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# The morphogenetic role of midline mesendoderm and ectoderm in the development of the forebrain and the midbrain of the mouse embryo

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## The morphogenetic role of midline mesendoderm and ectoderm in the development of the forebrain and the midbrain of the mouse embryo

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### SUMMARY

The anterior midline tissue (AML) of the late gastrula mouse embryo comprises the axial mesendoderm and the ventral neuroectoderm of the prospective forebrain, midbrain and rostral hindbrain. In this study, we have investigated the morphogenetic role of defined segments of the AML by testing their inductive and patterning activity and by assessing the impact of their ablation on the patterning of the neural tube at the early-somite-stage. Both rostral and caudal segments of the AML were found to induce neural gene activity in the host tissue; however, the de novo gene activity did not show any regional characteristic that might be correlated with the segmental origin of the AML. Removal of the rostral AML that contains the prechordal plate resulted in a truncation of the head accompanied by the loss of several forebrain markers.

However, the remaining tissues reconstituted *Gsc* and *Shh* activity and expressed the ventral forebrain marker *Nkx2.1*. Furthermore, analysis of *Gsc*-deficient embryos reveals that the morphogenetic function of the rostral AML requires *Gsc* activity. Removal of the caudal AML led to a complete loss of midline molecular markers anterior to the 4th somite. In addition, *Nkx2.1* expression was not detected in the ventral neural tube. The maintenance and function of the rostral AML therefore require inductive signals emanating from the caudal AML. Our results point to a role for AML in the refinement of the anteroposterior patterning and morphogenesis of the brain.

Key words: Prechordal plate, Ventral diencephalon, Neurulation, Patterning activity, Regionalisation, Mouse

### INTRODUCTION

Regionalisation of the neural plate in *Xenopus* and chick embryos has been shown to be regulated by both planar and vertical signals emanating from the gastrula organizer and its mesendodermal derivatives (Sharpe and Gurdon, 1990; Storey et al., 1992; Ruiz i Altaba, 1993; Storey et al., 1995; Lumsden and Krumlauf, 1996). In the mouse, recent findings suggest that the anterior identity of the neural axis may be specified independently of the organizer's derivatives and mediated by the activity of the visceral endoderm of the early embryo (Thomas and Beddington, 1996; Bouwmeester and Leyns, 1997; Varlet et al., 1997; Beddington and Robertson, 1998, 1999; Ding et al., 1998; Knoetgen et al., 1999; Sun et al., 1999). Mutations of the *Lim1* and *Otx2* genes encoding putative transcription factors, which are expressed in the visceral endoderm and also in the anterior mesendoderm, result in the loss of anterior neural structures (Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995; Ang et al., 1996). Embryological studies on ES cell↔embryo chimeras have provided a means to dissect genetically the relative role of the visceral endoderm and primitive-streak-derived anterior mesendoderm during anterior neural patterning (Rossant and Spence, 1998). Interestingly, the anterior deficiency of the *Otx2*

and *Lim1* in mutant embryos cannot be fully rescued in chimeras suggesting a requirement for wild-type primitive-streak-derived mesendoderm (Rhinn et al., 1998; Shawlot et al., 1999). In addition, recent analysis of the *Wnt3* null-mutation has demonstrated that, although anterior visceral endoderm markers are expressed and correctly positioned in the mutant embryo, the ectoderm lacks anteroposterior (AP) neural patterning (Liu et al., 1999). Collectively, these findings suggest that, following the initial specification of anterior identity by the extraembryonic tissues, subsequent morphogenetic interactions provided by the anterior mesendoderm are required for the complete development and patterning of the anterior neural structures.

Fate-mapping studies have shown that cells of the posterior epiblast of the early primitive streak mouse embryo contribute to the prechordal plate and the most anterior part of the ventral neural tube of the early-somite-stage embryo (Lawson et al., 1991; Tam et al., 1997). Cells of the head process that lie rostral to the node of the late gastrula embryo also contribute to the anterior midline mesendoderm and ventral tissues of the brain (Tam et al., 1982; Beddington, 1994; Sulik et al., 1994). In contrast to the avian embryo where the prechordal plate and the head process are distinct in their anatomy and tissue fate (Selleck and Stern, 1991; Seifert et al., 1993; Dale et al., 1999),

