Published in final edited form as:

Dev Dyn. 2006 September ; 235(9): 2507–2520. doi:10.1002/dvdy.20891.

Endogenous BMP antagonists regulate mammalian neural crest generation and survival

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Abstract

We demonstrate here that Chordin and Noggin function as Bone Morphogenetic Protein (BMP) antagonists in vivo to promote mammalian neural crest development. Using *Chrd* and *Nog* single and compound mutants, we find that Noggin has a major role in promoting neural crest formation, in which Chordin is partially redundant. BMP signaling is increased in dorsal tissues lacking Noggin, and is further increased when Chordin is also absent. The early neural crest domain is expanded with decreased BMP antagonism in vivo. Noggin and Chordin also regulate subsequent neural crest cell emigration from the neural tube. However, reduced levels of these BMP antagonists ultimately result in perturbation of neural crest cell derived peripheral nervous system and craniofacial skeletal elements. Such defects reflect at least in part a function to limit apoptosis in neural crest cells. Noggin and Chordin therefore function together to regulate both the generation and survival of neural crest cells in mammalian development.

Keywords

Chordin; Noggin; BMP; neural crest

INTRODUCTION

The neural crest is comprised of a multipotent, transient cell population that gives rise to a variety of cell types. These derivatives include nerve, muscle, cartilage, and bone tissues that comprise the greater part of the vertebrate face and peripheral nervous system (Le Douarin and Kalcheim, 1999). It is of both conceptual and pragmatic importance to elucidate the molecular control of neural crest development from many standpoints. Variation in neural crest development can facilitate evolutionary specializations of craniofacial structure and function, and neural crest deficiencies can result in certain human birth defects (neurocristopathies). Although many signaling molecules have been identified that promote various aspects of neural crest biology, including the Bone Morphogenetic Proteins (BMPs), their roles in vivo have been difficult to establish. Moreover, while some molecular functions in neural crest development are highly conserved, there is also strong evidence for interspecies differences (Aybar and Mayor, 2002). Here we take a genetic approach to explore the role of endogenous antagonists of BMP signaling in regulating neural crest development in the mouse.

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The development of the neural crest may be viewed as several discrete steps: induction of the neural crest region, followed by the delamination, migration, and differentiation of neural crest cells (NCCs) in target tissues. There is evidence from many experimental systems that the BMP signaling pathway has important roles in each of these processes. First, the neural crest is induced from the lateral edges of the neural plate via reciprocal interactions with adjacent surface ectoderm and underlying mesodermal tissues (Selleck et al., 1998). BMPs are expressed in multiple domains surrounding the dorsal neural tube (Furuta et al., 1997; Kanzler et al., 2000; Winnier et al., 1995), and graded BMP signaling compartmentalizes the ectoderm (Marchant et al., 1998; Nguyen et al., 1998). In avian and amphibian embryos, neural crest induction is achieved when (1) an intermediate level of BMP signaling is received by naïve ectoderm, and (2) when this competent region receives a second inductive (often posteriorizing) signal, such as FGF, WNT, or retinoic acid (Aybar and Mayor, 2002; Knecht and Bronner-Fraser, 2002).

Following induction, NCCs delaminate from the dorsal neural folds in order to migrate to their final destinations. In mouse, this epithelial-to-mesenchymal transformation is initiated in the rostral hindbrain around the five somite (5s) stage (Nichols, 1981), and subsequently extends bi-directionally along the anteroposterior axis. At any given level, active NCC generation lasts about 9 hours (Serbedzija et al., 1992). Studies in avian embryos suggest that BMP activity promotes delamination of NCCs, possibly via control of the small GTPase *rhoB* (Liu and Jessell, 1998), and that graded expression of the BMP antagonist *noggin* in the dorsal neural tube can directly regulate cell emigration promoted by BMP (Sela-Donenfeld and Kalcheim, 1999).

Finally, as NCCs transit from the neural tube to their final destinations, reciprocal interactions with their microenvironment influence cell migration and fate determination (Dorsky et al., 2000; Trainor and Krumlauf, 2000). BMP signaling has been implicated as one such regulator of each of these processes in multiple model systems (Aybar and Mayor, 2002; Christiansen et al., 2000; Knecht and Bronner-Fraser, 2002).

Several genetic studies have uncovered a requirement for BMP ligands in murine neural crest development. In specific genetic backgrounds, mice lacking an allele of *Bmp4* have mild craniofacial defects suggestive of cranial NCC deficiency (Dunn et al., 1997). Those completely lacking both *Bmp5* and *Bmp7* have craniofacial truncations and underdeveloped branchial arches due to defective maintenance or proliferation of migrating NCCs (Solloway and Robertson, 1999). Additionally, embryos lacking *Bmp2*, or ectopically expressing the BMP antagonist Noggin in the presumptive neural crest domain display multiple neural crest cell deficiencies (Kanzler et al., 2000; Ohnemus et al., 2002).

The potent influence of BMP activity on NCC development suggests that precise regulation of intercellular BMP signaling is important to achieve normal cell fates. Here, we elucidate the endogenous roles of BMP antagonists in mammalian neural crest biology, by analyzing mouse embryos carrying null alleles for *Chordin* (*Chrd*), *Noggin* (*Nog*), or both genes. We find that *Chrd* and *Nog* act with partial redundancy to limit BMP signaling in the nascent neural tube in vivo; in their absence, the expression of BMP transcriptional targets is increased in the dorsal neural tube during early stages of NCC development. The expression

of neural crest markers is expanded as well. In addition, we find that endogenous BMP antagonism by Chordin and Noggin limits the delamination of NCCs, and protects them from BMP-induced apoptosis during migration and differentiation.

RESULTS

Chordin and Noggin antagonize BMP signaling in the dorsal neural folds

Current models for neural crest cell specification suggest that an intermediate level of BMP signaling in the dorsal neural folds induces neural crest gene expression (Aybar et al., 2002). The secreted proteins Noggin and Chordin can antagonize BMP signaling when supplied exogenously, and can modulate BMP signaling when expressed in vivo (reviewed in Balemans and Van Hul, 2002). By assaying phosphorylation events indicative of BMP signal transduction, we have found that endogenous Noggin and Chordin act as redundant BMP antagonists during neurulation and organogenesis in mouse embryos (Y.P. Yang, R.M.A. and J.K., manuscript in preparation). Both genes are expressed in a spatiotemporal manner suggesting they might influence early neural crest formation. In addition to their common axial mesendoderm domain (Stottmann et al., 2001; Anderson et al., 2002) both genes are expressed in more dorsal domains as well. From E8.0, *Noggin* is expressed in the dorsal neural folds, and in the dorsalmost portion of the neural tube after closure (see Fig. 3A-C; McMahon et al., 1998). Meanwhile, *Chordin* is expressed at a very low, fairly even level throughout the neural plate and paraxial mesoderm, as indicated by section and whole-mount in situ hybridization and confirmed by RT-PCR of microdissected tissues (data not shown; Scott et al., 2000). Together, these considerations suggest that both of these proteins may regulate BMP signaling in the dorsal neural folds during neural crest generation.

If the principal function of both Chordin and Noggin in the neural plate is to bind and inhibit the activity of BMPs, it follows that a reduction in the allele dosage of *Noggin* and/or *Chordin* should result in increased expression of positive transcriptional targets of BMP signaling. We looked for changes in BMP signaling in the dorsal neural tube of mutants from three different genotypic classes: $Chrd^{-/-}$ mutants, $Nog^{-/-}$ mutants, and $Chrd^{-/-}$;$Nog^{-/-}$ double mutants by examining the expression of *Msx* genes, which are well-characterized positive transcriptional targets of BMPs (Arkell and Beddington, 1997; Iimura et al., 1994; Suzuki et al., 1997; Vainio et al., 1993). Importantly, increased levels of *Msx1* are correlated with increased production of NCCs in both avian and piscine embryos (Liu et al., 2004; Tribulo et al., 2003). At E8.5 (approximately 7-8 somite pairs) expression of *Msx1* and *Msx2* in $Chrd^{-/-}$ mutants was indistinguishable from wild-type (not shown). However, substantial up-regulation of these genes was detected in the dorsal neural tube of both $Nog^{-/-}$ and $Chrd^{-/-}$;$Nog^{-/-}$ mutants (Fig. 1B,C and data not shown). In addition, *Msx1* and *-2* expression is also expanded in the lateral plate mesoderm of $Chrd^{-/-}$;$Nog^{-/-}$ mutants (Fig. 1C). From these data, we conclude that BMP signaling in the dorsal neural tube is significantly increased in both $Nog^{-/-}$ and $Chrd^{-/-}$;$Nog^{-/-}$ mutants relative to wild-type.

