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Canonical BMP7 activity is required for the generation of discrete neuronal populations in the dorsal spinal cord

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SUMMARY

BMP activity is essential for many steps of neural development, including the initial role in neural induction and the control of progenitor identities along the dorsal-ventral axis of the neural tube. Taking advantage of chick in ovo electroporation, we show a novel role for BMP7 at the time of neurogenesis initiation in the spinal cord. Using in vivo loss-of-function experiments, we show that BMP7 activity is required for the generation of three discrete subpopulations of dorsal interneurons: dI1-dI3-dI5. Analysis of the BMP7 mouse mutant shows the conservation of this activity in mammals. Furthermore, this BMP7 activity appears to be mediated by the canonical Smad pathway, as we demonstrate that Smad1 and Smad5 activities are similarly required for the generation of dI1-dI3-dI5. Moreover, we show that this role is independent of the patterned expression of progenitor proteins in the dorsal spinal cord, but depends on the BMP/Smad regulation of specific proneural proteins, thus narrowing this BMP7 activity to the time of neurogenesis. Together, these data establish a novel role for BMP7 in primary neurogenesis, the process by which a neural progenitor exits the cell cycle and enters the terminal differentiation pathway.

KEY WORDS: Bone morphogenetic proteins, Neural tube, Interneurons, Primary neurogenesis, Smad proteins, Tissue patterning, Proneural bHLH proteins

INTRODUCTION

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β (TGFβ) superfamily of secreted proteins. During development, these proteins play multiple roles in organogenesis, cell type differentiation and tissue patterning. Moreover, altered BMP signalling is associated with cancer, skeletal and vascular diseases. The varied functions of BMP signalling are exemplified during neural tissue development. The first step that determines whether cells form neural or non-neural tissue (Stern, 2006) and the definition of the region from which the neural crest will be generated involve BMP signalling (Basch and Bronner-Fraser, 2006). Subsequently, after the neural tube (NT) closure, the dorsal-most part of the spinal cord (roof plate) becomes a source of BMP signals, and BMP-dependent activity has been proposed to provide the positional information that controls progenitor identities along the dorsoventral (DV) axis (reviewed by Helms and Johnson, 2003; Lee and Jessell, 1999; Liu and Niswander, 2005). Moreover, once neurons are generated, BMP signalling is again implicated in the outgrowth and guidance of axonal projections (reviewed by Sanchez-Camacho and Bovolenta, 2009).

Despite these diverse activities, the signalling pathway appears substantially linear. BMP ligands bind to a defined receptor complex composed of two transmembrane serine/threonine kinases, called type I and type II receptors. Ligand binding induces the formation of a heteromeric receptor complex in which the type II receptor phosphorylates, and thereby activates, the type I receptor. The activated type I receptor then propagates the signal through the Smad family of intracellular mediators. Smad proteins form a complex that moves to the nucleus where, together with additional DNA-binding proteins, they regulate transcription of target genes (Shi and Massague, 2003). In the case of BMPs, the various type I subunits phosphorylate one of three Smads – Smad1, Smad5 or Smad8 (Massague et al., 2005). However, BMPs can also signal through alternative non-canonical and completely transcription-independent cascades (Derynck and Zhang, 2003; Kang et al., 2009).

In the dorsal spinal cord of chick and mouse embryos, six distinct populations of dorsal interneuron subtypes (dIN; dI1-dI6) differentiate from non-overlapping domains of progenitors, arrayed along the dorsal-ventral axis. The initial description of a role for BMP in dorsal interneuron (dIN) generation came from studies in which purified active proteins or purified inhibitors were added ex vivo, in explants assays. These studies showed that several TGFβ/BMPs (including BMP4, BMP5, BMP7, Dsl1 and activin A/B) have the capacity to induce two types of dIN identified as Lhx2/9+ (dI1) and Islet1+ (dI3) (Liem et al., 1997; Liem et al., 1995). Subsequently, the description for an in vivo requirement of a TGFβ/BMP activity in dIN generation came from the analysis of GDF7, a distant member of the BMP subfamily. After neural tube closure, GDF7 is expressed in the roof plate, and GDF7 activity is required for the generation of a discrete sub-group of dIN (Lhx2a+, dI1A) (Lee et al., 1998). These studies raised the possibility of qualitative distinct activities for TGFβ/BMP proteins in the specification of particular subgroups of dIN, an idea that remains unresolved.

Furthermore, increasing the levels of BMP activity by the overexpression of two activated receptors, caBMP-RIa (ALK3) and caBMP-RIb (ALK6), increased the expression of dorsal progenitor markers such as Pax7 and the generation of selected dINs (dI1 and dI3) (Timmer et al., 2002). However, mice lacking these two same

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receptors (BMP-RIa and BMP-RIb) show a different phenotype in which dI1 are missing, numbers of dI2 are reduced, but dI3 are generated in normal numbers (Wine-Lee et al., 2004). All these data supported an in vivo role for BMP activity in NT development; however, they did not distinguish an early role for BMP in patterning from a later role in the specification of dorsal progenitors and differentiation of their progeny. This issue also remains unresolved.

On the other hand, several TGFβ proteins, of both the TGFβ/Activin and the BMP subfamilies participate in the control of neural stem cell self-renewal and differentiation. TGFβ signalling promotes cell cycle exit and neurogenesis, both in the spinal cord (Garcia-Campmany and Marti, 2007) and in discrete brain areas (Falk et al., 2008). BMPs can also promote cell cycle arrest in the dorsal spinal cord (Ille et al., 2007), although this role appears to be dependent on the developmental stage and the choice of the receptor (Panchision et al., 2001). Moreover, BMPs regulate the balance of neural stem cells quiescence and proliferation in the adult hippocampus (Mira et al., 2010).

Based on the observation that BMP7 is expressed in several progenitor domains of the dorsal spinal cord, at the time of primary neurogenesis, we set out to examine whether BMP7 signalling controls the generation of dorsal neuronal subtypes. Taking advantage of chick in ovo electroporation, we have individually knocked down BMP4 and BMP7, at the time primary neurogenesis has been initiated in dorsal progenitors. We show that BMP7 is required to sustain the differentiation of three dorsal interneuron subpopulations; dI1, dI3 and the more ventral dI5, whereas BMP4 activity only controls dI1 generation. Additionally, we show that this BMP7 activity is mediated by the canonical Smad pathway, using Smad1 and Smad5. Analysis of the BMP7 mice mutant shows the conservation of this activity in mammals. Furthermore, we show that BMP7 activity is not required for dorsal-ventral patterning, a role that might be associated with a different BMP, thus supporting qualitative distinct activities for BMP proteins. These data demonstrate a novel and specific role for BMP7 that might be relevant for the differentiation of other neuronal identities during CNS development and might also be relevant for the in vitro generation of neurons from stem cells.

MATERIALS AND METHODS

Mouse and chick embryos

Mice homozygous for the BMP7 null allele were as published (Dudley et al., 1995). Eggs from White-Leghorn chickens were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951). For chick embryo electroporation, plasmid DNA was injected into the lumen of neural tubes and electroporation carried out using an Intracel Dual Pulse (TSS10) electroporator.

For bromodeoxyuridine (BrdU) labelling, 0.5 μg/μl BrdU was injected into the neural tubes 40 minutes prior to fixation. Transfected embryos were allowed to develop to the indicated stages, then dissected, fixed and processed as indicated.