these structures in the mouse are contiguous, with no clear anatomical distinction and their developmental fate is not well characterised (for review, Tam and Behringer, 1997). However, at the early-neural-plate stage of the mouse embryo, the expression pattern of several genes seems to reveal a fine subdivision within the anterior axial mesendoderm. In the most rostral segment, underlying the prospective forebrain, *Gsc*, *Shh* and *Hnf3 $\beta$*  are expressed (Chiang et al., 1996; Filosa et al., 1997). By contrast, genes such as *Lim1*, *Otx2*, *Hnf3 $\beta$* , *Shh* and *Bmp7* are expressed in the rest of the anterior axial mesendoderm, which underlies the prospective midbrain and hindbrain (Acampora et al., 1995; Lyons et al., 1995; Matsuo et al., 1995; Ang et al., 1996; Chiang et al., 1996; Filosa et al., 1997; Belo et al., 1998; Perea-Gomez et al., 1999). At a more posterior level, the notochord that is derived from the node of the late gastrula expresses *Brachyury (T)*, *Hnf3 $\beta$* , *Shh* and *Noggin* (Wilkinson et al., 1990; Chiang et al., 1996; Filosa et al., 1997; McMahon et al., 1998).

It has been postulated that, in vertebrate embryos, the mesendoderm is instrumental in specifying different AP segments of the neural axis. Studies supporting this concept have demonstrated that transplantation of different segments of the axial mesendoderm can induce neural tissues displaying the morphological and molecular properties consistent with the segmental origin of the graft (Sharpe and Gurdon, 1990; Ruiz i Altaba, 1994; Foley et al., 1997). In the chick, there is inconsistency regarding the extent to which derivatives of the organizer, such as the head process or the prechordal mesoderm, possess neural-inducing or regionalising activity (Darnell et al., 1992; Dale et al., 1997; Foley et al., 1997; Pera and Kessel, 1997; Rowan et al., 1999). Transplantation experiments demonstrated that the prechordal plate possesses neuralising properties and produces signals that ventralise the prospective forebrain (Pera and Kessel, 1997). However, another study showed that prechordal tissues do not have neural-inducing ability but can confer more anterior character to posterior neuroectoderm (Foley et al., 1997). Investigations of the properties of defined regions of the head process showed that this tissue refines the rostrocaudal character rather than specifies regional differences within the neural plate (Rowan et al., 1999). Remarkably, AP polarity can be specified in zebrafish mutants, such as *floating head* and *no tail*, which exhibit defects in axial mesendoderm and in embryos following the ablation of the presumptive prechordal tissue (Halpern et al., 1993; Talbot et al., 1995; Grinblat et al., 1998). Similarly, homozygous *Hnf3 $\beta$*  mutant mouse embryos that lack the node and axial mesendoderm show appropriate AP patterning of the neural tube from the forebrain to the posterior end of the spinal cord (Ang and Rossant, 1994; Weinstein et al., 1994; Klingensmith et al., 1999). In the mouse, germ layer recombination explant experiments have shown that the mesendoderm from head-fold embryos (which includes some axial, paraxial and lateral tissues) can regulate the expression of two region-specific neural genes, *Otx2* and *En1*, within the epiblast tissue (Ang and Rossant, 1993; Ang et al., 1994). Despite these findings, the role of the mouse axial midline tissues during anterior neural development remains unresolved.

In the present study, we have undertaken an embryological approach to study the morphogenetic role of the axial tissues by analyzing the effects of removing defined segments of the anterior midline tissues (AML) on the development of specific

brain regions. We have found that removal of the rostral region of the AML severely affects the patterning of the forebrain, although some degree of AP patterning is observed in other regions of the brain. Interestingly, ablated embryos can reform tissue that resembles the prechordal plate and specifies ventral forebrain tissue. Furthermore, our results have shown that the caudal AML is required for the maintenance and the specification of the AML comprising the prechordal plate and presumptive ventral diencephalon. Analysis of gene expression of *Gsc*-deficient embryos demonstrated that *Gsc* activity is involved in the patterning of the ventral neural tube. In addition, transplantation of AML fragments to ectopic sites in recipient embryos has provided evidence of the neural inductive activity of the AML. Collectively, results of these experiments point to a role for AML in the maintenance and refinement of AP patterning in the neurulating mouse embryo.

