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Temporal control of BMP signalling determines neuronal subtype identity in the dorsal neural tube

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SUMMARY

The conventional explanation for how a morphogen patterns a tissue holds that cells interpret different concentrations of an extrinsic ligand by producing corresponding levels of intracellular signalling activity, which in turn regulate differential gene expression. However, this view has been challenged, raising the possibility that distinct mechanisms are used to interpret different morphogens. Here, we investigate graded BMP signalling in the vertebrate neural tube. We show that defined exposure times to Bmp4 generate distinct levels of signalling and induce specific dorsal identities. Moreover, we provide evidence that a dynamic gradient of BMP activity confers progressively more dorsal neural identities *in vivo*. These results highlight a strategy for morphogen interpretation in which the tight temporal control of signalling is important for the spatial pattern of cellular differentiation.

KEY WORDS: Morphogen, BMP, Patterning, Neural tube, Chick

INTRODUCTION

In many embryonic tissues, graded signals, usually referred to as morphogens, provide the positional information that governs the pattern of cellular differentiation. These signalling molecules diffuse from a localized source to form concentration gradients and induce distinct sets of genes in responding cells. A central issue is to understand how cells perceive and interpret the external morphogen in order to control differential gene expression.

Studies of Activin and Sonic Hedgehog (Shh) have provided evidence that different concentrations of a single ligand can provide positional information to a field of responding cells. How ligand concentration is interpreted in these two instances appears to differ, however. In the case of Activin, increasing concentrations of the ligand generate correspondingly higher levels of signalling activity and distinct thresholds of activity induce different sets of genes (Smith, 2009). By contrast, concentrations of Shh are converted into durations of signalling, with higher concentrations maintaining signalling activity for a longer period of time. In this case, target genes are sensitive to the level of signalling experienced over time (Dessaud et al., 2008).

Bone morphogenetic proteins (BMPs) are essential for the patterning of several embryonic tissues, including the mesoderm and neural tube (NT) (De Robertis and Kuroda, 2004). Whether BMPs act as classical morphogens in these tissues to instruct cell fate in a concentration-dependent manner remains unclear. Moreover, the dynamics of the transduction of ligand concentration into intracellular signalling activity are currently unknown. Here, we take advantage of the dorsal spinal cord to address these questions. In this region of the NT, six interneuron subtypes (dI1 to dI6) are generated from non-overlapping domains of progenitors arrayed along the dorsal-ventral (DV) axis (Fig. 1A). These domains can be identified by the expression of specific sets of transcription factors. The three most dorsal populations (dP1-3) are distinguishable in their expression of the proneural genes *Ath1* (dP1), *Ngn1* (dP2) and *Ash1* (dP3-5), which exhibit cross-inhibitory interactions (Gowan et al., 2001). Moreover, these three populations all express the transcription factor Olig3, which represses the expression of the homeobox gene *Lbx1*, an essential determinant of dI4-6 differentiation (Gross et al., 2002; Müller et al., 2005; Müller et al., 2002).

This transcriptional code appears to be established by diffusible signals, which include BMP family members, as well as Wnts and Activin, which emanate from the roof plate (RP) and dorsal ectoderm. Although Wnts regulate the expression of *Olig3* and Activin promotes the differentiation of dI3 (Timmer et al., 2005; Zechner et al., 2007), BMPs have been suggested to provide the positional information necessary to organize the pattern of dorsal interneuron generation. *Ex vivo*, BMPs promote the generation of dI1 and dI3 neurons (Liem et al., 1997) and *in vivo* gain-of-function experiments suggest that BMP signalling is able to induce dI1 and dI2-3 neurons at different thresholds of activity (Timmer et al., 2002). This has led to a model in which BMP signals act as morphogens to specify the spatial pattern of dI1-3 generation. Nevertheless, loss-of-function studies have, so far, provided only limited support for the role of BMP signalling in dorsal specification. In type I BMP receptor conditional mutants, dI1 are missing but dI2 and dI3 neurons are still generated (Wine-Lee et al., 2004). Moreover, how cells perceive and interpret exposure to BMP to acquire appropriate dorsal identities remains to be addressed.

In this study, we directly test the morphogen activity of BMP signalling in the NT. We provide evidence that different durations of exposure to the ligand generate distinct levels of intracellular signalling activity that induce specific dorsal neuron subtypes. Moreover, by monitoring the levels of signalling activity *in vivo* and blocking the pathway at various time points, we show that an endogenous gradient of BMP activity progressively specifies more dorsal identities in the NT. This suggests a model in which the control of ligand dynamics determines the levels of signalling in dorsal progenitors and thereby specifies dorsal interneuron subtype.

