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The specification of noradrenergic locus coeruleus (LC) neurones depends on bone morphogenetic proteins (BMPs)

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SUMMARY

The role of BMPs in the development of the major noradrenergic centre of the brain, the locus coeruleus (LC), was investigated. LC generation is reflected by initial expression of the transcription factors *Phox2a* and *Phox2b* in dorsal rhombomere1 (r1), followed by expression of dopamine-β-hydroxylase and tyrosine hydroxylase. *Bmp5* is expressed in the dorsal neuroepithelium in proximity to *Phox2*-expressing cells. BMP inhibition in stage 10 chick embryos resulted in the lack of LC neurones or in their generation at the dorsal midline, and loss of roof plate and rhombic lip, but it did not affect neural crest development. These results reveal late essential BMP functions in the specification of dorsal neuronal phenotypes in r1, including LC neurones, and in the development of dorsal midline structures.

Key words: Roof plate, Patterning, Dorsoventral, Hindbrain, BMP5, Autonomic neurons, Chick

INTRODUCTION

The generation and specification of noradrenergic neurones in the peripheral and central nervous system seems to be mediated by very similar transcriptional control mechanisms. In particular, the bHLH gene *Mash1* and the paired homeodomain protein *Phox2b* have been shown to be essential and sufficient for the generation of sympathetic ganglia and the locus coeruleus (LC), the major noradrenergic centre of the brain (Guillemot et al., 1993; Lo et al., 1998; Hirsch et al., 1998; Pattyn et al., 1999; Pattyn et al., 2000; Morin et al., 1997). In vivo studies have demonstrated that BMPs are essential for sympathetic neurone development (Reissmann et al., 1996; Schneider et al., 1999) and that the transcription factors Cash1 (the chick homologue of Mash1), Phox2a, Phox2b and dHand are downstream regulators of BMPs in the sympathetic lineage (Schneider et al., 1999; Howard et al., 2000). These observations have raised the question of whether the development of Mash1- and Phox2-dependent noradrenergic LC neurones also depends on BMPs.

BMPs and other members of the TGFβ superfamily of growth factors have been implicated in the specification of dorsal fates in the developing spinal cord and hindbrain. BMP signalling in early prepatterning events of the dorsal ectoderm leads to a subdivision of the ectoderm, including the neural plate (Mayor et al., 1999). At a later stage, BMP signalling is involved in the induction of dorsal midline phenotypes, the neural crest and the roof plate (Liem et al., 1995; Arkell and Beddington, 1997). The BMP signal is propagated from the epidermal ectoderm to the roof plate and roof plate-derived BMPs induce the generation of distinct classes of dorsal intereurones (Liem et al., 1997). In the absence of roof plate signals, dorsal interneurone specification is disturbed (Lee et al., 2000; Millonig et al., 2000).

Owing to the early embryonic death or redundant functions in BMP knockout mice, the role of BMPs in neural tube patterning remains unclear (Dudley et al., 1995; Luo et al., 1995; Winnier et al., 1995; Zhang and Bradley, 1996). However, BMP2 and BMP7 zebrafish mutants lack *Phox2*-positive LC neurones (Hynes and Rosenthal, 1999; Guo et al., 1999). However, the impairment of BMP signalling during early zebrafish development produces a repatterning of the ectoderm, resulting in the expansion of the neural plate and the lack of dorsal neural plate fates like neural crest (Nguyen et al., 1998; Barth et al., 1999). Thus, the lack of LC neurones in *Bmp2* and *Bmp7* deficient animals may be due to early, indirect effects of BMPs on neural plate patterning. However, in addition to an early dependence on BMP patterning at the neural plate stage, the generation of LC progenitor cells may also depend on later instructive signals from the roof plate. Such a sequential dependence on BMPs has been observed during the generation of sympathetic neurones involving BMPs in neural crest specification (Liem et al., 1997; LaBonne and Bronner-Fraser, 1998), delamination (Selleck et al., 1998; Sela-Donenfeld and Kalcheim, 2000) and in the specification of the noradrenergic neurone phenotype (Varley et al., 1995; Reissmann et al., 1996; Shah et al., 1996; Schneider et al., 1999).

To address the mechanism of action of BMPs in LC generation, we have analysed the correlation between BMP expression and LC generation and have interfered with BMP signalling at different stages of development. Our results

suggest that the specification of LC neurones depends directly or indirectly on BMPs that are present and produced in a graded fashion in the dorsal neural tube. We define the period of development during which dorsal identity is dependent on BMP signals and establish an essential in vivo role of BMPs in the differentiation or maintenance of the roof plate.

MATERIALS AND METHODS

Implantation of Noggin-expressing CHO cells or agarose beads loaded with Noggin into chick embryos

Noggin-expressing Chinese Hamster Ovary (CHO B3A4) cells were cultured in α-Modified Eagle Medium without nucleotides complemented with 10% dialysed foetal calf serum (FCS), 1% sodium pyruvate, 1% non-essential amino acids and 80 μM methotrexate. For implantation, a 90% confluent cell culture (10 cm dish) was harvested and centrifuged to form a soft pellet for implantation. For control experiments, CHO cells were cultured, harvested and implanted (see below), according to the same protocol.

Agarose beads (Affi-Gel blue beads, Biorad) were loaded with Noggin (1 mg/ml), bovine serum albumin (BSA) (1 mg/ml) or phosphate-buffered saline (PBS) as described previously (Schneider et al., 1999). Fertilised chicken eggs were incubated at 37°C until they reached the desired stages of development (stages 10-14) (Hamburger and Hamilton, 1951). The embryonic membranes were removed and the beads or Noggin-expressing CHO cells were implanted/injected lateral to the midbrain/hindbrain boundary. After further incubation, the embryos were fixed in 4% paraformaldehyde at 4°C for 24-48 hours, cryoprotected overnight in 30% (w/v) sucrose, embedded and sectioned. Consecutive 12-16 μm sections were selected and analysed using in situ hybridisation. The implanted cells were actively producing Noggin throughout the experimental period, as revealed by in situ hybridisation for Xnoggin (not shown).