## MATERIALS AND METHODS

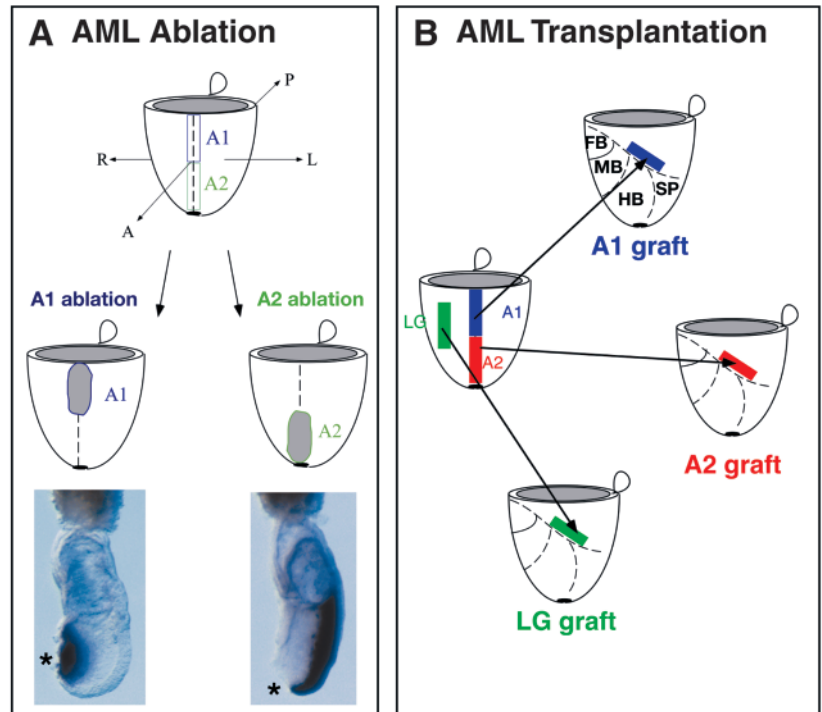
### Micromanipulation of mouse embryos

Mouse embryos were explanted from pregnant ARCs and *Gsc* mutant mice (Rivera-Perez et al., 1995; M. W. and R. R. B., unpublished) at 7.5 days postcoitum (dpc). Embryos from no allantoic bud to late bud stage (Downs and Davies, 1993), which display early signs of neural plate formation but no thickening of the anterior ectoderm, were selected for experiments. The AML that consists of the mesendoderm and the overlying ectoderm between the anterior edge of the neural plate and the node of the embryo was microsurgically ablated using finely drawn glass needles or electrolytically polished alloy needles. Three types of ablation were performed (Fig. 1A) to remove (i) the rostral segment of the AML associated with the prospective forebrain and rostral midbrain (A1 segment), (ii) the caudal segment of the AML associated with the caudal midbrain and the hindbrain (A2 segment) or (iii) the entire AML (A1+A2). The A1 or A2 fragments were approximately 175  $\mu$ m long (about half the length of the anterior side of the cylindrical embryo) and about 70  $\mu$ m wide, which is equivalent to one-tenth of the girth of the embryo. At this stage of development, the mesendoderm is about 10 cells in width and the cephalic neural plate spans about 200  $\mu$ m on either sides of the anterior midline of the embryo. Two other midline regions of the embryo were ablated in parallel experiments: the node with the adjacent germ layer tissues (Davidson et al., 1999) and the anterior one-third segment of the primitive streak (PS). Ablated embryos were harvested either immediately or after a period of development in vitro (4-7 or 24-30 hours) in Dulbecco's modified Eagle medium containing 75% rat serum (Tam, 1998). Embryos were fixed in 4% paraformaldehyde and examined for morphogenesis of anterior neural structures, axis morphology and somite number.