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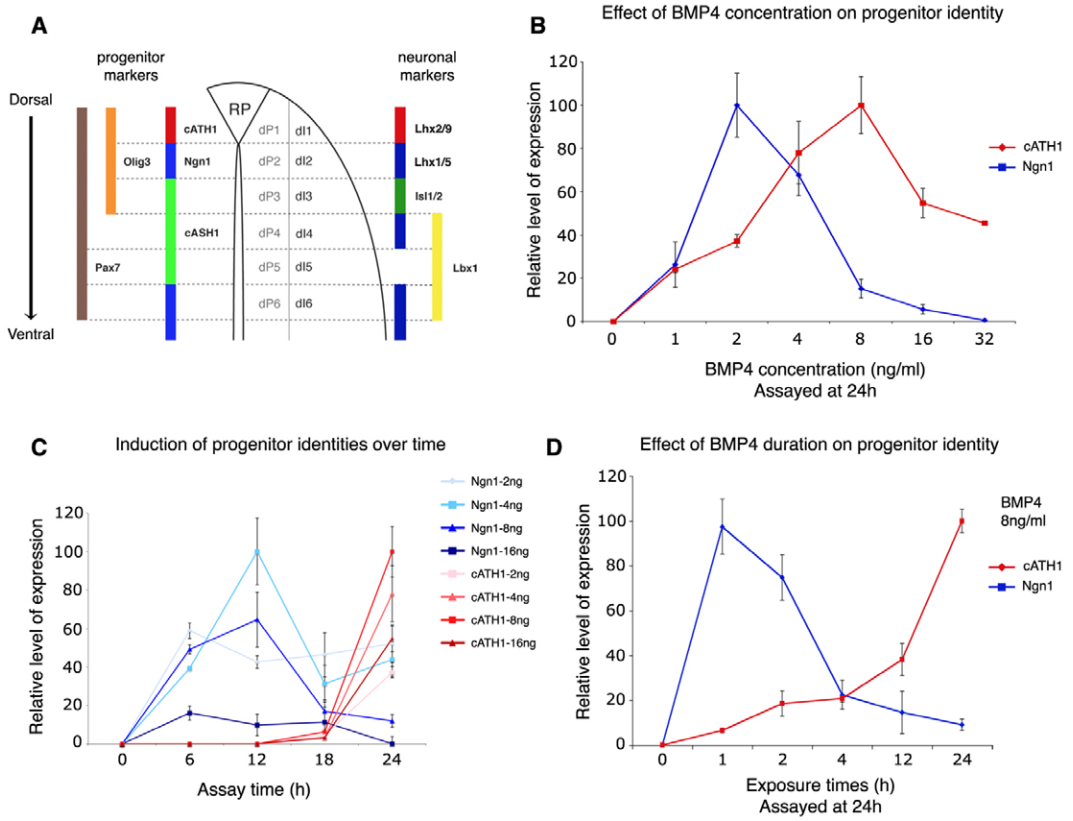

Fig. 1. *Ngn1* and chick *Ath1* are induced by different levels of BMP activity. (**A**) Progenitor and neuronal markers in the dorsal NT. (B-D) Comparison of the levels of Ngn1 and chick Ath1 expression measured by quantitative PCR in intermediate ([i]) explants cultured in the presence or absence of Bmp4. (**B**) Expression of Ngn1 and chick Ath1, measured by Q-PCR, in explants exposed to the indicated concentrations of Bmp4 for 24 hours. (**C**) Expression of Ngn1 and chick Ath1 exposed to the indicated concentrations of Bmp4 at 6, 12, 18 and 24 hours. (**D**) Explants exposed to 8 ng/ml Bmp4 for the indicated time periods before Bmp4 was removed and replaced with control media, Ngn1 and chick Ath1 expression was then assayed at 24 hours. Levels of expression are reported as a percentage of maximum fold induction in control conditions. Data are mean±s.e.m.

MATERIALS AND METHODS

Immunohistochemistry and *in situ* hybridization

For immunostaining, chick embryos were fixed for 1 hour in PFA, embedded and frozen in OCT, and cryosectioned at 12 μm. The primary antibodies to the following proteins were used: Lhx2/9 [LH2a/b (Liem et al., 1997)]; Olig3 (Müller et al., 2005); Lbx1 (Müller et al., 2002); Lhx1/5 (Lim1/2), Isl1/2, Lmx1b and Msx1/2 were purchased from DSHB; GFP and β-galactosidase were purchased from Biogenesis; the PSmad antibody was produced as a collaborative effort between the labs of Ed Laufer and Tom Jessell (both at Columbia University, NY, USA), and generously supplied to us. *In situ* hybridization has been described previously (Acloque et al., 2008). Chick probes for the following genes were used: *Ath1* (Gowan et al., 2001), *Ngn1* (Perez et al., 1999), *Ash1* (Jasoni et al., 1994), *Wnt1* (Hollyday et al., 1995) and *Bmp4* (Liem et al., 1995).

PSmad quantitation

Following immunostaining with the PSmad antibody, neural tubes were pictured and the dorsal 50% was divided into 10 equal bins. Average pixel intensity was then measured in each bin using Photoshop and plotted as a function of DV position.

Proliferation assays

EdU (100 μl of a 100 μM solution diluted in PBS) was introduced *in ovo* to embryos. These were processed after 1 hour of incubation and EdU staining was performed using the Click-iT EdU Alexa Fluor 594 Imaging kit (Invitrogen, C10084).

Constructs and in ovo electroporation

The Smad6-IRES-GFP construct was kindly provided by C. Stern (University College London, UK), and electroporated at 2 μg/μl. A minimum of four embryos were examined for each condition and at least 10 sections were analysed for each marker. Although all electroporations (EP) were performed similarly, some embryos showed much more mosaic transfection than others. The more mosaic transfections were used in Fig. 5B to assay whether intermingled neuronal populations could be observed.

The BRE-Luciferase construct was kindly provided by P. Ten Dijke (Leiden University Medical Center, The Netherlands). Luciferase assays in explants have been described previously (Dessaud et al., 2007). Results represent triplicates from at least two independent experiments.

RT-PCR on neural plate explants

Explants isolated from HH stage 10 chick embryos were cultured as described previously (Ericson et al., 1997). Bmp4 experiments were performed using recombinant proteins purchased from R&D (314-BP). Real-time RT-PCR (Q-PCR) was performed as described previously (Ribes et al., 2010). Expression levels are relative to Actin transcripts and normalized between sets of experiments. All results were obtained in duplicates from a minimum of three independent experiments. Unless otherwise specified, error bars correspond to s.e.m. The following primers were used: cath1 fwd, cgaccagctgcgtaatgtc; cath1 rev, tctgcagcgtctcgtacttg; ngn1 fwd, ctttgctgcacacccttaaac; ngn1 rev, ttggtgagtttggtgtcgtc; actin fwd, gagaaattgtgcgtgacatca; actin rev, cctgaacctctcattgcca.

