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Role of noggin as an upstream signal in the lack of neuronal derivatives found in the avian caudal-most neural crest

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Neural crest cells (NCCs) arising from trunk neural tube (NT) during primary and secondary neurulation give rise to melanocytes, glia and neurons, except for those in the caudal-most region during secondary neurulation (somites 47 to 53 in the chick embryo), from which no neurons are formed, either in vivo or in vitro. To elucidate this discrepancy, we have specifically analyzed caudal-most NCC ontogeny. In this region, NCCs emerge at E5/HH26, one day after full cavitation of the NT and differentiation of flanking somites. The absence of neurons does not seem to result from a defect in NCC specification as all the usual markers, with the exception of *Msx1*, are expressed in the dorsal caudal-most NT as early as E4/HH24. However, Bmp4-Wnt1 signaling, which triggers trunk NCC delamination, is impaired in this region due to persistence of noggin (*Nog*) expression. Concomitantly, a spectacular pattern of apoptosis occurs in the NT dorsal moiety. Rostral transplantation of either the caudal-most somites or caudal-most NT reveals that the observed features of caudal-most NCCs relate to properties intrinsic to these cells. Furthermore, by forced *Nog* expression in the trunk NT, we can reproduce most of these particular features. Conversely, increased Bmp4-Wnt1 signaling through *Nog* inhibition in the caudal-most NT at E4/HH24 induces proneurogenic markers in migratory NCCs, suggesting that noggin plays a role in the lack of neurogenic potential characterizing the caudal-most NCCs.

KEY WORDS: Apoptosis, Chick, Delamination, Msx1, Noggin, Wnt1, Neural crest, Neuronal differentiation

INTRODUCTION

Neural crest cells (NCCs) are a population of cells unique to vertebrates, which migrate from the dorsal aspect of the neural tube (NT) soon after its formation (Le Douarin and Kalcheim, 1999). It has been suggested that NCC specification occurs very early during development and involves the activity of Pax7, first detected at Hamburger and Hamilton stage 4^{+} ($HH4^{+}$) (Hamburger and Hamilton, 1951) in the epiblast layer (Basch et al., 2006). As development proceeds, *Pax7* expression gradually shifts to the neural folds where the NCCs will arise. A specific transcriptional program, resulting from a variety of signals secreted by the surrounding tissues, is then activated (Sauka-Spengler and Bronner-Fraser, 2008). Initially, a group of genes, often described as early markers, are activated in the prospective NCCs. These genes include several members of the Pax, Msx and Zic families. They collectively act to define a broad region containing cells competent to form the neural crest (NC). The subsequent generation of trunk NCCs requires the coordinated activity of *FoxD3*, *Snail2* and *Sox9*, considered as the trunk NCC 'specifying' genes. Their combined expression defines cells that manifest all the principal transcriptional and morphological characteristics of NCCs (Cheung et al., 2005). Once specified, NCCs delaminate from the dorsal neuroepithelium, undergoing an epithelial-to-mesenchymal transition (Duband, 2006). In addition, the onset of trunk NCC migration is thought to depend on Bmp-dependent Wnt activity coordinated by somitogenesis (Kalcheim and Burstyn-Cohen, 2005). All of these characteristics of NCC generation have been described principally in regions of primary neurulation. We recently showed that the same events occur more caudally in regions belonging to secondary neurulation (Osorio et al., 2009).

After detachment from the NT, NCCs migrate into the periphery along stereotypical pathways and arrive at specific locations, where they differentiate into a wide variety of cellular derivatives (Le Douarin and Kalcheim, 1999). Mesectodermal derivatives, like the craniofacial skeleton, derive only from the cephalic NC. Sensory neurons and glia derive from both the cephalic and trunk levels, whereas sympathetic neurons are trunk-specific. Melanocytes are produced along the entire rostrocaudal axis.

The most caudal region in both mammals and birds is characterized by the absence of motor nerves in the spinal cord and a lack of peripheral ganglia. In humans, this portion of the spinal cord constitutes the filum terminale, which extends through the sacrum to the first coccygeal vertebrae, whereas in birds it is located at the level of the pygostyle formed by the fusion of three to six caudal vertebrae (Catala et al., 2000). In the chick embryo, this part of the NT corresponds to the region of somites 47-53, which are the last pairs of somites to be formed during development. This 'caudal-most' part of the NT is formed at HH24 during the fourth day of embryonic development (E4). The lack of motor nerves arising from the spinal cord in this region has been described previously (Afonso and Catala, 2005). Concerning the absence of sensory ganglia and nerves, pioneer studies have shown that this is not due to a local lack of NCCs but rather to a restriction of their developmental potentials (Catala et al., 2000). Both in vitro culture and in vivo transplantation experiments have shown that these caudal-most NCCs give rise to Schwann cells and melanocytes, but never to neurons.

In the present study, our aim was to elucidate the mechanisms underlying the lack of neuronal derivatives that characterizes these caudal-most NCCs. We first determined the precise chronology of caudal-most NCC generation. Although the caudal-most NT is fully cavitated at E4/HH24 and adjacent somites are already differentiating, a very small number of NCCs are detected one day later, at E5/HH26. This is not due to a lack of specification of the NCCs. Instead, Bmp4-Wnt1 signaling, which is known to trigger

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trunk NCC delamination, is impaired in this region. In addition, an abnormal pattern of apoptosis is shown to take place at the same time (E4/HH24) in the dorsal half of the caudal-most NT. Both of these events undoubtedly contribute to the scarcity and delayed migration of caudal-most NCCs that in turn seem to culminate in their lack of neuronal potential. Results of heterotopic transplantation experiments of either the caudal-most somites or caudal-most NT into a more rostral region of younger embryos suggest that the features particular to caudal-most NCCs are the result of properties intrinsic to these cells. Furthermore, forced *Nog* expression in the trunk NT can reproduce the main characteristics observed for the caudal-most NCCs (scarcity and absence of neuronal derivatives), suggesting that impaired Bmp4 signaling is an event occurring upstream of the mechanisms operating in the caudal-most NT. Importantly, increased Bmp4-Wnt1 signaling, through inhibition of *Nog* in the caudal-most NT at E4/HH24, generates NCCs that express markers of specified neurogenic progenitors.

MATERIALS AND METHODS

Chick and quail embryos

Commercially available fertilized chick (*Gallus gallus domesticus*) and quail (*Coturnix coturnix japonica*) eggs were used. Embryos were staged according to E/HH and fixed in 4% paraformaldehyde. For sections (Leica cryostat, CM3050S), fixed embryos were cryoprotected and embedded in gelatin before freezing.

In ovo embryonic manipulations

Quail-chick transplantation experiments

Tails of E4/HH24 quail embryos were excised posteriorly to somite pair 46. After enzymatic dissociation, the rows of caudal-most somites and adjacent NT were isolated and transplanted into different E2/HH11-12 chick embryos. The caudal-most somites were transplanted bilaterally at the level of the posterior presomitic mesoderm (PSM) and the caudal-most NT was transplanted at the site of the endogenous NT, at the same level. Both types of grafts were performed over a length corresponding to ~ 4-5 prospective somites, keeping the correct anteroposterior (AP) and dorsoventral (DV) orientations. Chimeras were incubated for an additional 16-48 hours and then fixed.

