

Temporal progression of hypothalamic patterning by a dual action of BMP

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In the developing chick hypothalamus, Shh and BMPs are expressed in a spatially overlapping, but temporally consecutive, manner. Here, we demonstrate how the temporal integration of Shh and BMP signalling leads to the late acquisition of Pax7 expression in hypothalamic progenitor cells. Our studies reveal a requirement for a dual action of BMPs: first, the inhibition of GliA function through Gli3 upregulation; and second, activation of a Smad5-dependent BMP pathway. Previous studies have shown a requirement for spatial antagonism of Shh and BMPs in early CNS patterning; here, we propose that neural pattern elaboration can be achieved through a versatile temporal antagonism between Shh and BMPs.

KEY WORDS: Shh, BMP, Neural pattern, Hypothalamus, Gli, Chick

INTRODUCTION

In the developing vertebrate central nervous system (CNS), cellular fate and diversity are established through inductive interactions, mediated largely through only a few families of signalling ligands (Edlund and Jessell, 1999; Altman and Brivanlou, 2001; Ulloa and Briscoe, 2007). A key question is how this limited repertoire of signals can elicit the cellular diversity that is characteristic of the CNS? One way in which this can be achieved is through the ability of a cell to integrate inputs from multiple signalling pathways. In the ventral neural tube, for example, the transcriptional response of cells to a key ventralising signal, Shh, is modulated by both the Wnt and the BMP signalling pathways (McMahon et al., 1998; Liem et al., 2000; Patten and Placzek, 2002; Lei et al., 2006).

Much evidence has shown that the Hedgehog (Hh) and bone morphogenetic protein (BMP) signalling ligands play key roles in regulating cell identities in the neural tube (Ericson et al., 1995; Lee and Jessell, 1999; Ho and Scott, 2002; Chizhikov and Millen, 2005; Liu and Niswander, 2005; Ulloa and Briscoe, 2007). Through most of the neuraxis, BMPs and Shh, which emanate from dorsal and ventral regions, respectively, establish opposing spatial gradients that govern dorsoventral patterning by regulating cell-intrinsic factors (Edlund and Jessell, 1999; Briscoe et al., 2000; Ericson and Ericson, 2001; Liem et al., 2000; Patten and Placzek, 2002; Wijgerde et al., 2002; Jacob and Briscoe, 2003; Meyer and Roelink, 2003). However, although there is clear evidence that cells can integrate BMP and Shh signalling, the mechanisms that underlie this integrated response are less well defined. In particular, few studies have examined the interaction of Shh and BMP signalling at the level of the Gli genes. In the case of Shh, numerous lines of evidence have identified the Gli transcription factors as key mediators of the cellular response to Shh. Shh promotes ventral cell identities by antagonising the repressive activity of the Gli3 protein: in the absence of Shh, specific ventral cell types fail to form, but they can be rescued in Shh-null animals by the simultaneous inactivation of

Gli3 (Litingtung and Chiang, 2000; Persson et al., 2002; Wijgerde et al., 2002; Bai et al., 2004; Lei et al., 2004; Stamatakis et al., 2005; Blaess et al., 2006). However, although recent evidence shows that BMP signalling can maintain Gli3 expression in dorsal regions of the neural tube (Meyer and Roelink, 2003), no study has yet shown that BMP signalling can initiate the expression of Gli3.

In contrast to other regions of the neural tube, in the hypothalamus, Shh and BMPs are expressed in a spatially overlapping, but temporally consecutive manner (Dale et al., 1997; Dale et al., 1999; Ohyama et al., 2005; Manning et al., 2006). Here, we examine the mechanism by which this discrete signalling profile governs downstream Gli effectors and hypothalamic transcriptional signatures. Our studies show that Bmp7 operates in a dual manner to specify hypothalamic progenitors, first through a temporal antagonism of Shh-Gli activator function, which is mediated by Gli3 upregulation, and second, via Smad5 activation. We demonstrate that Shh signalling operates through the Gli activator (GliA) to prevent Pax7 expression. Bmp7 signalling upregulates Gli3 and we demonstrate that the Gli3 repressor (Gli3R) can derepress Pax7 expression, but inefficiently. Robust derepression requires both Gli3R and Smad5 activity. Together, our data reveal that BMPs can initiate Gli3 expression in the neural tube. In addition, they suggest that, in the CNS, neural pattern elaboration can be achieved through the temporal integration of antagonising Shh and BMP ligands.

MATERIALS AND METHODS

Immunolabelling

Chick embryos ($n=5-10$; each stage) and explants ($n=5-20$) were examined as described previously (Ohyama et al., 2005). Antibodies used were: 5E1 anti-Shh mAb (1:50); anti-Pax7 mAb (1:50); anti-Lim1 (4F8); anti-Msx1/2 (4G1: 1:50) (all from DSHB); Kyo2-60 anti-Nkx2.1 polyclonal antibody (1:2000); anti-Gsh polyclonal antibody (1:2000); NCL-Ki67-PKi67 anti-human (Novocastra, 1:3000); anti-Isl1 polyclonal antibody (1:800); AB3216 anti-RFP polyclonal antibody (Chemicon, 1:1000); anti-GFP polyclonal (BD 632576, 1:1000); anti-pSmad1/5/8 (Cell Signaling 95115, 1:100-1:500). Secondary antibodies were conjugated to Cy3, FITC (Jackson ImmunoResearch, 1:200), Alexa fluor 594 or Alexa fluor 488 (Molecular Probes, 1:500), and images taken using Spot RT software v3.2 (Diagnostic Instruments) or an Olympus FV-1000 confocal.