In situ hybridisation on sections

Nonradioactive in situ hybridisation was carried out as described (Reissmann et al., 1996). Antisense RNA probes specific for chicken Phox2a, TH, DBH (Ernsberger et al., 1995; Ernsberger et al., 2000), Lhx2a (Rodriquez-Esteban et al., 1998), Sox10 (Schneider et al., 1999), Wnt1 (kindly provided by Chaja Kalcheim), BMP5 (Oh et al., 1996), BMP7 (kindly provided from Brian Houston), Pax3 (Goulding et al., 1993) and Pax6 (kindly provided from T. Ogura) were used.

Viral stock preparation and injection into chick embryos

Virus stock of Phox2a-RCAS were prepared from supernatants of infected chick embryo fibroblasts (CEFs) as described (Morgan and Fekete, 1996). Fertilised virus-free chicken eggs were incubated until they reached the desired stages (stages 8 to 14). Using fine tungsten needles, the embryonic membranes were removed and the viral stock was transferred into the midbrain/hindbrain region by injection using fine glass capillaries attached to an aspirator tube (Sigma A-5177).

RESULTS

Generation and differentiation of LC neurones in the chick embryo

Phox2a and *Phox2b* are early markers for the LC (Morin et al., 1997; Pattyn et al., 1999; Pattyn et al., 2000). We have analysed the onset of Phox2 gene expression in rl and studied the timing of noradrenergic differentiation by the expression of *TH* and

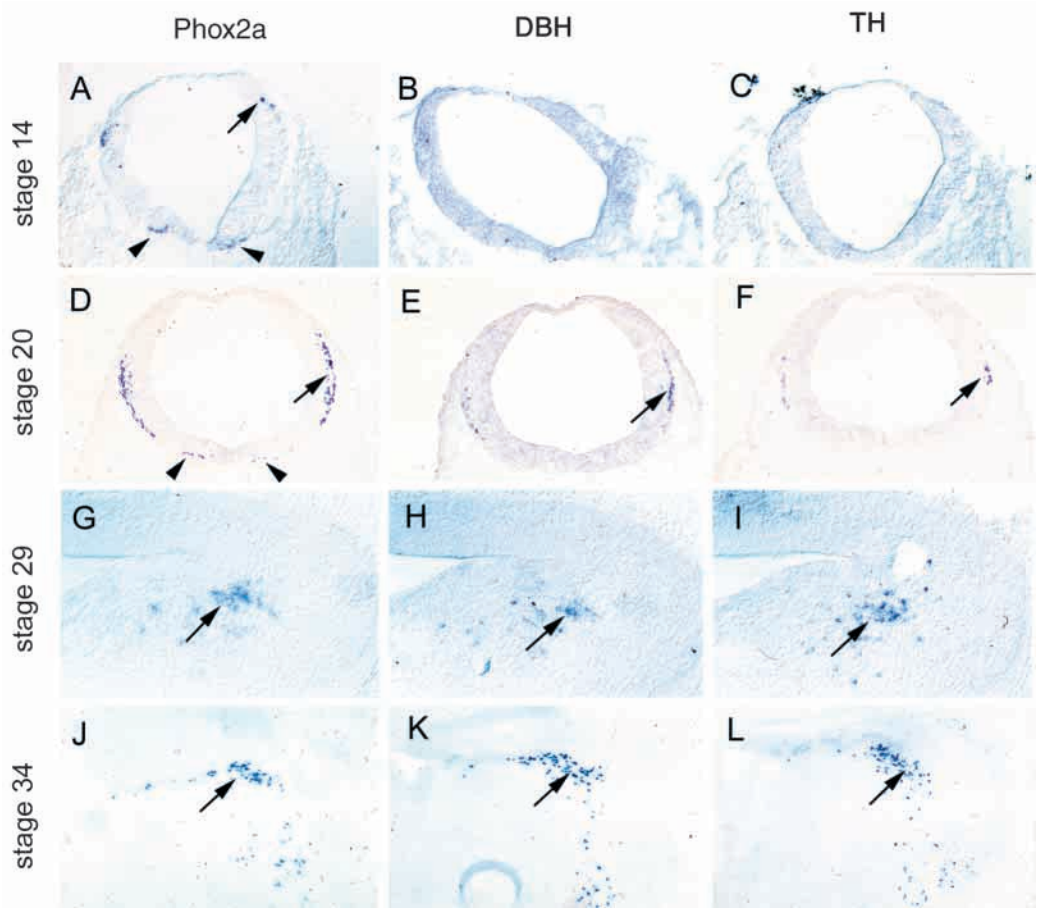

Fig. 1. Expression of *Phox2a* (A,D,G, J, arrow), *DBH* (B,E,H,K, arrow) and *TH* (C,F,I,L, arrow) in r1 at various stages of chick development. At stage 14, transcripts of *Phox2a* (arrow) are observed in the dorsal aspect of r1 (A), while no expression of *DBH* (B) and *TH* (C) is detected. (D-F) At stage 20, *Phox2a*, *DBH* and *TH* transcripts are found in a more ventral position (arrow) and become finally localised to a region (arrow) ventrolateral to the fourth ventricle at stage 29 (G-I) and stage 34 (J-K). Arrowheads in A,D indicate the ventrally localised trochlear nuclei.