DNA constructs

Pseudo-phosphorylated mutant versions of R-Smads (Smad-S/D) were generated by PCR, replacing the three serines at the C-terminal end by aspartic acid, and cloned into pCAGGS-ires-GFP and into pCAGGS-ires-H2B-RFP.

A BMP responsive element (BRE) was generated containing two copies of two distinct, highly conserved BMP-responsive elements (BRE) encompassing the genomic regions –1032/–1052 (SBE-3, SBE-2, GC'-5) and –1080/-1105 (CAGC-2, CAGC-1, GC'-3,4) of the natural human/mouse Id1 promoter (Korchynskyi and ten Dijke, 2002), and cloned into pGL3- (Promega) derived vectors tk-Luc or TATA-Luc for in vivo Luciferase transcription assays. Alternatively, the Luciferase reporter BRE-tk-Luc was replaced by GFP to monitor spatio-temporal BMP activity in vivo. The SBE-MLP:Luc (CAGA-12) was kindly gifted by P. ten Dijke (Leiden University Medical Centre, The Netherlands).

Short RNA hairpin (sh-RNA)-based expression vectors for RNA interference were generated using the pSuper RNAi system (OligoEngine). Pairs of custom 60-nucleotide (nt) oligonucleotides that contain unique 19-nt sequences for each mRNA were designed using the RNAi Design Tool (www.oligoengine.com) and purchased from Sigma. They were cloned into the pSHIN vector which contains the pSuper shRNA expression cassette and an independent eGFP-encoding cassette. Forward and reverse 19-nt sequences are shown in supplementary material Figs S4, S5.

Immunohistochemistry and in situ hybridization

For immunohistochemistry, embryos were fixed 2-4 hours at 4°C in 4% PFA in PB. For BrdU detection, sections were incubated in 2 N HCl for 30 minutes followed by 0.1 M $Na_2B_4O_7$ (pH 8.5) rinses, further PBT rinses and anti-BrdU incubation. Immunostaining was performed following standard procedures on either vibratome or cryostat sections. Several primary antibodies were generously gifted by labs of C. Birchmeier (Max-Delbrück-Center for Molecular Medicine, Berlin) (Lbx1, Tlx3, Olig3), M. Goulding (The Salk Institute, La Jolla, CA, USA) (Lbx1, Gsh1/2) and T. Jessell (Columbia University, NY, USA) (Lhx2/9). Monoclonal antibodies to BrdU, Islet1, Lhx1/5, Lmx1b and Pax7 were obtained from the Developmental Studies Hybridoma Bank (DSHB). Others were purchased from Invitrogen (GFP, Sox2, HuC/D) or BD Biosciences (cleaved caspase 3). Alexa488-, Alexa555- and Cy5-conjugated secondary antibodies were purchased from Invitrogen and Jackson Laboratories. After staining, sections were mounted, photographed using a Leica SP5 Confocal microscope, and processed with Adobe Photoshop CS3. Cell countings were carried out on pictures obtained from at least six different chick embryos per experimental condition, and five different mouse embryos for each genotype.

For in situ hybridisation, embryos were fixed overnight at 4°C in 4% PFA in PB, rinsed and processed for whole-mount RNA in situ hybridization following standard procedures using probes for chick Smad1, Smad5, Smad8, BMP4, BMP7, Atho1, Ngn1, Ascl1 (from the chicken EST project, UK-HGMP RC). The chick Ptf1a probe was obtained from T. Müller (C. Birchmeier's lab). Hybridized embryos were postfixed in 4% PFA, rinsed in PBT and vibratome sectioned and photographed on a Leica DMR microscope.

RT-quantitative real-time-PCR

EGFP-containing plasmid DNAs were electroporated and neural tubes dissected out 24 or 36 hours later. Single cell suspension was obtained by 10-15 minutes incubation in Tripsin-EDTA (SIGMA). GFP+ cells were sorted by flow cytometry using a MoFlo flow cytometer (DakoCytomation). Total RNA was extracted following the Trizol protocol (Invitrogen). Reverse transcription and real-time PCR were performed according to manufacturer's instructions (Roche) using a LC480 Lightcycler (Roche). Specific primers used for qPCR amplification of target genes were purchased (QuantiTec Primer Assays, Qiagen). Oligonucleotides specific for chick Gapdh were used for normalization. PCR amplifications were assessed from pools of electroporated neural tubes (15/pool), using 2-3 independent pools per experimental condition. Data represent mean standardised values±s.e.m.

Luciferase reporter assay

Transcriptional activity assays of pseudo-phosphorylated Smads or somitabun were performed in vivo. Chick embryos were electroporated with indicated DNAs or empty pCIG vector as control, together with the indicated firefly luciferase vector and a renilla luciferase reporter construct (Promega) for normalization. Embryos were harvested after 24 hours incubation in ovo and GFP-positive neural tubes were dissected and homogenized with a douncer in Passive Lysis Buffer on ice. Firefly- and renilla-luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega). Data are represented as mean±s.e.m. from at least two independent experiments (n=8-12 embryos per experimental condition).

Statistics

Quantitative data are expressed as mean±s.e.m. Statistical analysis was performed using the Statview software. Significance was assessed by performing ANOVA followed by the Student-Newman-Keuls test, except for experiments with somitabun, whose effects were examined using Student's *t*-test (**P*<0.05, ***P*<0.01 and ****P*<0.001).

RESULTS

BMP7 is expressed in specific domains of the dorsal spinal cord during dI1-dI6 neurogenesis

Using combinations of markers specific for each neuronal subtype (Fig. 1A), we determined that, in the chick spinal cord, neurogenesis of the six early born neuronal dI1-6 populations is accomplished in a narrow time window: it is initiated at Hamburger and Hamilton stage 18 (30/36 somites, 3 days of development) and by HH23 (>45 somites, 4 days of development) ~50% of dI1-6 neurons are generated (Fig. 1B). dI1-6 identities were not generated sequentially, but instead the number of neurons increased at a similar pace for each specific identity (Fig. 1B; supplementary material Fig. S1A-O). To search for the instructive signals that control the differentiation of dI1-dI6, we focused on BMP4/7 expression during this developmental time window. BMP4 expression was restricted to the roof plate at all stages examined (Fig. 1C). By contrast, high BMP7 expression was detected in several dorsal domains of progenitors from dI1 to dI5/6, but not in differentiated neurons, at the time when dI1-6 were being generated (Fig. 1C; supplementary material Fig. S2A,B,E,F).

To address where in the spinal cord the BMP pathway was active, we designed a BMP-responsive element (BRE)-tk-GFP reporter (see supplementary material Fig. S3A-D). Having first ensured that GFP expression was specifically due to the BMP-responsive element inserted upstream of the tk promoter (supplementary material Fig. S3A,B), we further tested the specificity of the BRE-tk-GFP reporter by analyzing its responsiveness to Smad proteins. Pseudo-phosphorylated Smad1, Smad5 and Smad8 (Smad1/5/8-SD, supplementary material Fig. S3C,D) were generated. The transcriptional activity of these proteins was evaluated by co-electroporation of Smad1/5/8-SD with the BMP reporter (BRE-TATA-Luc) and luciferase activity was assayed 24 hours post-electroporation (PE). Smad1-SD and Smad5-SD exhibited significantly increased transcriptional activity, whereas Smad8-SD appeared to function only as a weak activator (supplementary material Fig. S3E). Specificity of the reporter was shown by the lack of activation with Smad3-SD, a component of the TGFβ/Activin signalling pathway.