Electroporation experiments

For caudal-most electroporations, DNA was injected into the lumen of the NT-facing somite 45 in E4/HH24 embryos. Electrodes were positioned in order to target the dorsal region of the caudal-most NT and a Wave Stimulator (A-M Systems, Model 2100) delivered seven pulses of 35 V, 50 milliseconds each. The following expression plasmids were used: pcDNA3.1, containing mouse full-length *Bmp4* DNA in frame with a GFP coding sequence (0.8 μg/μl); pcDNA6.1 Gw/EmGFP-miR chick *Nog* (1 μg/μl), constructed as described in the manufacturer's instructions using BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogen, K4935-00; experimental details are available upon request); and pMiwIII, containing chick full-length *Wnt1* DNA (1.0 μg/μl) (Matsunaga et al., 2002), coelectroporated with pEGFP-N1 (GenBank, U55762; Promega, 6085-1; 0.4 μg/μl).

For trunk electroporations, pCIG containing full-length mouse *Nog* DNA in frame with a GFP coding sequence (0.5 μg/μl) was injected into the lumen of the NT located at the level of the posterior PSM of E2/HH11-12 embryos. Five pulses of 25 V and 50 milliseconds each were delivered.

Embryos were incubated for an additional 8-48 hours and only those correctly electroporated, as verified by GFP expression, were used for posterior analyses.

In situ hybridization (ISH)

ISH was performed according to Henrique et al. (Henrique et al., 1995). The following chick-specific riboprobes were used: *Bmp4* (Francis-West et al., 1994), *Cad6B* and *Cad7* (Nakagawa and Takeichi, 1995), *FoxD3* (Dottori et al., 2001; Kos et al., 2001), *Msx1* and *Msx2* (Coelho et al., 1991; Coelho et al., 1992), *Ngn1* and *Ngn2* (Perez et al., 1999), *Nog* (Reshef et al., 1998), *Pax3* (Goulding et al., 1993), *Snail2* (Nieto et al., 1994), *Sox9* (Cheung and Briscoe, 2003), *Sox10* (Cheng et al., 2000), *Uncx4.1* (Schrägle et al., 2004), *Wnt1* and *Wnt3a* (Megason and McMahon, 2002). The following mouse-specific riboprobe was used: mouse (m) *Nog* (McMahon et al., 1998).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Afonso and Catala, 2005), using the following primary antibodies: anti-phospho Histone H3 (pH3 ; Upstate Biotechnology, 06-570), anti-Isl1/2 (DSHB, 39.4D5), anti-N-cadherin (Sigma, FA-5), anti-NC1/HNK1 (Vincent et al., 1983; Tucker et al., 1984), anti-Pax7 (DSHB), anti-QCPN (DSHB), anti-TuJ1 (Chemicon, MAB1632), and anti-WRS (Reedy et al., 1998; Harris et al., 2008).

Nile Blue Sulfate (NBS) staining

NBS staining (Jeffs and Osmond, 1992), modified as described in Teillet et al. (Teillet et al., 1998), was used to detect cell death in whole embryos. The embryos were photographed and then fixed for TUNEL assay.

TUNEL

Detection of apoptotic cells was performed using the TUNEL assay, following the manufacturer's instructions (Roche, 12156792910).

Acquisition and analysis of images and art graphics

Whole-mount embryos were photographed using a Nikon DXM1200 camera coupled to a Leica MZFLIII microscope. Sections were photographed with an Evolution VF camera coupled to a Nikon Eclipse E800 microscope, using Image-Pro Plus software (Media Cybernetics) and OptiGrid System (Optem). Images were analyzed in Adobe Photoshop CS3.

RESULTS

Scarcity and delayed migration of caudal-most NCCs

By using migratory NCC markers such as *Cad7*, *Sox10* and HNK1, we investigated the early generation of caudal-most NCCs at the level of somites 47-53 in the chick embryo. Although the caudal-most NT was fully cavitated and flanked by differentiating somites at E4/HH24, we found no migrating NCCs at this stage (Fig. 1A,C,E). In fact, migrating caudal-most NCCs only appeared at E5/HH26 (Fig. 1B,D,F), as indicated by a few *Cad7*$^+$ cells found dorsal to the NT (Fig. 1B,B′), some *Sox10*$^+$ cells seen migrating underneath the dermomyotome (Fig. 1D,D′) and HNK1$^+$ cells located in the migration staging area (MSA) in front of dissociated somites (Fig. 1F). Results from electroporation with a GFP-coding plasmid confined to the caudal-most NT support these observations, revealing GFP-positive cells that are HNK1$^+$ and *Sox10*$^+$ (see Fig. S1 in the supplementary material). These first results highlight two main features of the caudal-most NCCs: scarcity and delayed migration. Indeed, in more anterior regions of the trunk undergoing either primary or secondary neurulation, the first migratory NCCs appear soon after somitogenesis at the level of the second to fourth last-formed somites (Tosney, 1978; Thiery et al., 1982; Teillet et al., 1987; Osório et al., 2009).

NCC specification in the caudal-most region

Several hypotheses might account for the scarcity and delayed migration of the caudal-most NCCs, one of which is a possible defect in their specification. We therefore analyzed the expression of a number of genes with known involvement in NCC specification (Sauka-Spengler and Bronner-Fraser, 2008). *Pax3* and Pax7 were detected in the dorsal aspect of the caudal-most NT as early as E4/HH24 (Fig. 2A-B″). By contrast, *Msx1* was not observed (Fig. 2C-C″), even at E5/HH26 (see Fig. S2A-A″ in the supplementary material). However, *Msx2* was already expressed at E4/HH24 (Fig. 2D-D″). In addition, *FoxD3*, *Snail2* and *Sox9* were also present in

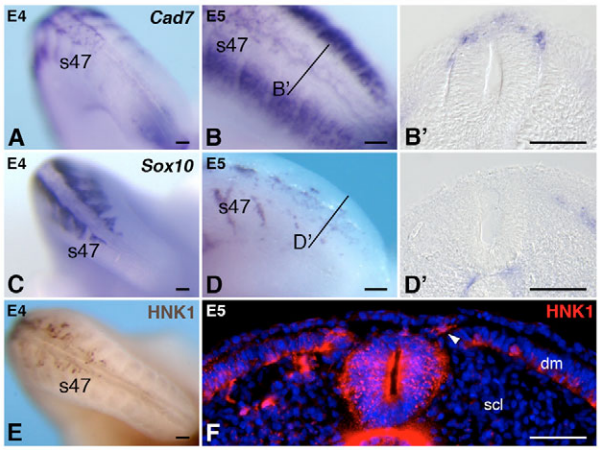

Fig. 1. Generation of caudal-most NCCs in the chick embryo. (**A-E**) Whole-mount detection of *Cad7* (A), *Sox10* (C) and HNK1 (E) in E4/HH24 embryos, showing the absence of NCCs in the caudal-most region posterior to somite 47 (s47). At E5/HH26, whole-mount preparations and cross-sections (at positions indicated) were hybridized for *Cad7* (B,B′) and *Sox10* (D,D′), revealing the presence of a few NCCs. (**F**) Cross-section immunolabeled with HNK1, showing NCCs located in the MSA (arrowhead). dm, dermomyotome; scl, sclerotome. Scale bars: 50 μm.

the dorsal aspect of the caudal-most NT at E4/HH24 (Fig. 2E-G″). We thus conclude that NCC specification has indeed occurred in the caudal-most NT at E4/HH24.

