Directed differentiation of neural cells to hypothalamic dopaminergic neurons

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Summary

Hypothalamic neurons play a key role in homeostasis, yet little is known about their differentiation. Here, we demonstrate that Shh and Bmp7 from the adjacent prechordal mesoderm govern hypothalamic neural fate, their sequential action controlling hypothalamic dopaminergic neuron generation in a Six3-dependent manner. Our data suggest a temporal distinction in the requirement for the two signals. Shh acts early to specify dopaminergic neurotransmitter phenotype. Subsequently, Bmp7 acts on cells that are ventralised by Shh, establishing aspects of hypothalamic regional identity in late-differentiating/postmitotic cells. The concerted actions of Shh and Bmp7 can direct mouse embryonic stem cell-derived neural progenitor cells to a hypothalamic dopaminergic fate ex vivo.

Key words: Shh, Bmp7, Chick, Dopamine, Hypothalamus

Development

Introduction

During development of the central nervous system (CNS), neurons are generated under the influence of secreted inductive signals (Liu and Joyner, 2001; Jessell, 2002). Signals derive from local cell groups and regulate the developmental potential of neural progenitor cells through the expression of cell-specific transcription factors (Edlund and Jessell, 1999; Briscoe and Ericson, 2001; Marquardt and Pfaff, 2001; Jessell, 2002). Recent studies have revealed, additionally, that neuronal subtype identity is malleable after cell cycle exit and can similarly be controlled through the restricted expression of transcription factors (Livet et al., 2002; William et al., 2003). Such postmitotic changes in transcription factor expression can, again, be elicited in response to localised inductive signals (Sockanathan et al., 2003).

The link between inductive signalling, transcription factor expression and neuronal fate is particularly well understood in the developing spinal cord (Edlund and Jessell, 1999; Briscoe and Ericson, 2001; Marquardt and Pfaff, 2001; Jessell, 2002). By contrast, far less is known of these events within the hypothalamus, a major component of the ventral diencephalon. Thus, despite their key role in mediating homeostasis in the adult, little is known about the differentiation of hypothalamic neurons. Studies in zebrafish embryos have suggested that ambient levels of Wnt activity determine early hypothalamic character (Kapsimali et al., 2004). However, the source, nature and mechanism of action of signals required to induce and specify neuronal identities within the hypothalamic anlage remains largely unclear. The tuberal (mid) hypothalamus is generally accepted to be induced by underlying prechordal mesoderm, a structure that is likely to initiate hypothalamic neuronal induction (Muenke and Beachy, 2000; Kiecker and Niehrs, 2001; Wilson and Houart, 2004); but to date, there has been little systematic analysis of hypothalamic neuronal differentiation in response to prechordal mesoderm-derived signals.

Sonic hedgehog (Shh) and bone morphogenetic proteins (Bmps) are expressed within prechordal mesoderm (Patten and Placzek, 2000) and interact to control development of ventral-most cells within the tuberal hypothalamus (Dale et al., 1997; Dale et al., 1999), cells that initially share an origin with the anterior floor plate (Patten et al., 2003; Placzek and Briscoe, 2005). Whether Shh and Bmps similarly act to establish hypothalamic neuronal identity is unclear. Hedgehog signalling is required cell-autonomously for the differentiation of some characteristics of hypothalamic neurons (Mathieu et al., 2002; Wilson and Houart, 2004), but is not sufficient to promote all aspects of hypothalamic neuronal character. Overexpression of Hedgehog proteins does not, for example, lead to the ectopic expression of the homeodomain (HD) transcription factor Nkx2.1 (Rohr et al., 2001; Wilson and Houart, 2004). As yet, no study has examined whether Bmp signalling contributes to the differentiation of hypothalamic neurons, nor established whether, and how, Shh and Bmps may cooperate to direct hypothalamic neuronal fate.

Here, we perform experiments in chick embryos to analyse the differentiation of tuberal hypothalamic neurons. Our studies identify a set of progenitor cells that give rise to hypothalamic dopaminergic (DA) neurons that transiently co-express tyrosine hydroxylase (Th) and the hypothalamic regional markers Nkx2.1 and Msx1/2. We show that sequential Shh and Bmp signals from the prechordal mesoderm control the neurotransmitter identity and regional characteristics of these cells, the two signals required in a temporally distinct

sequence. The induction of DA identity is initiated by Shh signalling; it occurs independently of, and precedes, induction of the hypothalamic regional markers Nkx2.1 and Msx1/2. Bmp7 acts on cells that are ventralised by Shh to induce these regional hypothalamic markers, and can elicit such hypothalamic characteristics in late-differentiating or postmitotic cells. In ovo electroporation studies reveal that Bmp7 and Shh cooperate to specify hypothalamic DA neuronal identity in a manner that depends on the transcriptional repressor Six3. Finally, we demonstrate that the combined action of Shh and Bmp7 can direct mouse embryonic stem (ES)-derived neural progenitor cells to a hypothalamic DA fate.

Materials and methods

Immunolabelling

Chick embryos (*n*=5-10; each stage) and explants (*n*=10-20) were examined as described previously (Patten et al., 2003). Antibodies used were: 68.5E1 anti-Shh mouse monoclonal antibody (mAb) (1:50); 4C7 anti-Foxa2/HNF3β mAb (1:50); 4F8 anti-Lim1 mAb (1:50); anti-Lim1/2 rabbit polyclonal Ab (T4) (1:4000); 73.4GN anti-En1 mAb (1:50); 4G1 anti-Msx/2 mAb (1:50); anti-Nkx2.2 rabbit polyclonal Ab (1:5000) (gifts of T. Jessell); Kyo2-60 anti-Nkx2.1 rabbit polyclonal Ab generated against the conserved amino acids sequence of rat and chick Nkx2.1 (GNMSELPPYQDTMR) as described (Lazzaro et al., 1991) (1:2000-4000); 50.5A5 anti-Lmx mAb; 74.5A5; anti-Nkx2.2 mAb (1:50); anti-Pax6 mAb (1:50); anti-Pax7 mAb (1:50); MAb318 anti-tyrosine hydroxylase (Th) mAb (Chemicon, 1:200); AB152 anti-Th rabbit polyclonal serum (1:200) (Chemicon); AB1585 anti-dopamine β hydroxylase (DβH) rabbit polyclonal Ab (Chemicon, 1:2000); anti-GAD67 rabbit polyclonal Ab (Chemicon, 1:1000-2000); anti-Six3 rabbit polyclonal Ab (1:200) (gift of G. Oliver); and anti-BrdU mAb (1:200; Novocastra). mAbs against Nkx2.2, Pax6, Pax7 and Lmx were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Appropriate secondary antibodies conjugated to Cy3 or FITC (Jackson Immunoresearch) were used (1:200). For BrdU/Th double labelling experiments, cryostat sections were labelled with anti-Th polyclonal Ab followed by FITC-conjugated anti-rabbit IgG. Sections were then treated with 2 N HCl at room temperature, rinsed in PBS and incubated with anti-BrdU mAb and Cy3 anti-mouse IgG. Images were taken using Spot RT software v3.2 (Diagnostic Instruments) and some images were also analysed using a Leica confocal microscope.

In situ hybridisation

Embryos and explants were processed for in situ hybridisation as described previously (Dale et al., 1999). The following template DNAs were used to generate digoxigenin-labelled antisense RNA probes: pcvhh encoding chick Shh (linearised with *Sal*I and transcribed with SP6 RNA polymerase); pBH2 encoding chick Bmp7 (linearised with *Xho*I and transcribed with T3 RNA polymerase); and psix3 encoding chick Six3 (linearised with *Xho*I and transcribed with T3 RNA polymerase). As controls, sense RNA probes were used.

Tissue dissection and explant culture

All embryos were staged according to Hamburger-Hamilton (Hamburger-Hamilton, 1951) and dissected in cold L15 medium (Gibco-BRL). Explant cultures were performed in collagen gels according to published techniques (Patten et al., 2003). Based upon fate-mapping analyses (Dale et al., 1999; Patten et al., 2003), prospective hypothalamic tissue was dissected out after Dispase treatment (1 mg/ml in L15 medium at room temperature, 5-15 minutes). For co-culture of hypothalamus and prechordal mesoderm, both dissected tissues were recombined in vitro. Explants were cultured for 2-3 days for the analysis of progenitor cells, 5-7 days for DA neurons and 13 days for the analysis of DβH expression (onset of culture designated as day 0). In BrdU labelling experiments, explants were incubated with 10 μM BrdU on day 1, 2, 3, 4, 5 or 6.

Real-time PCR analysis

Real-time quantitative RT-PCR analysis was performed using ABI Prism 7700 sequence detection system (Applied Biosystems). RNA in each sample was standardised using β-actin amplification as an internal control. Primers used were: *Shh*, forward primer 5′-CGG-CTTCGACTGGGTCTACT-3′ and reverse primer 5′-CGCTGCCAC-TGAGTTTTCTG-3′; the Taqman probe, 5′-CGAGTCCAAGGCGC-ACATCCACR-3′ (labelled with the reporter dye FAM on the 5′ nucleotide and the quenching dye TAMRA on the 3′ nucleotide); β-actin forward primer, 5′-GGTCATCACCATTGGCAATG-3′ and reverse primer, 5′-CCCAAGAAAGATGGCTGGAA-3′; the Taqman fluorogenic probe, 5′-TTCAGGTGCCCCGAGGCCCT-3′ labelled with the reporter dye VIC on the 5′ nucleotide and the quenching dye TAMRA on the 3′ nucleotide. Relative quantification of Shh mRNA was calculated using the comparative ΔCt method.