### Tracking the displacement of anterior midline tissues

Early-neural-plate stage embryos were labelled by microinjection of a diluted DiI solution (1:10 dilution in 0.2 M sucrose of a 2 mg/ml ethanolic stock solution, Molecular Probes) into the anterior midline tissues within the A1 or A2 segment. In A1-labelled embryos, midline tissues were ablated from the A2 region and, conversely, in A2-labelled embryos, A1 tissues were ablated. Some A1- and A2-labelled embryos were cultured as intact control. In addition, some embryos were labelled in both the A1 and A2 segments for an assessment of the relative movement of the tissues in the midline. All embryos were cultured for 6-7 hours in vitro and then fixed in 4% paraformaldehyde. They were examined under a Leica MZFL11 fluorescence dissecting microscope with a Rhodamine filter set (absorption at 574 nm). Bright-field and fluorescent images were captured using a SPOT2 digital camera (Scitech) and the digital images are electronically

**Fig. 1.** (A) Schematic diagram illustrating the ablation strategy of the AML of the late primitive streak to early neural plate stage embryos. The rostral AML, designated as A1, comprises the prechordal plate, the definitive endoderm and the overlying neuroectoderm of the forebrain and rostral midbrain. The caudal AML, designated as A2, consists of the midline tissues associated with the caudal midbrain and hindbrain. Lateral view of ablated embryos at the early neural plate stage shows the wound left behind (marked by asterisks) after the removal of A1 *Otx2*-expressing tissues and A2 *T*-expressing tissues. Arrows mark the AP (anteroposterior) and the L-R (left-right) embryonic axis. (B) Transplantation of A1 and A2 tissues and germ layer tissue from the lateral region (LG) of the late primitive streak to early neural plate stage embryo. The tissue fragments were grafted to the lateral region of stage-matched host embryos. The dashed lines on the host embryos demarcate the domains occupied by precursors of the brain and the spinal cord. FB, forebrain; MB, midbrain; HB, hindbrain; SP, spinal cord.



superimposed to show the location of the DiI-labelled cells in the anterior region of the embryo.

#### Tissue transplantation to test for inductive activity

Midline tissue fragments (A1 and A2) were tested for their ability to induce de novo neural gene expression by transplantation to ectopic sites. The donor tissues were isolated from no-allantoic-bud to late-bud stage embryos that express an X-linked *HMG-lacZ* transgene (Tam and Tan, 1992). Tissue transplantations were performed as described in Tam and Steiner (1999). The A1 or A2 fragments were transplanted to a site immediately lateral to the prospective neural plate of embryos at similar stages of development (Fig. 1B). As a control, a tissue fragment of similar size consisting of all three germ layers, was dissected from the lateral region (LG) that corresponds to the prospective surface ectoderm and neuroectoderm of the midbrain and hindbrain, and transplanted to the host embryo. Host embryos were cultured for 6 or 24–30 hours, then harvested and fixed with 4% paraformaldehyde. Embryos were stained with X-gal reagent (Progen) to reveal the  $\beta$ -galactosidase activity of the graft-derived cells and further analysed by whole-mount in situ hybridization and histology.

#### Whole-mount in situ hybridization and histology

Embryos were processed for whole-mount in situ hybridization according to the protocol of Wilkinson and Nieto (1993) with modifications. Riboprobes for *Lim1*, *Cer1*, *Six3*, *Fgf8*, *Hesx1*, *Otx2*, *Nkx2.1*, *En1*, *Hoxb1*, *Wnt1*, *Gsc*, *Shh*, *Hnf3 $\beta$*  and *T* genes were labeled with digoxigenin-11-UTP (Boehringer Mannheim) using the Ampliscribe kit (Epicentre Technologies). Prehybridization and hybridization solutions contained 1% SDS and 5 $\times$  SSC (pH 4.5) instead of CHAPS. Embryos were hybridized overnight with 2  $\mu$ g of probe per ml of hybridization solution. Hybridization and post-hybridization washes were carried out at 70°C for all the probes except for *Nkx2.1*, which was at 65°C. No RNase digestion was performed after hybridization and washes were carried out at high stringency without formamide. Some specimens were postfixed in 4% paraformaldehyde, embedded in paraffin or polyester wax and serially sectioned at 7  $\mu$ m for histological examination.