In situ hybridisation

Embryos and explants were processed as described previously (Ohyama et al., 2005; Manning et al., 2006; Borycki et al., 2000).

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Explant dissection and culture

pHyp and prechordal mesoderm were dissected with Dispase (1 mg/ml) for 15 minutes at room temperature and explants were cultured in collagen gels (Dale et al., 1999). Bmp7 and chordin proteins were prepared as described (Ohyama et al., 2005). To block Shh or BMP signalling, cyclopamine (600 nM) and/or dorsomorphin (4 μ M) were added into culture medium 24-36 hours after onset of culture and continued for up to 6 days.

Electroporation

Electroporation was performed as described (Ohyama et al., 2005). Plasmids used were: Gli1 (Sasaki et al., 1999); pCAGGS-GFP (Das et al. 2006); pCAGGS-RFP (Das et al., 2006); Smad5 (Ishida et al., 2000); Gli3 repressor (Gli3R) (Persson et al., 2002) and pRFPNAi-Luciferase (Luc RNAi) (Das et al., 2006). Gli3 RNAi plasmid was designed to target the following sequence: CCACATACATGGTGAGAAGAAA.

RESULTS AND DISCUSSION

In the developing hypothalamus, Shh and BMPs show temporally regulated wave-like expression patterns in ventral midline cells of the prechordal mesoderm, hypothalamic floor plate and in the ventro-lateral hypothalamus. In each cell group, Shh expression precedes that of Bmp7: they transiently overlap, before Shh is downregulated (Fig. 1A,B,E,I,J,M,Q,R,U) (Dale et al., 1997; Dale et al., 1999; Ohyama et al., 2005; Manning et al., 2006). Shh is retained at high levels only in the ventrolateral hypothalamus, in

cells that span the lumen, with nuclei at the ventricular zone (VZ) (Fig. 1Q,R). Two outstanding issues are whether ventrally derived Bmp7 temporally opposes Shh signalling to specify progenitors in the developing ventrolateral hypothalamus, and, if so, how this is achieved.

To begin to examine whether BMP signalling may temporally antagonise Shh signalling, we documented expression of recognised responses to the two signals, generating a profile of their spatiotemporal activities. The Shh-responsive genes *Ptc1* and *Gli1* (Epstein et al., 1996; Goodrich et al., 1996; Hynes et al., 1997; Marigo and Tabin, 1996) show a similar initial spatiotemporal profile to Shh itself: expression detected in ventral-dorsal waves, extending from the hypothalamic floor plate at stage 10 to ventrolateral cells at stage 15-17 (Fig. 1C,D,K,L); at these stages, expression of *Ptc1* and *Gli1* extends slightly more dorsally to that of Shh, a feature more pronounced at stages 22-25 (Fig. 1Q,S,T). The onset of expression of the BMP-responsive genes *Msx1/2* and *pSmad1/5/8* (Liem et al., 1995; Rios et al., 2004) in each cell group appears to correlate with the downregulation of Shh and Shh-responsive genes. *Msx1/2* and *pSmad1/5/8* are robustly detected in hypothalamic floor-plate cells at stage 15-17, then in ventrolateral cells by stage 22-25 (Fig. 1F,G,N,O,V,W). Together, this analysis is consistent with the idea that BMP might antagonise Shh signalling in a temporal manner.