RESULTS

The duration of exposure to Bmp4 distinguishes between dorsal cell fates

To define conditions that generate the distinct dorsal interneuron subtypes, we exposed intermediate ([i]) neural plate explants

isolated from Hamburger Hamilton stage 10 (HH stage 10) chick embryos to Bmp4. Control explants cultured in the absence of exogenous Bmp4 were comprised predominantly of $Pax7^+$ progenitors at 24 hours, and $Lbx1^+$ dI4-6 neurons at 48 hours (data not shown). By contrast, a 24-hour exposure to Bmp4 converted [i] explants into dorsal dP1-3 progenitors ($Olig3^+$) and migratory neural crest cells ($HNK1^+$) (supplementary material Fig. S1A). In order to discriminate between the different dorsal progenitor identities, we assayed by Q-PCR the expression of the proneural genes chick *Ath1* and *Ngn1*, expressed by dP1 and dP2 progenitors, respectively (Fig. 1A). The expression of *Ngn1* and chick *Ath1* peaked in explants exposed to low (2 ng/ml) and high (8 ng/ml) Bmp4 concentrations, respectively (Fig. 1B). This is consistent with previous results suggesting that higher concentration thresholds of Bmp4 induce more dorsal cell fates (Liem et al., 1997). However, the expression of chick *Ath1* was consistently detected in explants treated with low concentrations of Bmp4, indicating that concentration alone is not sufficient to discriminate between the generation of dP1 and dP2 cells.

Could differences in the dynamics of chick *Ath1* and *Ngn1* contribute to their differential expression? To test this, we monitored the timing of chick *Ath1* and *Ngn1* induction after exposure to Bmp4. Strikingly, *Ngn1* expression was induced by all concentrations of Bmp4 within 6 hours of culture. It reached a peak at 12 hours and then declined at high Bmp4 concentrations (8 and 16 ng/ml) after 18 and 24 hours of culture (Fig. 1C). By contrast, chick *Ath1* was strongly induced only after 24 hours. The delay in chick Ath1 induction could not be explained solely by a change in competence of explants over time, because, if addition of BMP was delayed by 24 hours, induction of chick Ath1 still required 18 hours of exposure to Bmp4 (supplementary material Fig. S1B).

The more rapid induction of *Ngn1* compared with chick *Ath1* prompted us to test whether different durations of exposure to the ligand could discriminate progenitor identity. Explants were exposed to 8 ng/ml Bmp4 for times ranging from 1 to 24 hours, then after the predetermined period of treatment, Bmp4-containing media was replaced with control media and gene expression was assessed, in all cases, at 24 hours (Fig. 1D). This revealed that short exposure times (1-2 hours) were sufficient to induce high levels of *Ngn1* expression. By contrast, only sustained Bmp4 treatment induced substantial chick *Ath1* expression. These data indicate that different durations of BMP exposure generate distinct and exclusive profiles of dorsal progenitor marker expression. Importantly, chick *Ath1* and *Ngn1* have been shown to exert cross-inhibitory interactions on each other (Gowan et al., 2001), suggesting that the induction of chick *Ath1* expression is responsible for the downregulation of *Ngn1*.

Longer durations of Bmp4 exposure generate higher levels of signalling

To address how the dynamics of Bmp4 exposure were transduced into intracellular signalling, we took advantage of a reporter of BMP activity consisting of a BMP responsive element (BRE) directing the expression of luciferase (Le Dréau et al., 2012). We first monitored the levels of signalling induced by different concentrations of Bmp4 (Fig. 2A). During the first 18 hours, intracellular signalling activity increased in response to all concentrations of Bmp4 and was approximately proportional to the concentration. From 18 hours, the profile of intracellular signalling differed depending on concentration. It continued to rise for the lowest concentration (2 ng/ml), plateaued for the intermediate concentration (4 ng/ml) and declined for the highest concentrations (8-16 ng/ml). These data indicate that different concentrations of Bmp4 generate distinct signalling profiles and that the highest concentrations trigger negative feedback within 24 hours (Akizu et al., 2010; Park, 2005).

We next monitored BRE-Luc activity in explants that had been exposed to 8 ng/ml Bmp4 for different durations (Fig. 2B). Explants were exposed to Bmp4 for 1-24 hours. After the period of treatment, Bmp4 was removed, replaced with control media and signalling activity subsequently assayed. To test the efficiency of ligand removal, the media used to wash out Bmp4 was transferred to naïve explants and BRE-Luc activity was measured. No significant BRE-Luc activity was found in explants that had received the transferred wash media (supplementary material Fig. S1D), suggesting that little if any BMP remained after the medium was exchanged. Strikingly, after Bmp4 removal, intracellular signalling activity was

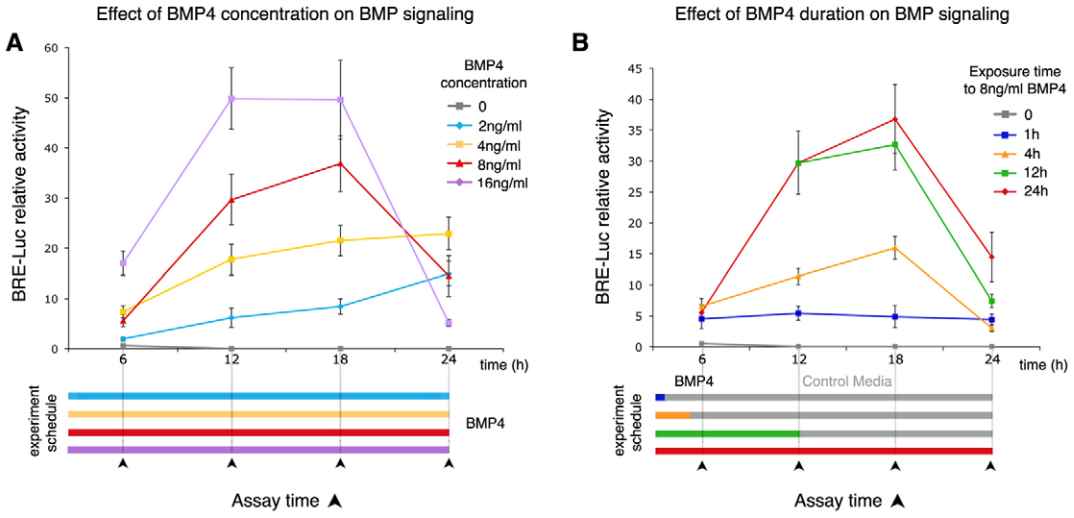

Fig. 2. Concentration and duration of BMP induce different profiles of intracellular signalling activity. (**A**,**B**) Intracellular BMP signalling activity measured with BRE-luciferase in [i] explants. HH stage 10 embryos were *in ovo* electroporated with BRE-Luc and a Renilla normalizing construct. After 2-3 hours of incubation, neural plate explants were dissected and exposed either to the indicated concentrations of Bmp4 (A) or to 8 ng/ml of Bmp4 for 1, 4, 12 and 24 hours (B). Reporter assays were performed at 6, 12, 18 and 24 hours, as indicated in the experimental schemes below each graph. Data are mean±s.e.m.