DiI labelling

Chick embryos were incubated to stage 15. The lipophilic carbocyanine dye, 1, 1-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) (Molecular Probes) was injected into the germinal zone of the lateral tuberal hypothalamus, targeting Shh+ cells. Embryos were either fixed immediately, or cultured to E6 and fixed in 4% paraformaldehyde (PFA), then analysed by immunolabelling for Shh and Th.

Proteins

Transient transfection of 293T cells were performed to obtain supernatants containing Shh, Bmp7 and chordin. The following plasmids were used: SHH-N IRES GFP, encoding the N terminus of Shh and green fluorescent protein (GFP); pdMb7, encoding a chimaera of dorsalin (pre-pro region) and chick Bmp7, with a Myc epitope inserted at the junction (gift of T. Jessell); and pMT11HA.1-chordin, encoding chick chordin with an HA epitope. Shh activity was evaluated by examining Nkx2.2 and Pax6 expression after exposure of chick lateral neural plate (LNP) explants. Shh-containing culture supernatant (1×) showed an activity equivalent to 3 nM Shh. In blocking experiments, anti-Shh IgG (20 μg/ml) was used as previously described (Ericson et al., 1996).

Bead implantation

Affigel beads (Pharmacia Biotech) were incubated with transfected culture supernatant, prepared as described above, at 4°C overnight. After a brief PBS wash, beads were implanted and embryos developed further.

Electroporation

Electroporation of HH stage 10-11 chick embryos was performed in ovo. Expression plasmids [either the repressor or the activator form of Six 3 (RDSix3, ADSix3) (Kobayashi et al., 2002)] were electroporated into the lateral mesencephalon. Efficiency of gene introduction was confirmed in separate experiments by co-electroporating a red fluorescent protein (RFP) construct. After electroporation, mesencephalic lateral neural tube was dissected and cultured, with factors, in collagen gels.

Differentiation of hypothalamic DA neurons using mouse ES cells-derived neural progenitor cells

Maintenance of undifferentiated ES cells (CCE), embryoid body (EB) formation, selection of nestin-positive cells, and neuronal differentiation were performed as described (Okabe et al., 1996; Lee et al., 2000). Nestin-positive cells were cultured in the presence of Shh and Bmp7, Shh alone or Bmp7 alone for 8 days. In control cultures, supernatant produced by 293T cells transfected with control plasmid DNA, or no DNA, was added to the culture medium. Differentiation was initiated by withdrawal of basic Fgf and the

signalling molecules, and supported through addition of ascorbic acid (200 µM, Sigma). Medium was changed every 2 days. After a further 8 days in culture, cells were fixed with 4% PFA and processed for immunolabelling.

Results

Nkx2.1+/Shh+ cells give rise to tuberal hypothalamic DA neurons

We first examined expression of Nkx2.1 in the developing chick hypothalamus. At stage 10-13, Nkx2.1+/Shh+ cells are detected broadly in the hypothalamus (Fig. 1A-D; data not shown). At stage 15, a downregulation of Shh occurs in ventral-most tuberal hypothalamic cells (Fig. 1B,E,F,R) (Barth and Wilson, 1995) (E. Manning, K.O. and M.P., unpublished), so that from stage 15, Nkx2.1/Shh expression defines ventral cells in the anterior hypothalamus (asterisk, Fig. 1B,R) and lateral cells in the tubero/mamillary hypothalamus (arrow, Fig. 1B,E,R). Within the tuberal hypothalamus, Shh is expressed at particularly high levels in cells in the germinal zone (Fig. 1F-

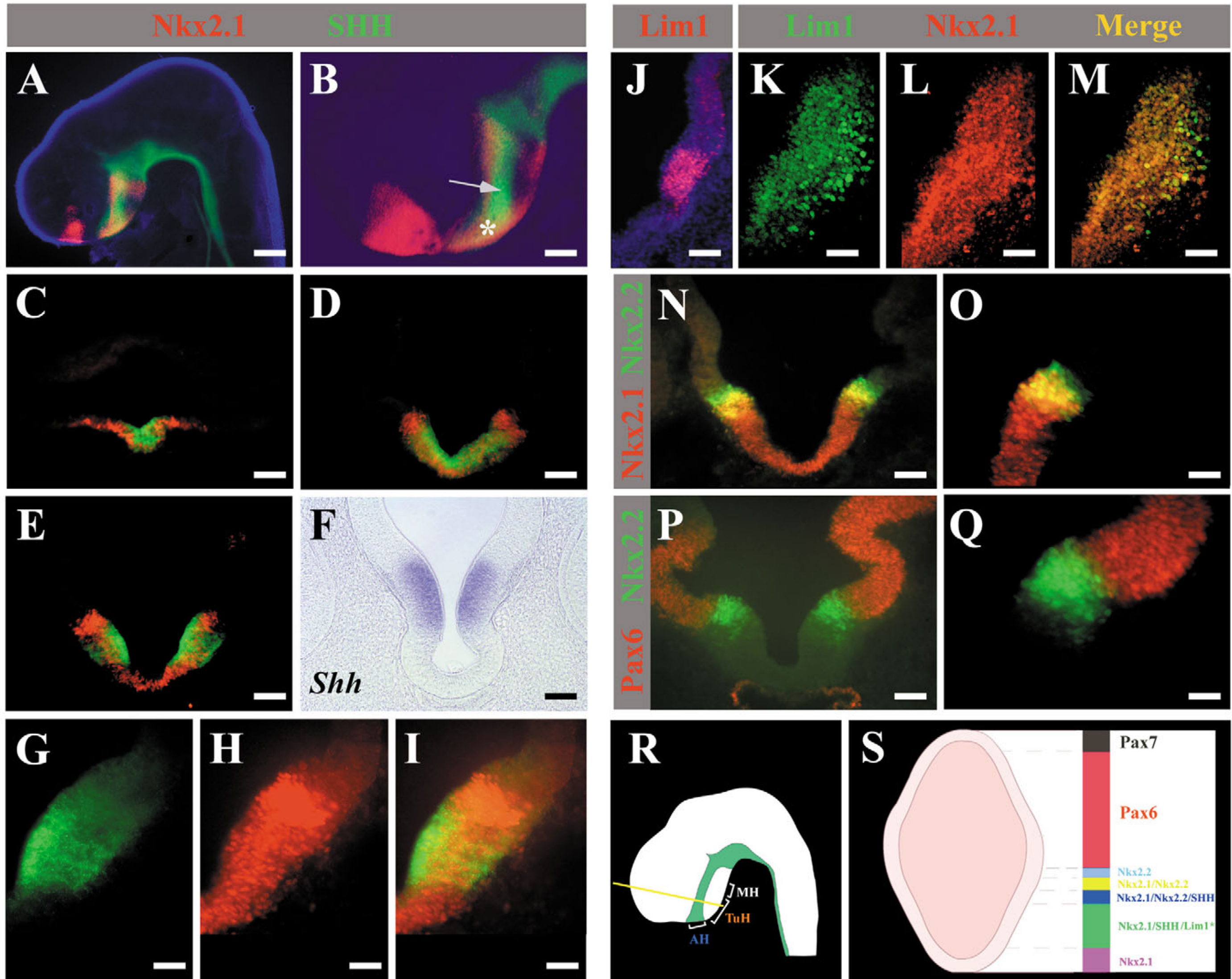

Fig. 1. Expression patterns of HD proteins and Shh in the tuberal hypothalamus. Whole-mount lateral views (A,B) or transverse sections through the tuberal hypothalamus (C-Q) of stage 10-15 embryos. (A,B) Double-label immunofluoresence to detect Shh (green) and Nkx2.1 (red) at stage15. In the diencephalon, Nkx2.1+/Shh+ progenitor cells occupy the anterior ventral hypothalamus (asterisk) and lateral tuberal hypothalamus (arrow) (see R). These populations have previously been defined as 'anterior-dorsal' hypothalamic cells (Mathieu et al., 2002). Blue shows DAPI counterstain. Telencephalic cells express only Nkx2.1 at this stage. (C-E,G-I) Double-label immunofluorescence reveals expression of Nkx2.1 and Shh at stage 10 (C), stage 13 (D) and stage 15 (E,G-I). From stage 15, Shh and Nkx2.1 are co-expressed by cells in the germinal zone I (G-I). (F) *Shh* mRNA expression in the germinal zone. (J-M) Double-label immunofluoresence shows expression of Lim1 and Nkx2.1. Lim1 is strongly expressed in lateral hypothalamic cells and overlaps extensively with Nkx2.1. (N,O) Nkx2.2 overlaps with Nkx2.1 and then extends more dorsally. (P,Q) Nkx2.2 expression is complementary to Pax6 expression. (R,S) Summary diagrams showing hypothalamic nomenclature, HD protein codes and Shh expression. Lim1* denotes high expression in lateral tuberal hypothalamic cells. For simplicity, weak Lim1 expression in more dorsal region is not shown in this diagram. AH, anterior hypothalamus; TuH, tuberal hypothalamus; MH, mammilary hypothalamus. Scale bars: 300 µm in A; 130 µm in B; 65 µm in C-F,J,N,P; 30 µm in G-I,O,Q; 25 µm in K-M.

I), and all Shh+ cells in this zone appear to co-express Nkx2.1 (Fig. 1G-I).

Comparison of Nkx2.1 with the progenitor markers Lim1, Nkx2.2, Pax6 and Pax7 reveals distinct subdivisions of Nkx2.1 domains within the tuberal hypothalamus at stage 15-22 – a ventral Nkx2.1+ domain, followed by (ventral to dorsal): Nkx2.1+/Shh+/Lim1+; Nkx2.1+/Shh+/Lim1+/Nkx2.2+; and Nkx2.1+/Lim1+/Nkx2.2+ domains. Dorsal to these lie Nkx2.2+/Lim1+ and Pax6+/Lim1+ domains (Fig. 1J-S). Previous studies have suggested that Nkx2.2+ and Pax6+ cells contribute to dorsal-most regions of the hypothalamus (Barth and Wilson 1995; Stoykova et al., 1996), confirming that Nkx2.1+/Shh+ regions of the tuberal hypothalamus can be defined as 'lateral' tuberal hypothalamic.