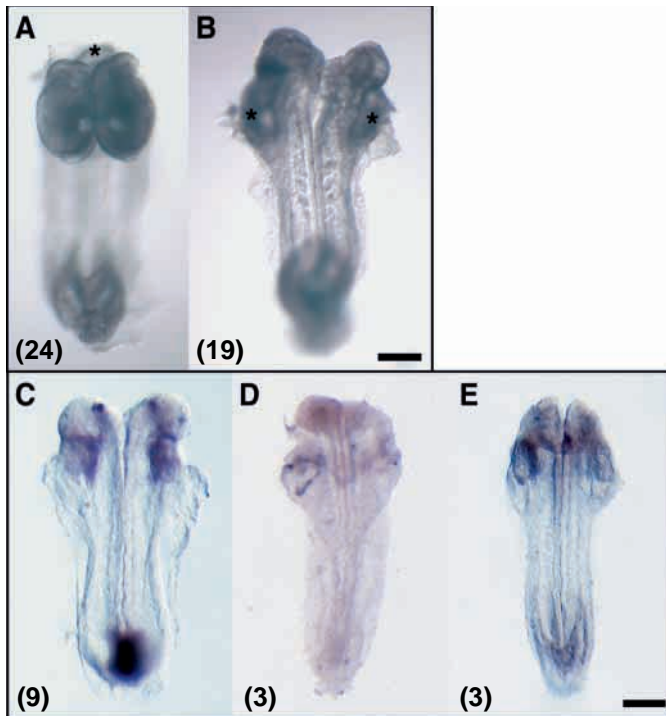
#### *Gsc* and *Gsc<sup>lacZ</sup>* mutant embryos

Embryos were collected at 7.5 dpc from mice of *Gsc<sup>+/-</sup>  $\times$  Gsc<sup>+/-</sup>* matings (Rivera-Perez et al., 1995). After 24–30 hours of in vitro development, yolk sacs of AML-ablated and control embryos were collected and digested for 3–4 hours in 50  $\mu$ l of buffered enzyme solution (50 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5% Tween 20, 200  $\mu$ g/ml Proteinase K (Amresco)) at 55°C followed by a heat treatment at 95°C for 5–10 minutes. The genotype (*Gsc<sup>+/+</sup>*, *Gsc<sup>+/-</sup>* or *Gsc<sup>-/-</sup>*) of the embryo was determined by PCR using specific primers for: wild-type allele (5'-GAGCTGCAGCTGCTCAACCAGCTGC-ACTGT-3' and 5'-TAGCATCGACTGTCTGTGCAAGTCC-3') and mutant allele (5'-AATCCATCTTGTTC AATGGCCGATC-3' and 5'-TTACAGCTAGCTCCTCTGTTGC-3'). The PCR was performed in 10 $\times$  PCR buffer (1.7 mg/ml BSA, 0.67 M Tris-HCl pH 8.8, 0.067 M MgCl<sub>2</sub>, 0.166 M (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>) containing 10% DMSO, 1.5 mM dNTP and 200 ng/ $\mu$ l of each of the four primers and 3–5  $\mu$ l of DNA preparation, for 35 cycles with annealing temperature set at 60°C. The size of the wild-type product was 743 bp and that of the mutant was 696 bp. Several litters were also collected from 7.5 dpc pregnant mice produced by *Gsc<sup>+lacZ</sup>  $\times$  Gsc<sup>+lacZ</sup>* matings. The *Gsc<sup>lacZ</sup>* allele was generated by insertion of the *nls-lacZ-SV40pA-flox neo* cassette into the second exon followed by CRE excision of the *neo* gene (M. W. and R. R. B., unpublished). AML-ablated and control embryos were X-gal stained to reveal *lacZ*-expressing cells.

## RESULTS

### The loss of AML disrupts morphogenesis of cephalic neural tube

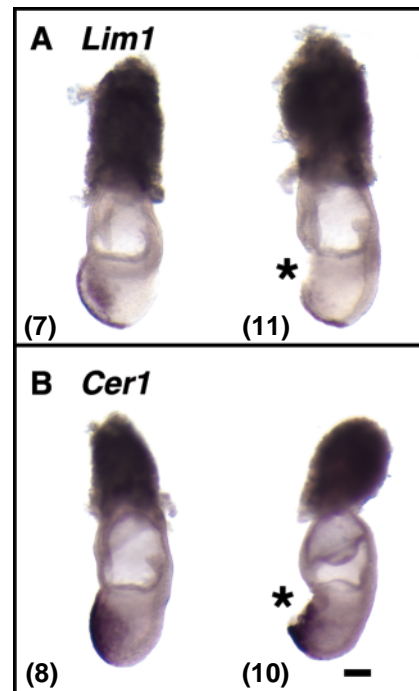
The morphogenesis of the neural tube was examined at early somite stage after removing different midline structures of the late gastrula, such as the PS, the node and the AML. Ablation of the anterior third of the PS did not affect the elongation or the morphogenesis of the neural tube but led to the loss of the