maintained at 18 hours, at least at the level reached during exposure to the ligand (Fig. 2B). Except for the 1-hour exposure condition, which remained constant, the levels of BRE-Luc activity dropped significantly between 18 and 24 hours, consistent with late-onset negative feedback. Together with the dynamics of gene induction (Fig. 1), these data indicate that BMP signalling 5- to 10-fold above basal levels, produced by 1-4 hours exposure to BMP, is sufficient to induce the expression of *Ngn1* at 6 hours. By contrast, longer times of exposure to Bmp4 induce higher levels of signalling and the expression of chick *Ath1* (Fig. 1D, Fig. 2B). Therefore, chick *Ath1* expression correlates with at least threefold more signalling activity than *Ngn1* [compare the 1-hour (blue) and 24-hour (red) conditions at the 24-hour time point in Fig. 2B]. Together, these data indicate that the level of intracellular signalling progressively increases in progenitors exposed to a constant concentration of Bmp4. By contrast, ligand removal leads to sustained signalling over a period of time, with the level of signalling determining differential gene expression.

A dynamic gradient of BMP activity in the dorsal spinal cord

To address how the *ex vivo* response of neural cells relates to the normal development of dorsal neural progenitors, we analysed the distribution of BMP signalling activity in the chick NT. To this end, we took advantage of an antibody recognizing the phosphorylated (active) forms of Smad1, Smad5 and Smad8 (Patterson et al., 2010) and performed immunostainings on HH stage 9 to HH stage 20 embryos. We focused our attention on dorsal progenitors at the brachial level (adjacent to somites 16 to 21). Fate-mapping data (Brown and Storey, 2000) suggest that brachial progenitors are localized in the caudal plate (caudal region of the neural plate, before NT closure) in HH stage 9-10 embryo and at the transition between somites and pre-somitic mesoderm in HH stage 12 embryos. The dorsal 50% of the neural plate or tube was divided into 10 equal bins and the average pixel intensity in each bin was plotted as a function of DV position (Fig. 3A). At HH stage 9, the PSmad staining in the neural plate was barely distinguishable from background (Fig. 3A, grey line). However at HH stages 10 and 11-12, a dorsal-to-ventral gradient was detected and its amplitude increased over time (compare the light-blue and blue lines) and along the anterior-posterior (AP) axis (compare the blue and dark-blue lines) in HH stage 12 embryos (Fig. 3A). At later time points (HH stages 16 and 20), the amplitude remained approximately constant but the slope steepened relative to the size of the NT (Fig. 3A; supplementary material Fig. S2A). These data suggest that dorsal progenitors are exposed to different dynamics of BMP activity such that more dorsal cells experience sustained and high level of signalling (supplementary material Fig. S2A).