Excess neural crest cells are generated in *Chrd;Nog* mutants

To determine the roles of Chordin and Noggin in vivo during NCC production in the mouse, we examined the expression of several transcriptional markers of the neural crest and of

migratory NCCs in mutant embryos. The transcriptional activator gene *Ap2α* (*Tcfap2a*; Mouse Genome Informatics) is expressed at the neural/surface ectoderm boundary, in presumptive, pre-migratory, and migrating NCCs, and in some epidermal domains (Fig. 1D) (Arkell and Beddington, 1997; Mitchell et al., 1991). Moreover, *Ap2α* is the earliest characterized marker of presumptive NCCs (Meulemans and Bronner-Fraser, 2004), is a competence factor for neural crest induction (Luo et al., 2003), and is necessary for normal differentiation of some NCC derivatives (Brewer et al., 2004; Schorle et al., 1996; Zhang et al., 1996). *Ap2α* expression in $Chrd^{-/-}$ mutants is indistinguishable from wild-type (data not shown). However, in $Nog^{-/-}$ and $Chrd^{-/-};Nog^{-/-}$ mutant embryos with 5-7 somites (s), *Ap2α* expression is increased in the dorsal region of midbrain, hindbrain, and trunk neural folds, surface ectoderm and head mesenchyme (Fig. 1E,F). Histological sections indicate that increased signal intensity is due primarily to up-regulation of *Ap2α* in both the dorsal neural tube and surface ectoderm (data not shown).

The HMG box transcription factor *Sox10*, like *Ap2α*, has roles both in production and development of NCCs (Honore et al., 2003). However, *Sox10* is expressed slightly later, and more specifically, in nascent NCCs (Fig. 1G) (Kuhlbrodt et al., 1998). Initially, *Sox10* is expressed in all neural crest progenitors, but later becomes restricted to melanocytes and glial cells of the peripheral nervous system. In contrast to the changes observed in *Ap2α* expression, we observed little or no alteration in *Sox10* trunk-level expression in any mutant class at developmental stages younger than 10 s, though expression was somewhat upregulated in the brain of the double null (Fig 1G-I and data not shown). However, at 11-12 somites, a few hours later in development, we observed a marked increase expression of *Sox10* in $Nog^{-/-}$ and $Chrd^{-/-};Nog^{-/-}$, but not in $Chrd^{-/-}$ mutant embryos (Fig 1J-L and data not shown). Furthermore, the axial extent of neural crest staining was expanded caudally; this result is considered with complementary data below.

In addition to the above results, embryos of each mutant class were also analyzed for expression of three additional neural crest markers: *Cad6, Crabp1*, and *Snail* (Inoue et al., 1997; Smith et al., 1992; Stoner and Gudas, 1989). NCC expression of each of these markers was increased in embryos older than 11-12 somites (data not shown). In summary, while it appears that the initial extent of neural crest cell induction *per se* may not be significantly affected, the NCC domain is subsequently expanded ectopically in $Nog^{-/-}$ and $Chrd^{-/-};Nog^{-/-}$ mutant embryos. Taken together, our results suggest that an increase in endogenous BMP signaling results in an expansion of the NCC population during early stages of mouse neural crest development.

Ectopic BMP2 signaling can induce some but not all neural crest markers *in vitro*

Our data show that both BMP signaling and NCC generation are increased in embryos with reduced BMP antagonism, consistent with the idea that BMP signaling promotes NCC formation. However, recent studies suggest that BMP signaling alone is insufficient to induce neural crest in the chick (Garcia-Castro et al., 2002). We therefore tested whether ectopic recombinant BMP could induce NCC markers in a manner similar to that of the mutant embryos. To mimic the environment of BMP antagonist-deficient embryos, we employed an explant culture system in which all tissue layers around the dorsal neural tube

were left intact, and BMP signaling levels were increased locally using beads saturated with recombinant BMP2. While BSA beads produced no effect in these experiments, BMP2 robustly induced *Msx1, Ap2α*, and *Cad6* after 7 hours of culture (Table 1, Fig. 2A-D, and data not shown). However, increased levels of NCCs expressing these markers were not observed migrating away from the region of induction. Moreover, neither *Sox10* nor *Snail* was induced by BMPs (Fig. 2E,F and data not shown). We tried a range of BMP concentrations, and both BMP4 and BMP2 proteins were tested, but no condition we tried resulted in clear induction of differentiating neural crest markers (data not shown). In some cases, as in the explant shown, BMP beads resulted in less Sox10 staining around the bead relative to the controls. This may reflect increased apoptosis of at least a subset of differentiating NCCs upon increased BMP exposure (see below; Figs. 7,8). Thus, although these in vitro experiments were not able to model all aspects of the NCC phenotypes in BMP antagonist mutant embryos, they confirm that increased BMP activity can increase at least some early markers of NCC induction in the mouse.

Precocious emigration of neural crest cells in *Chrd;Nog* mutants

In avian embryos, the delamination and emigration of NCCs from the dorsal neural tube occurs after neural tube closure, and appears to be regulated by dynamic changes in BMP signaling along the rostro-caudal axis (Sela-Donenfeld and Kalcheim, 1999). However, in the mouse, delamination and emigration commence prior to neural tube closure (Nichols, 1981; Nichols, 1986; Verwoerd and van Oostrom, 1979), raising the possibility that this process might be differently regulated in mammals. Since changes in *Noggin* expression in the dorsal neural tube correlate with delamination in the chick (Sela-Donenfeld and Kalcheim, 1999), we characterized the expression of *Nog* in the lateral neural plate/dorsal neural tube in mouse embryos with 7-20 somite pairs. Beginning around 10s, we observed that dorsal neural tube expression is stronger at more caudal levels, largely reciprocal to the increasing delamination of NCCs at more rostral levels (Fig. 3A-D). Thus a similar dynamic gradient of *Nog* expression exists in the mouse neural tube, although it is less pronounced than in the chick. Furthermore, down-regulation of *Nog* is coincident with the exodus of NCCs, as marked by *Sox10* expression (Fig 3D,E).

To determine the functional importance of BMP antagonists in choreographing delamination in mouse NCC development, we examined the timing of the initial migration of NCCs in $Nog^{-/-}$ and $Chrd^{-/-};Nog^{-/-}$ mutants. Migrating cells were observed peripheral to the dorsal neural tube in both mutant classes at axial levels much further caudal as compared to wild-type counterparts (Fig. 3F,G). Moreover, when equivalent axial levels are compared in stage-matched mutant and wild-type embryos, NCCs appear to have migrated further from the neural tube in mutants (eg Fig. 3F), suggesting both earlier delamination and increased outgrowth.

To further investigate the role of BMP antagonism during NCC emigration from the neural tube, we performed in vitro cell outgrowth assays. In these experiments, NCCs detach from explanted neural tube ectoderm and spread radially on gelatin-coated plastic. All cells migrating from trunk-level explants express AP2α protein, demonstrating their identity as migratory NCCs and not cells of mesodermal origin (Fig 3I). We tested the effect of elevated

BMP signaling on NCC outgrowth by supplementing culture medium with recombinant BMP2, and calculating a migratory index to quantify the extent of NCC outgrowth (see Experimental Procedures). We observed that BMP treatment results in a 55% increase in migratory index after 2 days of culture, as compared to BSA control treatment ($p<0.001$, Fig. 3H). Moreover, after 3 days, the migratory index of BMP treated explants was elevated by 79% over BSA treated explants ($P<0.001$, Fig. 3H). These data demonstrate that the neural tube ectoderm responds to BMP signaling with enhanced NCC outgrowth. We cannot exclude that some of the increased numbers of mesenchymal cells in the BMP treatment may also result from increased cell proliferation of migrating NCCs. These results obtained in vitro are consistent with the increased NCC outgrowth observed in vivo in embryos with decreased BMP antagonism.