**Fig. 2.** Headfold development of the early-somite-stage embryo is disrupted after AML ablation (A1+A2). (A,B) Dorsal view of (A) intact and (B) AML-ablated embryos. In the ablated embryo, the cardiogenic region is split and the heart develops as bifid tubes (asterisks) on the lateral sides of the truncated head. (C-E) Dorsal view of AML-ablated embryos indicates the loss of anterior neural markers *Fgf8* (C), *Six3* (D) and *Hesx1* (E). Expression in intact embryo is shown in Fig. 9A,D,G. The number in parentheses indicates the number of specimens analyzed. Embryos are orientated with anterior toward the top. Scale bar, 75  $\mu\text{m}$  for (A,B) and 100  $\mu\text{m}$  for (C-E).

paraxial mesoderm caudal to the 4-5th somite (data not shown). These PS-ablated embryos formed  $4.4 \pm 0.6$  ( $n=12$ ) pairs of somites significantly fewer in number than in control embryos ( $11.0 \pm 0.4$ ,  $n=7$ ). Consistent with the finding of Davidson et al. (1999), ablation of the node had no significant impact on the development of the cephalic part of the neural tube, although the overall development of the neural axis and the somites of the trunk was retarded. Node-ablated embryos developed intact axial mesendoderm underneath the headfolds but the notochord in the trunk was often abnormal or absent (Davidson et al., 1999). In contrast to the node- and PS-ablation, the removal of the entire AML that contains axial mesendoderm and ventral neuroectoderm of the prospective forebrain, midbrain and rostral hindbrain (A1+A2; Fig. 1A) resulted in the truncation of the cephalic neural tube (Fig. 2A,B). A longitudinally split head region was found in about 43% of the AML-ablated embryos (Fig. 2B-E).

A preliminary study of region-specific markers showed that AML-ablated embryos did not express the forebrain markers *Fgf8*, *Six3* and *Hesx1* (Fig. 2C-E, respectively). However, posterior neural tube developed normally and somite number ( $9.8 \pm 0.2$ ,  $n=19$ ) was similar to that of control embryos ( $10.3 \pm 0.4$ ,  $n=24$ ). These results show that morphogenesis of the cephalic neural tube is critically dependent on the presence of the AML.