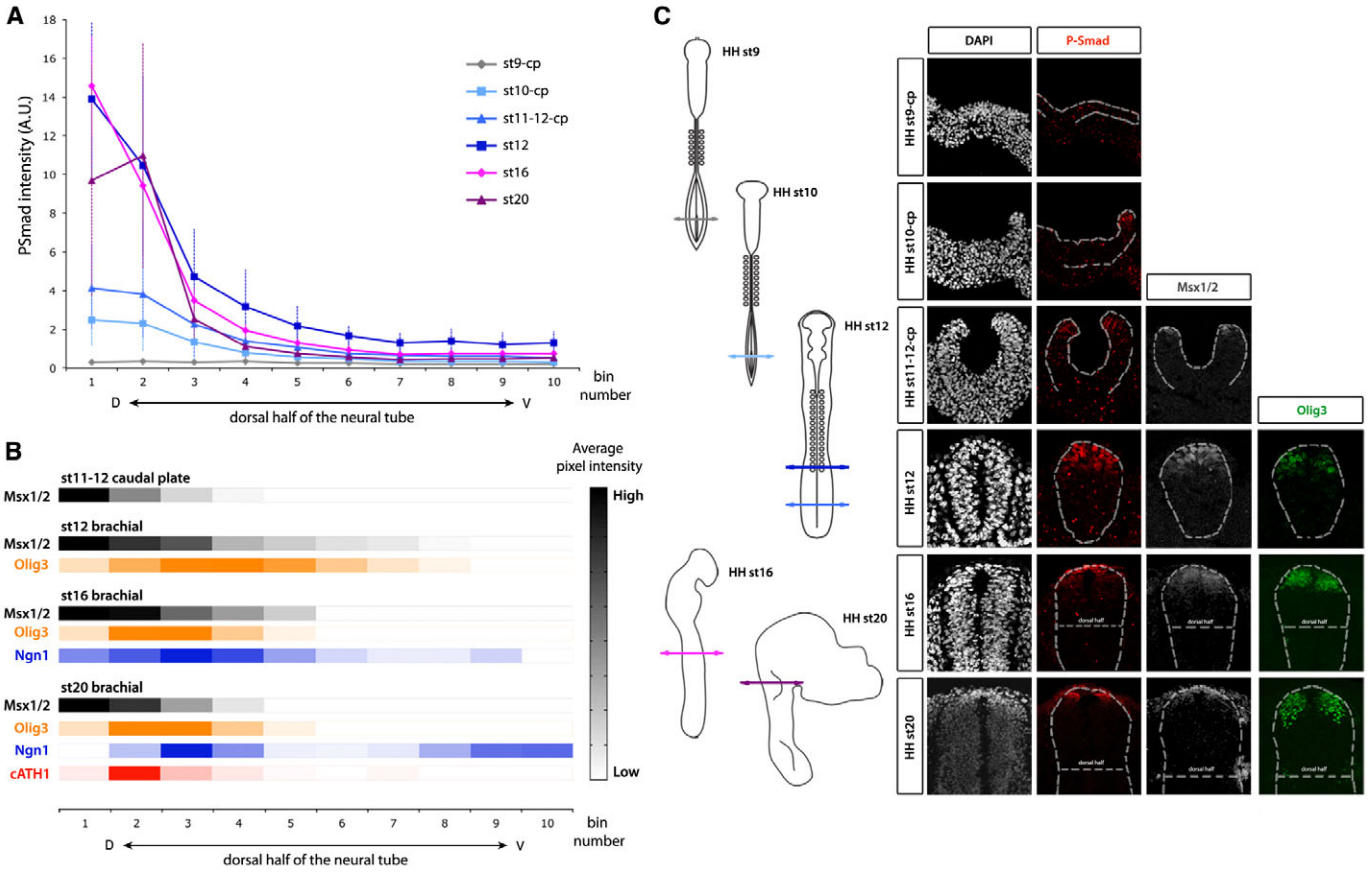

Fig. 3. BMP signalling displays a dynamic profile of activity during dorsal neural tube development. (**A**) Distribution of Phospho-Smad (PSmad) intensity along the DV axis of the dorsal NT. The average pixel intensity of PSmad staining was plotted as a function of the DV position in the dorsal half of the NT. Immunostaining for PSmad was performed on transverse sections of embryos taken at the indicated stages and AP positions. Error bars represent s.d. (**B**) The average pixel intensity following either immunostaining for Msx1/2 and Olig3 or *in situ* hybridization for Ngn1 and chick Ath1 was converted into a level of grey (and coloured to facilitate the reading) and plotted as a function of the DV position, matching the *x*-axis in A. (**C**) Left panel: schematics of the different embryonic stages, the double-headed arrows indicating the AP levels at which transverse sections were analysed. Right panel: transverse sections immunostained for P-Smad, Msx1/2 and Olig3 at the indicated stages and AP levels. cp, caudal plate; br, brachial.

The profile of BMP activity correlates with the appearance of dorsal markers

We next asked how the pattern of BMP activity was correlated with the distribution of dorsal markers (Fig. 3; supplementary material Fig. S2B). Importantly, Msx1/2, which is considered to be a direct target of the pathway (Suzuki et al., 1997), showed a distribution reminiscent of the PSmad gradient (Fig. 3B, greyscale, 3C). In parallel, we assayed the expression of Olig3, a marker of the dP1-3 population (see Fig. 1A). In contrast to Msx1/2, all cells expressed similar levels of Olig3 (Fig. 3B, orange scale, 3C). To gain more insight into the identity of dorsal progenitors, we monitored the expression of *Ngn1* and chick *Ath1* at different developmental stages (Fig. 3B, blue and red scales; supplementary material Fig. S2B). *Ngn1* was first detected at brachial level in HH stage 16 embryos, whereas chick *Ath1* was only expressed from HH stage 18. Moreover, *Ngn1* expression appeared first in a rather broad domain in the dorsal NT at HH stage 16 before becoming restricted to bin 3 [the ventral domain of *Ngn1* expression corresponds to dP6 progenitors (see Fig. 1A)], concomitant with the appearance of chick Ath1 in bin 2. These data are consistent with the expression dynamics measured in naïve explants (Fig. 1) and with the cross-repression between Ngn1 and chick Ath1 (Gowan et al., 2001). Ngn1 is sensitive to lower levels of BMP activity and can therefore be expressed earlier and in a broader domain than chick Ath1. Moreover, the evolution of the PSmad gradient suggests that chick Ath1 and Ngn1 expression might also depend on different durations of signalling: chick Ath1 is mainly expressed in bin 2 where signalling is high and constant, whereas Ngn1 is mainly expressed in bin 3 where signalling is lower and decreases after stage 12 (Fig. 3; supplementary material Fig. S2).

Dorsal identities are induced sequentially *in vivo*

The dynamic nature of the PSmad profile prompted us to test the requirement for BMP signalling at distinct developmental time points. We reasoned that later blockade of the BMP pathway would allow higher levels of signalling to be attained and therefore more dorsal populations to be induced. Thus, we decided to inhibit BMP signalling in a cell-autonomous manner by transfecting the chick NT with a plasmid expressing Smad6, an inhibitor of the BMP pathway (Hata et al., 1998). To test the efficiency of the Smad6 construct, we measured BRE-luciferase activity at different times following transfection of Smad6 or/and Bmp4 (supplementary material Fig. S3A). Smad6 reduced endogenous and ectopic Bmp4 induced BRE-luciferase activity by over 90% within 3 hours. By contrast, Smad6 had no effect on the level of Wnt signalling assayed with the TopFlash-Luc reporter (data not shown). In addition, the expression of the BMP targets Msx1/2 was severely reduced 12 hours after Smad6 transfection (supplementary material Fig. S3B). Importantly, the configuration used for electroporation (EP) rarely, if ever, targeted the RP. Consistent with this, expression of the RP markers Lmx1b, Wnt1 and Bmp4 was unaffected in all embryos analysed, suggesting that RP-derived signals were produced normally (supplementary material Fig. S3B,C; data not shown).