Depletion of migratory neural crest cells in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants

We assessed the ongoing development of NCCs in embryos deficient for BMP antagonists by examining the expression of BMP transcriptional targets and the distribution of NCCs in E9.5 mutants - a day after the initial NCCs were formed. Elevation of *Msx1* and *Msx2* expression in the dorsal neural tube persisted in both *Nog*$^{-/-}$ and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants, although no difference was observed in *Chrd*$^{-/-}$ mutant embryos (Fig 4A-C and data not shown). A substantial increase of *Sox10*-positive and *Ap2α*-positive NCCs was evident in affected classes of mutant embryos when compared to wild-type embryos (Fig 4D-L). Moreover, large ectopic aggregations of NCCs were observed superficial to the dorsal neural tube in both *Nog*$^{-/-}$ (McMahon et al., 1998) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants. These aggregations generally form in regions of the dorsal neural tube with clear dysmorphology, and express the NCC markers *Sox10*, *Ap2α*, *Crabp1*, and *Cad6* (Fig. 4E,F,H,I,K,L and data not shown)

In addition to levels of expression of NCC markers, BMP antagonist-deficient embryos displayed genotype-dependent differences in the distribution of NCCs after the initial migration from the dorsal neural tube. In *Nog*$^{-/-}$ mutants, expanded populations of migratory NCCs were spread more distantly from the dorsal neural tube (Fig 4H,K), consistent with an expansion of the NCC population at their source, and subsequent migration. For example, there are many more *Sox10* expressing cells around the midgut (mg) of *Nog*$^{-/-}$ embryos (Fig. 4H) relative to wild-type controls (Fig. 4G). However, in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos, the location of expanded populations of NCCs was biased more proximal to the dorsal neural tube (Fig. 4I,L). Thus, very few NCCs were seen in the midgut region of *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos relative to *Nog*$^{-/-}$ single mutants. These data suggest a defect in migratory behavior, differentiation, or survival of neural crest cells in the double null mutant class.

Because of this apparent depletion of migrating NCCs in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants, we wished to assess the consequences of reduced BMP antagonism upon formation of the craniofacial skeleton, which is derived in large part from NCCs (Jiang et al., 2002; Le Douarin and Kalcheim, 1999). However, the severe chondrogenesis defects of *Nog*$^{-/-}$ mutants (Brunet et al., 1998) obscure phenotypes specific to the NCC-derived skeleton, and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants die prior to skeletogenesis (Bachiller et al., 2000). We therefore

focused on craniofacial defects in *Chrd*$^{-/-}$;*Nog*$^{+/-}$ mutants. This genotypic class displays variable deletions of the ventral midline of the forebrain and overlying facial structures (Anderson et al., 2002). We previously documented a role for Chordin and Noggin in promoting outgrowth of the mandibular prominence of the first pharyngeal arch (PA1) in these mutants (Sottmann et al., 2001). The outgrowth of the mandibular prominence depends on a large influx of NCCs into the PA1. Although we did not assay NCC distribution per se in this study, the explanation we thought most likely to account for the reduced mandibular outgrowth was decreased NCC population of the mandibular prominence as a result of increased apoptosis, due largely to reduced expression PA1 expression of the NCC survival factor FGF8. Together with the results reported above, this raises the possibility that other cranial skeletal elements composed of NCC derivatives are also affected in these mutants.

Although the amount of cranial neural crest does not appear diminished at E8.5 in affected mutants (data not shown), skeletal elements derived from NCCs, such as the premaxillae and maxilla (as well as the mandible), are absent from the ventral midline in neonates (Fig. 5B,C). Lateral bones, such as the alisphenoid and zygomatic arch, are displaced toward and fused at the midline (Fig. 5E-G). In contrast, elements of the chondrocranium, derived from cranial mesoderm, are generally unaffected. These chondrocranial structures include the basioccipital, basisphenoid, and the otic capsule (Fig. 5B,C,E-G). From this analysis, we conclude that there is a more pervasive loss of NCC-derived skeletal elements in *Chrd*$^{-/-}$;*Nog*$^{+/-}$ embryos than truncation of the mandible. This defect is variable in its severity among mutant embryos, with ventromedial NCC derivatives being most susceptible to reduction. In contrast, mesodermally derived craniofacial skeletal elements are relatively unaffected.

To assess the consequences of a complete loss of Chordin and Noggin on the development of neural crest derivatives, we examined the cranial and dorsal root ganglia of the peripheral nervous system (PNS). To visualize the PNS we utilized an anti-neurofilament antibody, 2H3 (Fig. 6). While most cranial nerves are formed normally in *Nog*$^{-/-}$ mutants, the vagus (X) and glossopharyngeal (IX) plexuses are expanded, fused together, and generally disorganized (Fig. 6B). Furthermore, in *Nog*$^{-/-}$ mutants, dorsal root ganglia (DRG) appear expanded, and are not segmented properly (Fig. 6E). In contrast, most *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants retain only small clusters of 2H3-positive cells, presumably of the trigeminal ganglion (Fig. 6C). Although the expressivity of phenotypes in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutant embryos is somewhat variable, DRG are rarely formed. In most *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos, no 2H3-positive cells are found outside of the head (Fig. 6C). Even in the least affected mutant embryos, DRG are sparse (Fig. 6F). These data demonstrate that the organization and differentiation of PNS neurons requires attenuation of BMP signaling. We hypothesize that the more substantial defects observed in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutant embryos relative to *Nog*$^{-/-}$ mutants are a result of greater BMP signaling levels in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutant embryos.

Migratory NCCs have an interdependent relationship with the peripheral tissues through which they are migrating. While disrupted somite development in *Nog*$^{-/-}$ and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants may account for some defects of migration and differentiation, it is also possible that excessive BMP signaling itself may directly affect migrating NCCs. To more closely examine the effects of BMP signaling on the formation of DRGs, we devised a second type

of explant culture system. In these, E9.5 embryos were cut transversely with glass knives to produce segments spanning 5-6 somites, at trunk axial levels encompassing the forelimb bud. These segments were cut mid-sagittally along the ventral neural tube, and placed flat upon filter membranes: The dorsal neural tube thus lies along the central axis of the explant, while ventral portions are positioned laterally (see Experimental Procedures). These explants were cultured together with BMP2-, BMP4- or BSA-soaked beads. The results are presented in Table 2. BMPs, but not BSA, induced *Msx1* and diminished the lateral expression of *Ap2α* and *Sox10,* and also disrupted the neuronal structure of the DRG (Fig. 7A-C, and data not shown).

In these experiments, migrating NCCs may be either absent from peripheral tissues, or acquiring other fates. To distinguish between these alternatives, we prepared similar explants from mice in which NCCs are permanently lineage marked by β-galactosidase expression upon their origination at the dorsal neural tube (Chai et al., 2000; Stottmann et al., 2004). BMP2 bead application resulted in an absence of labeled cells in lateral tissues (Fig. 7D), indicating that the absence of NCC cells around BMP beads is not due to a change in cell fate, but rather due to impaired migration and/or cell death. Together with in vivo data, we conclude that elevated levels of BMP signaling disrupt the development of post-migratory, differentiating NCCs in the craniofacial skeleton and PNS.

Ectopic apoptosis of neural crest cells with decreased BMP antagonism

Because differentiated NCCs are present at reduced levels in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants during organogenesis, despite an increase in NCCs at E8.5, we examined cell death patterns in whole mount using the fluorescent dye Lysotracker (Abu-Issa et al., 2002). At E9.5, *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants display greatly increased cell death throughout the head mesenchyme, while *Nog*$^{-/-}$ mutants show slight increases in some mesenchymal domains (data not shown). To address whether NCCs were specifically dying, we incubated histological sections simultaneously with anti-AP2α antibody to identify NCCs, and TUNEL reagents to indicate apoptotic cells. In addition, the sections were labeled with anti-phosphohistone H3 antibody (p-hH3), which labels proliferating cells in metaphase (Hendzel et al., 1997). At the level of the otic placodes/second branchial arch, the amount of AP2α-positive neural crest cells is increased in *Nog*$^{-/-}$ mutants, and is even more dramatically increased in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants (Fig. 8A-C). Additionally, while few apoptosing NCCs (cells double-labeled by AP2α and TUNEL) were detected in wild-type embryos, an obvious increase was evident in *Nog*$^{-/-}$ mutants. In *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants, a substantial amount of apoptosing NCCs was seen, though apoptotic cells that were not co-labeled were also seen. Large differences in cell proliferation were not apparent. From these data we conclude that *Chrd* and *Nog* function redundantly to promote the survival of migratory NCCs and perhaps other cells in the pharyngeal region.