Lack of Bmp4 and Wnt1 signaling in the caudal-most NT

Both extrinsic and intrinsic mechanisms have been shown to be involved in the onset of NCC delamination at the trunk level, whereby the overall process seems to be modulated by dynamic changes in the adjacent somites (Kalcheim and Burstyn-Cohen, 2005). Signals emanating from epithelial somites inhibit *Nog* transcription in the dorsal NT and trigger a Bmp4-Wnt1 cascade, leading to NCC delamination (Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000; Burstyn-Cohen et al., 2004).

Since NCC specification in the caudal-most region of the chick embryo (at E4/HH24) is followed by a temporal lag and delayed migration of these cells (at E5/HH26), we analyzed the 'status' of both Bmp and Wnt pathways in this region. We observed that *Bmp4* is homogeneously distributed along the entire length of the caudal-most NT at E4/HH24 (Fig. 3A,A′). However, contrary to more anterior levels, *Nog* is not downregulated in the dorsal NT at the level of somites 47-53 as they form at E4/HH24 (Fig. 3B,B′) and continues to be expressed even after their complete dissociation at E5/HH26 (see Fig. S2B-B″ in the supplementary material). Interestingly, we did not detect *Wnt1* in the dorsal caudal-most NT (Fig. 3C,C′), even at E5/HH26 (see Fig. S2C-C″ in the supplementary material), whereas *Wnt3a* was expressed as soon as E4/HH24 (Fig. 3D,D′). These results show a lack of both Bmp4 and Wnt1 signaling in the dorsal caudal-most NT, which might contribute to the delayed caudal-most NCC migration.

Extensive cell apoptosis in the caudal-most NT at E4/HH24

In addition to the impairment in the signals normally triggering NCC delamination, other mechanisms, such as cell death, might account for the scarcity and delayed migration of the caudal-most NCCs.

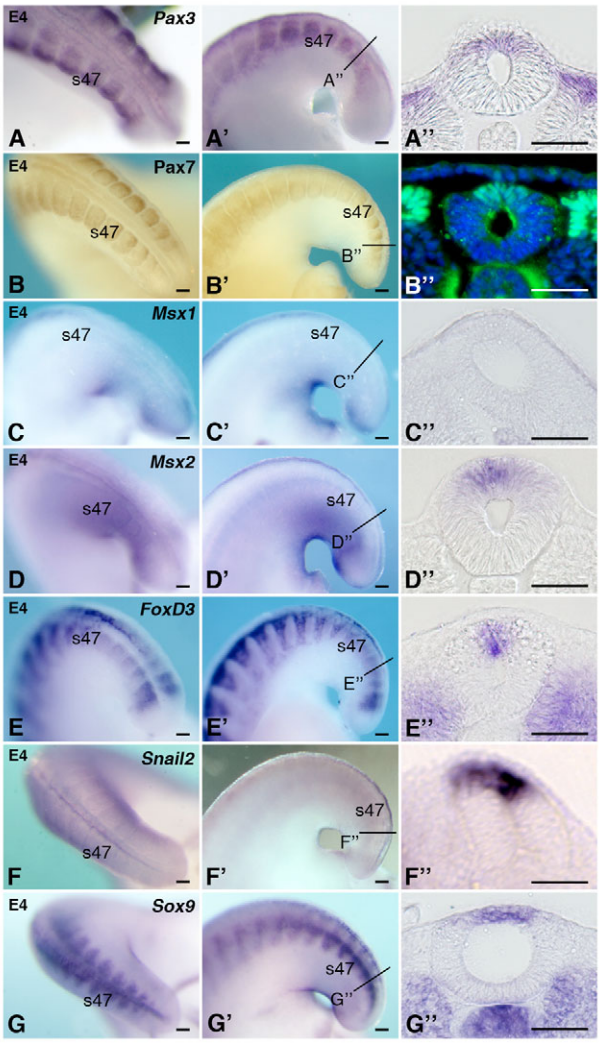

Fig. 2. NCC specification in the caudal-most NT in E4/HH24 chick embryos. Whole-mount ISH to detect *Pax3* in dorsal (**A**) and lateral (A′) views and cross-section (A″). Pax7 immunodetection in whole mount (**B**,B′) and cross-section (B″). Whole-mount ISH for *Msx1* (**C**,C′), *Msx2* (**D**,D′), *FoxD3* (**E**,E′), *Snail2* (**F**,F′) and *Sox9* (**G**,G′), and cross-sections caudally to the level of somite 47 (s47) (C″,D″,E″,F″,G″). All transcription factors, except for *Msx1*, are expressed in the dorsal region of the caudal-most NT at E4/HH24. Scale bars: 50 μm.

When compared with a more rostral region at an equivalent developmental stage (Fig. 4A,B), more pronounced cell death occurs along the caudal-most NT at E4/HH24, as shown by NBS staining (Fig. 4C) and TUNEL assay (Fig. 4D), which revealed massive apoptosis throughout the entire dorsal moiety of the caudal-most NT and the overlaying ectoderm. This is an exceptional and transient phenomenon, as no further apoptosis was observed in the caudal-most NT at E5/HH26 (Fig. 4E,F). To our knowledge, such apoptosis affecting the entire dorsal half of the NT has never been described at any level of the AP axis. Indeed, apoptotic cells within the trunk secondary NT showed no particular localization along the DV axis, like those in the primary NT (Hirata and Hall, 2000). Together with the lack of Bmp4-Wnt1 signaling, the massive apoptosis, which affects the dorsal moiety of the caudal-most NT at E4/HH24, must contribute to the observed drastic reduction in the number of NCCs formed in this region of the embryo.

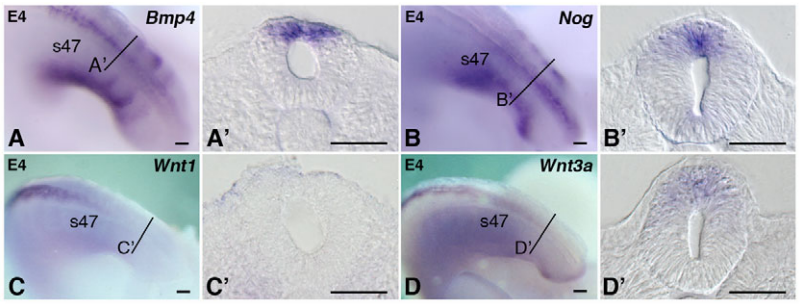

Fig. 3. Bmp4 and Wnt1 signaling in the caudal-most NT of E4/HH24 embryos. Whole-mount ISH to detect *Bmp4* (**A**), *Nog* (**B**), *Wnt1* (**C**) and *Wnt3a* (**D**) and cross-sections (A′,B′,C′,D′). *Bmp4* and its inhibitor *Nog* are both detected all along the dorsal caudal-most NT, facing somites posterior to somite 47 (s47) that are already differentiating (A-B′). (C,C′) *Wnt1* is not expressed, in contrast to *Wnt3a* (D,D′). Scale bars: 50 μm.

Caudal-most somites do not block trunk dorsal root ganglia (DRG) formation

The absence of *Wnt1* in the dorsal caudal-most NT was concomitant with the continued expression of *Nog*, despite maturation of the adjacent somites. We wondered whether these caudal-most somites were responsible for the lack of Bmp4-Wnt1 signaling observed in this region. We therefore performed heterotopic transplantation experiments, using the quail-chick chimera technique (Le Douarin, 1973). Quail caudal-most somites were grafted bilaterally at the level of the posterior PSM of E2/HH11-12 chick embryos (Fig. 5A′). Since NCC specification has already occurred at this level of the embryo (Sela-Donenfeld and Kalcheim, 1999), this allows us to evaluate the influence of caudal-most somites on NCC delamination and further development.

