Development 129, 3055-3065 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV2898

Distinct and cooperative roles for Nodal and Hedgehog signals during hypothalamic development

Juliette Mathieu[1], Anukampa Barth[2], Frederic M. Rosa[1], Stephen W. Wilson[2,*] and Nadine Peyriéras[1,*]

[1]U 368 INSERM, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France
[2]Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK
*Authors for correspondence (e-mail: peyriera@wotan.ens.fr and s.wilson@ucl.ac.uk)

Accepted 21 April 2002

SUMMARY

Despite its evolutionary conservation and functional importance, little is known of the signaling pathways that underlie development of the hypothalamus. Although mutations affecting Nodal and Hedgehog signaling disrupt hypothalamic development, the time and site of action and the exact roles of these pathways remain very poorly understood. Unexpectedly, we show here that cell-autonomous reception of Nodal signals is neither required for the migration of hypothalamic precursors within the neural plate, nor for further development of the anterior-dorsal hypothalamus. Nodal signaling is, however, cell-autonomously required for establishment of the posterior-ventral hypothalamus. Conversely, Hedgehog signaling antagonizes the development of posterior-ventral hypothalamus, while promoting anterior-dorsal hypothalamic fates. Besides their distinct roles in the regionalization of the diencephalon, we reveal cooperation between Nodal and Hedgehog pathways in the maintenance of the anterior-dorsal hypothalamus. Finally we show that it is the prechordal plate and not the head endoderm that provides the early signals essential for establishment of the hypothalamus.

Key words: Nodal, Hedgehog, Ventral diencephalon, Mesendoderm, Zebrafish

INTRODUCTION

The hypothalamus is the master regulator of endocrine and autonomic function within the brain. It develops from the ventral-most region of the anterior diencephalon. In zebrafish, fate mapping experiments have shown that hypothalamic precursors originate closer to the organizer than other forebrain derivatives and move rostrally within the neural plate to their final location in the anterior CNS (Woo and Fraser, 1995; Varga et al., 1999) (this study). Thus, early steps of hypothalamic development involve regulation both of the induction of hypothalamic identity and the migration of hypothalamic precursors. Although embryological manipulations have revealed the importance of mesodermal and/or endodermal tissues that underlie the brain in these processes, little is known of the molecular pathways involved in the specification and patterning of the hypothalamus.

Tissue ablation experiments have shown that organizer-derived mesendodermal tissues are a source of inducing signals required for hypothalamic development (reviewed by Muenke and Beachy, 2000; Kiecker and Niehrs, 2001). The secreted protein Sonic Hedgehog (Shh) is proposed to be one such signal as hypothalamic tissue is absent in mice lacking Shh function (Chiang et al., 1996) and increased Shh activity leads to ectopic expression of hypothalamic markers in fish (Barth and Wilson, 1995; Hauptmann and Gerster, 1996; Rohr et al., 2001). However, induction of the hypothalamus still occurs in fish that lack function of Smoothened, an essential transmembrane modulator of Hh activity (Chen et al., 2001; Rohr et al., 2001; Varga et al., 2001). This raises the possibility that at least in fish, Hh signaling may play a more important role in the maintenance and/or later development of hypothalamic tissue than in its induction.

Nodal signals are potentially involved in the specification of the hypothalamus, as evidenced from the hypothalamic defects seen in animals with compromised Nodal activity. These include fish carrying mutations in the genes encoding the Nodal ligands Squint (Sqt) (Heisenberg and Nüsslein-Volhard, 1997; Feldman et al., 1998) and Cyclops (Cyc) (Hatta et al., 1994; Rebagliati et al., 1998; Sampath et al., 1998), One-eyed-pinhead (Oep) (Schier et al., 1997; Strähle et al., 1997), a membrane-anchored protein of the EGF-CFC family essential for transduction of Nodal signals (Zhang et al., 1998; Gritsman et al., 1999), and Schmalspur/Fast1/FoxH1 (Sur) (Pogoda et al., 2000; Sirotkin et al., 2000), a transcriptional effector of Nodal signal transduction (Whitman, 1998). During gastrulation, *cyc* and *oep* are expressed both in mesendoderm and in overlying neuroectoderm (Rebagliati et al., 1998; Sampath et al., 1998; Zhang et al., 1998), suggesting that Nodal signaling may occur in both tissues. Indeed, Nodal activity is cell-autonomously required in the anterior axial mesendoderm (Schier et al., 1997) and in the prospective floor plate (Müller et al., 2000; Strähle et al., 1997). However, it has remained uncertain whether Nodal signals need to be received by

prospective hypothalamic cells (Hatta et al., 1994; Sampath et al., 1998). Alternatively, the hypothalamic defects in Nodal mutants might be a secondary consequence of the requirement for Nodal signals in mesendodermal tissues.

In the present study, we have been able for the first time to address the requirement for Nodal and Hh signaling within hypothalamic precursors by making use of reagents that activate or inhibit signaling in a cell autonomous fashion. We reveal an early regionalization of the hypothalamus anlage that strikingly correlates with gene expression domains. We demonstrate that reception of Nodal signals is indeed required for hypothalamic development, but only of the posterior-ventral region. By contrast, the Hh signaling pathway is not required for induction of the posterior-ventral hypothalamus and even inhibits the development of this region. Instead, Hh signaling favors the development of the anterior-dorsal hypothalamus. Furthermore, we show that when cells are compromised in their ability to receive both Nodal and Hh signals, then they are completely excluded from the developing hypothalamus, revealing a cooperative role for Nodal and Hh signals in hypothalamic maintenance.

MATERIALS AND METHODS

Fish strains

Embryos were obtained from natural spawning of wild-type (AB) or mutant zebrafish lines. The following mutant alleles were used: *oep*^{tz57} (Hammerschmidt et al., 1996), *cyc*^{b16} (Hatta et al., 1991), *sur*^{ty68b} (Brand et al., 1996), *sqt*^{cz35} (Heisenberg and Nüsslein-Volhard, 1997) and *smu*^{b641} (Barresi et al., 2000). Embryos lacking both maternal and zygotic Oep function were obtained by crossing *oep*$^{-/-}$ adults that had been rescued to viability by injection of *oep* RNA at the one-cell stage.

Microinjection and transplantation experiments

Synthetic mRNAs were transcribed in vitro using the SP6 mMessage mMachine^{TM} transcription kit (Ambion). MZ*oep* embryos were injected at the 16-cell stage into one marginal blastomere with combinations of *nls-GFP* (100 pg) and *nls-lacZ* (100 pg) RNA as lineage tracers, and *tar** (4 pg) and *XFD* RNA (200 pg). For transplantation experiments, donor embryos were injected at the one- to four-cell stage with *nls*-GFP and *lacZ* RNA (100 pg) as lineage tracers, either alone or in combination with *cPKA* (60 pg). *FAST1*^{SID} RNA (100 pg) was injected at the one- or 16-cell stage.

Cyclopamine treatment

Embryos were treated with 100 μM cyclopamine (TRC) from mid-blastula transition to 28 hpf (hours post fertilization) in the dark.

In situ hybridization and immunohistochemistry

In situ hybridization was carried out as described previously (Hauptmann and Gerster, 1994). The following mRNA probes were used: *shh* (Krauss et al., 1993), *nk2.1a* (Rohr et al., 2001), *emx2* (Morita et al., 1995), *gata5* (Rodaway et al., 1999), *axial* (Strahle et al., 1996), *gsc* (Stachel et al., 1993), *hgg1* (Thisse et al., 1994) and *bmp4* (Nikaido et al., 1997). β-galactosidase was revealed with a rabbit polyclonal antibody (Cappel) at 1:1000 dilution and a diaminobenzidine (DAB) staining. Mouse monoclonal anti-HU antibody (16A11 Interchim) was used at 1:200 dilution, and visualized by anti-mouse CY3 (affinity purified Fab fragments, Jackson) at 1:1000 dilution. Rabbit polyclonal anti-tyrosine hydroxylase (anti-TH Institut Jacques Boy SA, Reims France) was used at 1:750 dilution followed by a DAB staining.