DISCUSSION

In this study, we demonstrate that Chordin and Noggin perform critical functions as BMP antagonists during mammalian neural crest development. These factors function together to limit BMP signaling in and around the presumptive neural crest. We detect an expansion of

neural crest cells (NCCs) in the absence of *Nog* alone, but not in the absence of *Chrd* alone. However, when both antagonists are absent, we observe a much greater expansion of NCCs. In addition, NCCs leave the dorsal neural tube prematurely as a consequence of elevated BMP signaling levels in these mutants. Examination of neural crest derivatives in double mutants reveals deficiencies in both the peripheral nervous system and the craniofacial skeleton. Analysis of apoptosis demonstrates that NCCs are eliminated in $Chrd^{-/-};Nog^{-/-}$ at much higher levels than in wildtype, and at intermediate levels in $Nog^{-/-}$ mutants. Our results confirm positive roles for BMP signaling during mammalian NCC production and outgrowth. Moreover, they demonstrate in vivo that BMP levels must be held in check to ensure normal neural crest generation and survival in mouse embryos.

Chordin and Noggin antagonize BMP signaling during early phases of neural crest generation

Elevated BMP signaling in mouse embryos lacking *Noggin* or both *Chordin* and *Noggin* indicates that both factors function as BMP antagonists *in vivo* during early neural tube development in the mouse. *Chrd* appears to be expressed at quite low levels in and adjacent to the dorsal neural tube, including the remainder of the neural tube as well as paraxial mesoderm. Although significant changes in BMP signaling were not observed in $Chrd^{-/-}$ mutants in this study, there are two reasons to believe that Chordin is functionally important during early neural crest development. First, we have previously shown that $Chrd^{-/-}$ mutant explants have sensitized responses to ectopic BMP addition (Anderson et al., 2002). Secondly, while loss of Noggin results in increased BMP target gene expression in the dorsal neural tube, loss of both antagonists results in a much greater increase in that domain. Together, these data indicate that the widespread, low level of *Chrd* expression has a real functional significance, and that Chordin and Noggin have partially redundant functions in dorsal neural tube development. Furthermore, the expansion of dorsally expressed BMP target genes exhibited in these mutants illuminates a critical need for endogenous BMP antagonists to modulate BMP signaling levels in the dorsal neural tube and elsewhere. The co-localization of BMP antagonists with BMPs and BMP transcriptional targets indicates that these antagonists do not eliminate BMP signaling in the neural crest and dorsal neural tube. Rather, they help ensure proper BMP activity levels, and define temporal and spatial boundaries within the early neural plate.

BMP signaling and the formation of mammalian neural crest

BMPs have been hypothesized to be key factors in NCC induction based on their expression in tissues that induce NCCs, the ability of recombinant BMP to mimic this inductive activity, and the consequences of reducing BMP activity (Liem et al., 1995; Selleck et al., 1998). In support of this hypothesis, mouse and zebrafish mutants lacking *Bmp2* display severe deficiencies of NCCs (Kanzler et al., 2000). Additionally, loss of *Bmp7*/*snailhouse* or *Smad5*/*somitibun* results in a loss of trunk NCCs in zebrafish (Nguyen et al., 2000). However, very high levels of signaling result in specification of non-neural cells, and only intermediate levels of signaling result in neural crest (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Nguyen et al., 1998). Therefore, although BMP signaling is required for neural crest induction, some degree of BMP attenuation is required. As such, a simple linear relationship between BMP signaling levels and extent of neural crest cell generation

does not hold. Similarly, although *Msx1* is an extensively characterized transcriptional target of BMPs (Suzuki et al., 1997; Yamamoto et al., 2000), it has been shown by Tribulo and colleagues (2003) that *Msx1* may have a non-linear relationship with BMP signaling; inhibition of BMP signaling promotes *Msx* transcription.

Our results provide further evidence that BMPs provide a positive signal for generation of neural crest in mouse. Moreover, they demonstrate a necessity for BMP antagonism in regulating the production of NCCs, in which Chordin and Noggin function with partial redundancy. The lack of general neural crest phenotypes with loss of *Chrd* alone suggests that BMP signaling must be elevated above a defined threshold to affect NCC development. Since we observe only positive correlations between loss of BMP antagonists and both *Msx1* and NCC marker expression, this suggests that the BMP pathway is not maximally activated in the absence of Chordin and Noggin. This may result from other redundant mechanisms of BMP antagonism. These possibilities, which are not mutually exclusive, include autoregulatory negative feedback upon expression of BMPs and BMP pathway components, the presence of other secreted BMP antagonists (Balemans and Van Hul, 2002), and the inhibition of BMP signaling via crosstalk from FGF or MAPK signaling (Aubin et al., 2004; De Robertis and Kuroda, 2004).

In *Chrd;Nog* mutant embryos, we saw a spatial expansion in the trunk region of Sox10 expression, a definitive NCC marker, after approximately the 10 somite stage. However, we did not see such an expansion earlier, at a time and place when *Ap2a* was expanded in the dorsal neural tube. Clearly, generation of NCCs was increased in the double mutant as well as in *Noggin* mutants. However, since *Ap2a* marks presumptive neural crest at the dorsal neural tube, as well as migrating mesenchymal NCCs, it is possible that neural crest induction per se was not significantly increased with the loss of the BMP antagonists. In an explant assay to study the ability of BMP to induce NCCs in mammalian tissue, we saw up-regulation of *Ap2a*, but no induction of later, downstream NCC markers. In avian embryos, the CNS tissue adjacent to the presumptive neural crest is competent to form neural crest after endogenous neural crest ablation; this regulative capacity lasts for up to 6 hours after the onset of neural crest cell emigration (Scherson et al., 1993). Because our explants were prepared from 5-7 somite stage embryos and NCC emigration can first be detected at the 5 somite stage (Nichols, 1981), these explants were likely still to be competent for NCC induction. One explanation is that our BMP beads were unable to sufficiently replicate the endogenous alterations in BMP activity that occur in the absence of BMP antagonist(s). For example, it is possible that the relevant BMP activity increased in the antagonist mutants is a complex mixture of individual BMPs, which in turn is able to increase NCC formation; such a mixture might simply not be adequately reproduced by adding recombinant BMP2 or BMP4. However, our BMP bead applications created increased BMP signal transduction in explants that reflected the increase we saw in the dorsal neural tubes of the mutants, at least as judged by target gene expression. Accordingly, an alternative explanation for the lack of increased differentiating NCCs in the BMP treated explants is that BMP signaling may promote a permissive environment for NCC specification, but is insufficient as an inductive signal on its own.

Recently, evidence has been presented that Wnt signaling is necessary and sufficient for induction of avian NCCs (Garcia-Castro et al., 2002). Recombinant Wnt3a was able to induce NCCs in vitro; however, it remains to be determined if Wnt can induce NCC in the absence of BMP signaling. Thus a further possibility is that an additional cytokine, such as Wnt, would be required in our culture system to get robust increases in NCC formation. With the availability of recombinant Wnt proteins, further studies will reveal whether exogenous Wnt can synergize with exogenous BMP to promote ectopic NCC formation.

Temporal regulation of BMP signaling in the dorsal neural tube has been proposed to control the timing of NCC emergence in avian embryos, such that cells become migratory as *Nog* expression diminishes rostro-caudally (Sela-Donenfeld and Kalcheim, 1999). In mice also, reduction in *Nog* expression spreads caudally in a wave that is reciprocal to initiation of NCC migration, although less dramatically than in the chicken. Given that we observe premature and more extensive NCC migration in $Nog^{-/-}$ and $Chrd^{-/-};Nog^{-/-}$ mutants than in wild-type, and that NCC outgrowth explants exhibit increased migration in the presence of exogenous BMP, our data support such a mechanism for mouse as well.