Most of the chimeras collected at 16-48 hours post-transplantation (hpt) were morphologically normal (21/25), and the grafts were always found between somites 22 and 28, validating their initial location. No change in *Sox10* expression was found at 16 hpt at the level of the graft, as indicated by anti-QCPN immunolabeling (Fig. 5B), and NCCs were found dorsally to the NT and in the MSA (Fig. 5B′; *n*=3). At 24 hpt, a defect in the segmented pattern of migration of NCCs facing the grafted caudal-most somites was apparent (Fig. 5C,C′; *n*=4). We observed that *Wnt1* expression in the dorsal NT was not modified (Fig. 5D,D′; *n*=3), indicating that Bmp4 activity continued in the presence of the grafted caudal-most somites. At 48 hpt, a continuous, non-segmented expression of *Sox10* (Fig. 5E,E′) was observed (*n*=3). In spite of this, DRG, formed by Isl1/2$^+$ postmitotic neurons (Avivi and Goldstein, 1999), developed at the graft level (Fig. 5F,G; *n*=4). Interestingly, the DRG facing the grafted caudal-most somites were irregularly segmented, smaller and more dorsally located than the normal DRG, as was found following the transplantation of a series of posterior half-somites at the place of the entire somites (Kalcheim and Teillet, 1989). However, normal striped *Uncx4.1* expression (Schrägle et al., 2004) indicated that the caudal-most somites presented anterior and posterior compartments (Fig. 5H,H′), as did the more rostral somites.

In conclusion, caudal-most somites provide the required signals for trunk NCC delamination and do not seem to be responsible for the lack of Bmp4-Wnt1 signaling occurring in the NT of the caudal-most region.

Rostral transplantation of the caudal-most NT does not restore DRG formation

To further investigate the role of the somitic environment in the defective Bmp4-Wnt1 signaling occurring in the caudal-most NT, we grafted the quail caudal-most NT at the posterior PSM level of E2/HH11-12 chick embryos (Fig. 5A″), thereby confronting it with all of the steps of trunk somitogenesis.

Most of the chimeras that survived up to E4/HH24 were morphologically normal (21/26), with some embryos presenting fused somites at the midline just prior to and/or after the level of the graft, revealing a local interruption of the NT due to the lack of growth of the grafted caudal-most NT. The transplant was generally found in the trunk region between somites 20 and 25, justifying its initial location. We first analyzed NCC generation from the graft. At 24 hpt, no quail *Sox10*$^+$ cells were observed (Fig. 5I,I′; *n*=5), even when the adjacent somites were well formed. At 48 hpt, quail HNK1$^+$ cells were detected dorsally to the NT (Fig. 5J,J′). They seemed more numerous than NCCs of the caudal-most region in situ at an equivalent stage. However, no DRG were ever detected (Fig. 5J; *n*=5). It should be noted that quail DRG were still absent in similar chimeras at E12, where only quail melanocytes and glial cells had differentiated (Catala et al., 2000). Moreover, we found that some quail NCCs at 48 hpt were WRS$^+$ (Fig. 5K), corresponding to early melanocyte precursors (Reedy et al., 1998). In addition, at 24 hpt, *Wnt1* expression in the dorsal region of the ectopically grafted caudal-most NT was not restored (Fig. 5L,L′; *n*=3), indicating continued impairment of Bmp4 signaling.

Taken together, the results of the rostral transplantation of either the caudal-most somites or the caudal-most NT indicate that Bmp4-Wnt1 signaling defects and a lack of neuronal potential of the caudal-most NCCs are properties intrinsic to these cells.

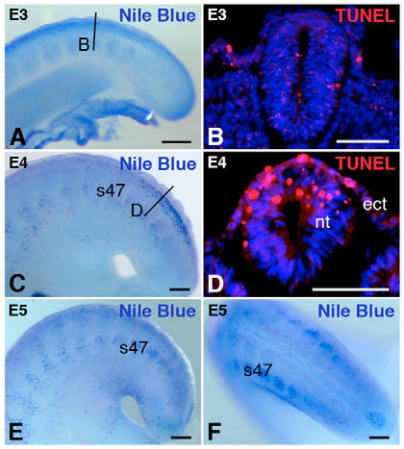

Fig. 4. Cell apoptosis in the caudal-most NT. (**A-F**) NBS staining and TUNEL assay. (A,C,E,F) NBS staining in whole embryos at E3/HH18 (A), E4/HH24 (C) and E5/HH26 (E,F). Note the intense level of apoptosis detected caudally to somite 47 (s47) at E4/HH24. (B,D) TUNEL assay on cryosections at E3/HH18 (B) and E4/HH24 (D) at equivalent regions. Apoptosis affects the dorsal moiety of the caudal-most NT (nt) and the overlaying ectoderm (ect) at E4/HH24. Scale bars: 50 μm.