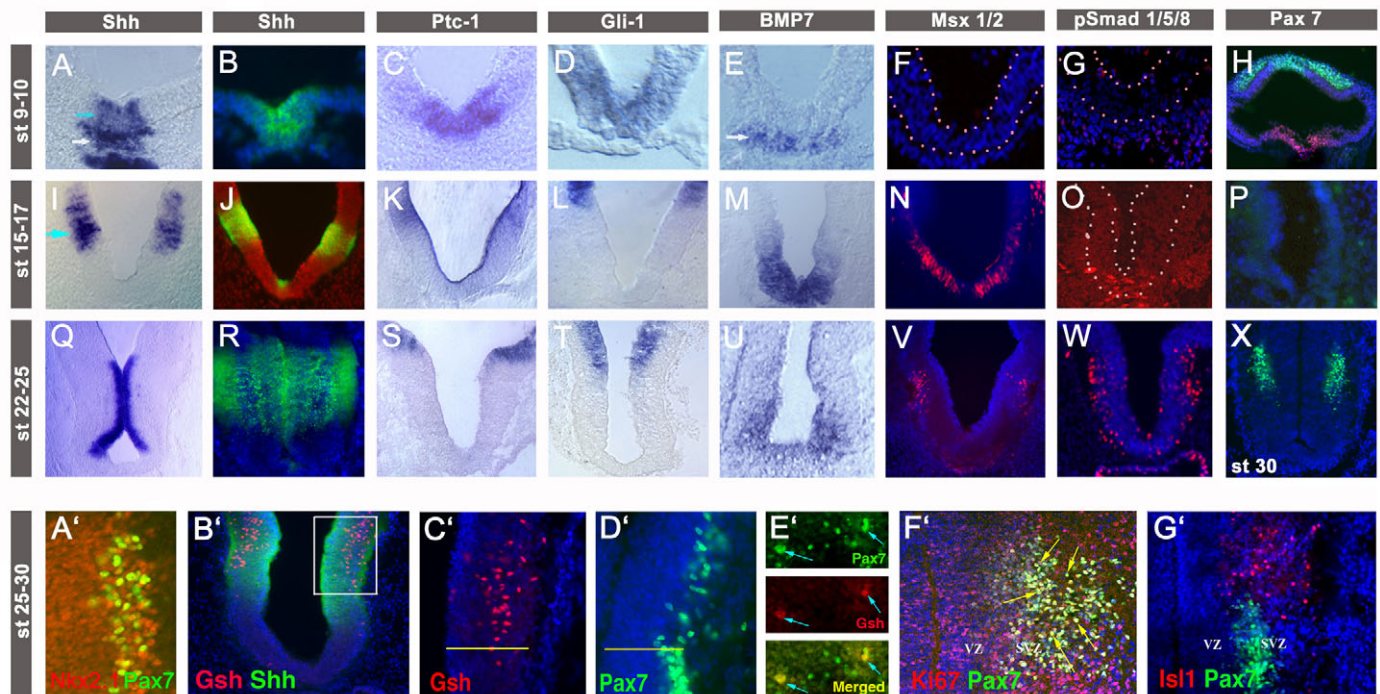


Fig. 1. Temporal expression of Shh, BMP signal pathway components and Pax7 in the chick hypothalamus. (A-H) At stage 9-10, Shh, Ptc1 and Gli1 are detected in prechordal mesoderm (white arrow, A) and/or hypothalamic floor plate (blue arrow, A). Bmp7 is detected in prechordal mesoderm (arrow, E), but Bmp7, Msx1/2 and pSmad1/5/8 are not detected in the hypothalamic floor plate. Pax7 is detected in roof plate cells (H), but not floor-plate cells, marked by expression of Nkx2.1 (red). (I-P) At stage 15-17, Shh is expressed in Nkx2.1+ ventrolateral cells (arrow, I; Nkx2.1 expression shown in red in J); Ptc1 and Gli1 show some overlap with Shh, but extend more dorsally. Expression of Bmp7, Msx1/2 and pSmad1/5/8 is detected in hypothalamic floor plate cells. (Q-W) At stage 22-25, ventrolateral cells that span the neuroepithelium express Shh. Expression is largely detected in cell bodies and end-feet. *Shh* mRNA appears to be largely confined to VZ cells. Ptc1 and Gli1 are detected dorsal to Shh-expressing cells. Bmp7, Msx1/2 and pSmad1/5/8 are detected in ventrolateral cells, which now express Pax7. (X) Robust expression of Pax7 is detected in ventrolateral cells at stage 30. (A') Pax7+ cells form in Nkx2.1+ cells located furthest from the VZ. Many Pax7+ cells co-express Nkx2.1, but a minority of Pax7-single positive cells can be detected. (B'-G') Pax7 marks basal progenitors of the ventrolateral hypothalamus. Gsh+ cells are located at the VZ/SVZ boundary. The majority of Gsh+ cells lie just medial to Pax7+ cells, but some cells co-express Gsh and Pax7 (arrows, E'). Pax7+ cells co-express Ki67 (F', arrows), and are distinct from postmitotic Isl1+ cells (G').

We reasoned, then, that additional progenitor markers that are usually dorsally restricted in the posterior CNS might show ventral expression patterns within the hypothalamus, and focused on expression of the general 'dorsal' progenitor marker Pax7. Prior to stage 16, Pax7 is expressed at the forebrain roof plate, but is not observed in the ventral or ventrolateral hypothalamus (Fig. 1H,P). By contrast, by stage 25, Pax7 expression is detected in roof-plate cells (shown in Fig. 4E), but, additionally, a new domain of expression is detected in a set of progenitor cells in the ventrolateral Nkx2.1+ hypothalamus (Fig. 1A',D'). At stage 25, the ventrolateral Pax7+ cells occupy Shh-negative regions of the subventricular zone (SVZ), and are located within outermost regions of a broader Nkx2.1+ domain, and just lateral to Gsh+ cells (Fig. 1A'-D'); many cells co-express Nkx2.1 and Pax7, and some cells show co-expression of Gsh and Pax7 (Fig. 1A',E'). By stage 30, Pax7+ cells

occupy a distinct domain (Fig. 1X). Double-labelling of Pax7 and a proliferation marker, Ki67, demonstrates that at stage 30, many Pax7+ cells are proliferating progenitors (Fig. 1F'). Consistent with this observation, they are segregated from postmitotic Isl1+ cells (Fig. 1G'). Together, these data show that Pax7 is upregulated in proliferating basal progenitors of the ventrolateral hypothalamus.