BMP antagonism is required for survival of migratory NCCs

Neural crest cells encounter a diversity of signals in the tissues through which they move, influencing migratory route, fate specification and survival (Paratore et al., 2002). Although excess NCCs are produced in embryos with diminished BMP antagonism, many structures derived from NCCs are diminished. For instance, we saw dramatic reductions in craniofacial bones derived from the neural crest, while bones of somitic origin were relatively unaffected. Similarly, the sensory neurons of the peripheral nervous system, also derived from NCCs, were severely reduced in *Chrd;Nog* mutants by midgestation. Neither of these effects is observed in $Chrd^{-/-}$ or $Nog^{-/-}$ single mutants. Excessive BMP signaling may impact aspects of NCC terminal fate determination in different ways. First, we have demonstrated that high levels of peripheral BMP signaling can interfere with the migration of neural crest cells in culture. Secondly, NCCs encountering BMPs near the dorsal neural tube, and in more peripheral routes and destinations, may be incorrectly instructed to undergo apoptosis (see below) and inappropriate differentiation. While BMP2 has demonstrated neurogenic effects upon neural crest stem cells in isolation (Shah et al., 1996), the integration of multiple environmental signals, as well as community effects, in vivo are likely to have alternative influences (Dorsky et al., 2000; Paratore et al., 2002).

BMP antagonists also protect migratory NCCs from apoptosis mediated by BMP. $Nog^{-/-}$ mutants show modestly increased levels of apoptosing NCCs relative to wild-type littermates. However, we observed a high level of apoptosis in migrating NCCs in $Chrd^{-/-};Nog^{-/-}$ embryos. These results suggest that survival of NCCs is globally impaired only when BMP levels are relatively high, as when both antagonists are absent.

Both Chordin and Noggin are expressed in several domains that could allow them to exert a protective influence on migratory cranial NCCs. During organogenesis stages, Chordin is broadly expressed at very low levels (Scott et al., 2000; data not shown), with higher levels in the notochord and dorsal foregut endoderm (Bachiller et al., 2000; Stottmann et al., 2001). Besides the dorsal neural tube, Noggin is expressed in the notochord, dorsal foregut

endoderm and anterior neural ridge; in addition, Noggin appears to be expressed in some migrating cranial NCCs in mouse embryos (McMahon et al., 1998; Stottmann et al., 2001; Anderson et al., 2002). Thus, it is possible that some of the effects we observe are direct consequences of BMP antagonists on NCCs, while others are indirect effects that alter the tissue environment and/or the activity of other cytokines. We cannot distinguish at present which of these individual domains of Chordin or Noggin expression are required for normal development of NCC derivatives; this would require tissue-specific ablation of each gene in the suspected domains.

Our results support data from the chick system that BMP signaling can lead to apoptosis in hindbrain NCCs (Graham et al., 1994). Further work in the chick has shown that some migrating NCCs express *Noggin*, implying that Noggin may protect them from BMP mediated cell death (Smith and Graham, 2001). Our experiments suggest that the high level of BMP antagonism provided synergistically by both Noggin and Chordin fulfills this protective role. In addition, Chordin and Noggin promote rostral expression of *Shh* (Anderson et al., 2002), which in turn is essential for cranial neural crest survival (Ahlgren and Bronner-Fraser, 1999; Dunn et al., 1995). Thus, Chordin and Noggin may protect NCCs from apoptosis induced directly by elevated BMP signaling, and indirectly by maintaining Shh signaling.

BMP antagonism and craniofacial diversity

It has been postulated that the evolution of the neural crest has allowed the emergence of many features specific to vertebrates—particularly the characteristics of the face and neck that allow efficient predation (Gans and Northcutt, 1983). Developmental potential and plasticity of the neural crest is suggested by the striking diversity of specialized faces among vertebrates. Increasing evidence indicates that the molecular environments through which NCCs migrate profoundly influence their fates (Richman and Lee, 2003). Furthermore, transplantation experiments have demonstrated that NCCs themselves provide many cell autonomous and non-autonomous signals that direct craniofacial development (Schneider and Helms, 2003). The potent influence of BMPs upon many aspects of neural crest development, and the wide variety of neural crest phenotypes we observed as alleles of BMP antagonists were deleted in the various single and double mutants, suggests that NCC development is very sensitive to modulations in BMP signaling. Variations in BMP levels have been shown to regulate beak morphology in chick embryos via modulating outgrowth of the frontonasal mass (Wu et al., 2004; Abzhanov et al., 2004), which involves extensive colonization by NCCs. Moreover, levels of BMP4 expression in the embryonic frontonasal mass correlate with variations in beak morphology among species of Darwin’s finches (Abzhanov et al., 2004). Similarly, species variations in the rostral expression of BMP antagonists could facilitate morphological change in craniofacial structures, for example by altering levels of apoptosis during NCC colonization and proliferation of the facial prominences. Species-specific changes in BMP antagonist expression levels have been observed; for example, we have seen that the rostrocaudal gradient of *Nog* expression in the mouse dorsal neural tube is not as robust as seen in chick. Additionally, it is apparent that axial expression of *Chordin* in chick is greater than *Nog* expression, which is converse to the situation in mouse (our observations) (Chapman et al., 2002; Streit and Stern, 1999). Also,

alternative regulation of *Chordin* expression in zebrafish relative to Xenopus, suggests that different species may use BMP antagonists for unique roles (Miller-Bertoglio et al., 1997). Thus the control of BMP signaling via modulation of BMP antagonism is potentially an engine utilized by evolution to revise and redefine adaptive craniofacial features.

EXPERIMENTAL PROCEDURES

Mice

Chrd$^{-/-}$;*Nog*$^{+/-}$ and *Nog*$^{+/-}$ mice were maintained on an outbred genetic background as described (Anderson et al., 2002), and mutant embryos were generated by interbreeding. Wild-type embryos were obtained from timed matings of ICR mice (Harlan Sprague Dawley). Embryonic day (E) 0.5 is defined as noon of the day following plug detection. Embryos bearing *Wnt1-Cre* (Danielian et al., 1998) and *R26R* (Soriano, 1999) alleles, which together mark neural crest lineages, were generated as previously described (Chai et al., 2000; Stottmann et al., 2004).

Gene expression assays

Whole mount *in situ* hybridization was performed as described (Anderson et al., 2002). For section in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde (PFA), equilibrated in 30% sucrose, and embedded in OCT. 16 μm sections were re-fixed in 4% PFA, washed with Phosphate Buffered Saline (PBS), and treated with 1 μg/ml proteinase K (Gibco) for 5 min. Sections were rinsed with PBS and re-fixed for 5 min with 4% PFA. Following pre-incubation for 2 hr at 65°C with hybridization buffer (Anderson et al., 2002), samples were hybridized overnight with DIG-labeled riboprobe. Sections were then rinsed with 5X SSC, washed with 0.2X SSC for 1 hr at 65°C, washed with 100 mM Maleic acid, 150 mM NaCl, pH 7.5 at room temperature. Following pre-incubation with MAB + 0.2% blocking reagent (Roche) for 2 hr, this buffer was supplemented with anti-DIG antibody (Roche) at a 1:5000 dilution. Sections were washed with MAB and color was developed with BM purple substrate per manufacturer's instructions (Roche). The following DIG-labeled riboprobes were used: *Ap2α* (Mitchell et al., 1991); *Crabp1* (Stoner and Gudas, 1989); *Cad6* (Inoue et al., 1997); *Snail* (Smith et al., 1992); *Msx1* (Hill et al., 1989); *Chrd* (Klingensmith et al., 1999); and *Sox10* (Kuhlbrodt et al., 1998). β-galactosidase staining was performed using standard techniques (Hogan et al., 1994).

Antibody staining

For antibody staining, samples were fixed with 4% PFA, and pre-incubated in antibody buffer (10mM Tris-Cl, pH7.4, 100mM $MgCl_2$, 0.5% Tween-20, 1%BSA, 5%FBS) (Hogan et al., 1994). Embryos were blocked in PBSMT (PBS, 0.1% TritonX100, 5% Carnation powdered milk), and incubated in 1:200 Neurofilament antibody (2H3; Developmental Studies Hybridoma Bank) overnight. Embryos were washed in several changes of PBSMT and incubated overnight with 1:500 Cy3-conjugated antibody (Jackson ImmunoResearch). Embryos were washed in several changes of PBSMT, several changes of PBS, and were then dehydrated through a methanol gradient and imaged in 1:2 benzyl alcohol: benzyl benzoate (BABB). AP2α antibody (3B5; Developmental Studies Hybridoma Bank) was used at a

1:200 dilution. For fluorescent detection, a Cy3-conjugated secondary antibody was used at a 1:500 dilution, and samples were imaged with Zeiss LSM510 confocal microscope.