DBH (Fig. 1). This analysis demonstrated an initial appearance of Phox2a-positive cells in the dorsal aspect of rl at stage 13, followed by the expression of *Phox2b* at stage 16, *DBH* at stage 17 and *TH* at stage 18. The *Phox2*-positive cells are initially generated dorsally but are observed later in a more ventral location (Fig. 1).

Correlation of BMP expression with the generation of Phox2-positive cells in rl

Bmp4 and *Bmp7* are expressed in the dorsal hindbrain before and during neural crest specification (Graham et al., 1994; Watanabe and Le Douarin, 1996; Schultheiss et al., 1997; Streit et al., 1998; Streit and Stern, 1999; Wall and Hogan, 1995; Liem et al., 1995). Neural crest cells, specified in rl at stage 8+ (Nieto et al., 1994), emigrate between stage 9 and 11 at this axial level (Nieto et al., 1994; Tosney, 1982; Wingate and Hatten, 1999). Thus, the onset of *Phox2* gene expression at stage 13/14 occurs much later than the BMP-dependent neural crest specification. Therefore, we analysed the expression pattern of TGFβ family members, before and during the onset of *Phox2* expression in rl. Strong *Bmp5* expression was observed at stage 10 and 11 in the rostral hindbrain (Fig. 2A,B), extending in r1 up to the caudal limit of isthmic *Fgf8* expression (Fig. 2B). On sections, we observed graded *Bmp5* expression in the dorsal neural tube of rl in a relatively broad area at stage 10 and 11 (Fig. 2C,D; see Fig. 4C). At stage 14, *Bmp5* was strongly expressed in dorsal r1 (Fig. 2E), whereas *Bmp7* was expressed in the epidermal ectoderm that is closely apposed to the neural tube during early development (Fig. 2F). We were unable to detect *BMP4* expression in r1 between stage 10 and 14. Thus, the expression of *Bmp5* (and *Bmp7*) in rl is compatible with a late role of these factors in the specification of the dorsally generated LC neurones.

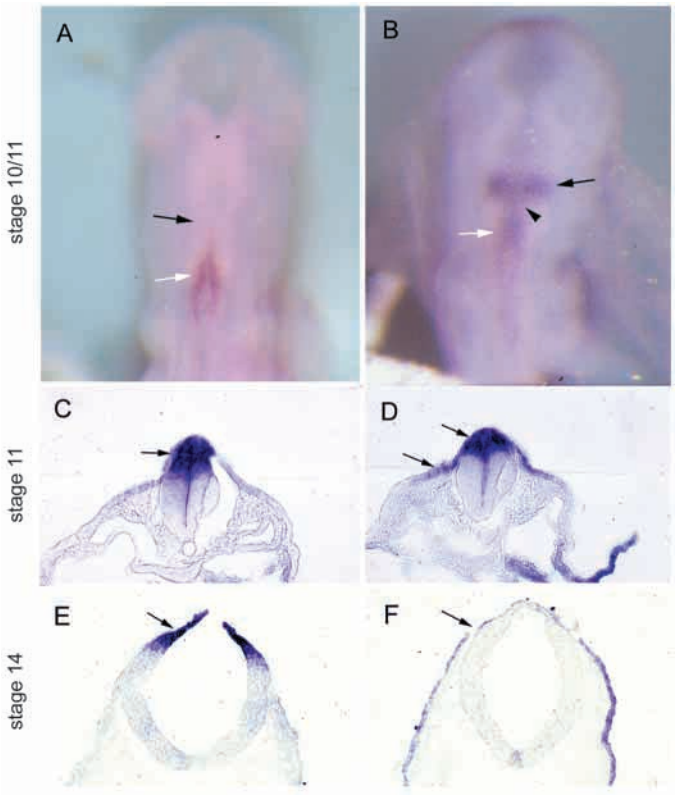

Fig. 2. Expression pattern of *Bmp5* (A-E) and *Bmp7* (F) in r1 during early stages of chick development. (A) Whole-mount in situ hybridisation for *Bmp5* at stage 10 demonstrates expression in the rostral hindbrain, including r1 (white arrow, *Bmp5* expression; black arrow, midbrain-hindbrain-constriction). (B) Simultaneous detection of *Bmp5* and *Fgf*8 at stage 11 by whole-mount in situ hybridisation demonstrates an overlap (arrowhead) of *Bmp5* (white arrow) and *Fgf8* (black arrow) expression in r1. (C,D) Transcripts of *Bmp5* (arrows) are found at stage 11 throughout the dorsal aspect of r1, at both rostral (C) and caudal (D) regions. Note that there also seems to be expression in the epidermal ectoderm (D). At stage 14/15, transcripts of *Bmp5* (arrow) are restricted to the roof plate and dorsal edges of the neural tube (E), while *Bmp7* expression (arrow) is restricted to the overlying epidermal ectoderm (F). Whole-mount in situ hybridisation was used for the detection of BMP5 in C,D, resulting in the collapse of the ventricle when compared with hybridisation of sections (Fig. 4C).

BMP signalling is essential for the development of the LC

To investigate the physiological role of BMPs in r1, we blocked their action by applying the BMP antagonist Noggin (Zimmermann et al., 1996). Noggin-producing CHO cells or Noggin-soaked beads were implanted in the presumptive LC region close to the neural tube at stages 10 to 11. The effect of Noggin treatment was analysed by *Phox2* and *DBH* in situ hybridisation at early stages (HH 15 to 29) and in a few cases that survived until E8.

