organization and sequence similarities in the paired domain Pax genes can be subdivided into subgroups, which share common expression domains. Pax-3 and Pax-7 form such a paralogous group (Mansouri et al., 1996). Both these genes are expressed at the neural plate border and later, on the dorsal neural tube in mouse and chick embryos and at least Pax-3 in frog embryos (Mansouri et al., 1996, Basch et al. submitted, Bang et al., 1997). Interesting, while expression of Pax-3 precedes expression of Pax7 in mouse, the opposite is true in chick embryos (Mansouri et al., 1996, Basch et al., submitted). Mutations in the murine Pax-3 seem to affect migration of neural crests in a non-cell autonomous manner (Epstein et al., 1991). In contrast, Pax7 null mice exhibit a loss of craniofacial structures that are neural crest derived. Double mutant mice for Pax-7 and Pax-3 would prove whether the

phenotypes observed for each individual gene are attenuated by functional redundancy.

In chick embryos, we show the onset of Pax-7 expression at stage 4+, which precedes the expression of Pax-3. Pax-7 expression is restricted to a very discreet region of the epiblast that has the potential to generate neural crest. This expression pattern suggests that Pax-7 could be the earliest marker for neural crest identified so far in chick embyos. In contrast, Pax-3 is expressed in a more ventral territory that comprises not only prospective neural crest but also neural plate. Functional experiments using morpholinos to inhibit Pax-7 translation suggest that Pax-7 is required at early stages for proper specification of the neural crest (Chapter 2) .

Figure 1: Selected stages of chick development

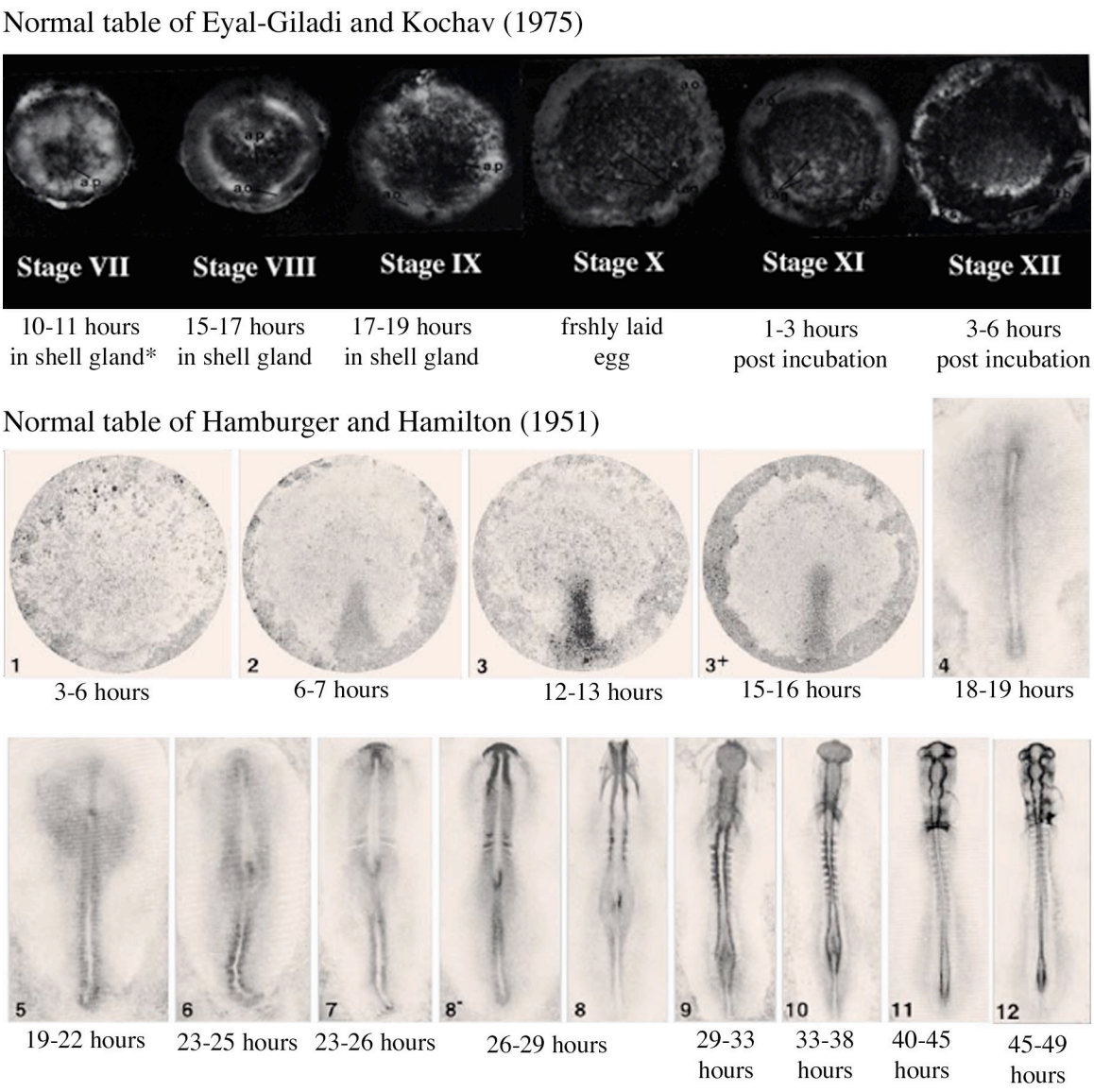

Fig. 1, Top Row: Early stages of chick development according to Eyal-Giladi and Kochav (Eyal-Giladi and Kochav 1971). Before the egg is laid, time is measured in hours after entering the shell gland which takes place about 20 hours after fertilization. **Bottom rows:** Early stages of developments according to Hamburger and Hamilton (Hamburger and Hamilton 1951). Time is measured as hours of incubation.

Figure 2: Neurulation and neural crest migration

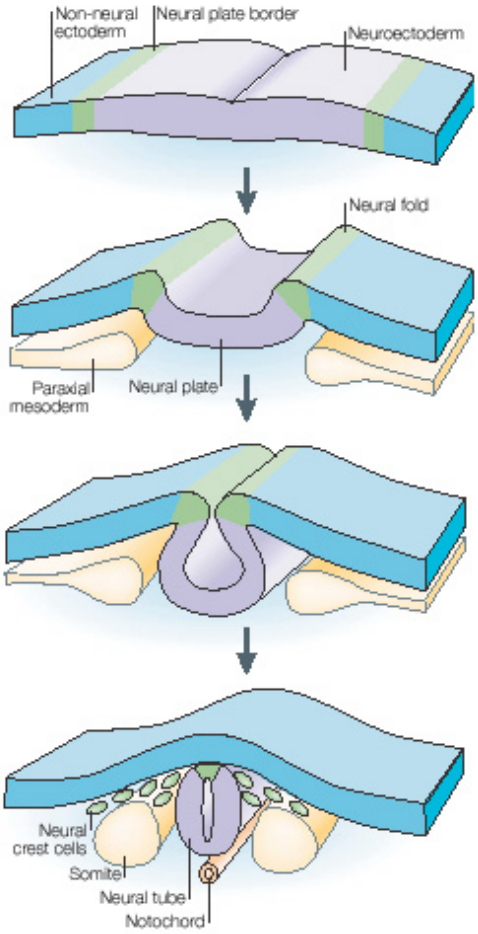

Fig. 2, The neural plate border (green) is induced by signaling between the neuroectoderm (purple) and the non-neural ectoderm (blue) and from the underlying paraxial mesoderm (yellow). During neurulation, the neural plate borders (neural folds) elevate, causing the neural plate to roll into a neural tube. Neural crest cells (green) delaminate frm the neural folds ordorsal neural tube, depending on the species and axial level (From Gammill and Bronner-Fraser, 2003).

Figure 3: Neural crest precursors are multipotent

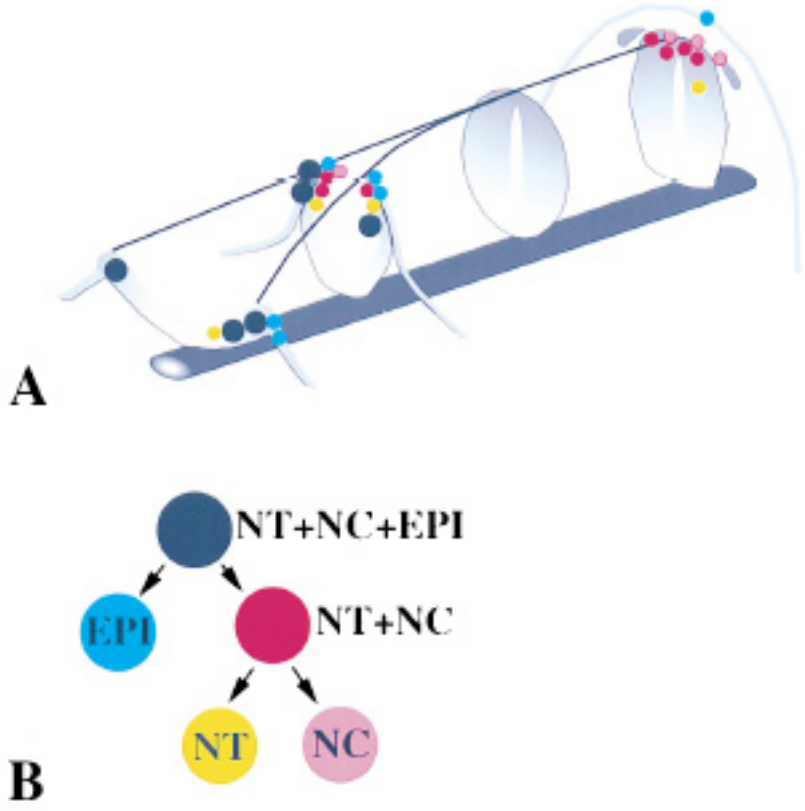

Fig. 3, A: After injection of a single cell (black) in the neural folds, the progeny is located within all three ectoderm derivatives, neural tube (NT), neural crest (NC), and epidermis (EPI). Only after neural tube closure are “tripotent” black cells no longer observed. Some precursors (red) within the dorsal neural tube form both neural tube and neural crest cells. **B:** summary of deduced ectodermal linages (from Selleck and Bronner-Fraser 1996).

Figure 4: Early Neural Crest Markers

Fig. 4, Left: Slug In situ hybridization on a stage 10 HH chick embryo. At this stage Slug is expressed in early migratory cranial neural crest and in neural crest precursors on the neural tube. **Right:** Pax-7 (purple) and Pax-3 (cyan) double *in situ* hybridization on a stage 8 chick embryo. Both genes are expressed in the neural folds, Pax-7 expression being more anterior and dorsal than Pax-3.

Table 1: Genes expressed in premigratory neural crest (adapted from Gammill and Bronner-Fraser 2003)

GENE	Mouse	Chick	Fish	Frog	NP	NF	EPI
Ap2	x	x	x	x		x	x
Crestin			x			x	
eif4a2				x	x	x	
FoxD3	x	x	x	x		x	
Id2		x				x	
Mes1b				x	x	x	
Msx-1	x	x	-	x		x	x
Msx2	x		-			x	x
Msxb/c	-	-	x		-		x
c-myc				x	x	x	
Nbx				x	x	x	
Notch1	x	x	x	x	x	x	
Pax-3	x	x	x	x	x	x	
Pax-7	x	x	x		x	x	
rhob	x	x				x	
slug	-	x		x		x	
Snail	x	-	x	x		x	
Sox9	x		x	x		x	
Sox10	x	x	x	x		x	
Twist	x	x		x		x	
Zic1	x	x	x	x	x	x	
Zic2	x	x	x	x	x	x	
Zic3	x	-	x	x	x	x	
Zic5				x	x	x	
Zicr1				x	x	x	

Table 1: -, the gene is not expressed in the neural crest in that organism. **X,** the gene is expressed in the neural plate (NP), the neural folds (NF) or the non-neural ectoderm (EPI).

Chapter 2:

Specification of Neural Crest Occurs During Gastrulation and Requires Pax7

Martín L. Basch, Marianne Bronner-Fraser and Martín I. García-Castro

ABSTRACT

Neural crest cells have stem cell properties and are important for development of the vertebrate craniofacial skeleton and peripheral ganglia. Despite the interest in neural crest diversification, little is known about what initiates crest formation. To address this issue, we screened neural crest markers at early stages and found Pax7 to be expressed in a symmetric domain flanking the primitive streak in gastrulating chick embryos, in a region fated to be neural crest. We show that the presumptive Pax7 domain is already specified to form neural crest; it generates neural crest when explanted under defined non-inducing conditions *in vitro*. Blocking Pax7 translation prevents specification *in vitro* and blocks expression of the neural crest markers Slug, Sox10 and HNK-1 *in vivo*. Taken together, these data suggest that neural crest specification initiates much earlier than previously assumed and that Pax7 plays a critical role in crest formation during gastrulation and neurulation.

INTRODUCTION

Neural crest cells originate along the border between the neural plate and the epidermis (His, 1868), migrate extensively and generate a wealth of derivatives, including neurons and glia of the peripheral nervous system, neuroendocrine cells, melanocytes, as well as most of the bone and cartilage of the head skeleton (Le Douarin and Kalcheim, 1999). Although melanocytes, neurons and glia arise at all axial levels, only cranial neural crest forms bone and cartilage. This cell population accounts for a high percentage of human birth defects (e.g., cleft palate in 1 in 500 to 2000 live births) (Wilkie and Morris-Kay, 2001).

While the mechanisms of neural crest migration and differentiation have received a great deal of attention, comparatively less is known about their origin and induction (Knecht and Bronner-Fraser, 2002). In amphibians and chick, interactions between the neural plate and adjacent tissues (non-neural ectoderm or mesoderm) induce neural crest cells (Moury and Jacobson, 1989; Selleck and Bronner-Fraser, 1995) and elicit a molecular program characteristic of these cells as they migrate and differentiate . In amniotes, neural crest induction studies have been largely based upon an *in vitro* assay where "naïve" intermediate neural plate tissue is induced by non-neural ectoderm (Selleck and Bronner-Fraser, 1995; Dickinson et al., 1995) or defined factors (Basler et al., 1993; Liem et al., 1995; García Castro et al., 2002) to generate neural crest. Signals such as BMPs and Wnts can act as epidermal inducers of neural crest (Liem et al., 1995, García

Castro et al., 2002; Saint Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997). In avians, most studies have focused on the induction of trunk neural crest cells using the open neural plate of stage 10 embryos. While the competence of this trunk tissue to generate neural crest cells declines after stage 10 (Basch et al., 2000), our knowledge of when neural crest induction begins *in vivo*, particularly at cranial levels, is very limited.

METHODS

In situ hybridization and immunohistochemistry

Whole mount *in situ* hybridization (Henrique et al., 1995) and immunohistochemistry (García-Castro et al., 2002) were performed as previously described. For double *in situ* hybridization, fluorescein and digoxigenin labeled probes were added simultaneously and sequentially incubated with antibody. Alkaline-phosphatase coupled anti-fluorescein antibody was added first and color was developed by addition of BCIP. After fixation, embryos were incubated with an anti-digoxigenin coupled with alkaline phosphatase and color was developed by addition of NBT/BCIP or BM purple. HNK-1 antibody was obtained from American Type Culture and Pax7 from Developmental Studies Hybridoma Bank.

Morpholino electroporation

Lysamine labeled morpholino oligonucleotides were obtained from Gene Tools, LLC. A cPax7 5'UTR antisense morpholino oligonucleotide (5'-TCCGTGCGGAGCGGGTCACCCCC-3'), a cPax3 5'UTR antisense morpholino oligonucleotide (5'-CCAGCGTGGTCATCGCGGCGGCGC-3') and a cPax7 5'UTR antisense morpholino oligonucleotide carrying 5 mismatches as a control (5'-TCgGTcCGGAGccGGTgACaCCC-3'). Morpholinos were stored at -80°C at a concentration of 1.2 μM and diluted 1:1 in 10%sucrose just before injection. St 4 embryos were explanted onto filter paper rings and placed ventral side up on

drops of 2% agarose in water and morpholinos were introduced into embryos via electroporation (Funahashi et al 1999). They were injected onto the prospective neural crest forming region of the epiblast followed by 2 square electroporation pulses of 5-10 mV at 25 msec. After electroporation, embryos were placed in thin albumin for 1-2 hours to recover and then prepared for dissection or incubation in modified Newculture (Stern and Bachvarova 1997).

***In vitro* translation**

Full length cPax7, cPax3 or *Xenopus* EF1 mRNA was transcribed using mMessage mMachine (Ambion). Proteins for each mRNA were translated using Rabbbit Reticulocyte Lysate Nuclease-treated (Promega) according to manufacturer instructions. In each reaction, 2 μl of morpholino oligonucleotides of varying concentrations were added and incubated for 15 minutes at room temperature before adding ^{35}S-methionine. Protein samples were stacked in a 4% polyacrylamide gel and separated by 10% polycacrylamide gels. Gels were rinsed in water, fixed for 20' in 40% methanol/20% glacial acetic acid. After fixing, gels were enhanced in 1M sodium salicylate for 10 minutes. Gels were rinsed in water before drying under vacuum for 30 minutes at 80°C.

Embryo dissection and tissue culture

St 3 and st 4 chick embryos were dissected in Ringer's solution using tungsten or glass needles. The lower layers were removed with careful dissection and the explanted epiblast tissue was placed in PB1 solution for 1-2 hours before embedding it in collagen gels for culture. Collagen gels were prepared by mixing 90 μl of rat type I collagen (Collaborative Research, Waltham, Massachusetts) with 10 μl of 10XDMEM and 4.5 μl of 7.5% sodium bicarbonate. The collagen gels were covered with 300 μl of defined F12/N2 serum free medium and the tissue was cultured for 40-48 hours in a gassed tissue incubator.

RESULTS

To examine early events in neural crest formation in the chick embryo, we analyzed the expression of known neural crest markers at progressively earlier developmental stages. We found that expression of Pax7 (Fig. 1), a transcription factor of the paired box family (Chi and Epstein 2002), correlates with the presumptive neural crest domain in gastrulating embryos and is expressed much earlier than conventional neural crest markers like Slug (Nieto et al., 1994) or SoxE (Cheung and Briscoe 2003) genes. We first detected Pax7 by both mRNA and protein expression at stage 4+ (in 30% of embryos), when the primitive streak has reached its full length. It appeared as two bilaterally symmetric oblique bands lateral to Hensen's node, extending diagonally ~400 μm towards the primitive streak. By stage 5, Pax7 expression is observed in all embryos. The rostral-most portion of the Pax7 expression domains is rostral to Hensen's node (~150μm) and reaches ~300 μm caudal to it. From stage 6 onward, Pax7 expression superimposed onto the newly formed neural folds, anterior to the primitive streak and posterior to the forebrain. Interestingly, the orthologue Pax3 (Bang et al., 1997) was expressed more caudally and medially than Pax7 (Fig. 2), suggesting that the patterns of Pax3/7 in the chick are the reciprocal of those described in mouse (Mansouri et al., 1996).

Neural crest induction has typically been assumed to occur at the neural plate border. Therefore, we compared the expression of Pax7 to known markers of presumptive epidermis, neural plate, ectodermal placode and the neural plate

border Streit et al., 1998; Streit and Stern, 1999) (Fig. 3). Dlx5 was used as a marker for the presumptive ectodermal placodes, Gata2 for the presumptive epidermis and Sox2 for the presumptive neural region. At stage 4+, there was no overlap between Dlx5 and Pax7, though by stage 5, the Dlx5 domain appeared to spread caudally in a crescent shape such that the caudal portions abutted the most lateral domains of Pax7. Gata2 was primarily expressed in the caudal portion of the embryo at stage 4+ with little overlap with Pax7; by stage 5, the Pax7 domain lay adjacent to a Gata2 positive domain. Similarly, Sox2 expression in the presumptive neural plate initially abutted only a subset of the Pax7 domain but subsequently (by stage 6) came to lie largely within the two stripes of Pax7 expression, though extending far more anteriorly. The neural plate border markers BMP4 and Msx-1 overlapped with Pax7 in lateral and caudo-lateral domains, respectively. These results suggest a partial, but not complete, overlap of Pax7 with the neural plate border by stage 5.

The expression of Pax7 in the neural folds raised the intriguing possibility that its earliest expression during gastrulation marks the region fated to give rise to neural crest. In support of this idea, the Pax7 expression domain of stage 4+ embryos is consistent with the data of previous fate maps of the neural crest. To further characterize the neural crest forming region before the onset of Pax7 expression, we performed focal DiI injections on stage 4 embryos. Cells labeled in the presumptive Pax7 domain (i.e., ~300 microns lateral to the primitive streak) were later incorporated into the dorsal neural folds/neural crest (Fig. 4). In contrast, the region above Hensen's node formed forebrain, lateral regions close to the area opaca formed ectoderm and the regions immediately adjacent to

the primitive streak gave rise to mesoderm (Fig. 4), consistent with previous fate maps (García-Martínez et al., 1993; Rex et al., 1997).

We found that cells in the presumptive Pax7 domain were already specified toward a neural crest fate. Here, we define "specification" as the ability of a particular tissue to form neural crest cells in the absence of external influences when placed in a neutral environment. We explanted various pieces of epiblast from stage 4 embryos and tested their ability to generate neural crest cells when cultured in isolation. A thin strip of epiblast tissue was dissected perpendicular to the primitive streak and 250 μm caudal to Hensen's node. The lower layer was removed and the epiblast strip was cut into 14 pieces (~100 μm wide), explanted onto collagen gels, and cultured for 48 hours (Fig 5b). We found that only the fragments ~300 μm lateral to and on both sides of the primitive streak were able to generate migratory HNK-1+, Pax7+ neural crest cells (n = 8/10 embryos). We further tested neural crest specification at stage 3 and found that similar to stage 4, only regions midway between the primitive streak and area opaca generated neural crest cells (Figure 5b). These results suggest that this specific domain of epiblast, corresponding to the presumptive Pax7 expressing domain, is already specified to form neural crest cells as early as stage 3, prior to overt Pax7 expression (at stage 4+ to 5).

In order to test whether Pax7 was required to form neural crest at these early stages, we turned to a loss-of-function approach. We designed antisense morpholino oligonucleotides to specifically prevent the translation of Pax7 (MoPax7). First, we tested the specificity of the morpholino designed against

Pax7 using an *in vitro* translation assay. We found that increasing concentrations of the MoPax7 reduced Pax7 protein expression in a dose-dependent fashion whereas a Pax3 morpholino had no effect (Fig. 6). Translation of an unrelated protein (EF1a) was unaffected in the presence of either Pax-3 or Pax-7 morpholino.

We next showed that Pax7 expression was required for specification of neural crest cells (Fig. 7). We electroporated stage 4 embryos with MoPax7 or control mismatched morpholino, and explanted the "presumptive "neural crest domain into collagen gels and cultured them for 48 hours. While very few explants treated with MoPax7 produced neural crest cells (n = 4/26), the majority of explanted epiblast tissues electroporated with control morpholinos (containing 5 mismatches from the original MoPax7 sequence) generated migratory HNK-1+ neural crest cells (n = 21/27), suggesting that Pax7 is essential for neural crest specification.