Co-transfection of the BRE-tk-GFP reporter with activated Smad forms Smad1/5 (Smad1/5-S/D-ires-RFP) increased GFP expression in a cell-autonomous manner (supplementary material Fig. S3D), indicating that it responded to activation of the BMP-regulated Smads. Electroporation of Smad8-S/D did not result in any obvious changes in BRE-tk-GFP reporter activity.

Next, we analyzed the activity of the BRE-tk-GFP reporter at development stages of primary dorsal neurogenesis. Twenty-four hours PE at HH stage 11 (13 somites, 2 days of development),

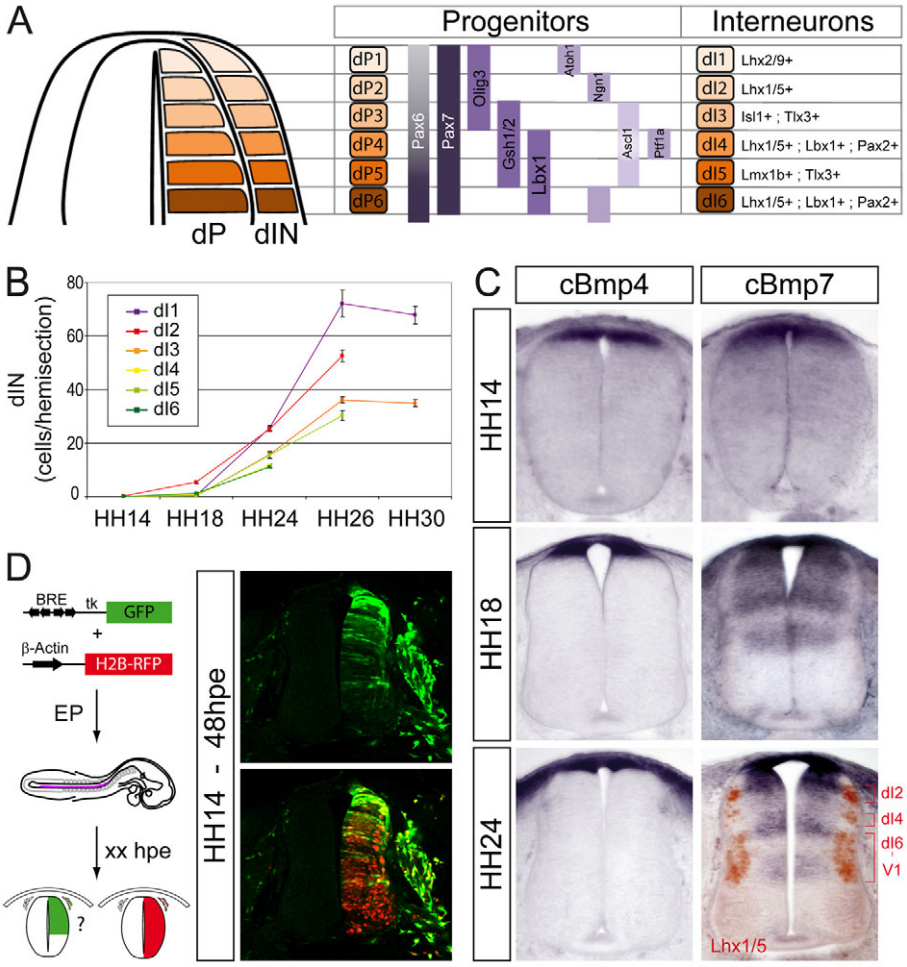

Fig. 1. BMP7 is expressed throughout the dorsal neural tube at the time of dorsal neurogenesis. (**A**) Schematic representation of a dorsal neural tube during primary neurogenesis, indicating markers specific for each progenitor population (dP1-dP6) and each neuron subgroup (dI1-dI6). (**B**) Quantitative data show mean±s.e.m. total number of marker-expressing cells by hemisection, at the indicated stages. Onset of neurogenesis is at HH18 (30/36 somites), by HH23 ~50% of neurons are born, final numbers are reached at HH26. (**C**) Expression of chick BMP4 and chick BMP7 at the indicated stages. BMP4 expression is restricted to the roof plate, whereas BMP7 is expressed through the dorsal half of the spinal cord. (Bottom right) Combined immunohistochemistry with Lhx1/5 to identify dI2, dI4 and dI6-V1. (**D**) Schematic representation of the experiment allowing visualization of the endogenous BMP activity in the NT. Electroporation of the BMP reporter BRE-tk-GFP shows the extent of BMP activation (green cells), compared with the control pCAGGS_ires_H2B-RFP vector expressed by every EP cell (red cells).

reporter activity was restricted to the dorsalmost part of the spinal cord. By contrast, late electroporations (HH14, 22 somites), showed GFP expression in the dorsal half of the spinal cord, marked by Pax7 expression (supplementary material Fig. S3B). Moreover, 48 hours PE at HH14 (HH stage 23/24), GFP expression extended throughout the dorsal and intermediate spinal cord (Fig. 1; supplementary material Fig. S3B). These data indicate that canonical BMP signalling is active at these stages of spinal cord development in a broad region of the dorsal and intermediate neural tube, and prompted us to investigate the role of BMP7 at these stages.

BMP7 activity is required for the generation of dI1, dI3 and dI5 neurons

To test the requirement of BMP4 and/or BMP7 activities at the time of primary neurogenesis, we independently knocked down each ligand, by electroporation of short-hairpin RNA (sh-RNA) that was specific for chick BMP4 and BMP7. Embryos were electroporated at HH14 (22 somites), before the onset of dI1-6 neurogenesis. Analysis by qPCR of target RNA in FAC-sorted transfected neural cells indicated that chick BMP4 and chick BMP7 RNA levels were significantly reduced at ~24 hours PE by the sh-BMP4-b and sh-BMP7-a constructs, respectively (supplementary material Fig. S4A-D). This timing resulted in the reduction of endogenous BMP signalling as neurogenesis was initiated. Knock-down experiments performed with sh-BMP4-b showed that BMP4 was required for the generation of Lhx2/9+ dI1 neurons but not for any of the other dorsal subpopulations (only those embryos in which the roof plate was not affected were analysed) (Fig. 2A,B; supplementary material Table S1). Increasing the concentration of sh-BMP4-b led to a similar phenotype (Fig. S4E; supplementary material Table S1). By contrast, electroporation of sh-BMP7-a showed that BMP7 activity was required for the generation of dI1 (Lhx2/9+), dI3 (Islet1+ or Tlx3+/Lmx1b–) and dI5 (Tlx3+/Lmx1b+) neurons (Fig. 2A). Quantification of the ratio of neuronal subtypes on the electroporated versus the non-electroporated side of the spinal cord indicated a reduction of ~50% in the generation of dI1, dI3 and dI5 (Fig. 2B; supplementary material Table S1). Apoptosis was not significantly increased as assessed by cleaved caspase 3 staining (data not shown). Increasing the concentration of sh-BMP7-a appeared to reduce the generation of dorsal neurons dI1-dI6, although dI1, dI3 and dI5 were again the most affected populations (supplementary material Fig. S4F, Table S1). These data reveal qualitative distinct activities for BMP4 and BMP7 in vivo.