We next assessed the consequences of a blockade of BMP signalling on dP1-3 progenitors and their corresponding neurons. EP of Smad6 at HH stage 8 led to a reduction of the entire dP1-3/dI1-3 population without affecting the proliferation rate (Fig. 4; supplementary material Fig. S3B,C). We then blocked the pathway at successively later stages. Whereas the expression of Olig3 was progressively recovered (Fig. 4A), we observed striking differences between the subpopulations of dorsal progenitors. The number of dI3 neurons generated was substantially reduced only when Smad6 was transfected at HH stage 8, later transfections resulted in a progressive recovery of the population (Fig. 4B). By contrast, the numbers of dI2/dP2 cells were only fully recovered after HH stage 18 EP, whereas dI1/dP1 cells were always strongly reduced after Smad6 transfection (Fig. 4B; Fig. 5A,B). These data are consistent with a model in which later blockades of signalling allow higher levels of BMP activity to be reached and therefore more dorsal populations to be specified. Importantly, unlike the removal of Bmp4 in our *ex vivo* experiments, which resulted in sustained levels of signalling (Fig. 2B), transfection of Smad6 led to a marked

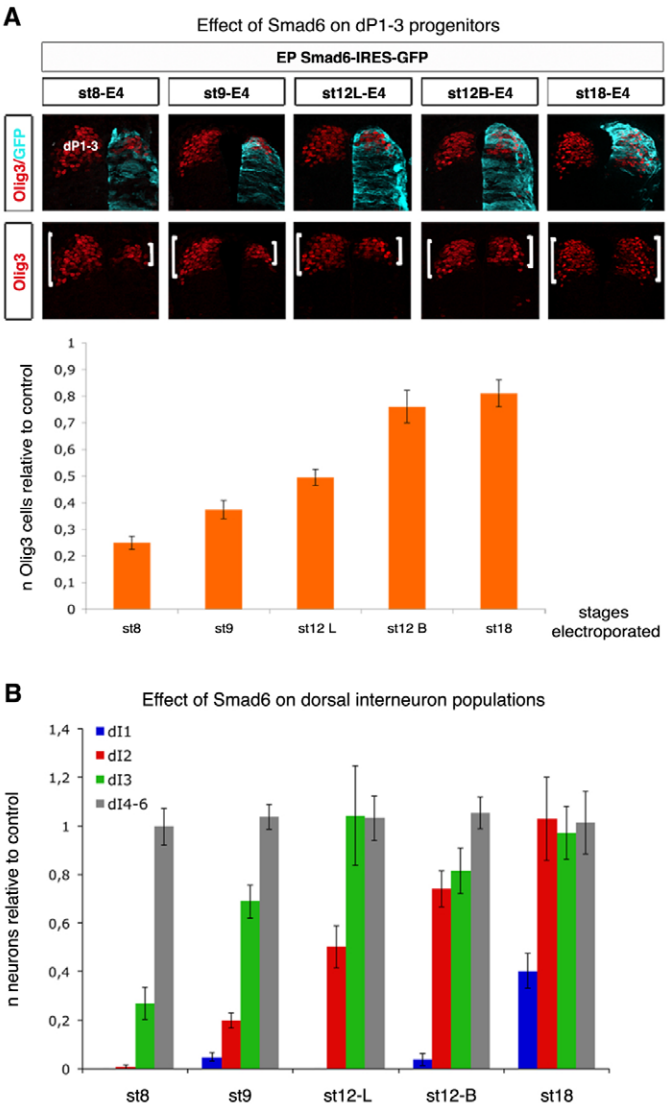

Fig. 4. Dorsal progenitors and interneurons require different durations of BMP signalling. (**A**) Upper panel: expression of Olig3 (dP1-3) at E4, after electroporation (EP) of Smad6 at the indicated stages (transfected side is on the right of each image). Brackets indicate the extent of Olig3 expression. Lower panel: the graph shows the relative numbers of Olig3 cells after Smad6 EP at the indicated stages. The ratios were calculated by counting the number of Olig3 cells expressing Smad6 and normalizing this to the number in control GFP-transfected embryos. All sections were taken at brachial levels, except for HH stage 12 for which both lumbar (L) and brachial (B) levels were analysed. (**B**) Relative numbers of each dorsal neuronal subtype after Smad6 EP at the indicated stages (the ratios were calculated as for Olig3 in A). Dorsal interneuron populations were identified as follows: dI1, Lhx2/9^{+}; dI2, Lim1/2^{+}Lbx1^{-}; dI3, Isl1/2^{+}; dI4-6, Lbx1^{+}. Data are mean±s.e.m.