In addition to its requirement for specification, we went on to show that Pax7 was required for neural crest formation *in vivo*. We introduced morpholinos unilaterally into the presumptive neural crest region of stage 4 using *in vivo* electroporation, with the opposite side serving as an internal control. The embryos were allowed to further develop for 24 hours in modified New culture. Consistent with the *in vitro* translation assay, *in vivo* MoPax7-electroporated cells had a dramatic reduction of immunocytochemically detectable Pax7 (Fig. 8). Furthermore, electroporation with MoPax7 resulted in clear alterations of the neural crest markers Slug (n=14/21 embryos) and Sox10 (n=17/22 embryos) on

the electroporated (right) side of the embryos. Both premigratory and migrating cranial neural crest cells appeared to be depleted and the axial level of the reduction exactly correlated with the location of the introduced MoPax7. In contrast, embryos electroporated with control morpholinos showed no obvious alterations of Slug (n =1/ 10 embryos) or Sox10 (n = 1/12 embryos) expression in most cases (Fig. 9). In addition to the phenotypes observed for Slug and Sox10, we also found that MoPax7 treated embryos displayed a reduction of migratory HNK1+ cells on the electroporated side (Fig. 10). Consistent with this, Pax7 null mice are reported to have defects in cranial neural crest derivatives Bang et al. 1997). These results show that early Pax7 protein is required for the expression of the neural crest markers Slug, Sox10, and HNK-1, supporting a role for Pax7 in neural crest formation during gastrulation/early neurulation. In contrast to Pax7, early Pax3 expression is not required for neural crest induction Bang et al., 1997).

Finally, we examined whether Pax7 function altered the expression of markers for the neural plate or its border. Embryos electroporated with MoPax7 showed no significant alterations in Sox2 (n = 0/12 embryos), BMP4 (n = 1/23 embryos) or Dlx5 (n = 0/11 embryos) expression (Fig. 11). These results suggest that Pax7 is selectively required for presumptive neural crest, but it is not essential for formation of neural plate or border tissue.

DISCUSSION

Neural crest cells are derived from the ectoderm and classically have been thought to originate via interactions between neural and non-neural ectoderm and/or mesoderm (Knecht and Bronner-Fraser, 2002). Here, we present the surprising result that discrete regions of epiblast from stage 3-4 embryos are already specified to form neural crest, suggesting that all necessary components are present in this tissue to elicit crest formation. Since this 'neural crest' domain is flanked by presumptive epidermis and presumptive mesendoderm, it is possible that interactions between these tissues account for the induction. However, at this stage, prospective mesendoderm is flexible in fate and transiently expresses neuronal markers Sox3 (McLArren et al., 2003) and Zic1 (unpublished observation), complicating our understanding of its nature.

Interestingly, the domain containing presumptive neural crest cells lies caudal to the neural plate according to recent fate maps of stage 4+ (García-Martínez et al., 1993; Rex et al., 1997).

Furthermore, the presumptive Pax7 domain is specified to generate neural crest well before a proper neural territory exists. Therefore, the established role of interactions between neural and non-neural ectoderm in neural crest formation might be required at later stages to maintain an already induced and specified neural crest territory, marked by Pax7 expression. Our results suggest that formal establishment of a neural plate border is not requisite for neural crest specification or induction, and that border formation and neural crest induction may be separable events. Consistent with this possibility, over-expression of

Dlx5 creates an ectopic neural plate border but does not induce neural crest (McLarren et al., 2003)

In summary, the present study shows that neural crest specification occurs earlier than previously anticipated, in a region of epiblast cells midway between the primitive streak and area opaca of stage 3-4 embryos. Moreover, Pax7 marks this presumptive neural crest territory, including both cranial and trunk neural crest, from stage 4+ onward and is required for neural crest formation at these early stages. Our results establish Pax7 as the earliest known marker for neural crest cells in the avian embryo and place the start of the induction process at or before gastrulation. This alters our current thinking regarding the mechanisms underlying neural crest induction and specification and establishes a new role for Pax7 in these processes.

ACKNOWLEDGEMENTS:

We thank Drs. Scott Fraser, Andrew Groves, Laura Gammill, Vivian Lee, John Sechrist and Tanya Moreno for helpful comments to the manuscript.

Figure 1: Early expression of Pax-7

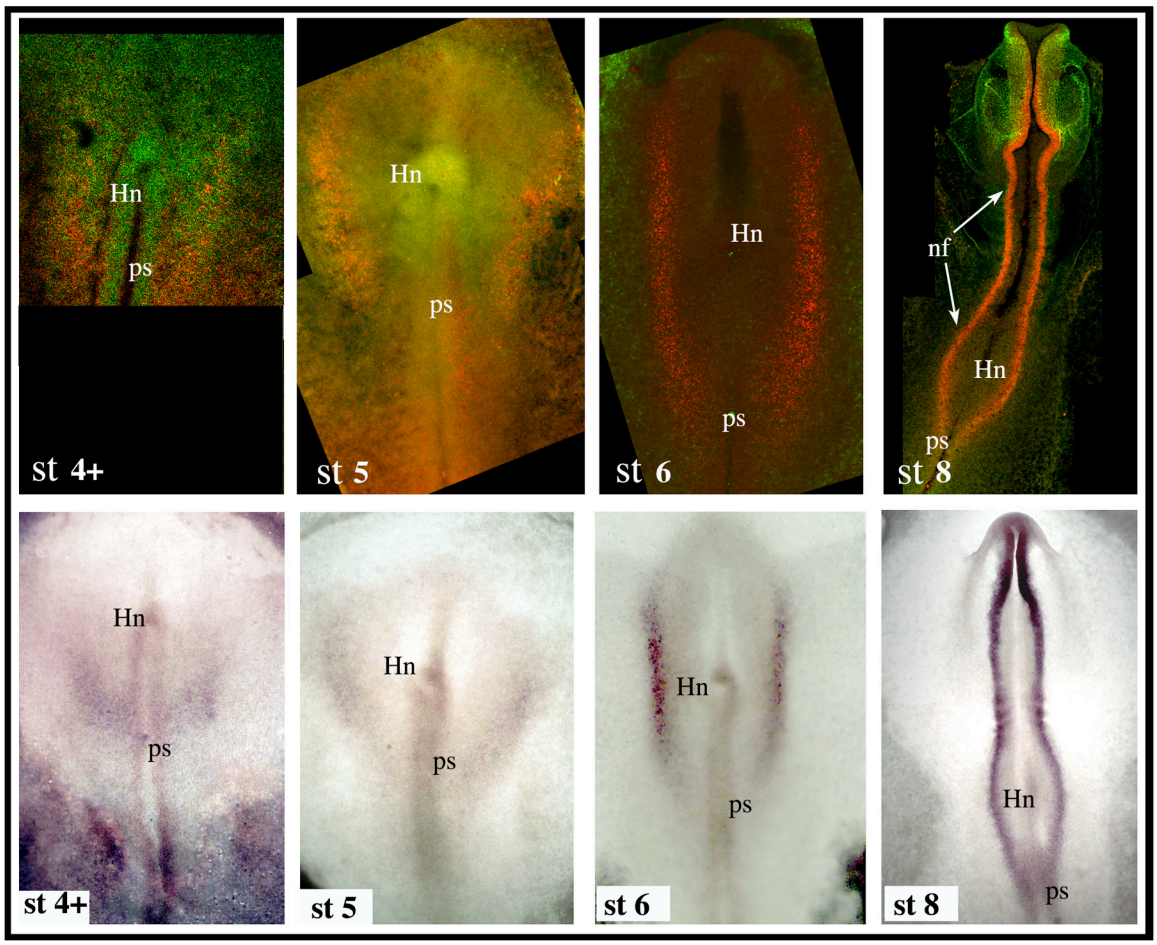

Fig. 1, Early expression of Pax7 in presumptive neural crest from gastrulation to neurulation. **Top row:** Pax7 immunostaining shown in red at st4+, 5, 6 and 8. Green staining is shown for contrast. **Bottom row:** Pax7 *in situ* hybridization shown at the same stages.

Figure 2: Pax-7 Expression compared to Pax-3

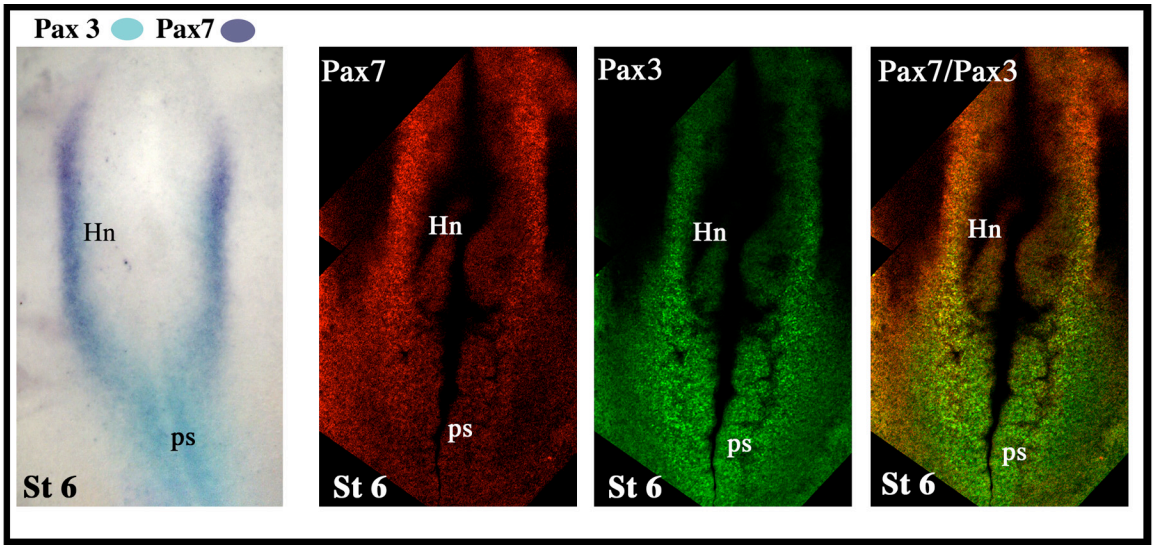

Fig. 2, left panel: double *in situ* hybridization of Pax7 (purple) and Pax3 (cyan) in a st6 embryo. Right panels: Pax7 (red), Pax3 (green) and Pax7/Pax3 immunostaining at st6.

Figure 3: Pax-7 and neural, epidermis and border markers

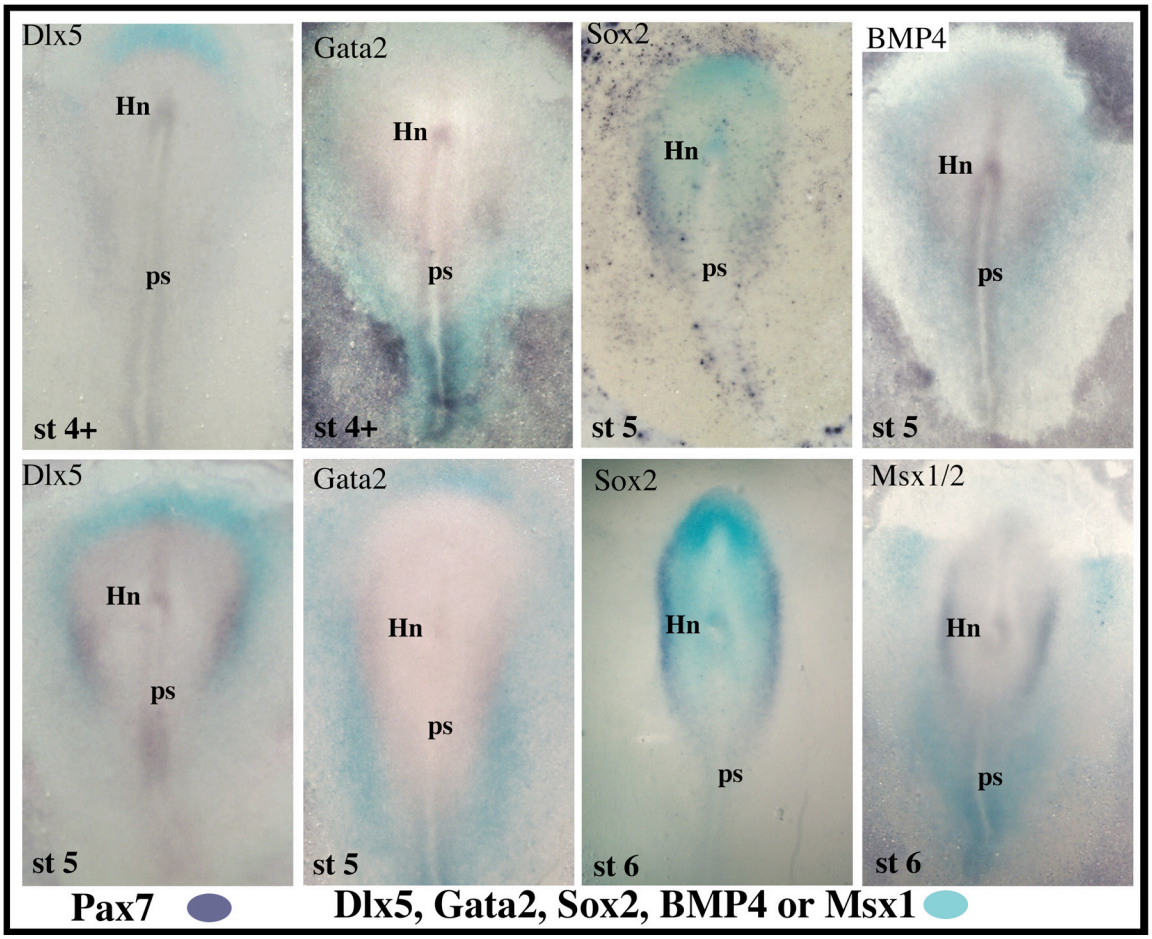

Fig. 3, double *in situ* hybridizations comparing Pax7 (purple) early expression with expression of early neural, non neural and border markers (cyan): Dlx5, Gata2, Sox2, BMP4 and Msx1/2. **Hn:** Hensen's node, **ps:** primitive streak.

Figure 4: DiI labeling of the prospective neural crest at st.4

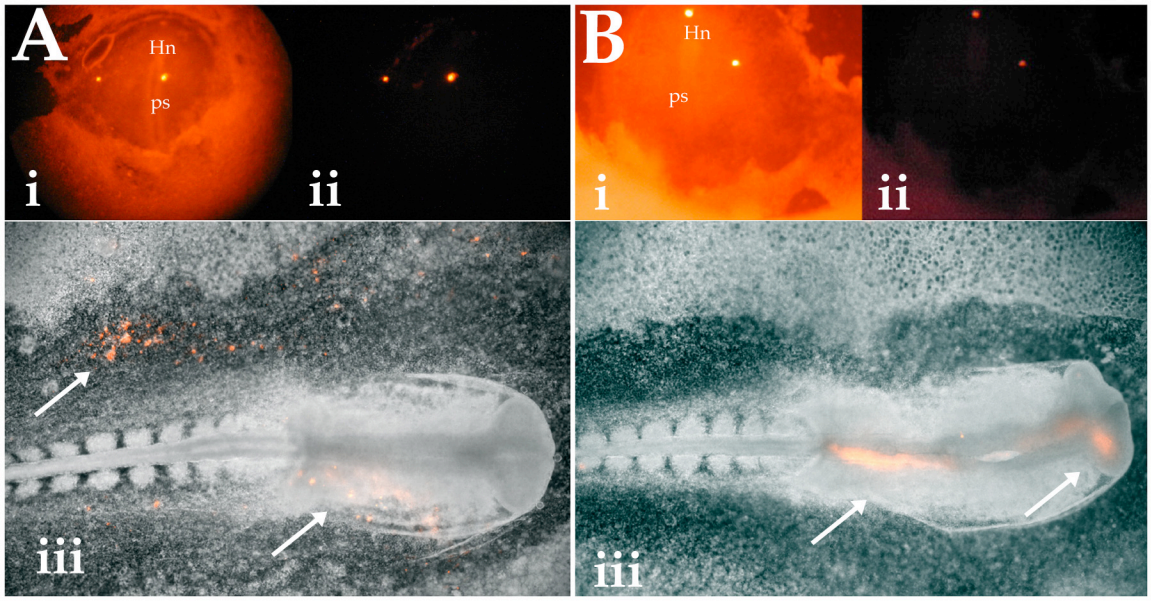

Fig. 4, DiI labeling of the presumptive Pax-7 domain. **A:** labeled cells lateral and medial to the Pax-7 prospective domain become epidermis and mesoderm respectively. **B:** labeled cells in the prospective pax-7 expression domain become neural crest. Cells anterior to Hensen's node become forebrain. **i:** position of the labeled cells in the embryo. **ii:** fluorescence image showing the focal injections of DiI. **iii:** position of the labeled cells after 24 hours. **Hn:** Hensen's node, **ps:** primitive streak.

Figure 5: Early specification of neural crest

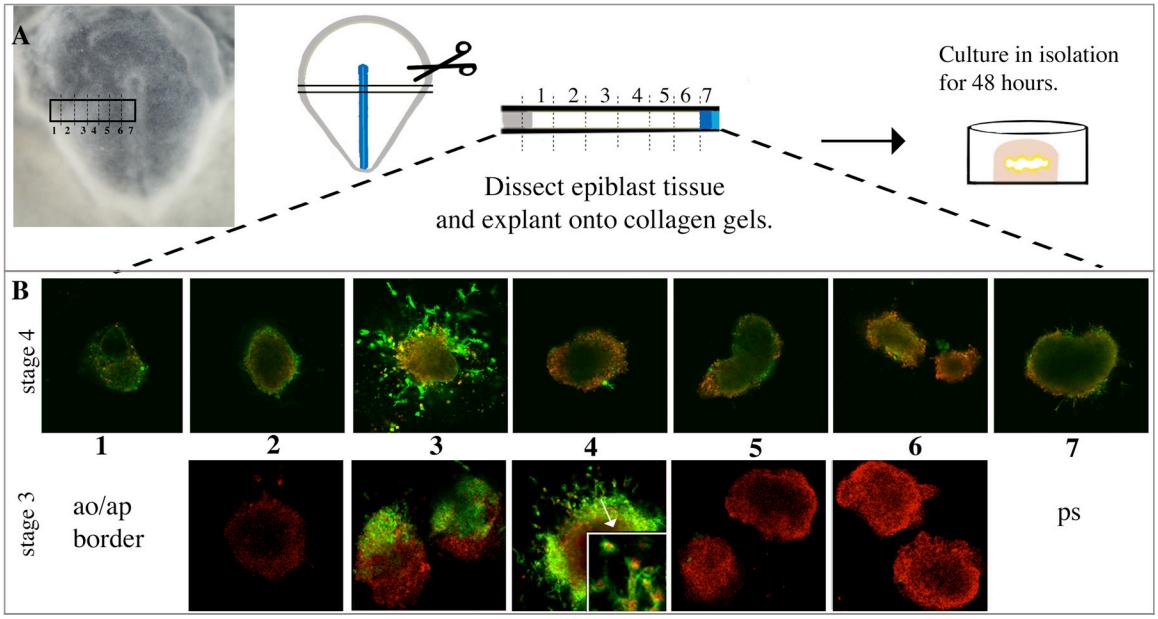

Fig. 5, A region of the early epiblast is specified to generate neural crest in gastrulating embryos. **A**, a strip of epiblast perpendicular to the primitive streak was dissected into 14 pieces (7 from each half of the embryo of each about 100 μm wide). After 40-48 hours in culture, the tissues were immunostained for Pax7 (red) and HNK-1 (green). **B,** result of the experiment described in A. Top row: explants from a st 4 embryo Bottom row: explants from a st 3 embryo. Inset shows higher magnification of $HNK1^{+}$, $Pax7^{+}$ migratory cells. **ao:** area opaca, **ap:** area pellucida, **ps**: primitive streak.

Figure 6: Inhibition of Pax-7 translation *in vitro*

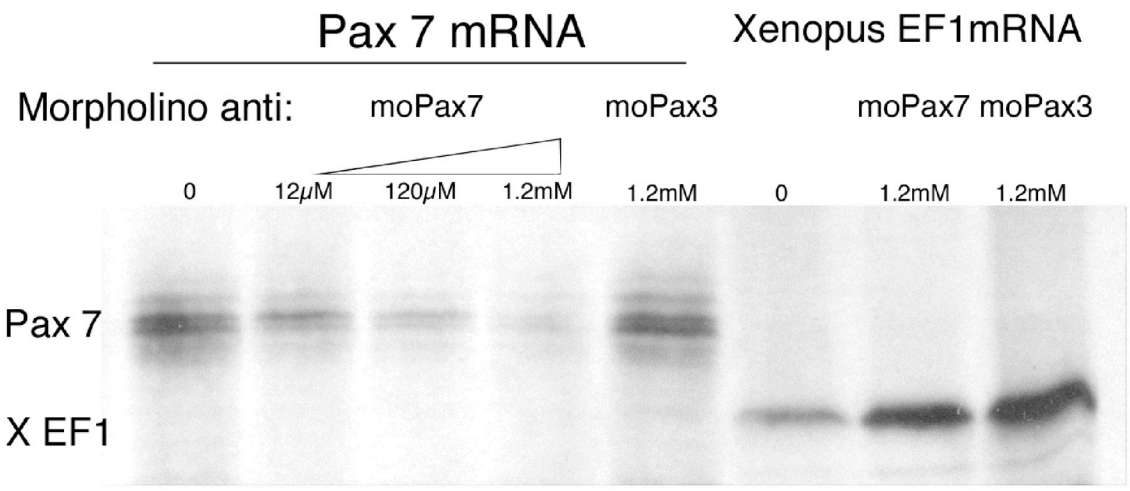

Fig. 6, *in vitro* translation of Pax7 in the presence of increasing concentrations of morpholinos against Pax7. Inhibition of Pax-7 translation is specific. Morpholinos against Pax-3 do not block Pax-7 translation.