We examined whether a defined class of neurons arise from progenitor cells within the lateral tuberal hypothalamus. In rodents, A12 tubero-infundibular dopaminergic (DA) neurons arise from the ventral lobe of the tuberal hypothalamus (Altman and Bayer 1978; Daikoku et al., 1986). Similarly, we find that tyrosine hydroxylase (Th)-immunoreactive neurons appear in the lateral tuberal hypothalamus at embryonic day 5 (E5) (Fig. 2A,A′,B,B′ and data not shown). These neurons do not co-express dopamine β hydroxylase (DβH), defining them as dopaminergic, not noradrenergic. To test whether DA neurons arise from progenitor cells within the Nkx2.1+/Shh+ domain, we fate-mapped the Nkx2.1+/Shh+ germinal zone of the lateral tuberal hypothalamus. At E6, cells that had been DiI-labelled at stage 15 (Fig. 2C,D) continued to reside within the Shh+ territory (Fig. 2E). Furthermore, many Th+ cells were labelled with DiI (Fig. 2F, arrows). Thus, many tuberal DA neurons originate in the lateral tuberal hypothalamus and differentiate in situ.

We next examined whether we could distinguish hypothalamic DA neurons from other classes of DA neurons. At E5-E6, many Th+ hypothalamic DA neurons in the lateral tuberal hypothalamus continue to co-express Nkx2.1 (Fig. 2G,H) and Lim1 (Fig. 2I). Furthermore, Msx is co-expressed with Nkx2.1 in the ventrolateral hypothalamus between E3 and E6 (Fig. 2J-L). No Nkx2.1+/Msx+/Th+ neurons were detected in other parts of the CNS, including the anterior hypothalamus, at E5-E6 (data not shown). These studies show that, at least transiently, co-expression of Nkx2.1/Msx/Th defines lateral tuberal hypothalamic DA neurons.

Prechordal mesoderm triggers the induction of hypothalamic DA neurons

Previously, we have found that prechordal mesoderm governs ventral tuberal hypothalamic cell identity (Dale et al., 1997),

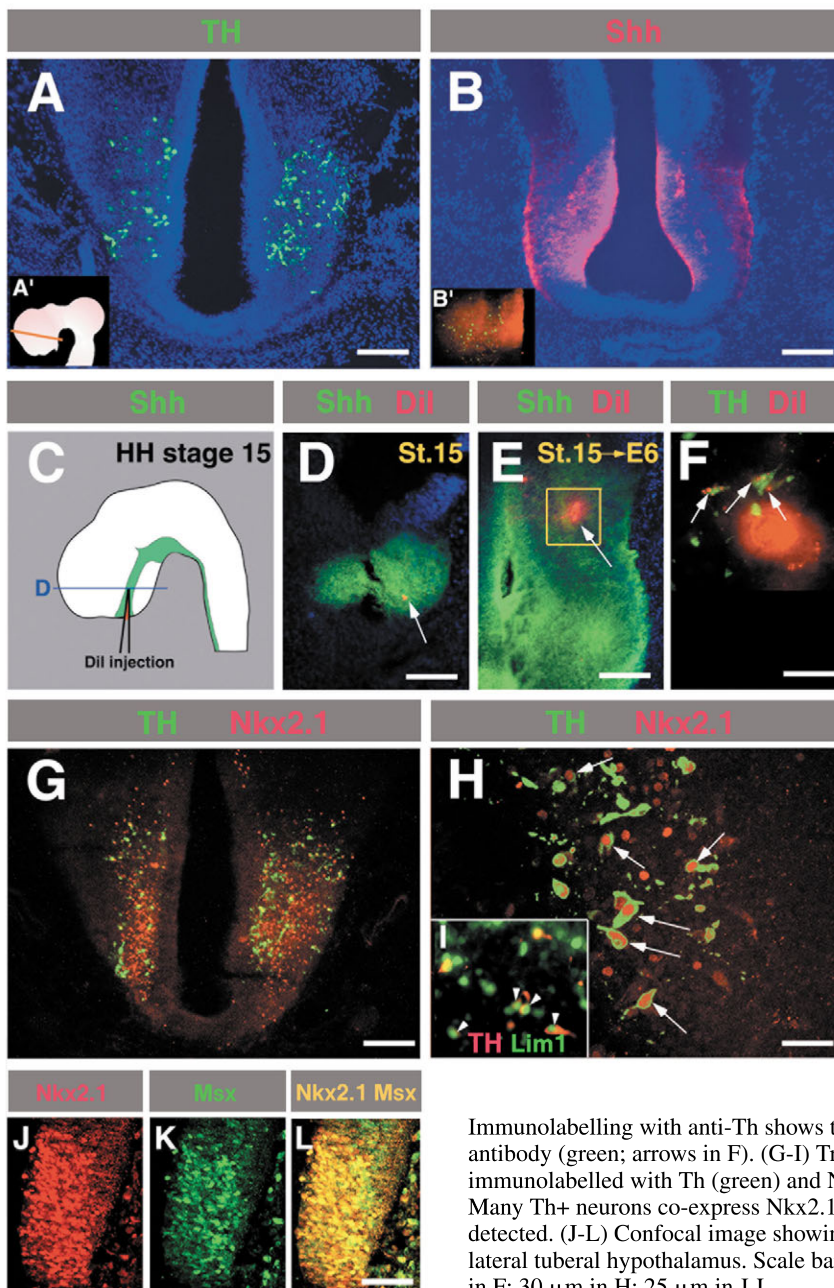

Fig. 2. Nkx2.1+/Th+ hypothalamic DA neurons are generated from Nkx2.1+/Shh+ progenitors in the lateral tuberal hypothalamus. (A,B) Transverse sections through embryonic day 6 (E6) tuberal hypothalamus (shown in A′), immunolabelled with Th (green) (A), Shh (red) (B) or both (B′). Th and Shh show extensive overlap. Blue shows DAPI counterstain. (C-F) Fate mapping of germinal zone of lateral tuberal hypothalamus. (C) Schematic, showing DiI labelling at stage 15. (D-F) Transverse sections through DiI-labelled embryos, fixed immediately (D) or developed to E6 (E,F). Immunolabelling with anti-Shh confirms that lateral tuberal cells are targeted at stage 15 (arrow, D) and remain in situ at E6 (arrow, E). Immunolabelling with anti-Th shows that DiI-labelled cells (red) co-label with anti-Th antibody (green; arrows in F). (G-I) Transverse sections through E6 tuberal hypothalamus, immunolabelled with Th (green) and Nkx2.1 (red) (G,H) or Th (red) and Lim1 (green) (I). Many Th+ neurons co-express Nkx2.1. Likewise, extensive co-expression of Th and Lim1 is detected. (J-L) Confocal image showing overlapping expression of Nkx2.1 and Msx1 in the lateral tuberal hypothalamus. Scale bars: 140 μm in A,B,G; 60 μm in D; 120 μm in E; 20 μm in F; 30 μm in H; 25 μm in J-L.

but no study has yet examined whether prechordal mesoderm is sufficient to trigger the differentiation of hypothalamic neurons. To test this, we first examined whether prechordal mesoderm could induce Nkx2.1+/Shh+ cells. In stage 4-5 lateral neural plate (LNP) explants (blue square in Fig. 4) cultured alone for 2 days, no cells expressed Nkx2.1 or Shh (Fig. 3A), although Lim1/2, Pax6 and Pax7 were detected (Fig. 3B-D). By contrast, co-culture of prechordal mesoderm with LNP explants induced Nkx2.1+/Shh+ cells in the neural explant (Fig. 3E; data not shown), many of which co-expressed Lim1 (Fig. 3F). Nkx2.1+/Shh+ and Nkx2.1+/Lim1+ cells were induced some distance from the prechordal mesoderm, whereas cells expressing Nkx2.1 alone were induced immediately adjacently (brackets in Fig. 3E,F). These latter cells co-expressed Foxa2 and Bmp7, which transiently mark ventral tuberal hypothalamic cells (Dale et al., 1997; Dale et al., 1999) (Fig. 3G,H,O). This shows that prechordal mesoderm triggers the induction of ventral tuberal hypothalamic cells in immediately adjacent tissue, and the induction of Nkx2.1+/Shh+ cells more distantly. To address whether signals from prechordal mesoderm can directly trigger the induction of Nkx2.1+/Shh+ cells, LNP explants were cultured at a distance from prechordal mesoderm. In this contact-independent context (Tanabe et al., 1995), Nkx2.1+/Shh+ cells that co-expressed Lim1 were induced (Fig. 3I,J) but Foxa2+/Bmp7+ ventral tuberal hypothalamic cells were not detected (Fig. 3K,L).

To determine whether prechordal mesoderm can induce the differentiation of Nkx2.1+/Msx+ hypothalamic DA neurons, stage 5 prospective hypothalamic (pHyp) explants (green square in Fig. 4F) were cultured alone or with prechordal mesoderm for 5-6 days. In the absence of prechordal mesoderm, many Th+ neurons differentiated, but none expressed Nkx2.1 or Msx (Fig. 3M; data not shown).

