Inhibition of Wnt/Axin/β-catenin pathway activity promotes ventral CNS midline tissue to adopt hypothalamic rather than floorplate identity

Marika Kapsimali[1], Luca Caneparo[2], Corinne Houart[2] and Stephen W. Wilson[1,*]

[1]Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK
[2]MRC Centre for Developmental Neurobiology, New Hunt's House, King's College London, London SE1 9RT, UK
*Author for correspondence (e-mail: s.wilson@ucl.ac.uk)

Accepted 17 September 2004

Development 131, 5923-5933
Published by The Company of Biologists 2004
doi:10.1242/dev.01453

Summary

Ventral midline cells in the neural tube form floorplate throughout most of the central nervous system (CNS) but in the anterior forebrain, they differentiate with hypothalamic identity. The signalling pathways responsible for subdivision of midline neural tissue into hypothalamic and floorplate domains are uncertain, and in this study, we have explored the role of the Wnt/Axin/β-catenin pathway in this process. This pathway has been implicated in anteroposterior regionalisation of the dorsal neural tube but its role in patterning ventral midline tissue has not been rigorously assessed.

We find that *masterblind* zebrafish embryos that carry a mutation in Axin1, an intracellular negative regulator of Wnt pathway activity, show an expansion of prospective floorplate coupled with a reduction of prospective hypothalamic tissue. Complementing this observation, transplantation of cells overexpressing *axin1* into the prospective floorplate leads to induction of hypothalamic gene expression and suppression of floorplate marker gene expression. Axin1 is more efficient at inducing hypothalamic markers than several other Wnt pathway antagonists, and we present data suggesting that this may be due to an ability to promote Nodal signalling in addition to suppressing Wnt activity. Indeed, extracellular Wnt antagonists can promote hypothalamic gene expression when co-expressed with a modified form of Madh2 that activates Nodal signalling. These results suggest that Nodal signalling promotes the ability of cells to incorporate into ventral midline tissue, and within this tissue, antagonism of Wnt signalling promotes the acquisition of hypothalamic identity. Wnt signalling also affects patterning within the hypothalamus, suggesting that this pathway is involved in both the initial anteroposterior subdivision of ventral CNS midline fates and in the subsequent regionalisation of the hypothalamus. We suggest that by regulating the response of midline cells to signals that induce ventral fates, Axin1 and other modulators of Wnt pathway activity provide a mechanism by which cells can integrate dorsoventral and anteroposterior patterning information.

Key words: Axin, Floorplate, Hypothalamus, Nodal, Wnt, Zebrafish, *mbl*

Introduction

Ventral midline central nervous system (CNS) cells originate in proximity to the organiser from where they extend along the anteroposterior (AP) axis of the forming neural tube to generate hypothalamic fates rostrally and floorplate caudally (Dale et al., 1999; Mathieu et al., 2002; Patten et al., 2003; Varga et al., 1999). The movements and signalling activity of axial midline neural tissue (and underlying axial mesendoderm) affect the subsequent development of the entire ventral CNS (Jessell, 2000; Wilson and Houart, 2004). Although hypothalamic and rostral floorplate cells originate from the same region of the embryo and share molecular characteristics at early developmental stages (Dale et al., 1997; Dale et al., 1999), specific regional character becomes evident as these cells extend along the AP axis of the forming CNS. By the end of gastrulation, the most rostral diencephalic ventral midline cells lose expression of genes specific to floorplate identity and acquire hypothalamic character (Barth and Wilson, 1995; Dale et al., 1997; Dale et al., 1999; Mathieu et al., 2002; Rohr et al., 2001). Thus, in all vertebrates, two distinct domains can be distinguished in the ventral CNS: rostral is hypothalamic tissue which contains preoptic (anterior) and infundibular (posterior) regions which primarily have neuroendocrine and autonomic functions; and caudal is the floorplate which is flanked by motorneurons and ventral interneurons. The eventual fate of floorplate tissue is uncertain but during development, this structure plays crucial roles in the induction and/or patterning of neurons, glia and axons (Briscoe and Ericson, 2001; Ruiz i Altaba et al., 2003; Strähle et al., 2004). The rostral limit of the floorplate (and caudal limit of the hypothalamus) is usually considered to be located in the diencephalon, ventral to the zona limitans intrathalamica (Rubenstein et al., 1998; Wilson and Houart, 2004).

The different origin and behaviour of ventral midline cells compared to neural plate tissue destined to form more dorsal CNS structures raises the question of whether the same

signalling pathways establish AP pattern in these two CNS domains. Indeed, in fish, at stages when the prospective alar neural plate is receiving signals that influence its anterior character (see Erter et al., 2001; Houart et al., 2002), prospective hypothalamic cells are still located caudal to the eye field (Mathieu et al., 2002; Varga et al., 1999; Woo and Fraser, 1995) and have yet to reach their final destination, the ventral anterior tip of the forming neural tube. Thus, it is unclear if signals that promote anterior identity in the lateral neural plate/dorsal neural tube, also establish hypothalamic (anterior) identity in ventral CNS midline tissue.

The signalling pathways responsible for subdivision of the ventral CNS into hypothalamic and floorplate domains are uncertain (reviewed by Wilson and Houart, 2004). Nodal and Hedgehog (Hh) signals have essential roles in the induction of the ventral CNS, but both pathways influence the entire axis and there is little evidence to suggest involvement in AP regional patterning. Nodal and Hh ligands are expressed in the organiser and subsequently in the axial mesendoderm and axial neuroectoderm, and these signals act in a parallel or cooperative manner to induce the entire ventral midline and adjacent CNS tissue (reviewed by Ruiz i Altaba et al., 2003; Strähle et al., 2004). For example, in zebrafish, the Nodal-related signal Cyclops and its downstream effector Madh2 can induce *shh* expression in the neuroectoderm and promote floorplate fate (Muller et al., 2000; Tian et al., 2003). In addition, Nodal signalling is cell-autonomously required for the establishment of posterior-ventral (PV) hypothalamus and acts indirectly (through specification of the prechordal plate and PV hypothalamus) to promote dorsal-anterior hypothalamic fate (Mathieu et al., 2002; Rohr et al., 2001). Similarly, mutations affecting Hh signalling disrupt both hypothalamic and floorplate specification (e.g. Chen et al., 2001; Chiang et al., 1996; Rohr et al., 2001; Varga et al., 2001).

Experimental evidence from the chick implicates Bmp signalling in the AP regionalisation of the ventral neural tube. The Bmp antagonist Chordin probably generates a permissive environment for Shh-mediated induction of the floorplate in regions of low Bmp signalling activity (Patten and Placzek, 2002) whereas Bmp7 from the prechordal mesendoderm acting together with Shh is proposed to promote hypothalamic/rostral ventral midline identity (Dale et al., 1997; Dale et al., 1999). However, in zebrafish, Bmp signalling influences prospective dorsoventral (DV) rather than AP patterning of the rostral neural plate (Barth et al., 1999; Hammerschmidt et al., 2003) and abrogating Bmp activity has little effect upon the initial specification and regional subdivision of midline neural tissue into hypothalamic and floorplate domains (Barth et al., 1999). These results do not exclude the possibility that Bmps play a role in AP patterning of the ventral CNS midline of zebrafish, but do raise the possibility that other signalling pathways may have more critical roles in the allocation of hypothalamic versus floorplate fates.

The Wnt/Axin/β-catenin signalling pathway is a candidate to regulate AP patterning in the ventral CNS midline given its role in AP regionalisation of other domains of the neural plate. The activity of various Wnt agonists and antagonists is thought to generate graded Wnt signalling activity (high caudally and low rostrally), which contributes to the establishment of early AP subdivisions of the neural plate (Kiecker and Niehrs, 2001a; Yamaguchi, 2001). For instance, abrogation of activity of the Wnt pathway transcriptional repressors Tcf3/Headless and Tcf3b results in the loss of anterior CNS fates (Dorsky et al., 2003; Kim et al., 2000). Conversely, abrogation of Wnt8 activity results in enlargement of the forebrain and reduction or absence of more caudal neural tissue (Erter et al., 2001; Lekven et al., 2001; Nordstrom et al., 2002).

Subsequent to the initial regionalisation of the neural plate, Wnt/β-catenin signalling has additional roles in the refinement of AP patterning within discrete domains of the CNS. For instance, within the dorsal forebrain, *masterblind/axin1* mutant (*mbl*) zebrafish embryos show a reduction or absence of telencephalon and eyes and expansion of dorsal diencephalic fates (Heisenberg et al., 1996; Masai et al., 1997). Axin1 is a cytoskeletal scaffolding protein that participates in the assembly of kinases responsible for β-catenin degradation (Dajani et al., 2003) and so it is likely that the forebrain phenotype of *mbl* embryos is due to overactivation of Wnt signalling in the anterior neural plate (Heisenberg et al., 2001; Houart et al., 2002; Van de Water et al., 2001). Supporting this interpretation, activity of the Secreted Frizzled Related Protein Tlc emanating from the anterior border of the neural plate (ANB) promotes telencephalic identity through local inhibition of Wnt signalling (Houart et al., 2002). Similar roles for Wnts and Wnt antagonists in the regional patterning of forebrain derivatives are proposed in other species (Braun et al., 2003; Gunhaga et al., 2003; Lagutin et al., 2003). To date, however, most analyses have focused upon the role of Wnts and Wnt antagonists in the AP regionalisation of the dorsal CNS and the possibility that this signalling pathway influences AP identity of ventral CNS structures has not been thoroughly investigated.