In 13% of the embryos treated with Noggin-producing CHO cells at stage 10 to 11 (n=38) a clear-cut loss-of-function phenotype, devoid of LC neurones, was observed at HH 15 to 17 (Fig. 3D), and the hindbrain was noticeably smaller than normal. The other phenotype, observed in 63% of the embryos, consisted of embryos where LC neurones were observed in a continuous zone of cells across the dorsal region of the neural tube, indicating that the two dorsal domains of the r1 have fused (Fig. 3E,F). The staining for *DBH* unequivocally identifies the ectopic cells as LC neurones. In control embryos that received normal CHO cells, PBS-soaked or BSA-soaked beads, we did not observe a loss or a dorsal shift of LC neurones (n=25; Fig. 3A-C). The Noggin-bead implantation produced only the weaker phenotype with dorsally located LC neurones (Fig. 3E,F), which can be explained by a lower amount of Noggin released from the beads when compared with the CHO cells.

Differential effects of Noggin treatment on LC, roof plate and neural crest development in r1

Next, we investigated at what embryonic stage LC development is dependent on BMPs. Two phases of BMP-dependent dorsal neural tube patterning have been distinguished: an initial specification of the early dorsal midline fates neural crest and roof plate, followed by roof plate-derived BMP signalling that controls dorsal neuronal fates at later stages. *Wnt1* is initially expressed in the dorsal neuroepithelium of r1 and later on in the rhombic lip, but not in the roof plate (Rodriquez and Dymecki, 2000; Wingate, 2001), whereas *Bmp5* is expressed in the roof plate (below). Using *Wnt1* and *Bmp5* as dorsal midline markers (Parr et al., 1993; Robert et al., 1989; Hill et al., 1989; Arkell and Beddington, 1997; Lyons et al., 1995; Lee et al., 1998), and

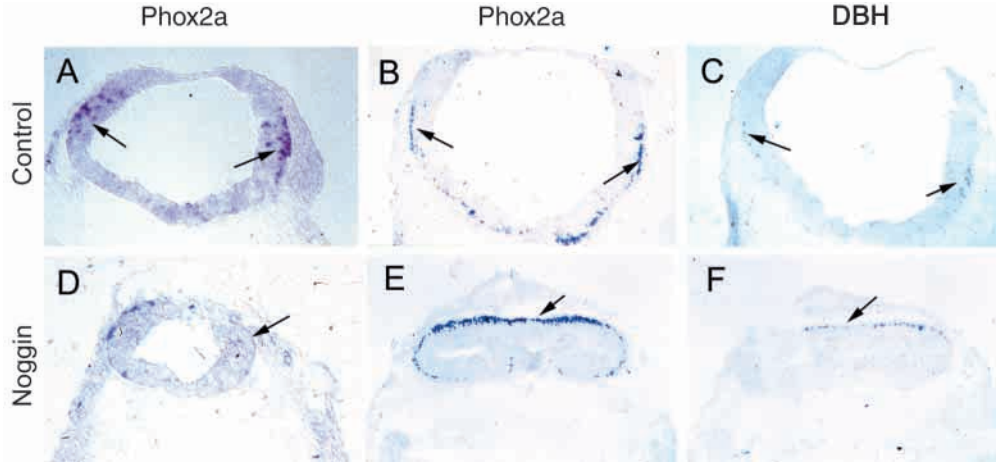

Fig. 3. Noggin affects development of LC neurones. Control embryos (A-C) and Noggin-treated embryos (D-F) were analysed for the expression of *Phox2a* (A,B,D,E, arrow) and *DBH* (C,F, arrow). Noggin-expressing CHO cells (D) or Noggin-soaked beads (E,F) were implanted into stage 10 chick embryos. *Phox2a* and *DBH* expression (arrow) is either lost on the Noggin-treated side (D, stage 17) or detected at the dorsal midline of r1 (E,F, stage 22).

Sox10 as neural crest marker (Kuhlbrodt et al., 1998; Schneider et al., 1999), we extend previous data that neural crest cells have been specified in r1 and are in the migratory phase at stage 10 (Fig. 4) (Nieto et al., 1994; Tosney, 1982). At stage 11 all *Sox10*-expressing neural crest cells had left rostral r1 (not shown).

Noggin-treatment at stage 10 and 11 does not affect the generation and migration of neural crest cells. Control and experimental embryos display a similar pattern of trigeminal neural crest cells in the r1 region (Fig. 4D,G). This finding demonstrates that the specification of LC neurones and neural crest cells are distinct events and can be distinguished by different periods of BMP dependency. By contrast, we observed a lack of the dorsal midline structures in Noggin-treated embryos, which is evident from changes in morphology and marker gene expression. *Wnt1* and *Bmp5* are expressed in dorsal r1 at the onset of Noggin treatment (Fig. 4B,C) and become restricted to dorsal midline structures between stages 10 and 14 (Fig. 2E; not shown). The inhibition of BMPs results in a hindbrain of even thickness without dorsal roof plate specialisation and with a lack of *Wnt1* and *Bmp5* expression, which are expressed in control embryos in rhombic lip and roof plate (compare Fig. 4E,F with Fig. 4H,I).