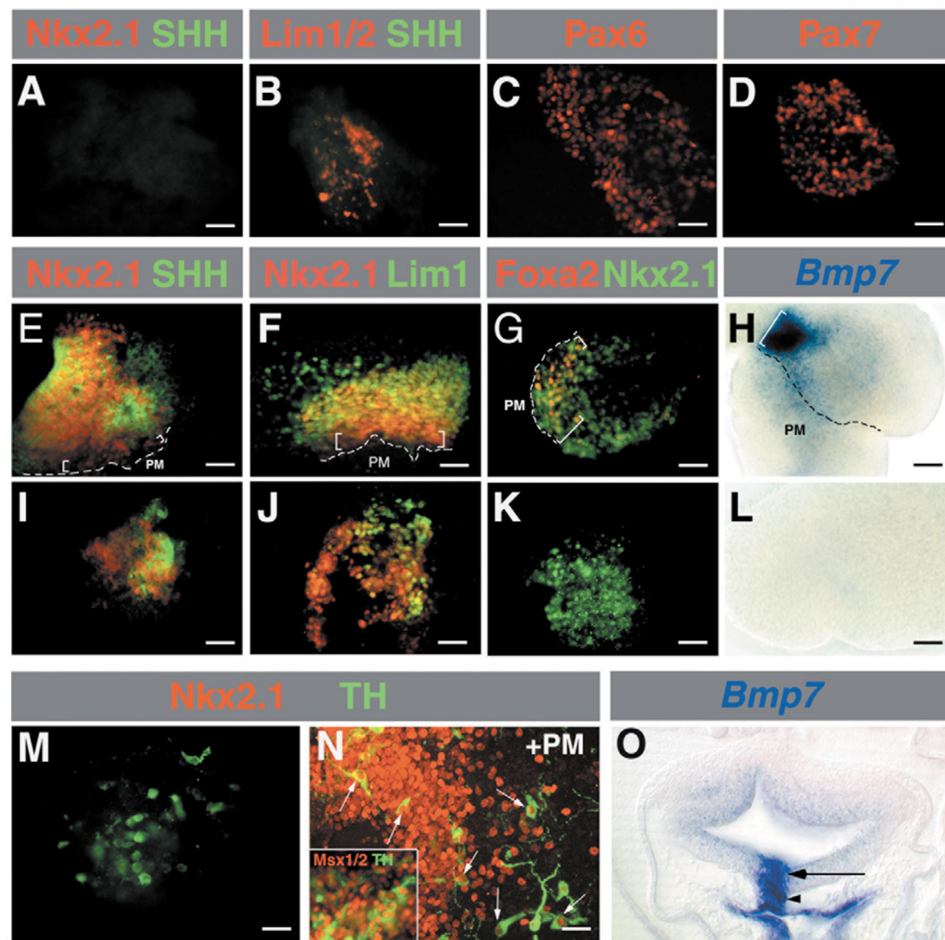

Fig. 3. Induction of Nkx2.1+/Shh+ cells and Nkx2.1+/Msx/2+/Th+ DA neurons can be triggered by prechordal mesoderm in a contact-independent manner. (A-D) Stage 4-5 LNP explants (blue box, Fig. 4F) cultured alone express Lim1/2, Pax6 and Pax7 but not Nkx2.1 or Shh. (E-H) In stage 4-5 LNP explants co-cultured in contact with stage 5+ prechordal mesoderm, Nkx2.1+/Foxa2+/Bmp7+ ventral tuberal hypothalamic cells are detected (white brackets) at the boundary with prechordal mesoderm (broken line). Nkx2.1+/Shh+/Lim1+ lateral tuberal hypothalamic progenitor cells are detected adjacent to these. (I-L) In stage 5 LNP explants co-cultured at a distance from HH stage 5+ prechordal mesoderm, Nkx2.1+/Shh+/Lim1+ lateral tuberal hypothalamic cells are detected, but no Nkx2.1+/Foxa2+/Bmp7+ ventral tuberal cells are found. (M,N) Th+ neurons, but not Nkx2.1+/Th+ neurons, differentiate in a stage 5 pHyp explant (green box, Fig. 4F) cultured alone (M). Nkx2.1+/Msx1/2+/Th+ neurons differentiate in stage 5 pHyp explant co-cultured with prechordal mesoderm (N). (O) Transverse section of stage 10 embryo showing expression of Bmp7 in the ventral tuberal hypothalamus (arrow) and prechordal mesoderm (arrowhead). Scale bar: 40 μm in A-L; 30 μm in M,N.

By contrast, when cultured with prechordal mesoderm, Nkx2.1+/Msx+ hypothalamic DA neurons with long neurites differentiated in the neural explant (Fig. 3N).

Progressive specification of hypothalamic characteristics

To address when different aspects of hypothalamic cell identity are specified, we analysed cell differentiation in pHyp explants isolated at progressive stages of development (Fig. 4A,F,K). Stage 4 pHyp explant cultures express Pax6 and Lim1 (Fig. 4B,C) but not Nkx2.1, Nkx2.2 or Shh (Fig. 4B-E). Stage 5 pHyp explants express Pax6, Lim1, Shh and Nkx2.2, but not Nkx2.1 (Fig. 4G-J). Stage 6 pHyp explants express Nkx2.1, and as in vivo, many Nkx2.1+ cells co-express Shh and Lim1 (Fig. 4L-O). In addition, an upregulation of Msx is initiated in Nkx2.1+ cells (Fig. 2L; Fig. 4O inset). Together, these analyses show a progressive specification of lateral tuberal hypothalamic cells; first Shh+/Lim1+ cells are specified; subsequently, Nkx2.1/Msx expression is upregulated in Shh+/Lim1+ progenitors.

To ascertain how such cultures would progress, we cultured progressive stages of pHyp explants for 6-7 days. No Th+ neurons were detected in stage 4 pHyp cultures (not shown). Th+ neurons were detected in stage 5 pHyp explant cultures, but these did not co-express Nkx2.1 or Msx1 (Fig. 3M). However, in stage 6 pHyp explants, many Nkx2.1+/Msx+ DA neurons were detected (Fig. 4P and inset). In addition, six3, a marker previously implicated in hypothalamic specification (Lagutin et al., 2003) was detected generally within the explants (Fig. 4S), and a subset of Nkx2.1+ DA neurons co-expressed GAD65/67, a marker that distinguishes hypothalamic from mesencephalic DA neurons (Daikoku et al., 1986; Kim et al., 2002) (data not shown). No Th+ cells co-expressed En1 or Foxa2, markers of mesencephalic DA neurons (Hynes et al., 1995; Kim et al., 2002) (Fig. 4Q; data not shown), or Pax6 a marker of thalamic DA neurons (Vitalis et al., 2000) (Fig. 4R). Similarly, no co-expression of DA and DβH was detected (data not shown). Together, these results show that hypothalamic Nkx2.1+/Msx+ DA neurons can differentiate in vitro, but are only specified at stage 6.

Shh is required, but not sufficient, to induce Nkx2.1+/Shh+ cells and hypothalamic DA neurons