To test whether ventrally derived Bmp7 upregulates Pax7 expression in the ventrolateral hypothalamus, we first co-cultured prospective hypothalamic (pHyp) explants (Fig. 2A) with prechordal mesoderm (PM), a source of Bmp7 (Fig. 1E), for 5-6 days. Many Pax7+ cells were detected, the majority of which co-expressed Nkx2.1, and were co-expressed with, or adjacent to, Gsh+ cells ($n=6$; Fig. 2B,G). By contrast, pHyp explants cultured alone expressed Nkx2.1 and Gsh but not Pax7 ($n=15$; Fig. 2C,H,K). Bmp7 mimicked the activity of prechordal mesoderm: in explants exposed

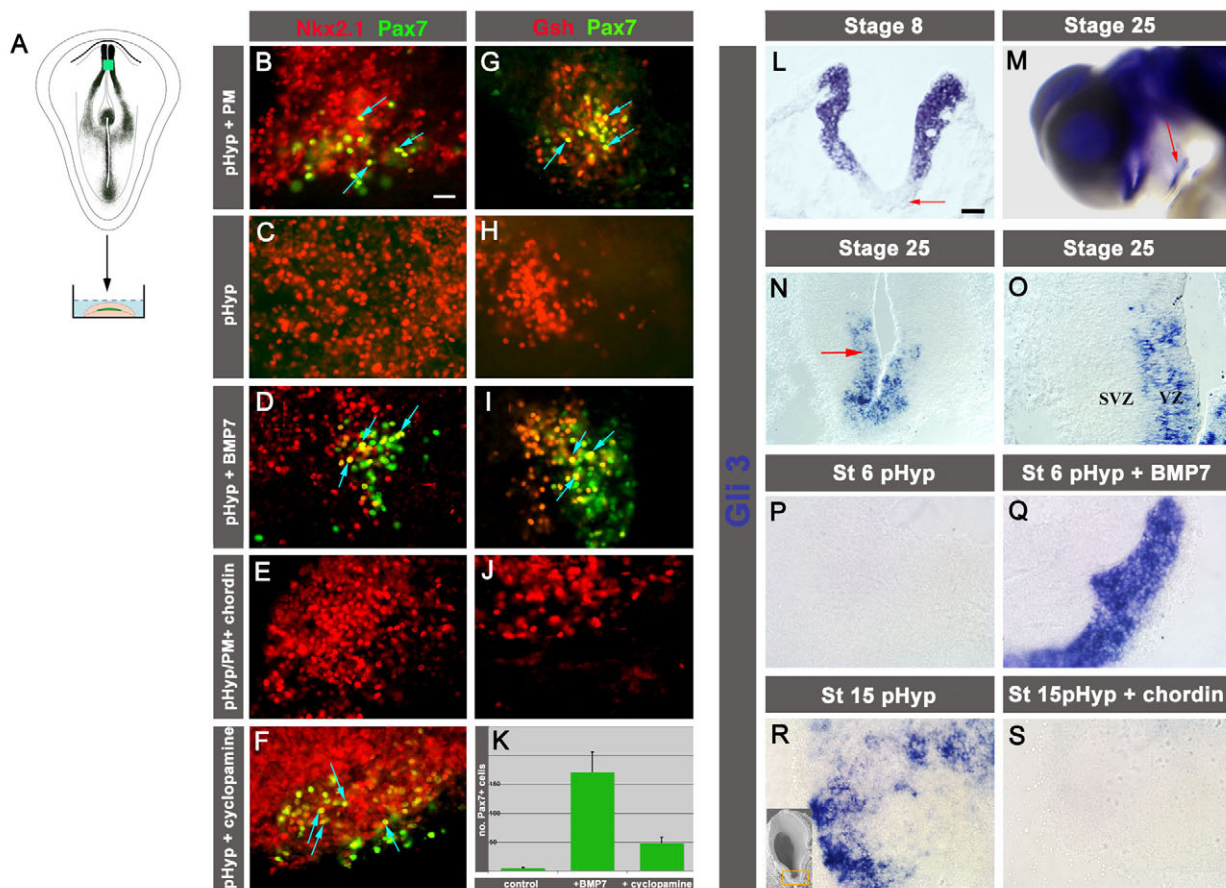


Fig. 2. Bmp7 upregulates Gli3 and specifies Pax7+ basal progenitors. (A) Schematic showing pHyp dissection (green box) from stage 5-7 embryos and culture in collagen. (B-J) Sections through explants, analysed by double-colour immunofluorescent labelling for expression of Nkx2.1/Pax7 (B-F) or Gsh/Pax7 (G-J). Pax7+ cells, either adjacent to, or co-expressed with, Nkx2.1 and Gsh (arrows), are detected in the presence of prechordal mesoderm (PM; B,G), Bmp7 (D,I) and cycloamine (F). (H,I) Quantitative analysis of pHyp explants cultured with (I) or without (H) Bmp7. Quantitative analysis reveals that expression of Pax7 does not occur at the expense of Gsh+ cells (mean number of Gsh+ cells: in control=150±5.8, in explants with Bmp7=156±8.2; mean number of Pax7+ cells: in control=0.4±0.2, in explants with Bmp7=171±9.1). (F) Pax7+ cells, many of which co-express Nkx2.1 are found in pHyp explants cultured in the presence of cycloamine. (K) Quantitative analysis on pHyp explants cultured alone, with Bmp7 or with cycloamine. Bars show mean number of Pax7+ cells/section. Cycloamine can significantly increase the number of Pax7+ cells (control=4.9±1.3 cells; cycloamine=47.7±10.2 cells; $P<0.0024$; unpaired *t*-test), but approximately threefold more Pax7+ cells are detected with Bmp7. (L-S) Gli3 expression in vivo (L-O) and in pHyp explants (P-S). (L) At stage 8, Gli3 is not detected in the ventral/ventrolateral hypothalamus (arrow). (M-O) At stage 25, Gli3 expression is detected in ventral and ventrolateral regions of the hypothalamus, expression confined to VZ/SVZ cells (arrows in M,N). (P,Q) Gli3 expression is not detected in a stage 6 pHyp explant cultured alone (P), but is upregulated in the presence of Bmp7 (Q). (R,S) Stage 15 ventral hypothalamic explants, dissected from region shown (box in inset, R). Gli3 expression is detected when ventral hypothalamic regions are explanted at stage 15 and cultured to a stage 25 equivalent (R). Expression is abolished when explants are cultured with chordin (S).