Explant culture

For neural crest induction assays, E8.5 embryos were dissected free of extraembryonic membranes in PBSw (PBS + 0.1% Tween-20) and transferred to culture medium (DMEM, 10%FBS, 1X non-essential amino acids, 1X sodium pyruvate; Gibco). Using glass knives, 3-5 somite embryos were cut transversely; segments (caudal to hindbrain) were placed on 0.1um Nucleopore membranes (Whatman #110405). Protein-saturated beads (Anderson et al., 2002) were added, and membranes were floated on medium in a humidified 37°C incubator with 5%CO_2/ 95%air. Recombinant human BMP2 and BMP4 (Wyeth) were used at 10 ug/ml sepharose beads (BioRad).

NCC outgrowth cultures were performed as reported (Huang et al., 1998). BSA (100 ug/ml) or BMP2 (100 or 200 ug/ml) was added to the medium after 24 hr, following attachment of the explant to the dish. Explants were photographed and measured daily, for 4 days. The migration rate of cultured NCCs was gauged by calculation of a “migratory index” for each sample (Huang et al., 1998; Moase and Trasler, 1990). Here, overall outgrowth area per unit of neural explant perimeter was calculated daily for 3 days, and was normalized at the start of differential protein treatment (day 2). Using NIH image software, three independent measurements were taken and averaged. Statistical significance was assessed by the un-paired student’s t-test. For dorsal NCC migration explants, glass knives were used to cut E9.5 embryos into transverse sections, then to remove tissues ventral to the neural tube. Explants cut longitudinally along the floor plate were placed dorsal-side-up on filters, and cultured, as described above, with protein-saturated beads for 24 hours.

Neural crest cell apoptosis and proliferation analysis

Embryos were embedded in paraffin and sectioned using established techniques (Hogan et al., 1994). Antigen un-masking was performed by incubation in 10mM citric acid, pH 6.0 at 100°C for 10 min. Apoptosis was detected using the fluorescent In Situ Cell Death Detection Kit (Roche #1684795), based on the TUNEL reaction, following supplied instructions. Sections were then stained simultaneously for AP2α and phospho-Histone H3 (Upstate Biotechnology), a metaphase marker. Cy5-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG antibodies at 1:500 dilution were used for fluorescent detection (Jackson ImmunoResearch). Coverslips were mounted with GelMount (BioMedia Corp.) and imaged via confocal microscopy.

Whole-mount cell death analysis was performed as described using nile blue sulfate or Lysotracker Red (Abu-Issa et al., 2002; Anderson et al., 2002).

Histological and skeletal analysis

Histological analysis was conducted on 8μm paraffin or cryo-embedded sections using established protocols (Hogan et al., 1994). Counterstaining of sections processed for lacZ expression was via Hematoxylin-Eosin staining. Skeletal tissues were prepared and visualized as previously described (Stottmann et al., 2001).

ACKNOWLEDGEMENTS

We thank E. Meyers and B. Hogan for helpful comments on the manuscript, and M. Bronner-Fraser for useful discussion. M. Berrong, J. Goodwin, and A. Lawrence provided technical assistance. We thank T. Oliver, T. Williams and A. Moon for helpful technical advice. We are grateful to Genetics Institute/ Wyeth for BMP protein. The 2H3 antibody, developed by J. Dodd and T. Jessell, and the 3B5 antibody, developed by T. Williams, were obtained from the Developmental Studies Hybridoma Bank maintained by the Univ. of Iowa, Department of Biological Sciences, Iowa City, IA 52242. *Wnt1*-cre mice were kindly supplied by A. McMahon, via S. Orkin. This research was supported by NIH awards to JK (R01DE13674 and P01HD39948).

Grant sponsor: NIH - NIDCR (DE13674) and NICHD (HD39948)

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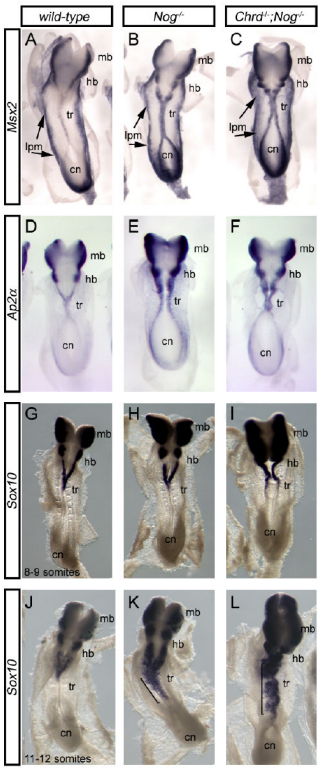

Figure 1.

Increased BMP signaling and increased neural crest cell generation in *Nog*$^{-/-}$ and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants. (A-C) *Msx2* expression used as a measure of BMP signaling in the dorsal neural tube. Compared to wild-type 8-9 somite embryos (A), increased *Msx2* expression is observed in the dorsal neural tube of *Nog*$^{-/-}$ (B) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (C) mutants at all axial levels. In *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants, *Msx2* expression is increased in the lateral plate mesoderm (lpm) as well. (D-F) *Ap2α* expression at the neural epidermal boundary and in neural crest cells. Compared to wild-type 6-7 somite embryos (D), expression is increased in *Nog*$^{-/-}$ (E) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (F) mutants. (G-L) *Sox10* expression in neural crest cells. At the 8-9 somite stage, *Sox10* expression is unchanged in *Nog*$^{-/-}$ mutants (H), and slightly expanded in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants (I). At the 11-12 somite stage, expression is dramatically increased in both *Nog*$^{-/-}$ (K) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (L) mutant embryos. This region of expanded *Sox10* expression in the trunk is indicated by brackets. Abbreviations: mb, midbrain; hb, hindbrain; tr, trunk; cn, caudal neuropore.

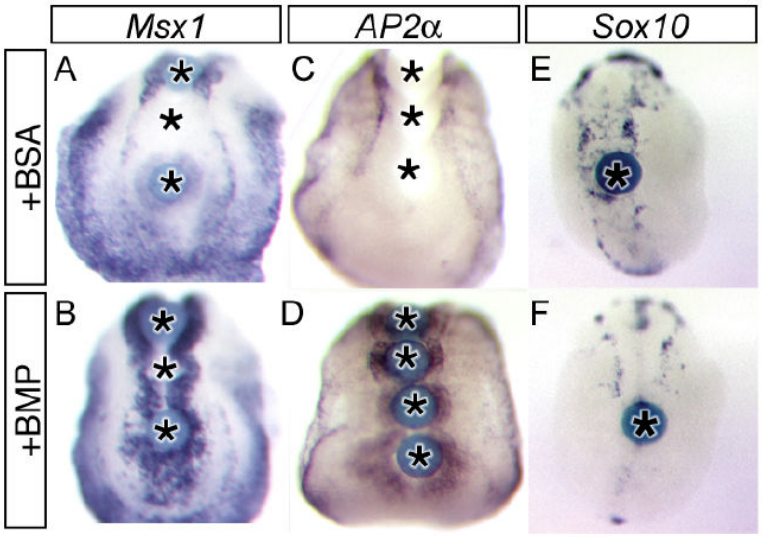

Figure 2.

BMP is not sufficient for neural crest cell induction in vitro. In wild-type explants of 5-7 somite embryos cultured for 7 hours with protein-soaked beads, BMP2, but not BSA, elicited increased amounts of *Msx1* (B) and *Ap2α* Bone morphogenetic protein (BMP) is not sufficient for neural crest cell induction in vitro. In wild-type explants of five- to seven-somite embryos cultured for 7 hr with protein-soaked beads, BMP2, but not bovine serum albumin (BSA), elicited increased amounts of Msx1 (B) and Ap2α adjacent to protein-soaked beads. adjacent to protein-soaked beads. Increased *Sox10* expression (E, F) was not observed with either treatment. Indeed, *Sox10* expression sometimes was decreased locally around the BMP beads (F), possibly due to increased cell death of NCC derivatives (see text). Bead locations during culture are indicated by asterisks.