Figure 7: Inhibition of Pax-7 prevents NC specification

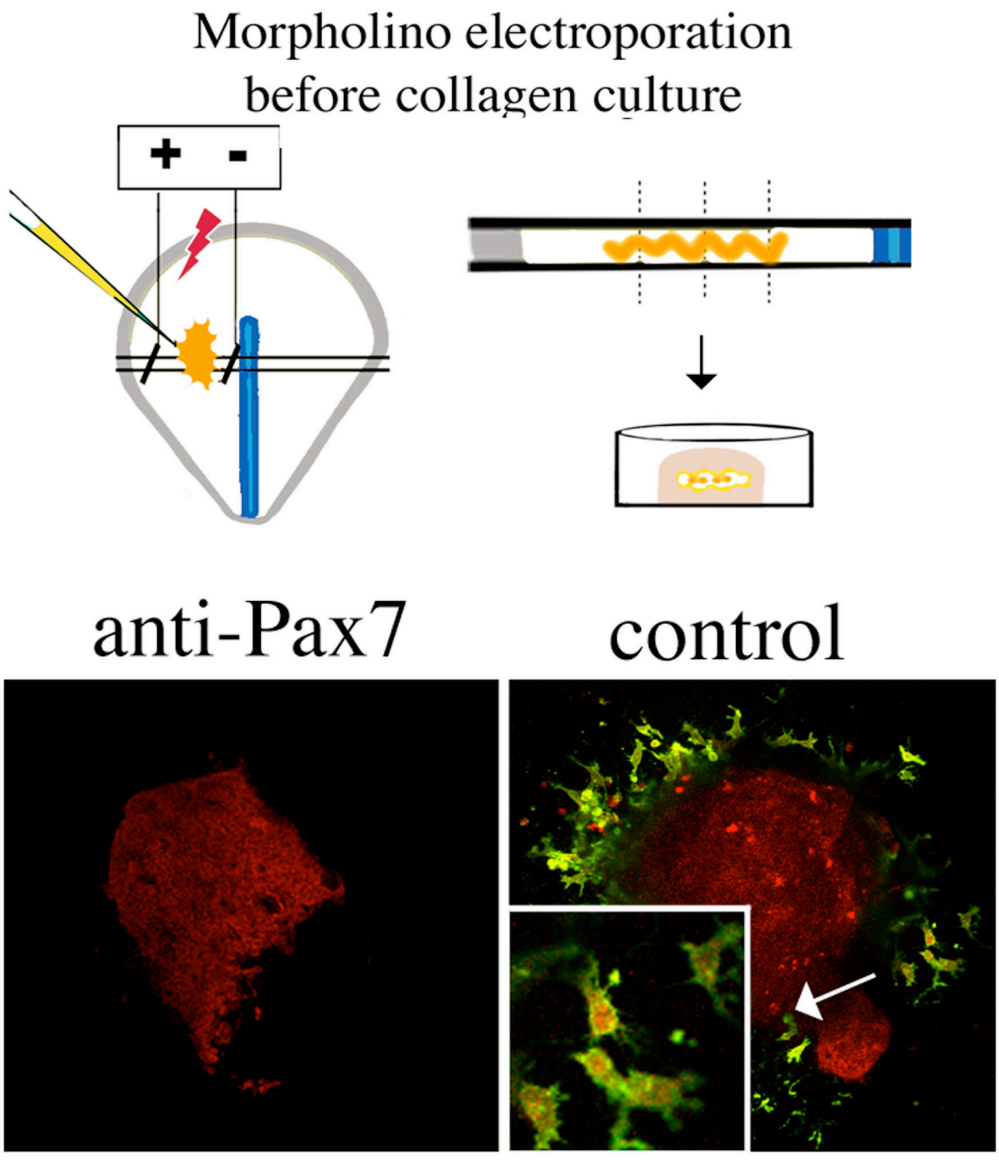

Fig. 6, st4 embryos were electroporated with morpholinos against Pax7 or morpholinos against Pax7 carrying 5 mismatches. The prospective neural crest forming region of the epiblast was dissected After 40-48 hours in culture, the tissues were immunostained for Pax7 (red) and HNK-1 (green).

Figure 8: Inhibition of Pax-7 translation *in vivo*

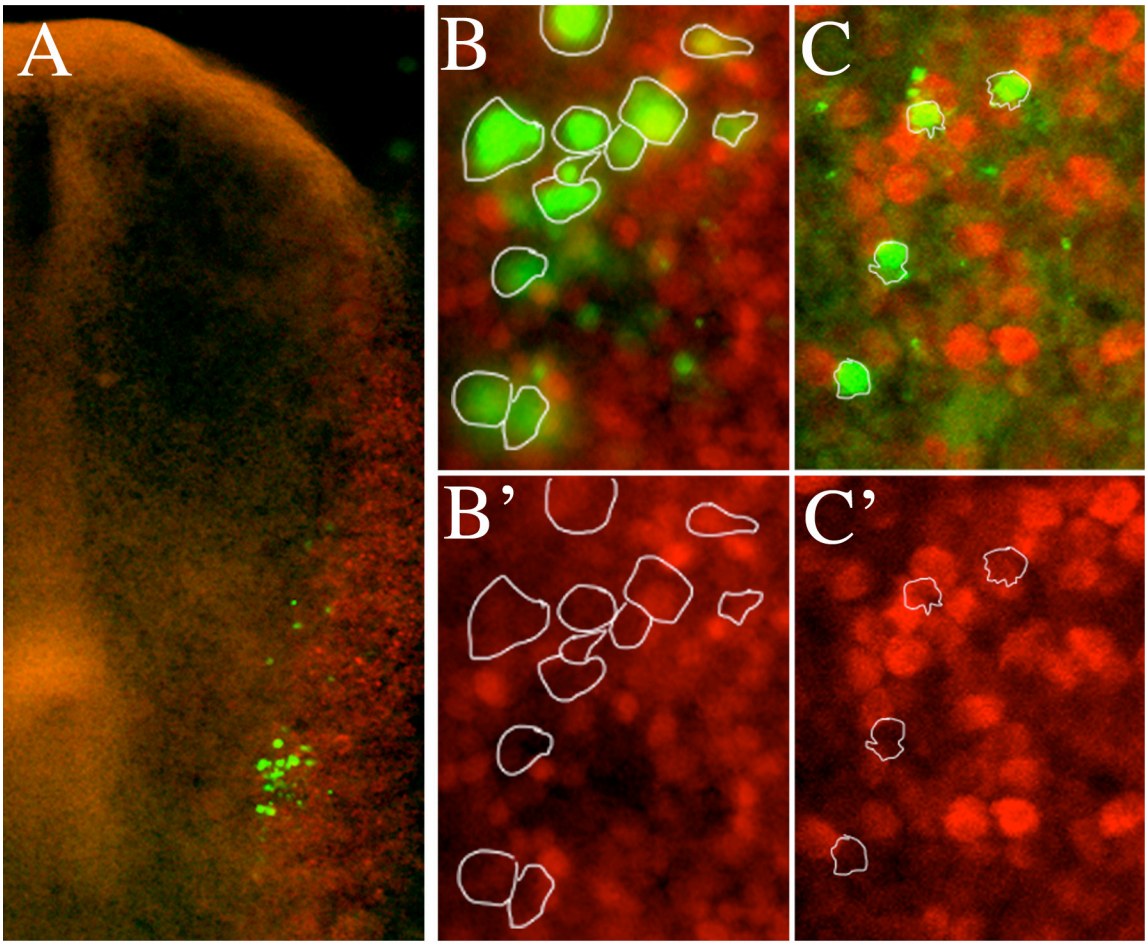

Fig. 8, Unilateral electroporation of MoPax7 at stage 4 down-regulates expression of neural crest markers (arrows) *in vivo*. **S**tage 6 (A, B, B') and stage 8 embryos (C, C') treated with MoPax7 (green) show Pax7 downregulation (red). **B, C,** Close up to electroporated cells (outlined) surrounded by Pax7 expresisng cells shown with and without (**B', C')** green signal.

Figure 9: MoPax-7 prevents expression of NC markers

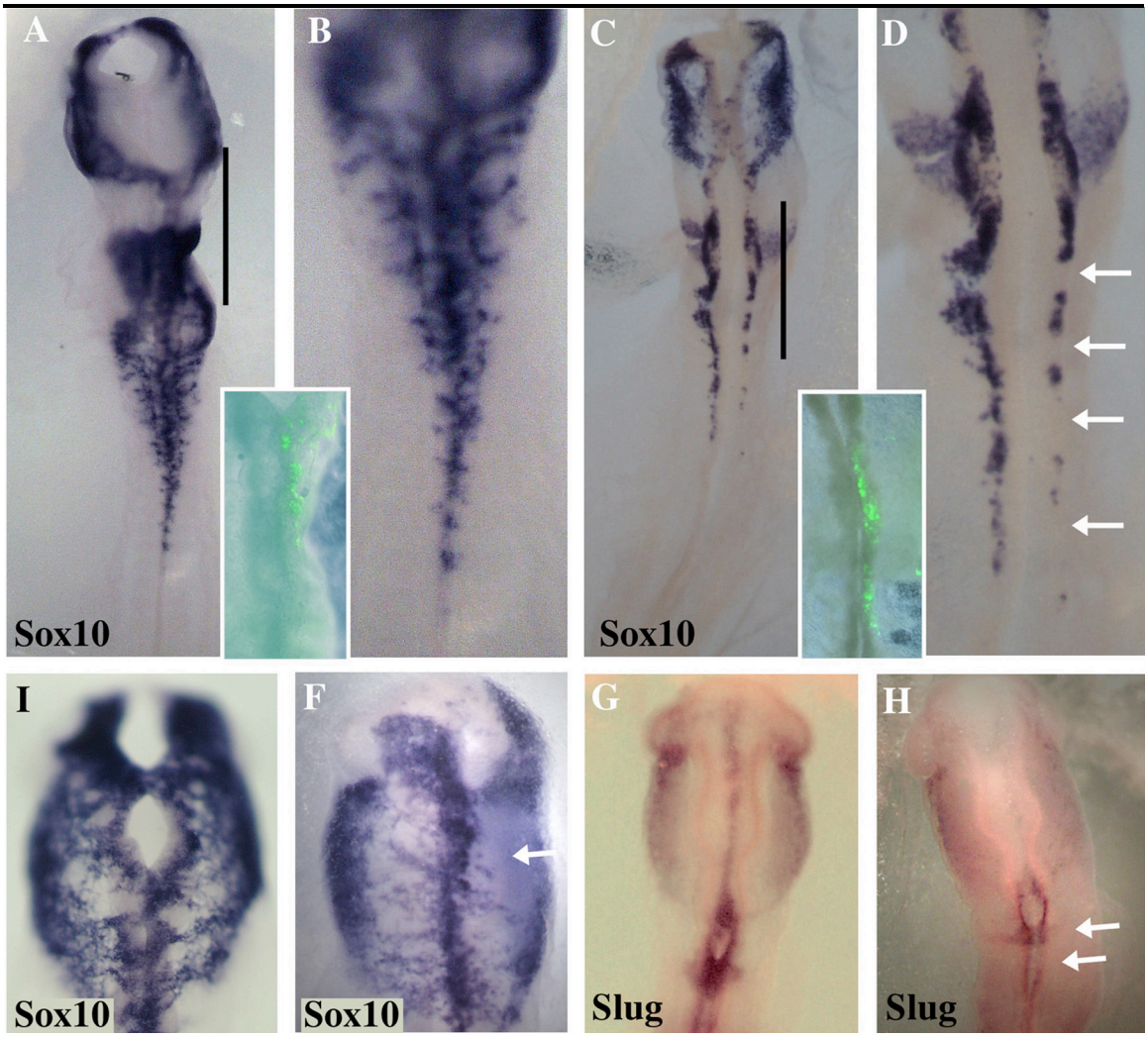

Fig. 9, Expression of Sox10 (**A, B, I**) and Slug (**G**) is symmetric in embryos electroporated with control morpholinos, while MoPax7 treated embryos display unilateral diminished expression (arrows) of pre- and migratory neural crest markers Sox10 (**C, D, F**) and Slug (**H**) in the treated side (midbrain to anterior trunk levels). Bars correspond to the area shown in insets. Inset shows morpholino distribution before *in situ* hybridization.

Figure 10: MoPax-7 prevents expression of NC markers

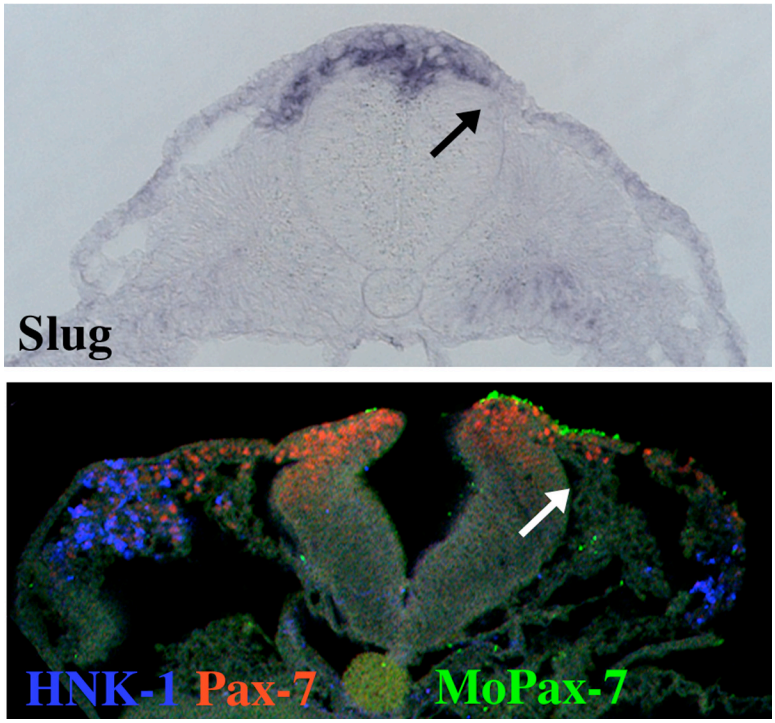

Fig. 10, Top: section of a MoPax7 electroporated embryo showing reduction of Slug at the vagal crest level. **Bottom:** section of a MoPax7 electroporated embryo showing reduction of Pax7 (red), and HNK-1 (green) at midbrain level. Unilateral reduction of head mesenchyme, Slug, HNK-1+ cells, and Pax7+ cells were detected along the entire midbrain (≥220 μm).

Figure 11: MoPax-7 do not affect the expression of epidermis, neural or border markers

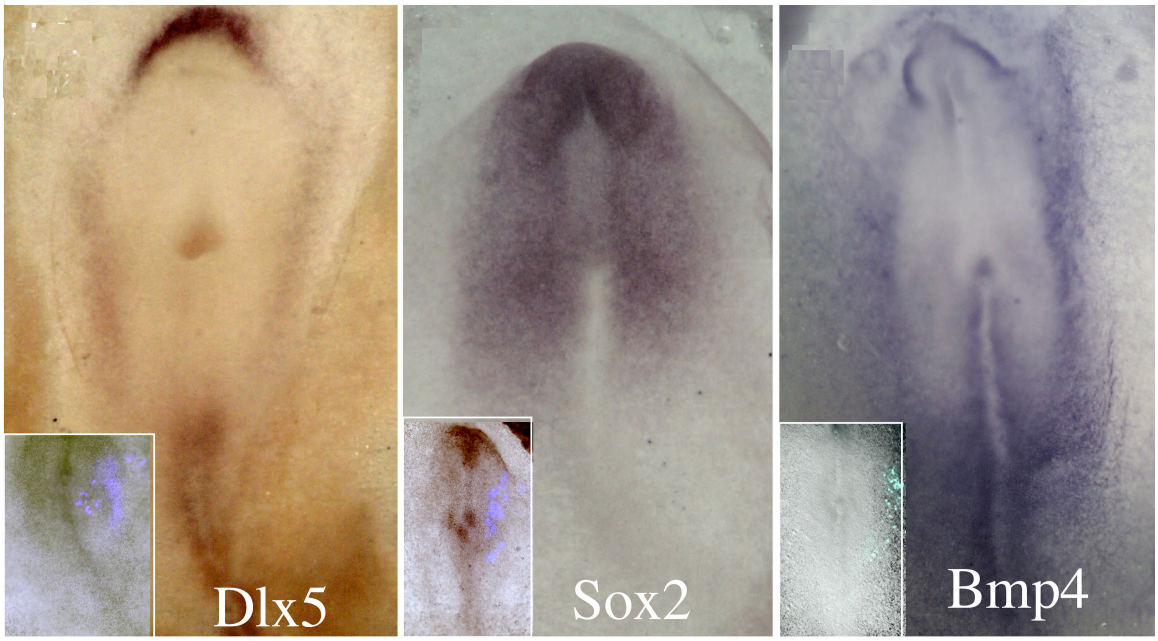

Fig. 11, Morpholinos against Pax-7 do not affect the expression of epidermal (Dlx-5) neural (Sox2) or border (BMP4) markers. Insets show the distribution of MoPax-7 before *in situ* hybridization.

Chapter 3:

Timing and Competence of Neural Crest Formation

Martín L. Basch, Mark A.J. Selleck and Marianne Bronner-Fraser

Developmental Neuroscience, 22, 217-27, 2000

ABSTRACT

Neural crest cells can be induced by an interaction between neural plate and ectoderm. To clarify the timing and nature of these inductive interactions, we have examined the time of competence of the neural plate to become neural crest as well as the time of neural fold specification. The neural plate is competent to respond to inductive interactions with the non-neural ectoderm for a limited period, rapidly losing its responsive ability after stage 10. In contrast, non-neural ectoderm from numerous stages retains the ability to induce neural crest cells from competent neural plate. When neural folds are explanted to test their ability to produce neural crest without further tissue interactions, we find that folds derived from all rostrocaudal levels of the open neural plate are already specified to express the neural crest marker, Slug. However, additional signals may be required for maintenance of Slug expression, since the transcript was later down-regulated *in vitro* in the absence of tissue interactions. Taken together, these results suggest that there are multiple stages of neural crest induction. The earliest induction must have occurred by the end of gastrulation, since the newly formed neural fold population is already specified to form neural crest. However, isolated neural folds eventually down-regulate Slug, suggesting a second phase that maintains neural crest formation. Thus, induction of the neural crest may involve multiple and sustained tissue interactions.

INTRODUCTION

The neural crest is a transient population of embryonic cells derived from the ectoderm and defined by their migratory behavior and ability to form numerous derivatives. The ectoderm cells destined to generate neural crest precursor cells undergo an epithelial-to-mesenchymal conversion and then migrate to diverse sites in the embryo where they differentiate into multiple derivatives such as neurons and glia of the peripheral nervous system, melanocytes of the skin, and much of the craniofacial skeleton (Le Douarin, 1982).

A number of fate mapping studies have shown that in the early embryo, prospective neural crest cells are located at the border between neural tissue and the adjacent non-neural (epidermal) ectoderm (Rosenquist, 1981). For instance, in regions of the embryo where the neural plate is open, neural crest cells lie at the lateral margin of the neural plate within the elevating neural folds. Lineage analyses of individual neural fold cells at this time have shown that single cells can form epidermal, neural crest and neural tube derivatives (Selleck and Bronner-Fraser, 1995), suggesting that these cells are not yet committed to a particular fate but remain multipotent in their ability to form ectodermal derivatives. Our recent finding that a notochord, or Shh-expressing cells, grafted adjacent to the neural folds can prevent neural crest formation (Selleck et al., 1998), further supports the idea that neural crest cells are not committed to their fate at this time.

Around the time of neural tube closure, notochords and Shh-secreting cells are no longer able to perturb neural crest formation (Artinger and Bronner-Fraser, 1992; Selleck et al., 1998). In contrast, the BMP antagonist, noggin, is able to inhibit neural crest formation, suggesting that BMPs are required for neural crest formation at this time. The expression of BMP-4 and –7 in the dorsal neural tube at this stage is consistent with a role for these molecules in neural crest generation.

After neural tube closure, neural crest progenitors are located within the dorsal neural tube, at least in chick embryos. Lineage analysis of the closed neural tube has shown that (i) single cells can contribute progeny to both neural crest and central nervous system derivatives, and (ii) single cells can contribute to a variety of different neural crest derivatives. These results demonstrate that at least some neural crest precursors are multipotent at this stage and that even after neural tube closure, neuroepithelial cells are not fully committed to a neural crest fate.

Given that neural crest cells arise at the border of the neural and non-neural ectoderm, it is likely that interactions between these two cell populations are responsible for aspects of neural crest formation. In both amphibians and avians, transplantation of neural plate into epidermis or vice versa results in the formation of neural crest derivatives at the border between the juxtaposed tissues; interestingly both epidermis and neural plate can contribute to the newly formed neural crest derivatives (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Dickinson et al., 1995). However, the finding that BMP-4 and BMP-7 are able to substitute for non-neural ectoderm in inducing neural crest cells

(Liem et al., 1995) raises the question of what the role of neural plate-epidermis interactions is in normal development.

At an extreme, epidermis might induce neural crest cells simply because it contains BMPs and that neural plate-epidermis interactions do not play a role in normal neural crest development. An alternative possibility is that neural plate-epidermis interactions are required for maintained BMP expression in the dorsal neural tube. A third scenario is that the epidermis is required for neural crest induction, but in a BMP-independent fashion. If neural plate-epidermis interactions are important during normal development, little is known about the timing of the interaction. For example, when is the neural plate competent to respond to induction by the ectoderm and when is the epidermal ectoderm able to function as an inducer?

In the present study, we have examined, 1) the time at which naïve neural plate and ectoderm are capable of functioning as inducers/responders when juxtaposed *in vitro* or *in vivo*; and 2) the time at which neural folds cells are specified to form neural crest. Our results suggest that induction of the neural crest occurs continually and over a long period of time starting during gastrulation and persisting past the time of neural tube closure.

MATERIALS AND METHODS

Isolation of tissues for grafting or collagen gel culture

Fertile chicken eggs (White leghorn) were incubated for 28-72 hours to obtain stage 8-20 embryos. Intermediate neural plate explants were taken from stage 8-20 donor embryos; explants included approximately the ventral two-thirds of the caudal neural plate excluding the floor plate; on average explants were approximately 100 x 50 μm in dimension. Neural plates from stages 8-10 were dissected in 0.1% trypsin/ Ca^{++}-Mg^{++} free Tyrode's whereas neural plates of older stages were dissected in the presence of 1 mg/ml Dispase (Boehringer Mannheim) and rinsed twice with Ca^{++}-Mg^{++} free PBS and then were allowed to recover in Modified Eagle's Medium (MEM) with 15% horse serum and 10% embryo extract for 20 minutes to 2 hours on ice prior to grafting or embedding in collagen gels. Approximately 200 μm square pieces of presumptive non-neural ectoderm were dissected from a region near the area pellucida/area opaca border of stage 8-15 embryos in the presence of 0.1% trypsin/ Ca^{++}-Mg^{++} free Tyrode's. Recombinants were made by wrapping the recovered neural plates in the non-neural ectoderm directly after it was dissected, then placing it in Modified Eagle's Medium (MEM) with 15% horse serum and 10% embryo extract. Both recombinants and isolated pieces of non-neural ectoderm alone were kept on ice for approximately 20 minutes to 1 hour in Modified Eagle's Medium (MEM) with 15% horse serum and 10% embryo extract before embedding in collagen gels.

Neural fold fragments, 100 to 200μm in length, were isolated from the three levels of the open neural plate (rostral, middle and caudal) of stage 10 embryo. Dissections were performed in a solution of trypsin (0.1% in calcium- and magnesium- free Tyrode's saline) to ensure complete removal of mesoderm and non-neural ectoderm from the ectoderm fragments. The neural fold isolates were transferred to medium for 30 minutes on ice prior to embedding in collagen gels.

In vitro growth of explanted tissues

Collagen matrix gels were prepared as previously described (Tessier-Lavigne et al. 1987; Artinger and Bronner-Fraser, 1993), except that only 7-10 μl of bottom collagen and 3-5 μl of top collagen was used to ensure efficient penetration of digoxigenin-labeled probes in subsequent whole mount *in situ* hybridization steps. Cultures were grown in F-12 media plus N-2 supplements (Gibco-BRL) for 0 to 48 hours in a gassed tissue culture incubator.

Neural plate grafts

Eggs and embryos were prepared for *in ovo* manipulations using standard methods (see Selleck et al., 1995). Neural plate tissues were grafted into stage 8 - 20 (Hamburger and Hamilton, 1951) host embryos by gently peeling up a small region of the non-neural ectoderm creating a pocket in which the donor tissue could be inserted. Eggs were sealed with adhesive tape and incubated for 16 to 18 hours in a forced draft, humid 38˚C incubator.

Whole mount *in situ* hybridization

Digoxigenin-labeled riboprobes were synthesized *in vitro* using standard protocols (Nieto et al., 1996). In situ hybridization was performed with Slug (Nieto et al., 1994) and BMP-4 (Duprez et al., 1996) probes as described previously (Henrique et al., 1995). Embryos were fixed once the color reaction had reached completion and photographed in whole mount. Some of the embryos were subsequently embedded in gelatin for cryostat sectioning as described in Sechrist et al., 1995.