In mammals, this may have hitherto been unnoticed because of early lethal phenotypes in mice lacking members of the BMP family, particularly BMP4 and BMP5/7 (Solloway and Robertson, 1999; Winnier et al., 1995). However, mice lacking BMP7 exhibit renal dysplasia and anophthalmia but survive to birth (Dudley et al., 1995), thus allowing for the analysis of spinal cord development. To test the requirement for BMP7 in dIN differentiation, the spinal cords of 10.5 dpc mice littermates (*Bmp7*$^{+/+}$, *Bmp7*$^{+/-}$ and *Bmp7*$^{-/-}$) were analysed with markers specific for each dI1-dI6 subpopulations (Fig. 3). Brachial and thoracic spinal cords were analysed separately, though results were identical at each level. Results show a reduction in the total numbers of dI1 (Lhx2/9+), dI3 (Islet1+ or upper Tlx3+) and dI5 (lower Tlx3+) neurons in the absence of BMP7 signalling (Fig. 3A-F), whereas the numbers for dI2 (Lhx1/5+Lbx1–), dI4 (upper Lhx1/5+Lbx1+ cells or upper Pax2+) and dI6 (lower Lhx1/5+Lbx1+ cells) are largely unaffected (Fig. 3G-L). Quantitative data showed total numbers for each dINs subpopulation on each genotype analysed +/+, +/– and –/– (Fig. 3M). Apoptosis was not significantly increased in BMP7 mutant mice, as analysed by caspase 3 staining (data not shown). These data demonstrate the requirement of BMP7 for the generation of discrete neuronal subtypes. These results contrasted with the previous observation that neurons expressing the marker combination LH2A/B/TAG1, identified as commissural neurons, were generated in normal numbers in the absence of BMP7 (Butler and Dodd, 2003). However, this previous analysis was restricted to the dorsalmost cells close to the roof plate, when compared with our analysis that included all Lhx2/9+ cells within the NT.

To begin to address whether this role for BMP7 in neurogenesis was mediated by the canonical BMP/Smad pathway, we took advantage of a mutant version of Smad5 (somitabun, Sbn). This construct encodes a dominant-negative version of Smad5 that was reported to inhibit endogenous Smad5 activity and the activity of the related Smad1 protein in zebrafish (Hild et al., 1999), and that specifically and significantly reduced the transcriptional activity of a BRE-tk-Luciferase reporter after electroporation into the chick

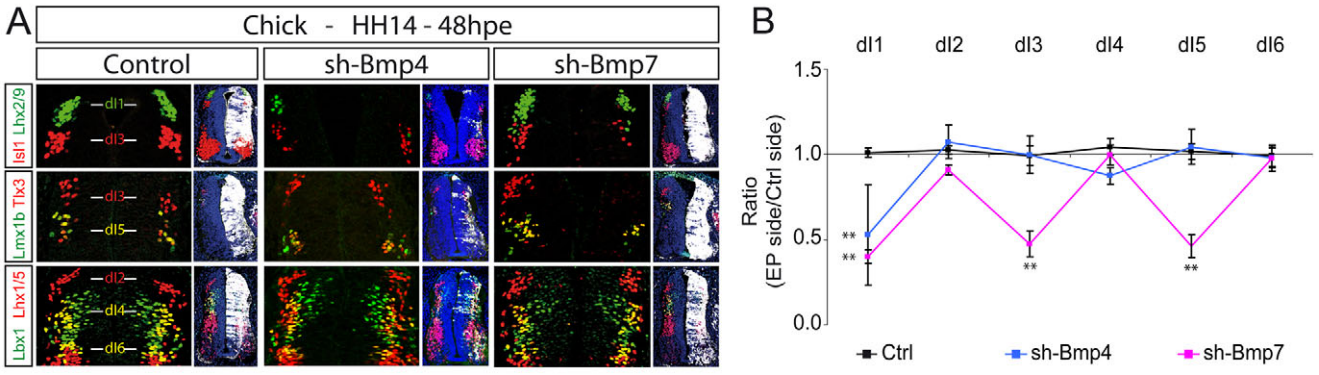

Fig. 2. BMP7 is required for the generation of dI1, dI3 and dI5 in the chick NT. (**A**) Selected images show dI1-6 neuronal subgroups, identified by the expression of the indicated markers (dI1, Lhx2/9+; dI2 Lhx1/5+Lbx1–; dI3, Tlx3+Lmx1b–; dI4, upper Lhx1/5+Lbx1+; dI5, Tlx3+Lmx1b+; dI6, lower Lhx1/5+Lbx1+). Selected images after electroporation of a control vector expressing GFP only (control), sh-BMP4b or shBMP7a at stage HH14 (22 somites), and analysed 48 hours later (E4). GFP (white) and DAPI (blue) are shown as indicators of electroporation and global neural tube morphology, respectively. (**B**) Quantitative data show mean ratios±s.e.m. of marker-expressing cells on the EP versus control non-EP side. BMP4 activity is required for dI1 generation, BMP7 activity is required for dI1, dI3 and dI5.

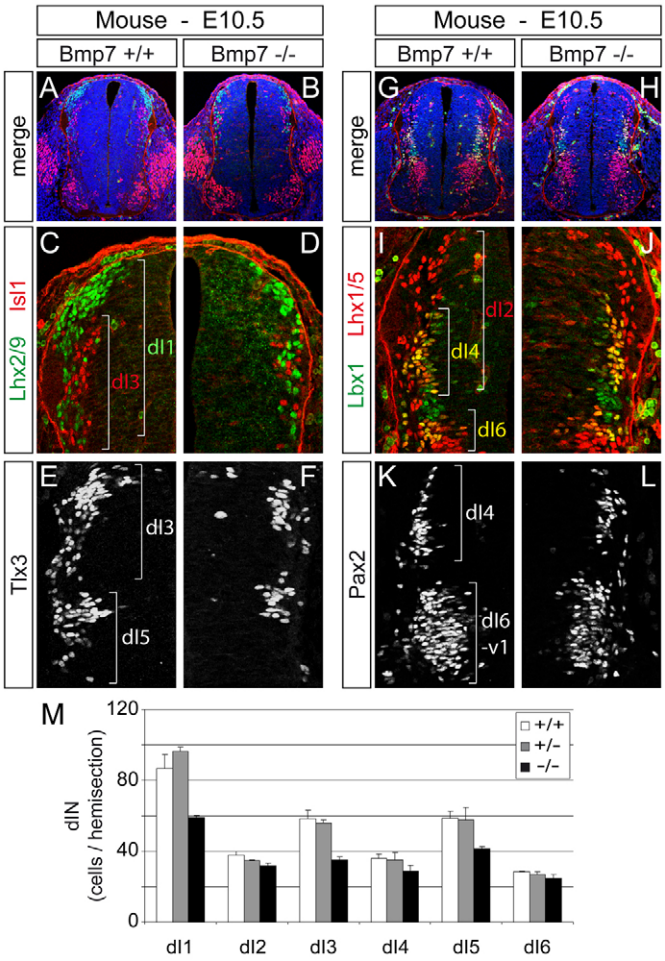

Fig. 3. BMP7 is required for the generation of dI1, dI3 and dI5 in the mouse NT. Images show brachial sections of 10.5 dpc mouse littermates of indicated genotypes; DAPI (blue) shows global neural tube morphology. (**A-F**) Markers identify dI1, dI3 and dI5 as follows: Lhx2/9+, dI1 cells; Islet1+, dI3 cells; Tlx3+, dI3 and dI5 cells. (**G-L**) Markers identify dI2, dI4 and dI6 as follows: Lhx1/5+Lbx1–, dI2; upper Lhx1/5+Lbx1+, dI4 cells; lower Lhx1/5+Lbx1+, dI6 cells; Pax2+, dI4 and dI6-V1. (**M**) Quantitative data show mean±s.e.m. of total number of marker-expressing cells by hemisection, in brachial sections of 10.5 dpc littermates with the indicated genotypes.

neural tube (supplementary material Fig. S4G). EP of Sbn mimicked the phenotype obtained by knockdown of BMP7 (supplementary material Fig. S4H, Table S1), providing further support for the role of BMP signalling in the differentiation of specific dorsal subtypes and implicating the canonical BMP pathway in this process. This observation prompted us to test for the expression and function of Smad proteins in NT development.