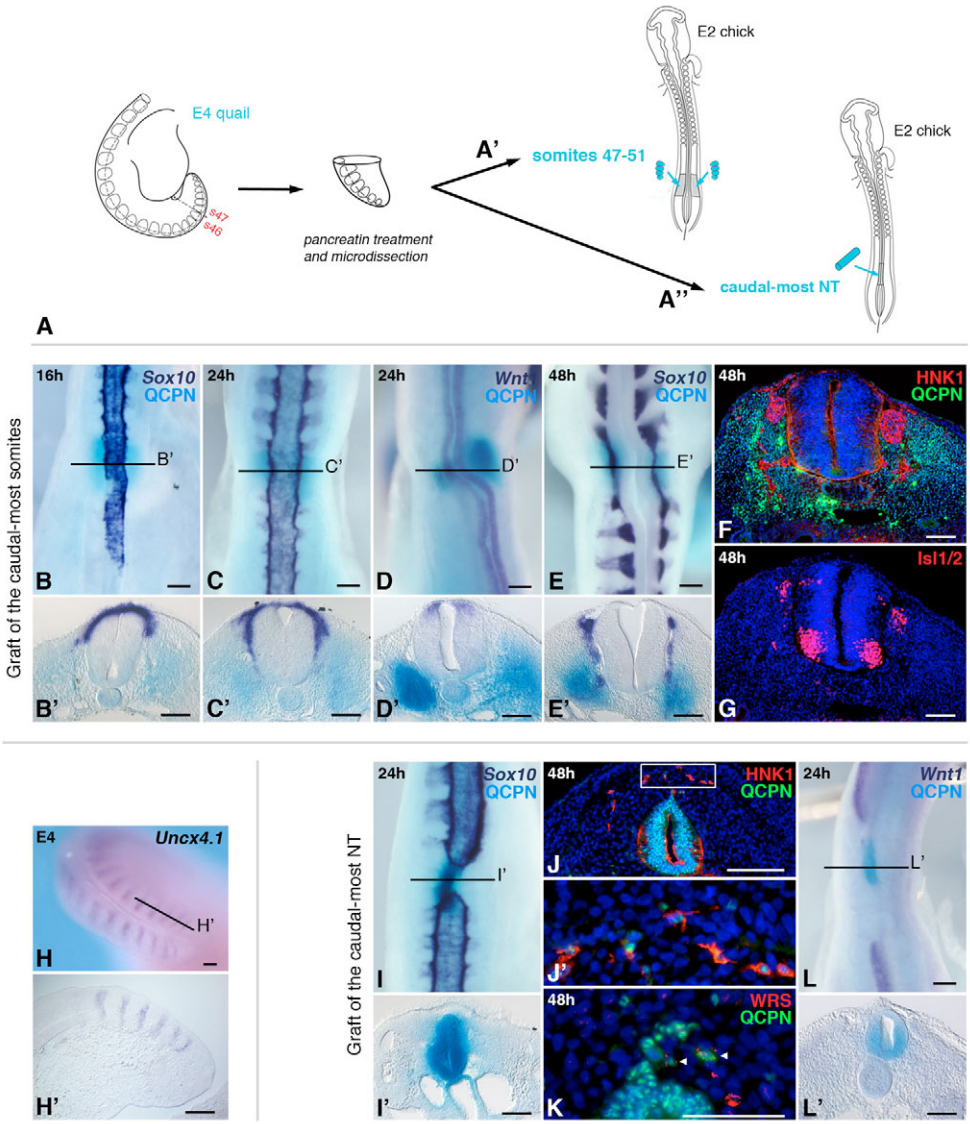

Fig. 5. Rostral transplantation of quail caudal-most somites or NT into E2/HH11-12 chick embryos. (**A**) Schematic representation of the heterotopic transplantations of caudal-most somites (A′) and caudal-most NT (A″) and respective results (**B-H′** and **I-L′**). (B,C,E) *Sox10* and QCPN in whole chimeras 16 (B), 24 (C) and 48 (E) hpt. *Sox10*$^+$ cells are located dorsal to and close to the NT at the level of the graft (B′,C′,E′). (D) Chimera labeled for *Wnt1* and QCPN 24 hpt and cross-section in the graft region (D′). (F) Immunohistochemistry for HNK1 and QCPN, showing sensory ganglia facing the transplanted caudal-most somites at 48 hpt. (G) These ganglia present Isl1/2$^+$ postmitotic neurons. (H) Whole-mount ISH to detect *Uncx4.1* and parasagittal section (H′) revealing two distinct compartments in the caudal-most somites. (I) *Sox10* and QCPN in whole chimera 24 hpt of quail caudal-most NT. No quail NCCs are detected at the level of the graft (I′). Forty-eight hpt, double labeled HNK1-QCPN quail NCCs are detected dorsal to the grafted NT (J,J′). (K) Some quail NCCs are WRS$^+$ (melanocyte progenitor cells; arrowheads). (L) Whole-mount ISH to detect *Wnt1* and QCPN 24 hpt and cross-section at graft level (L′); *Wnt1* is not restored. Scale bars: 50 μm.

Forced expression of *Nog* in the trunk NT mimics the main features of a caudal-most NCC 'phenotype'

The results obtained so far suggest that the maintenance of *Nog* expression in the caudal-most NT is a crucial upstream event in the molecular mechanisms directing caudal-most NCC development. In order to strengthen this hypothesis, we analyzed the effect of forced *Nog* expression in the trunk NT located at the level of the posterior PSM of E2/HH11-12 chick embryos, where NCC specification has already occurred. In contrast to the experiments in which Nog-secreting cells grafted close to the dorsal NT prevented NCC migration from the neuroepithelium (Sela-Donenfeld and Kalcheim, 1999), our aim was to evaluate the effect of maintaining *Nog* expression in the pre-migratory NCCs themselves. At 8 hours post-electroporation (hpe), we found *Sox10*$^+$ NCCs along the entire length of the electroporated NT (Fig. 6A,B; *n*=6). Interestingly, these cells remained dorsal to the NT at the level of both epithelial and dissociating somites and did not migrate ventrally (Fig. 6B′,B″), as NCCs do in control GFP-electroporated embryos (see Fig. S3A-B″ in the supplementary material). Moreover, delaminated GFP$^+$ NCCs did not express *Nog*, suggesting that they were influenced by Nog emanating from the adjacent NT (Fig. 6C,C′; *n*=4). At 24 and 48 hpe, NCCs remained dorsal to the NT and in the MSA, with no formation of DRG (Fig. 6D-G; *n*=5). In addition, *Cad6B* expression was decreased at 12 hpe (Fig. 6H,I; *n*=3), whereas at 24 hpe N-Cad was upregulated in both the dorsal NT and the NCCs overlaying the NT, but absent from NCCs located in the MSA (Fig. 6J,K; *n*=3). Nevertheless, some *Cad7*$^+$ NCCs were present dorsally at 48 hpe (Fig. 6L-M; *n*=3). In addition, *Msx1* (Fig. 6N; *n*=4), *Wnt1* (Fig. 6O; *n*=4) and *FoxD3* (Fig. 6P; *n*=3) were absent from the electroporated region at 24 hpe.

We went further in our analysis by examining the fate of the NCCs generated under these conditions. We found that some of the cells were in fact neuronal precursors *Ngn1*$^+$ (Fig. 6Q-R; *n*=3) (Perez et al., 1999). However, anti-TuJ1 and anti-Isl1/2 immunolabeling showed differentiating neurons only inside the NT (Fig. 6S,T; *n*=5). More importantly, a great number of the migratory NCCs were TUNEL$^+$ and massive apoptosis also occurred in the electroporated NT in a non cell-autonomous manner (Fig. 6U; *n*=5).

In conclusion, forced *Nog* expression in the dorsal trunk NT does not completely block NCC migration. However, the possibility that the migration process had begun before effective ectopic *Nog* expression occurred must be considered. In addition, forced *Nog*