To define the time period during which LC and roof plate development are dependent on BMPs, we applied Noggin at different time points after stage 10. At stage 11, generation of LC neurones was prevented in one out of five Noggin-treated embryos. After Noggin-application at stage 12, we never observed a complete loss of LC neurones, while in 66% of the embryos, a dorsal shift of LC neurones and loss of roof plate occurred (n=9). After Noggin treatment at stage 13-14 (n=11), only a few LC neurones were located in the dorsal midline, mainly in the rostral part of r1 (27% of embryos), while LC development was unaffected in the caudal region. These embryos also lacked *Wnt1* and *Bmp5* expression in the rostral part of r1, but not in the caudal part (data not shown). Thus, it appears that LC neurones are specified by BMPs in r1 up to stage 11, and that from stage 12 onwards LC neurones no longer depend on BMPs. By contrast, the development of other dorsal phenotypes, including the roof plate and rhombic lip, depends on BMPs from stage 12 up to stage 13/14 in rostral

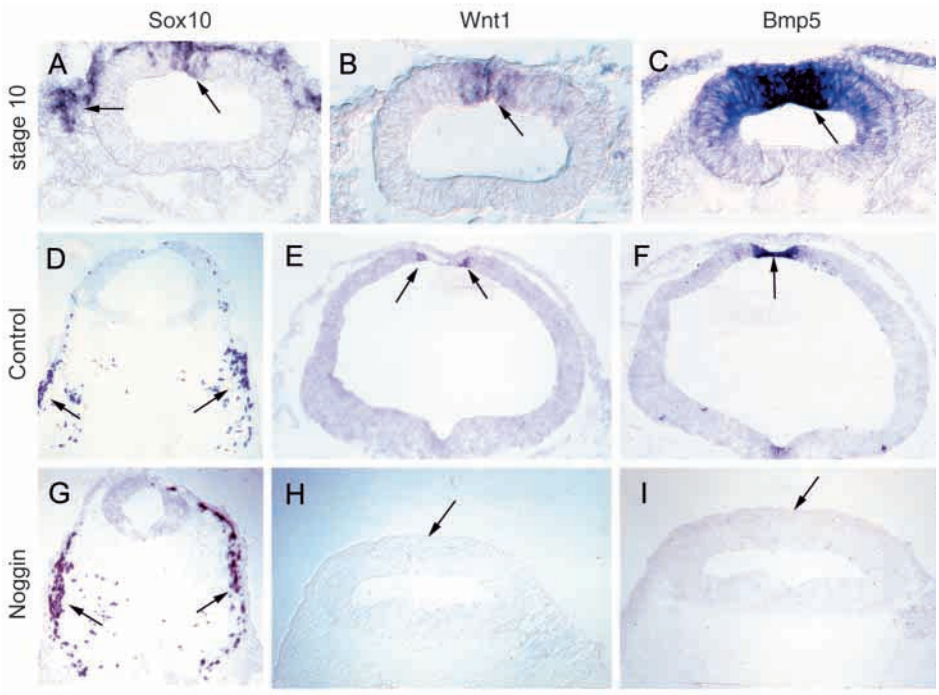

Fig. 4. Noggin application does not interfere with neural crest specification, but is required for the development of the roof plate and rhombic lip. Expression of the neural crest marker *Sox10* (A,D,G, arrows) and of dorsal midline markers *Wnt1* (B,E,H, arrow) and *Bmp5* (arrow, C,F,I) in control embryos at stage 10 (A-C). The effect of implantation of control CHO cells (D-F) and of Noggin-expressing CHO cells (G-I) was analysed at stage 22. Note that in Noggin-treated embryos *Sox10* expression is not affected (G), while expression of *Wnt1* and *Bmp5* is completely lost at the dorsal midline (arrows, H,I).

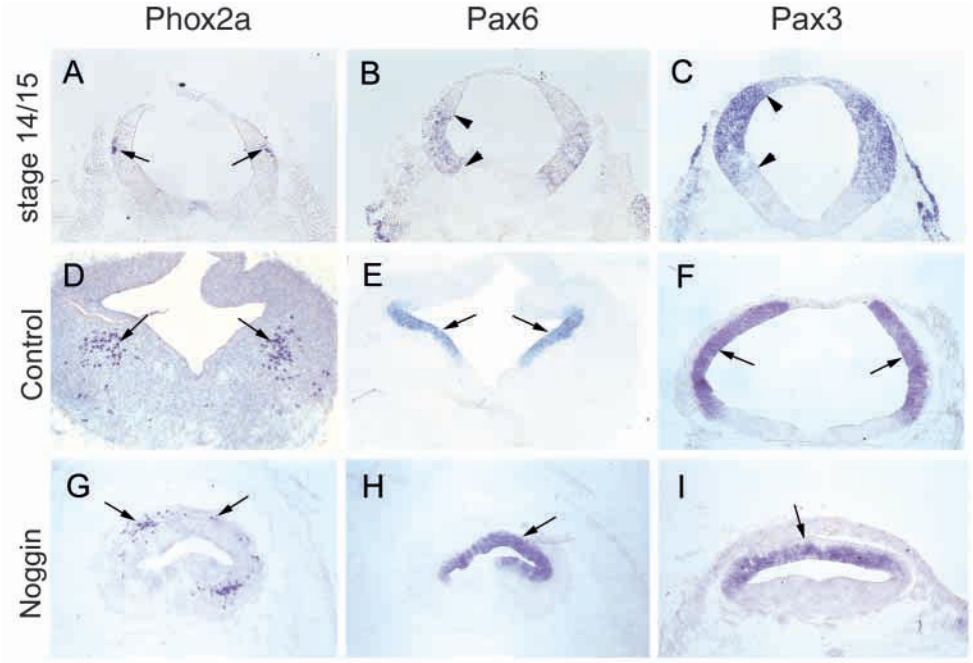

Fig. 5. Expression of *Phox2a* (A,D,G, arrow), *Pax6* (B,E,H, arrows) and *Pax3* (C,F,I arrows) was analysed in control embryos at stage 14/15 (A-C), stage 26/27 (D,E) and stage 22 (F), and in Noggin-treated embryos at stage 26/27 (G,H) and stage 22 (I). (A-C) Wild-type embryos; (D-F) control embryos treated with CHO cells; (G-I) Noggin-expressing CHO cell treatment. Note that *Phox2a* expression at stage 14/15 is observed within the *Pax3* and *Pax6* domain (compare A-C). In controls, at stage 26/27, expression of both *Phox2a* and *Pax6* is localised to ventrolateral regions of r1 (D,E). Pax-3 expression at stage 22 is observed in a large region that excludes dorsal midline and most parts of the ventral region (F). After application of Noggin-expressing cells, *Phox2a* and *Pax6* expression is observed in the dorsal region of the neural tube (G,H). *Pax3* expression is now expressed in the entire dorsal region (I).

r1. This suggests that dorsal BMPs have several distinct functions during this time period: controlling the fate of LC progenitors and the maintenance or differentiation of the dorsal midline structures.