Smad1/5/8 are expressed in discrete and partially overlapping domains in the chick spinal cord

At the onset of dI1-dI6 neurogenesis (stage HH18, 30/36 somites) Smad1 and Smad5 were both broadly expressed in the ventricular zone, although at a higher level in the dorsal spinal cord (Fig. 4A,E). Smad8 on the other hand was restricted to narrow domains in the ventral- and the dorsal-most parts of the spinal cord (Fig. 4I). At stage HH24, Smad1 expression appeared to increase in cells transiting from the progenitor to the mantle zone, particularly in the domains corresponding to dI1, dI3 and dI5 neurons (Fig. 4B-D). Smad5 expression, however, remained restricted to the ventricular zone and showed a dorsal-to-ventral gradient (Fig. 4F-H). Both Smad1 and Smad8 were expressed in the motoneurons, although not at all axial levels (Fig. 4B,J).

This analysis indicated that Smad1 and Smad5 are expressed at the appropriate time and location to transduce a BMP7 signal in differentiating dI1, dI3 and dI5 cells. Of particular interest we noticed the high expression level of Smad1 at the transition zone of dP1, dP3 and dP5 (Fig. 4C,D; supplementary material Fig. S2C,D,G,H), the region where progenitors are withdrawing from the cell cycle, and particularly in those progenitor subgroups for which we observed the requirement of BMP7 activity. However, the in vivo role of Smad1 and Smad5 at this stage of neural development had not been analysed owing to the early lethality of the mutant mice (Chang et al., 1999; Tremblay et al., 2001; Yang et al., 1999). Thus, taking advantage of the temporal and spatial control afforded by chick in ovo electroporation, we next sought to test the in vivo requirement of Smad activity during neurogenesis of dorsal neurons.

Smad1/5 activity is required and sufficient for the generation of dI1, dI3 and dI5 neurons

To test the activity of individual Smads, we generated shRNAs specific for chick Smad1, Smad5 and Smad8 sequences (supplementary material Fig. S5A). qPCR analysis of FAC-sorted cells transfected with each sh-RNA was used to select sh-RNAs that reduced the levels of the relevant Smad RNA (supplementary material Fig. S5B-D). We then used selected shRNAs (sh-Smad1b, sh-Smad5a and sh-Smad8b) to assess the role of each individual Smad during dorsal neurogenesis. Knockdown at the time of neurogenesis, showed that lowering the levels of Smad8 reduced the numbers of dI1 neurons generated, but did not affect any of the other dorsal subpopulations, dI2-6 (Fig. 5A,B; supplementary material Table S1). This phenotype is consistent with the restricted domain of Smad8 expression (Fig. 4I-K). By contrast, Smad1 activity was required for the generation of all dorsal identities dI1-dI6, although dI1, dI3 and dI5 neurons were significantly more sensitive to the loss of Smad1 function (Fig. 5A,B; supplementary material Table S1). Knockdown of Smad5 activity resulted in a similar phenotype (Fig. 5A,B; supplementary material Table S1). Moreover, the combined knockdown of Smad1 and Smad5 resulted in a quantitatively similar loss of dI1, dI3 and dI5 neurons (supplementary material Fig. S5E, Table S1). Strikingly, we also observed that the loss of specific neuronal subpopulations caused by reduced BMP7/Smad activity, was not compensated by an increase in the production of other neuronal subtypes (Figs 2, 3, Fig. 5A,B). We reasoned that the minor increase in apoptosis observed after knock-down of either Smad1, Smad5 or Smad8 activities (assessed by staining for cleaved caspase 3, supplementary material Fig. S6D) would not be sufficient to account for the observed loss in dI1, dI3 and dI5 neurons.

To test whether the Smad activity was sufficient to promote the neural differentiation, we electroporated Smad1-, 5- or 8-SD individually at HH14 (22 somites) and analysed dINs generation 48 hours later. This analysis revealed that either Smad1-SD or Smad5-SD activity was sufficient to increase the numbers of dI1, dI3, and dI5 generated, whereas Smad8-SD did not (Fig. 5C,D; supplementary material Table S1). Consistent with the in vivo loss-of-function data, overexpression of Smad1 or Smad5 did not affect significantly the differentiation rate of dI2, dI4 or dI6 neural subtypes (Fig. 5C,D). Together, these data suggest that Smad1 and Smad5 have similar activities during neurogenesis of specific subpopulations of dorsal interneurons.

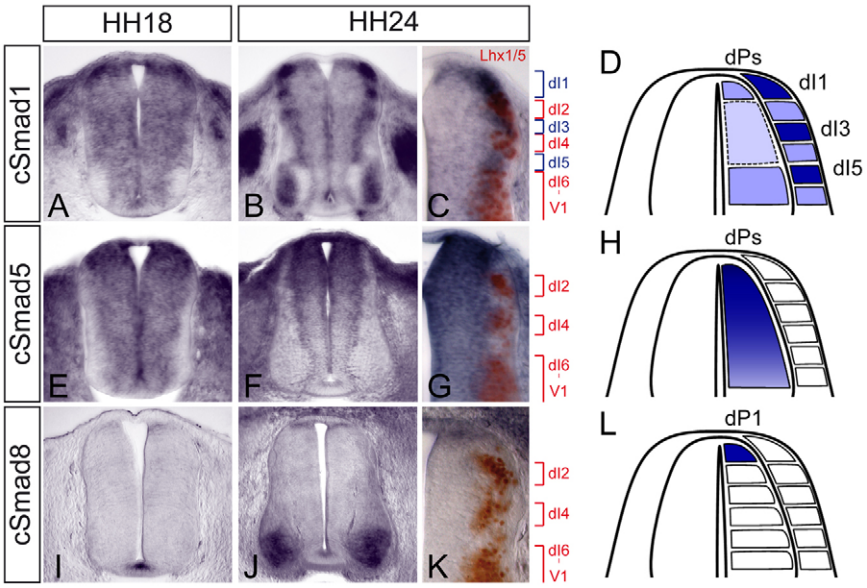

Fig. 4. Smad1, Smad5 and Smad8 are expressed in discrete and partially overlapping domains of the spinal cord. In situ hybridization for chick Smad1, chick Smad5 and chick Smad8 on sections from the prospective forelimb level, at the indicated stages. (**A-C**) Smad1 is expressed in the VZ at HH18 (30/36 somites) and in the VZ and transition zone at HH24. Double labelling with Lhx1/5 identified Smad1 highly expressing cells as dI1, dI3 and dI5. Smad1 is also expressed in a subset of motoneurons and in the DRGs. (**E-G**) Smad5 expression is restricted to the VZ both at HH18 (30/36 somites) and HH24. (**I-K**) Smad8 expression in the floor plate at HH18 (30/36 somites) and close to the roof plate at HH24. Smad8 is also expressed in motoneurons. (**D**,**H**,**L**) Schematic drawings represent a summary of each Smad expression pattern.