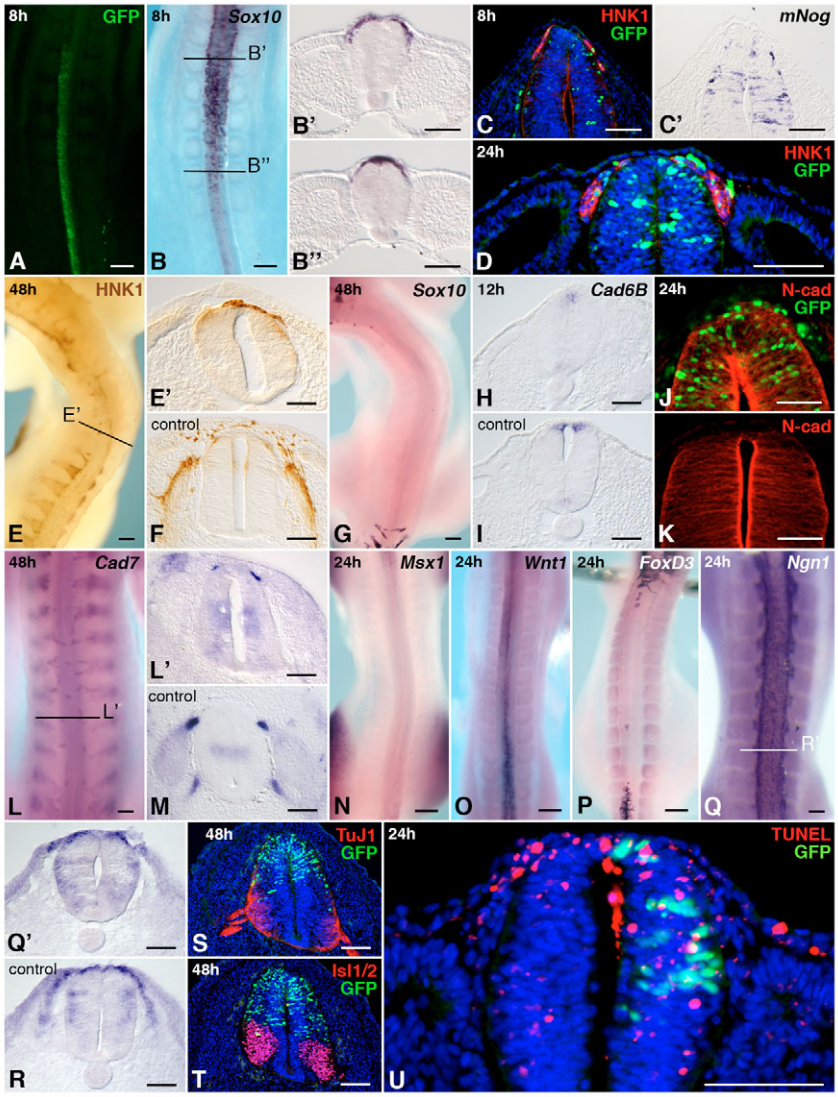

Fig. 6. Forced *Nog* expression in the trunk NT of E2/HH11-12 chick embryos. Whole-mount GFP expression (**A**) and ISH for *Sox10* (**B**) 8 hpe. Cross-sections showing NCCs located dorsally to the NT at dissociating (B′) and epithelial (B″) somite levels. Cross-section immunolabeled with HNK1 (**C**) and ISH for m*Nog* (C′) 8 hpe. Cryosection labeled with HNK1 and GFP 24 hpe (**D**). Whole mount and cross-section labeled with HNK1 48 hpe (**E**,E′), with control cross-section (**F**). (**G**) Whole-mount *Sox10* expression. ISH to detect *Cad6B* 12 hpe (**H**) and control (**I**) cross-sections. Immunodetection of N-Cad 24 hpe (**J**) and control (**K**) cross-sections. ISH to detect *Cad7* in whole mount (**L**) and cross-section (L′) 48 hpe and control cross-section (**M**). *Msx1* (**N**), *Wnt1* (**O**) and *FoxD3* (**P**) 24 hpe, all absent in the electroporated NT. *Ngn1* in whole mount (**Q**) and cross-section (Q′) 24 hpe and control cross-section (**R**). (**S**,**T**) Immunodetection of TuJ1 and Isl1/2 in adjacent cross-sections 48 hpe. (**U**) TUNEL assay on cryosection shows massive apoptosis in the electroporated NT and NCCs 24 hpe. Scale bars: 50 μm.

expression abolishes NCC ventral migration and prevents ganglia formation. Thus, it mimics the main features of the caudal-most NT (absence of *Msx*1 and *Wnt1*, and apoptosis) and the specific caudal-most NCC ‘phenotype’ (scarcity and lack of neuronal derivatives). However, some differences concerning *Cad6B*, N-Cad and *FoxD3* expressions were observed.

Nog inhibition in the caudal-most NT can induce neurogenic NCCs

In order to scrutinize more deeply the role of Bmp4, Nog and Wnt1 signaling in defining the features observed in the caudal-most NCCs, we experimentally modified their gene expression by in ovo electroporation in the dorsal caudal-most NT at E4/HH24. Twenty-four hours after *Bmp4* or miRNA-*Nog* electroporation, we found an increased number of HNK1$^+$ (Fig. 7A,B,E,F) and *Sox10*$^+$ (Fig. 7C,C′,G,G′) cells migrating dorsoventrally. Most of the HNK1$^+$ cells were GFP-negative, indicating a non cell-autonomous effect (Fig. 7A,B,E,F). *Wnt1* expression was restored in the dorsal aspect of the electroporated caudal-most NT (Fig. 7D,D′,H,H′). As expected, *Nog* had disappeared after miRNA-*Nog* electroporation (Fig. 7I,I′). In the *Wnt1*-electroporated embryos, we also found that NCC number had increased in a non cell-autonomous manner (Fig. 7J,K). A large number of *Sox10*$^+$ NCCs were found migrating through the dorsal pathway (Fig. 7L,L′). In addition, *Msx1* expression was partially restored (Fig. 7M,M′) and *FoxD3* expression enlarged (Fig. 7N,N′). The number of NCCs increased further 48 hours after miRNA-*Nog* electroporation (Fig. 7O,O′). The same observation was made after *Bmp4* or *Wnt1* overexpression (data not shown). Importantly, *Nog* inhibition generated caudal-most NCCs expressing *Ngn1* (Fig. 7P,P′) and *Ngn2* (Fig. 7Q,Q′), corresponding to neuronal precursors. Thus, restoration of Bmp4-Wnt1 signaling in the caudal-most NT, through *Nog* inhibition, confers upon caudal-most NCCs the potential for neuronal differentiation.

DISCUSSION

In the present study, we have analyzed the properties of the NCCs in the caudal-most region of the avian embryo. Our results show that the drastic decrease in cell number and delayed migration that distinguish the caudal-most NCCs from more rostral ones are most probably the result of an abnormal *Nog* expression and the consequent lack of Bmp4-Wnt1 signaling in the caudal-most dorsal NT. Defective NCC delamination is concomitant with massive cell apoptosis in the dorsal moiety of the NT, that together provide an explanation for the lack of neuronal NCC derivatives forming at the most caudal level of the embryonic AP axis. These features can be