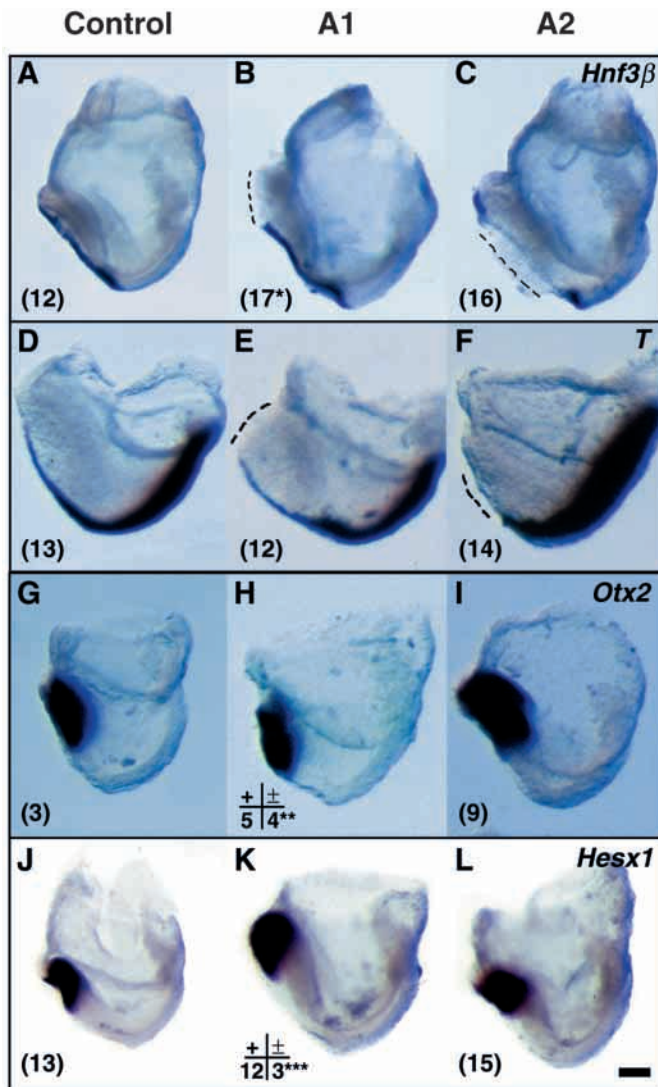


**Fig. 3.** The ablation of the A1 segment results in the loss of the anteriormost mesendoderm that expresses *Lim1* and *Cer1* activity. (A) *Lim1* expression in intact embryo (left) and A1 embryo (right). (B) *Cer1* expression in intact early neural plate stage embryo (left) and A1 embryo (right). Asterisks mark the ablated region. The number in parentheses indicates the number of specimens analyzed. Scale bar, 100  $\mu\text{m}$ .

### Ablation deletes the axial tissues without significant loss of the neural progenitor population

In order to understand the relative role of different segments of the AML on cephalic development, microsurgical ablation was performed on early neural plate stage embryos to remove either the axial tissues associated with the prospective forebrain and rostral midbrain (A1 segment) or those associated with the prospective caudal midbrain and the hindbrain (A2 segment; Tam, 1989; Fig. 1A). Embryos with deleted A1 segment (designated as A1 embryos) lose the anteriormost *Lim1*- and *Cer1*-expressing mesendoderm (Shawlot et al., 1998; Perea-Gomez et al., 1999) when examined immediately after ablation (Fig. 3A,B) and the *Hnf3 $\beta$* -expressing anterior midline tissues, but not the *T*-expressing tissues immediately anterior to the node (at 4-6 hours; Fig. 4A,B,E). The majority (90%) of embryos with deleted A2 segment (designated as A2 embryos) completely lost *Hnf3 $\beta$* -expressing tissues in the A2 region (Fig. 4A,C) and *T* activity was absent in the midline anterior to the node (Fig. 4D-F). These results demonstrate that the microsurgery has ablated the specific AML segment and that the missing tissues were not reconstituted 4-6 hours following ablation.

A1 and A2 embryos were also examined 4-6 hours after ablation for the expression of *Otx2* and *Hesx1* (Thomas et al., 1995). Both gene activities were detected in the anterior ectoderm of all ablated embryos examined, although *Otx2* and *Hesx1* expression were diminished in 4/9 and 3/15 A1 embryos, respectively (Fig. 4G-L). These results indicate that

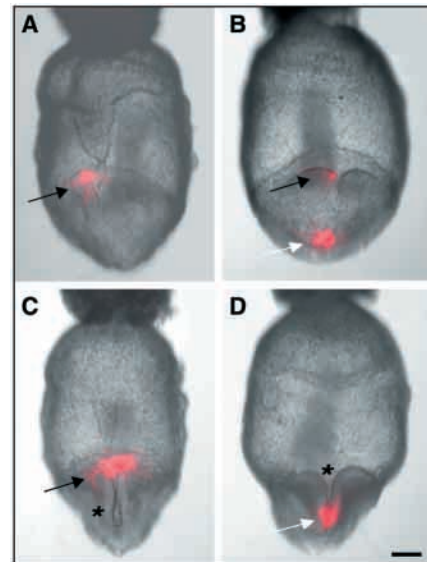


**Fig. 4.** Gene expression in axial mesendoderm and anterior neural tissues 4-6 hours after A1 or A2 ablation. (A,D,G,J) *Hnf3β*, *T*, *Otx2* and *Hesx1* expression in intact embryos. (B,C) The ablated regions (dashed lines) lack expression of *Hnf3β*. (E,F) As expected, A2 but not A1 embryo lacks the *T*-expressing cells immediately anterior to the node. (H,I,K,L) Ablation of either A1 or A2 segment does not alter the expression of *Otx2* and *Hesx1* suggesting that the prospective tissues for the forebrain and rostral midbrain are not significantly reduced by the ablation. The number in parentheses indicates the number of specimens analyzed. The inset shows the number of specimens displaying (+) normal or (+/-) diminished expression. \*Three A1 embryos show weak *Hnf3β* activity in the healed wound. \*\*Four A1 embryos display reduced *Otx2* expression domain. \*\*\**Hesx1* expression is absent in one headfold in three A1 embryos. Embryos are orientated with anterior to the left. Scale bar, 20  $\mu$ m.

the ablation has not significantly depleted anterior neural progenitors.