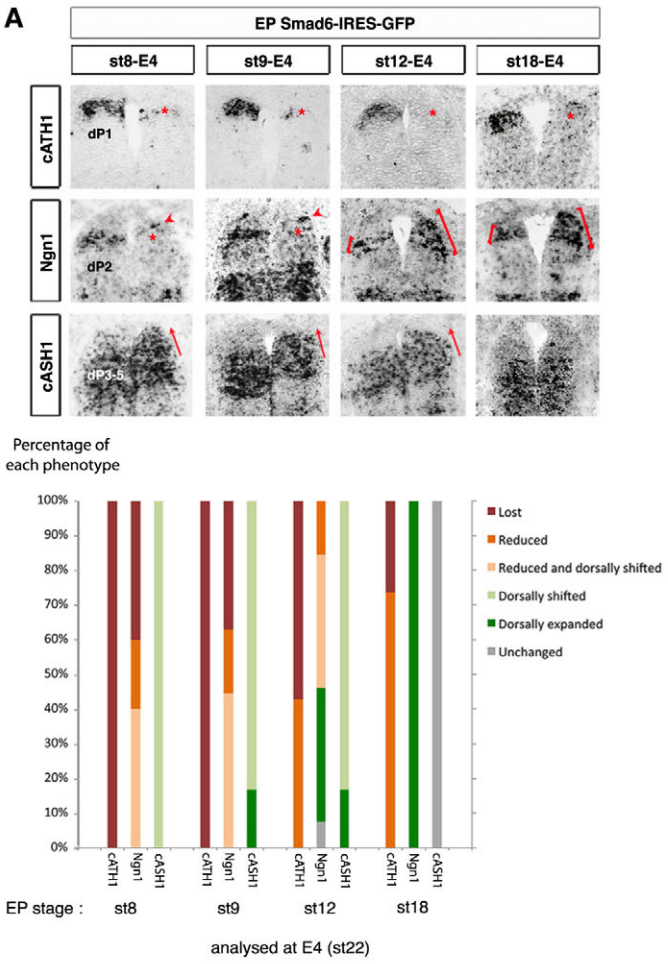

Fig. 5. Blockade of BMP signalling leads to respecification of dorsal interneurons. (**A**) Upper panel: expression of chick *Ath1* (dP1), *Ngn1* (dP2) and chick *Ash1* (dP3-5) at E4, after electroporation (EP) of Smad6 at the indicated stages (transfected side is on the right of each image). Asterisks, arrowheads, arrows and brackets indicate loss, ectopic, dorsal shift and dorsally expanded gene expression, respectively. Lower panel: the spatial distribution of dorsal progenitor markers shown in the upper panel was classified and the percentage of each phenotype was plotted for each marker and stage electroporated. (**B**) Transverse sections immunostained with the indicated markers at E4 following EP with Smad6 at HH stages 8, 9 and 12. dI4-6, dI3 and dI2 interneurons are seen at their endogenous position (brackets) but are also detected ectopically (arrowheads) in the most dorsal part of the NT. Ectopic dI3 and dI2 appear intermingled with non-transfected dI1 neurons. In the lower panel, Lbx1 appears ventrally in grey, labelling dI4-5-6 neurons, and overlaps with red Lhx1/5 labelling in dI4 and dI6 neurons. Thus, dI2 corresponds to the red-only cells, as indicated by the white brackets.

reduction in BMP activity (supplementary material Fig. S3A). This suggests that specification of dI1-3 progenitors relies not only on different levels but also on different durations of BMP signalling.

We then turned our attention to the spatial distribution of dI1-3 subtypes in the different BMP blockades. We reasoned that BMP blockade at successively later stages would affect the position of generation of dI4-6, dI3 and dI2 populations differently. They should be detected at ectopic dorsal positions, and the later BMP signalling is blocked the smaller the expected ectopic shift and the closer the position of these domains to wild type. Consistent with this, inhibition of the BMP pathway at HH stage 8 resulted in a dorsal shift of dP3-5 progenitors (Fig. 5A) and dI4-6 neurons (supplementary material Fig. S4D), these populations progressively occupying their normal position as BMP signalling was blocked later (Fig. 5A; supplementary material Fig. S4D). Moreover, although dI3 and dP2/dI2 populations were greatly reduced after inhibition of BMP signalling at HH stages 8 and 9, respectively (Fig. 4B), the cells that kept these identities were located more dorsally than normal (Fig. 5A, arrowheads; supplementary material Fig. S4B,C). As the BMP pathway was blocked at later stages, dI3 and dP2/dI2 were found at progressively more ventral positions. In addition, consistent with cross-inhibition from chick Ath1 (Gowan et al., 2001), dP2/dI2 were expanded dorsally when Smad6 was transfected at HH stages 12 and 18 (Fig. 5A, brackets; supplementary material Fig. S4B).

To gain better insight into the putative ventralization of cell identities, we took advantage of mosaic transfections of Smad6 and focused on the dI1 domain. This resulted in Smad6 transfected cells intermingled with non-transfected dI1 neurons. When Smad6 was transfected at HH stage 8, dI4-6 neurons appeared dorsally shifted but were never intermingled with dI1 (Fig. 5B). Nevertheless, in this condition, the chick Ash1 domain reached the most dorsal part of the NT (Fig. 5A). A possibility therefore is that dI1 progenitors are respecified as dP4-6 progenitors but fail to differentiate in the most dorsal part of the NT. By contrast, mosaic EP of Smad6 at HH stages 9 and 12 led to the generation of dI3 and dI2 neurons intermingled with non-transfected dI1, respectively (Fig. 5B). These data suggest a model in which the increase in BMP activity successively confers to the most dorsal progenitors the identity of dI3, dI2 and finally dI1 neurons.

DISCUSSION

BMP signals have been shown to play an important role in the generation and patterning of dorsal interneurons in the spinal cord. Here, we provide evidence that duration of exposure to the ligand discriminates between dorsal progenitor subtypes. Moreover, *in vivo* analysis of the BMP activity profile and blockade of the pathway at different stages suggest a model in which dorsal progenitors are specified sequentially over time along the DV axis.

The *ex vivo* experiments indicate that different Bmp4 exposure times generate distinct levels of BMP signalling, which in turn

induce the different dorsal interneuron populations (Figs 1, 2). This does not rule out the idea that graded distributions of ligand are important for signal interpretation, but instead emphasizes that the key parameter is the level of intracellular signalling. This is likely to be achieved, at least in part, by restricting ligand availability *in vivo*. Thus, BMP antagonists in the dorsal NT are likely to help establish intracellular signalling activity at the appropriate levels at different positions *in vivo*. Moreover, different combinations of ligand concentration/duration may lead to similar expression profiles. Consistent with this, a 1-hour exposure to Bmp4 could induce expression of chick Ath1 as long as the concentration was high enough (supplementary material Fig. S1C). A crucial step will be to measure the distribution of BMPs and BMP antagonists in the NT, and to define the profile of free BMP ligands at the relevant stages of development.

An interesting feature of BMP signalling in response to short exposure times is its ability to be maintained over a long period (Fig. 2B). This mode of action is reminiscent of Activin, another member of the TGFβ family. A short exposure to Activin (10 minutes) is sufficient to trigger nuclear translocation of Smad2 (effector of the pathway) and maintain it for up to 4 hours after treatment (Bourillot et al., 2002). The basis of this memory was suggested to rely on accumulation of signalling liganded receptor within intracellular vesicles (Jullien and Gurdon, 2005). A correlate of this is that cells can switch only to the expression of a higher dose response gene, the so-called 'ratchet effect'. Our data indicated that a 1-hour exposure to Bmp4 was sufficient to maintain BMP activity for over 20 hours after treatment, suggesting that the two pathways share common properties. Whether the molecular mechanism is similar remains to be determined. In addition, Wnt signalling has also been suggested to influence the stability of Smad proteins (Fuentealba et al., 2007). As Wnt ligands are expressed in the RP, they might also contribute to the duration of BMP signalling in dorsal progenitors.