In this study, we have examined the ability of Wnt/Axin/β-catenin signalling to influence AP identity of ventral CNS midline tissue in developing zebrafish embryos. We find that the abrogation of Axin1 activity in *mbl* embryos results in expansion of floorplate at the expense of hypothalamic tissue. Conversely, exogenous Axin1 is able to suppress floorplate and induce hypothalamic markers in ventral CNS midline tissue of the midbrain and hindbrain. These results suggest that hypothalamic identity is promoted by suppression of Wnt signalling. We also explore the possibility that activation of Nodal signalling combined with suppression of Wnt signalling promotes expression of hypothalamic markers. Finally, mutant analyses and mis-expression studies suggest that within the hypothalamus, the levels of Wnt/Axin1/β-catenin signalling activity contribute to the AP regionalisation of this structure.

Materials and methods

Fish strains

Embryos were obtained from natural spawning of wild-type (tue or tupTL) or *axin1*^{tm213} (Heisenberg et al., 2001) zebrafish lines. At stages prior to exhibiting obvious morphological defects, *mbl* embryos were identified by the presence of consistent phenotypes in 25% of embryos from crosses of two heterozygous parents.

Microinjection and transplantation experiments

Synthetic mRNAs were transcribed in vitro using the mMessage mMachineTM transcription kit (Ambion). For transplantation experiments, donor embryos injected at the 2-4-cell stage with *gfp* or nuclear *gfp* (100 pg) RNA as lineage tracer and test RNA: zebrafish *axin1* (200-300 pg) (Heisenberg et al., 2001), *dkk1* (150 pg) (Shinya et al., 2000), *wnt8b* (100-200 pg) (Kelly et al., 1995), human *lrp6Δc*

(400 pg) (Tamai et al., 2000), zebrafish *hdl/tcf3a/tcf7/1a* (100-120 pg) (Dorsky et al., 2003; Kim et al., 2000), *tlc* (200-400 pg) (Houart et al., 2002), *sfrp3/frzb1* (500-700 pg) (Agathon et al., 2003). All reagents were tested for activity by assessing phenotypes following widespread overexpression. As expected, overexpression of *wnt8b* posteriorised embryos whereas injection of *axin1* at the 2-4-cell stage gave bigger eyes (80%) or ventralised (20%) phenotypes (n=510) consistent with suppressed Wnt activity. Other Wnt antagonists gave similar overexpression phenotypes.

RNA encoding constitutively active Madh2 (Madh2CA/Smad2CA) (Muller et al., 2000) was injected at 10-100 pg in donor embryos. Cells from donors embryos injected with 20-40pg *madh2CA* RNA integrated in the floorplate/PV hypothalamus and/or the notochord (50%), whereas those with higher amounts of *madh2CA* (40-80 pg) formed aggregates in the mesendoderm of the host embryos (80% of the transplanted embryos) or died. As a result, 25 pg of *madh2CA* RNA was co-injected with other RNAs in order to facilitate integration of the transplanted cells in the ventral neural tube.

All test RNA-expressing cells were taken from the animal pole epiblast of late blastula to 40% epiboly embryos and transplanted to ectoderm adjacent to the shield of mid-gastrula (50-65% epiboly)-stage hosts. At this stage, donor epiblast cells are not committed to specific fates and readily incorporate into most tissue/cell types in recipient hosts. For transplantation, donor and host embryos were mounted in 3% or 0.5% methyl-cellulose in embryo medium, and viewed with a fixed stage Nikon Optiphot or a Leica fluo microscope. Cells were removed from donor embryos by suction using a mineral oil-filled glass micropipette attached to a 50 µl Hamilton syringe (Houart et al., 1998) and gently aspirated into recipient hosts.

In situ hybridization, immunohistochemistry and histology

In situ hybridization was carried out as described previously (Hauptmann and Gerster, 1994). Probes used were: *dlx3* (Akimenko et al., 1994), *emx2* (Morita et al., 1995), *foxa1/fkd7* and *foxb1.2/fkd3* (Odenthal and Nusslein-Volhard, 1998), *foxa2/axial* (Strahle et al., 1993), *hgg1* (Thisse et al., 1994), *nk2.1a* (Rohr et al., 2001), *ntl* (Schulte-Merker et al., 1994), *pax2.1/pax2a* (Krauss et al., 1991), *rx3* (Chuang et al., 1999), *shh* (Krauss et al., 1993). GFP was revealed with a rabbit polyclonal antibody (AMS Biotechnology) at 1:1000 dilution and diaminobenzidine (DAB) staining. Some stained embryos were embedded in JB4 resin (Polyscience) and sectioned with a Leica microtome (10-14 µm).

Results

Abrogation of wild-type Axin1 function leads to altered expression of midline markers in the neural plate

The scaffolding protein Axin1 is a potent intracellular inhibitor of Wnt/β-catenin signalling (Jones and Bejsovek, 2003), and we reasoned that if Wnt signalling plays a role in AP regionalisation of the CNS ventral midline, then abrogation of Axin1 function should disrupt AP patterning of this tissue. We therefore analysed gene expression along the ventral CNS midline of *mbl* embryos that carry a point mutation in the GSK3β-binding domain of Mbl/Axin1 (Heisenberg et al., 2001). *mbl* embryos have excessive Wnt signalling activity in the neural plate and although forebrain patterning is disrupted,

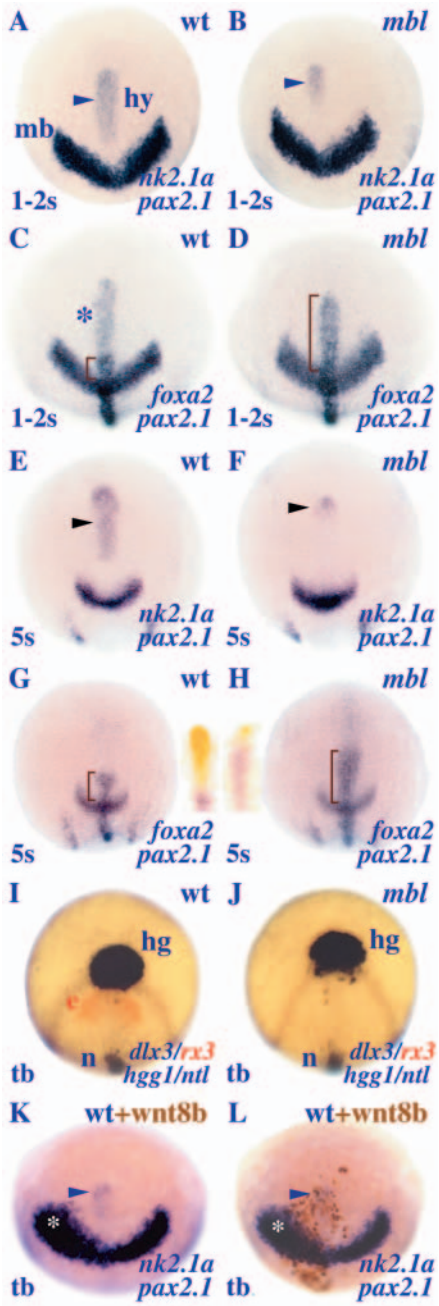

Fig. 1. *mbl* embryos show altered AP regionalisation of the ventral midline of the neural plate. Dorsal views of wild-type and *mbl* embryos with anterior on the top. In this and other figures, where indicated, stage is shown bottom left, genes analysed by in situ hybridization are shown bottom right and the experimental procedure is top right. Arrowheads point to hypothalamic (*nk2.1a*) gene expression. *pax2.1* is used as a marker of the prospective midbrain in A-H and K-L. The asterisk indicates the prospective hypothalamic domain of reduced *foxa2* expression in a wild-type embryo (C) and brackets indicate the AP length of the prospective floorplate domain rostral to the midbrain in wild-type (C,G) and *mbl* (D,H) embryos. The inset panel between G and H shows *nk2.1a* expression in the prospective hypothalamus in yellow and *foxa2* in the prospective floorplate in blue. The margin of the neural plate, the hatching gland, the prospective notochord and eye field (red) are shown by *dlx3, hgg1, ntl* and *rx3* expression domains in I,J. The *mbl* embryo lacks *rx3* expression in the eye field. (K-L) Transplanted cells overexpressing *wnt8b* (brown) suppress *nk2.1a* expression (blue, arrowhead) and expand rostrally *pax2.1* expression (white asterisk) in a wild-type host embryo. Abbreviations: tb, bud stage, e, prospective eye, hg, *hgg1* expression in the prechordal plate, hy, hypothalamus, mb, midbrain, n, prospective notochord; s, somite stage; wt, wild type.

mutants show no evidence of cyclopia or other ventral fusions suggesting that ventral CNS tissue is specified and extends normally along the entire axis.

nk2.1a is the earliest specific hypothalamic marker in zebrafish with expression first detected as a stripe of cells in the medial anterior neural plate of wild-type embryos by the end of epiboly (Rohr et al., 2001) (Fig. 1A). Strikingly, in the neural plate of *mbl* embryos, *nk2.1a* expression is reduced to about half its normal AP length, suggesting that presumptive hypothalamic tissue is reduced compared to wild-type siblings (Fig. 1A,B,E,F). As neural plate size is not obviously affected in mutant embryos (Heisenberg et al., 2001) (Fig. 1I,J and data not shown), this suggests that neural plate midline tissue may alter its AP regional character in response to enhanced Wnt signalling. Supporting the idea that enhanced Wnt signalling antagonises hypothalamic development, local expression of exogenous Wnt8b suppressed expression of *nk2.1a* (Fig. 1K,L; *n*=13/18, and data not shown).