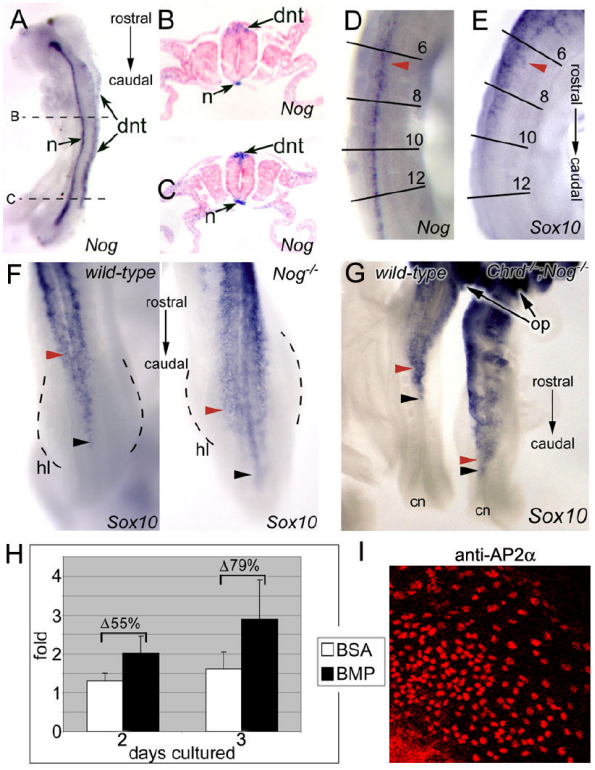

Figure 3.

Precocious delamination of NCCs in *Nog*$^{-/-}$ and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos. (A-D) Increasing NCC delamination (rostral to caudal) is reciprocal to *Nog* expression gradient. (A) Graded *Nog* expression in dorsal neural tube (dnt) of 10-somite embryo as detected by in situ hybridization. Expression in notochord (n) is not graded, and serves as an internal control. Rostral (B) and caudal (C) sections of embryo shown in panel A. Dorsal neural tube expression is stronger at more caudal levels. (D) In ~12s embryos, *Nog* expression is diminished rostrally, beginning at the level of the 6th–8th somite pair (red arrowheads). (E) *Sox10* expression is observed in NCCs emigrating from the dorsal midline beginning at the axial levels where *Nog* expression is diminished in the neural tube. (F) Dorsal aspect of trunk region of 22s wild-type and *Nog*$^{-/-}$ mutant embryos labeled with *Sox10* to mark NCCs. Both non-migrating (black arrows) and migrating NCCs (red arrows) are seen further caudally in *Nog*$^{-/-}$ mutants than in wild-type littermates (compare to axial level of hindlimb, hl, outlined by dashed lines). (G) *Sox10* expression in wild-type and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ littermates. Axial levels of NCC specification (black arrows) and migration (red arrows) are displaced further caudally in mutants (compare distance to closing lip of caudal neuropore). Exact stage matching by somite counting was not possible due to aberrant somite development in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants. (H) Treatment of cultures with recombinant BMP2 (n = 32) increased the migration index of neural crest cells by 54.4% after 2 days of culture, and by 79.4% after 3 days of culture, relative to explants cultured in BSA-treated medium (n = 18) ($p < 0.001$ for both comparisons). BMP concentrations of 100ng/ml and 200ng/ml gave results with no statistically significant difference, and were therefore pooled. (I) Antibody staining of NCC outgrowth cultures for AP2α. All migratory cells from trunk explants

express AP2α, All migratory cells from trunk explants express AP2α, indicating their identity as NCCs. Core of tissue at the lower left is a neural tube explant.

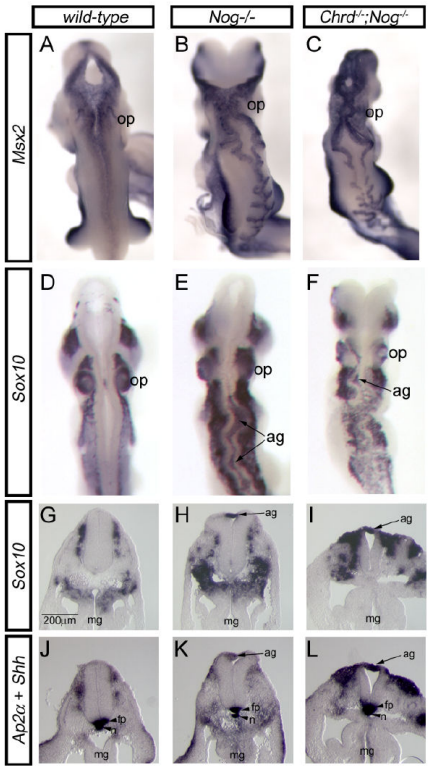

Figure 4.

BMP signaling and neural crest phenotypes at E9.5. Wild-type (A,D,G,J), *Nog*$^{-/-}$ mutant (B,E,H,K), and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (C,F,I,L) mutant embryos. (A-C) *Msx2* expression as and indicator of BMP signaling in the dorsal neural tube. (B) *Nog*$^{-/-}$ and (C) *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutant embryos display increased *Msx2* expression the dorsal midline. (D-F) *Sox10* expression in NCCs. (E) *Nog*$^{-/-}$ and (F) *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants display an increase in dorsal NCCs; in both classes, ectopic aggregates (ag) of NCCs accumulate at the dorsal midline. (G-L) Transverse section in situ hybridization for *Sox10* (G-I) and *Ap2α* + *Shh* (J-L) at the forelimb level. Increased amounts of NCCs are seen in (H,K) *Nog*$^{-/-}$ and (I,L) *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants. Distribution of NCCs in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants is biased more proximally to the dorsal neural tube than in *Nog*$^{-/-}$ mutants. For example, note in *Nog*$^{-/-}$ mutants more *Ap2α* and *Sox10* positive cells around the midgut (mg), whereas in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ more labeled cells are located dorsally. Ectopic aggregates are observed superficial to the dysmorphic dorsal neural tube of both mutant classes.

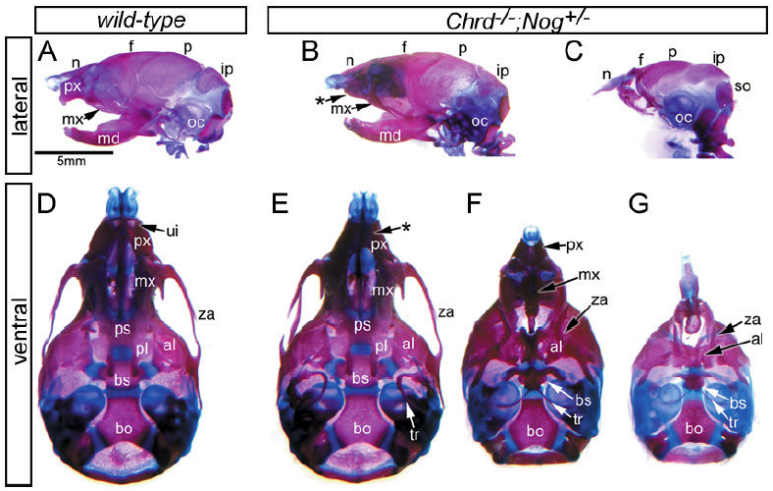

Figure 5.

Deficiency of skeletal neural crest derivatives in *Chrd*$^{-/-}$;*Nog*$^{+/-}$ mutants. Lateral (A-C) and ventral (D-G) views of wild-type (A,D) and *Chrd*$^{-/-}$;*Nog*$^{+/-}$ mutant (B,C,E-G) skulls. Affected mutants have a variable skull phenotype (arranged left to right with increasing severity) and show a corresponding lack of neural crest-derived craniofacial skeletal elements, such as upper incisors (ui; position marked by asterisk in mutants), medial premaxillae (px) and palate, and presphenoid (ps). Lateral structures such as the tympanic rings (tr) zygomatic arches (za), maxillae (mx), and alisphenoids (al) are displaced medially (tympanic rings removed from wild-type for clarity; compare E and F). Other abbreviations: nasal, n; frontal, f; parietal, p; interparietal, ip; supraoccipital, so; otic capsule, oc; mandible, md; palatine, pl; basoccipital, bo; basosphenoid, bs.