to Bmp7, Pax7 expression was upregulated in and adjacent to Nkx2.1+ and Gsh+ cells ($n=5$; Fig. 2D,I,K). Conversely, when pHyp explants were co-cultured with PM in the presence of chordin, a Bmp7 inhibitor, no expression of Pax7 was detected in Nkx2.1 or Gsh+ cells ($n=6$; Fig. 2E,J). These data suggest that Bmp7 can upregulate Pax7 expression in Nkx2.1+/Gsh+ ventrolateral progenitors.

To begin to address the mechanism of Pax7 upregulation by Bmp7, we examined whether exposure of pHyp explants to cyclopamine, a Shh-signalling inhibitor (Incardona et al., 1998), can mimic Bmp7 activity. Although not as efficient as Bmp7, cyclopamine significantly upregulated Pax7 ($n=10$; Fig. 2F,K). Together, these experiments suggest that BMP exerts its effect, at least in part, by antagonising Shh signalling.

The antagonistic effects of Shh and Bmp7 on Pax7 expression are reminiscent of dorsoventral spinal cord patterning, where BMPs contribute to the establishment of neural progenitor domains in dorsal and intermediate regions by opposing ventrally derived Shh

and repressing ventral progenitor identity (Liem et al., 1995). The mechanisms by which BMP and Shh signals antagonise each other's function are diverse, but one point of intersection is at the Gli transcription factors. The Gli3 repressor (Gli3R) opposes Shh-GliA signalling, governing dorsal identity through the repression of Nkx2.2+ and derepression of Pax7 (Persson et al., 2002). It is believed that in the prospective spinal cord, BMPs elicit their effects on dorsoventral patterning by maintaining Gli3 expression (Meyer and Roelink, 2003), but the factors that govern initial expression of Gli3 remain unclear.

We therefore examined whether there is a correlation in BMP signalling and Gli3 expression in the hypothalamus. At stage 8-13, Gli3 is not detected in the ventral hypothalamus, although strong expression is detected in the dorsal and intermediate forebrain (Fig. 2L). However, just prior to Pax7 upregulation, Gli3 expression is observed, in a dynamic fashion in the ventral and ventro-lateral hypothalamus (Fig. 2M-O). To test whether Bmp7 can direct Gli3 expression, pHyp explants were cultured alone ($n=8$) or with Bmp7