To assess whether the reduction in prospective hypothalamic tissue is correlated with an increase in prospective floorplate tissue, we analysed expression of *foxa2* in *mbl* embryos. *foxa2* is initially expressed throughout the ventral axial neural tissue including both the prospective hypothalamus and prospective

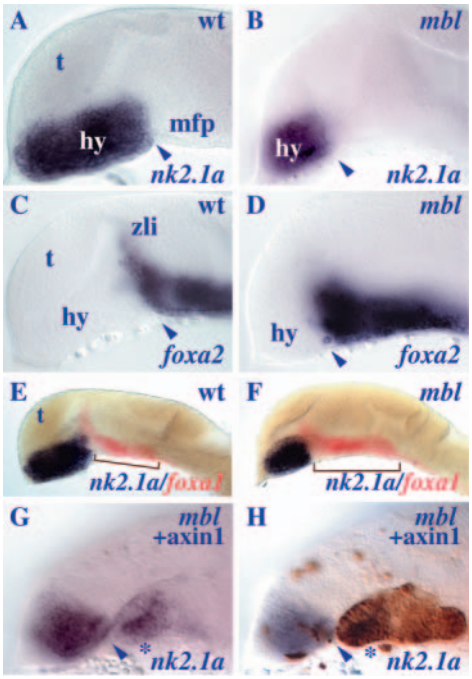

Fig. 2. *mbl* embryos show a reduction in the size of the hypothalamus coupled with an expansion in posterior diencephalic/midbrain floorplate. Lateral views of brains of embryos between 25 and 31 somites with anterior to the left. (A-F) The *mbl* embryos (B,D,F) show reduced hypothalamic tissue and rostral expansion of floorplate-expressed genes compared to the wild-type embryos (A,C,E). Arrowheads point at the rostral limit of floorplate marker expression (A-D). Brackets show the AP expansion of diencephalic/midbrain *foxa1* expression (red) in the *mbl* (F) compared to the wild-type embryo (E). (G-H) Transplanted cells overexpressing *axin1* (brown) restore *nk2.1a* expression (blue, asterisk) in the rostral ventral neural tube of an *mbl* embryo. Abbreviations: hy, hypothalamus; mfp, medial floorplate; t, telencephalon; zli, zona limitans intrathalamica; wt, wild type.

medial and lateral components of the floorplate (Barth and Wilson, 1995; Odenthal et al., 2000), but expression is progressively downregulated in the prospective hypothalamus (compare *foxa2* expression in Fig. 1C,G with *nk2.1a* expression in Fig. 1A,E) (Barth and Wilson, 1995; Dale et al., 1997). Thus from about 5-somite stages, *nk2.1a* and *foxa2* show complementary expression in the ventral neural tube with *nk2.1a* being restricted to the prospective hypothalamus and the anterior limit of *foxa2* expression coinciding with the anterior edge of the floorplate (Fig. 1G inset, Fig. 2A,C).

In *mbl* embryos, the reduction of *nk2.1a* expression is accompanied by persistent rostral expression of *foxa2*. Thus at 1-2 somites, a midline domain of intense *foxa2* expression extends much further rostrally to the prospective midbrain than in wild-type embryos (Fig. 1C,D). By 5-somite and later stages, complementarity of expression between *nk2.1a* and *foxa2* is observed but *foxa2* expression extends considerably further rostrally within the neural plate of *mbl* compared to wild-type embryos (Fig. 1E-H).

The development of axial neural tissue is dependent upon signals from underlying mesendoderm (Camus et al., 2000; Dale et al., 1997; Kiecker and Niehrs, 2001b) and so changes in the AP regionalisation of the neural plate could be due to altered Axin1 activity in the neural plate itself or in the underlying axial tissues. We did not observe any major differences in expression of axial mesendodermal markers in *mbl* embryos (see Heisenberg et al., 2001) and the regionalisation of this tissue into prechordal plate and prospective notochord domains appears to be unaffected (Fig. 1I,J and data not shown). Thus the AP patterning phenotype is predominantly restricted to the ectoderm, supporting the idea that the primary requirement for Axin1 in CNS patterning is within the neural plate.

Abrogation of wild-type Axin1 function leads to expansion of floorplate at the expense of hypothalamic tissue

In order to assess whether the early changes in neural plate expression domains are reflected in altered patterning of the ventral CNS at later stages, we analysed the ventral neural tube of *mbl* embryos at one day of development. Reduction of *nk2.1a* expression and rostral expansion of *foxa2* and *foxa1* expression is preserved, confirming that hypothalamic tissue is reduced and floorplate tissue is expanded in the ventral neural tube of *mbl* embryos (Fig. 2A-F). The extent of reduction of hypothalamic tissue was variable from relatively mild phenotypes (Fig. 2F), to an almost complete loss of tissue (not shown). Together these results show that the reduced activity of Axin1 in *mbl* embryos results in expanded floorplate development in the anterior ventral midline of the neuroectoderm at the expense of hypothalamic fates.

Axin1 is sufficient to promote hypothalamic and suppress floorplate markers in the ventral neural tube

To further investigate the hypothesis that Axin1 activity within ventral CNS cells promotes hypothalamic identity, we transplanted wild-type epiblast cells or epiblast cells overexpressing *axin1* in the ectoderm adjacent to the organiser of *mbl* embryos at 50-65% epiboly. From within

this domain, precursors of both anterior floorplate and hypothalamus extend rostrally along the AP axis of the ventral CNS midline during gastrulation (Mathieu et al., 2002; Woo and Fraser, 1995). Both transplanted wild-type and *axin1* overexpressing cells integrated into the hypothalamus and floorplate of *mbl* embryos (Fig. 2G-H and data not shown; *n*=23/23 and *n*=29/29 *mbl* embryos receiving transplants of *gfp* and *axin1+gfp* overexpressing cells respectively). Furthermore, when wild-type or *axin1* overexpressing transplanted cells incorporated into the rostral ventral neural tube, they expressed *nk2.1a* (*n*=9/11 and 17/18 respectively) and these *mbl* embryos often had expanded hypothalamic gene expression compared to the *mbl* mutants that did not receive transplants (*n*=6/11 and *n*=14/18; compare Fig. 2G,H with 2B). These results show that restoration of Axin1 activity in the anterior ventral neural tube of *mbl* embryos is sufficient to restore hypothalamic marker gene expression.

Given the reduction of hypothalamic tissue and the restoration of hypothalamic gene expression by exogenous Axin1 in *mbl* embryos, we next asked if Axin1 is sufficient to promote anterior/hypothalamic identity at the expense of floorplate fate in the ventral CNS midline. To address this question, we transplanted epiblast cells overexpressing *axin1* in the prospective floorplate of 60-65% epiboly stage wild-type embryos. Control transplants of GFP-expressing cells integrated into the ventral neuroepithelium but did not affect the expression of floorplate or hypothalamic markers (Fig. 3A,B and data not shown). In contrast, in the majority of cases (*n*=58/83 embryos), many transplanted *axin1+* cells ectopically expressed *nk2.1a* in the posterior ventral neural tube where floorplate, motor neurons or ventral interneurons should form (Fig. 3C,D). The ectopic *nk2.1a* expression in *axin1* overexpressing transplants was observed in grafts that incorporated in the ventral midbrain and hindbrain but not in the ventral spinal cord nor in lateral or dorsal regions of the neural tube (Fig. 3D). Ectopic *nk2.1a* expression was also observed in transplanted cells overexpressing *axin1* in the midbrain floorplate of a minority of *mbl* embryos (*n*=4/11, data not shown). Unlike the hypothalamic marker *nk2.1a*, expression of the dorsal anterior forebrain marker *foxg1* was unaffected in *axin1*-expressing transplants in the ventral CNS even when the grafts extended laterally to the floorplate (*n*=12/12, Fig. 3J,K).