Examination of embryos

In situ hybridizations of neural plate and/or ectoderm recombinants fragments or whole embryos were photographed using Kodak Ektachrome 160T slide film. Color slides were subsequently imported into Adobe Photoshop using a Kodak SprintScan slide scanner. Photographic plates were printed on a Kodak XLS color printer.

RESULTS

Competence of the intermediate neural plate to form neural crest

Neural crest cells can arise via an inductive interaction between neural and non-neural ectoderm. Previous experiments have demonstrated that neural plate from both stage 4 and stage 8-10 embryos can form neural crest in response to interactions with the ectoderm (Selleck et al., 1995; Dickinson, et al., 1995). To define the time window during which the intermediate neural plate is competent to generate neural crest, we varied the age of the donor tissue from stage 8 to stage 20. In all cases, host ectoderm was derived from stage 10 embryos. Donor grafts were always taken from the same rostrocaudal position (adjacent to the future forelimb) regardless of the age of the embryo. Neural crest formation was assayed by analyzing the expression of Slug, a zinc finger transcription factor that is the earliest known neural crest marker in the chick (Nieto et al., 1994). Slug is expressed on premigratory neural crest cells as well as early migrating neural crest; however, it is down-regulated at later times of migration.

Grafts *in vivo*

Quail intermediate neural plate tissue was grafted underneath the non-neural ectoderm of host embryos near the area pellucida/opaca border, at presumptive forelimb levels (Figure 1). At the time of grafting, no Slug expression could be observed in the donor neural plate. Quail donor tissue

could be recognized by staining with the quail specific antibody QCPN, allowing distinction between donor and host cells. Embryos were allowed to develop an additional 16 hours after grafting and then analyzed for Slug expression.

Robust Slug expression was noted in donor grafts derived from stage 8, 9 and 10 embryos (Figure 2 A-C). By stage 11, a drop-off in Slug staining was noted such that only 50% of the grafts were Slug positive (Figure 2 D-F). After stage 11, no Slug staining was observed in grafted tissue (Table I) ranging from stage 12-20. This suggests that the naïve neural plate loses competence to respond to induction by the non-neural ectoderm after stage 10-11.

Recombinants *in vitro*

The grafts described above juxtapose neural plate with non-neural ectoderm *in vivo*; however, there is also some mesoderm underlying the ectoderm. Thus, one cannot exclude the possibility that mesoderm also may play a role in inducing the naïve neural plate to form neural crest or to imparting competence to respond to an ectodermal signal.

In order to examine the competence of neural plate to respond to ectoderm under defined cultures, the two tissues were recombined in collagen gels *in vitro* using a defined medium without growth factors (Figure 3). The stage of the donor tissue was varied from stage 9 to stage 15 whereas stage 10 host ectoderm was used for all recombinants. Neural crest formation was assayed by expression of Slug 16 hours after explantation.

In the culture medium used in the present study, Slug expression was detected in explants derived from stage 9 and 10 neural plate co-cultured with ectoderm. There was some variability in the results depending upon the efficacy

of juxtaposition of the explants, but a majority of explants at these stages had Slug expression (Figure 4A). In contrast, donor neural plate derived from stage 11, 12 (Figure 4B),13 , 14 or 15 embryos never expressed Slug. These results confirm the *in vivo* findings and suggest that the competence of the naïve neural plate to respond to induction by the ectoderm is lost after stage 10.

Competence of the non-neural ectoderm to induce neural crest

The above results demonstrate the neural tissue has a distinct window of competence in its ability to respond to induction by the ectoderm. A salient question is whether the ectoderm also has a defined period during which it has inductive capacity. In order to test this possibility, competent stage 10 intermediate neural plate was recombined with ectoderm from donor embryos at stage 8, 10, 12 and 20. Both ectoderm and neuroectoderm was derived from the same rostrocaudal level, adjacent to the future forelimb region. Slug expression was noted at each of the host stages utilized (data not shown), suggesting that the ectoderm maintains its inductive ability for prolonged periods of time.

Effects of the mesoderm on neural crest formation from the neural plate

Although interactions between the neural plate and ectoderm are sufficient to elicit neural crest induction, this does not rule out the possibility that other tissues may participate in neural crest formation as well. The mesoderm underlies the open neural plate and ectoderm, and remains in contact with these tissues during the course of neural tube closure. To test whether or not

mesoderm has the ability to induce neural crest markers in naïve neural tissue, competent intermediate neural plate from stage 10 embryos was co-cultured in collagen gels with segmental plate mesoderm derived from stage 8, 10 and 12 embryos (n = 4 co-cultures per stage). In no case was Slug expression detectable in these cultures (data not shown), suggesting that mesoderm at these stages does not possess neural crest inducing capacity.

Time of neural fold specification to form neural crest

To better understand the time at which neural crest induction occurs, we analyzed the ability of neural fold tissue from different rostrocaudal levels of the open neural plate to give rise to neural crest cells after isolation under defined conditions. Here, we define a tissue as "specified" if it follows a particular developmental pathway in the absence of other embryonic signals, for example, when isolated from the embryo and cultured in a neutral medium (Slack, 1991).

Neural folds were dissected from three levels along the rostrocaudal axis of the open neural plate of stage 10 embryos resulting in the isolation of rostral, middle and caudal neural folds (Figure 5). The caudal-most neural folds represent the newly formed neural folds since they arise just above Hensen's node, the site of gastrulation. The neural folds were subsequently grown in collagen gels in a defined medium and fixed at 6 hour intervals up to 18 hours after explantation. Neural crest formation was assayed by analyzing Slug expression.

Immediately after explantation, the neural folds from all rostrocaudal levels expressed no Slug transcripts (Table II and Figure 6). In "rostral" neural

folds, Slug expression manifested itself by 6 hours in some explants and by 12 hours in all explants (Figure 6). Interestingly, by 18 hours after explantation of "rostral" neural folds, Slug expression appeared to be down-regulated since no explants expressed Slug at this time point. By contrast, Slug expression was delayed in both "middle" and "caudal" neural folds compared to "rostral" levels and was first observed at 18 hours post-explantation (Table II). This delay likely reflects the temporal gradient of rostrocaudal development.

The above observations suggest that Slug expression is specified at all levels of the open neural folds, since they autonomously express Slug after explantation in a neutral environment. It is interesting to note, however, that Slug is not maintained at 18 hours of culture. This is in marked contrast to the situation *in vivo*, where Slug is first expressed in the neural folds and expression is maintained in the closed neural tube until after the time of neural crest emigration. This may indicate that prolonged interactions of the neural folds with other embryonic tissue are necessary to maintain Slug expression.

In addition to Slug, we analyzed BMP-4 expression in explants examined at 12 hours post-explantation. At this time point, BMP-4 is expressed at all levels (rostral, middle, and caudal) whereas Slug is only expressed in rostral neural fold explants. In addition, BMP-4 expression is more intense at caudal than rostral levels (data not shown). This may indicate that BMP-4 expression in the neural folds precedes that of Slug.

DISCUSSION

Time of competence of neural plate to respond to induction by ectoderm

Although it is clear that neural crest cells are an induced population (Selleck and Bronner-Fraser, 1995; Dickinson et al., 1995), the exact timing and molecular nature of inductive interaction has not been defined. To clarify these questions, we have examined the time of competence of the neural plate to become neural crest and the time of specification of the neural folds. Our results indicate that there is defined window of time during which naïve neural plate can be induced to generate neural crest cells. We previously found that both stage 4 and stage 8-10 neural plate could respond to non-neural ectoderm by forming neural crest (Selleck and Bronner-Fraser, 1995; Dickinson et al., 1995). Here, we find that there is a sharp decline in this competence after stage 10 as assayed both *in vivo* and *in vitro*. This suggests that initial induction of neural crest cells is likely to be completed within the open neural plate of stage 10 embryos. However, secondary interactions (see below) are likely to be important for further development of the neural crest population. Since development proceeds in a rostral to caudal progression, the timing of inductive interactions varies according to axial level. For the purpose of this study, we have focussed on a single level (adjacent to the future forelimb).

Our data support the idea that inductive interactions may be time-limited in the early development of the neural crest. Whereas juxtaposition of ectoderm and early neural plate induces the formation of neural crest cells (Selleck and

Bronner-Fraser, 1995), interactions using slightly older neural tissues leads to the expression of neural crest cells plus dorsal tube markers like *Wnt-1* and *Wnt-3a* (Dickinson et al., 1995). Thus, induction of particular markers is stage-dependent. Similarly, addition of BMPs to early neural plates can induce neural crest markers (Liem et al., 1995) whereas addition to later neural tubes elicits differentiation of roof plate cells and dorsal sensory neurons (Liem et al., 1997). Thus, neural plate/epidermal interactions may induce, in a temporally distinct sequence, multiple dorsal properties in the developing spinal cord. Taken together, these results demonstrate that the competence of the neural plate to respond to induction changes as a function of time.

Time of competence of the ectoderm to induce neural crest

In contrast to the rapid decline of the neural plate to respond to induction by the ectoderm after stage 10, the ectoderm maintains inducing ability over a much broader time period. In fact, we found that ectoderm derived from stage 20 embryos was able to efficiently induce neural crest cells. This indicates that the window of competence to induce and respond are not necessarily matched. One possibility is that a continuous ectodermal signal has different effects on the neural tube with time such that early ectoderm induces neural crest whereas later ectoderm maintains neural crest. Although the nature of the ectodermal signal remains unclear, it is unlikely to be mediated by BMPs alone, since they are expressed in the early ectoderm but are down-regulated in ectoderm and then up-regulated in the neural folds as they elevate and close to form the neural

tube (Watanabe and LeDouarin, 1997). In contrast to the ectoderm, we noted no obvious inductive ability by segmental plate mesoderm.

Timing of neural crest induction and maintenance of Slug

We find that the neural fold population is specified to autonomously express Slug at all rostrocaudal levels of the open neural folds. Thus, initial induction of Slug has already occurred in the earliest neural plate and, therefore, is likely to have occurred during gastrulation. However, Slug is not maintained at later time points in explanted neural folds. This suggests that a later signal, perhaps derived from contact with the ectoderm, may be necessary for maintenance of the Slug expression in the neural folds. These results raise the interesting possibility that induction of the neural crest starts during gastrulation but requires continuous interactions for maintenance of the neural crest precursor pool.

Because the neural folds, themselves, may be a heterogeneous population, we cannot rule out the possibility that inductive interactions can take place within the explanted neural folds. In this scenario, individual neural fold cells may be unspecified but distinct such that interactions between unequal neural fold cells (e.g., one more ectodermal in character and another more neural in character) induce neural crest cells. Thus, we can conclude that the neural fold is specified as a population, but not necessary at the single cell level.

Little is known about the actual function of the transcription factor, Slug, in neural crest formation. Recent studies of Slug in a rat bladder carcinoma cell line (Savagner et al., 1997) point to a role in regulating desmosome assembly in

these epithelial cells. Ectopic expression of Slug in *Xenopus* embryos does not itself induce neural crest formation (Labonne and Bronner-Fraser, 1998). However, over-expression of Slug in the presence of a Wnt signal yields robust neural crest formation (Labonne and Bronner-Fraser, 1998). Therefore, Slug may be an immediate consequence of the initial induction of the neural crest. Recent experiments in the frog (LaBonne and Bronner-Fraser, submitted) suggest that Slug function is required both early for neural crest emigration and later for continued migration and differentiation of this population. In support of this idea, antisense oligonucleotide knock-out of Slu*g* transcripts in the early chick embryo blocks neural crest emigration (Nieto et al., 1994).

Role of BMP4 in neural crest production

BMPs have been shown to be sufficient to substitute for the non-neural ectoderm in inducing neural crest cells (Liem et al., 1995). Recent experiments suggest that BMPs may function at a secondary stage in neural crest development. Initially expressed in the ectoderm, BMPs are down-regulated in the ectoderm and subsequently expressed in the elevating neural folds and recently closed dorsal neural tube (Watanabe and LeDouarin, 1997). Accordingly, inhibition of BMPs by noggin fails to block initial neural crest induction when BMPs are expressed in the ectoderm, but rather blocks neural crest production at the time they are expressed within the dorsal neural tube (Selleck et al., 1998).

Our preliminary experiments suggest that BMP-4 is up-regulated in the isolated neural folds just prior to up-regulation of Slug. This supports the idea

that BMPs within the neural folds may play a role in the onset of Slug expression. An interesting possibility is that BMPs may be induced in the neural folds via an inductive interaction between non-neural and neural ectoderm. It remains to be determined if molecules other than BMPs may also effect Slug induction.

CONCLUSIONS

These experiments clarify the time of neural crest specification and the window of competence during which neural plate cells can be induced to form neural crest. Our results show that there is a rapid drop off after stage 10 such that naïve neural plate loses its competence to respond to induction by the ectoderm. In contrast, non-neural ectoderm from numerous stages retains the ability to induce neural crest cells from competent neural plate. Interestingly, in the endogenous neural fold population, we found that neural folds at all levels of the open neural plate were already specified to form neural crest. Thus, when explanted in culture, they expressed the neural crest marker Slug in the absence of further interactions. However, we noted that other signals were required to maintain Slug expression. This leads us to speculate that there are multiple stages of neural crest induction. The earliest induction must have occurred by the end of gastrulation, since the newly formed neural folds are already specified to form neural crest. However, it is clear that isolation of the neural folds leads to down-regulation of Slug, suggesting a second phase that maintains neural crest formation. Thus, induction of the neural crest may involve a multiple processes and molecular signals.

TABLE 1: Competence of the neural plate to respond to induction by the ectoderm

Donor Stage	No. of Embryos	Slug in Vivo	No. explants	Slug In Vitro
8	6/6	+	6/6	+
9	6/6	+	6/6	+
10	6/6	+	6/6	+
11	3/6	+/-	0/5	-
12	0/6	-	0/5	-
13		-	0/5	-
14	0/6	-	0/5	-
15	0/6	-	0/5	-
16	0/4	-		
18	0/4	-		
28	0/4	-		

Table 1,Naïve neural plate from donors ranging from stage 8 – 20 were grafted underneath the non-neural ectoderm. Their ability to express Slug is indicated by + or -. Figures after the dash represent the number of embryos examined per stage.

TABLE 2: Specification of Neural Folds to Produce Neural Crest Cells

	Time after explantation			
Level	**0 hr**	**6hr**	**12hr**	**18hr**
Rostral	0/6	2/7	5/7	0/8
Middle	0/6	0/6	0/6	5/8
Caudal	0/7	0/8	0/7	6/8

Table 2, Neural folds were dissected from rostral, middle or caudal levels of the open neural plate and assayed for their ability to express Slug transcripts at indicated times after explantation. The ratio represents the number of Slug-expressing explants over the total number of explants examined.

Figure 1: Grafts *in vivo* (schematic)

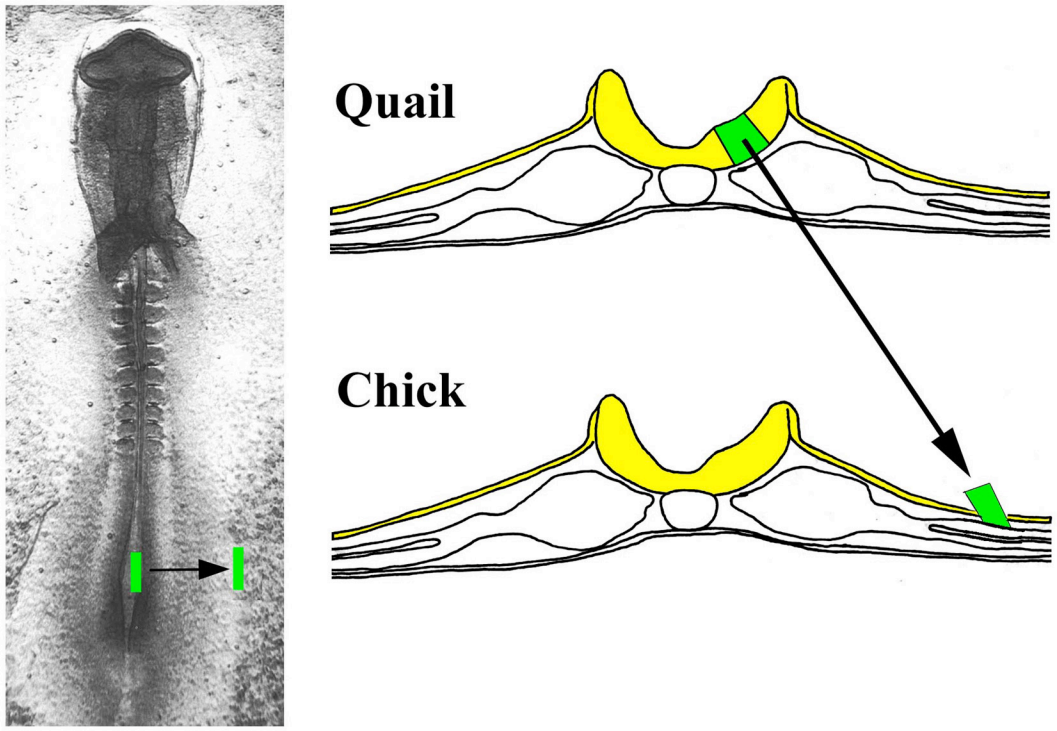

Figure 1. Intermediate neural plate (green) or intermediate neural tube tissue from stage 8 –20 donor quail embryos were isolated and grafted into stage 10 host chick embryos. On the left is shown a stage 10 embryo with the location of the graft indicated in green. Donor grafts were derived from this rostrocaudal level at all stages (adjacent to the future forelimb). Schematic diagrams (right) of transverse sections illustrate the position from which the donor graft was derived and where it was placed. Grafted tissue (indicated in green) was placed through the ectoderm between the area opaca and area pellucida.

Figure 2: Grafts *in vivo*

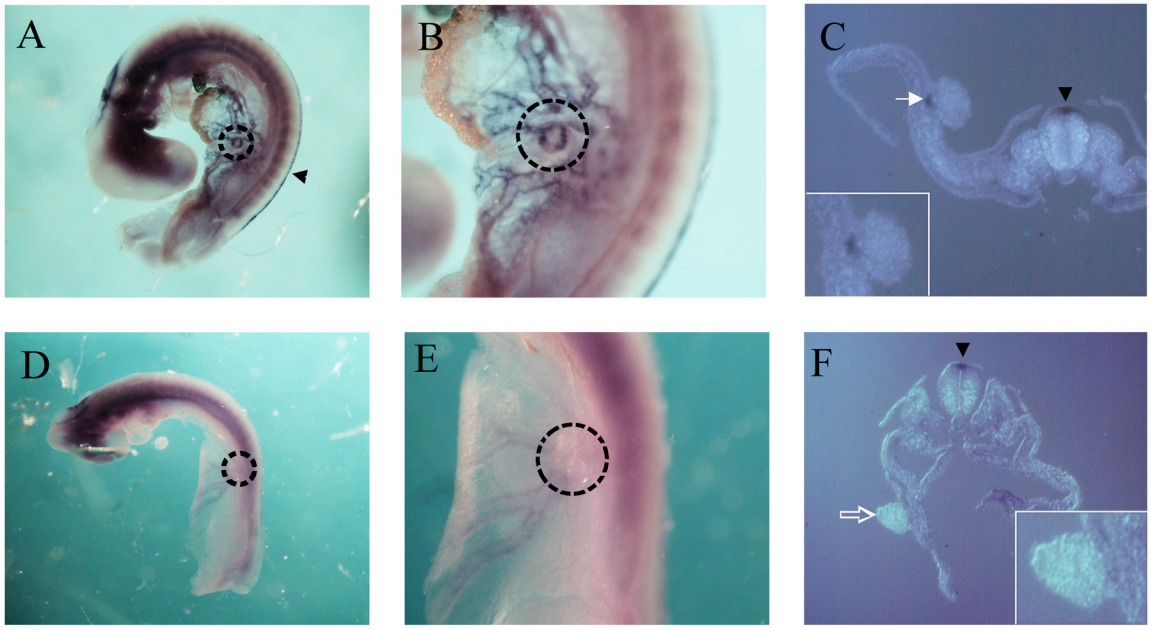

Figure 2. *Intermediate neural tube loses competence to induce Slug expression around stage 11*. **(A-C)** In situ hybridization showing Slug expression in intermediate neural tube derived from a stage 10 embryo (A-C). (**A, B**) Whole mount views at low and high magnification, with the graft indicated by dashed circle, which is Slug-positive. Black arrowhead indicates the dorsal neural tube, which is Slug-positive. (**C**) In transverse section, Slug expression is seen in the region of the graft (white arrow) as well as in the dorsal neural tube (black arrowhead. (**D-F**) In situ hybridization showing Slug expression in intermediate neural tube derived from a stage 11 embryo. (**D, E**) Whole mount views at low and high magnification, with the graft, which is Slug-negative, indicated by a dashed circle. (**F**) In transverse section, Slug expression is only seen in the dorsal neural tube (black arrowhead) but not in the donor graft (white empty arrow). The darker staining of the blood vessels in A,B versus D,E is due to the inclusion of more extraembryonic membrane in the former as well as some differences in host age.

Figure 3: NP/EPI recombinants (schematic)

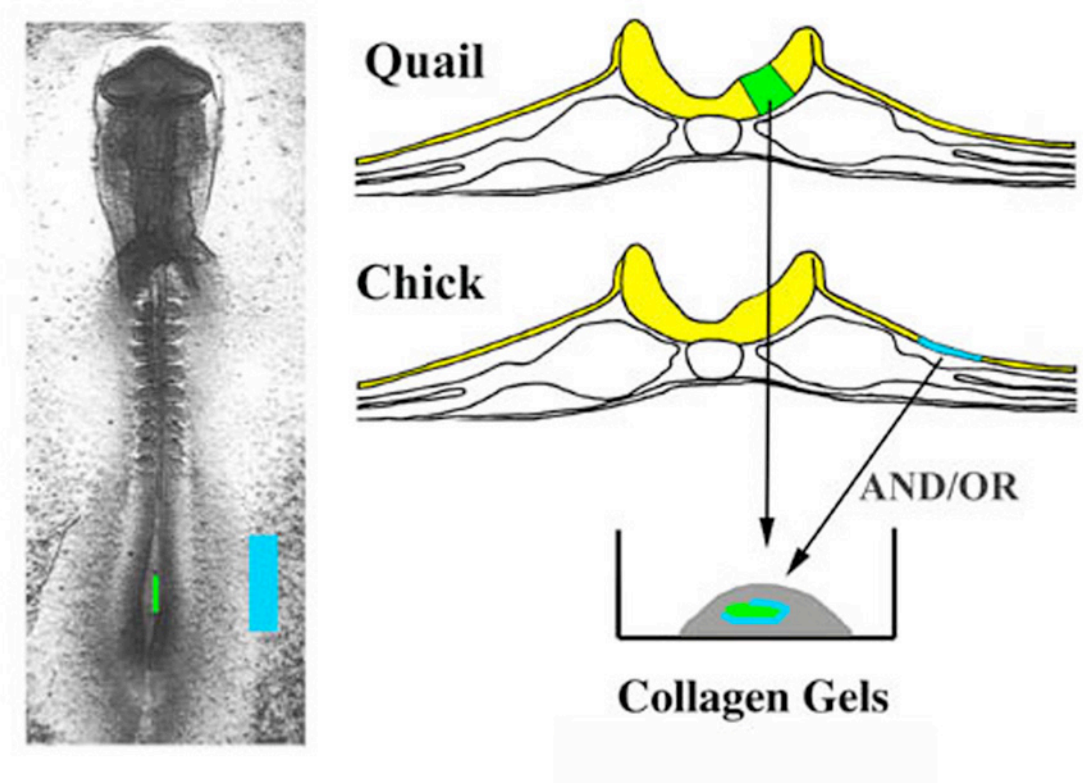

Figure 3. Schematic diagram illustrating the procedure for isolating intermediate neural plate/tube (indicated in green) from stage 8-15 quail embryos and combining them with ectoderm (indicated in blue) from stage 10 chick embryos. The recombinants were cultured for 16-18 hours in collagen gels in defined medium, fixed, and processed for Slug expression.

