CHAPTER 2

Neural Crest Inducing Signals

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Abstract

The formation of the neural crest has been traditionally considered a classic example of secondary induction, where signals form one tissue elicit a response in a competent responding tissue. Interactions of the neural plate with paraxial mesoderm or nonneural ectoderm can generate neural crest. Several signaling pathways converge at the border between neural and nonneural ectoderm where the neural crest will form. Among the molecules identified in this process are members of the BMP, Wnt, FGF and Notch signaling pathways. The concerted action of these signals and their downstream targets will define the identity of the neural crest.

Introduction

The neural crest is a transient population of embryonic cells that originate at the border between the neural plate and the prospective epidermis. Around the time of neural tube closure, neural crest cells undergo an epithelial to mesenchymal transition and initiate extensive migrations throughout the embryo. Shortly after migration to their final position, neural crest cells differentiate to form a wealth of derivatives. The mechanisms of migration and differentiation of neural crest have been studied extensively.[1,2] In contrast, little is known about the embryological origins of the neural crest, the signaling events that lead to their formation and the molecular nature of the interactions that generate them.

Formation of neural crest has traditionally been considered a classic example of secondary induction where signals from one tissue elicit differentiation in a competent, responding 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.[3-6] Interestingly, some of these experiments have shown that both neural plate and epidermis can generate neural crest when combined, suggesting the existence of bidirectional inductive events.

Over the past decade, researchers have been studying the nature of the tissue interactions and molecular signals involved in neural crest induction. Current evidence suggests that both the ectoderm and mesoderm can contribute inductive signals.[5-8] TGF-β, FGF, Wnt and Notch signaling pathways have all been shown to play an essential role in this process. The convergence of these signals at the border between the neural and non neural ectoderm apparently triggers transcriptional events that lead to neural crest specification. The origin and nature of these signals, as well as the evidence for their role in neural crest formation, are described below.

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Signals from the Ectoderm

The localization of neural crest precursors at the border between neural plate and epidermis suggests a potential role for interactions between these two tissues in induction of the neural crest. The grafting experiments of Rollhäuser-ter Horst in amphibians showed that gastrula ectoderm generated both neural and neural crest cells when grafted to the neural folds.[9,10] The juxtaposition of these tissues Axolotl embryos, generated neural crest at the newly formed border.[3] By grafting tissues in from pigmented embryos into albino hosts, they observed that the newly induced neural crest cells originated from both the neural plate and the epidermis. Interestingly, while tissue from the neural plate formed mostly melanocytes, the epidermis tissue formed spinal and cranial ganglia. The role of neural plate and epidermal interactions in neural crest induction was later confirmed in vivo in other organisms by analogous transplantation experiments in chick, fish and *Xenopus* embryos, with essentially similar results.[5,6,11] In vitro cocultures of epidermis and neural plate tissue from both chicken and frog embryos showed that the interactions between these two tissues are sufficient to generate neural crest.[4,6,12] However, the competence of the chick neural plate to respond to signals from the ectoderm is lost by 1.5 days of development (stage 11 HH staging according to Hamburger and Hamilton),[13] suggesting that inductive interactions that lead to neural crest formation may be temporally limited in the chick embryo.[14]

Signals from the Mesoderm

One of the first experiments to address the issue of neural crest induction showed that portions of the archenteron roof of amphibian gastrulae had the capacity to induce neural tissue and neural crest when grafted into the blastocoel of a host embryo. If the grafted tissue was derived from 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.[15] The ability of paraxial mesoderm to induce neural crest was confirmed later by recombination experiments in vitro, both in amphibians[16] and in chick embryos.[17] 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.[18] 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 or 'neuralized' *Xenopus* animal caps (explants of naïve ectodermal tissue that have been exposed to neuralizing molecules).[19] However, recent findings in zebrafish embryos suggest that signals from the mesoderm are dispensable for neural crest formation.[20] Zebrafish mutants in which mesoderm formation was blocked expressed normal neural crest markers and formed neural crest derivatives. The authors proposed that in the absence of mesoderm, the ectoderm is able compensate for the missing vertical signals from the mesoderm.

The neural crest originates adjacent to three embryonic tissues, the non neural ectoderm, the neural plate and the underlying mesoderm. All these tissues are potential sources of inductive signals and their interactions are responsible for neural crest formation. Many of the experiments presented above are based on tissue recombinations and in vitro manipulations. While these experiments have proved extremely useful in testing the necessityof tissue interactions and have helped identify candidate molecules involved in neural crest induction, they may not reflect the complexity of the interactions that take place in vivo.