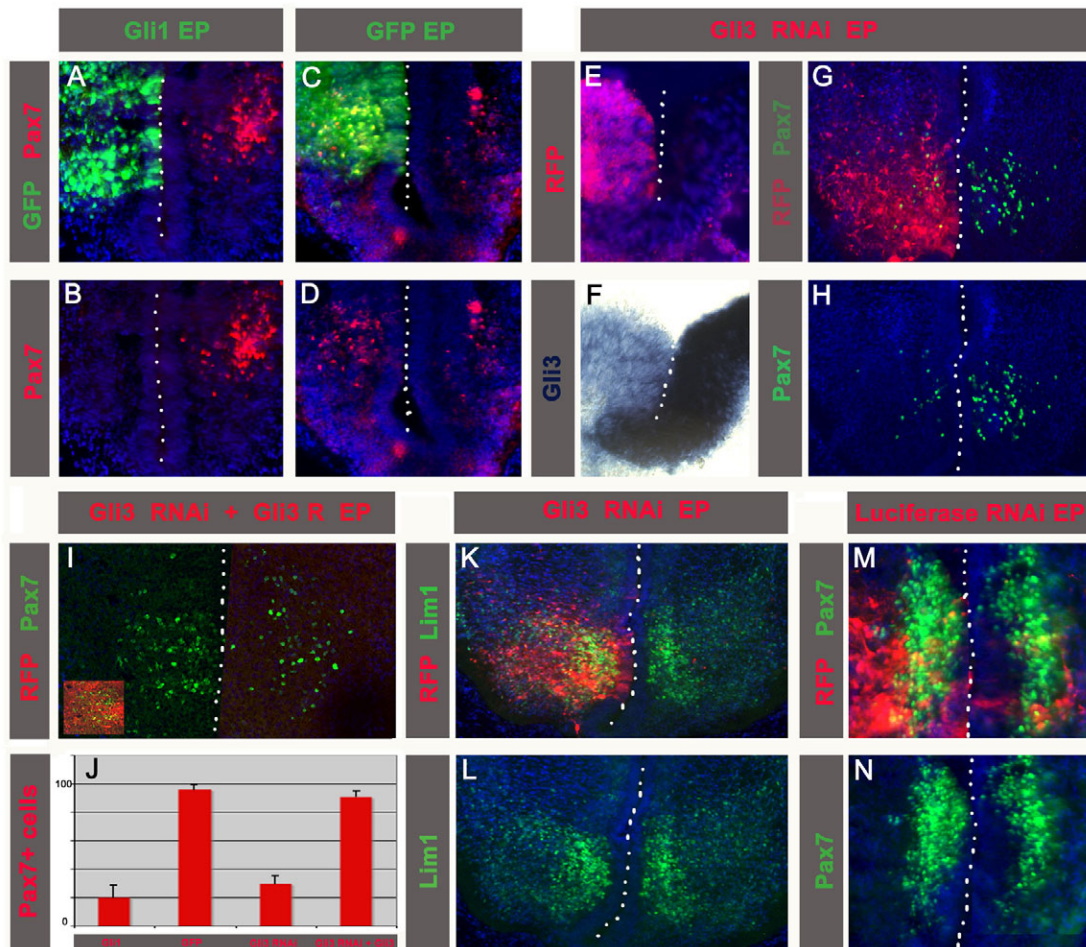


Fig. 3. Antagonistic actions of GliA and GliR regulate Pax7 expression. (A-I;K-N) Transverse section through hypothalamus of chicks electroporated at stage 10-12 and developed until stage 25-30. White dots indicate lumen; electroporated sides are to the left of the lumen in images A-N. (A-D) Electroporation of Gli1 (GliA) abolishes Pax7 expression (A,B). A control GFP construct has no apparent effect on Pax7 (C,D). (E-H) Electroporation of a Gli3 RNAi construct leads to the downregulation of Gli3 (E,F; adjacent sections). (G,H) Electroporated cells show a marked downregulation of Pax7; neighbouring non-electroporated cells develop with normal Pax7+ identity. (I) Co-electroporation of Gli3 RNAi and a Gli3R shows rescue of Pax7; i.e. similar numbers of Pax7+ cells are detected on non-electroporated and electroporated sides. (J) Percentage of Pax7+ cells on electroporated versus non-electroporated sides of the hypothalamus. (Gli1 overexpression=20±8 (s.e.m.); GFP=97±3; Gli3 RNAi=27±6; Gli3 RNAi + Gli3=93±4). Residual Pax7 expression detected after Gli1 overexpression or Gli3 knockdown appears to be in non-electroporated cells (e.g. G). (K,L) Gli3 RNAi has no effect on Lim1+ expression. (M,N) A control luciferase RNAi construct does not affect Pax7 expression.

($n=7$) for 3-4 days. In the presence of Bmp7, Gli3 expression was detected (Fig. 2P,Q). Conversely, when stage 15 pHyp explants were cultured alone, until a stage 25 equivalent, Gli3 was detected. When identical stage 15 pHyp explants were cultured with chordin, Gli3 showed a marked downregulation ($n=4$ each; compare Fig. 2R with 2S), indicating that BMP is required to upregulate Gli3 in the hypothalamus.

The late onset of expression of Gli3 raises the possibility that a temporally controlled antagonism of Shh and BMP signalling causes the differentiation of SVZ cells to a Pax7+ progenitor fate. To test this, we first examined whether Shh-GliA function prevents the onset of Pax7 expression, by electroporating a Gli1 constitutive activator (GliA) construct (Sasaki et al., 1999) into the ventrolateral hypothalamus. Gli1 overexpression prevented Pax7 upregulation ($n=5$), whereas a control GFP-alone vector showed no significant effect ($n=5$ each; Fig. 3A-D,J). We next tested whether, conversely, there is a requirement for Gli3 repressor function for Pax7 expression, by performing Gli3 knockdown experiments. A Gli3 siRNA knockdown construct (Das et al., 2006), efficacious in reducing *Gli3* expression (Fig. 3E,F), was electroporated into the hypothalamus at stage 10-12 and embryos were analysed at stage 25-30. When Gli3 was knocked down, the number of Pax7+ progenitors was markedly reduced ($n=6$; Fig. 3G,H,J); this effect could be reversed through the simultaneous electroporation of Gli3

RNAi and a Gli3 expression vector ($n=6$; Fig. 3I,J). The knockdown of Gli3 did not abolish hypothalamic cells, as expression of an additional hypothalamic marker, Lim1 (Ohyama et al., 2005), was unaltered by Gli3 knockdown ($n=4$; Fig. 3K,L). A luciferase knockdown construct did not affect Pax7+ cells ($n=4$; Fig. 3M,N). Together, these data suggest that Gli3 is required for Pax7 expression in the ventral hypothalamus.

Our experiments support the idea that the upregulation of Pax7 requires the temporal antagonism of Shh signalling by BMP-mediated upregulation of Gli3, but do not indicate whether this is sufficient. To examine this, we asked whether Gli3R can upregulate Pax7 in the hypothalamus, targeting electroporations into regions of the anterior/medial hypothalamus in which neither Gli3 nor Pax7 are normally observed. These experiments revealed that Gli3R can upregulate Pax7, but in an inefficient and spatially restricted manner. Ectopic Pax7 expression was detected only when the Gli3R construct was electroporated into regions close to endogenous Pax7+ domains, and then only in a subset of embryos (6/9; Fig. 4A,B).