The prechordal mesoderm expresses Shh at

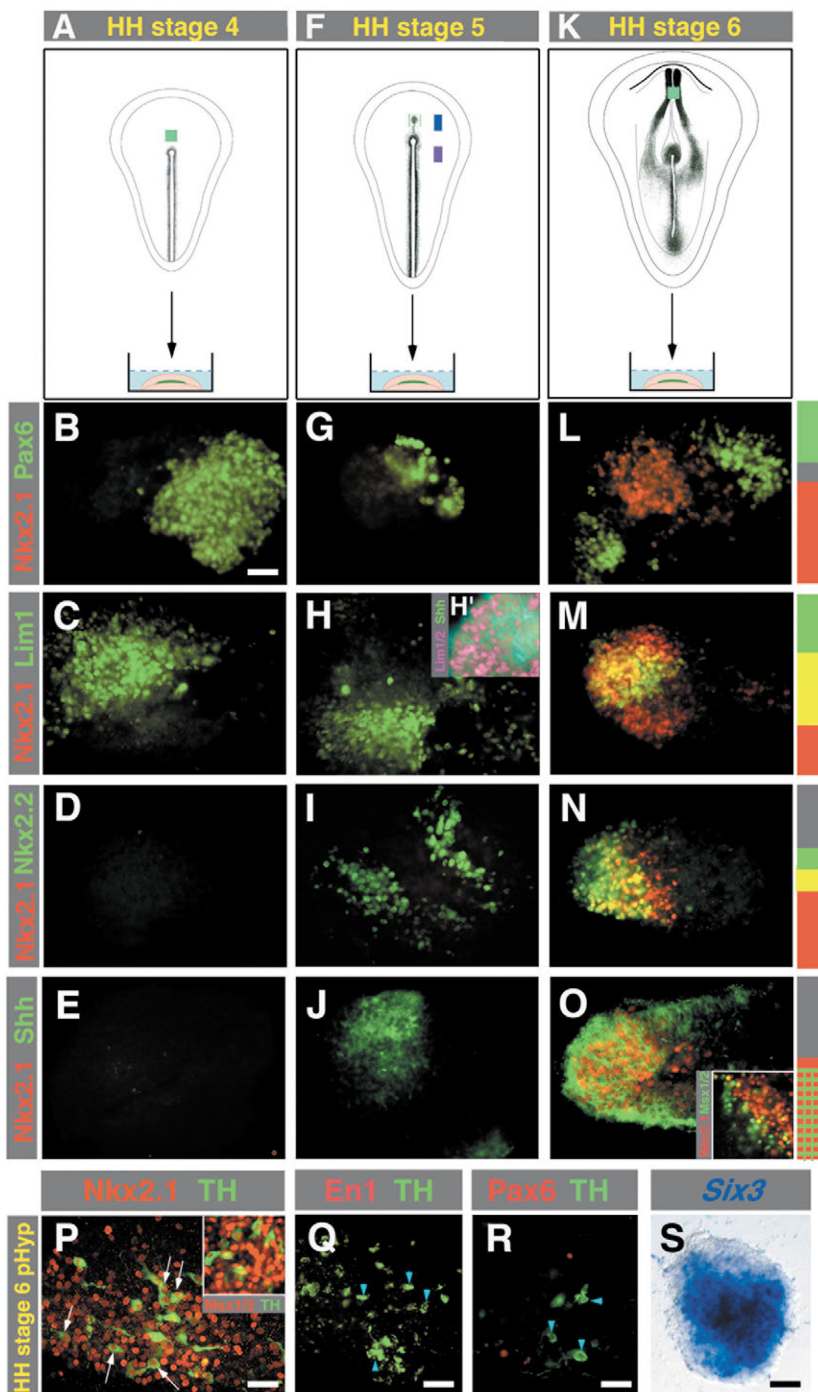

Fig. 4. Progressive specification of Nkx2.1+/Msx1/2+/Th+ DA neurons. (A,F,K) Schematic of stage 4-6 embryos, showing regions dissected. Prospective hypothalamic (pHyp) regions (green) and anterior (blue) or posterior (purple) lateral neural plate are indicated. (B-E,G-J,L) pHyp explants, cultured for 2-3 days and analysed by immunofluorescent labelling. Co-expression of Shh and Lim1 is detected in HH stage 5 explants (H′). Nkx2.1 expression is detected only in stage 6 explants and is exclusive to Pax6 (L). From HH stage 6, Nkx2.1+/Lim1+, Nkx2.1+/Nkx2.2+, Nkx2.1+/Shh+, Nkx2.1+/Msx1/2+ cells are specified, as in vivo (M-O; see Fig. 1, Fig. 2L). (P-R) Immunofluorescent analysis of stage 6 pHyp explants cultured for 6-7 days. Many Nkx2.1+/Msx1/2+/Th+ neurons differentiate (arrows in P; inset in P). Th+ neurons (arrowheads) do not co-express En1 (Q) or Pax6 (R). (S) Widespread expression of Six 3 is detected within stage 6 pHyp explants after 40 hours culture. Scale bars: 40 μm.

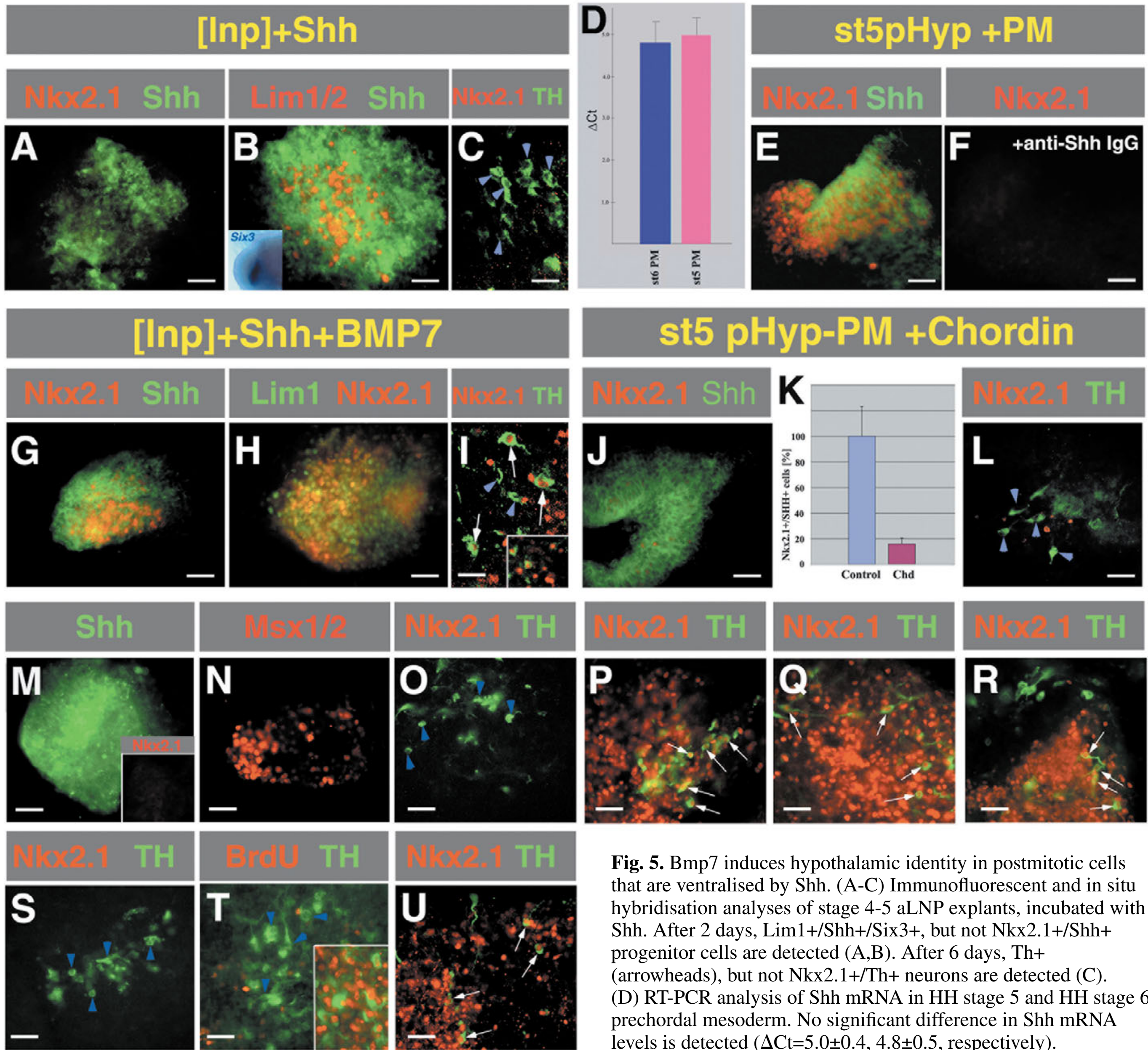

Fig. 5. Bmp7 induces hypothalamic identity in postmitotic cells that are ventralised by Shh. (A-C) Immunofluorescent and in situ hybridisation analyses of stage 4-5 aLNP explants, incubated with Shh. After 2 days, Lim1+/Shh+/Six3+, but not Nkx2.1+/Shh+ progenitor cells are detected (A,B). After 6 days, Th+ (arrowheads), but not Nkx2.1+/Th+ neurons are detected (C). (D) RT-PCR analysis of Shh mRNA in HH stage 5 and HH stage 6 prechordal mesoderm. No significant difference in Shh mRNA levels is detected (ΔCt=5.0±0.4, 4.8±0.5, respectively). (E,F) Recombinate explants of stage 4-5 pHyp and prechordal mesoderm, incubated alone (E) or with anti-Shh IgG (F). No Nkx2.1+ cells are detected in the presence of anti-Shh IgG. (G-I) Shh and Bmp7 cooperate to induce Nkx2.1+/Shh+progenitors and Nkx2.1+/Th+ neurons. Stage 4-5 aLNP explants, incubated with Shh and Bmp7. After 2 days, Nkx2.1+/Shh+ (G) and Nkx2.1+/Lim1+ (H) progenitors are detected. Msx+/Th+ neurons are detected on day 5 (I, inset) and Nkx2.1+/Th+ neurons detected on day 7 (I, white arrows). Th+ (blue arrowheads) neurons can also be detected. (J,K) Chordin blocks prechordal mesoderm-mediated specification of Nkx2.1+/Shh+ progenitors. (J) A stage 5 pHyp explant, co-cultured with prechordal mesoderm in the presence of chordin. Very few Nkx2.1+/Shh+ cells are detected. (K) Significantly more Nkx2.1+/Shh+ cells form in control cultures compared with cultures treated with chordin (control, 100%±23.2%; +Chd, 15.9%±4.3%, P<0.0001; unpaired t-test). (L) A stage 5 pHyp explant co-cultured with prechordal mesoderm and chordin for 6 days. Th+ cells differentiate (blue arrowheads), but these do not co-express Nkx2.1. (M) Stage 5 aLNP explants, incubated with Shh. After 22 hours, Shh but not Nkx2.1 (M, inset) was induced. (N) Stage 5 aLNP explants, incubated with Bmp7. After 22 hours, Msx/2 was detected but no Nkx2.1 was observed. (O) Stage 5 aLNP explants, transiently incubated with Bmp7 (day 0-1), then Shh (day1-7). On day 7, Th+ cells differentiate but they do not co-express Nkx2.1 (blue arrowheads). (P) Stage 5 aLNP explant, transiently incubated with Shh (day 0-1), then Bmp7 (days 1-7). On day 7, Nkx2.1+/Th+ cells differentiate (arrows). (Q) Stage 5 aLNP explant, transiently incubated with Shh (day 0-1), then transiently incubated with Bmp7 (day1-2). After 7 days, Nkx2.1+/Th+ cells differentiate (arrows). (R) Stage 5 aLNP explants, transiently incubated with Shh (day 0-1), then transiently exposed to Bmp7 (day 5-6). On day 7, Nkx2.1+/Th+ cells differentiate (arrows). (S) Stage 5 aLNP explants, transiently incubated with Shh (day 0-1), then transiently exposed to Bmp7 (day 6-7). On day 7, Th+ cells differentiate but they do not co-express Nkx2.1 (blue arrowheads). (T) Stage 5 pHyp explants, incubated with BrdU on day 5. On day 7, no BrdU+/Th+ cells are observed (blue arrowheads). Inset: stage 5 pHyp explants, incubated with BrdU on day 3. On day 7, BrdU+/Th+ cells are observed. (U) Stage 5 pHyp explants, transiently incubated with Bmp7 on day 5. On day 7, Nkx2.1+/Th+ cells differentiate (arrows). Scale bars: 40 μm in A,B,E-G,J,L,M-U,W; 30 μm in C,I.

stage 4-5 (Marti et al., 1995; Dale et al., 1999; Patten et al., 2003). To examine the role of Shh in hypothalamic neuronal differentiation, we exposed LNP explants to Shh. After 2 days, Shh+ cells that co-expressed Lim1/2 and Six3 were detected, but none co-expressed Nkx2.1, suggesting that Shh signalling leads to a partial, but incomplete, specification of lateral tuberal hypothalamic cells. Similarly, after 6 days, Th+ neurons were generated, but they did not co-express Nkx2.1 or Msx (Fig. 5C, blue arrowheads; data not shown). To exclude the possibility that prechordal mesoderm-mediated induction of Nkx2.1+/Msx+ hypothalamic DA neurons is mediated by particularly high levels of Shh that are not achieved in our in vitro experiments, we measured levels of *Shh* mRNA in prechordal mesoderm by real-time quantitative RT-PCR. No significant increase in *Shh* was detected within prechordal mesoderm at stage 5, when Nkx2.1 is not specified, and stage 6-7, when Nkx2.1 is specified (Fig. 5D).