Histology

Stained embryos were embedded in JB4 resin (Polyscience) and sectioned with a glass knife (3 μm), or mounted in 7.5% gelatin, 15% sucrose for cryostat sectioning (10 μm). Alternatively, embryos hybridized with *shh* and *nk2.1a* probes were embedded in polyester wax (BDH Microscopy) to make serial sections (6 μm), before immunodetection of the riboprobes.

RESULTS

Expression of *shh* and *emx2* defines complementary domains of the hypothalamus

Although the homeobox gene *nk2.1a* is expressed throughout the hypothalamus (Rohr et al., 2001) (Fig. 1K), other markers reveal that this region of the diencephalon is subdivided at early developmental stages. *shh* is initially expressed throughout the prospective hypothalamus, but by mid-somite stages, expression is reduced posteriorly and ventrally, and consolidated anteriorly and dorsally (Barth and Wilson, 1995) (Fig. 1B,E,H). *emx2* expression increases in the posterior-

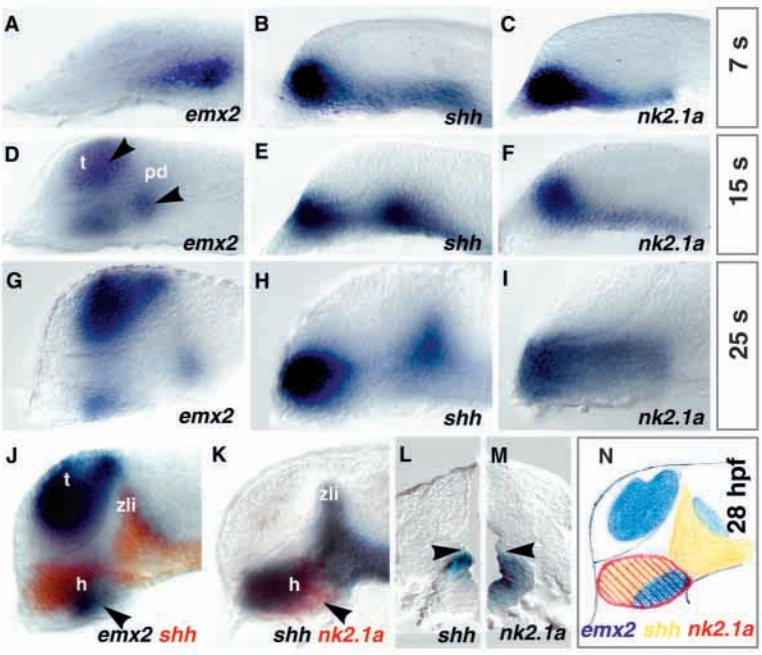

Fig. 1. *shh* and *emx2* expression sites define complementary domains of the hypothalamus. (A-K) Lateral views of brains of wild-type embryos with anterior towards the left showing expression of *nk2.1a*, *shh* and *emx2* either in dark blue or red, as indicated (bottom right). Stages are indicated on the far right. Arrowheads in D show additional sites of *emx2* expression in the posterior diencephalon and dorsal telencephalon. In J,K, the arrowheads indicate the PV hypothalamic region that expresses *nk2.1a* and *emx2* but not *shh*. (L-M) Frontal serial sections through the hypothalamus, dorsal towards the top. The dorsal limit of *nk2.1a* and *shh* expression domain coincide (arrowheads). (N) Schematic view of *nk2.1a*, *shh* and *emx2* expression in the 28 hpf brain. *nk2.1a* expression encompasses both *shh* and *emx2* hypothalamic domains. h, hypothalamus; pd, posterior diencephalon, t, telencephalon; zli, zona limitans intrathalamica.

ventral (PV) hypothalamus around the same time that *shh* expression fades (Fig. 1A,D,G), and by day 1 of development, *shh* and *emx2* expression domains demarcate complementary subdivisions of the *nk2.1a*+ hypothalamic neuroepithelium (Fig. 1J-N). The domain of *shh* expression in the anterior dorsal (AD) hypothalamus is a site of production of early-born neurons (Barth and Wilson, 1995) that are likely to contribute to retro- and supra-chiasmatic nuclei (Puelles, 1995). By contrast, the zone of *emx2* expression in the tuberal and mammilary regions shows no early neurogenesis and is likely to give rise to the infundibulum and other posterior and ventral hypothalamic nuclei. We used these various markers to investigate the roles of Nodal and Hh signaling pathways in induction and patterning of the hypothalamus.

Progressive loss of PV and then AD hypothalamus in Nodal pathway mutants

Mutations that severely reduce Nodal signaling lead to a complete failure of hypothalamic development and severe cyclopia (Schier and Shen, 2000; Rohr et al., 2001) (Fig. 2F). However, Nodal pathway mutants with less severe phenotypes possess ventral diencephalic tissue and retain AD hypothalamus, while PV hypothalamus is reduced or absent.

Schmalspur (*sur*) mutants, devoid of FoxH1/Fast1 activity, retain *shh* expression in the AD hypothalamus, while *emx2* expression in the PV hypothalamus is reduced (Fig. 2A). *MZsqt* embryos, which are devoid of maternal and zygotic Sqt function, exhibit a forebrain phenotype of variable expressivity. Mildly affected *MZsqt* embryos resemble *sur*$^{-/-}$ embryos (Fig. 2B), whereas in more severely affected *MZsqt* embryos, hypothalamic *emx2* expression is lost while *shh* and *nk2.1a* expression is retained (Fig. 2C,D). Finally, hypothalamic *shh* and *emx2* staining is absent altogether in the most severely affected *MZsqt* mutant embryos (Fig. 2E). All hypothalamic markers were also absent in *cyc*$^{-/-}$, maternal and zygotic *oep* (*MZoep*) or in embryos injected with RNA encoding dominant-negative FAST1 (FAST1^{SID}) (Fig. 2F, and data not shown), confirming previous observations (Müller et al., 2000; Rohr et al., 2001).

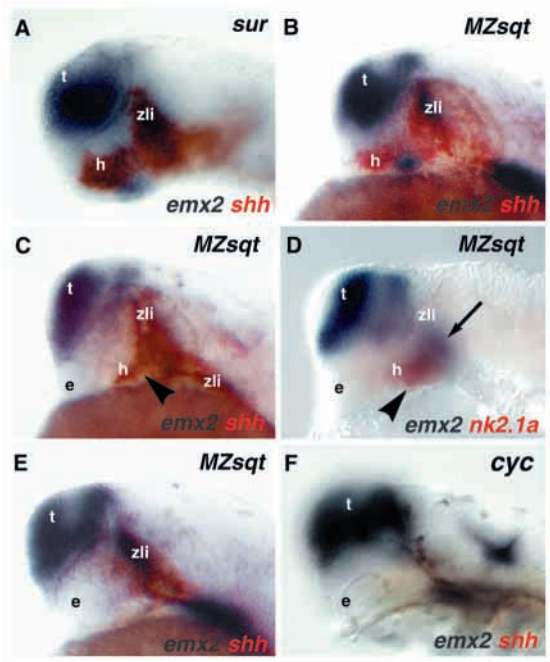

Fig. 2. Progressive loss of PV and then AD hypothalamus in Nodal pathway mutants. (A-F) Lateral views of hypothalamic gene expression (indicated bottom right) in 28 hpf brains of embryos of various genotypes (indicated top right). Arrowheads in C and D indicate remaining *shh* and *nk2.1a* expression in the hypothalamus of *MZsqt* embryos. The arrow in D indicates posterior diencephalic expression of *emx2*. e, eye; h, hypothalamus; t, telencephalon; zli, zona limitans intrathalamica.

Together, these data indicate that the PV hypothalamus is more sensitive than AD hypothalamus to reduction in Nodal signaling and raise the possibility of a differential requirement for Nodal signaling in the formation of the two hypothalamic subdomains. Although these results confirm that Nodal activity is required for hypothalamic development, they do not address which cells must receive the Nodal signals, or for what process Nodal signaling is required. To address these issues, we investigated the requirements for Nodal signaling within mesendodermal cells and within neuroectodermal cells, and attempted to establish what events are mediated by Nodal activity in these two tissues.