BMP7 activity is not required for dorsal patterning of the spinal cord

The observation that the absence of BMP7/Smad activity resulted in the loss of particular neuronal identities without the increase in alternative neuronal subpopulations, prompted us to check for the identity of the corresponding progenitors.

To that end, we took advantage of the patterned expression of transcription factors that identify sub-groups of dorsal progenitors (Kriks et al., 2005; Muller et al., 2005; Muller et al., 2002; Timmer et al., 2002). Electroporation of sh-BMP7 or sh-Smad1/5 at HH14 (22 somites), did not alter the expression of Pax7 (dP1-dP6), Gsh1/2 (dP3-dP5) or Olig3 (dP1-dP3) (Fig. 6A-I and data not shown). The identity of the roof-plate was also conserved, as shown by the expression of Lmx1b (Fig. 6A-D), together indicating that the canonical BMP7 activity is not required at these stages to maintain the patterned expression of progenitor proteins in the dorsal neural tube.

To further test the genetic requirement of BMP7 activity for the patterned expression of dorsal progenitor proteins, we analysed 10.5 dpc mice mutant for BMP7. Similar to the results observed after knock-down of BMP7 in chick embryos, the expression of Pax7, Gsh1/2 and Olig3 was identical in $Bmp7^{+/+}$ and $Bmp7^{-/-}$ (Fig. 6J-T), supporting the idea that BMP7 activity is dispensable for dorsal patterning of the NT, and thus narrowing the activity of BMP7 to the time of neurogenesis.

The promotion of neurogenesis is strongly associated with the expression of proneural bHLH proteins (Bertrand et al., 2002). cAth1 expression is restricted to dP1 and its activity is both required and sufficient for dI1 differentiation (Gowan et al., 2001). Moreover, BMP signalling regulates cAth1 expression as reported (Wine-Lee et al., 2004). Smad1/5 knockdown resulted in the reduction of cAth1 expression (Fig. 7A,B), whereas Smad1/5-S/D overexpression led to more cAth1-expressing cells located in the transition zone (Fig. 7C,D). The lateral extent of cAth1 expression corresponded to the expanded domain where dI1 were generated after the overexpression of Smad proteins (Fig. 7C,D, see Fig. 5C). Additionally, the cAscl1 expression domain (dP3-5) was regulated by Smad activity; cAscl1 was reduced in knock down experiments, but expanded by Smad overexpression (Fig. 7I-L). This is consistent with previously published evidence that BMP signalling regulates mAscl1 expression, both in the peripheral and the central nervous system in mouse (Alvarez-Rodriguez and Pons, 2009; Lo et al., 1997). By contrast, neither cNgn1 (dP2 and dP6), nor cPtf1 (dP4) expression was altered by the Smad activity (Fig. 7E-H,M-P). However, in some, but not all, embryos, cNgn1 expression appeared dorsally expanded in the absence of Smad activity (Fig. 7F). This is likely to be secondary to the loss of cAth1 inhibitory action, as would be expected due to the reported cross-repression in between these factors (Gowan et al., 2001), but did not result in a significant increase in generation of dI2 neurons.

DISCUSSION

In this study, we provide evidence for a new role of BMP7 in primary neurogenesis in the dorsal spinal cord. BMP7 activity is required for the generation of three discrete dINs: dI1, dI3 and also the more ventral dI5, the generation of which has never been associated to BMP signalling so far. Interestingly, we show that this activity is specific to BMP7 and not to the related BMP4, demonstrating qualitative distinct activities for the different BMPs, in vivo. In an attempt to study the downstream signalling pathway, we show that this BMP7 role in neurogenesis is mediated through the canonical Smad1/5 transcriptional activity. Moreover, this study shows that, both in the chick and the mouse embryos, BMP7 activity is not required for the patterned expression of progenitor proteins, thus narrowing the temporal activity of BMP7 to the time of primary neurogenesis, and strongly suggesting that distinct BMP-related signals regulate patterning of neural progenitors.

While this manuscript was under revision, two independent studies reported similar roles for the BMP pathway in dorsal NT development, although analysed by the study of the inhibitory Smads. On the one hand, Xie and colleagues (Xie et al., 2011) found that, in the dorsal spinal cord, Smad6 promotes neuronal differentiation by repressing the Wnt canonical pathway, and thus resulting in the reduced proliferation rate of neural progenitors. On the other hand, Hazen and colleagues (Hazen et al., 2011) showed that electroporation of Smad6 or Smad7, at the time of dIN neurogenesis, results in a reduction of dI1 and dI3, without the compensatory increase of dI4 neurons. These data strongly support our findings, as well as the requirement for a finely tuned regulation of BMP activity during primary neurogenesis.