Neural patterning defects in BMP-deficient dorsal rl

The loss of the roof plate in Noggin-treated embryos, together with the dorsal location of LC neurones in the weaker phenotype, indicates a change in neural patterning in the dorsal rl. To address this issue, we examined the expression of Pax transcription factors that monitor the early subdivision of the neural tube into ventral and dorsal halves (St-Onge et al., 1995). At stage 14/15, *Pax6* is expressed in both the ventral and dorsal aspect of r1 (Fig. 5B). During further development, the expression becomes localised to a more ventral position (Fig. 5E) similar to the *Phox2a*-expressing region (Fig. 5D). The expression of *Pax3* defines dorsal neural progenitors in r1 (Fig. 5C,F) and expression is observed in the dorsal part of r1 at stage 10 (not shown, Fig. 8). Similar to *Phox2a* and *Pax6* expression, expression of *Pax3* expands to a more ventral position during further development but remains expressed in the dorsal aspect of r1 (Fig. 5C,F) and the first *Phox2a*-positive LC neurones are observed within the dorsal *Pax3/Pax6* co-expression domain in r1 (compare Fig. 5A-C). After Noggin application at stage 10 to 11, *Pax6* and *Pax3* expression was shifted to the dorsal midline (Fig. 5H,I). If the dorsal domains characterised by the exclusive expression of *Pax3* are lost, the dorsalmost progenitor cell identity would correspond approximately to the region where *Pax3* and *Pax6* overlap in wild-type embryos.

We then examined the impact of BMP inhibition and the change in patterning on the generation of specific classes of neurones. We investigated the development of *GATA2*-expressing ventral neurones (Fig. 6E) and of *Lhx2*a-positive dorsal interneurones. *Lhx2a*-expressing cells in the spinal cord are generated dorsally and migrate then to a ventral position. Their development depends on roof plate signals (Lee et al., 1998). In r1, *Lhx2a*-positive cells were first detected at stage 16 in a dorsal position and appear to be intermingled with *Phox2a*-positive cells up to stage 23 (Fig. 6A,C), but their final

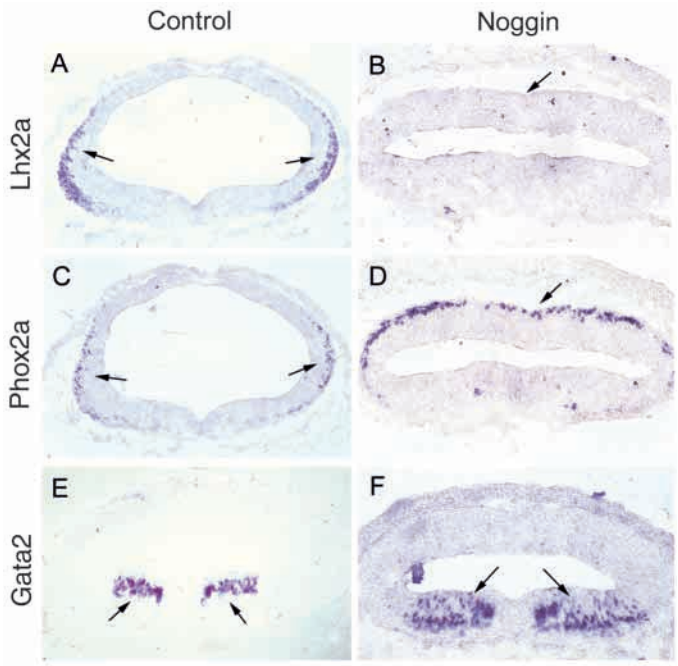

Fig. 6. Differential BMP dependence of dorsal *Phox2a*-positive LC neurones, *Lhx2a*-positive neurones and ventral *GATA2* neurones in r1. *Lhx2a*-positive neurones (A, arrows) have a similar expression pattern to *Phox2a*-expressing LC neurones (C, arrows) during the early stages of chick r1 development (stage 22). Implantation of Noggin-producing CHO cells results in a complete loss of *Lhx2a* expression at stage 22 (B, arrow), while in a parallel section, *Phox2a*-positive cells are present in the dorsal midline (D, arrow). By contrast, at stage 22, the *GATA2* expression pattern of ventral neurones (E, arrow) is unchanged after Noggin-treatment (F, arrow).