Figure 4: NP/EPI recombinants

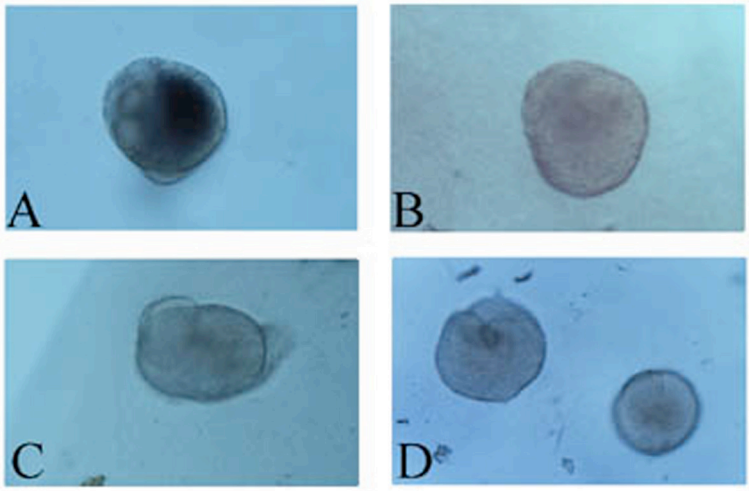

Figure 4. *Slug expression in intermediate neural tube/ectoderm conjugates*. (**A**) Stage 10 intermediate neural tube plus stage 10 ectoderm has Slug expression. (**B**) Stage 10 intermediate neural tube cultured alone has no Slug expression. (**C**) Stage 12 intermediate neural tube plus stage 10 ectoderm has no Slug expression. (**D**) Stage 10 ectoderm cultured alone has no Slug expression.

Figure 5: Specification of neural folds (schematic)

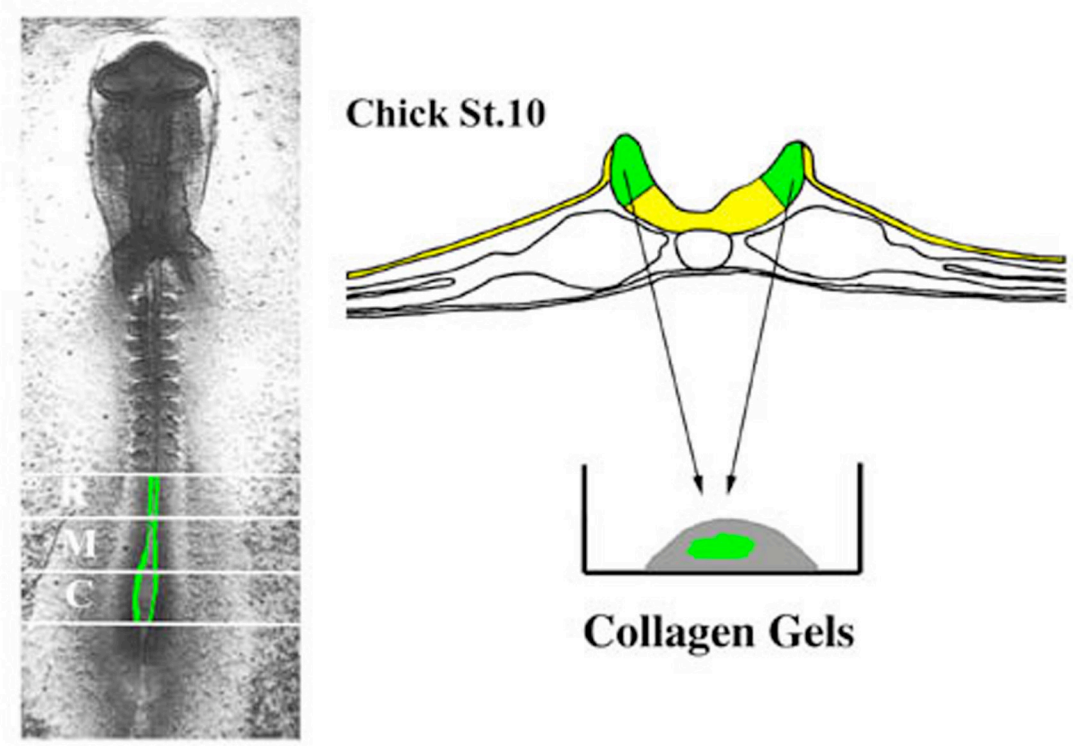

Figure 5. *Schematic diagram illustrating the subdivision of neural folds in order to test specification in isolated conditions.* Open neural plates from stage 10 chick embryos were divided rostrocaudally into three segments, rostral (R), middle (M) and caudal (C). Neural folds form each segment were isolated and cultured in collagen gels in defined medium, prior to staining for Slug expression.

Figure 6: Specification of neural folds

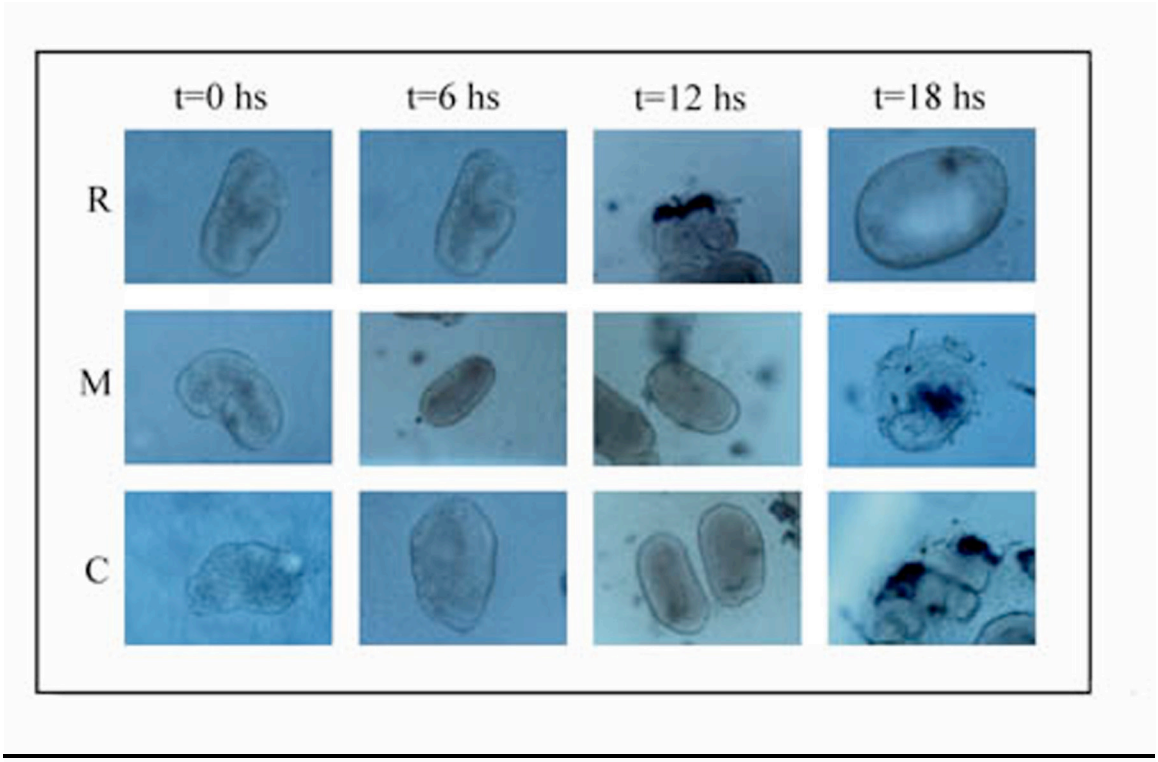

Figure 6. *Timing of Slug expression in neural fold explants*. Explants were prepared as described in Fig. 5 and cultured for 0, 6, 12 or 18 hours prior to fixation and staining for Slug expression. Slug was detected after 12 hours of culture in rostral explants, and after 18 hours of culture in middle and caudal explants. Note that by 18 hours of culture, rostral explants no longer have Slug expression.

Chapter 4:

Inhibition of Notch Signaling Promotes Early Differentiation of Neural Crest and Neural Tube

INTRODUCTION

The neural crest is a transient population of embryonic cells that originates at the border between the neural plate, which forms the central nervous system, and the non-neural ectoderm, which generates the epidermis. During or after the closure of the neural tube, neural crest cells go through an epithelial to mesenchymal transition and migrate extensively throughout the embryo to eventually differentiate into many derivatives, including neurons and glia of the peripheral nervous system, melanocytes, cartilage and secretory cells from the adrenal gland.

Before the onset of migration the neural crest precursors are a heterogeneous population of cells located on the dorsal aspect of the neural tube, or at the edges of the closing neural plate, the neural folds. Lineage analyses have revealed that this population of precursor cells is multipotent. When single cells of the dorsal neural tube were labeled with an intracellular fluorescent dextran, their progeny contributed cells to the neural crest and to the central nervous system (Bronner-Fraser and Fraser, 1988). Furthermore, when similar labeling experiments were performed on cells of the open neural folds (before neural tube closure), labeled progeny was found in the epidermis in addition to neural crest and neural tube (Selleck and Bronner-Fraser, 1995). These experiments suggest that there are at least two consecutive events in the segregation of the ectodermal lineage. Epidermis segregates from the neural/neural crest fates prior to neural tube closure, and the neural crest and neural fates segregate around the time of neural crest migration. One hypothesis is that the segregation of the epidermal lineage from the neural crest/CNS could occur as a mechanical consequence of

the tube closure and the subsequent separation of neural crest and neural tube fates as the results of changes in cell adhesion properties necessary for the onset of neural crest migration (Selleck and Bronner-Fraser, 1996; LaBonne and Bronner-Fraser, 1999). Alternatively, these events could be the result of fate decisions taking place at the border of the neural plate, whereby signaling events between adjacent cells mediate the acquisition of epidermal and neural or neural crest fates.

In birds, the border of the neural plate is established by the concerted action of FGFs, BMPs and BMP antagonist signals such as chordin and noggin (Streit et al., 1999; Pera and Kessel, 1999). In addition, Wnt signals have been proposed to mediate early fate decisions between neural and epidermal fates (Wilson et al., 2001). In Xenoups, the border of the neural plate is positioned in response to a BMP gradient, and a Wnt signal is later required for the induction of neural crest (LaBonne and Bronner-Fraser, 1998). It has been proposed that the Notch signaling pathway also plays a role in the formation of the neural crest in avians and amphibians, as well as in fish (LaBonne and Bronner-Fraser, 1998; Endo et al., 2000; Cornell and Eisen, 2000)

Activation of the Notch pathway plays a pivotal role in fate decisions during neurogenesis through lateral inhibition. This process has been best characterized by studies of neurogenesis in drosophila. During fly neurogenesis, a group of cells in defined positions of the ectoderm acquire the potential to become neural which is reflected by expression of a group of bHLH proneural genes. Usually, when one of these cells adopts a neural fate, it signals to the neighboring cells through Delta, a Notch ligand. Activation of the Notch signaling pathway in the neighboring cells leads to a repression of the proneural

genes and eventually to the acquisition of epidermal fates. Both Notch and its ligands, are transmembrane proteins and therefore Notch signaling requires direct cell-cell interactions (reviewed in Martinez-Arias, 1998).

Several lines of evidence suggest that Notch signaling could be involved in early fate decisions of the neural crest precursors. It has recently been shown that Notch mediates fate decisions between neural crest derivatives. Delta-1 overexpression experiments both *in vitro* and *in vivo* suggest that activation of Notch signaling can promote formation of glia at the expense of neurogenesis during the formation of neural crest derived ganglia (Wakamatsu et al., 2000). Similarly, a transient exposure of neural crest stem cells to soluble active forms of Delta is sufficient to promote the loss of neurogenic capacity and accelerated glial differentiation in these cells (Morrison et al., 2000). In chick embryos, overexpression of cNIC, an activated form of Notch, prevents the expression of neural crest markers while ectopic activation or inhibition of Notch signaling leads to a decrease in the epidermal expression of BMP-4 (Endo et al., 2002). In zebrafish embryos, the cells at the edge of the neural plate can become Rohon-Beard primary sensory neurons or neural crest. Neurogenin-1 (ngn-1), is a zebrafish homolog of the basic helix-loop-helix (bHLH) proneural genes in drosophila and it is required for the formation of primary neurons. A recent study proves that Delta/Notch signaling leads to inhibition of ngn-1, and therefore prevents the formation of Rohon-Beard cells, favoring the formation of neural crest cells instead. These data suggest that although Notch/Delta can repress neurogenic fates it is not actively promoting the formation of neural crest (Cornell and Eisen, 2002)

In the present study, we ask whether Notch signaling plays a role in the fate decisions responsible for the segregation of the ectodermal lineages that will eventually lead to the formation of neural crest cells. First, we addressed this issue by analyzing the expression patterns of cNotch-1 and one of its ligands, cDelta-1. Next, we show that a soluble form of Delta-1 can act as a dominant negative, and finally we block Notch signaling both *in vivo* and *in vitro* at the time of ectodermal lineage segregation. Our preliminary data suggests that inhibition of the Notch pathway promotes an early differentiation of neural crest precursors both *in vivo* and *in vitro.*

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METHODS

In situ hybridization and immunohistochemistry

Whole mount *in situ* hybridization was performed as previously described (Henrique et al., 1995). cNotch-1, cDelta-1 and cSlug fragments were used as templates for the mRNA probes. Color reaction was developed by addition of NBT/BCIP. Immunohistochemistry was perfomed on sections or in cultured explants as described (García Castro et al., 2002).

Injection of DiI labeled Delta-Fc of Fc expressing cells

293T Delta-Fc or 293T Fc expressing cells were grown in STO medium (DMEM +10% heat inactivated Fetal Bovine Serum). At 80% confluence cells were treated with Trypsin/EDTA 1x (Gibco) and recovered for 1 hour in STO + 20% serum at room temperature. Excess serum was washed by addition of Ringers solution to 50ml and cells were spun 3 to 5 minutes at 1000-1500 rpm. DiI solution was prepared by dissolving 50 μg CM-DiI (Molecular Probes) in 10 μl ethanol, and adding 500 μl of 10% sucrose and 1ml of Ringers solution. 300 μl of DiI solution was incubated with the pellet of cells 15 minutes at room temperature followed by addition of 45 ml of ringers solution and centrifugation of the cells 3 to 5 minutes 1000 to 1500 rpm. Pellets were resuspended in 1 ml of Ringers with 6 μl of 35%BSA. Cells were spun $\leq$ 3500 rpm, all supernatant was removed and cells were resuspended by mechanical agitation. The cells were loaded on glass needles with a Hamilton syringe. Injections were done either at

the sides or in the lumen of the neural tube of St 8-10 chick embryos at the level of the open neural plate using a picospritzer .

Conditioned media and Western blot

Delta-Fc and Fc conditioned media were prepared as described (Morrison et al. 2000). 20 μl of each conditioned medium were run through a 8% polyacrylamide gel. The proteins were electrotransfered onto a nitrocellulose membrane and probed with a Goat Anti-human IgG, Fc, Fragment Specific antibody (Jackson Immuno Research), diluted 1:1000. An anti-goat coupled with alkaline phosphatase was used as a secondary antibody, and protein detection was visible through a color reaction with NBT/BCIP.

Transfection of luciferase reporter and luciferase activity

A JH26 construct (CBF-1 enhancer upstream of a SV40 promoter driving expression of luciferase) and a CSK-LacZ construct were co-transfected into N113 cells (C2C12 cells that carry the Notch receptor and intracellular components of the Notch pathway) using Lipofectamine Reagent (invitrogen) according to manufacturer instructions. Luciferase activity was detected using a Luciferase Assay System (Promega) and quantified in an Optocomp I luminometer (MGM instruments)

Embryo dissection and tissue culture

White Leghorn chick eggs were incubated for 36-39 hours to obtain stages 9 and 10 embryos. Neural folds were dissected using tungsten needles in a $Ca^{++,/}$ Mg^{++} free Tyrodes solution. The dissected tissue was allowed to recover for an hour in PB1 medium containing 6μl of 35% BSA per ml before embedding it in collagen gels for culture. Collagen gels were prepared by mixing 90μl of rat type I collagen (Collaborative Research, Waltham, Massachusetts) with 10μl of 10XDMEM and 4.5 μl of 7.5% sodium bicarbonate. The collagen gels were covered with 300μl of defined F12/N2 serum free medium and the tissue was cultured for 40-48 hours in a gassed tissue incubator.

RESULTS

c-Notch and c-Delta1 expression pattern during neural crest precursors fate decisions

The expression of cNotch and its ligands in chicken embryo has been characterized during neurogenesis and somite formation. However, expression in embryos at a time when neural crest precursors fate is being narrowed has not been established. Here we show the early expression pattern of cNotch and one of its ligands, cDelta-1 (Figs. 1 and 2). cNotch expression starts around stage 3 in the caudal portion of the forming primitive streak and its expression is maintained in the prospective mesoderm first and in the presomitic and somatic mesoderm later. The expression on the neural folds is turned on caudally when the folds begin to form around stage 6. By stage 9 cNotch is expressed on the neural folds and dorsal neural tube throughout the entire AP axis of the embryo, with the exception of the forebrain and the most caudal regions of the open neural plate. cDelta expression also begins around stage 3 in the posterior primitive streak. By stages 4 and 5 it is expressed in the primitive streak, the epiblast surrounding it and in the ingressing mesoderm. By stage 6, the mesodermal and epidermal expression of cDelta is restricted to the posterior of the embryo around the area where Hensen's node is regressing. At these stages, Delta seems to be absent form the neural plate. Between stages 8 and 9 expression of cDelta is upregulated on the neural folds and dorsal neural tube

along the entire AP axis of the embryo. Serrate1 and Serrate 2 expressions were not detected at early stages.

Delta-Fc acts as an inhibitor of the Notch pathway

The expression patterns of cNotch and cDelta at stages 9 and 10 intersect at the level of the open neural plate at the time of tube closure. Based on these expression patterns, it is possible that epidermal Delta-1 expressing cells at the border of the open neural plate can signal to the adjacent neural plate cells expressing cNotch. The timing of this interaction correlates with the segregation of the epidermal lineage from the neural/neural crest fates. To further investigate this possibility, we took advantage of cells expressing a soluble form of Delta-1 to manipulate Notch signaling at the time of neural tube closure.

Delta is a transmembrane ligand of the Notch receptor. It has been postulated that in order to properly activate the Notch pathway Delta needs to be presented to the receptor in its membrane bound form. The Delta-Fc construct is a soluble form of the Delta extracellular domain fused to the Fc portion of immunoglubulins. Anti Fc-antibody induces oligomerization (clustering), which is required for this soluble ligand to activate Notch signalling upon binding. Unclustered Delta-Fc or Delta-Fc clustered with an excess of antibody can act a dominant negative of Notch signaling. In these conditions, binding of the ligand to the receptor can occur, but the subsequent internalization and activation are prevented (Hicks et al, 2002). We designed an *in vitro* assay to test the effect of

the Delta-Fc soluble protein in cells lines transfected with a downstream reporter of Notch activity (fig. 3). First, we obtained conditioned medium from Delta-Fc and Fc expressing 293T cells, and confirmed the presence of proteins of the expected sizes in a Western Blot. Next, we analyzed the effects of different ratios of Delta-Fc/anti-Fc-antibody in the activation of the Notch pathway. N113 cells (C2C12 modified cells containing the Notch receptor and intracellular components of the Notch pathway) were transfected with a CBF-luciferase reporter and cultured for 24 hours in the presence of Fc-CM, Delta Fc-CM, Delta Fc-CM/ Fc 1:100 or Delta Fc-CM/ Fc 1:500. A lacZ reporter was cotransfected in all cases to standardize luciferase measurements. Fig. 4 shows relative luciferase measurements obtained for the four conditions. Only cells grown in the presence of Delta Fc-CM/ Fc 1:100 were able to activate the Notch pathway. Cells grown in the presence of Delta Fc-CM or Delta Fc-CM/ Fc 1:500 provided luciferase measurements similar to cells grown in the presence of Fc-CM. These results suggest that Delta-Fc CM might be acting as a dominant negative. The soluble form of non-clustered Delta can bind to but not activate the Notch receptor.

Inhibition of Notch signaling promotes early neuronal differentiation *in vivo*

To investigate the role of the Notch pathway in the fate of neural crest precursors, we injected DiI labeled 293T cells expressing a Delta-Fc or an Fc construct either in the lumen or at the sides of the closing neural tube of stages 9 and 10HH embryos. After injection, embryos were allowed to develop until they

reached stage 16-17HH. By this stage, the injected cells could be detected at the level of the flank just caudal to the forming forelimb. We first analyzed the injected embryos by whole mount *in situ* hybridization with the neural crest markers Slug and Cad7. Neither of these markers seemed to be affected in the embryos injected with the Delta-Fc expressing cells (not shown). Second, we analyzed the expression of HNK-1, Hu and Tuj-1 in sections. While expression of the neural crest marker HNK-1 seemed unaffected, we found expression of the neuronal differentiation markers Hu and Tuj-1 significantly increased in the embryos injected with the Delta-Fc expressing cells, compared to the controls (injected with Fc expressing cells) (not shown). Furthermore, we detected early migratory neural crest cells that were expressing Hu (fig. 5). These preliminary results suggest that Delta-Fc expressing cells are promoting early neuronal differentiation both in the neural tube and in early migratory neural crest.

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Inhibition of Notch signaling promotes early neuronal differentiation *in vitro*

Next, we tested the effect of the Delta-Fc expressing cells on explanted neural folds. Neural folds of stage 10 embryos were dissected and grown for 30 hours on a monolayer of 293T cells expressing either Delta-Fc or Fc. After fixation the tissues were stained for HNK-1 and Hu/TuJ1. Fig. 6 shows colocalization of HNK-1 and Hu staining on cells from explants grown on the Delta-Fc expressing cells, whereas cells from explants grown on control cells expressing Fc have very little Hu staining which does not coincide with HNK-1 positive cells. Interestingly, there seem to be a total of more Hu positive cells in

the explants grown on top of the Delta-Fc secreting cells. These results support the idea that Delta-Fc is promoting an early differentiation of neurons in the neural tube and from migratory neural crest.