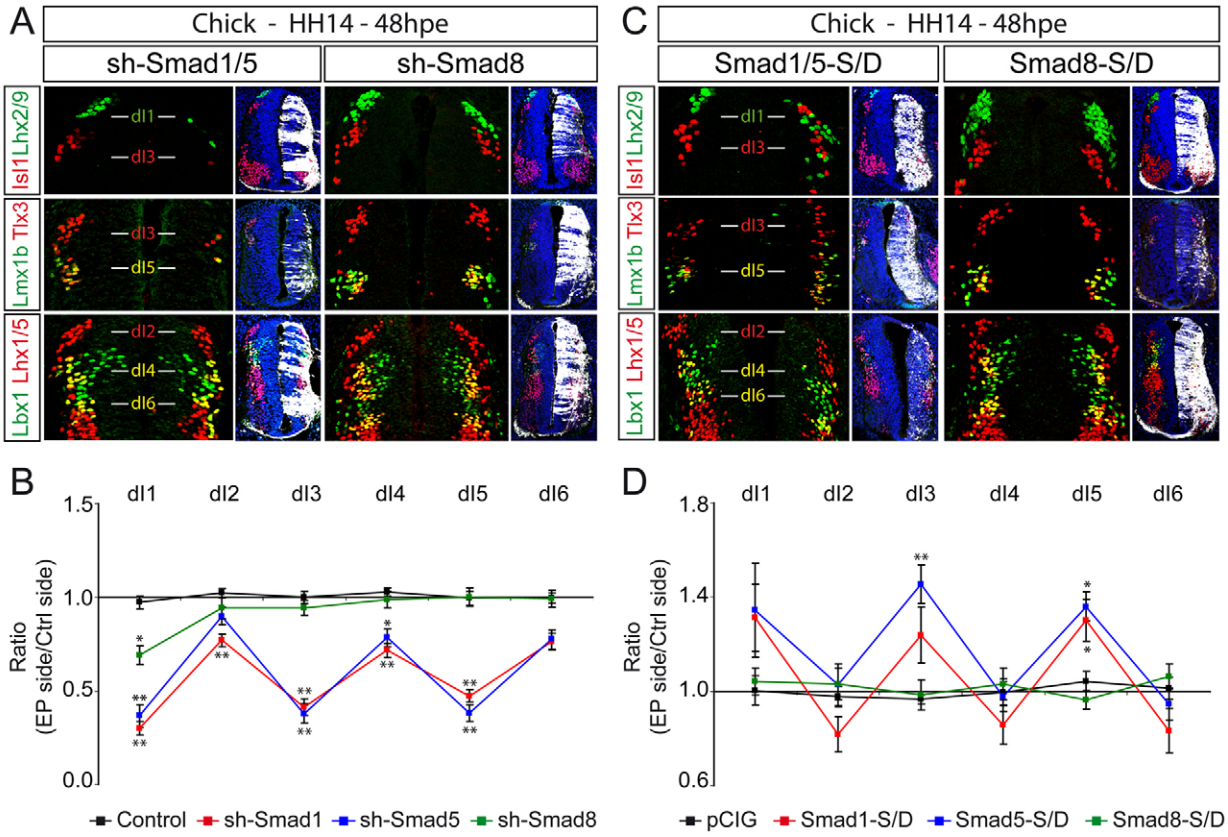

Fig. 5. Smad1/5 activity is required and sufficient for the generation of dI1, dI3 and dI5 neurons. (**A**) In vivo loss-of-function of Smad1/5/8 by sh-RNA EP shows that the activity of individual Smads is required for neurogenesis. dI1-dI6 were identified by immunohistochemistry with the indicated markers (dI1, Lhx2/9+; dI2, Lhx1/5+Lbx1–; dI3, Tlx3+Lmx1b–; dI4, upper Lhx1/5+Lbx1+; dI5, Tlx3+Lmx1b+; dI6, lower Lhx1/5+Lbx1+). Embryos were electroporated at stage HH14 (22 somites) and analysed 48 hours PE (E4). GFP (white) and DAPI (blue) are shown as indicators of electroporation and global neural tube morphology, respectively. (**B**) Quantitative data show mean ratios±s.e.m. of marker-expressing cells in EP versus control non-EP side. Smad8 activity is required for dI1 generation; Smad1/5 activity is required for dI1, dI3 and dI5. (**C**) In vivo gain-of-function of Smad1/5/8 by EP of pseudo-phosphorylated Smads shows that the activity of individual Smads is sufficient to enhance neurogenesis of specific identities. (**D**) Quantitative data show mean ratios±s.e.m. of marker-expressing cells in EP versus control non-EP side. Smad1/5 activity is sufficient for the enhanced generation of dI1, dI3 and dI5 neurons.

Our results suggest that activities of Smad1 and Smad5 might be similar in the dorsal spinal cord at these stages. Given the high degree of homology shared by these three members of the BMP-Smad family (Smad1, Smad5 and Smad8), it has been of interest to understand the extent to which they function interchangeably in vivo. Our knock-down and gain-of-function experiments suggested that Smad1 and Smad5 functions are largely interchangeable during spinal cord neurogenesis. This was further supported by the observation that Smad1-SD overexpression rescued Smad5 loss of function, and conversely Smad5-SD overexpression showed the capacity to rescue the sh-Smad1 phenotype fully (supplementary material Fig. S5F,G). However, in early embryonic mouse development, despite the robust co-expression of *Smad1*, *Smad5* and *Smad8* in extra-embryonic tissues, loss of either *Smad1* or *Smad5* resulted in distinct phenotypes (Tremblay et al., 2001). Moreover, in zebrafish embryos, the dorsalized phenotype of the *Bmp2b* mutant can be rescued by Smad1, but not by Smad5 (Dick et al., 1999), thus indicating unique roles played by Smad1 and Smad5 in this context. Whether Smad1 and Smad5 have unique but complementary roles or instead redundant functions during spinal cord neurogenesis will require further experiments and a better understanding of their mechanisms of action.

Data presented in this study make a clear distinction between patterning and neurogenesis as two processes separately controlled by BMPs. We observed that the loss of specific neuronal subpopulations was not compensated by an increase in the production of other neuronal subtypes, an observation that prompted us to analyse the expression of patterning proteins. Both in the chick and the mouse embryo, our data show that BMP7 is not required for the patterned expression of progenitor proteins in the dorsal spinal cord, suggesting that dorsal patterning might be associated with other ligands, such as BMP4. Thus, it is plausible that the unchanged progenitor identities prevented the changes in cell fate, and thus the compensation by alternative neuronal identities. Furthermore, the observation that BMP7 activity is not required for progenitor identity, but is required for neuronal identity, narrowed the window of BMP7 activity to the time of neurogenesis.

Our data also showed that the knock-down of canonical BMP/Smad activity causes a reduction in the total number of neurons generated during this time period. Further analysis revealed a slight, but consistent, cell-autonomous reduction in the proliferation rate of the neural progenitors after knock-down of Smad1 and Smad5 (supplementary material Fig. S6A-C and data