BMPs

Dorsalin-1, a TGF-β family member, is present in the dorsal neural tube. Purified dorsalin-1 added to explants of intermediate neural plate (portions of the neural plate between the ventral midline and the neural folds) is sufficient to induce migratory neural crest cells.[21] Such intermediate neural plate tissue is considered naïve in the sense that it has not received signals to specify it as dorsal or ventral. Although dorsalin-1 is expressed only transiently after neural tube closure, this observation suggested that other TGF-b family members expressed in the epidermis adjacent to the neural plate may also be involved in the induction of neural crest.

The first candidate molecules identified in the epidermis were BMP-4 and BMP-7. Like dorsalin-1, the addition of these molecules to intermediate neural plate explants could induce neural crest in the absence of epidermis, leading to the proposal that BMPs may be the epidermal signal responsible for neural crest induction.[4] 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 pattern 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.[22] Furthermore, BMP-4 expression is downregulated in the epidermis adjacent to the closing neural folds and, instead, becomes strongly expressed on the neural folds themselves. This expression pattern may suggest a role for BMP-4 in the maintenance rather than the initial 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.[23] In addition, they block neural crest emigration from the neural tube.[24]

Mutations in different members of the BMP family and their antagonists in mice suggest that these molecules are not absolutely required for neural crest formation. 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.[25] BMP-7 homozygous null mice present some craniofacial skeletal defects but they are more likely related to bone formation rather than neural crest.[26] 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.[27] Mice carrying homozygous mutations for the BMP antagonists Noggin[28] or follistatin[29] do not exhibit defects in neural crest formation. While the normal expression pattern of BMPs could not account for the possibility of functional redundancy,[22] 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 that other unidentified molecules (e.g., other TGF-β family members) stimulate 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.[8,16] 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.[16] Interestingly, over-expression 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.[8,30] 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.[31-33] A direct effect of the BMP pathway on neural crest development is hard to infer from these mutants because the defects associated with these mutations are not exclusively neural crest related, and rather affect the main axis of the embryos, Interestingly, zebrafish bmp2b is functionally more similar to *Xenopus* BMP-4 than

zebrafish bmp4.[34] 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.[31] However, the BMP gradient may simply set the position of the neural plate border rather than directly inducing neural crest cells.

Wnts

Several lines of evidence suggest that members of the Wnt (wingless/INT) family of secreted glycoproteins can act as neural crest inducers.[8,35-37] Both Wnt-1 and Wnt-3a are expressed in the dorsal neural tube. 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.[38] However, some neural crest cells form in these animals, suggesting that these molecules may influence later events such as proliferation rather than the initial formation of neural crest. Furthermore, it has been shown that neural crest arise in vitro in the absence of Wnt1 and Wnt3a.[12] Recent evidence suggest that Wnt signals in the dorsal neural tube are critical for neural crest cell differentiation into sensory ganglia.[39] Thus, Wnt signals in the ectoderm other than Wnt1 and Wnt3a may be responsible for early steps in neural crest formation.

Wnt family members are strong inducers of neural crest markers when injected in neuralized animal caps. Over-expression 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.[35] Because Wnt signaling can result in cell proliferation,[40] the authors repeated the experiment blocking cell proliferation at gastrula stages and obtained 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.[8,36,41]

Experiments in chick embryos have shown that Wnt signals are necessary for neural crest formation. Injection of cells expressing a dominant negative Wnt1 construct adjacent to the neural folds blocks expression of the neural crest marker *Slug*. Moreover, addition of soluble Wnts to intermediate neural plate explants generated migratory neural crest cells, suggesting that Wnt signals are sufficient to induce neural crest. The generation of neural crest cells in vitro occurs in a defined minimum medium lacking additives. In contrast, BMP-4 was unable to induce neural crest in these explants in defined medium without additives, suggesting that its effects might be the result of synergistic actions with other signaling molecules. In chick, Wnt6 is expressed in the ectoderm at the correct time and place to be involved in neural crest induction. Taken together, these data suggest that Wnt is an epidermal inducer of neural crest in chick embryos.[37] Similar experiments in zebrafish confirm the requirement for Wnts in neural crest induction.[42] Using a transgenic zebrafish line that expressed an inducible inhibitor of the canonical Wnt pathway, the authors defined a critical period for Wnt signaling in the induction of neural crest. However, this approach globally eliminated Wnt signaling in the whole embryo and although in a stage controlled manner, Wnt signals are important for other developmental processes that may have an indirect effect on neural crest formation. Wnt8 is expressed adjacent to the prospective neural plate, and thus is a good candidate for the neural crest inducer in zebrafish. Consistent with this idea, blocking Wnt8 function by injection of antisense morpholino oligonucleotides resulted in the loss of early neural crest markers.