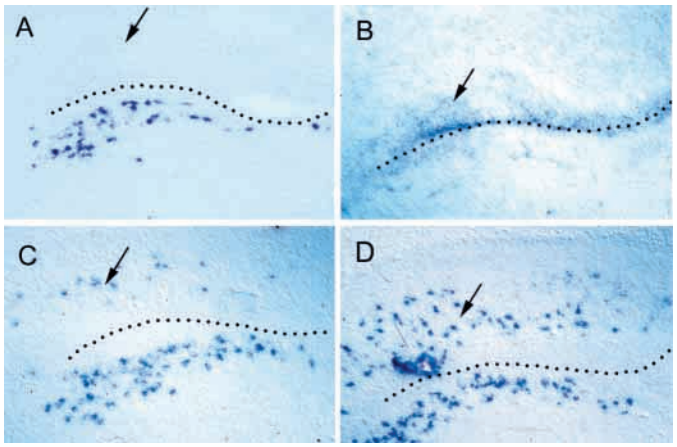

Fig. 7. Phox2a is sufficient to induce noradrenergic neurones in r1. (A) At E8 *DBH* expression in wild-type embryos is not detected dorsal to the fourth ventricle (B, arrow). After injection of *Phox2a*-expressing RCAS vectors at stage 9, the dorsolateral alar plate, dorsal to the ventricle, is infected as visualised by the RT expression pattern (arrow). (B,C) In this infected E8 embryo, ectopic *DBH* (C) and *TH*-positive (D) neurones are observed dorsal to the fourth ventricle (arrows). The ventricle is indicated by a broken line.

location in r1 and the caudal hindbrain is dorsolateral to *Phox2a/Phox2b*-postive cells (data not shown). Whereas the location and area of ventral *GATA2*-positive neurones was not affected by the inhibition of BMPs (Fig. 6F), *Lhx2a* expression was either completely absent or greatly reduced in all Noggin-treated embryos, including embryos where *Phox2a*-positive cells are present in the dorsal midline (compare Fig. 6B with 6D), or after Noggin-treatment at stage 13/14. We analysed whether the loss of dorsal neural cell types in Noggin-treated embryos might result from increased apoptosis. As only a few TUNEL-positive apoptotic cells were detected in Noggin-treated embryos, similar to the wild type at stages 17 to 22, this appears not to be the case (data not shown).

Taken together, these results suggest an essential role of BMP family members for the generation of several dorsally generated neuronal subpopulations, including LC neurones.

Phox2a is sufficient to elicit the generation of noradrenergic cells in rl

The BMP-dependent LC specification and dorsoventral patterning in the dorsal rl raises the question of whether cell populations that are specified for another dorsal phenotype by their position in the BMP gradient would still be able to switch their fate to that of a noradrenergic neurone by the forced expression of Phox2 genes. Overexpression of *Phox2a* (Stanke et al., 1999) in r1 resulted in the induction of *Phox2b*, *TH* and *DBH* at E8 dorsal to the ventricle (Fig. 7C,D). Thus, only a few cells were able to respond to forced expression of Phox2 genes in rl. Most progenitor cells, and in particular all ventrally located cells, did not respond by the acquisition of a noradrenergic phenotype and ectopic noradrenergic markers were only observed when we virally infected r1 before stage 10. The results suggest that the patterning and prespecification in rl restricts the potential of the vast majority of the cells.

DISCUSSION

These studies have examined the generation of the LC in the chick embryo. The onset of Phox2 gene expression in dorsal r1 occurs in the vicinity of *Bmp5* expressing cells in dorsal neural folds and roof plate. The inhibition of BMPs at stage 10 by Noggin prevents the generation of LC neurones or results in a dorsal midline localisation of LC neurones. The effects can be explained by BMP functions in the dorsoventral patterning of r1. Interestingly, this patterning function seems to include the generation and/or maintenance of the Lhx2a-positive neuronal cell population and the dorsal BMP signalling centres, the roof plate and rhombic lip.

The LC develops between E2 and E6 in the chick embryo (Yurkewicz et al., 1981). We used Phox2 genes as early markers for noradrenergic neurones to re-evaluate the generation of the LC. The first *Phox2*a-positive cells in r1 were observed at stage 13/14 in a dorsal position that co-expresses Pax3, a marker for dorsal neuronal progenitors (St-Onge et al., 1995), and Pax6. Around E5, the domains of *Phox2a, Pax3* and *Pax6* expression are observed at a more ventrolateral position. This may be explained by the differential growth of dorsal versus ventral halves of the neural tube or by changes in the expression of dorsoventral patterning genes *Pax3* and *Pax6* and the ventral migration of *Phox2a*-positive cells (Hemond and Glover, 1993). Ventral translocations of dorsal interneurones have been observed in the spinal cord (Lee et al., 2000) and explained by tangential migration (Leber and Sanes, 1990).

The expression of BMP family members in ectoderm and neural folds suggested that BMPs are involved in LC specification. We identified *Bmp5* as a likely candidate in LC generation, owing to its strong and dorsoventrally graded expression in r1 during stages 10 and 14, at the time when LC neurones are specified and are first detectable. This is in agreement with recent fate-mapping studies at stage 10 that identified the origin of the LC in dorsal r1 (P. Aroca and L. Puelles, personal communication), within the area of *Bmp5* expression.

The specification of dorsal cell types in the spinal cord requires a sequence of BMP signals, initiated in the epidermal ectoderm and propagated to the dorsal roof plate. Similarly, in the hindbrain, neural crest and roof plate are specified by early BMP signalling (Tosney, 1982; Nieto et al., 1994; Wingate and Hatten, 1999) (this study). The previously demonstrated absence of LC neurones in zebrafish mutants devoid of BMPs (Guo et al., 1999) reflects an early requirement of BMPs for neural plate patterning and is paralleled by the expansion of neural plate, the lack of neural crest and many dorsal neural tube fates. Mayor et al. have proposed, that the role of BMPs may reflect the maintenance of fates initially specified at the neural plate stage rather than a late specification event (Mayor et al., 1999). In this study we identified a later, BMP-dependent LC neurone specification period in r1. Interfering with BMP signalling at stage 10, after neural crest is specified, resulted in two phenotypes, i.e. embryos devoid of LC neurones or with LC neurones at the dorsal midline. These two different phenotypes most probably reflect the variations in the local concentrations of Noggin in different embryos. Our results demonstrate that LC progenitors are specified by BMPs up to stage 10 and suggest an essential role of late BMP signals for the development of dorsal phenotypes. In particular, we also

demonstrate an essential role of BMPs for the roof plate and rhombic lip. While the dorsal midline markers *Wnt1* and *Bmp5* disappeared in Noggin-treated embryos, expression of *Pax3* and *Pax6* was shifted to the dorsalmost position, normally devoid of *Pax3* and *Pax6* expression. This suggests that while dorsal regions are lost, generic dorsoventral patterning is maintained.