To test whether, nonetheless, Shh signalling is required for the differentiation of Nkx2.1+/Msx+ hypothalamic DA neurons, prechordal mesoderm-pHyp conjugates were grown with anti-Shh IgG (Ericson et al., 1996). In the presence of anti-Shh IgG, both Nkx2.1+/Shh+ cells and Nkx2.1+/Msx+/Th+ neurons were eliminated in comparison with controls (Fig. 5E,F; data not shown). Together, these experiments show that Shh is required, but not sufficient, to induce Nkx2.1+/Shh+ cells and Nkx2.1+/Msx+ hypothalamic DA neurons.

Cooperative action of Shh and Bmp7 in the differentiation of hypothalamic DA neurons in vitro

Previous analyses have shown that Bmp7 is expressed by prechordal mesoderm from stage 5-6 (Dale et al., 1999; Vesque et al., 2000), and have suggested that it is required for the induction of Nkx2.1 in ventral tuberal hypothalamic cells (Dale et al., 1997). These findings, together with the correlation between Bmp7 signalling and Msx expression observed in other tissues (Lee and Jessell, 1999), prompted us to ask whether Bmp7 plays a key role in hypothalamic neuronal identity, acting with Shh to induce Nkx2.1+/Msx+ hypothalamic DA neurons. Incubation of LNP explants for 2 days in the presence of both Shh and Bmp7 induced Nkx2.1+/Shh+ cells (Fig. 5G), many of which co-expressed Lim1 (Fig. 5H). After 5-7 days, Nkx2.1+/Msx+ DA neurons were generated (Fig. 5I and inset). Neither cell type was generated in LNP explants exposed to Bmp7 alone (data not shown). These experiments show that Bmp7 can cooperate with Shh to induce Nkx2.1+/Shh+ cells and Nkx2.1+/Msx+ hypothalamic DA neurons. However, the LNP shows a restricted competence to differentiate with hypothalamic character: Shh and Bmp7 could induce hypothalamic cells in LNP taken anterior to Hensen's node, but not in LNP from more posterior regions (purple square in Fig. 4F; data not shown).

To test the requirement for Bmp7 in hypothalamic DA neuronal specification, conjugate explants of prechordal mesoderm-pHyp were incubated with chordin, a Bmp inhibitor (Piccolo et al., 1996). In 2-day cultures exposed to chordin, Shh+ cells were detected, but there was a dramatic decrease in the number of Nkx2.1+/Shh+ cells (compare Fig. 5E with 5J). Likewise, in 6-day cultures, Th+ cells were detected, but no Nkx2.1+ hypothalamic DA neurons differentiated (Fig. 5L; compare with Fig. 3N). These cells do not default to A13 thalamic, to A8-A10 mesencephalic DA or to hindbrain noradrenergic fates (not shown), suggesting they are immature hypothalamic neurons that have initiated an incomplete differentiation programme.

Bmp7 can induce hypothalamic regional markers in late-differentiating/postmitotic cells that are ventralised by Shh signalling

A remaining issue is the mechanism of co-operation of Shh and Bmp7. Shh and Bmp7 signalling could induce distinct characteristics of progenitors that, together, specify hypothalamic identity. Alternatively, Bmp7 could induce hypothalamic character in cells that are ventralised by Shh signalling. To distinguish these possibilities, we asked whether there is a temporally distinct requirement for the two signalling molecules in hypothalamic DA neuronal specification by exposing explants only transiently to either Shh or Bmp7, and altering the order of their addition.

We first asked how transient exposure of explants to Shh or Bmp7 alone affects cell fate. In anterior LNP (aLNP) explants (Fig. 4F, blue square) exposed transiently to Shh, a downregulation of Pax6 and Pax7, and a concomitant upregulation of Shh was observed (Fig. 5M: compare with Fig. 3A; data not shown). However, no expression of Nkx2.1 was detected (Fig. 5M, inset). In aLNP explants exposed to Bmp7, Pax6, Pax7 and Shh were unaffected (data not shown), Msx was upregulated (Fig. 5N), but Nkx2.1 was not (data not shown). Thus, a transient addition of Shh and Bmp7 is sufficient to alter cell fate within aLNP, but neither alone can upregulate Nkx2.1, nor induce Nkx2.1+/Msx+ hypothalamic cells.

aLNP explants were next transiently exposed to Bmp7 under conditions in which Msx was upregulated. Subsequently, Shh was added to the cultures. Under these conditions, Pax6 and Pax7 were downregulated, and Shh was upregulated; transiently, Shh+/Msx+ cells were detected (not shown). However, although after 7 days, Th+ neurons were detected, Nkx2.1+/Msx+/Th+ neurons were not (Fig. 5O; data not shown). Thus, Bmp7 and Shh do not appear to operate in independent parallel pathways, mediating distinct characteristics that together induce hypothalamic identity.

By contrast, in explants exposed transiently to Shh and then to Bmp7, Nkx2.1+/Msx+/Th+ neurons were detected (Fig. 5P; data not shown), suggesting that Bmp7 can induce hypothalamic characteristics in cells that are ventralised by Shh signalling. Both sustained and transient exposure of explants to Bmp7 resulted in the appearance of hypothalamic DA neurons (Fig. 5P,Q). Remarkably, addition of Bmp7 on days 1-2 or 5-6 of culture resulted in the differentiation of equivalent numbers of Nkx2.1+/Th+ neurons (Fig. 5P-R). Only when Bmp7 was added on day 6 were no Nkx2.1+/Th+ neurons detected (Fig. 5S).

The ability of Bmp7 to induce Nkx2.1 when added to day 5 cultures led us to examine whether Bmp7 signalling can act directly on differentiating or postmitotic DA neurons to induce Nkx2.1 in the neurons themselves. To address this, we examined when Th+ neurons become postmitotic in pHyp explants. Stage 5 or stage 7 pHyp explants were cultured and BrdU added to parallel cultures on successive days. When explants were incubated with BrdU on days 2 or 3, BrdU+/Th+ neurons were detected on day 7 (Fig. 5T, inset; not shown). By

contrast, when explants were incubated with BrdU on day 5, Th+ neurons were detected, but none of these co-labelled with BrdU (Fig. 5T). Despite the fact that Th+ neurons are differentiating or postmitotic by day 5, addition of Bmp7 to stage 5 pHyp explants on day 5 resulted in the appearance of Nkx2.1+/Th+ neurons on day 7 (Fig. 5U). Together, these data indicates that Bmp7 can act on differentiating or postmitotic DA neurons to induce hypothalamic regional identity.

Bmp7 induces Nkx2.1+/Shh+ cells in vivo in a Six3-dependent manner

We tested the requirement in vivo for Bmp7 for the induction of Nkx2.1+/Shh+ cells. Beads soaked with chordin were implanted adjacent to the prospective ventral tuberal hypothalamus of stage 5 embryos, and embryos developed until stage 15 (Fig. 6). In embryos exposed unilaterally to chordin, Nkx2.1+ expression was abolished from the neural tube ipsilateral to the bead (Fig. 6A,B), showing that Bmp7 is necessary for the induction of Nkx2.1+/Shh+ cells in vivo.

To determine whether Shh and Bmp7 can induce ectopic Nkx2.1+/Shh+ cells in vivo, beads soaked in Shh and Bmp7 were placed adjacent to the lateral anterior neural plate of stage 5 embryos, and embryos developed until stage 15. In control embryos, Nkx2.1+/Shh+ cells are restricted to the anterior ventral, and the lateral tuberal, hypothalamus, but are not found elsewhere (Fig. 1A, Fig. 6C,D). By contrast, in embryos exposed to Shh and Bmp7, ectopic Nkx2.1+/Shh+ cells are detected (Fig. 6D,D′). Beads soaked in either Bmp7 alone or Shh alone did not induce Nkx2.1+/Shh+ cells (data not shown).

Notably, in these experiments, ectopic induction of Nkx2.1+/Shh+ cells was confined to the dorsal telencephalon, the optic vesicles and the anterior pituitary (Fig. 6D; data not shown). These sites correlate with expression of the homeobox transcriptional repressor, Six3 (Bovolenta et al., 1998), which has previously been shown to govern Nkx2.1 expression (Lagutin et al., 2003). To examine whether Shh and Bmp7 induce hypothalamic cells in a Six3-dependent manner, we first examined the correlation in Six3 expression with the response of HH stage 5 LNP cells at different levels along the anteroposterior axis (Fig. 4F). aLNP explants that respond to Shh and Bmp7 by differentiating to a hypothalamic cell fate express Six3, whereas pLNP explants that do not differentiate into hypothalamic fates do not express Six3 (not shown).