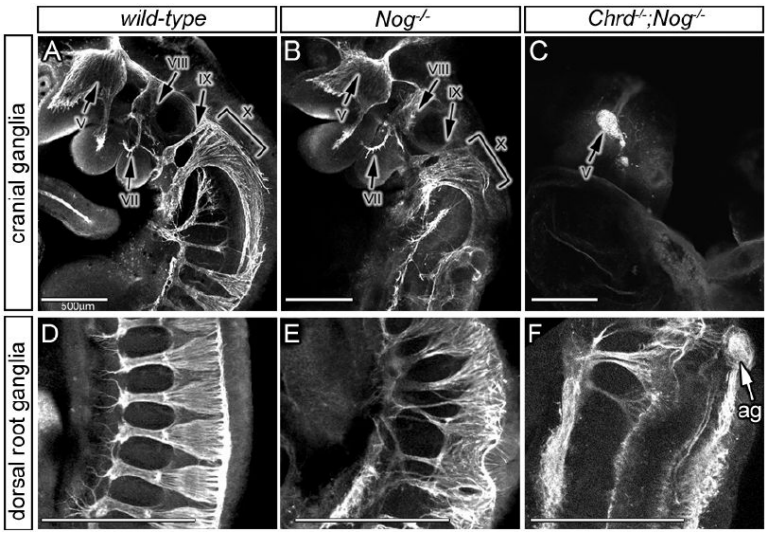

Figure 6.

Aberrant peripheral nervous system development with decreased BMP antagonism. (A-C) Lateral aspect of fluorescent neurofilament staining in E10.5 mutant embryos. Wild-type (A). In *Nog*$^{-/-}$ mutants (B) the vagus (X) is expanded and is indistinct from glossopharyngeal (IX). Severe *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants (C) lack most neurofilament immunoreactivity, but a rudimentary portion of the trigeminal ganglion (V) remains. (D-F) Higher magnification of dorsal root ganglia from mutant embryos. Wild-type (D). In *Nog*$^{-/-}$ mutants (E) DRGs are present, but are disarrayed and indistinct. In mild *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants (F), most DRGs are absent. Note neurofilament-positive aggregation (ag) dorsal to neural tube.

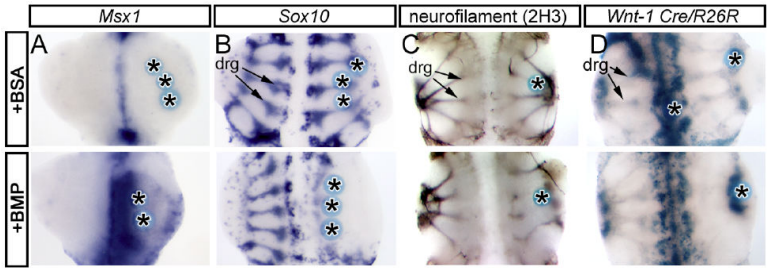

Figure 7.

Ectopic BMP disrupts dorsal root ganglia formation in explants. E9.5 trunk explants cultured for 24 hours with beads incubated in BSA (top row) or BMP (bottom row). Asterisks indicate bead positions during culture. The left side of each explant serves as an internal control. (A) *Msx1* expression at the dorsal midline. *Msx1* expression is not elicited by BSA-coated beads, but is induced to high levels by BMP beads. (B) *Sox10* expression in NCCs contributing to the peripheral nervous system. The distribution of *Sox10*-expressing cells is not affected by BSA beads, but is strongly disrupted by BMP2 beads. Arrows point to examples of dorsal root ganglia (drg). (C) NCC-derived sensory neurons stained for neurofilament (2H3) are not disrupted by BSA beads, but are significantly disrupted by BMP4-coated beads. (D) Genetic NCC-lineage tracing in explants prepared from *Wnt1-Cre;R26R* embryos; NCCs are stained blue. BSA beads has no effect, but BMP beads disrupts NCC contribution to peripheral tissue.

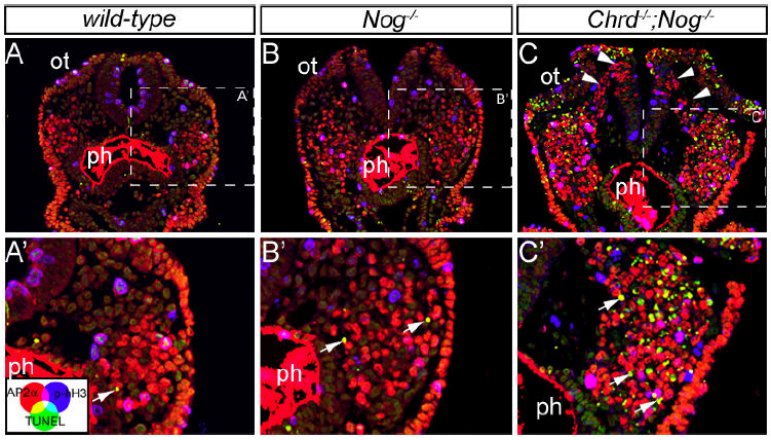

Figure 8.

Chrd and *Nog* protect migratory neural crest cells from BMP-mediated apoptosis. Transverse sections of 11-12s wild-type (A), $Nog^{-/-}$ mutant (B), and $Chrd^{-/-};Nog^{-/-}$ mutant (C) embryos, taken at the level of pharyngeal arch 2. (A'-C') Higher magnification of corresponding samples shown in A-C, as indicated by hatched boxes. Apoptotic TUNEL-positive cells are indicated in green, proliferating phospho-histone H3-positive cells in blue, and AP2α-positive cells in red, as in figure key (inset, A'). The number of NCCs and extent of NCC apoptosis (yellow, examples indicated by arrows) is increased in $Nog^{-/-}$ mutants, and much more so in $Chrd^{-/-};Nog^{-/-}$ mutants. Additionally, $Chrd^{-/-};Nog^{-/-}$ mutants show ectopic domains of AP2α-positve cells in the neural tube and streaming into the periphery (C, arrowheads). Differences in cell proliferation rates were not apparent at this stage. Positive signal in lumen of pharynx is specific staining of non-cellular material. Abbreviations: ot, otic placode, ph, pharynx.

Table 1

Induction of neural crest cell markers in explants

	+BSA				+BMP			
Marker	**decr.**	**∅**	**incr.**	***n*=**	**decr.**	**∅**	**incr.**	***n*=**
Ap2α	0	15	0	15	0	1	23	24
Cad6	0	6	0	6	0	0	7	7
Snail	0	4	0	4	0	4	0	4
Sox10	0	10	0	10	0	15	0	15
Msx1	0	12	1	13	0	0	14	14

Trunk explants from 5-8s embryos were cultured for 7 hours with BSA or BMP2 protein-soaked beads, then analyzed by in situ hybridization. Abbreviations: decr., decreased expression; ∅, no change; incr., increased expression.

Table 2

Ectopic BMP disrupts dorsal root ganglia development in explants

	+BSA			+BMP2			+BMP4		
Marker	**decr.**	**∅**	***n*=**	**decr.**	**∅**	***n*=**	**decr.**	**∅**	***n*=**
Ap2α	0	5	5	4	1	5	-	-	-
Sox10	0	10	10	12	1	13	-	-	-
2H3	0	5	5	2	4	6	3	3	6
R26R	0	4	4	8	2	10	-	-	-

	incr.	**∅**	***n*=**	**incr.**	**∅**	***n*=**	**incr.**	**∅**	***n*=**
Msx1	0	4	4	3	0	3	4	0	4

Trunk explants from E9.5 embryos were cultured for 24 hours with BSA (1mg/ml), BMP2 (10ug/ml), or BMP4 (10ug/ml) protein-soaked beads. Effects were assessed with in situ hybridization, immunohistochemistry (2H3), and lacZ staining (R26R). Abbreviations: decr., decreased expression; ∅, no change; incr., increased expression.