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 interneurones (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 (Rodriguez-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 (CEF) 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 r1 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+, are emigrated between stage 9 and 11 at this axial level (Nieto et al., 1994), migrate 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 rl up to the caudal limit of ischmic 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 rl (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 rl 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.

BMP signalling is essential for the development of the LC

To investigate the physiological role of BMPs in rl, 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 rl 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 rl

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 neural crest fates at later stages. Wnt1 is initially expressed in the dorsal neuroepithelium of rl and later on in the rhombic lip, but not in the roof plate (Rodriguez 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
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
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 r1**

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 r1. 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 Lhx2a-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. 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-positive 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 r1**

The BMP-dependent LC specification and dorsoventral patterning in the dorsal r1 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 r1. 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 r1 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 Phox2a-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.

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 former 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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BMPs in central noradrenergic neurone development