To test directly whether Six3 acts as a competence factor for Shh and Bmp7 to induce hypothalamic cells, we electroporated a Six3 repressor expression construct, RD Six3, into the mesencephalon in ovo. Electroporated lateral neural tube cells were immediately explanted, and a subset examined to establish that exogenously introduced Six3 could be detected (Fig. 6E,F). The remaining explants were exposed in vitro to Shh and Bmp7. After a 7-day culture, we detected many Nkx2.1+/Th+ DA neurons in RD Six3-electroporated tissue (Fig. 6G). By contrast, in tissue electroporated either

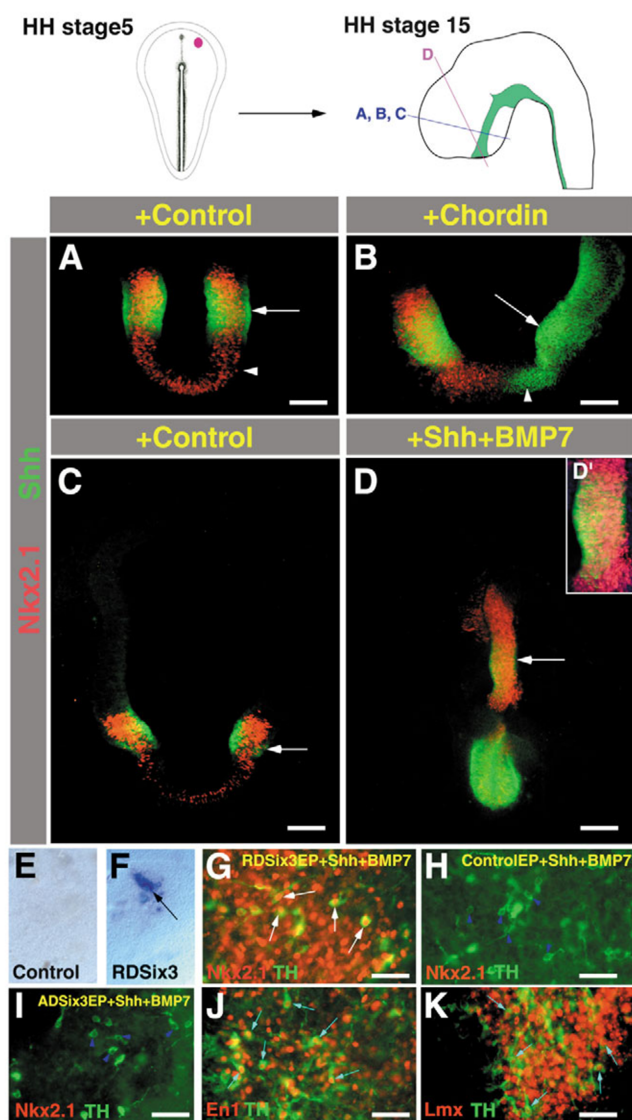

Fig. 6. Shh and Bmp7 are necessary and sufficient to induce Nkx2.1+/Shh+ cells and Nkx2.1+/Th+ neurons in a Six3-dependent manner in vivo. (A-D) Transverse section through tuberal hypothalamus (A-C) or anterior hypothalamus/dorsal telencephalon (D) of HH stage 15 embryos, immunolabelled to detect Nkx2.1 and Shh. (A) Control embryo showing Nkx2.1+/Shh+ cells in the lateral tuberal hypothalamus (arrow) and Nkx2.1+ ventral tuberal cells (arrowhead) after implantation of a control bead (bead has been displaced). (B) A chordin (Chd)-soaked bead implanted at stage 5, adjacent to the prechordal mesoderm/ventral tuberal hypothalamus. Nkx2.1 expression is abolished from lateral tuberal hypothalamic cells (arrow). Concomitantly, Shh expression is expanded (arrowhead in D). (C,D,D′) Implanting a Shh/Bmp7-soaked bead at stage 5 (schematic above: bead displaced laterally) results in ectopic Nkx2.1+/Shh+ cells in the dorsal telencephalon (arrow in D,D′). Ventral cells in the anterior hypothalamus also co-express Shh and Nkx2.1. (C) Normal expression of Nkx2.1+/Shh+ lateral hypothalamic progenitors (arrow). (E,F) Electroporation of the RD Six3 construct results in the ectopic expression of Six3 in mesencephalic lateral neural tube explants (F, arrow). No Six3 expression is detected in control (RFP) electroporated explants (E). (G,H) Nkx2.1+/Th+ cells are induced in RD Six3-electroporated lateral mesencephalic explants, exposed to Shh and Bmp7 (G, arrows), whereas mock-electroporated explants give rise only to Nkx2.1–/Th+ cells after exposure to Shh and Bmp7 (H, blue arrowheads). (I-K) In AD Six3-electroporated mesencephalic tissue, exposed to Shh and Bmp7, Th+ cells do not co-express Nkx2.1 (I, blue arrowheads) but co-express En1 and Lmx (J,K, light blue arrows). Scale bars: 75 μm in A,B; 150 μm in C,D; 40 μm in G-K.

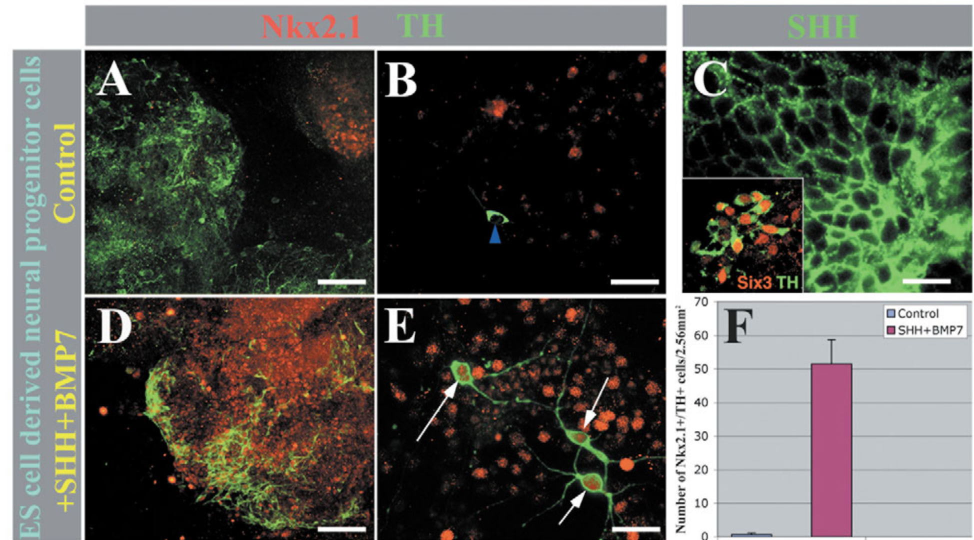

Fig. 7. Shh and Bmp7 direct mouse ES cell-derived neural progenitor cells to an Nkx2.1+/Th+ DA fate. (A-E) Mouse ES cell-derived neural progenitor cells, cultured without (A-C) or with (D,E) Shh and Bmp7. In control cultures, Th+, Six3+, Nkx2.1+ and Shh+ (A-C) cells are observed but no Nkx2.1+/Th+ neurons are detected. In the presence of Shh and Bmp7, many Nkx2.1+/Th+ cells (arrows in E) are found (D,E). (F) Quantification of Nkx2.1+/Th+ neurons after directed ES cell differentiation. The average number of Nkx2.1+/Th+ cells in cultures shows a significant increase after exposure to Shh and Bmp7 (control 0.66±0.42 cells/2.56mm^2; +Shh+Bmp7 51.44±7.27 cells/2.56mm^2, P<0.0001; unpaired t-test), whereas the average of total number of Th+ cells does not show any significant difference (P>0.05, unpaired t-test; data not shown). Scale bar: 150 μm in A,D; 40 μm in B,C,E.

with a control vector, or with an activator form of Six3 (AD Six3), and then exposed to Shh and Bmp7, Th+ cells appeared, but none co-expressed Nkx2.1 (Fig. 6H,I). These neurons co-expressed En1 and Lmx, suggestive of mesencephalic DA character (Fig. 6J,K). Together, these data suggest that Shh and Bmp7 induce hypothalamic fate in a Six3-dependent manner, and show that repressor activity is required for Six3 function.

Co-operation of Shh and Bmp7 can direct mouse ES cell-derived neural progenitor cells to a hypothalamic DA fate

Finally, we asked whether Shh and Bmp7 are sufficient to direct the differentiation of mouse embryonic stem (ES) cell-derived neural progenitor cells into Nkx2.1+/Th+ hypothalamic DA neurons. A mouse ES cell line (CCE) was enriched for nestin-positive neural progenitor cells (Okabe et al., 1996). During the ex vivo expansion stage, neural progenitor cells were exposed to Shh and Bmp7. In control cultures, Nkx2.1+ and Shh+ cells differentiated, but no cells co-expressed Nkx2.1 and Shh (data not shown). By contrast, Nkx2.1+/Shh+ progenitors were detected in cultures exposed to Shh and Bmp7 (data not shown). After a further 8 days, we detected Nkx2.1+, Th+ and Shh+ cells in control cultures. Many of the Th+ neurons co-expressed Six3; however, none co-expressed Nkx2.1 (Fig. 7A-C). By contrast, in cultures treated with Shh and Bmp7, many Nkx2.1+/Th+ DA neurons differentiated (Fig. 7D-F). Thus, by recapitulating the developmental genetic programme, it is possible to generate hypothalamic progenitors and differentiated hypothalamic DA neurons from ES cells-derived neural progenitor cells ex vivo.

Discussion

Shh and Bmp7 are sequentially required for the differentiation of hypothalamic DA neurons

Here, we have examined the nature, and mechanism of action, of signals required to induce and maintain hypothalamic DA neurons. Our studies show a sequential action of Shh and Bmp7.