To assess whether the ectopic induction of hypothalamic markers was correlated with a loss of endogenous ventral neural tube markers in the Axin1 transplants, we examined the expression of genes endogenous to the floorplate and ventral neuroepithelium. In the majority of embryos, expression of *foxa1* and *foxa2*, which label the medial floorplate and medial plus lateral floorplate respectively (Odenthal et al., 2000), was absent in many cells overexpressing *axin1* that incorporated into the prospective floorplate of wild-type embryos (Fig. 3E-I, *n*=11/20 and *n*=21/26 showing obvious gaps in *foxa1* and *foxa2* expression respectively). Enhanced Axin1 activity in the ventral CNS midline therefore results in the suppression of floorplate markers. Altogether these results reveal that Axin1 activity promotes anterior (hypothalamic) at the expense of posterior (floorplate) marker gene expression in cells positioned within the ventral CNS midline.

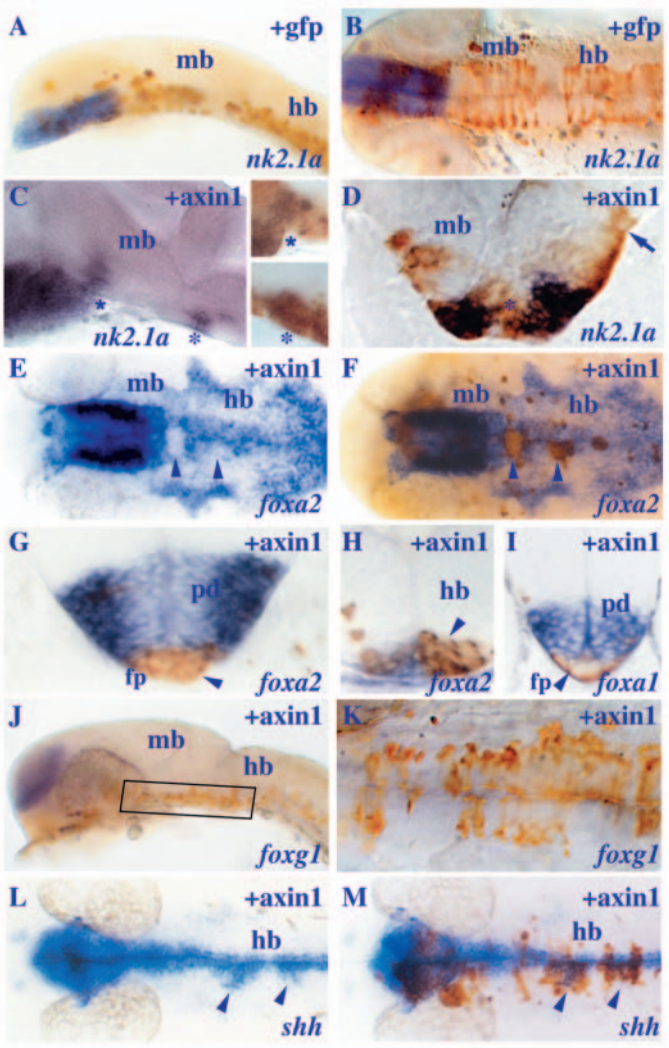

Fig. 3. Exogenous Axin1 promotes hypothalamic and suppresses floorplate marker gene expression. Lateral (A,C,J) and dorsal (B,E,F,K,L,M) views and 14 μm transverse sections (D,G-I) of brains of embryos between 22 and 28 somites in which cells (brown) expressing various constructs (top right) were transplanted into the prospective hypothalamus/anterior floorplate of 60-65% epiboly stage hosts. Sections are at the level of the ventral midbrain (D,G,I) and the hindbrain (H). (A-B) *gfp*-expressing cells (brown) integrate in the ventral midbrain and hindbrain but do not express *nk2.1a*. (C-D) Cells overexpressing *axin1* (brown, asterisks in the insets) in the midbrain and hindbrain of two different embryos ectopically express *nk2.1a* (blue, asterisks). The arrow points to the absence of ectopic *nk2.1a* expression in more dorsal cells overexpressing *axin1* (brown) in the midbrain. (E-I) Floorplate marker expression (blue) is downregulated in *axin1* overexpressing cells (brown, arrowheads). The expression of *foxa2* is shown in the same embryo after in situ hybridization (E) and after immunohistochemistry to reveal the *axin1* overexpressing cells (brown, F) and in transverse sections (G,H). The arrowheads point to sites of downregulation of *foxa2* or *foxa1* in the transplanted cells in the midline of the midbrain (E-G,I) and hindbrain (H). (J-K) *axin1* overexpressing cells do not ectopically express *foxg1* in the posterior ventral neural tube of the embryo (J) and in higher magnification (K). (L-M) Exogenous *axin1* expands *shh* expression (arrowheads) lateral to the CNS midline shown after in situ hybridization (L) and after immunohistochemistry to reveal the transplanted cells (M). Abbreviations: mb, midbrain; hb, hindbrain; fp, floorplate; pd, posterior diencephalon.

Intracellular inhibition of Wnt/β-catenin signalling promotes hypothalamic identity

Axin1 inhibits canonical Wnt signalling and so our favoured hypothesis to explain the results described above is that suppression of Wnt signalling promotes hypothalamic identity. To test this idea, we assessed whether other Wnt antagonists could promote hypothalamic identity. Indeed, cells overexpressing *hdl/tcf3a,* a potent intracellular repressor of Wnt canonical/β-catenin activity (Kim et al., 2000) often expressed ectopic *nk2.1a* expression when they incorporated into the ventral CNS (Fig. 4A,B, *n*=7/12). Surprisingly, however, when cells overexpressing the secreted Wnt/Axin/β-catenin antagonists Dkk1 (Glinka et al., 1998; Niehrs et al., 2001), Tlc (Houart et al., 2002) or Frzb1 (Agathon et al., 2003) were transplanted in the prospective floorplate of 60-65% epiboly stage hosts, *nk2.1a* was not ectopically expressed (*n*=21/21, *n*=9/9, *n*=25/25; Fig. 4D and data not shown).

Altogether these results show that intracellular antagonists of Wnt/β-catenin signalling promote hypothalamic gene expression within ventral CNS midline tissue. What then, might account for the differences in the ability of intracellular and extracellular Wnt antagonists to promote hypothalamic gene expression in the prospective floorplate?

Activation of Nodal signalling promotes incorporation of cells into the CNS midline

Although we always transplanted cells to the same region (the prospective hypothalamus/anterior floorplate) of host embryos, we noticed a difference in the distribution of the cells expressing different reagents one day later. *axin1*+ cells had a high incidence of integrating into medial regions of the floorplate rather than the adjacent neuroepithelium (*n*=28/83 predominantly in the medial floorplate (e.g. Fig. 4C); *n*=39/83 in the medial floorplate and adjacent cells (e.g. Fig. 3C lower inset and Fig. 3F,G,H) and *n*=16/83 dispersed more widely in the ventral CNS (e.g. Fig. 3C upper inset and Fig. 3D,K). In contrast, *gfp*+, *dkk1*+ and *sfrp*+ cells tended to integrate in the neuroepithelium of the ventral neural tube lateral to the most medial domain of the floorplate of the host embryos (Fig. 3B, Fig. 4D and data not shown, *n*=31/31 for *dkk1* and *n*=34/34 for *sfrp* genes, respectively). As Nodal signalling is essential for cells to form the medial floorplate and posterior/ventral hypothalamus (Schier, 2003), we hypothesised that the exogenous Axin1 in the transplanted cells may facilitate Nodal activity. Indeed, in certain assays, Axin1 can function as an adapter for Smad/Madh proteins to facilitate Nodal signalling (Furuhashi et al., 2001).