Reception of Nodal signals is not required in prospective hypothalamic cells for rostrally directed movement within the neural plate

The hypothalamic phenotype of Nodal pathway mutants has been proposed to be the consequence of defects in two processes, specification of the hypothalamic identity and rostral movement of hypothalamic precursors into the anterior neural plate (Varga et al., 1999; Rohr et al., 2001). To determine which features of hypothalamic development are compromised when prospective hypothalamic cells are unable to receive Nodal signals, we generated chimeric embryos in which prospective hypothalamic cells could not receive Nodal signals. To do this, we transplanted *MZoep* mutant cells, which are unable to transduce Nodal signals (Gritsman et al., 1999), into the presumptive hypothalamus of wild-type embryos, just rostral to the shield (Fig. 3A). This approach allowed us to address the cell-autonomous requirement for Oep-dependent Nodal signaling in hypothalamic precursor cells.

During gastrulation, we observed no obvious difference in the behavior of wild-type and *MZoep* cells and by bud stage, both cell groups had moved to the animal pole and were found in the anterior neural plate above the prechordal plate mesendoderm (Fig. 3B,C). At later stages, it was evident that both wild-type and *MZoep* cells reached the anterior limit of the basal forebrain, just ventral to the optic recess (Fig. 3D,E). The transplanted cells were positioned within the *nk2.1a* expression domain confirming that both wild-type and *MZoep* cells had contributed to the hypothalamus (Fig. 3G,H; Table 1).

Although Nodal signaling is absolutely dependent on Oep during gastrulation in zebrafish (Gritsman et al., 1999; Chen and Schier, 2001), recent in vitro experiments have suggested that in some species, a reduced level of Nodal signaling may occur through Alk7 in the absence of Oep-related proteins (Reissmann et al., 2001). Therefore we overexpressed *Fast1*^{SID} in prospective hypothalamic cells as a second approach to abrogate Nodal signaling in these cells. As with *MZoep* cells, *FAST1*^{SID}-expressing cells still moved rostrally and contributed to the hypothalamus (Fig. 2I). These data indicate that despite

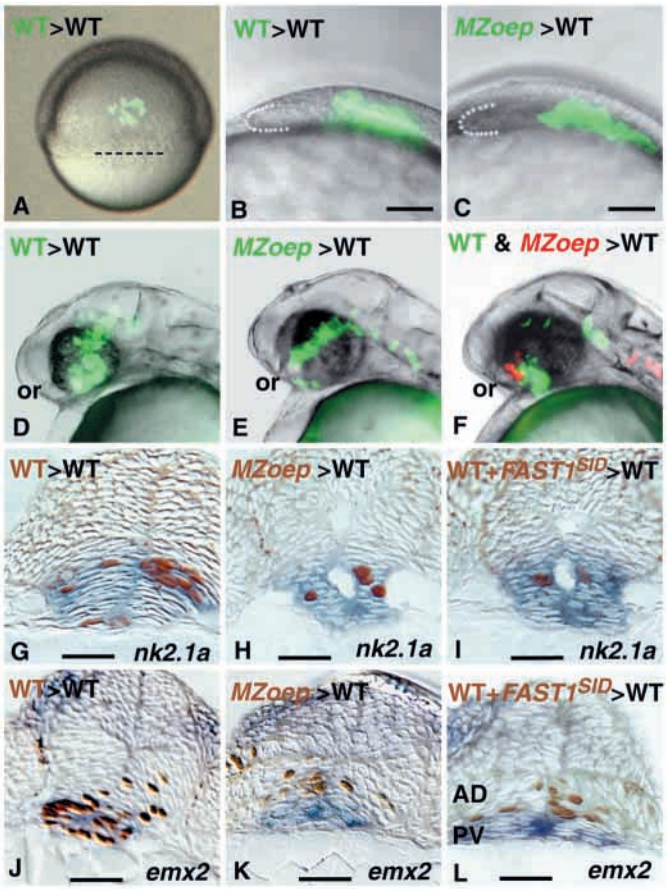

Fig. 3. Reception of Nodal signals is required for cells to contribute to the PV but not the AD hypothalamus. Embryos in which wild-type and/or *MZoep* cells, or wild-type cells expressing *Fast1*^{SID}, were transplanted into the prospective hypothalamus of wild-type hosts are shown. Genotypes are indicated in the top left-hand corner (donor>host). (A-F) Live embryos in which transplanted cells are visualized with GFP (green) or rhodamine-dextran (red). (A) Dorsal view of a shield stage embryo with rostral upwards. The blastoderm margin is indicated by broken line. At this stage, hypothalamic precursors are close to the shield, some distance from the front of the neural plate. (B-C) Lateral views of bud stage embryos with anterior to the left. The anterior limit of the prechordal mesendoderm is indicated by white dots. By this stage, hypothalamic precursors have reached the front of the neural plate. (D-F) Lateral views of brains of 28 hpf embryos with anterior towards the left. (G-L) Frontal sections through the hypothalami of 28 hpf embryos, showing β-gal+ transplanted cells (brown) in relation to the expression of various hypothalamic markers (indicated in the bottom right-hand corner). AD and PV, anterior dorsal and posterior ventral hypothalamus; or, optic recess. Scale bars: 60 μm.

an inability to perceive Nodal signals, neuroectodermal cells can move rostrally within the neural plate and contribute to the hypothalamus.

Oep is cell-autonomously required for cells to contribute to the PV but not to the AD hypothalamus

Although no difference was initially detected in the distribution of transplanted hypothalamic wild-type and *MZoep* cells, a marked difference in the location of the cell groups was apparent by 28 hpf (hours post fertilization) (Fig. 3D-F). While wild-type cells colonized the entire hypothalamus (Fig. 3D,G,J), *MZoep* and *Fast1*^{SID} cells colonized only dorsal hypothalamus (Fig. 3E,H,I) and were strictly excluded from the *emx2*-expressing PV hypothalamus (Fig. 3K,L, Table 1). Co-transplantation of both wild-type and *MZoep* cells confirmed that by 28 hpf, these two populations had segregated with only the wild-type cells contributing to the PV hypothalamus (Fig. 3F). These data show that cell autonomous reception of Nodal signals is a necessary requirement for cells to contribute to the PV hypothalamus, but not to the AD hypothalamus.

Table 1. *oep* is required cell-autonomously in the PV but not AD hypothalamus

Donor genotype	Injected RNA	Marker	%	*n*
Wild type	–	*nk2.1a*	100	14
Wild type	–	*emx2*	100	32
MZoep	–	*nk2.1a*	100	7
MZoep	–	*emx2*	0	26
Wild type	*Fast1*^{SID}	*nk2.1a*	100	14
Wild type	*Fast1*^{SID}	*emx2*	0	13

Wild-type cells (+/– *Fast1*^{SID} RNA as indicated in the second column) or *MZoep* cells (donor genotype indicated in the first column) were transplanted into the presumptive hypothalamus of a wild-type host embryo (see Fig. 3A). The recipients were observed on day 1 of development. All of them had a chimeric hypothalamus with 10 to 40 cells originating from the donor embryo. The recipients (*n* indicates the number used) were processed for in situ hybridisation (marker gene indicated in the third column). The percentage indicates the proportion of recipients exhibiting cells in the expression domain of the marker gene.

The cell-autonomous requirement for Nodal signaling in the PV hypothalamus explains why this territory is severely reduced or absent in Nodal pathway mutants. However, the fact that cells incapable of receiving Nodal signals can still contribute to the AD hypothalamus suggests that the loss of this territory in Nodal mutants is secondary to other phenotypic defects. The possibility that we investigate below is that Nodal signaling is required for development of axial mesendoderm and that this tissue mediates AD hypothalamic development.

Activation of the Nodal pathway cell non-autonomously rescues the AD but not the PV hypothalamus of MZ*oep* embryos

Oep activity is required for specification of axial mesendodermal cells (Schier et al., 1997) and so the hypothalamic defects in Nodal-deficient embryos may be secondary to defects in the underlying mesendoderm. We have shown that cells incapable of receiving Nodal signals can contribute to AD hypothalamus in a wild-type environment. One possible explanation of this result is that the transplanted cells receive signals other than Nodal proteins from the underlying mesendoderm. To address whether the mesendoderm is involved in AD hypothalamic development, we restored this tissue in embryos in which cells were unable to perceive Nodal signals. To do this, we activated the Nodal signaling pathway in a subset of cells in *MZoep* embryos, using an activated form (Tar*) of a putative Nodal receptor (Taram-a) (Renucci et al., 1996; Peyrieras et al., 1998).