Alternatively, it is possible that the effect we observed both *in vivo* and *in vitro* could be mediated by interactions with the 293T cells in a Notch independent way. To address this possibility we decided to test the effects of the Delta Fc-CM and Delta Fc-CM/anti-Fc 1:100 on stage 10 neural fold explants. We dissected neural folds form stage 10 chick embryos and cultured them for 38 hours in collagen gels in a defined medium to which we added either Delta Fc-CM, Delta Fc-CM/anti-Fc 1:100 or Fc-CM. We then stained the explants with HNK-1 and Hu/TuJ1. The neural folds grown in the presence of clustered Delta-Fc showed no significant difference in the expression of these markers when compared to controls grown in the presence of Fc-CM (not shown). However, in the explants grown in the presence of unclustered Delta-Fc we observed a significantly larger number of migratory cells leaving the explant which were positive for the neural crest marker HNK-1 and for the neuronal differentiation markers Hu and Tuj1 (Fig. 6).

Taken together, these data presented above suggest that inhibiting the Notch pathway at the level of the open neural plate by stage 10 not only promotes early differentiation of neural crest precursors, but it also stimulates migration and/or proliferation of these cells.

DISCUSSION

Neural crest precursors are localized in the neural folds and closing neural tube and are multipotent. Lineage analysis has shown that this population of cells can contribute to the epidermis, neurons of the CNS and neural crest. As the neural tube closes their fate becomes more restricted but not yet committed as they can still become migratory neural crest cells or part of the central nervous system. Our analysis of cNotch-1 and cDelta-1 expression patterns at the time of fate restriction suggests that these molecules could be involved in fate decisions taking place in the neural folds.

Our experimental approach to test the function of the Notch receptor at the time of ectodermal lineage segregation consisted of inhibition of the Notch pathway using a soluble form of a Notch ligand that can act as a dominant negative. While we did not detect a bias in fate of the neural crest precursors, we found an early differentiation of neuronal cell types both in the neural tube and in migratory neural crest. This result is consistent with an inhibition of the Notch pathway in light of the traditional role of Notch as a suppressor of neurogenesis.

Because the role of the Notch pathway in neural crest lineage decisions remains unclear, we are developing a different strategy to address this issue. We have designed expression vectors that carry a constitutively active form of Notch (the Notch intracellular domain, or Nic) or a dominant negative form of the Notch downstream effector suppressor of Hairless (dnSu(H)). The expression of both constructs is driven by a chicken actin promoter and a CMV enhancer and they are upstream of an IRES-GFP sequence. Electroporation of these constructs in the open neural plate of stages 6-8 embryos will allow us to ectopically

activate or inhibit the Notch pathway on the developing neural folds. We plan to analyze the effects of this overexpressions by looking at neural epidermal and neural crest markers (both by *in situ* hybridization and immunohistochemistry in whole mounts and in sections). These constructs will also allow us to confirm the data we obtained with cell injections and Delta-Fc conditioned media.

Another approach will consist of blocking the Notch pathway *in vivo* with several chemical inhibitors of Notch (MG132 from Calbiochem, MW167 from Enzyme systems). Again, the analysis would be carried out by looking at the expression of different ectodermal, neural and neural crest markers both by *in situ* hybridization and immnochistochemistry. .

Figure 1: *cNotch-1* expression pattern

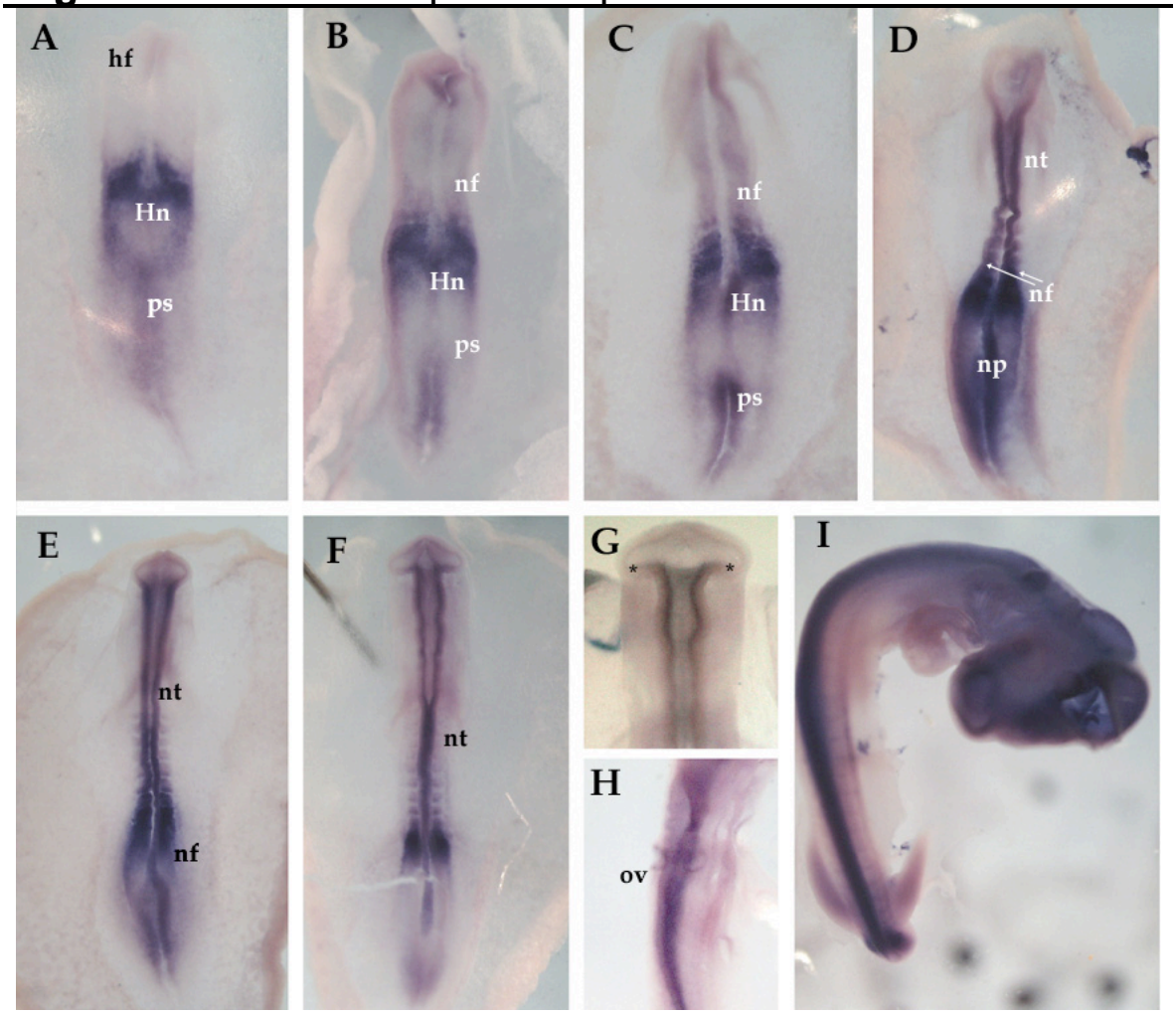

Fig 1, cNotch-1 expression pattern in early chick embryos. **A:** at stage 7HH cNotch-1 is expressed in the primitive streak and caudal segmental plate. **B, C:** stage 7^{+}/8 and 8HH, cNotch-1 expression continues in the primitive streak and it extends to the presomitiv mesoderm. Faint expression can be detected on the dorsal neural folds. **D, E:** stages 8+ and 9HH. As expression continues on the primitve streak it also becomes strong on the dorsal neural folds and neural tube. **F:** stage 10HH. Expression of cNotch-1 in the mesoderm is restricted to the last forming somites in a segmental pattern. It is strongly expressed on the dorsal neural folds and dorsal neural tube. **G:** close up of a stage 10HH head. The sharp boundary of expression at the forebrain level is marked by *. **H:** cNotch-1 stains the otic vesicle. **I:** stage 16HH cNotch-1 expression is maintained on the dorsal neural tube at late stages. **Hn;** Hensen's node. **ps**, primitive streak. **hf;** head folds. **ov**; otic vesicle.

Figure 2: *cDelta-1* expression pattern

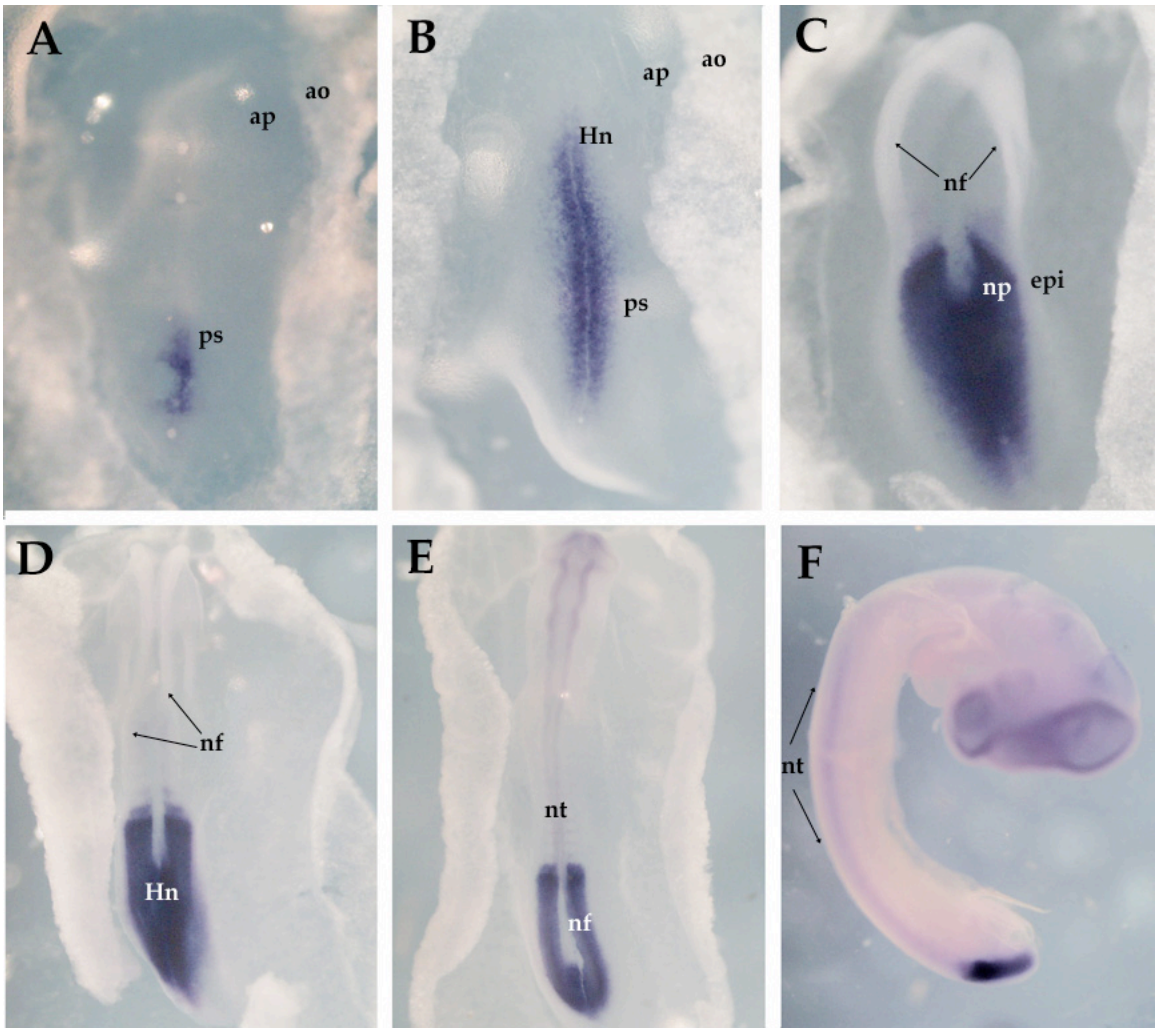

Fig 2, cDelta-1 expression pattern in early chick embryos. **A,B:** during gatrulation cDelta-1 is strongly expressed in the primitive streak, the epiblast surrounding it and the ingressing mesoderm. **A:** stage 3+**, B:** stage 4 **C:** at stage 7 expression continues on the segmental plate but it is absent from the node **D:** by stage 9 expression cDelta-1 expression begins on the caudal neural folds and is very strong in the segmental plate and newly form somites. **E:** at stagge 10 cDelta-1 is strongly expressed in the dorsal neural tube, neural folds and caudal presomitic mesoderm. **F:** at later stages expression is restricted to the caudal tip of the embryo and the dosal aspect of the neural tube **ao:** areao opaca; **ap:** area pellucida; **ps:** primitive streak; **Hn:** Hensen's node; **nf:** neural folds; **np:** neural plate; **epi**: epidermis; **nt:** neural tube

Figure 3: Schematic of the experimental design

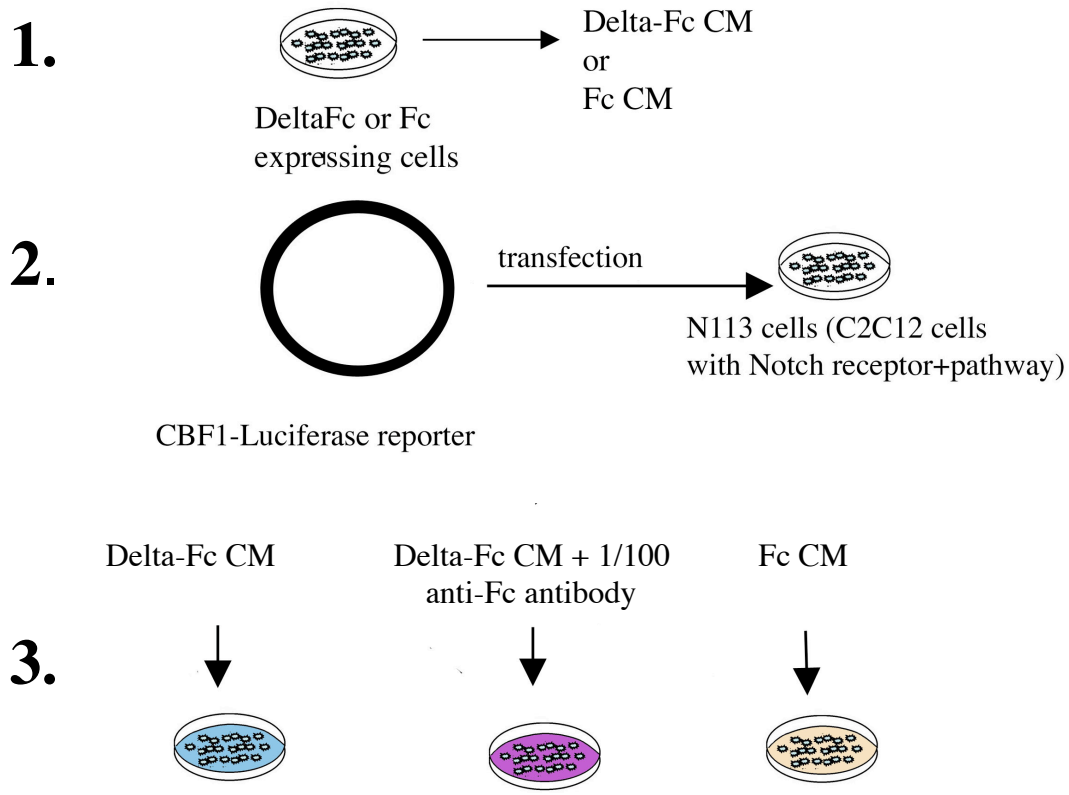

Fig 3, N113 cells were transfected with a CBF-1 luciferase reporter and a LacZ reporter. Following transfection, the cells were grown in the presence of Delta-Fc conditioned medium, Delta-FC conditioned medium crosslinked with anti-Fc antibody, or Fc conditioned medium for 24 hrs before measuring luciferase activity.

Figure 4: Delta-Fc acts as a dominant negative

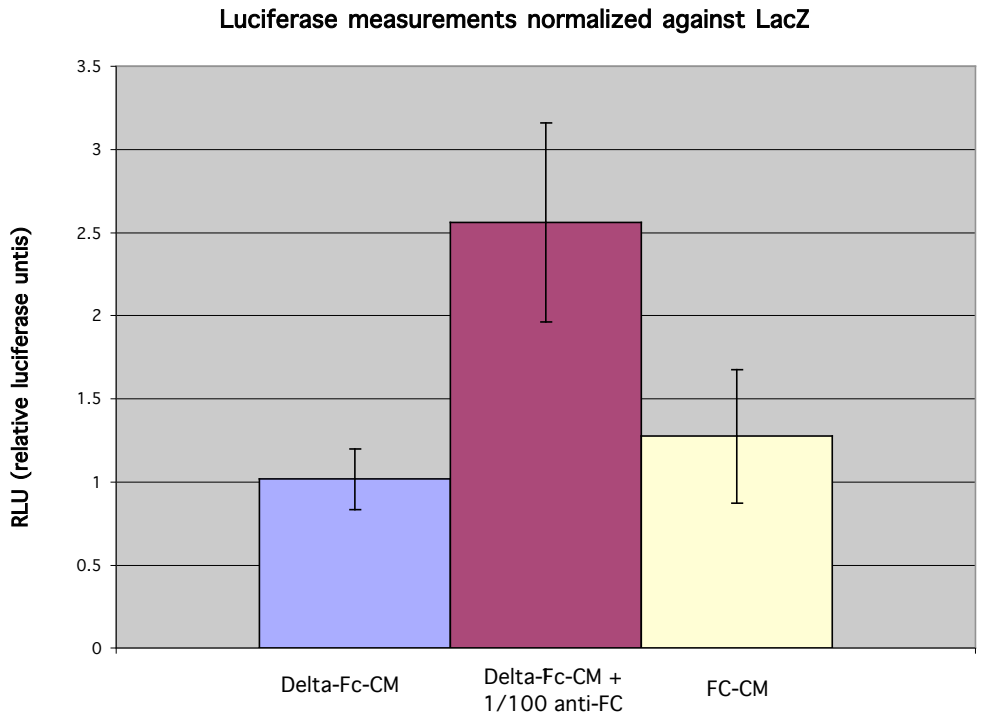

Fig 4, The graphic shows the results of two experiments done by triplicate. Luciferase activity was normalized against LacZ. In another experiment we included a population of cells treated with Delta-Fc CM crosslinked with 1/500 anti-Fc antibody. Luciferase activity in that case was not significantly different than the values for Fc or Delta Fc alone (not shown).

Figure 5: Inhibition of *Notch in vivo*

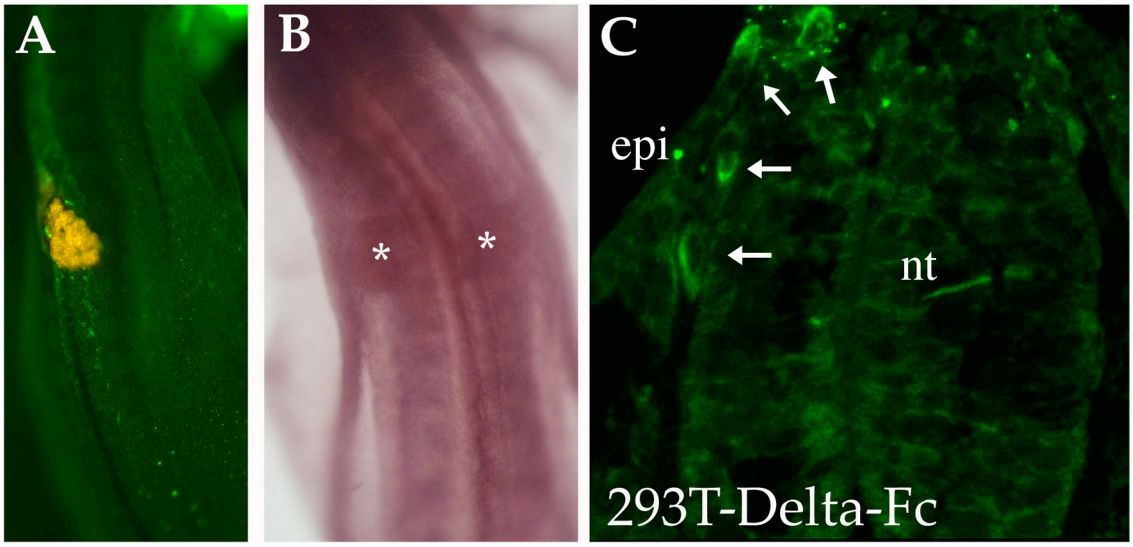

Fig 5, DiI labeled DeltaFc or Fc expressing 293T cells were injected in the open neural plate or at the sides of the neural folds of stage 10 HH embryos **(A,B). C:** Embryo injected with Delta-Fc expressing cells, 24 hours after injection. Early neural crest migrating cells are expressing the neuronal differentiation marker Hu (green) as indicated by arrows. Normally Hu is not observed until the end of neural crest migration after neural crest cells condense to form ganglia. **nt:** neural tube, **epi:** epidermis .

Figure 6: Inhibition of *Notch in vitro*

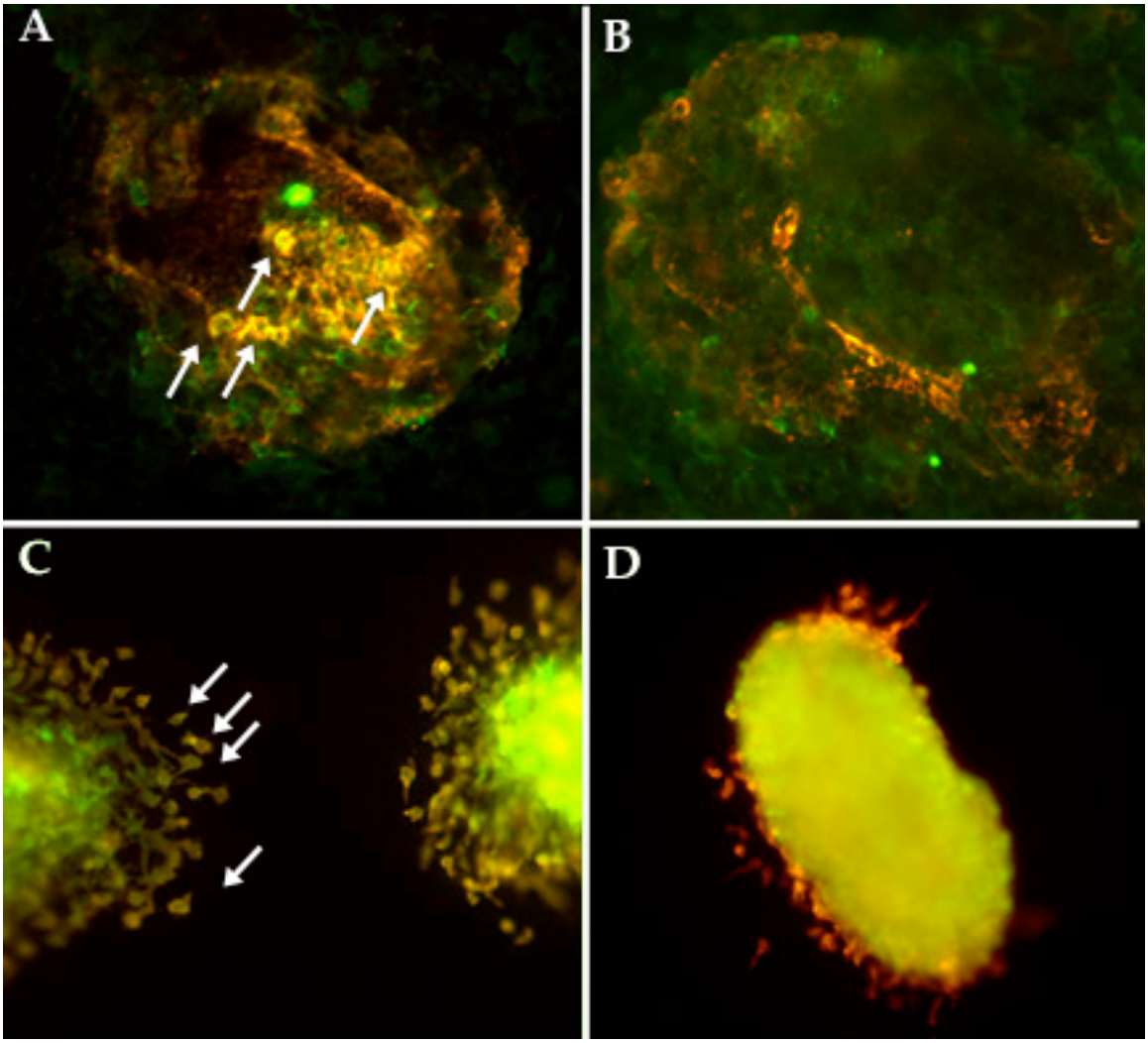

Fig 5, A, B: stage 10 HH dorsal neural folds were explanted and grown for 36 hours on top of a monolayer of Delta-Fc cells **(A)** or Fc expressing 293T cells. In the explants grown on Delta-Fc cells, **(A)** neural crest cells (expressing HNK-1, red), have differentiated into neurons as shown by coexpression of Hu (green) (arrows). **C, D:** stage 10 HH dorsal neural folds were explanted onto collagen gels and grown in the presence of Delta-Fc **(C)** or Fc conditioned media **(D).** Explants were stained for HNK-1 (red) and Hu/tuJ1 (green). Explants grown in the presence of Delta-Fc CM **(C)** have migratory HNK-1 positve, Hu positive cells (arrows). Neural crest cells from explants grown in control conditioned medium are HNK-1 positive (red) but do not express Hu. **(D)**

Chapter 5:

Discussion

INDUCTION OF THE NEURAL CREST

The formation of neural crest has traditionally been considered a classic example of induction where signals from one tissue elicit differentiation in a responding competent tissue. This assumption was largely based on the observation that neural crest can be generated *de novo* by the juxtaposition of epidermis with "naïve" regions of the neural plate or paraxial mesoderm both *in vivo* and *in vitro* (Moury and Jacobson, 1991; Liem et al., 1995; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996). Interestingly, these experiments have shown that both neural plate and epidermis can generate neural crest when combined, suggesting the existence of bidirectional inductive events. While a great deal of attention was placed on the epidermis and mesoderm as the potential source of inducers, very little is known about signals from the neural plate that can induce neural crest in competent ectoderm.