To explore the possibility that exogenous Axin1 influences Nodal signalling, we analysed the behaviour of ventral CNS cells with activated Nodal signalling. To do this we transplanted cells expressing a constitutively active form of the Nodal pathway effector Madh2 (Madh2CA) (Muller et al., 2000) into the prospective hypothalamus/anterior floorplate. From this position, cells expressing a high level of *madh2CA* RNA (40-80 pg) moved out of the ectoderm and predominantly incorporated into axial mesendoderm (data not shown). However, cells expressing less *madh2CA* (20-40 pg), behaved in a similar way to Axin1-expressing cells, preferentially incorporating into the most medial floorplate (Fig. 4E and compare to 4C). Furthermore, expression of low levels of

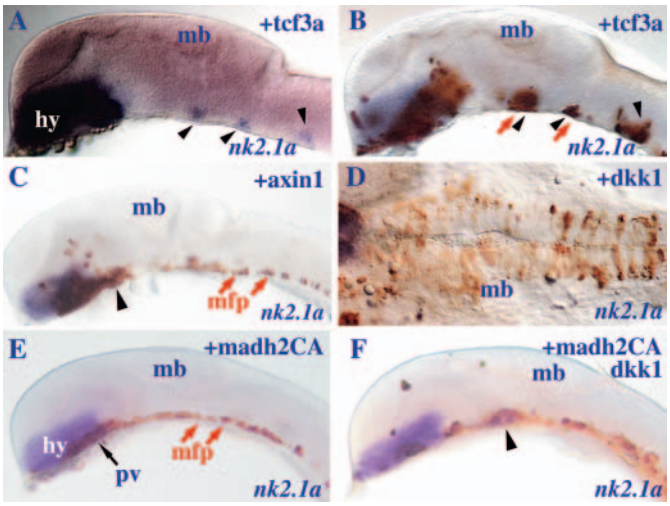

Fig. 4. Suppression of Wnt signalling in combination with activation of Nodal signalling promotes hypothalamic marker gene expression. Lateral (A,B,C,E,F) and ventral (D) views of brains of embryos at 24-somite stage with anterior to the left in which cells (brown) overexpressing *hdl/tcf3a* (A,B), *axin1* (C), *dkk1* (D), *madh2CA* (E) or *madh2CA+dkk1* (F) were transplanted into the prospective hypothalamus/anterior floorplate at 60-65% epiboly. A and B show the same embryo before and after immunohistochemistry for Gfp. In A,B,C,F, black arrowheads point to ectopic *nk2.1a* expression in the medial floorplate and/or adjacent cells. In B,C,E, red arrows point to cells that integrated in the medial floorplate. In D, Dkk1+ cells (brown) mainly integrate in more lateral positions in the neuroepithelium compared to the Axin1+ and Madh2CA+ cells that integrate in the medial floorplate (brown, red arrows) of the embryos in C and E respectively. Abbreviations: hy, hypothalamus; mb, midbrain; mfp, medial floorplate; pv, posterior-ventral hypothalamus.

madh2CA + *axin1* led to many cells entering the mesendoderm (not shown) suggesting that the addition of exogenous Axin1 facilitates Madh2 activity. Axin1 and Madh2CA also both promote the lateral expansion of medial floorplate markers (Muller et al., 2000) (Fig. 3L,M, *n*=12/20). Thus, both Nodal signalling and Axin1 promote the acquisition of midline identity and enhance the ability of donor cells to incorporate into the ventral CNS midline.

Activation of Nodal signalling in combination with inhibition of Wnt signalling promotes hypothalamic identity

In light of the possible influence of Axin1 on both Nodal and Wnt pathways, we next assessed whether activation of Nodal signalling coupled with the activity of other extracellular Wnt antagonists promotes hypothalamic identity. To do this, we transplanted cells expressing *madh2CA* alone or with *dkk1*, or with RNA encoding a C-terminal truncated form of the human Lrp6 (Lrp*Δc*) Wnt co-receptor, in which the Axin-binding domain is deleted, thus preventing the translocation of Axin to the membrane and consequently enhancing β-catenin degradation (He et al., 2004; Tamai et al., 2000). Cells overexpressing *madh2CA* alone did not induce any ectopic *nk2.1a* expression and had no effect on the regional subdivision of the ventral neural tube into floorplate and hypothalamic domains (Fig. 4E). In contrast, embryos containing cells co-expressing *dkk1* + *madh2CA* or *lrpΔc* + *madh2CA* showed

ectopic patches of *nk2.1a* expression in the posterior ventral neural tube (Fig. 4F and data not shown; *n*=11/18 and *n*=5/10 respectively). Control transplants expressing the ligand Wnt8b in combination with Madh2CA showed no ectopic *nk2.1a* expression (*n*=19/19, data not shown). Altogether, these results suggest that Nodal signalling promotes the incorporation of cells into ventral midline tissue, and that within this tissue, antagonism of Wnt signalling promotes the expression of hypothalamic markers.

Compromised Axin1 activity results in loss of rostral hypothalamic tissue and expansion of posterior diencephalic/midbrain floorplate

Although Axin1 activity promotes hypothalamic identity, a small domain of *nk2.1a*-expressing hypothalamic tissue is retained in *mbl* embryos that are compromised in Axin1 function (Fig. 2B). To examine the identity of this remaining hypothalamic tissue, we analysed expression of markers of regional identity within the hypothalamus. In wild-type embryos, *rx3* is expressed in the anterior hypothalamus from early somitogenesis (Chuang et al., 1999) (Fig. 5A) and *shh* expression is consolidated in the anterior-dorsal hypothalamus by mid-somite stages (Fig. 5C). In contrast, *emx2* is expressed in the posterior and ventral hypothalamus by mid-somite stages (Fig. 5E) (Mathieu et al., 2002). In *mbl* embryos, *emx2* is expressed at the anterior tip of the ventral neural tube similar to *nk2.1a* (Fig. 5F compare with Fig. 2B) whereas *rx3* expression and the most anterior domain of *shh* expression is lost (Fig. 5B,D). Wild-type Axin1 activity is therefore required for the establishment of anterior hypothalamic identity. Consistent with this observation, transplantation of *gfp*+ or *axin1*+ cells restored *rx3* expression in the hypothalamus of *mbl* embryos (*n*=6/12 and 7/12; Fig. 5G,H and data not shown). However, when Axin1-expressing cells incorporated into floorplate domains of wild-type embryos, they expressed the caudal hypothalamic marker, *emx2* (Fig. 5I,J).

Within the posterior diencephalon and midbrain, floorplate markers are expressed in a broader domain of the ventral neural tube than within hindbrain and spinal cord. This domain of floorplate-marker gene expression is expanded along the AP axis of the ventral neural tube of *mbl* embryos (Fig. 2E,F) indicating that the loss of anterior hypothalamic fate is correlated with an expansion of rostral floorplate tissue.

Wnt/β-catenin signalling influences the patterning of the hypothalamus along its AP axis

Enhanced Wnt pathway activity in *mbl* embryos is correlated with a loss of anterior hypothalamic fate suggesting that Wnt signalling may play a role in the regionalisation of the hypothalamus. To further explore this possibility, we locally manipulated levels of Wnt activity by transplanting cells expressing *wnt8b* into the prospective hypothalamus of wild-type embryos. *wnt8b* expression is upregulated in *mbl* embryos and Wnt8b activity is thought to play a role in the AP patterning of the alar/dorsal forebrain (Houart et al., 2002; Kim et al., 2002). *wnt8b*-expressing cells failed to express *rx3* when located in the anterior hypothalamus and suppressed *rx3* expression in adjacent host hypothalamic tissue (*n*=22/29, Fig. 6A-C). Complementing this, expression of the caudal hypothalamic marker *emx2* was rostrally expanded in the presence of transplanted *wnt8b* (or *wnt8b* + *madh2CA*)-expressing cells in the hypothalamus (*n*=16/22; *n*=18/21; Fig. 6D-F and data not shown). Thus, expression of exogenous *wnt8b* in the presumptive hypothalamus leads to expansion of posterior at the expense of anterior hypothalamic marker gene expression.

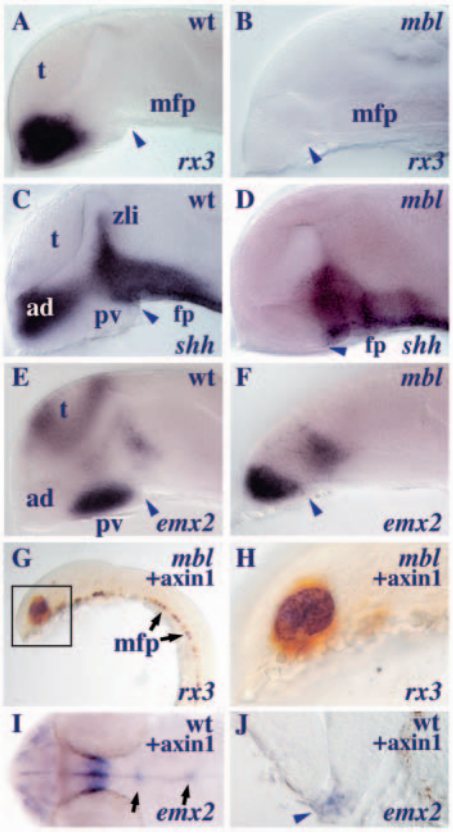

Fig. 5. *mbl* embryos lose rostral but retain caudal hypothalamic marker gene expression. (A-H) Lateral views of brains of embryos at about 31 somites with anterior to the left. (A-D) The hypothalami of the *mbl* embryos (B,D) do not express *rx3* (B) and lose the anterior domain of *shh* expression (D) compared to the wild-type embryos (A,C). (E-F) The *mbl* embryo (F) retains expression of the hypothalamic marker *emx2* which is expressed in the caudal hypothalamus of the wild-type embryo (E). Arrowheads point at the caudal limit of the hypothalamus. (G-H) Transplanted cells overexpressing *axin1* (brown) that have incorporated into the rostral ventral CNS restore *rx3* expression (blue) in the *mbl* embryo (G). (H) A higher magnification view of the transplant (boxed) of this embryo. (I-J) Some Axin1-overexpressing cells that incorporate into the floorplate domain of a wild-type host ectopically express *emx2* (arrows). The transverse section (indicated by the left arrow in I) shows ectopic *emx2* expression in the floorplate (arrowhead in J). Abbreviations: ad, anterior-dorsal hypothalamus; fp, floorplate; mfp, medial floorplate; pv, posterior-ventral hypothalamus; t, telencephalon; zli, zona limitans intrathalamica.