In addition to their epidermal expression, members of the Wnt family are expressed throughout the embryo in domains that are compatible with a role in neural crest induction, including the dorsal neural tube[35] and the paraxial mesoderm.[41] In fact, the ability of paraxial mesoderm to induce neural crest markers in neuralized animal caps is lost in the presence of a dominant negative form of Wnt8, suggesting that Wnt signaling mediates the inducing ability of paraxial mesoderm.[7,8,41]

FGFs

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 the transient expression of several neural crest markers.[7] 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.[30] In a subsequent study it was shown that FGFs ability to induce neural crest in frog embryos was dependent on Wnt signaling.[8]

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.[43] Interestingly, recombinants of Hensen's node and neuralized animal caps 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.[41]

Notch

Experiments in chick, frog and zebrafish suggest a role for Notch signaling in neural crest induction.[44-48] In chick embryos, Notch ligands are expressed in non neural ectoderm adjacent to the Notch expression domain in the neural plate. Over-expression of an activated form of Notch results in down-regulation of the neural crest marker *Slug* and a reduction in the number of HNK-1+ migratory neural crest cells in the head region.[46] Surprisingly, inhibition of Notch signaling through electroporation of a dominant negative form of the Notch ligand Delta (Delta^{stu}) had the same effect. Gain and loss of function experiments negatively modulated the levels of BMP-4 in the neural plate border region. Over-expression of a BMP-4 construct was able to rescue the loss of Slug after the Delta^{stu} electroporation but not after Notch activation. The authors proposed that Notch acts upstream of BMP-4 in the specification of neural crest, but that loss of neural crest by Notch activation is independent of BMP-4. In addition, the effect of Notch on BMP-4 transcript levels was mediated by Deltex, (another component of the Notch pathway), in a non canonical manner independent of suppressor of hairless. These experiments suggest that Notch signaling could be acting on neural crest formation through parallel pathways at different times.[45] Because both activation and inhibition of Notch signaling result in the loss of neural crest markers, the authors conclude that a threshold level of Notch activation is required in order to achieve BMP-4 expression and subsequently proper neural crest specification. Similar experiments in Xenopus also revealed somewhat different results. In frogs, activation of the Notch pathway leads to a decrease in the levels of BMP-4 and subsequently to an expansion of neural crest markers.[44] Inhibiting the pathway results in an expansion of BMP-4 expression and a reduction in neural crest markers. Over-expression of hairy2, a downstream target of Notch, has the same effect as activating the Notch pathway, suggesting that the effects of Notch on BMP-4 levels and neural crest induction are mediated by this bHLH transcription factor (Glavic et al 2004). The apparent discrepancies between chick and frog could be explained by different requirements of BMP-4 at the border between neural plate and non neural ectoderm for neural crest induction, the timing at which the manipulations were performed, or both. In zebrafish, the evidence for a role of Notch in neural crest formation comes from the analysis of mutants. Fish carrying a mutation in the deltaA gene (*dlAdx2*) generate an excess of Rohon-Beard sensory neurons in the trunk at the expense of neural crest cells. These findings suggest a role for Notch in fate decisions amongst common precursors of these two cell types.[48] Interestingly, depletion of neurogenin-1 in deltaA mutants restores expression of neural crest markers in the trunk, suggesting that the main function of Notch is to repress the sensory neuron fate rather than specifying neural crest fate.[47] It is worth noting that in contrast to chick and frog, Notch signaling in zebrafish has little effect on cranial neural crest and the major effects were observed in the trunk region. This observation could have evolutionary implications regarding the origins of the neural crest at different rostrocaudal levels, and the conservation of the inducing signals.

Neural Crest Induction Is a Multistep Process

Neural crest induction in *Xenopus* requires inhibition of BMP signaling to set the epidermal, neural and border fates within 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.[8,16,30] 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.[23] This result suggests that BMP signals are required for the maintenance of specified neural crest. In addition, isolated caudal neural folds of stage 10HH embryos begin to express Slug after 18 hours in culture in the absence of any further signals, suggesting that neural crest cells are specified long before the expression of specific markers. However, this expression is transient.[14] 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.

Conclusions

From the data presented above, it is clear that interactions between the epidermal ectoderm and/or mesoderm with the neural plate can generate neural crest. Members of the wingless/INT (Wnt), bone morphogenetic proteins (BMPs), Notch and fibroblast growth factors (FGF) families have been shown to participate in the process of neural crest induction. The data gathered from different organisms is still not enough to propose a unified model for neural crest induction. Even though all vertebrates seem to use the same set of signals for neural crest formation, the precise hierarchy and timing in which these signals are received by the responding tissues seems to differ slightly in the different model organisms. These discrepancies however, may just reflect the differences in the experimental approaches used to study each organism, rather than an intrinsic difference in the process of neural crest induction. 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. 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.

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. The concerted action of multiple signaling pathways at the border between neural and nonneural ectoderm defines the domain from which the neural crest will form. Ultimately, the identity of the neural crest will be established by the combination of the downstream targets of the converging signals in this territory.

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