We then asked, whether in addition to LC neurones other neuronal subtypes would also be affected in Noggin-treated embryos. The expression of the transcription factor *Lhx2a* identifies a dorsal neuronal population in spinal cord (Lee et al., 1998) and hindbrain (present study), whereas *GATA2* is expressed by ventral r1 neurones (Bell et al., 1999). Interestingly, *Lhx2a* expression, which initially shows a similar expression pattern to Phox2a, was completely eliminated in the absence of BMP signalling, whereas *GATA2* expression was not affected.

The proposed change in cellular identity would explain the smaller size of the r1 in Noggin-treated embryos. As Wnt genes are essential for the proliferation of dorsal neuronal progenitors (Dickinson et al., 1994; Ikeya et al., 1997), the lack of *Wnt* expression may also account for the decreased size of r1. Proliferative effects have been also described for *Bmp7* in the hindbrain (Arkell and Beddington, 1997). An alternative explanation would be the death of cells in the absence of BMP-dependent specification and maintenance. The observed lack of significantly increased numbers of TUNEL-positive cells favours a change in cellular identity according to BMP levels.

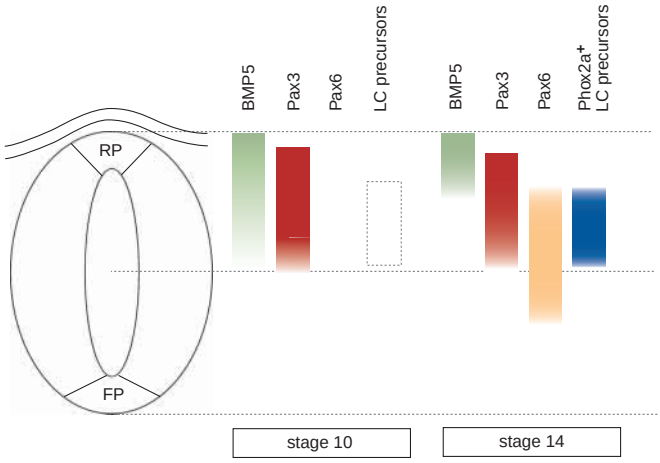

Fig. 8. A gradient of BMPs (green) produced in the dorsal half of r1 controls the specification of different cellular phenotypes. We propose that at stage 10/11, after neural crest cells have been specified and left the neuroepithelium, the specification of the precursors for noradrenergic LC neurones (broken line) and other dorsal neurones depends on BMPs. We propose that LC precursors are localised within the BMP5 and Pax3 expression domains at stage 10. Pax6 expression is not observed at this stage (Li et al., 1994). The first Phox2a-positive LC precursors (blue) are detected at stage 13/14 within the Pax3/Pax6 co-expression domain in the dorsal compartment of r1. BMPs are also required in r1 for the differentiation and/or maintenance of the dorsal midline structures, the roof plate (RP) and rhombic lip. This model differs from the classical morphogen scenario, as the BMP gradient is not generated by diffusion from a signalling centre but by a gradient of BMP synthesis. FP, floor plate.

The results we observed after BMP inhibition could be explained by a primary effect on roof plate development and the subsequent lack of several roof plate-derived signals that are responsible for the generation of different dorsal phenotypes (Lee et al., 1998). Alternatively, a BMP gradient in dorsal r1 might be responsible for dorsoventral patterning and the subsequent generation of different phenotypes at distinct BMP concentration thresholds. The latter model is supported by the demonstration of a *Bmp5* expression gradient in r1, and by the lack of correlation between the absence of roof plate and the lack or dorsal shift of LC neurones in Noggin-treated embryos (i.e. embryos without roof plate either lack LC neurones or have LC neurones in the dorsal position). Further supporting evidence is provided by the weak BMP-signalling zebrafish mutant *somitabun*, where ectopic LC neurones are generated in dorsal positions (Guo et al., 1999). To address the question of whether the fate of non-LC progenitors is irreversibly determined, Phox2a was expressed in r1. The observed Phox2a-induced generation of a low number of ectopic noradrenergic neurones suggests that uncommitted precursor cells represent a small minority of dorsal populations. The absence of *Lhx2a*-positive neurones in Noggin-treated embryos that contain LC neurones may be due to their generation at a more dorsal location in the BMP gradient or, more likely, due to their specification at a later time point. As Noggin might antagonise the action of several BMPs, our results do not exclude the possibility that, besides BMP5, other BMP family members are involved in LC development. However, *Bmp5/Bmp7* double knockout mice lack *Wnt1* expression in the hindbrain neuroepithelium, similar to what is observed after Noggin treatment (Solloway and Robertson, 1999).

In conclusion, the present study defines the timing and location of early LC neurogenesis in the chick embryo (Fig. 8). It provides evidence for the importance of BMP signals in the generation of LC neurones and other dorsal cell populations in r1. The results strongly support the notion of a late action of a dorsal BMP gradient in the generation of dorsal neuronal phenotypes. We also demonstrate the BMP dependence of the dorsal midline structures roof plate and rhombic lip, as well as the differential timing of BMP-dependence for different dorsal hindbrain phenotypes.

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