Discussion

In this study, we found that Axin1 activity is required for AP regionalisation of the ventral CNS. In *mbl* embryos with compromised Axin1 function, the anterior hypothalamus is absent, posterior hypothalamic markers are expressed at the rostral tip of the ventral CNS and the floorplate is expanded.

Complementary to this, exogenous Axin1 promotes expression of hypothalamic markers at the expense of floorplate markers. We suggest that Axin1 modulates the response of ventral CNS cells to signals, such as Nodals and Hhs, that induce ventral CNS identity. In doing this, it is part of the mechanism by which there is coordination between the signals that specify AP and DV identity in the CNS. Although our data suggest that Axin1 may influence Nodal signalling, it is likely that the primary role for this protein is to suppress Wnt/β-catenin signalling. This is supported by observations that the transcriptional repressor of Wnt signalling, Hdl/Tcf3a (Kim et al., 2000) can mimic the activity of Axin1, and that exogenous Wnt8b reduces and posteriorises the hypothalamus. Altogether our data suggest that levels of activity of the canonical Wnt/Axin/β-catenin signalling pathway determine the response of axial midline cells to signals that induce ventral CNS identity.

Axin1/Wnt signalling regulates AP regionalisation of the ventral CNS

Our data show that Wnt/Axin/β-catenin signalling must be suppressed for ventral CNS cells to adopt hypothalamic rather than floorplate identity. Furthermore, within the nascent hypothalamus Wnt signalling promotes posterior at the expense of anterior identities. Taken together, it appears that Wnt signalling influences both the initial subdivision of the ventral CNS into hypothalamic and floorplate domains, and subsequently influences regionalisation within the hypothalamic subdomain (promoting posterior at the expense of anterior identity). A similar mechanism is likely to operate in dorsal regions of the CNS (reviewed by Wilson and Houart, 2004) where early-acting Wnt signals are proposed to contribute to the initial regional subdivision into forebrain, midbrain, hindbrain and spinal cord domains, whereas later-acting Wnts and Wnt antagonists locally modulate levels of signalling within individual domains, thereby further refining cell fate decisions.

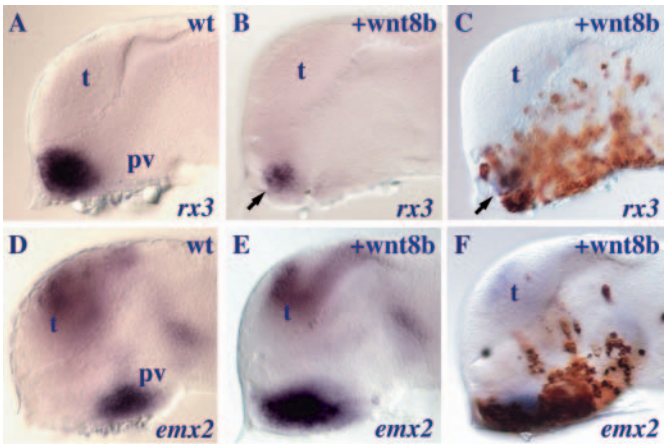

Fig. 6. *wnt8b* overexpression promotes expression of posterior hypothalamic markers. Lateral (A-F) views of brains of wild-type embryos (A,D) and embryos in which cells overexpressing *wnt8b* (B,C,E,F) have incorporated into the hypothalamus. C and F show the same embryos as B and E after immunocytochemistry to reveal the positions of cells expressing *wnt8b*. The arrows in B,C point to decreased *rx3* expression (B,C) compared to control (A). Abbreviations: pv, posterior-ventral hypothalamus; t, telencephalon.

In our experiments, exogenous Axin1 only induced hypothalamic markers at the expense of floorplate in the midbrain and hindbrain and did not do so in all expressing cells. Furthermore posterior rather than anterior hypothalamic markers were induced in these experiments. A reasonable interpretation of these findings is that the increased levels of Axin1 activity only partially suppress Wnt signalling, neither sufficiently to induce markers of anterior hypothalamus nor sufficiently to suppress floorplate markers in the caudal CNS. This interpretation is also supported by the observation that exogenous Axin1 was less efficient at inducing *nk2.1* in the floorplate of *mbl* embryos (which have enhanced Wnt signalling) than wild-type embryos.

Axin1 may promote Nodal signalling and integration into midline tissue

Our data show that activation of Nodal signalling promotes the incorporation of epiblast cells into the MFP and PV hypothalamus. This is consistent with previous data showing that cells unable to receive Nodal signals are excluded from the PV hypothalamus (Mathieu et al., 2002), and that Nodal signalling is required for formation of MFP (Strahle et al., 2004). One unexpected observation was that exogenous Axin1 promotes incorporation of cells into midline neural tissue in a manner similar to reagents that activate Nodal signalling. Indeed, Axin1 also promotes the cell-autonomous lateral expansion of *shh* expression as does Madh2CA (this study) (Müller et al., 2000). Furthermore, co-expression of *axin1* together with low levels of *madh2CA* leads to exclusion of cells from the CNS and incorporation into mesendoderm, phenocopying the consequences of expression of high levels of *madh2CA* alone. Altogether these results suggest that Axin1 may facilitate Nodal signalling in addition to its well-established role in antagonising Wnt signalling (Jones and Bejsovec, 2003; Tolwinski and Wieschaus, 2004). Indeed biochemical studies have suggested a mechanism by which this could occur as Axin1 can bind and promote the activity of Madh proteins functioning in the Nodal signalling cascade (Furuhashi et al., 2001). It is also possible that Tcf/Lef proteins could directly modulate the transcriptional activity of the Nodal pathway Madh proteins (Labbe et al., 2000; Nishita et al., 2000). Embryos completely lacking Axin1 function have not been generated in fish but in mice, such animals show multiple axes (Zeng et al., 1997), a phenotype unlikely to occur if Nodal signalling was severely compromised. Therefore, although Axin1 may facilitate Nodal signalling, it is unlikely to be an essential signal transduction component of this pathway.

What is the source of Wnts and their inhibitors that influence ventral CNS development?

Although our studies provide compelling evidence that Wnt/β-catenin signalling influences the AP regionalisation of the ventral neural tube, we have not identified the source of Wnts that promote posterior development nor do we know the exact timing at which modulation of the pathway influences fate decisions. One challenge for resolving this issue is that it is not known when the fate choice between hypothalamic and floorplate identity is made. At early stages, all axial midline neural cells appear to express similar genes (Dale et al., 1997) and it is not until late during gastrulation that hypothalamus-

specific markers are first expressed (Dale et al., 1999; Rohr et al., 2001). This suggests that fate decisions may only be determined once the axial cells have extended along the CNS midline. However, there is no evidence to rule out the possibility that fate decisions are initiated at earlier stages than this, when the precursor populations are located close to the organiser, prior to the extension movements of gastrulation. Given that many other fate decisions are being made in and around the organiser from the onset of gastrulation, or even earlier, then hypothalamic/floorplate fate decisions could be initiated around this time. If so, there are both Wnt ligands, such as Wnt8, and Wnt antagonists, such as Dkk1, that could influence levels of Wnt activity in and adjacent to the domain of floorplate and hypothalamic precursors (Erter et al., 2001; Foley et al., 2000; Kiecker and Niehrs, 2001a; Pera and De Robertis, 2000).

Hypothalamic versus floorplate identity – different inducing signals or different responses to the same signals?

There are two classes of model to explain how ventral midline CNS tissue acquires either hypothalamic or floorplate identity (Wilson and Houart, 2004). The first proposes that signals from underlying axial mesendoderm vary along the AP axis and that depending upon the nature of the signals received, the midline neural tissue differentiates either with floorplate or with hypothalamic identity (Dale et al., 1997; Dale et al., 1999; Patten et al., 2003; Placzek et al., 2000). The second class of models proposes that the signals from underlying axial mesendoderm are the same along the entire axis and that it is intrinsic AP differences within the neural ectoderm that determine the responses to these signals (Ericson et al., 1995; Kobayashi et al., 2002; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997). As we discuss below, these two models are not mutually incompatible, and indeed it is likely that elements of both models are correct.