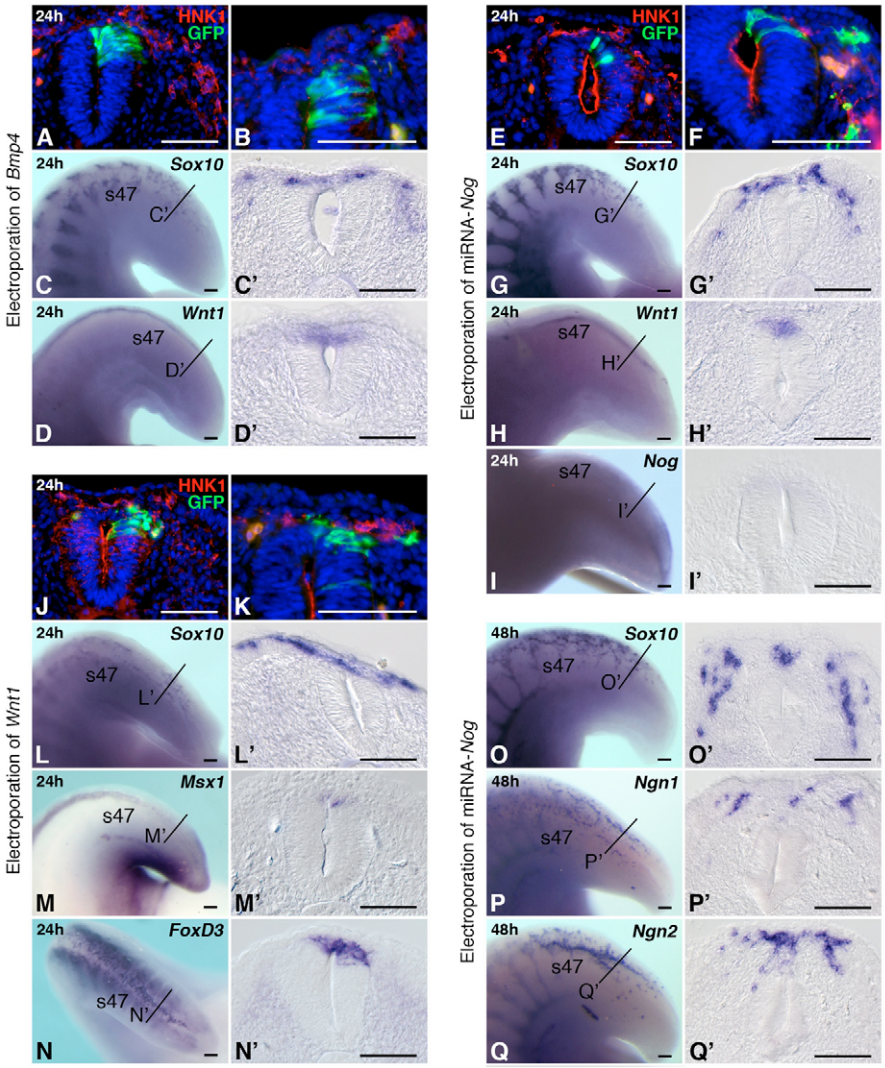

Fig. 7. Increased Bmp4 and Wnt1 signaling in the dorsal caudal-most NT of E4/HH24 chick embryos. Twenty-four hpe of *Bmp4* (**A-D'**), miRNA-*Nog* (**E-I'**) and *Wnt1* (**J-N'**). (A,B,E,F,J,K) Cryosections labeled with HNK1 and GFP, showing numerous NCCs close to the dorsal caudal-most NT. Whole-mount ISH for *Sox10* (C,G,L) and cross-sections (C',G',L'). *Wnt1* expression is restored in the dorsal aspect of the caudal-most NT after *Bmp4* (D,D') or miRNA-*Nog* (H,H') electroporation. *Nog* is not detected in the caudal-most NT electroporated with miRNA-*Nog* (I,I'). Electroporation of *Wnt1* in the dorsal caudal-most NT partially restores *Msx1* expression (M,M') and increases *FoxD3* expression (N,N'). Forty-eight hpe of miRNA-*Nog* (**O-Q'**). ISH to detect *Sox10* (O,O'), *Ngn1* (P,P') and *Ngn2* (Q,Q'). s47, somite 47. Scale bars: 50 μm.

reproduced at the mid-level of the trunk by *Nog* overexpression. Moreover, increased Bmp4-Wnt1 signaling through *Nog* inhibition in the caudal-most NT confers upon caudal-most NCCs the potential for neuronal differentiation.

Absence of neuronal derivatives in the caudal-most region: a consequence of the delayed NCC emigration?

A correlation between the timing of NCC migration from the dorsal NT, their migration pathways and the types of derivatives that they form has long been suggested (reviewed by Le Douarin and Kalcheim, 1999). The first cells that delaminate at the trunk level travel into the ventral pathway between the NT and the anterior half of each somite in a segmented manner. These mainly give rise to neurons and glia of both the sensory and sympathetic nervous systems. By contrast, later migrating NCCs travel in a uniform and unsegmented fashion into the dorsal pathway between the somites and the overlying ectoderm and generate skin melanocytes. Migration into this last pathway is delayed by ~24 hours in the chick embryo (Erickson et al., 1992). In light of such correlations, the 'temporal lag' observed in the caudal-most region between NCC specification at E4/HH24 and their migration at E5/HH26, associated with the lack of neuronal derivatives, led us to propose that early migrating NCCs, which normally give rise to neurons, are lacking at this level. NCCs that delaminate at E5/HH26 most probably correspond to the late migrating population that principally gives rise to melanocytes.

Mechanisms of elimination of early migrating NCCs

Absence of the early migrating NCC population might be the result of the massive cell apoptosis which occurs in the dorsal caudal-most NT. This cellular event shows high temporal precision and occurs at E4/HH24 (see Fig. 4), with no observable apoptotic cells by E5/HH26 (see Fig. S2 in the supplementary material). This argues in favor of a specific elimination of the early migrating NCC population, with those NCCs arriving later being unaffected. In our study, we were unable to identify the cause of this massive apoptosis. The relation between defective NCC migration and neuroepithelial cell death remains unclear. One possibility is that defective Bmp4-Wnt1 signaling prevents the delamination of NCCs following their specification, thereby rendering the cells incapable of survival, as has been reported in other studies (Vogel and Weston, 1988; Cheung et al., 2005). Neurogenic cell death could also be a direct consequence of the absence of Wnt1, described as a cell survival factor in distinct cell types (He et al., 2004; You et al., 2004; Almeida et al., 2005). Other roles for Wnt1 must also be considered, as it promotes the expansion of NCCs (Ikeya et al., 1997; Megason and McMahon, 2002; Dunn et

al., 2005). The increase in NCC number obtained in our electroporation experiments in the caudal-most NT is consistent with both putative Wnt1 activities. According to our results, these might involve the activity of *Msx1* and/or *FoxD3*, both induced in response to ectopic *Wnt1* expression. In fact, both of these genes have been reported to play an important role in the maintenance of NCC progenitors, by protecting pre-migratory or early migrating NCCs from apoptosis (Ishii et al., 2005; Lister et al., 2006; Teng et al., 2008). It is important to mention that *FoxD3* is already present in the dorsal caudal-most NT at E4/HH24, in contrast to *Msx1*, which is lacking (see Fig. 2 and Fig. S1 in the supplementary material). This indicates that the lack of Bmp4-Wnt1 signaling has no consequence on the initial expression of *FoxD3*, in contrast to that of *Msx1*.

Pre-specification or pluripotency of NCCs in the caudal-most region?

Several studies in both chick and zebrafish embryos have shown that late migrating NCCs are able to counterbalance the loss of the early migrating NCCs (Raible and Eisen, 1996; Baker et al., 1997), highlighting the high level of plasticity of these cells, as well as the role of the environment in determining their fate (Le Douarin et al., 2004). However, in the caudal-most region, the NCCs generated later do not compensate for the loss of the early migrating population, as they do not contribute to the formation of neuronal derivatives. A similar observation has been reported in other experimental situations (Maynard et al., 2000). Cumulating evidence suggests that NCCs constitute a heterogeneous population of cells containing both pluripotent and fate-restricted progenitors (Harris and Erickson, 2007). The existence of 'pre-specified' lineages, an early migrating lineage endowed with neurogenic potential and a later migrating lineage with melanogenic ability, has already been described (Erickson and Goins, 1995; Henion and Weston, 1997; Reedy et al., 1998). In light of such observations, the early migrating population of NCCs that is absent in the caudal-most region most probably corresponds to the population of cells with neurogenic potential. In addition, the fact that no neuronal derivatives are formed even when the caudal-most NT is transplanted into a more rostral somitic environment indicates that this is an intrinsic property of all caudal-most NCCs. It also suggests that the signals required for the establishment of this neurogenic potential are not effective in the dorsal caudal-most NT. Although formal in vivo evidence of such a pre-specified population of neurogenic progenitors is still lacking in the chick embryo, its existence has been demonstrated in zebrafish and mouse (Raible and Eisen, 1994; Wilson et al., 2004).