Injection of *tar** RNA cell-autonomously rescued the formation of endoderm and axial mesendoderm (see below) in *MZoep* mutant embryos. By 28 hpf, *tar** expressing cells were

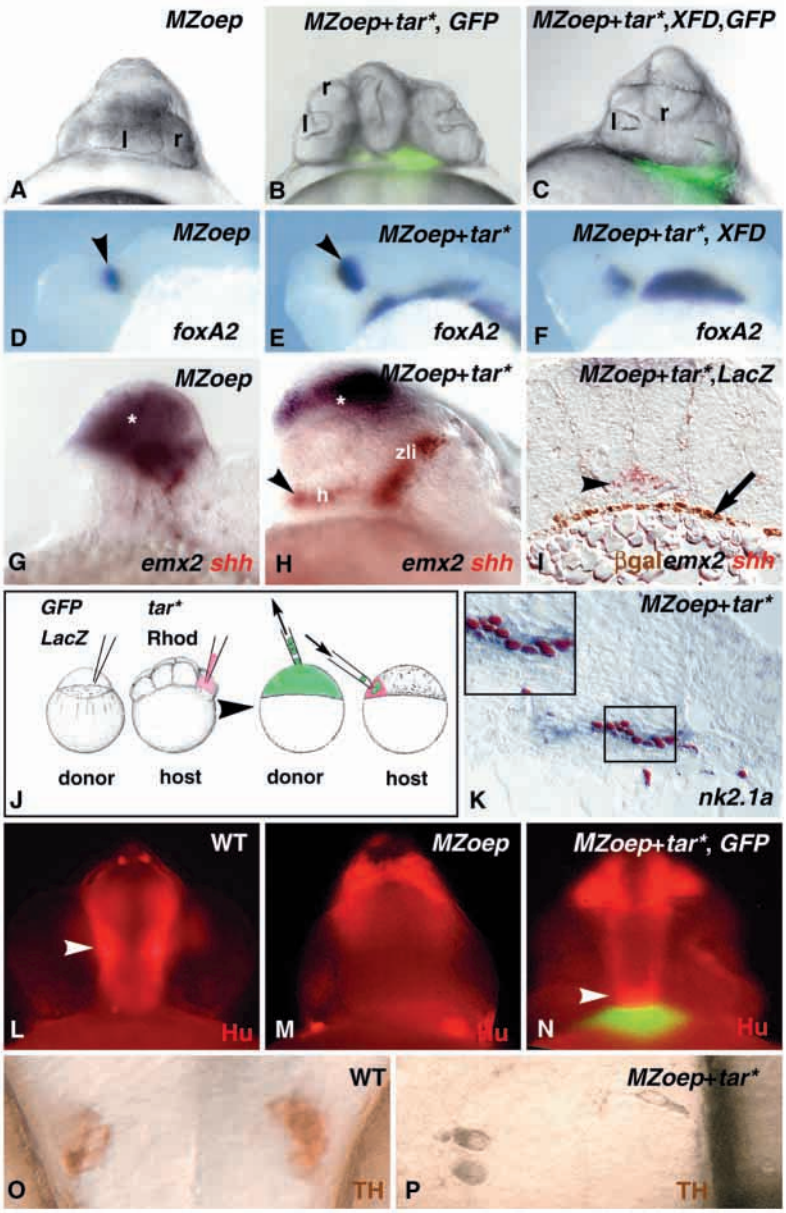

Fig. 4. Mesendoderm can rescue AD hypothalamus and neurogenesis in *MZoep* embryos. Views of wild-type embryos, *MZoep* embryos or *MZoep* embryos injected in one blastomere at the 16-cell stage with either *tar** RNA or *tar*+XFD* RNA together with *nls-GFP* or *nls-lacZ* RNA as lineage tracers. Genotype+injected RNA indicated top right. Probes and antibodies indicated in the bottom right-hand corner. (A-C) Frontal views of live embryos with GFP labeled rescued mesendoderm visible (green). Cyclopia is rescued in B. (D-F) Lateral views of brains with anterior towards the left showing expression of the endodermal marker *foxa2*. Expression in the brain (arrowhead) is at the zona limitans intrathalamica. (G-I) Lateral views (G,H) and transverse section (I) of brains showing absence/presence of hypothalamic markers. The rescued AD hypothalamus (black arrowheads) and the β-gal-positive endodermal *tar**-expressing cells (arrow) are indicated. Expression of *emx2* in G,H (asterisks) is in the dorsal brain (primarily telencephalon). (J) Schematic showing experimental approach in which *MZoep* cells expressing *GFP* and *lacZ* RNA are transplanted into the *tar**-expressing domain of a *tar** mRNA injected *MZoep* host. (K) Transverse section of a brain from an embryo that underwent the experimental procedure outlined in J. The transplanted cells (brown nuclei) contribute to the rescued hypothalamus stained with *nk2.1a* (blue). (L-N) Frontal views of embryos labeled to show Hu immunoreactive neurons in the ventral forebrain (white arrowheads). In N, neurons are restored above the rescued mesendoderm (GFP green). (O,P) Ventral views of brains labeled to show TH immunoreactive ventral diencephalic neurons. These neurons are absent in *MZoep* embryos (not shown) but are restored in the mesendoderm-rescued embryo shown in P. h, hypothalamus; l, lens; r, retina, t, telencephalon, zli, zona limitans intrathalamica.

lying beneath the brain (Fig. 4B,E,I) and cell-autonomously expressed the endodermal marker *axial/foxA2* (Fig. 4E, Table 2 and data not shown). Furthermore cyclopia was rescued in the injected embryos (Fig. 4B), suggesting that midline neural tissue had successfully migrated into the eye field separating left and right optic primordia.

The presence of mesendodermal tissues in *MZoep* embryos rescued ventral diencephalic expression of *shh* and *nk2.1a* (Fig. 4H,I and data not shown), suggesting that interaction with mesendoderm could induce AD hypothalamic markers in forebrain cells incapable of perceiving Nodal signals. By contrast, mesendoderm was unable to induce hypothalamic *emx2* expression (Fig. 4H,I), consistent with the conclusion that development of PV hypothalamic identity requires cell autonomous reception of Nodal signals.

One caveat of these results is that we could not be certain that the prospective hypothalamic cells had not inherited injected RNA at a level below that which we could detect by lineage tracers. If this was the case, the restoration of AD hypothalamus could be due to activation of Nodal signaling in the prospective hypothalamic cells themselves. To assess this possibility, we transplanted cells from donor *MZoep* embryos, which could therefore not inherit any injected RNA, into rescued host *MZoep* embryos. Sixty-two percent of the rescued chimeric embryos (n=29) showed a contribution of the transplanted cells to the hypothalamus (Fig. 4K) confirming that cells incapable of receiving Nodal signals can form AD hypothalamus in *MZoep* embryos with rescued mesendoderm.

Mesendoderm can rescue ventral diencephalic neurogenesis in *MZoep* embryos

The experiments described above indicate that mesendodermal signals can restore AD hypothalamic markers in *MZoep* embryos but the presence of these early markers does not allow assessment of whether differentiation of this tissue occurs. To address this point, we investigated whether the neurons that differentiate along the dorsal boundary of the hypothalamus are generated in mesendoderm-rescued *MZoep* embryos.

The RNA-binding protein Hu is expressed in most/all of the neurons that differentiate along the tract of the postoptic commissure (TPOC) and the enzyme tyrosine hydroxylase (TH) is initially expressed in a few neurons near to the TPOC (Patel et al., 1994; Guo et al., 1999). Immunoreactivity to both markers was completely absent in the ventral brain of *MZoep*

Table 2. Rescue of genes expression in *MZoep* embryos following *tar or *tar*+XFD* injection**

	Injected RNA			
	*tar**		*tar*+XFD*	
Marker/stage	% rescue	*n*	% rescue	*n*
shh/28 hpf*	9†	180	0	81
emx2/28 hpf*	0	180	0	81
Hu/28 hpf*	9†	43	–	–
TH/28 hpf*	6†	35	–	–
foxa2/28 hpf‡	100	33	100	26
gata5/shield	100	13	100	18
gsc/shield	100	32	0	33
shh/shield	72	33	–	–
bmp4/80%§	86	58	0	46
hgg1/80%	100	9	0	18

*Expression in the ventral diencephalon.
†Only a fraction of the *MZoep* mutants exhibited rescued AD hypothalamus upon localised TAR* expression. Sorting of injected embryos at the end of gastrulation according to the position of the injection relative to the dorsal side, showed that rescue of ventral CNS patterning only occurred in embryos that had been injected dorsally.
‡Endodermal expression
§Mesendodermal expression.