ABOUT THE SIGNALS INVOLVED, AND HOW WE STUDY THEM

Several lines of evidence suggest that Wnts, BMPs and FGFs play an important role in neural crest specification (Mayor et al., 1997; Garcia Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Liem et al., 1997; Monsoro-Burq et al., 2003). In chick embryos, this evidence usually comes from manipulation of these signaling pathways *in vivo*, resulting in downregulation of neural crest specific markers, or from *in vitro* experiments in which some of these signals are

sufficient to induce formation of neural crest cells when added to explants of intermediate neural plate (neural plate tissue of intermediate dorsoventral character) (Liem et al., 1997; Selleck et al., 1998; García-Castro et al., 2002). What do these experiments really tell us? The *in vivo* experiments take advantage of the accessibility of the chick embryo during the folding of the neural plate. Researchers analyze the effects of implanting beads coated with antagonists of signaling molecules in the young neural folds, and look several hours later at the expression of neural crest markers as readout of the experiment. Downregulation of neural crest markers is often interpreted as a requirement for the signal studied in neural crest induction. What this analysis fails to distinguish though, is whether these signals are required for the initial events of neural crest specification and/or whether they are only required to maintain the specified state of the otherwise induced neural crest. The *in vitro* experiments test for sufficiency of signals involved in neural crest induction. These types of experiments are similar to neuralized animal cap assays done in *Xenopus*. The assumption is that the ectodermal tissue tested is "naïve" in that it has not yet received signals that could bias its fate toward neural crest. The candidate inducer molecules are added to intermediate neural plate, which does not generate neural crest when cultured alone. The detection of migratory cells that are positive for neural crest markers is interpreted as sufficiency of the signal to induce neural crest. The caveat of this experiment is the nature of the tissue tested. By open neural plate stages, the intermediate neural plate has already received signals from the floor plate and the epidermis that specify aspects of its dorsoventral character (see Lee and Jessell, 1999 for a review). In addition, intermediate regions of the neural tube can generate neural crest *in vivo*

following ablation of the neural folds (Scherson et al., 1993). These data cast a shadow of doubt on the "naïve" state of the intermediate neural plate. The generation of neural crest after addition of BMPs or Wnts could be the result of interactions of these signals with others preexisting in the tissue. While the use of intermediate neural plate in these studies has proven to be useful in the identification of candidate molecules for neural crest induction, a similar *in vivo* role for these molecules cannot be inferred from these types of experiments alone.

NEURAL CREST INDUCTION IS A MULTI-STEP PROCESS

Neural crest induction in *Xenopus* has been explained by a model in which a gradient of BMP signaling initially acts to specify epidermal, neural and border fates in the ectoderm. The ectoderm at the border between epidermis and neural plate is then competent to respond to a second signal that enhances and maintains neural crest induction. Both Wnt and FGF signals have been proposed to play a role in this process (Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998). Induction of neural crest occurs during or shortly after neural induction and the formation of the neural plate. In chick embryos, there is also evidence pointing to the existence of several steps in the induction of neural crest. *In vivo* and *in vitro* experiments have shown that neural crest formation has temporally distinct periods of sensitivity to the BMP antagonist Noggin. Addition of Noggin prevents specification of neural crest when added to

neural folds of the closing neural tube, but not when added to neural folds at the level of the open neural plate of stage 10 HH chick embryos (Selleck et al., 1998) This result suggests that BMP signals are required for the maintenance of the specified neural crest. In addition, we have shown that caudal neural folds of stage 10HH begin to express Slug after 18 hours in culture. This result suggests that neural crest are specified long before the expression of specific markers. However, expression of neural crest markers is lost in the absence of further signals (Chapter 3). Taken together, these data suggest that neural crest induction requires at least an initial specification event and subsequently, the sustained action of further signals for its maintenance.

EARLY SPECIFICATION OF NEURAL CREST

Neural crest originate at the border of the neural plate, where signaling interactions between non-neural ectoderm, neural plate and underlying mesoderm could converge to specify the fate of the neural crest precursors. The fact that combinations of these tissues can recapitulate neural crest induction both *in vivo* and *in vitro* does not imply a requirement for their interactions (or the signaling molecules that mediate them) in the initial specification events of neural crest *in vivo*. Most studies of neural crest induction in chick have focused on the specification of neural crest in newly formed neural folds, at the caudal levels of stage 10 HH embryos (Selleck et al., 1998; García-Castro et al., 2002; Cheung and Briscoe, 2003). Because chick embryos develop in a rostrocaudal sequence, cranial neural crest cells are already migrating in these embryos. The

mechanisms that initiate neural crest specification in general and cranial crest in particular have remained largely unexplored. We have shown that in chick embryos neural crest is already specified by the beginning of gastrulation and before the formation of the neural plate (Chapter 2). A recent study suggests that by stage VIII EG&K, the epiblast has already received signals that specify neural and epidermal fates (Wilson and Edlund, 2001). The model proposed for this early neural induction involves the repression of BMP expression by FGFs in the epiblast. Wnt signals present in the prospective non-neural ectoderm release the inhibition of BMPs by FGFs .The absence of Wnt in the prospective neural cells leads to the inhibition of BMPs and therefore to neural induction. According to this model, interactions between neural and non-neural specified ectoderm could still account for the induction of neural crest in chick embryos. The formal establishment of a neural tissue, characterized by the formation of a columnar neuroepithelium however, is a much later event which takes place after head mesoderm starts to ingress through Hensen's node (stage 5 HH). It seems unlikely that neural crest could be specified by interactions of differentially specified ectoderm in the absence of a differentiated neural plate. Alternatively, the same signals (or some of their interactions) that specify neural and epidermal fates could be responsible for the specification of neural crest fates, in the same way that *Xenopus* ectodermal fates were proposed to be specified through a BMP gradient (Mancilla and Mayor, 1996). In our study, we showed that specification of neural crest fates has already happened at stage 3 HH. If indeed neural crest specification can happen through the same signals that prepattern the ectoderm at stage VIII EG&K, further experiments need to be done, analyzing neural crest markers in cultured explants from these young embryos. Evidence of such an

early specification would suggests that induction of neural crest in amniotes and amphibian embryos could follow a general conserved mechanism as it was suggested for neural induction (Wilson and Edlund, 2001).

NEURAL CREST AND NEURAL PLATE BORDER: ONE BUT NOT THE SAME

Neural crest cells were first described in the avian embryo by His (His 1868) as band of particular material lying between the presumptive epidermis and the neural tube. In chick embryos, the earliest known markers for neural crest are not expressed until stage 8 HH, when they are visible at the tips of the inward-rolling neural folds at head levels (Nieto et al., 1994). The neural folds represent the edges of the neural plate and are in close contact with the adjacent non-neural ectoderm. The expression patterns of these markers together with the fact that neural crest can be induced by neural/non-neural interactions led to the common belief that neural crest originates at the border between these two tissues (see Knecht and Bronner-Fraser, 2002 for a review). However, there is no evidence of when or where neural crest cells form.

In this study, we propose the paired box transcription factor Pax-7 as the earliest marker for neural crest in chick embryos. Pax-7 expression initiates at stage 4^{+}HH in a discreet domain that seems lateral and caudal to the proposed border of the neural plate according to recent fate map experiments (figure 1, Chapter 2. compare to Fernández-Garré et al., 2002). From stage 6 onwards, when the head folds become recognizable, the expression of Pax-7 correlates

with the neural plate epidermis border. We performed focal DiI injections in the prospective domain of Pax-7 expression at stage 4, and observed that labeled cells were later incorporated into the dorsal neural folds/neural crest. Moreover, blocking Pax-7 function prevents specification of neural crest both *in vivo* and *in vitro* but does not affect the expression of neural, epidermal or border markers (see Chapter 2). In addition, a recent study has shown that formation of an ectopic neural/non-neural border is not sufficient to specify neural crest fates (McLarren et al., 2003 but also Woda et al., 2003). Finally, in chick embryos, neural crest does not originate from the anterior border of the neural plate, instead this region gives rise to ectodermal placodes (Couly and Le Douarin, 1990). Taken together, these data suggest that the specification of neural crest fates might occur through signaling events distinct from the ones required for the formation of a border between neural and non-neural ectoderm.

PAX-7 AND NEURAL CREST SPECIFICATION

Our analysis of the early expression pattern of Pax-7 suggested that it could play a role in the early specification of neural crest. Pax-7 is first expressed in chick embryos at stage 4+ HH, as two symmetric oblique bands lateral to Hensen's node. From stage 6 onwards, expression Pax-7 correlates with the neural plate border. We showed that expression of Pax-7 is required for the formation of neural crest, both *in vivo* and *in vitro*. Using a morpholino-based loss of function approach, we prevented the specification of neural crest in

prospective neural crest explants. Similarly, our *in vivo* experiments showed a downregulation of neural crest markers after electroporation of morpholinos against Pax-7 in stage 4 HH embryos. Taken together, these results suggest that Pax-7 could be a direct target of the signals that initially specify the neural crest domain. The lack of Pax-7 expression in the anterior border of the neural plate is consistent with the fact that neural crest does not originate in that region. However, this expression pattern suggests that neural crest specification requires other signals in addition to those that specify neural territories (the presence of repressor in the anterior border?), or that the these events occur through different spatial or temporal actions of the same signals. Several recent reports favor the idea of different temporal requirements for these signals in the specification of neural crest. At stage XII (EG&K), Wnt8 is expressed in a ring around the epiblast consistent with a role in the specification of non-neural ectoderm (other Wnts are weakly expressed or absent). By stage 4 HH however, Wnt8c and Wnt11 are expressed in paraxial mesoderm posterior to Hensen's node and excluded from the anterior neural plate (Skromme and Stern, 2001; Nordstrom et al., 2002). In addition, Wnt signals from the prospective paraxial mesoderm at stage 5 are responsible for the expression of Pax-3. Pax-3 and Pax-7 form a group of equivalence among Pax genes; their sequence and expression pattern is very similar although Pax-7 expression is more anterior, dorsal and earlier than Pax-3 in the chick embryo. In mouse embryos these patterns of expression are reciprocal (Chapter 2; Bang et al., 1997). Furthermore, it has been shown that a FGF signal is present in the posterior neural plate of stage 4 HH embryos, and is required to confer posterior character (Nordstrom et al., 2002). Collectively, these data suggest that specification of neural crest and the onset of Pax-7 expression

could be mediated by the same signals that specify neural cells, albeit acting later in development. According to our results and the reported expression patterns of Wnt signals, specification of neural crest could occur sometime after stage XII EG&K and before stage 3 HH. If this were the case, it would be contrary to Edlund's idea of similar events leading to the formation of neural crest in frogs and amniotes (Wilson and Edlund, 2002).

To further understand the role of FGFs, Wnts and BMPs in the induction of Pax-7 expression and in the specification of the neural more research needs to be done. Specification assays like the ones described in Chapter 2 should be repeated in the presence of inhibitors of the three pathways, and combinations of them. Addition of noggin, the Wnt inhibitor CK-1 and the fgfr inhibitor SU5402 to early explants that are specified to become neural crest would test for the necessity of these signals in the specification process. Likewise, it would be interesting to address if one or more combinations of these signals are capable of inducing Pax-7 expression and/or neural crest from ectoderm that will not express Pax-7 and is not specified to become neural crest by stage 3HH.

At stage 10 HH, Pax-7 is strongly expressed in the dorsal neural folds and tube. However, it is absent (or expressed at very low levels) in the intermediate neural plate or floor plate. However, addition of Wnts or BMPs to intermediate neural plate seems to be sufficient to induce neural crest. How are this signals acting? One possibility is that Wnts or BMPs can induce expression of Pax-7 in the explants preceding the formation of neural crest. Alternatively, these molecules could be inducing the formation of a tissue with epidermal or mesodermal characteristics in the intermediate neural tube, and neural crest formation could be secondary to an interaction between these tissues. One way

to discriminate between these possibilities would be to analyze whether mesodermal or epidermal markers are upregulated following the addition of Wnts and/or BMPs to the explants. In addition test whether Pax-7 expression is upregulated in intermediate neural plate/epidermal recombinants and also prevent expression of Pax-7 in either or both tissues and analyze if their interaction can still generate neural crest.

An alternative scenario for the regulation of Pax-7 expression is that the onset of its expression is regulated by another type of diffusing molecules originated at the primitive streak, or a combination of this molecule(s) with the ones described above. A diffusible signal from the streak could account for the discreet domain of expression of Pax-7 and it would be consistent with the timing of the onset of expression. If this hypothesis was valid then Pax-7 expression and neural crest specification could be independent from the formation of a neural/non-neural border. However, we cannot eliminate the possibility that Pax-7 expression could be a consequence of tissue interactions. Even in the specification assays described in Chapter 2, we cannot rule out that the tissue we isolate and is specified to become crest, actually might contain a salt and pepper mix of neural and ectodermal precursors. If we could analyze the specification state of individual cells within the explant we could test whether or not interactions between different cell types are indeed required for neural crest specfication and/or Pax-7 expression.

CONCLUSIONS

Neural crest induction is a multi-step process that requires the concerted actions of multiple signaling pathways. The initial events that lead to the specification of the neural crest precursors take place during or before the onset of gastrulation. We found a discreet region in the epiblast of stage 3 and 4 HH, chick embryos that expresses neural crest markers when isolated in culture for 40 hours. This region of the ectoderm is slightly posterior to the tip of the primitive streak and intermediate between the midline of the embryo and the limit between the area opaca and area pellucida. Focal DiI injections in this region revealed that it gives rise to anterior neural crest. Surprisingly, this area of the embryo does not coincide with the proposed border of the neural plate at stage 4HH.

Based on our analysis of its early expression pattern, we propose that the paired box transcription factor Pax-7 is the earliest marker known for neural crest in chick embryos. Furthermore, we demonstrate an early requirement of Pax-7 for the specification of neural crest fates both *in vivo* and *in vitro*. Interestingly, Pax-7 does not seem to be necessary for the formation of the border between neural and non-neural ectoderm.

Neural crest cells can be induced by an interaction between neural plate and ectoderm. To clarify the timing and nature of these inductive interactions, we have examined the time of competence of the neural plate to become neural crest as well as the time of neural fold specification. The neural plate is

competent to respond to inductive interactions with the non-neural ectoderm for a limited period, rapidly losing its responsive ability after stage 10 HH. In contrast, non-neural ectoderm from numerous stages retains the ability to induce neural crest cells from competent neural plate.

The segregation of the epidermal and neural crest/neural lineages in the dorsal neural folds occurs around the time of neural tube closure. Neural and neural crest fates are separated by the onset of neural crest migration. We began to address the role of Notch in fate decisions of the neural crest precursors. Our preliminary data suggests that inhibition of Notch signaling promotes early neuronal differentiation both in the neural tube and in early migratory neural crest.

Appendix 1:

Pax7 and Ear Development

INTRODUCTION

The vertebrate ear develops from a thickening of the ectoderm at the neural plate border, the otic placode. Around stage 10+ this epithelium invaginates and becomes visible as an otic vesicle adjacent to the hindbrain at the level of rhombomeres 5 and 6 (Baker and Bronner-Fraser, 2001; Streit, 2001 for a review). The induction of the otic placode is a complex multi-step process. The tissues and some of the molecular players involved in this induction have been identified but it remains unclear what are the initial interactions that originate the otic placode. The ectoderm is competent to respond to signals from the hindbrain (such as Wnt8c and FGF-3, (Ladher et al., 2000; Vendrell et al., 2000), and from the underlying mesoderm (such as FGF-19 Ladher et al., 2000) to induce the invagination of the otic placode and/or expression of otic markers. However, none of these signals alone seem to be sufficient or essential for formation and proper patterning of the otic vesicle. It is likely that the initial steps of otic induction involve the interaction of signals from the hindbrain and the mesoderm on the responding adjacent ectoderm. Studies have shown that as early as the 5 somite stage this region is specified to express early otic markers such as the pair box homeodomain gene Pax2 and by stage 10 the ectoderm is committed to form and epithelial vesicle (Groves and Bronner-Fraser, 2000).

Here, we report that the member of the paired box family of transcription factors Pax7 is necessary for proper formation of the otic vesicle. This finding adds one more component to the list of molecules involved in the process of otic development.

RESULTS

During our efforts to characterize the role of the pair box transcription factor Pax7 in neural crest induction, we analyzed the effects of blocking its translation on the early chick embryo. We electroporated morpholino olignonucleotides specifically designed against Pax7 in the neural crest forming region of stage 4 embryos. We allowed the embryos to develop for 24 hours after electroporation and then look for specific neural crest markers. We detected the fluorescently labeled morpholinos along the neural fold of the electroporated side, frequently along the entire A/P axis of the embryo. Surprisingly, the effect on neural crest was not the only phenotype we observed. In the majority of the embryos (57%, n=35), we noticed the absence of the otic vesicle on the electroporated side (fig. 1). Embryos electroporated with control morpholinos or with morpholinos designed against Pax3 had normal otic vesicles on both sides. Interestingly, when we electroporated morpholinos against Pax7 together with morpholinos against Pax3, we noticed a decrease in the incidence of the phenotype (36%, n=44). However, this may be simply due to a dilution of the effective concentration of the morpholinos against Pax7. In all the embryos that displayed the phenotype, we were able to detect the morpholino oligonucleotides on the dorsal neural tube at the level of the hindbrain, and in some cases on the ectoderm where the oitc vesicle should have formed. Taken together, these data suggest that the absence of a morphologically distinct otic vesicle is a specific consequence of the inhibition of Pax7 translation.

To further understand the role of Pax7 in ear development, we looked at two markers of the otic placode in Mo7 electroporated embryos. Analysis of Pax2 and Sox10 expression revealed that these markers are still present on the ectoderm over where the otic cup should form but there is no morphological distinction of the otic cup epithelium. On the contralateral side, these markers present their normal expression pattern lining the epithelium of the otic vesicle (fig. 2)

DISCUSSION

The formation of the otic placode is the result of an inductive process that involves integration of signals from the hindbrain and mesoderm in the responding ectoderm. The placode is first morphologically visible as an otic vesicle around st11. In this study, we have identified the paired box transcription factor Pax7 as a necessary component for oitc vesicle formation. Inhibition of Pax7 expression results in the absence of a morphologically distinct otic cup. However, some of the early otic placode markers are still expressed on the ectoderm of embryos that lack otic vesicles. Based on our data, it is hard to establish whether Pax7 is actually required for the induction of the otic placode or for the morphogenetic movements that generate the invagination of the otic epithelium.

One possibility is that the phenotype we observed is due to mechanical constrains on the invagination process. It would be interesting to test this hypothesis by looking at adhesion molecules in embryos that lack Pax7. Another explanation would be that Pax7 is required for the generation of inductive

signals from the hindbrain to the ectoderm. Because this may be a direct or indirect effect and we still need to identify direct downstream targets of pax7, we can compare the expression of local hindbrain markers at the level of the otic placode in control versus Mo7 electroporated embryos (members of the Eph/Ephrin ligands, Hox genes, etc). Another experimental approach to investigate the potential roles of Pax7 in otic development is overexpression and missexpression of Pax7 to test for sufficiency. The non-neural ectoderm form several A/P levels of the embryo is competent to respond to inductive signals from the hindbrain to induce the otic placode. WhilePax7 expression is absent in the most anterior region of the dorsal neural tube, the ectoderm adjacent to this region is competent to form an otic vesicle (Groves and Bronner-Fraser 2000). Overexpression of Pax7 in this domain could induce ectopic ears. It is not likely however that Pax7 alone would be sufficient to induce an ectopic ear, given that Pax7 is expressed throughout the entire A/P axis of the embryo caudal to the midbrain. Instead, downstream targets of Pax7 could interact with more local signals (perhaps from the mesoderm underlying the hindbrain) to elicit otic induction.