Variations along the AP axis in the mechanisms triggering NCC delamination

Despite maintained levels of *Nog* expression and lack of Bmp4-Wnt1 signaling in the caudal-most NT, NCCs are still able to delaminate at this level in the chick embryo (see Fig. 1). This suggests that mechanisms other than those based on Bmp-dependent Wnt signaling, currently proposed for the trunk (Shoval et al., 2007), are operating in this region. It is tempting to consider the existence of a correlation between the type of mechanisms involved in NCC delamination and the nature of their derivatives. In such a way, early emigrating NCCs contributing to neuronal derivatives would be dependent on Bmp4-Wnt1 activities, whereas those migrating later and giving rise to melanocytes would not. In addition, whilst we did demonstrate that *Nog* overexpression in the trunk NT was able to mimic the main features of the caudal-most NT and NCCs, we also ascertained some important differences. In this experimental situation, we found decreased *Cad6B* expression in prospective NCCs, whereas N-Cad was upregulated both in the dorsal NT and in the NCCs located dorsally to it (see Fig. 6). In the caudal-most region however, although *Nog* expression is maintained, *Cad6B* is found in the prospective NCCs, whereas N-Cad is absent from these cells (data not shown). This supports our statement that mechanisms governing NCC delamination in the caudal-most region of the embryo are specific to this region. It should be noted that specific Bmp signaling-independent and Ets1 activity-dependent mechanisms have already been reported in the control of cranial NCC delamination (Théveneau et al., 2007).

In spite of the obvious changes in the adhesion properties of the prospective NCCs induced by ectopic *Nog* expression in the trunk NT, we still found some delaminating NCCs. This is puzzling in the light of recent data showing that *Nog* overexpression maintains N-Cad, causing an almost complete failure of NCC delamination (Shoval et al., 2007). However, one possibility to explain this discrepancy might be of technical order: in our experiments, we have used a concentration of 0.5 μg/μl of DNA for *Nog* electroporation, whereas Shoval and colleagues used a DNA concentration of 3-5 μg/μl. In fact, it seems that the effect of *Nog* is dose-dependent, as we were able to completely block trunk NCC delamination by using a 2.0 μg/μl concentration of *Nog* expression plasmid (our unpublished data).

The Bmp-dependent Wnt signaling model, first described in relation to the onset of trunk NCC delamination (Kalcheim and Burstyn-Cohen, 2005), could be more complex than previously believed. In fact, the results of our heterotopic transplantation experiments raise important questions concerning the specific role of the epithelial somites in the regulation of Wnt1 signaling by *Nog* downregulation. Firstly, we found that *Wnt1* was not restored in rostrally transplanted caudal-most NT (see Fig. 5L,L′) and secondly, caudal-most somites did not prevent *Wnt1* expression in the trunk NT (see Fig. 5D,D′). This suggests that the dorsal NT itself, in particular the caudal-most NT, plays an important role in NCC delamination by its own ability to respond to the somitic signal(s).

Nature of the signals involved in the generation of distinct NCC lineages

One main conclusion from our study concerns the identity of the Wnt signal involved in melanocyte specification. Although the implication of Wnt signaling in the generation of melanocytes has been clearly demonstrated, previous studies have been unable to discriminate the exact nature of the signal involved (Jin et al., 2001; Ikeya et al., 1997; Dorsky et al., 1998; Hari et al., 2002). Our results in the chick embryo suggest that the generation of the later migrating NCCs, and thus melanocyte progenitors, specifically depends on a Wnt3a, and not Wnt1, signal. This is supported by other data showing that Wnt3a but not Wnt1 promotes melanocyte differentiation at the expense of other derivatives (Dunn et al., 2005). Nevertheless, we observed a large number of NCCs migrating through the dorsal pathway after *Wnt1* overexpression in the caudal-most NT (see Fig. 7). Ectopic *EphB2* or *EDNRB2* expression was recently shown to be sufficient to induce dorso-lateral migration of non-melanogenic NCCs (Harris et al., 2008). Ectopic *Wnt1* expression might produce a similar effect. In fact, NCCs migrating along the dorsal pathway under these conditions are not WRS^{+} (data not shown), indicating that they are not melanoblasts. Wakamatsu et al. (Wakamatsu et al., 1998) previously reported the brief presence of some NC-derived neuronal cells on the dorsal pathway, before their removal by an episode of apoptosis.

The nature of the signals controlling neuronal differentiation has been more difficult to identify. Our results in the caudal-most region suggest that both Bmp4 and Wnt1 are required for the generation of

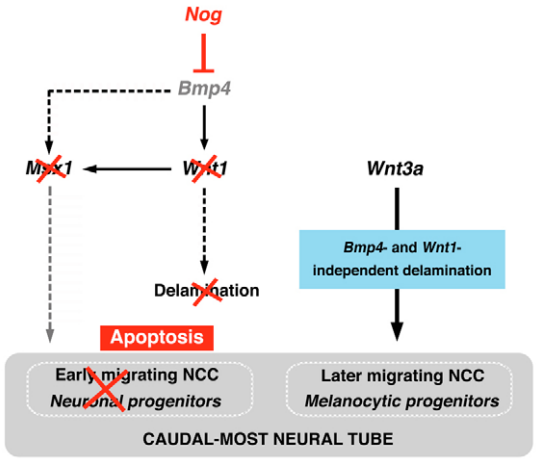

Fig. 8. A model for the lack of neuronal NCC derivatives in the caudal-most region of the chick embryo. Caudal-most NCCs are characterized by their scarcity and delayed migration. Maintenance of *Nog* expression impairs Bmp4 signaling, thus leading to the absence of its targets *Msx1* and *Wnt1*. This impairment is concomitant with a massive dorsal NT apoptosis at E4/HH24 that culminates in the elimination of the early migrating NCC population that would contribute to neuronal derivatives. By contrast, later migrating NCCs mainly constituting melanocytic progenitors specifically require *Wnt3a*. Delamination of these cells involves a mechanism other than one that depends on Bmp4-Wnt1 signaling.

early migrating NCCs and thus neuronal derivatives. In the chick and mouse embryos, Bmp and Wnt signaling has been implicated in the generation of sympathetic and sensory neuronal derivatives (Huber, 2006; Sommer, 2006). In addition, Wnt1 seems to have an instructive influence on the sensory fate in multipotent NCCs (Kleber et al., 2005; Lee et al., 2004). Importantly, we have shown that increased Bmp4-Wnt1 signaling in the caudal-most NT induces NCCs to be specified as neurogenic progenitors (see Fig. 7). At 48 hpe, the longest survival time of the electroporated embryos that we were able to obtain, we observed no definitive neuronal differentiation. It should be noted here that in order to target the caudal-most NT, our electroporation experiments needed to be performed at E4/HH24. This is precisely the stage at which a massive apoptosis is occurring and thus a selective elimination of early migrating NCCs might already have been underway. In addition, in the zebrafish embryo, early migrating NCCs endowed with the ability to give rise to DRG neurons require appropriate environmental factors to express this intrinsic ability (Raible and Eisen, 1996). Thus, we cannot exclude the possibility that an asynchrony between NT and adjacent somite maturation contributes to the absence of peripheral ganglia in the caudal-most region.

Conclusions

NCCs arising from the caudal-most region of the avian embryo, located from somites 47 to 53, are characterized by their inability to give rise to neuronal derivatives. In the present work, we addressed the mechanisms underlying this particular feature. We have found that caudal-most NCC development, and in particular their delamination, is governed by specific mechanisms unique to this level of the AP axis. The results obtained led us to propose that the early migrating NCCs that normally give rise to neurons are absent from this region (Fig. 8). This is the consequence of continued expression of *Nog* and subsequent lack of Bmp4-Wnt1 signaling occurring in the caudal-most NT, concomitant with extensive cell death. Indeed, inhibition of *Nog* in the caudal-most NT is sufficient to induce NCC expressing neurogenic markers. Thus, *Nog* acts as an upstream signal for the lack of neuronal derivatives from the caudal-most NCCs. In addition, delamination of the later migrating population of NCCs that generates melanocytes is independent of Bmp4-Wnt1 signaling.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/cgi/content/full/136/10/1717/DC1

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