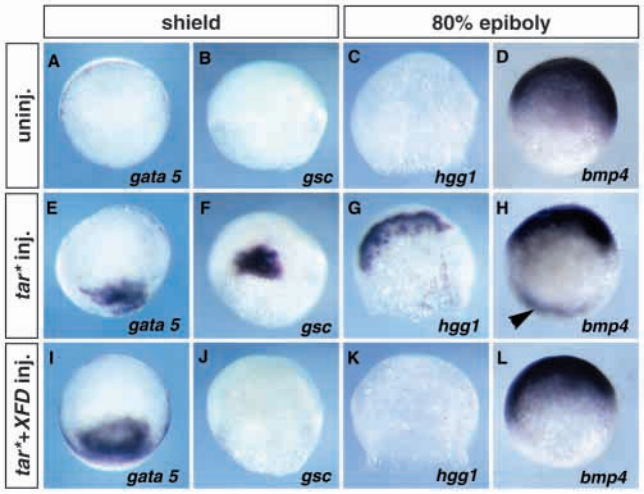

Fig. 5. Expression of endodermal and prechordal plate markers in *MZoep* gastrula following *tar** and *tar*+XFD* RNA injections. Views of *MZoep* gastrulae (stage indicated above and genes analyzed in the bottom right-hand corner). (A,D,E,H,I,L) Animal pole views with ventral towards the top; (B,C,F,G,J,K) dorsal views with the animal pole towards the top. (A-D) Uninjected embryos. (E-H) *tar** RNA-injected embryos. (H) The rescued prechordal plate expression of *bmp4* is indicated (arrowhead in H) and the remaining *bmp4* expression is in the non-neural ectoderm. (I-L) *tar*+XFD* RNA injected embryos.

embryos (Fig. 4M and data not shown) but was restored in *MZoep* embryos with rescued mesendoderm (Fig. 4N,P; Table 2). The neurons were positioned closer to the ventral midline than in wild-type embryos, consistent with the lack of PV hypothalamus in the mesendoderm-rescued *MZoep* embryos. These results indicate mesendoderm rescues the formation of the AD hypothalamus and restores neurogenesis in *MZoep* embryos.

The prechordal plate is required for rescue of the AD hypothalamus

Although it is widely accepted that tissues underlying the anterior neural plate influence the development of this tissue, the exact roles played by the different mesodermal and endodermal tissues remain less clear (Stern, 2001). In the experiments described above, we have shown that *tar**-expressing cells can restore AD hypothalamic development in *MZoep* embryos. To better understand this rescue, we assessed the identity of the mesendodermal tissues rescued by *tar** expression and attempted to dissect their relative roles in ventral brain patterning.

MZoep embryos lack expression of both general endodermal markers and markers of the prechordal plate mesendoderm (Gritsman et al., 1999). Upon injection of *tar**, markers of both of these tissues were recovered. We observed cell-autonomous restoration of endodermal expression of *gata5* and *foxa2* (Fig. 4E, Fig. 5E and data not shown) and prechordal plate expression of *gsc*, *bmp4* and *hgg1* (Fig. 5F-H, Table 2 and data not shown).

To distinguish between the hypothalamic patterning activity of the general endoderm and the prechordal plate, we generated *MZoep* embryos in which only general endoderm was restored. To do this, we co-expressed *tar** together with *XFD*, which encodes a dominant negative form of the *Xenopus* FGF receptor 1 (Amaya et al., 1993). Using this co-expression approach, the rescue of prechordal plate markers was abolished (Fig. 5J-L), while general endodermal markers were still present (Fig. 4F, Fig. 5I) and endodermal cells migrated apparently normally beneath the anterior brain (Fig. 4C).

MZoep embryos that possessed endoderm but no prechordal plate still exhibited cyclopia (Fig. 4C) and lacked expression of all hypothalamic markers (Table 2 and data not shown). This suggests that it is the prechordal plate and not the endoderm that is necessary for restoration of hypothalamic development in *MZoep* embryos. An alternative possibility is that although endoderm is present, its functional properties may be altered by XFD expression. Therefore to complement these studies, we examined hypothalamic development in *casanova* (*cas*) mutant embryos that lack endoderm (Alexander et al., 1999) but retain prechordal plate mesendoderm. In $cas^{-/-}$ embryos, hypothalamic development appeared normal (data not shown), suggesting that signals from the prechordal plate are sufficient to mediate hypothalamic development in the absence of general endoderm. Altogether, these studies indicate that the prechordal plate is both necessary and sufficient to mediate hypothalamic development.

Hh signaling promotes early AD hypothalamic development but may suppress PV hypothalamic development

Previous studies have implicated the Hh pathway in hypothalamic development both in fish (Karlstrom et al., 1999; Rohr et al., 2001; Varga et al., 2001) and in other species (Chiang et al., 1996; Patten and Placzek, 2000). As Hh genes are expressed both in the axial mesendoderm and in the ventral diencephalon, then Hh signals are candidates for mediating the prechordal plate rescue of AD hypothalamus in *MZoep* embryos. Indeed, expression of *tar** cell-autonomously restores *shh* expression to the prospective axial mesendoderm of *MZoep* embryos (Fig. 6F, Table 2 and data not shown),

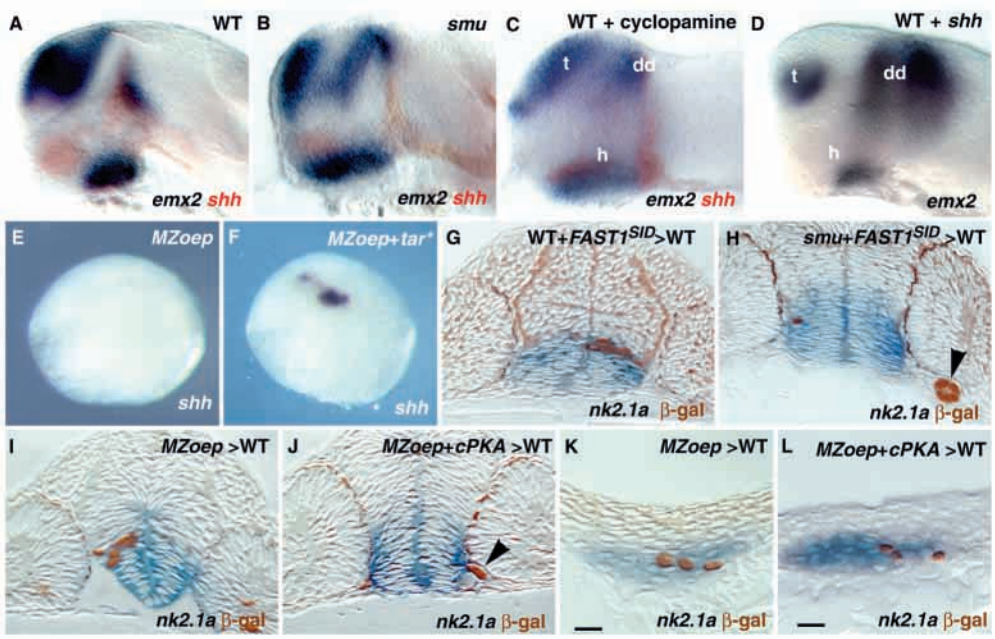

Fig. 6. Hh and Nodal signals have both discrete and cooperative roles in hypothalamus formation. In all panels, genotype + injected RNA/drug treatment is shown in the top right-hand corner, genes/proteins visualized are indicated in the bottom right-hand corner. (A-D) Lateral views of 28 hpf brains. (E-F) Dorsal views of shield stage *MZoep* embryos showing induction of *shh* expression upon *tar** RNA injection. (G-L) Sections through the hypothalami of wild-type embryos in which β-gal-labeled cells (brown) were transplanted into the prospective hypothalamus at shield stage. (G-J) Sections through 28 hpf brains. The arrowheads in H,J indicate cells that have been expelled to the mesenchyme around the eyes. This was never observed for *MZoep* cells or cells expressing *Fast1*^{SID} and thus does not depend on the depth at which cells were transplanted into the epiblast. (K,L) Sections through bud stage neural plates. Cells were found at various positions along the anteroposterior axis within the *nk2.1a* expression domain. dd, dorsal diencephalon; h, hypothalamus; t, telencephalon. Scale bars: 25 μm.

indicating that Hh signals are likely to be present in these embryos. To further investigate the role of the Hh pathway in hypothalamic development, we assessed the expression of hypothalamic markers in embryos with compromised Hh signaling. *smu*$^{-/-}$ embryos have a reduced hypothalamus in which markers expressed along the dorsal edge of the hypothalamus, such as *nk2.2*, are reduced or absent (Rohr et al., 2001; Varga et al., 2001). Supporting such observations, *shh* expression in the AD hypothalamus is reduced in *smu*$^{-/-}$ embryos (Fig. 6B).