The finding that Bmp7, but not Gli3R, can robustly upregulate Pax7 suggests a complex action of Bmp7. We therefore asked whether the upregulation of Pax7 could be more robustly achieved through the simultaneous activation of the BMP signalling pathway and repression of the Shh signalling pathway. A Smad5 activator

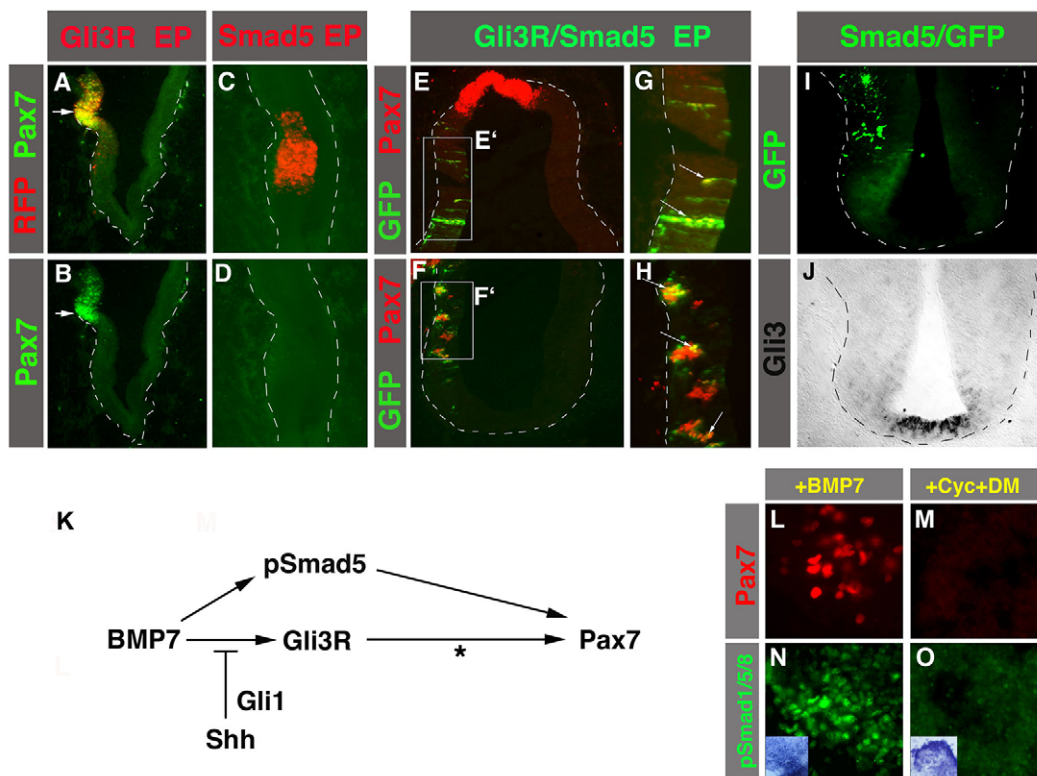


Fig. 4. Dual BMP action specifies Pax7+ ventral progenitors in the hypothalamus. (A-J) Transverse sections through stage 25 hypothalamus, after electroporation at stage 10. (A,B) Co-electroporation of Gli3R and an RFP expression plasmid. Ectopic Pax7+ cells are observed (white arrows), but are restricted to the dorsal region of the hypothalamus, close to endogenous sites of Pax7 expression at the roof plate. (C,D) Co-electroporation of Smad5 and RFP. No ectopic Pax7+ cells are detected. (E-H) Co-electroporation of GFP, Gli3R and Smad5. Ectopic Pax7+ cells are detected throughout the dorsoventral axis. G and H show high-power views of boxed regions (E',F'). White arrows show cells co-expressing Pax7 and GFP. (I,J) Co-electroporation of Smad5 and GFP. No ectopic expression of Gli3 is detected. (K) Model for the specification of Pax7+ ventral progenitors in the developing hypothalamus. Asterisk (*) indicates that Gli3R de-represses Pax7. (L-O) Stage 6 pHyp explants cultured with Bmp7 or a combination of cyclopamine (Cyc) and dorsomorphin (DM). No expression of Pax7 or pSmad1/5/8 is detected with cyclopamine and dorsomorphin. Inset shows that expression of Gli3 is detected under both conditions.

(Smad5A), a downstream signalling component of BMPs (Ishida et al., 2000), was electroporated either alone or with Gli3R. Smad5A alone was unable to induce ectopic Pax7⁺ cells ($n=5$; Fig. 4C,D), although it was able to efficiently induce Msx expression (data not shown). However, co-electroporation of Gli3R and Smad5A resulted in a very robust induction of Pax7⁺ cells, ectopic Pax7 expression detected in electroporated cells throughout the dorsoventral axis ($n=8/9$; Fig. 4E-H). These data suggest that pSmad5 and Gli3R may operate in non-linear pathways, both of which are required for Pax7 expression. In support of this, examination of Smad5-electroporated embryos showed, indeed, that Gli3 is not upregulated by Smad5 ($n=9$; Fig. 4I,J). Together, these data suggest the idea that a dual action of BMP signalling specifies Pax7⁺ basal progenitors: a Smad5-independent Bmp7-Gli3R pathway opposes the Shh-Gli1A pathway to de-repress Pax7 in the ventrolateral hypothalamus; in addition, BMP signalling via pSmad5 is required to activate Pax7 (Fig. 4K).