Whether cells integrate the signalling activity over the whole period or instead become competent to interpret it only at a certain time window is an important issue. The observation that 2 ng/ml and 8 ng/ml Bmp4 conditions generate similar levels of signal at 24 hours (Fig. 2A), but the higher concentration induces a higher level of chick Ath1 expression, favours the idea that the signal is integrated over the whole period. Moreover, signalling activity decreases for the 8 ng/ml condition between 18 and 24 hours (Fig. 2A), suggesting that although chick Ath1 expression requires a high level of signalling to be reached, it can later be sustained by lower levels. Nevertheless, we cannot exclude the possibility that competence also evolves over time. Work by Tucker et al. performed in the zebrafish embryo (Tucker et al., 2008) shows that the BMP activity gradient is stable over time but rostral regions are competent to respond before caudal ones. In our case, explants exposed to Bmp4, even to a high concentration, do not express chick Ath1 before the 24-hour time point (Fig. 1C). Consistent with this, although the amplitude of endogenous BMP activity reaches a maximum at HH stage 12 (Fig. 3A), chick Ath1 expression is detected at all AP levels only from HH stage 20 (supplementary material Fig. S2). These data raise the possibility that chick Ath1 expression relies not only on a threshold of BMP activity but also on a competence window. To address this, we compared the induction of chick Ath1 in explants exposed to 8 ng/ml Bmp4 from the start of the experiment with explants that were matured in control media for 24 hours before exposure to BMP (supplementary material Fig. S1B). This indicated that induction of chick Ath1 was still delayed when BMP was added to matured explants, albeit it took 18 hours rather than 24 hours to induce expression. This suggests that induction of chick Ath1 by BMP is intrinsically delayed compared with Ngn1.

Moreover, the dynamics of the BMP signalling gradient *in vivo* suggest that the distinct dorsal progenitors also require different durations of signalling (Fig. 3). Indeed, blocking the BMP pathway at different stages led to strikingly different phenotypes. Early blockade resulted in a loss of the whole population of dI1-3 neurons, while later inhibition successively allowed the generation of dI3 and dI2 neurons, with dI1 being strongly reduced in all conditions analysed (Fig. 4B). This suggests that specification of dI1-3 progenitors relies not only on different levels but also on different durations of BMP signalling. Thus, dI1, dI2 and dI3 require progressively lower levels and shorter durations of signalling. These findings provide an explanation to previous BMP loss-of-function studies that failed to produce a complete loss of the dI1-3 population (Chesnutt et al., 2004; Wine-Lee et al., 2004). In the latter case, the BMP pathway was manipulated after NT closure and resulted in a loss of dI1, a reduction of dI2 but had no effect on dI3 cells, a phenotype similar to our EP at HH stage 12. This strengthens the conclusion that dI3, and to some extent dI2, are specified in the caudal neural plate from HH stage 8 and do not then require further BMP input. Nevertheless, it should be noted that from HH stage 18, BMP7 is expressed in characteristic stripes along the DV axis and is required for the differentiation of dI1, dI3 and dI5 neurons, without affecting the pattern of progenitor domains (Le Dréau et al., 2012). This change of activity parallels observations in zebrafish mesoderm development (Pyati et al., 2005; Pyati et al., 2006) in which BMP signalling was shown to be required for ventral mesoderm formation until early gastrulation. However, from mid-gastrulation, BMP is needed to repress ventrolateral fates in favour of ventral-most cells.

In addition, our data suggest that dorsal interneurons dI3, dI2 and dI1 are sequentially induced in the dorsal spinal cord by increasing levels of BMP signalling. This is suggested by the timing of expression of dP2/dI2 and dP1/dI1 *ex vivo* (Fig. 1C) and *in vivo* (supplementary material Fig. S2B). Furthermore, mosaic transfections suggest that progenitors fated to become dI1 instead adopt dI3 identity when BMP signalling is blocked at HH stage 9, and dI2 identity when BMP is blocked at HH stage 12 (Fig. 5B). Taken together, these results suggest that dorsal progenitors are successively fated as dI3, dI2 and finally dI1.

BMP antagonists secreted by the notochord (Liem et al., 2000) (data not shown) might help shape the gradient of signalling, thus establishing the conditions for the generation of discrete populations of dorsal interneurons. Nevertheless, other signals are likely to take part in the specification of dI1-3 neurons. Wnt1/3a secreted by the RP have been implicated in both proliferation and specification of dorsal progenitors (Megason and McMahon, 2002; Zechner et al., 2003; Zechner et al., 2007). In particular, Wnt signalling was shown to promote the expression of the transcription factors Msx1/2 and Olig3, both of which are involved in the generation of dorsal interneurons (Bai and Joyner, 2001; Liu et al., 2004; Müller et al., 2005; Zechner et al., 2007). Thus, BMP and Wnt signals are likely to cooperate during the establishment of DV identities.

More generally, our study provides new insight into the mechanisms of morphogen interpretation. Specifically, the activity of BMP signalling in the generation of distinct dorsal neuronal subtypes can be compared with the mechanism of ventral neuron specification by graded Shh signalling. In both cases, the duration of exposure to the morphogen is important and there is evidence that negative feedback influences both signalling pathways. However, the dynamics of the two pathways differ. Peak Shh signalling appears to

be reached within 6 hours (Dessaud et al., 2010), whereas peak BMP signalling takes 12-18 hours and the *in vitro* data indicates that signal gradually increases during this time (Fig. 2A). This suggests a mechanism in which the duration of BMP exposure is transformed into distinct levels of intracellular signalling that then control differential gene expression. We propose that the transcriptional network downstream of the BMP pathway (Gowan et al., 2001) is responsible for the interpretation of signalling. Together, these data therefore emphasize the temporal component in the interpretation of morphogens and reveal diversity in the strategies adopted in the provision of positional information to developing tissues.

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The authors declare no competing financial interests.

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