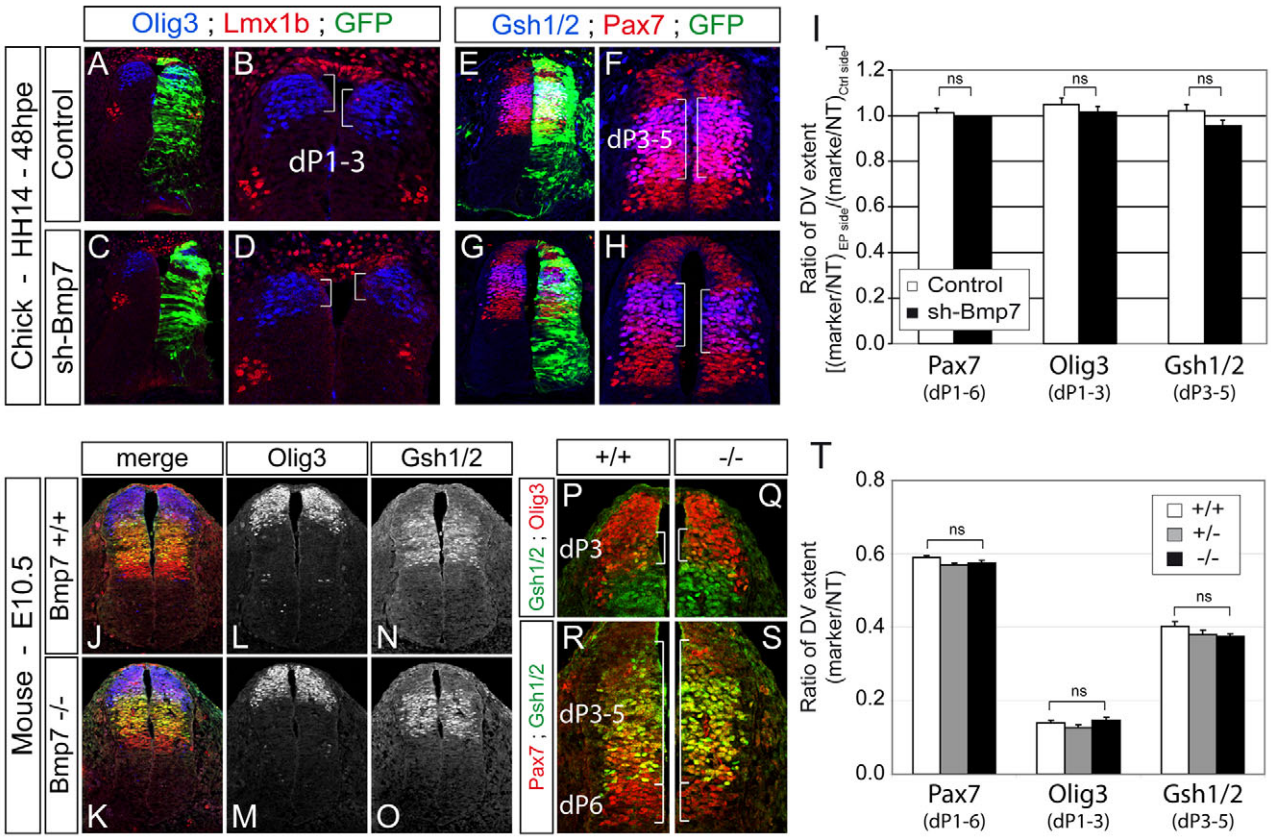

Fig. 6. BMP7 activity is not required for the patterned expression of progenitor proteins. (**A-H**) Chick embryos electroporated at HH14 (22 somites) were analysed 48 hours PE for the expression of markers to identify dorsal progenitor populations (dP) and roof-plate cells. No obvious change was observed on patterning of the dorsal neural tube (Pax7+), of dP1-3 progenitors (Olig3+) or of dP3-5 progenitors (Gsh1/2+) after electroporation of sh-BMP7 or control. Lmx1b+ roof-plate cells did not appear to be affected by control or sh-BMP7 EP, whereas dI5 Lmx1b+ cells were reduced in number only after sh-BMP7 EP (D). GFP (green) is shown as an indicator of electroporation. (**I**) Quantitative data show mean ratios±s.e.m. of dorsal-ventral extents of patterning markers to the whole dorsal-ventral extent of the neural tube side (using DAPI) in EP and control non-EP sides of the spinal cord. ns indicates non-significant statistical differences. (**J-S**) Selected images show brachial sections of 10.5 dpc mouse littermates of indicated genotype. Markers identify progenitor cells as follows: Pax7+, dP1-6 cells; Olig3+, dP1-3 cells; Gsh1/2+, dP3-5 cells. No obvious difference in the DV extent of these patterning markers has been observed between *Bmp7*$^{+/+}$ and *Bmp7*$^{-/-}$. (P-S) Higher magnifications of double-labelled sections show no obvious differences in the overlapping region of Olig3-Gsh1/2 and Pax7-Gsh1/2 expression. (**T**) Quantitative data show mean ratios±s.e.m. of dorsal-ventral extents of patterning markers to whole dorsal-ventral extent of the neural tube (using DAPI) in brachial sections of 10.5 dpc littermates of the indicated genotype. ns, non-significant statistical differences.

not shown). This indicates that Smad1/5 activity is required for the maintenance of neural cells as committed progenitors, and that the loss of canonical BMP/Smad activity forces cell cycle exit and premature neurogenesis. As a consequence, the total number of neurons generated in the absence of BMP7-Smad1/5 activity might be reduced, which further prevented the compensation by alternative neuronal identities.

Interestingly, loss-of-function experiments of BMP7/Smad resulted in the reduction of all six dIN populations, yet the decrease was much more important and significant for dI1, dI3 and dI5, precisely those progenitor populations expressing high levels of Smad1. Additionally, we noted that the generation of dIN deriving from progenitor domains expressing proneural proteins Ath1 (dP1) or Ascl1 (dP3 and dP5) alone are highly dependent on the level of BMP signalling, whereas dIN populations emerging from progenitor domains expressing proneural proteins Ngn1 (dP2 and dP6) and Ptf1a (dP4) are barely affected by the reduction of BMP/Smad activity. Together, this suggested that the way BMP activity regulates the generation of a particular dIN population relies not only on the highly restricted expression of the Smad1 effector, but also on the specific proneural bHLH factor expressed by the corresponding progenitor domain.

Similarly, we show that canonical BMP activity regulated the expression of Ath1 and Ascl1 in the dorsal spinal cord, but did not regulate the expression of Ngn1 and Ptf1a. Selective regulation of specific proneural proteins might additionally contribute to the spatially restricted BMP/Smad activity in dorsal spinal cord, and it would be worth defining whether the expression of these bHLH factors is directly regulated by Smad activity. Additional BMP-dependent post-transcriptional downregulation of Ath1 protein has been shown in cerebellar granule neuron progenitors and primary medulloblastoma cells (Zhao et al., 2008), and might also be operating in the NT. Whether the activated Smads interact directly with specific bHLH proteins in the dorsal spinal cord to regulate target genes remains a possibility, as a direct association of Ngn1 and Smad1 has been reported in the developing mouse telencephalon (Sun et al., 2001).

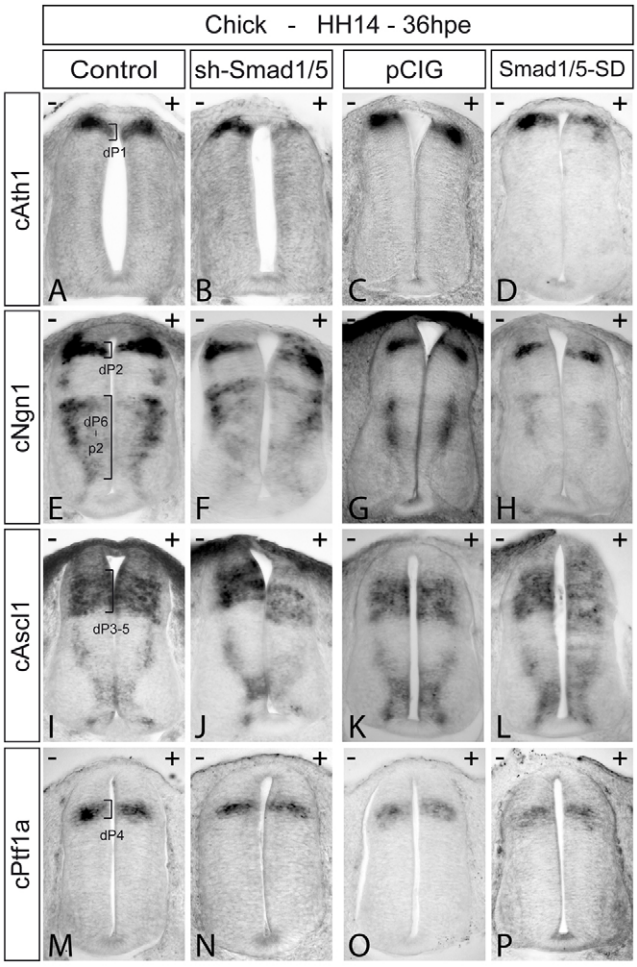

Fig. 7. Canonical Smad activity regulates the expression of discrete proneural genes. (**A-P**) In vivo loss- and gain-of-function experiments reveal that the expression of bHLH proneural proteins is differentially regulated by the BMP canonical activity. Electroporated side of the neural tube is towards the right, as indicated (+). Expression of chick Ath1 (A-D), Ngn1 (E-H), Ascl1 (I-L) and Ptf1a (M-P) were analysed 36 hours PE in HH14 (22 somites) embryos electroporated with a sh-RNA control, sh-Smad1/5, the empty pCIG vector and Smad1/5-SD. Expression of chick Ath1 and chick Ascl1 are reduced after sh-Smad1/5 EP, and expanded after Smad1/5-SD EP. Expression of chick Ngn1 and chick Ptf1 are largely resistant to changes in the level of BMP activity.

Together, these data demonstrate an unexpected role for BMP7 signalling in controlling the rate of neuronal differentiation in the dorsal spinal cord. This regulation of neurogenesis by canonical BMP signalling might be crucial for balancing the self-renewal and differentiation of progenitors, thus ensuring the well-ordered generation of the appropriate numbers of neuronal subtypes in this region of the CNS. The genetic interaction between the BMP/Smad pathway and some but not all proneural bHLH proteins could provide an underlying mechanism that explains the specificity of the effects we observed. Importantly, the role of BMP signalling in neurogenesis is independent of the early role of BMP in patterning the dorsal neural tube, revealing a novel function for this signalling pathway that may be relevant to the development of other regions of the CNS, as well as for the in vitro differentiation of neural stem cells.

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Competing interests statement
The authors declare no competing financial interests.

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