At early stages, Shh ventralises prospective lateral tuberal hypothalamic progenitors, upregulating expression of Shh itself in Lim1+ progenitors and specifying DA neuronal generation. Incubation of prechordal mesoderm/pHyp explants with anti-Shh IgG results in a failure to specify Shh or Th; conversely, Shh is sufficient to downregulate Pax6 and Pax7, and to specify Shh+/Lim1+ progenitor cells and Th+ neurons in LNP explants. A number of lines of evidence suggest that induction of Shh and Th occurs through the Shh signalling pathway. Shh signalling components, including Gli1 and Gli2 are expressed in lateral tuberal hypothalamic progenitor cells (M.P., unpublished), and *Gli2*-null mice lack Shh expression in the lateral tuberal hypothalamus (Matise et al., 1998). Similarly, the Th promoter region has binding sites for Gli1/Gli2 (Schimmel et al., 1999) and Th+ DA neurons fail to differentiate in *Gli2*-null mice (Matise et al., 1998).

By contrast, late aspects of hypothalamic regional identity, evidenced by the upregulation of Nkx2.1 and Msx in cells that are specified as DA neurons, is imposed by Bmp signalling. Neither Shh nor Bmp7 is sufficient to induce Nkx2.1 in aLNP explants but addition of Shh and Bmp7 results in the robust induction of Nkx2.1+/Msx+ hypothalamic DA neurons. Conversely, addition of chordin to prechordal mesoderm/pHyp recombinates prevents prechordal mesoderm from inducing hypothalamic DA neurons.

A number of lines of evidence show that Bmp7 imposes hypothalamic identity on cells that are ventralised by Shh signalling. The transient exposure of progenitor cells first to Bmp7 and then to Shh results in the induction of Msx and Shh, with some progenitor cells co-expressing Msx/Shh. However, these cells do not differentiate into hypothalamic Nkx2.1+/Msx+ DA neurons. This argues against a model in which Bmp7 and Shh signalling function in parallel to induce distinct characteristics that, together, specify hypothalamic identity. Instead our data indicate that Bmp7 acts on cells that are ventralised by Shh to promote the hypothalamic regional

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identity of DA neurons. First, Nkx2.2 and Shh+/Lim1+ progenitor cells that require Shh signalling alone are specified before Nkx2.1+/Shh+/Lim1+ cells that require both Shh and Bmp7. Second, Bmp7 can induce the expression of Nkx2.1/Msx in either cells that are about to exit the cell cycle, or in postmitotic neurons that are committed to a DA neuronal fate. Although our experiments do not indicate whether the action of Bmp7 occurs exclusively at this stage, these results add to the growing body of evidence that signalling molecules that act early in embryogenesis to govern the developmental potential of neural progenitor cells can elicit regional changes in neural identity postmitotically (Livet et al., 2002; William et al., 2003; Sockanathan et al., 2003). Finally, in vivo, the onset of expression of Bmp7 in prechordal mesoderm occurs some hours after that of Shh (Dale et al., 1999; Vesque et al., 2000; Patten et al., 2003).

Our studies show that Shh signalling plays a dual role in the differentiation of hypothalamic DA neurons, controlling neurotransmitter phenotype and acting with Bmp7 to control regional character. This is distinct from the role of Shh signalling in the differentiation of midbrain DA neurons: analysis of Lmx1b-deficient mice reveals that two independent signalling pathways govern midbrain DA neuronal differentiation, a Shh-dependent pathway that controls neurotransmitter phenotype and a Shh-independent region-specific pathway (Sakurada et al., 1999; Smidt et al., 2000). Together, these results suggest that Shh controls pan-dopaminergic character, but that region-specific character is controlled through complex pathways that either cooperate with Shh, as shown here, or operate independently of Shh.

Co-operative signalling by Bmp7 and Shh

In the posterior CNS, Bmps at the dorsal epidermal ectoderm and roof plate oppose and constrain the expression and actions of Shh emanating from ventral tissues (Lee and Jessell, 1999; Liem et al., 2000; Patten and Placzek, 2002). By contrast, in this study we show that Msx/2, a read-out of active Bmp signalling, is expressed in the Shh-expressing lateral tuberal hypothalamus. This unusual expression of Msx within a Shh-expressing region suggests the existence of a molecular mechanism to prevent Shh downregulating in the presence of active Bmp signalling. How this mechanism operates remains unproven, but a likely transcriptional candidate is Nkx2.1. Our studies show that Nkx2.1 is induced by Bmp7 signalling, and show a correlation in Nkx2.1 expression and the maintenance of Shh expression. In support of this interpretation, *Nkx2.1*-null embryos lack Shh expression in the hypothalamus (Sussel et al., 1999). As our data shows that Shh expression in hypothalamic explants precedes that of Nkx2.1, these results together support a cell-autonomous role for Nkx2.1 in the maintenance of Shh expression. Further support for a role for HD factors in regulating Shh expression derives through a recent dissection of the Shh enhancer element, SFPE2, which reveals that a HD site is required for Shh transcription in the posterior spinal cord (Jeong and Epstein, 2003). Thus, our studies suggest that Bmp signalling may play a dual role in the hypothalamus, inducing regional identity and maintaining Shh expression.

Shh and Bmp7 act with Six3 to establish hypothalamic identity

Our data suggest that Shh and Bmp7 can suffice to promote hypothalamic DA neurons, but only in tissue that has acquired an earlier competence. Blockade of Bmp7 signalling abolishes the ability of prechordal mesoderm to induce Nkx2.1+/Th+ neurons in pHyp explants. Under these conditions, the prechordal mesoderm induces Th+ neurons, but these do not adopt characteristics of DA neurons from other regions. Such Th+ neurons do not default to A13 thalamic, A8-A10 mesencephalic DA fates or to a hindbrain noradrenergic fate. Instead, our data suggest that these display the characteristics of immature hypothalamic neurons that have initiated an incomplete differentiation programme, as evidenced through their continued expression of Lim1 and Six3.

Previous studies in mice have suggested that Six3 not only marks hypothalamic cells (Bovolenta et al., 1998), but is required for hypothalamic cell differentiation: Nkx2.1 expression is absent in the hypothalamus of Six3-null mice (Lagutin et al., 2003). Our studies support and extend these findings, showing that Shh and Bmp7 induce hypothalamic neuronal identity only in the presence of Six3 repressor activity. First, the ability of Shh and Bmp7 to induce Nkx2.1+/Shh+ cells and Nkx2.1+/Th+ neurons correlates, in vivo and in vitro, with Six3 expression. Second, ES cell-derived neural cells that express Shh and that can respond to Bmp7 by upregulating Nkx2.1 in Th+ neurons also express Six3. Finally, exposure to Shh and Bmp7 of lateral mesencephalic tissue electroporated with a Six3-repressor, but not a Six3-activator, construct results in the induction of Nkx2.1+ DA neurons.

Six3 directly binds to the Wnt1 promoter to repress its expression (Lagutin et al., 2003), suggesting that a key function of Six3 is to abolish expression of Wnt1. Intriguingly, recent studies in zebrafish have provided evidence that levels of Wnt activity are crucial to the acquisition of hypothalamic identity, suggesting that lower levels of Wnt activity are required to promote hypothalamic fates versus more posterior fates (Kapsimali et al., 2004). Together, these analyses suggest that Six3 represses Wnt, and that ambient levels of Wnt activity

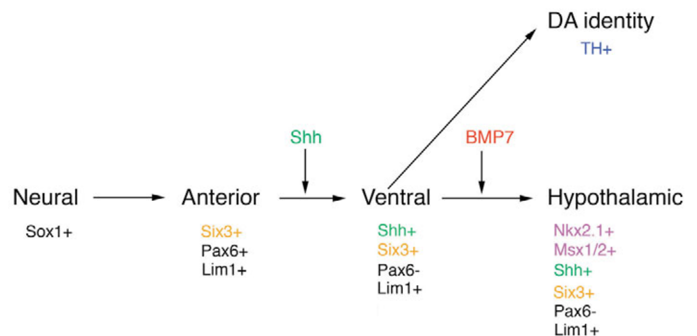

Fig. 8. Model for the differentiation of hypothalamic DA neurons. Our data suggest a model for the specification of hypothalamic DA neurons. An early patterning step promotes expression of Six3+ cells, potentially affecting ambient levels of Wnt activity (Lagutin et al., 2003; Kapsimali et al., 2004). Shh acts to ventralise Six3+ cells, downregulating Pax6 and upregulating Shh. Cells at this point are specified to a forebrain-like/incomplete hypothalamic DA neurotransmitter identity, but do not exhibit definitive aspects of hypothalamic regional identity. Subsequently, Bmp7 acts to induce hypothalamic regional identity on Shh+ Six3+ cells, upregulating Nkx2.1 and Msx1/2.

promote an early hypothalamic competence/fate that dictates the ability of Shh and Bmp7 to govern later aspects of hypothalamic identity (Fig. 8).

Finally, although our studies are restricted to the analysis of hypothalamic DA neurons, they may have general implications for neuronal differentiation in the hypothalamus. In posterior regions of the neural tube, the integrated action of multiple signalling pathways specifies neuronal identity. There, the spatial and temporal integration of signals, including Shh, Bmps and retinoids, elicit particular neuronal fates. A growing body of evidence shows that, in the posterior neural tube, secreted signals can regulate the transcriptional profile, and hence developmental potential, both of neural progenitor cells and of postmitotic neurons. Our studies reveal that in the hypothalamus, Shh and Bmp7 are both expressed ventrally, but sequentially. Our studies show that the sequential signalling of Shh and Bmp7 can specify neurons with hypothalamic DA character, and show that Bmp7 can induce hypothalamic regional identities in late differentiating or postmitotic cells. This raises the possibility that other hypothalamic neuronal identities may be similarly specified by the sequential action of Shh and Bmp7.

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