Analysis of *mbl* embryos favours the idea that signals from axial mesendoderm are largely unaffected in the mutants, but the responsiveness of ectoderm to these signals is perturbed. This study and others have found neither major changes in the prechordal plate or prospective notochord nor phenotypes, such as cyclopia or defective induction of floorplate to suggest that axial mesendodermal signals are perturbed. Conversely, there is ample evidence to indicate that disrupted Axin1 activity within the neural plate is responsible for defective regionalisation of this tissue. For instance, restoration of Axin1 activity within anterior neural plate cells of *mbl* embryos is sufficient to restore hypothalamic marker gene expression. Axin1 only promotes hypothalamic markers in medial (prospective ventral) neural plate cells, whereas in more lateral (prospective dorsal) domains, it promotes expression of alar plate markers (Heisenberg et al., 2001; Houart et al., 2002). It appears therefore that Axin1 activity modulates the response of medially positioned neural plate cells to axial signals, such as Nodals and Hhs that induce ventral CNS identity.

There are many mechanisms by which levels of Wnt activity are modulated within the forming neural plate, including spatially and temporally localised expression of a variety of intracellular and extracellular agonists and antagonists of signalling. Although many of the factors modulating levels of Wnt activity are intrinsic to the neural plate (Houart et al., 2002; Dorsky et al., 2003; Kim et al., 2000; Kim et al., 2002), there are several secreted Wnt antagonists, such as Dkk1 (Kiecker and Niehrs, 2001b; Mukhopadhyay et al., 2001), expressed in mesendodermal tissues underlying the rostral neural plate. This implies that the level of Wnt pathway activity, and hence the differential responsiveness of ventral neural tissue to axial mesendodermal signals, may in part be determined by secreted proteins that themselves originate in the mesendoderm. Thus both the signals produced by mesendoderm and the responsiveness of ventral neural tissue varies along the AP axis of the nascent neural plate, and both are likely to contribute to the regionalisation of axial CNS in response to signals that induce ventral fate.

One intriguing possibility is that intracellular modulators of Wnt/β-catenin signalling, such as Axin1, could influence levels of pathway activity to some extent independent of extracellular ligands. Within the prospective forebrain, the transcriptional repressor activity of Tcf proteins is crucial for regionalisation of the neural plate (Dorsky et al., 2003; Kim et al., 2000). Wnt ligand signalling leads to alleviation of Tcf-dependent repression but, at least in theory, transcriptional or translational regulation of intracellular Wnt pathway proteins independent of Wnt ligand activity could also lead to changes in levels of nuclear Tcf-dependent repression.

Although our study has focused upon the subdivision of axial neural tissue into hypothalamic and floorplate domains, there is likely to be further regionalisation within the floorplate itself. For instance, in fish, various mutant conditions differentially affect anterior versus posterior floorplate (Amacher et al., 2002; Schier et al., 1997), and this fact may reflect different origins of anterior and posterior parts of the floorplate and/or variation in the AP extent of expression of various floorplate markers (Dale et al., 1999; Le Douarin and Halpern, 2000; Patten et al., 2003).

We thank many colleagues for providing reagents, Masa Tada, Tetsuhiro Kudoh and members of our research groups for comments and advice throughout the course of this work and Carole Wilson and her team for care of fish. This research was supported by grants from the BBSRC, Wellcome Trust and European Community to S.W. and from the CNRS to M.K. S.W. is a Wellcome Trust Senior Research Fellow.

References

Agathon, A., Thisse, C. and Thisse, B. (2003). The molecular nature of the zebrafish tail organizer. *Nature* **424**, 448-452.

Akimenko, M. A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to distal-less: part of a homeobox gene code for the head. *J. Neurosci.* **14**, 3475-3486.

Amacher, S. L., Draper, B. W., Summers, B. R. and Kimmel, C. B. (2002). The zebrafish T-box genes no tail and spadetail are required for development of trunk and tail mesoderm and medial floor plate. *Development* **129**, 3311-3323.

Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755-1768.

Barth, K. A., Kishimoto, Y., Rohr, K., Seydler, C., Schulte-Merker, S. and Wilson, S. W. (1999). Bmp activity establishes a gradient of positional information throughout the entire neural plate. *Development* **126**, 4977-4987.

Braun, M. M., Etheridge, A., Bernard, A., Robertson, C. P. and Roelink,

H. (2003). Wnt signaling is required at distinct stages of development for the induction of the posterior forebrain. *Development* **130**, 5579-5587.

Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr. Opin. Neurobiol.* **11**, 43-49.

Camus, A., Davidson, B. P., Billiards, S., Khoo, P., Rivera-Perez, J. A., Wakamiya, M., Behringer, R. R. and Tam, P. P. (2000). The morphogenetic role of midline mesendoderm and ectoderm in the development of the forebrain and the midbrain of the mouse embryo. *Development* **127**, 1799-1813.

Chen, W., Burgess, S. and Hopkins, N. (2001). Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity. *Development* **128**, 2385-2396.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Chuang, J. C., Mathers, P. H. and Raymond, P. A. (1999). Expression of three Rx homeobox genes in embryonic and adult zebrafish. *Mech. Dev*. **84**, 195-198.

Dajani, R., Fraser, E., Roe, S. M., Yeo, M., Good, V. M., Thompson, V., Dale, T. C. and Pearl, L. H. (2003). Structural basis for recruitment of glycogen synthase kinase 3beta to the axin-APC scaffold complex. *EMBO J.* **22**, 494-501.

Dale, J. K., Vesque, C., Lints, T. J., Sampath, T. K., Furley, A., Dodd, J. and Placzek, M. (1997). Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. *Cell* **90**, 257-269.

Dale, K., Sattar, N., Heemskerk, J., Clarke, J. D., Placzek, M. and Dodd, J. (1999). Differential patterning of ventral midline cells by axial mesoderm is regulated by BMP7 and chordin. *Development* **126**, 397-408.

Dorsky, R. I., Itoh, M., Moon, R. T. and Chitnis, A. (2003). Two tcf3 genes cooperate to pattern the zebrafish brain. *Development* **130**, 1937-1947.

Ericson, J., Muhr, J., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog: a common signal for ventral patterning along the rostrocaudal axis of the neural tube. *Int. J. Dev. Biol*. **39**, 809-816.

Erter, C. E., Wilm, T. P., Basler, N., Wright, C. V. and Solnica-Krezel, L. (2001). Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo. *Development* **128**, 3571-3583.

Foley, A. C., Skromne, I. and Stern, C. D. (2000). Reconciling different models of forebrain induction and patterning: a dual role for the hypoblast. *Development* **127**, 3839-3854.

Furuhashi, M., Yagi, K., Yamamoto, H., Furukawa, Y., Shimada, S., Nakamura, Y., Kikuchi, A., Miyazono, K. and Kato, M. (2001). Axin facilitates Smad3 activation in the transforming growth factor beta signaling pathway. *Mol. Cell. Biol*. **21**, 5132-5141.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.

Gunhaga, L., Marklund, M., Sjodal, M., Hsieh, J. C., Jessell, T. M. and Edlund, T. (2003). Specification of dorsal telencephalic character by sequential Wnt and FGF signaling. *Nat. Neurosci.* **6**, 701-707.

Hammerschmidt, M., Kramer, C., Nowak, M., Herzog, W. and Wittbrodt, J. (2003). Loss of maternal Smad5 in zebrafish embryos affects patterning and morphogenesis of optic primordia. *Dev. Dyn.* **227**, 128-133.

Hauptmann, G. and Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and *Drosophila* embryos. *Trends Genet.* **10**, 266.

He, X., Semenov, M., Tamai, K. and Zeng, X. (2004). LDL receptor-related proteins 5 and 6 in Wnt/{beta}-catenin signaling: arrows point the way. *Development* **131**, 1663-1677.

Heisenberg, C. P., Brand, M., Jiang, Y. J., Warga, R. M., Beuchle, D., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P. et al. (1996). Genes involved in forebrain development in the zebrafish, Danio rerio. *Development* **123**, 191-203.

Heisenberg, C. P., Houart, C., Take-Uchi, M., Rauch, G. J., Young, N., Coutinho, P., Masai, I., Caneparo, L., Concha, M. L., Geisler, R. et al. (2001). A mutation in the Gsk3-binding domain of zebrafish Masterblind/Axin1 leads to a fate transformation of telencephalon and eyes to diencephalon. *Genes Dev.* **15**, 1427-1434.

Houart, C., Westerfield, M. and Wilson, S. W. (1998). A small population of anterior cells patterns the forebrain during zebrafish gastrulation. *Nature* **391**, 788-792.

Houart, C., Caneparo, L., Heisenberg, C., Barth, K., Take-Uchi, M. and Wilson, S. (2002). Establishment of the telencephalon during gastrulation by local antagonism of Wnt signaling. *Neuron* **35**, 255-265.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.

Jones, W. M. and Bejsovek, A. (2003). Wingless signalling: an axin to grind. *Curr. Biol.* **13**, R479-R481.

Kelly, G. M., Greenstein, P., Erezyilmaz, D. F. and Moon, R. T. (1995). Zebrafish wnt8 and wnt8b share a common activity but are involved in distinct developmental pathways. *Development* **121**, 1787-1799.

Kiecker, C. and Niehrs, C. (2001a). A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. *Development* **128**, 4189-4201.