In striking contrast to the loss of AD hypothalamus, we observed an expansion of *emx2* expression in the hypothalamus of *smu*$^{-/-}$ embryos. This expansion of PV gene expression into the AD hypothalamic domain may also account for the previous observation of rostral extension of *emx1* expression in the ventral brain (Varga et al., 2001). As *smu*$^{-/-}$ embryos retain a small amount of Hh signaling mediated through maternal Smu protein (Chen et al., 2001; Lewis and Eisen, 2001; Varga et al., 2001), we complemented these studies by using the Hh pathway inhibitor cyclopamine (Incardona et al., 1998) to block Hh activity. Wild-type embryos treated with cyclopamine exhibited a phenotype very similar to *smu*$^{-/-}$ embryos in which PV hypothalamus was expanded and AD hypothalamus was reduced (Fig. 6C).

If loss or severe reduction of Hh activity leads to expansion of PV hypothalamus and reduction of AD hypothalamus, then we might expect the opposite phenotype if Hh activity is increased. We therefore overexpressed *shh* in wild-type embryos and assessed effects upon hypothalamic markers. Previous studies have shown that general or dorsal hypothalamic markers are expanded in *shh*-injected embryos (Barth and Wilson, 1995; Rohr et al., 2001). By contrast, *shh* overexpression leads to severe reduction or occasionally loss, of the PV hypothalamic marker *emx2* (Fig. 6D).

Altogether, these results reveal distinct roles for Nodal and Hh signals in the development of hypothalamic sub-domains. PV hypothalamic development requires reception of Nodal signals but may be inhibited or limited by Hh signals. By contrast, there is no absolute requirement for AD hypothalamic cells to perceive Nodal signals, whereas Hh activity favors the development of this region of the ventral diencephalon.

Cells unable to perceive Hh signals are compromised in their ability to participate in hypothalamic development

Although AD hypothalamic development is promoted by Hh activity, our results do not formally show that it is the hypothalamic cells that need to receive Hh signals. To begin to address the requirement for Hh signaling in the hypothalamic precursors, we transplanted cells compromised in their ability to activate the Hh signaling pathway cell-autonomously, through overexpression of constitutively active PKA (cPKA) (Concordet et al., 1996), into wild-type embryos and assessed their ability to contribute to the hypothalamus.

By day one of development, cPKA-expressing cells were found in both hypothalamic sub-domains (data no shown), suggesting that activation of the Hh signaling pathway is not an absolute requirement for cells to contribute to the hypothalamus. This supports the observation that some AD hypothalamic tissue is retained in *smu* and cyclopamine-treated embryos. However, in contrast to wild-type cells, some cPKA-expressing cells were excluded from the hypothalamus and found in the head mesenchyme (not shown, but see below). Below, we further explore the possibility that Hh signaling is required for cells to remain integrated and viable within the hypothalamus.

Hh and Nodal pathways cooperate to enable cells to maintain hypothalamic fate

Given that prospective hypothalamic cells are exposed both to

Nodal and Hh signals, we considered it possible that abrogation of both pathways may reveal interactions between the pathways. We therefore used two different approaches to cell autonomously inhibit both Nodal and Hh signaling and then assessed the ability of such cells to contribute to the hypothalamus.

Through both experimental approaches, we found that cells unable to transduce Nodal and Hh signals were compromised in their ability to contribute to the entire hypothalamus. First, transplanted *smu*$^{-/-}$ cells expressing *FAST1*^{SID} were found both in the AD hypothalamus and in the head mesenchyme (n=12 out of 12, Fig. 6H), while wild-type cells expressing *FAST1*^{SID} colonized the hypothalamus exclusively (n=23 out of 24, Fig. 6G). *smu* cells may retain some ability to transduce Hh signals through maternal Smu, and so as a second strategy, we transplanted *MZoep* cells expressing cPKA in wild-type hosts. In this situation, none of the transplanted cells colonized the hypothalamus and instead, all were found in the head mesenchyme (n=12 out of 12, Fig. 6J). This exclusion is unlikely to be due to a toxic effect of cPKA as expressing cells transplanted elsewhere integrated successfully into the eye and telencephalon.

The absence of Nodal and Hh signaling-compromised cells from the hypothalamus could be due to these cells being expelled from the prospective hypothalamus at any time following transplantation. To investigate this point, we assessed the location of the transplanted cells at the end of gastrulation. In 40 of 53 cases, the transplanted cells were within the prospective hypothalamus (Fig. 6K,L). This indicates that cells compromised in their ability to activate the Nodal and Hh signaling pathways can still move rostrally within the neural plate and occupy the prospective hypothalamus. It further suggests that such cells are excluded from the hypothalamus at later developmental stages, although we have no direct evidence of how this is achieved. Thus, the ability to activate the Nodal and Hh signaling pathways is essential for cells to be maintained within the maturing hypothalamus.

DISCUSSION

During early CNS development, prospective hypothalamic cells move rostrally within the neural plate, acquire hypothalamic identity and adopt region-specific hypothalamic fates. In this study, we have elucidated the roles of the Nodal and Hh pathways in regulating these events. Although all steps of hypothalamic development are blocked in the absence of Nodal activity, we have shown that the regionalization of the hypothalamus is the only event for which cell-autonomous reception of Nodal signals is an essential requirement. If cells cannot perceive Nodal signals, then they do not contribute to the PV hypothalamus. By contrast, Nodal signaling compromised cells can move within the neural plate and contribute to the AD hypothalamus. We suggest that these aspects of hypothalamic development are, at least in part, indirectly regulated by Nodal activity in the prechordal plate. In support of this interpretation, Hh signaling may be dispensable for establishment of the PV hypothalamus but promotes the development of the AD hypothalamus. Finally, when cells are compromised in their ability to receive both Nodal and Hh signals, then they are entirely excluded from the hypothalamus. This suggests that in addition to discrete roles for Nodal and Hh signaling in AD and PV hypothalamic development, these pathways cooperate to enable cells to contribute to the maturing hypothalamus.

Nodal signaling is required for PV hypothalamic development

The *nk2.1a*-expressing hypothalamic neuroepithelium is divided into at least two major subdomains: the *emx2*-expressing PV hypothalamus and the *shh*-expressing AD hypothalamus. Our studies provide two sets of data, indicating that cell-autonomous reception of Nodal signals is required for cells to contribute to PV hypothalamus. First, cells compromised in their ability to transduce Nodal signals, either because of the absence of Oep or because of overexpression of dominant-negative Fast1, are excluded from PV hypothalamus. Second, restoration of Nodal signaling in mesendoderm beneath CNS tissue that cannot perceive Nodal signals fails to rescue PV hypothalamus. By contrast, full restoration of Nodal signaling in both mesendoderm and neural ectoderm leads to complete rescue of CNS development (Gritsman et al., 1999). Cyc is the Nodal protein most likely to be responsible for establishment of PV hypothalamic identity as it is expressed beneath the neural plate throughout gastrulation and subsequently within the prospective hypothalamus itself (Rebagliati et al., 1998; Sampath et al., 1998).