Figure 1: MoPax-7 prevent the formation of the otic vesicle

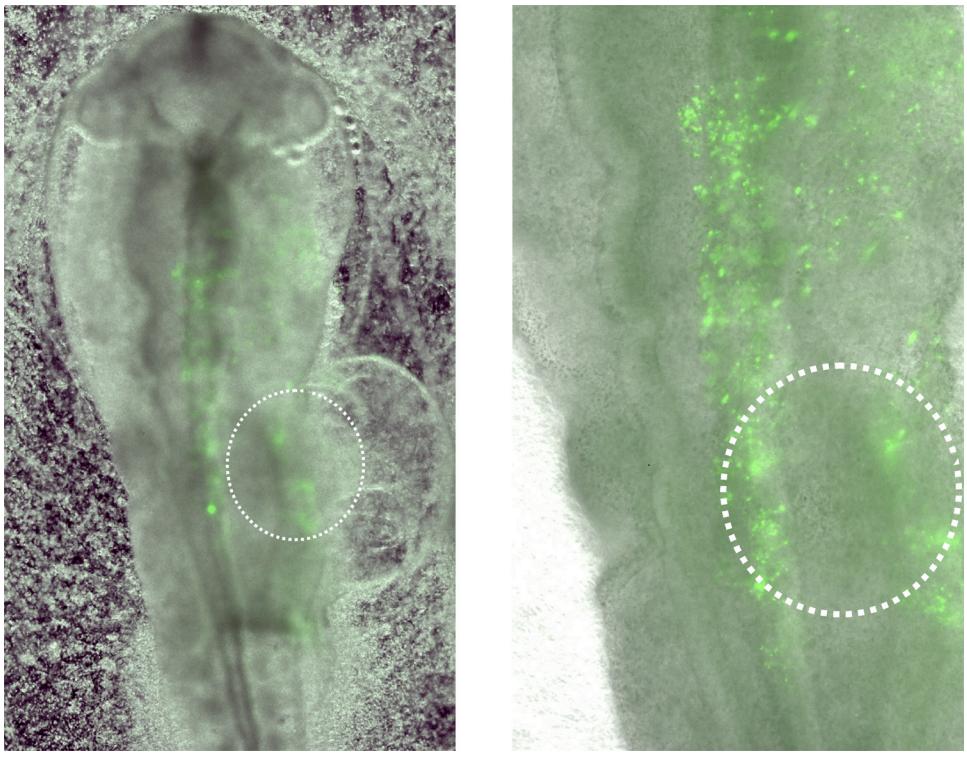

Fig 1, Embryos were electroporated unilaterally with morpholinos against Pax-7 (green) at stage 4. A normal otic vesicle formed on the control (unelectroporated) side. No visible otic vesicle formed on the electroporated side (circle). Embryos electroporated with morpholinos control or morpholinos against Pax-3 develop normal otic vesicles in both sides (not shown)

Figure 2: Otic placode markers are present in Mo-Pax-7 embryos

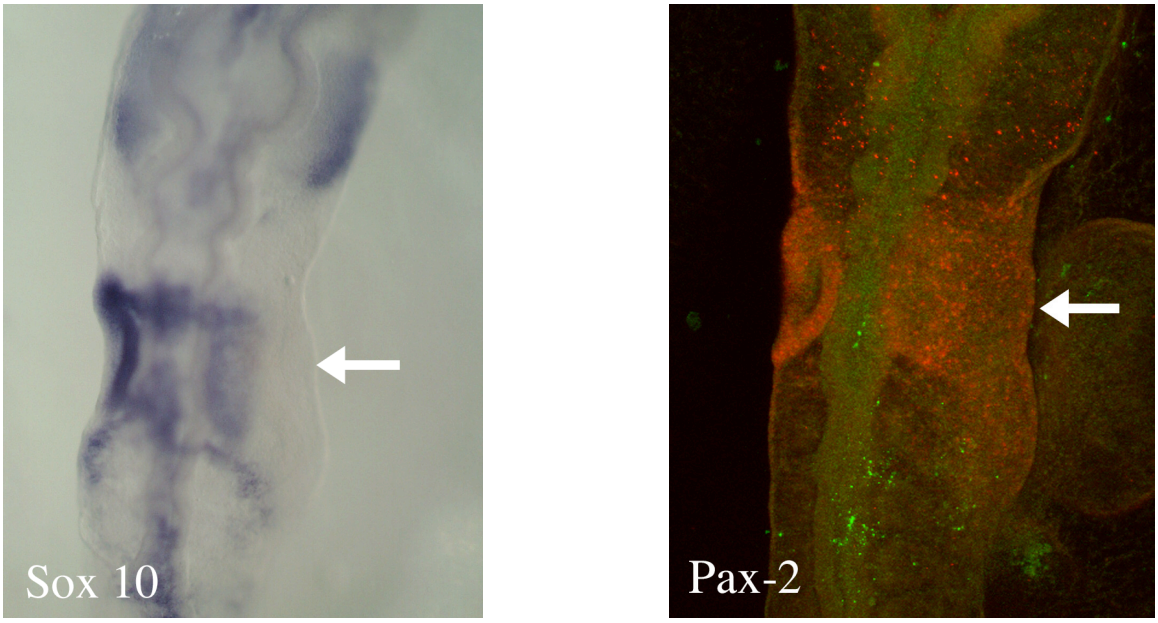

Fig 2, Left: Sox 10 *in situ* hybridization on embryo electroporated unilaterally at stage 4 HH with morpholinos against pax-7. Arrow points to electroporated side, where otic vesicle is absent and Sox 10 expression is diffuse over the otic epithelium. On the contralateral side, Sox 10 expression is lining the epithelium of the otic vesicle. **Right:** Pax-2 immunostaining (red) of an embryo electroporated unilaterally at stage 4 HH with morpholinos against Pax-7 (green). Arrow points to Pax-2 expression on the electroporated side, in the absence of an otic vesicle.

Appendix 2:

Supplementary Materials and Methods

RNA AND PROTEIN METHODS

Whole Mount Single In Situ Hybridization (from Henrique et al., 1995)

Day 1

1. Fix embryos in 4% paraformaldehyde 2hrs @ rt or O/N @ 4ºC
2. Wash twice in Ptw
3. Wash with 50% MeOH/Ptw, then 100% MeOH twice, can store at this point at –20ºC (I don't find the dehydration step necessary, although it can help to reduce background)
4. Rehydrate embryos through 75%, %0%, 25% MeOH/Ptw (allowing embryos to settle), and washing twice with Ptw.
5. At this point I transfer the embryos to 4ml dram vials, and I carry on all the following steps in these tubes.
6. Treat embryos with 10μg/ml proteinase K in Ptw; for chick embryos, time of incubation in min=stage number. (For embryos stage 10 and younger I don't find this step necessary, specially if the probe is good and the expression is superficial. For embryos younger than stage 7 I find it can disturb more than it helps.)
7. Remove proteinase K, rinse briefly (care!) with Ptw, and post fix for 20 min in 4% HCHO +0.1% glutaraldehyde, in Ptw
8. Rinse and wash once with Ptw
9. Rinse once with 1:1 Ptw/hyb mix. Let embryos settle
10. Rinse with 1 ml hyb mix. Let embryos settle.
11. Replace with 1ml hyb mix and incubate >1hour at 65ºC (for most probes 70ºC works better)
12. Add 1ml pre-warmed hyb mix @~1μg/ml DIG-labelled RNA probe. Place back at 65ºC or 70ºC. Incubate O/N

Day 2

1. Rinse twice with prewarmed hyb mix
2. Wash 2 x 30' @ 70ºC in hyb mix (actually, the longer you wash and the more changes of solution you make, the better).
3. Wash 10' @ 70ºC in 1:1 Hyb mix/Mabt

4. Rinse 2x with Mabt
5. Wash 1 x 15′ with 1.5ml Mabt
6. Incubate 1hour with Mabt + 2% Boehringer blocking reagent (BBR)
7. Incubate >1hour in Mabt +2%BBR +20 heat inactivated serum
8. Incubate O/N @ 4ºC in Mabt +2%BBR +20%HI serum + 1/2000 of AP-anti-DiG antibody.

Day 3

1. Rinse 3 x with Mabt
2. Wash 3 x >1hour with Mabt (the more washes and the larger the volume, the better. I try to do at least 6 washes > 1/2 hour each. If not in a hurry, a last wash O/N is good)
3. Wash 2x10′ with Mabt
4. Incubate in the dark with Mabt + 4.5μl NBT (75mg/ml in dimethyl formamide) + 3.5μl/ml BCIP (50mg/ml in 70%DMF) until color develops.
5. Rinse in Mabt or Ptw and refix in 4%HCHO/0.1 glutaraldehyde (or just 4%PF).
6. Rinse 2x in Ptw and store in Ptw with 0.02% sodium azide.

Solutions

HybMix:	For 50ml
Formamide: 50%	25ml
SSC (20x, ph5 w/citric acid): 1.3X	3.25ml
EDTA (0.5M, pH 8): 5mM	0.5ml
tRNA: 50μg/ml	125μl
Tween-20 (10%): 0.2%	1ml
CHAPS (10%): 0.5%	2.5ml
Heparin (50mg/ml): 100μg/ml	100μl
H_2O	17.5ml

5X MAB	For 200ml
Maleic Acid	11.6g
NaCl	8.7g

H_2O	~185ml

Add Tween to the 1X MAB, to a final concentration of 0.1%

NTMT	For 50ml
5M NaCl	1ml
2M TrisHCl pH9.5	2.5ml
2M $MgCl_2$	1.25ml
10% Tween-20	0.5ml
H_2O	40.25ml

Whole Mount Double In Situ Hybridization (my modification of the previous protocol).

This protocol works well with two probes, one DIG-labelled mRNA and one Fluorescein labeled mRNA. The strongest or more robust probe should be fluorescein labeled.

Day 1

- Same as above, but add both probes simultaneously. Add ~1μg/ml of each probe.

Day 2

- Same as above BUT, add AP-anti-Fluorescein antibody 1/2000

Day 3

- Same as above BUT, always develop the first color (weakest probe, fluorecein labeled , first) with NBT+BCIP or BMP purple (actually, BM purple work very nice on young embryos).
- After color developed, fix in 4% PF or HCHO+glutsraldehyde, 1 or 2 hours at room temperature.
- Rinse and wash in Ptw or MABT
- Dehydrate the embryos in a series of MeOH/Ptw (or MABT). This step is VERY important in order to kill the alkaline phosphatase.
- Rehydrate the embryos in MABT

- Usually there is no need to block the embryos further, but I incubate them for an hour in MABT +2%BBR +20%HI Serum.
- Add the anti-DIG-AP antibody 1/2000 in MABT+2%BBR+20% HI serum, incubate O/N at 4ºC

Day 4

- Same as Day 3 in previous protocol BUT, develop second color using only BCIP. It takes a long time to develop. Sometimes developing @ 37^0C helps but BCIP might form blue precipitates. Its best to increase 1.5 folds BCIP concentration and develop at room temperature.

Table 1: In situ probes used

Probe	digested	RNApol	Probe	digested	RNApol
Slug	EcorI	T7	Gata-2	NcoI	T7
BMP2	HindIII	T3	Dlx-5	BamHI	T7
BMP4	XbaI	T3	Ganf	NotI	T7
BMP5	EcoRV	T3	Wnt6	EcoRI	T3
BMP7	XhoI	T3	Wnt8c	BamHI	T7
cNotch-1	SalI	T7	Pax-7 (fl)	BamHI	T7
cDelta-1	NotI	T3	Pax-3 (fl)	SalI	T3
cSerrate-1	HindIII	T7	Pax-7 300bp	SalI	T7
cSerrate2	EcoRI	T3	Sox2	SalI	T7
Sox10	EcoRI	T3	Msx-1	SalI	SP6

Whole mount immunohistochemistry (Fluorescence)

- Fix embryos as required for primary antibody
- Rinse 2X in Ptw
- Block >1h in Ptw + 5% heat treated serum
- Add primary antibody O/N @ 4^0C or 4 hr @ rt
- Wash extensively in Ptw
- Add secondary antibody O/N @ 4^0C or 4 hr @ rt
- Wash extensively in Ptw

Whole mount immunohistochemistry (HRP)

- Fix embryos as required for primary antibody
- Rinse 2X 30′ in Ptw 30

- Replace PBS with 0.25% H2O2 in PBS 2-3 hours
- Rinse 2X 30′ in PBS
- Rinse 2X 30′ in PBT (PBS + 0.2%BSA, 1% Triton X-100 and 0.01% thimerosal; thimerosal only if solution is to be stored)
- Rinse 30′ in PBT + 5% heat treated serum
- Add primary antibody O/N @ 4^0C
- Rinse 2X 30′ in PBT
- Rinse in PBT/HT serum
- Incubate with secondary antibody O/N @ 4^0C
- Rinse 2X 30′ in PBS
- Rinse in 0.1M Tris, pH 7.4 + 0.1M NaCl
- Incubate 10′ in Tris containing 1mg/ml Diaminobencidine (DAB, 10mg/40ml)
- Add H2O2 to a final concentration of about 0.001%. After label has appeared, rinse rapidly in tap water to stop the reaction. The reaction takes a little to start but once it does, it proceeds rapidly.
- Rinse in Tris

Rinse 2X 30′ in PBS. Store

Immunohistochemistry on slides

- PBS /0.1%BSA + 10% HT serum to block (1/2 w/coverslips)
- Wash with PBS/BSA + 0.1% triton
- 1^{ary} ab O/N (cofver slips, humidified chamber, 4ºC)
- 3X5' PBS
- 2^{ary} 1-2 hr @ rt (under cover slips, `1:200)
- 3X5' PBS + 1X5' H_2O
- 1 drop of GelMount, cover slip

(after gel mount let slides still 1 hr, then store horizontally @ 4ºC. Wait 1 week before storing them vertically)

DAPI staining**:** working solution in water 1-5 μg/ml. Alternatively, 1:2000 of a 4mg/ml stock solution can be added to fluormount-G

Table 2: Primary antibodies used:

Antibody	Species/type	From	dilution	comments
QCPN	Mouse IgG1	Hybridoma Bank	1:1 /1:10	Perinuclear staining on quail cells.
HNK-1	Mouse IgM	Hybridoma Bank	1:100/1:300	Migratory neural crest
Hu	Mouse IgG2b	Molecular Probes	1:250/1:500	Differentiated neurons
Tuj1	Mouse IgG2a	BABCO	1:500/1:1000	Neuronal filaments
Pax-2	Rabbit polyclonal	Zymed	1:2000	Early marker for the otic placode
Pax-3	Mouse IgG2a	Hybridoma Bank	1:100	Very nice staining. Detectable from stage 5 in neural plate and folds
Pax-7	Mouse IgG1	Hybridoma Bank	1:100	Very nice antibody.
Slug 62.1E6	Mouse IgG1	Hybridoma Bank	1:10/1:300	Very inconsistent. Only got it to work after short fixations in the colds, in neural fold explants. Never in whole mount or sections.
GFP	Mouse	Molecular Probes	1:250	
Msx2 4G1	Mouse IgG1	Hybridoma Bank	Tried a range of dilutions	Never detected signal
AP2 3B5	IgG2b	Hybridoma Bank	Tried a range of dilutions	Never detected signal

Table 3: Secondary antibodies used:

Company	Dilution	type	Conjugate	
Jackson Immunoresearch	1:200/1:400	Donkey anti-rabbit Donkey anti-mouse IgG Donkey anti-mouse IgM	Rodamine red (RRX), 570nm Cy2, 490nm (green) Cy5, 647nm (far red)	
Molecular probes	1:1000 (slides) 1:2000 (embryos)	anti IgM anti-IgG1 anti-IgG2a anti-IgG2b	Alexa 488 Alexa 568, 594 Alexa 633	I used a neutravidin tertiary(350nm, blue) from Molecular probes against the biotinilated secondary.
Zymed	1:100/1:300	anti IgM anti-IgG1 anti-IgG2a anti-IgG2b	FITC, 480nm TRITC, 546nm Biotin HRP	

MORPHOLINOS, IN VITRO TRANSLATION, ELECTROPORATION AND DiI INJECTIONS

Lysamine labeled morpholino oligonucleotides were obtained from Gene Tools, LLC. A
MoPax7 cPax7 5'UTR (5'-TCCGTGCGGAGCGGGTCACCCCC-3')
MoPax3 cPax3 5'UTR (5'-CCAGCGTGGTCATCGCGGCGGCGC-3')
MoPax75M cPax7 5'UTR (5'-TCgGTcCGGAGccGGTgACaCCC-3')
Morpholinos were stored at -80^0C in 5μl aliquots of 10mg/ml ~ 1.2mM (before electroporation MO were diluted 1:1 in 10% sucrose => working concentration ~0.6mM)

Morpholino Translation Inhibition In Vitro

Capped Pax-7 full length mRNA, lin w/ XbaI, SP6 (Ambion message machine)
Capped Pax-3 full length mRNA, lin w/ XbaI, T7

1μl RNA
1μl MO (several different diluitions, from stock solution, to 0)
0.5μl Rnasin
0,5μl aa mix –Met
3μl lysate (Nuclease treated Rabbit Reticulocyte Lysate (Promega)

15' @ RT

Then add:
14μl lysate
1μl aa mix –Met
1μl ^{35}S-Methionine

90' 30ºC

Proteins were stacked in a 4% polyacrylamide stacking gel and separated in a 10% polyacrylamide gel. Gels were rinsed once in water, them fixed for 20' in 40% Methanol/20% glacial acetic acid. After fixing, gels were rinsed in water and then

enhanced in 1M sodium salicylate for 10 minutes. Gels were rinsed in water before drying at 80^0C under a vacuum for 30 minutes. Dried gels were exposed at -80^0C.

Morpholino electroporation

St 4 embryos were explanted onto filter paper rings and placed ventral side up on drops of 2% agarose in water. Morpholinos were injected onto the prospective neural crest forming region of the epiblast, by placing the needle between the hypoblast and the epiblast. Parallel platinum wires were placed at the sides of the embryo, touching the paper but not the embryo itself. Electroporation was done by 2 square pulses of 5-15 mV at 25 msec Embryos were allowed to recover on top of the agarose drops for 30 sec to 1 minute before carefully rinsing them with Ringers and placing them in thin albumin for 1-2 hours to recover and then prepared for dissection or incubation in modified Newculture.

DiI injections

10% sucrose (fresh sn)
CM-DiI (50μg)
10μl 100% EtoH
+90μl 10% sucrose
spin 5'
Supernatant into fresh tube
Needles were loaded with the DiI solution, and the same setup used for electroporation was used to inject DiI in the prospective neural crest forming region of stage 4 HH embryos.

CELL AND TISSUE CULTURE METHODS

Embryo dissections

All dissections were done using tungsten needles. Tungsten needles can be made from tungsten wire of approx 0.3mm in diameter. I sharpened the needles electrolitically by connecting the needle holder with the wire to a 10-12V DC power source and immersing the tip of the needle and the indifferent electrode into a 10N NaOH solution.
Explant the embryo onto a sylgard coated dish filled with Ringers solution, and pin it using stainless steel minutien pins (Fine science tools).
There are two alternatives for the dissection:
1)Let the embryo sit in dispase on ice for about 15' (time varies depending on the stage of the embryo). Then remove the embryo and dissect.
2)Fill the sylgard dish with Ca++/Mg++ free Tyrode's solution containing 0.1% trypsin, and dissect.

Dissected tissues can be allowed to recovered in PB1 solution containing 6μl/ml of 35% BSA on ice for up to 2 hours.
Usually the tissue is then placed on a fibrinectin coated dish or a collagen gel in filtered F12/N2 medium (98ml Hams F-12 (Gibco), 1ml N2 supplements (Gibco), 1ml 100X pen/strep, 1ml glutamin)

Solutions

PB1
5.97 g/L NaCl
0.2 g/L KCl
1.142 g/L NaH_2PO_4
0.19 g/L KH_2PO_4
0.04 g/L Sodium Pyruvate
1 g/L Glucose
0.1 g/L $MgCl_2$-6 H_20
0.14 g/L $CaCl_2$-$2H_20$
0.06 g/L Penicillin
0.05 g/L Streptomycin
0.01 g/L Phenol Red

Collagen gels
90μl collagen type I
10μl 10X DMEM (vortex)
4.5 μl 7.5%$NaHCO_3$
keep in ice

Tyrodes Saline 10X
(bold excluded for Ca/Mg free)
80 g NaCl
2g KCl
2g $CaCl_2$ (2.7 if there are $2H_2O$)
0.5 g NaH_2PO_4
2g $MgCl_26H_20$
10g glucose
H_20 to 900 mL
Autoclave or filter

Fibronectin: 20μg/ml in PBS, Ringers, or medium. Leave in incubator for >1 hour, rinse 3x5' before use.

BMP-4: 50ng/ml

Dispase, (1.5 mg/ml)
Make 1 M Hepes, pH to 7.5 which is the optimal pH for Dispase,then autoclave it.
Make media:
This must be done in the hood.
Take a bottle of 500 ml DMEM and add 5 ml of HEPES . (The final pH is 8) . 50 ml aliquots into 50 ml tubes. Freeze
Make Dispase:
Put 0.075 g of dispase into the 50 ml tube of media.
Vortex
Make 1ml, and 5 ml aliquots.

Protocol for passage of cells

- Start with a dish of confluent cells
- 2 or 3 x Ringers (rinse)
- 1ml trypsin (0.25% Trypsin-EDTA, GIBCO)(make sure it covered all the cells, then remove)
- 3' @ rt, hit the plate to loosen cells
- Resuspend cells in1 ml STO, 1/10 or 1/3 to a fresh dish
- For Delta-Fc or Fc 293T expressing cells, add 200μg/ml Hygromicin B to select against the loss of the constructs

STO MEDIUM:

500ml DMEM (high glucose)
10% Fetal Bovine Serum (gibco) (thaw and heat inactivate 20' @ 56°)
5 ml of 100X Pen/Strep

Protocol to store cells

- After tryspination (as above)
- Resuspend in 1ml/dish, collect all dishes and add serum to 20% final
- Spin ~ 1000-1500 rpm
- ~ 10 dishes in 2.5 ml of STO (for 5 ml final volume)
- Final solution: 10% DMSO + 50% HT FBS + STO (20% DMSO in 2.5 ml HT FBS + 2.5 ml of cells in STO)
- In ice immediately
- NEVER add the cells to the DMSO nor DMSO directly to the cells
- Aliquot the final solution into cryotubes and set in a proper container w/ MEOH into -80. Transfer next day to liquid N_2.

HT FBS: Heat treated Fetal Bovine Serum (Gibco). Heat inactivate for 20′ @ 56ºC

Protocol to inject cells

- Trypsin
- +STO +20% serum (final) ~1 hr @ rt (or 37°)
- Take the volume to 50ml w/ Ringers to get rid of the serum (can rinse 2X)
- Spin 1000-1500rpm, 3'to5'
- Prepare DiI w/ 25μl EtoH in 500μl of sucrose 10% in 1ml of Ringers (final volume 1.5ml)
- 300μl of DiI / dish (add to the pellet of cells). Mix w/ pipette and leave 15' @ rt
- Add 45 ml of Ringers, rinse, spin @ 1000-1500 RPM 3'-5'
- Resuspend in 1ml of Ringers and transfer to an epp tube (Ringers + 6μl/ml of 35%BSA)
- spin 3500, 3'-5', remove all liquid and resuspend (against a rack)

Preparation of Delta-Fc or Fc Conditioned Media

- After cells are confluent, change media to DMEM high glucose P/S
- Grow for 5 days
- Add medium to centricon-10 or -30 (you can previously add 6μl/ml of 35% BSA)
- Spin twice
- Can reconcentrate in microcon-30 or centricon-30 (milipore) tubes to achieve 50X concentration
- Store @ 4ºC for upto 4 days

Explant tissues on collagen gels (let tissues recover on PB1 + 6μl/ml BSA 35%)

Add 30μl of CM per ml of culture media (F12/N2/P/S)

For Delta-Fc to actcivate Notch signaling, add 1/100 -Fc antibody to the culture media

Lipofectamine Transfection

- Mix together: Tube A: 60 ng-6μgDNA + 840μl optimem (Gibco)
  Tube B: 56μl lipofectamine (Invitrogen) + 840μl optimem
- Mix A and B, leave at rt for 30'
- Wash cells in optimem
- Add 6.4 ml optimem to cells, add the lipofectamine/DNA mix
- Leave on cells~5 hours
- Remove media, add new growth media
- Next day, passage cells 1:3 or up to 1:6. Can begin to check for transfection

Appendix 3:

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