Kiecker, C. and Niehrs, C. (2001b). The role of prechordal mesendoderm in neural patterning. *Curr. Opin. Neurobiol*. **11**, 27-33.

Kim, C. H., Oda, T., Itoh, M., Jiang, D., Artinger, K. B., Chandrasekharappa, S. C., Driever, W. and Chitnis, A. B. (2000). Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. *Nature* **407**, 913-916.

Kim, S. H., Shin, J., Park, H. C., Yeo, S. Y., Hong, S. K., Han, S., Rhee, M., Kim, C. H., Chitnis, A. B. and Huh, T. L. (2002). Specification of anterior neuroectoderm patterning by Frizzled8a-mediated Wnt8b signalling during late gastrulation in zebrafish. *Development* **129**, 4443-4455.

Kobayashi, D., Kobayashi, M., Matsumoto, K., Ogura, T., Nakafuku, M. and Shimamura, K. (2002). Early subdivisions in the neural plate define distinct competence for inductive signals. *Development* **129**, 83-93.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991). Expression pattern of zebrafish pax genes suggests a role in early brain regionalization. *Nature* **353**, 267-270.

Krauss, S., Concordet, J. P. and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.

Labbe, E., Letamendia, A. and Attisano, L. (2000). Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways. *Proc. Natl. Acad. Sci. USA* **97**, 8358-8363.

Lagutin, O. V., Zhu, C. C., Kobayashi, D., Topczewski, J., Shimamura, K., Puelles, L., Russell, H. R., McKinnon, P. J., Solnica-Krezel, L. and Oliver, G. (2003). Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. *Genes Dev*. **17**, 368-379.

Le Douarin, N. M. and Halpern, M. E. (2000). Discussion point. Origin and specification of the neural tube floor plate: insights from the chick and zebrafish. *Curr. Opin. Neurobiol.* **10**, 23-30.

Lekven, A., Thorpe, C., Waxman, J. and Moon, R. (2001). Zebrafish *wnt8* encodes two Wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. *Dev. Cell* **1**, 103-114.

Masai, I., Heisenberg, C. P., Barth, K. A., Macdonald, R., Adamek, S. and Wilson, S. W. (1997). Floating head and masterblind regulate neuronal patterning in the roof of the forebrain. *Neuron* **18**, 43-57.

Mathieu, J., Barth, A., Rosa, F. M., Wilson, S. W. and Peyrieras, N. (2002). Distinct and cooperative roles for Nodal and Hedgehog signals during hypothalamic development. *Development* **129**, 3055-3065.

Morita, T., Nitta, H., Kiyama, Y., Mori, H. and Mishina, M. (1995). Differential expression of twozebrafish emx homeoprotein mRNAs in the developing brain. *Neurosci. Lett.* **198**, 131-134.

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D. W., Glinka, A., Grinberg, A., Huang, S. P. et al. (2001). Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev. Cell* **1**, 423-434.

Müller, F., Albert, S., Blader, P., Fischer, N., Hallonet, M. and Strahle, U. (2000). Direct action of the nodal-related signal cyclops in induction of sonic hedgehog in the ventral midline of the CNS. *Development* **127**, 3889-3897.

Niehrs, C., Kazanskaya, O., Wu, W. and Glinka, A. (2001). Dickkopf1 and the Spemann-Mangold head organizer. *Int. J. Dev. Biol.* **45**, 237-240.

Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H. and Cho, K. W. (2000). Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. *Nature* **403**, 781-785.

Nordstrom, U., Jessell, T. M. and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. *Nat. Neurosci.* **5**, 525-532.

Odenthal, J. and Nüsslein-Volhard, C. (1998). fork head domain genes in zebrafish *Dev. Genes Evol.* **208**, 245-258.

Odenthal, J., van Eeden, F. J., Haffter, P., Ingham, P. W. and Nusslein-Volhard, C. (2000). Two distinct cell populations in the floor plate of the zebrafish are induced by different pathways. *Dev. Biol.* **219**, 350-363.

Patten, I. and Placzek, M. (2002). Opponent activities of Shh and BMP signaling during floor plate induction in vivo. *Curr. Biol.* **12**, 47-52.

Patten, I., Kulesa, P., Shen, M. M., Fraser, S. and Placzek, M. (2003). Distinct modes of floor plate induction in the chick embryo. *Development* **130**, 4809-4821.

Pera, E. M. and de Robertis, E. M. (2000). A direct screen for secreted proteins in Xenopus embryos identifies distinct activities for the Wnt antagonists Crescent and Frzb-1. *Mech. Dev.* **96**, 183-195.

Pera, E. M. and Kessel, M. (1997). Patterning of the chick forebrain anlage by the prechordal plate. *Development* **124**, 4153-4162.

Placzek, M., Dodd, J. and Jessell, T. M. (2000). Discussion point. The case for floor plate induction by the notochord. *Curr. Opin. Neurobiol.* **10**, 15-22.

Rohr, K. B., Barth, K. A., Varga, Z. M. and Wilson, S. W. (2001). The nodal pathway acts upstream of hedgehog signaling to specify ventral telencephalic identity. *Neuron* **29**, 341-351.

Rubenstein, J. L. R., Shimamura, K., Martinez, S. and Luis Puelles, L. (1998). Regionalization of the prosencephalic neural plate. *Annu. Rev. Neurosci.* **21**, 445-477.

Ruiz i Altaba, A., Nguyen, V. and Palma, V. (2003). The emergent design of the neural tube: prepattern, SHH morphogen and GLI code. *Curr. Opin. Genet. Dev.* **13**, 513-521.

Schier, A. F. (2003). Nodal signaling in vertebrate development. *Annu. Rev. Cell Dev. Biol.* **19**, 589-621.

Schier, A. F., Neuhauss, S. C., Helde, K. A., Talbot, W. S. and Driever, W. (1997). The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. *Development* **124**, 327-342.

Schulte-Merker, S., van Eeden, F. J., Halpern, M. E., Kimmel, C. B. and Nusslein-Volhard, C. (1994). no tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. *Development* **120**, 1009-1015.

Shimamura, K. and Rubenstein, J. L. (1997). Inductive interactions direct early regionalization of the mouse forebrain. *Development* **124**, 2709-2718.

Shinya, M., Eschbach, C., Clark, M., Lehrach, H. and Furutani-Seiki, M. (2000). Zebrafish Dkk1, induced by the pre-MBT Wnt signaling, is secreted from the prechordal plate and patterns the anterior neural plate. *Mech. Dev.* **98**, 3-17.

Strahle, U., Blader, P., Henrique, D. and Ingham, P. (1993). Axial, a zebrafish gene expressed along the developing body axis, shows altered expression in cyclops mutant embryos. *Genes Dev.* **7**, 1436-1446.

Strahle, U., Lam, C. S., Ertzer, R. and Rastegar, S. (2004). Vertebrate floor-plate specification: variations on common themes. *Trends Genet.* **20**, 155-162.

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P. and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407**, 530-535.

Thisse, C., Thisse, B., Halpern, M. E. and Postlethwait, J. H. (1994). Goosecoid expression in neurectoderm and mesendoderm is disrupted in zebrafish cyclops gastrulas. *Dev. Biol.* **164**, 420-429.

Tian, J., Yam, C., Balasundaram, G., Wang, H., Gore, A. and Sampath, K. (2003). A temperature-sensitive mutation in the nodal-related gene cyclops reveals that the floor plate is induced during gastrulation in zebrafish. *Development* **130**, 3331-3342.

Tolwinski, N. S. and Wieschaus, E. (2004). Rethinking WNT signaling. *Trends Genet.* **20**, 177-181.

Van de Water, S., van de Wetering, M., Joore, J., Esseling, J., Bink, R., Clevers, H. and Zivkovic, D. (2001). Ectopic Wnt signal determines the eyeless phenotype of zebrafish masterblind mutant. *Development* **128**, 3877-3888.

Varga, Z. M., Wegner, J. and Westerfield, M. (1999). Anterior movement of ventral diencephalic precursors separates the primordial eye field in the neural plate and requires cyclops. *Development* **126**, 5533-5546.

Varga, Z. M., Amores, A., Lewis, K. E., Yan, Y. L., Postlethwait, J. H., Eisen, J. S. and Westerfield, M. (2001). Zebrafish smoothened functions in ventral neural tube specification and axon tract formation. *Development* **128**, 3497-3509.

Wilson, S. W. and Houart, C. (2004). Early steps in the development of the forebrain. *Dev. Cell* **6**, 167-181.

Woo, K. and Fraser, S. E. (1995). Order and coherence in the fate map of the zebrafish nervous system. *Development* **121**, 2595-2609.

Yamaguchi, T. P. (2001). Heads or tails: Wnts and anterior-posterior patterning. *Curr. Biol.* **11**, R713-R724.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry, W. L., III, Lee, J. J., Tilghman, S. M., Gumbiner, B. M. and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell* **90**, 181-192.