Cells unable to transduce Nodal signals not only fail to express the PV hypothalamic marker *emx2*, but are excluded from the presumptive PV hypothalamus and occupy a territory fated to its AD counterpart. This suggests differences in cell surface properties between PV hypothalamic cells that have received Nodal signals and the *MZoep* or Fast1^{SID} cells that have been unable to activate Nodal signaling. This observation contributes to a growing body of evidence that Nodal signaling regulates not only cell fate decisions, but coordinately also regulates cell behaviors (Carmany-Rampey and Schier, 2001; David and Rosa, 2001).

The cell surface proteins that are regulated by Nodal activity are largely unknown, although subtractive screens and other approaches are beginning to identify candidates that include cadherins, ephrins and various other proteins (Dickmeis et al., 2001; Liu et al., 2001). Indeed, the exclusion of *MZoep* cells from the PV hypothalamus appears very similar to the exclusion of cells from specific hindbrain domains that occurs when Eph/Ephrin signaling is disrupted (Mellitzer et al., 2000; Cooke et al., 2001) highlighting the Eph/Ephrin signaling pathway as a potentially interesting target for Nodal activity in the ventral forebrain.

Hh signaling promotes AD and limits PV hypothalamic development

Although AD hypothalamus is absent in embryos with severely compromised Nodal signaling, cells unable to receive Nodal signals are still able to contribute to this region of the brain. The absence of AD hypothalamus in Nodal pathway mutants is therefore likely to be due to Nodal-dependent regulation of other factors involved in AD hypothalamic development. Among these factors is Shh. Expression of *shh*

is reduced or absent in Nodal-pathway mutants (Krauss et al., 1993; Rohr et al., 2001) and is restored upon activation of the Nodal signaling pathway. Shh also induces dorsal hypothalamic markers (Barth and Wilson, 1995) and such markers are reduced or absent in Hh pathway mutants (Rohr et al., 2001; Varga et al., 2001). The regulation of Hh signaling by the Nodal pathway may in part be through direct regulation of *hh* gene expression by transcriptional effectors of Nodal activity (Müller et al., 2000).

An unexpected finding was that although AD hypothalamus is severely reduced in embryos with compromised Hh activity, PV hypothalamus is actually expanded. Hh signaling must therefore limit the extent of PV tissue. We do not know how this happens, although the expansion of *emx2* expression towards the AD hypothalamus in *smu* mutants suggests a change in allocation of regional fates within the hypothalamus. Indeed, the expansion of *emx* gene expression domains in both the hypothalamus and in the telencephalon of Hh pathway mutants (this study) (Varga et al., 2001) suggests that high levels of Hh activity may more generally inhibit *emx* gene expression and limit the extent of *emx*-expressing CNS domains. We suggest that *emx* genes in the forebrain behave like class 1 homeobox genes within the spinal cord, for which expression is inhibited when Hh activity is above a certain threshold (Lee and Pfaff, 2001). Indeed, *emx2* expression is absent from the hypothalamus at early stages when *shh* is expressed throughout the axial mesendoderm and neuroectoderm, and *emx2* expression only appears concordant with the downregulation of *shh* in the PV hypothalamus.

Whether there is an absolute requirement for Hh activity in hypothalamic induction remains uncertain. In vitro studies have shown that Hh activity can induce hypothalamic markers in naïve neuroepithelium (Dale et al., 1997) and mice lacking Shh function appear to lack a hypothalamus (Chiang et al., 1996). However, it has not been assessed if there is a direct requirement for Shh activity in hypothalamic precursors in mice, nor has it been assessed if PV hypothalamic development is initiated in *shh*-null mice. In fish, as *smu* embryos retain some Hh activity mediated by maternal Smu, it remains possible that an early, perhaps low level of Hh activity is required for induction of the hypothalamus, even if later high levels of Hh activity inhibit PV hypothalamic development. However, in the absence of Nodal signaling, Hh activity is unable to induce early hypothalamic marker (Rohr et al., 2001), suggesting that Hh signaling alone is not sufficient to induce hypothalamus.

A cooperative role for Nodal and Hh signaling

Although AD hypothalamic cells do not need to be able to transduce Nodal signals, Nodal signaling does contribute to the development of this region. Most obviously, it does so through the regulation of Hh activity but several of our experiments indicate a Hh-independent role for Nodal signals in AD hypothalamic development. Notably, we find that cells compromised in their ability to perceive both Hh and Nodal signals are excluded from the entire hypothalamus. As abrogation of either pathway alone fails to produce such a severe phenotype, then we conclude that both pathways cooperate to enable cells to contribute to the maturing hypothalamus. These same cells are not compromised in their ability to express early hypothalamic markers and it is only subsequent to hypothalamic induction that they are excluded from the brain. It is intriguing that the Nodal/Hh signaling-compromised cells are excluded from the brain altogether rather than incorporating into more dorsal regions of the diencephalon. This again suggests differences in cell-surface properties, in this case between hypothalamic cells and more dorsal diencephalic cells. Perhaps analogous to this situation in the ventral forebrain is the observation that during normal development, ventral midline cells in the hindbrain appear unable to leave the floorplate and enter the basal plate, although more dorsal cells can freely move within the DV axis of the neural tube (Fraser et al., 1990).

The prechordal plate mediates hypothalamic development

Although tissues underlying the neural plate are believed to have essential roles in the induction and patterning of CNS structures (Muenke and Beachy, 2000), evidence regarding the exact roles of specific mesendodermal cell populations is often conflicting or confusing. In part, this is due to apparent differences in activities of mesendodermal derivatives in different species and uncertainty in identification of homologous cell populations between species (Stern, 2001). The prechordal mesendoderm is a derivative of the organizer that gives rise to a loosely defined population of cells beneath the neural plate, termed the prechordal plate. It has been proposed that prechordal mesendoderm is essential for establishment both of anterior and of ventral CNS (Kiecker and Niehrs, 2001). Our study provides evidence that the prechordal plate mediates the specification of the anterodorsal hypothalamus. Most notably, hypothalamic tissue is absent in MZ*oep* embryos with rescued anterior endoderm but lacking prechordal plate, whereas anterior-dorsal hypothalamus is induced when prechordal plate is present, whether or not anterior endoderm is absent. These observations indicate that in fish, it is the prechordal plate and not the anterior endoderm that mediates hypothalamic development, by regulating the movements of cells in the overlying neural plate or by mediating inductive events.

The prechordal plate expresses numerous secreted proteins that could affect overlying neural tissue, including (in fish) Wnt8b and the Wnt antagonist Dkk, the Nodal protein Cyc, the Hh proteins Shh and Twhh, and the BMP proteins Bmp4, Bmp7 and Admp. Nodal signaling is required for development of the prechordal plate itself (Schier et al., 1997) and we have shown that Nodal signals are required for development of the PV hypothalamus. As discussed above, Hh proteins initially likely to originate from the prechordal plate and subsequently from the CNS itself, also promote hypothalamic development. The roles of other secreted proteins produced in the prechordal plate remain less certain (reviewed by Kiecker and Niehrs, 2001).

We are grateful to L. Bally-Cuif, P. Herbomel, C. Houart, S. Schneider-Maunoury and Z. Varga for comments and discussions; our colleagues in the community for reagents; and F. Chelghoum, F. Bouallague and the UCL Fish Facility team for fish care. This study was supported by INSERM, ARC (N. P., F. R.), AFM, ACI (F. R.) and the Wellcome Trust (S. W.). N. P. is a CNRS Senior Research Fellow and S. W. is a Wellcome Trust Senior Research Fellow.

REFERENCES

Alexander, J., Rothenberg, M., Henry, G. L. and Stainier, D. Y. (1999). Casanova plays an early and essential role in endoderm formation in zebrafish. *Dev. Biol.* **215**, 343-357.

Amaya, E., Stein, P. A., Musci, T. J. and Kirschner, M. W. (1993). FGF signalling in the early specification of mesoderm in Xenopus. *Development* **118**, 477-487.

Barresi, M. J., Stickney, H. L. and Devoto, S. H. (2000). The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity. *Development* **127**, 2189-2199.

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.

Brand, M., Heisenberg, C. P., Warga, R. M., Pelegri, F., Karlstrom, R. O., Beuchle, D., Picker, A., Jiang, Y. J., Furutani-Seiki, M., van Eeden, F. J. et al. (1996). Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* **123**, 129-142.