To test this hypothesis, we prevented signalling of both Bmp7 and Shh in pHyp explants. We reasoned that if BMP signalling acts exclusively through Shh antagonism, the effect of cyclopamine should be dominant over that of dorsomorphin, an ALK2/3/6 inhibitor that blocks pSmad (Yu et al., 2008), and Pax7⁺ cells should be retained. pHyp explants were exposed to a combination of cyclopamine and dorsomorphin ($n=9$), and analysed for expression of Gli3, pSmad5 and Pax7. Only Gli3 was upregulated in the presence of cyclopamine and dorsomorphin (Fig. 4M,O), whereas all three were detected in pHyp explants exposed to Bmp7 (Fig. 4L,N; $n=9$). Scattered expression of pSmad5, and no expression of Gli3 were detected in pHyp explants cultured alone (not shown). Together, these results support our proposed model for a dual action of Bmp7 is mediating Pax7 upregulation in the hypothalamus.

In conclusion, our analysis makes a number of key points. First, it provides a novel insight into how cellular diversity can be achieved within the embryo in response to a limited repertoire of signalling molecules. Our study shows that, in addition to the well-accepted view that BMP signal opposes Shh activity in a spatial manner (Liem et al., 1995; McMahon et al., 1998; Liem et al., 2000; Briscoe and Ericson, 2001; Meyer and Roelink, 2003), ventrally derived Bmp7 signalling can oppose Shh signalling in a temporal manner to specify ventral progenitors within the hypothalamus. The deployment of the two signals in this versatile temporal manner in turn leads to novel modules of transcription factor expression, in order to achieve elaborate cellular diversity (Edlund and Jessell, 1999; Ingham and Placzek, 2006; Blaess et al., 2006). Our work adds to the growing body of data suggesting that cell fate in the neural tube is governed through the temporal integration of, and adaptation to, signalling ligands (Stamatakis et al., 2005; Blaess et al., 2006; Dessaud et al., 2007; Patthey et al., 2008; Tucker et al., 2008).

Our data suggest, further, that Pax7 is normally suppressed in early progenitors that are exposed to Shh signalling, but that a BMP-mediated upregulation of Gli3 leads to a repression of Shh signalling and subsequent de-repression of Pax7 expression. Our study is the first to demonstrate an upregulation of Gli3 by Bmp7. Previous experiments have shown that BMPs can maintain Gli3 expression within dorsal regions of the presumptive spinal cord (Meyer and Roelink, 2003); our study extends these observations. A key question is how does BMP upregulate Gli3 expression within the ventral hypothalamus? Several reports indicate epistatic relationships between BMP and Wnt signalling (e.g. Fuentealba et al., 2007), and recent data have suggested that Wnt signalling can

induce, or maintain, Gli3 expression (Alvarez-Medina et al., 2008). However, we find no evidence that a canonical BMP/Wnt signalling pathway is sufficient to induce Gli3 in the hypothalamus: first, overexpression of pSmad5 does not lead to Gli3 upregulation; second, inhibition of pSmad signalling by dorsomorphin does not eliminate Gli3 expression; third, although Wnt8b is expressed within a region of the hypothalamus (Hollyday et al., 1995), its expression does not match precisely that of pSmad1/5/8. Our *in vitro* analyses, however, suggest some complexity to the upregulation of Gli3 by Bmp7, as induction is limited to a small region within pHyp explants, favouring the interpretation that only specific cell types are competent to respond to BMP by upregulating Gli3. Potentially, different competence factors enable different responses to pSmad5 signalling, making it difficult to exclude entirely some role for pSmad activity in Gli3 upregulation. A second possibility, though, is that Gli3 upregulation is mediated by a distinct signalling pathway. Earlier reports raise the possibility that non-canonical BMP signalling pathways may operate in the CNS (Panchision et al., 2001). Alternatively, Gli3 upregulation may be governed by a Tbx2-mediated pathway. In previous work, we have demonstrated that a BMP-Tbx2 pathway leads to a downregulation of Shh and then of Shh signalling pathway elements, including Ptc1 and Gli1/2 (Manning et al., 2006). Future experiments will determine whether Tbx2 leads to Gli3 upregulation.

Our experiments reveal, however, that the repression of Shh signalling and upregulation of Gli3 expression are insufficient to drive Pax7, which instead requires additional BMP signalling. This suggests that a dual action of BMP signalling specifies Pax7⁺ basal progenitors in the ventrolateral hypothalamus: a Smad5-independent Bmp7-Gli3R pathway opposes the Shh-Gli1A pathway, and, in addition, a Smad5-dependent BMP signalling pathway is required to activate Pax7 (Fig. 4K).

Generally, our studies show insights into how complexity of cell patterning can be achieved within the CNS through the temporal restriction of key signalling factors. The sequential temporal exposure of hypothalamic progenitors to the 'ventralising' influence of Shh and subsequently to the 'dorsalising' influence of Bmp7 establishes specific transcription factor codes, in this case directing the differentiation of ventrolateral Pax7⁺ progenitors. Here, we focus on the importance of this temporal sequence in the specification of Pax7⁺ hypothalamic progenitors. However, they are clearly only a fraction of the basal progenitor pool: the existence of Ki67/Pax7⁻ progenitors implies that there are other late-arising ventral hypothalamic progenitors. Future studies will reveal whether they are also specified by the temporal antagonism of Shh and BMP signalling.

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