Carmany-Rampey, A. and Schier, A. F. (2001). Single-cell internalization during zebrafish gastrulation. *Curr. Biol.* **11**, 1261-1265.

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.

Chen, Y. and Schier, A. F. (2001). The zebrafish Nodal signal Squint functions as a morphogen. *Nature* **411**, 607-610.

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.

Concordet, J. P., Lewis, K. E., Moore, J. W., Goodrich, L. V., Johnson, R. L., Scott, M. P. and Ingham, P. W. (1996). Spatial regulation of a zebrafish patched homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development* **122**, 2835-2846.

Cooke, J., Moens, C., Roth, L., Durbin, L., Shiomi, K., Brennan, C., Kimmel, C., Wilson, S. and Holder, N. (2001). Eph signalling functions downstream of Val to regulate cell sorting and boundary formation in the caudal hindbrain. *Development* **128**, 571-580.

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.

David, N. B. and Rosa, F. M. (2001). Cell autonomous commitment to an endodermal fate and behaviour by activation of Nodal signalling. *Development* **128**, 3937-3947.

Dickmeis, T., Mourrain, P., Saint-Etienne, L., Fischer, N., Aanstad, P., Clark, M., Strahle, U. and Rosa, F. (2001). A crucial component of the endoderm formation pathway, CASANOVA, is encoded by a novel sox-related gene. *Genes Dev.* **15**, 1487-1492.

Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schier, A. F. and Talbot, W. S. (1998). Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature* **395**, 181-185.

Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-435.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W. S. and Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* **97**, 121-132.

Guo, S., Wilson, S. W., Cooke, S., Chitnis, A. B., Driever, W. and Rosenthal, A. (1999). Mutations in the zebrafish unmask shared regulatory pathways controlling the development of catecholaminergic neurons. *Dev. Biol.* **208**, 473-487.

Hammerschmidt, M., Pelegri, F., Mullins, M. C., Kane, D. A., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C. P. et al. (1996). Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, Danio rerio. *Development* **123**, 143-151.

Hatta, K., Kimmel, C. B., Ho, R. K. and Walker, C. (1991). The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* **350**, 339-341.

Hatta, K., Puschel, A. W. and Kimmel, C. B. (1994). Midline signaling in the primordium of the zebrafish anterior central nervous system. *Proc. Natl. Acad. Sci. USA* **91**, 2061-2065.

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

Hauptmann, G. and Gerster, T. (1996). Complex expression of the zp-50 pou gene in the embryonic zebrafish brain is altered by overexpression of sonic hedgehog. *Development* **122**, 1769-1780.

Heisenberg, C. P. and Nüsslein-Volhard, C. (1997). The function of silberblick in the positioning of the eye anlage in the zebrafish embryo. *Dev. Biol.* **184**, 85-94.

Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. *Development* **125**, 3553-3562.

Karlstrom, R. O., Talbot, W. S. and Schier, A. F. (1999). Comparative synteny cloning of zebrafish you-too: mutations in the Hedgehog target gli2 affect ventral forebrain patterning. *Genes Dev.* **13**, 388-393.

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

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 actvity in Zebrafish embryos. *Cell* **75**, 1431-1444.

Lee, S. K. and Pfaff, S. L. (2001). Transcriptional networks regulating neuronal identity in the developing spinal cord. *Nat. Neurosci.* **4**, 1183-1191.

Lewis, K. E. and Eisen, J. S. (2001). Hedgehog signaling is required for primary motoneuron induction in zebrafish. *Development* **128**, 3485-3495.

Liu, Q., Marrs, J. A., Chuang, J. C. and Raymond, P. A. (2001). Cadherin-4 expression in the zebrafish central nervous system and regulation by ventral midline signaling. *Dev. Brain Res.* **131**, 17-29.

Mellitzer, G., Xu, Q. and Wilkinson, D. G. (2000). Control of cell behaviour by signalling through Eph receptors and ephrins. *Curr. Opin. Neurobiol.* **10**, 400-408.

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

Muenke, M. and Beachy, P. A. (2000). Genetics of ventral forebrain development and holoprosencephaly. *Curr. Opin. Genet. Dev.* **10**, 262-269.

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.

Nikaido, M., Tada, M., Saji, T. and Ueno, N. (1997). Conservation of BMP signaling in zebrafish mesoderm patterning. *Mech. Dev.* **61**, 75-88.

Patel, C. K., Rodriguez, L. C. and Kuwada, J. Y. (1994). Axonal outgrowth within the abnormal scaffold of brain tracts in a zebrafish mutant. *J. Neurobiol.* **25**, 345-360.

Patten, I. and Placzek, M. (2000). The role of Sonic hedgehog in neural tube patterning. *Cell Mol. Life Sci.* **57**, 1695-1708.

Peyrieras, N., Strahle, U. and Rosa, F. (1998). Conversion of zebrafish blastomeres to an endodermal fate by TGF-beta-related signaling. *Curr. Biol.* **8**, 783-786.

Pogoda, H., Solnica-Krezel, L., Driever, W. and Meyer, D. (2000). The zebrafish forkhead transcription factor FoxH1/Fast1 is a modulator of nodal signaling required for organizer *formation*. *Curr. Biol.* **10**, 1041-1049.

Puelles, L. (1995). A segmental morphological paradigm for understanding vertebrate forebrains. *Brain Behav. Evol.* **46**, 319-337.

Rebagliati, M. R., Toyama, R., Haffter, P. and Dawid, I. B. (1998). cyclops encodes a nodal-related factor involved in midline signaling. *Proc. Natl. Acad. Sci. USA* **95**, 9932-9937.

Reissmann, E., Jornvall, H., Blokzijl, A., Andersson, O., Chang, C., Minchiotti, G., Persico, M. G., Ibanez, C. F. and Brivanlou, A. H. (2001). The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev.* **15**, 2010-2022.

Renucci, A., Lemarchandel, V. and Rosa, F. M. (1996). An activated form of type I serine/threonine kinase receptor TARAM-A reveals a specific signalling pathway involved in fish head organiser formation. *Development* **122**, 3735-3743.

Rodaway, A., Takeda, H., Koshida, S., Broadbent, J., Price, B., Smith, J. C., Patient, R. and Holder, N. (1999). Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-beta family signals and discrimination of mesoderm and endoderm by FGF. *Development* **126**, 3067-3078.

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.

Sampath, K., Rubinstein, A. L., Cheng, A. M., Liang, J. O., Fekany, K., Solnica-Krezel, L., Korzh, V., Halpern, M. E. and Wright, C. V. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* **395**, 185-189.

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.

Schier, A. F. and Shen, M. M. (2000). Nodal signalling in vertebrate development *Nature* **403**, 385-389.

Sirotkin, H. I., Gates, M. A., Kelly, P. D., Schier, A. F. and Talbot, W. S. (2000). fast1 is required for the development of dorsal axial structures in zebrafish. *Curr. Biol.* **10**, 1051-1054.

Stachel, S. E., Grunwald, D. J. and Myers, P. Z. (1993). Lithium perturbation and goosecoid expression identify a dorsal specification pathway in the pregastrula zebrafish. *Development* **117**, 1261-1274.

Stern, C. D. (2001). Initial patterning of the central nervous system: how many organizers? *Nat. Rev. Neurosci.* **2**, 92-98.

Strahle, U., Blader, P. and Ingham, P. W. (1996). Expression of axial and sonic hedgehog in wildtype and midline defective zebrafish embryos. *Int. J. Dev. Biol.* **40**, 929-940.

Strähle, U., Jesuthasan, S., Blader, P., Garcia-Villalba, P., Hatta, K. and Ingham, P. W. (1997). one-eyed pinhead is required for development of the ventral midline of the zebrafish (Danio rerio) neural tube. *Genes Func.* **1**, 131-148.

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.

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. and Postlethwait, J. H. (2001). Zebrafish smoothened functions in ventral neural tube specification and axon tract formation. *Development* **128**, 3497-3509.

Whitman, M. (1998). Smads and early developmental signaling by the TGFbeta superfamily. *Genes Dev.* **12**, 2445-2462.

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

Zhang, J., Talbot, W. S. and Schier, A. F. (1998). Positional cloning identifies zebrafish one-eyed pinhead as a permissive EGF-related ligand required during gastrulation. *Cell* **92**, 241-251.