#### The pattern of displacement of cells in the midline is not affected by tissue ablation

During the 6-7 hours of in vitro development after dye labelling, A1-labelled ectodermal and mesodermal cells of the intact



**Fig. 5.** The displacement of the A1 and A2 tissues in (A,B) intact embryo, (C) A2 embryo and (D) A1 embryo. DiI labelling is examined by fluorescence microscopy after 6-7 hours of in vitro development. Black arrow, labelled A1 cells; white arrow, labelled A2 cells, asterisk, the deleted region. Embryos are shown in en face view. Scale bar, 60  $\mu$ m.

**Table 1.** *Gsc* expression analysis of anterior midline (AML) tissues, at 6 hours of culture in vitro, in ablated embryos and after transplantation.

		<i>Gsc</i> expression: no. (%) of embryos		
		Normal	Reduced	Absent
(A) AML-ablated embryo				
A1 Ablation	<i>n</i> =35	17 (48.5)	13 (37.1)	5 (14.3)
A2 Ablation	<i>n</i> =42	6 (14.3)	14 (33.3)	22 (52.4)
control	<i>n</i> =37	35 (94.6)	1 (2.7)	1 (2.7)
(B) AML-grafted tissue				
A1 Graft	<i>n</i> =8	0	3	5
A2 Graft	<i>n</i> =8	7	1	0
LG graft	<i>n</i> =7	0	0	7

A1, rostral AML fragment; A2, caudal AML fragment; LG, lateral germ layers; see Fig. 1.

embryo were displaced anteriorly to the anterior margin of the headfolds (Fig. 5A; *n*=5). The labelled endodermal cells were displaced more rostrally beyond the neural plate to the region underlying the prospective cardiogenic mesoderm. A2-labelled cells were distributed to the prospective midbrain and upper hindbrain of the embryo (*n*=6; data not shown). The A1- and A2-labelled cells remained separate from one another at the end of the 6-7 hours of in vitro development (Fig. 5B; *n*=8), suggesting that during the initial phase of the formation of the headfolds, cells from the two different segments of the AML do not intermingle when they are displaced anteriorly. The normal pattern of displacement of the A1 cells was not affected by A2 ablation (Fig. 5C; *n*=5). Following A1 ablation, A2-labelled cells did not migrate anteriorly to take the place of the A1 cells but remained in the caudal margin of the ablation (Fig. 5D; *n*=6).

**Table 2. The expression of molecular markers in anterior mesendoderm and ventral neuroectoderm in intact and AML-ablated embryos**

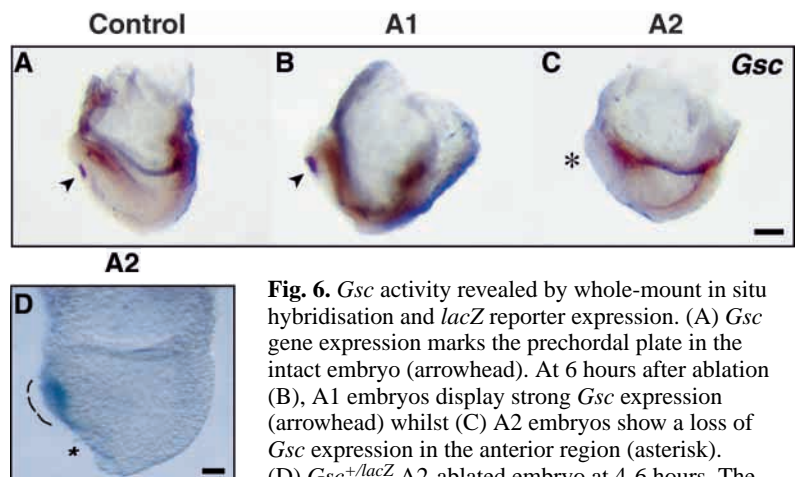
	Control	Ablation		Somite no.	Gene expression (% of embryos)			
		A1	A2		Increased	Normal	Reduced	Absent
<i>Gsc</i>	18			10.2±0.5	–	100	–	–
		23		11.4±0.4	70	17	–	13
<i>Shh</i>	16		14	10.1±0.5	–	7	–	93
		20		10.2±0.4	–	100	–	–
<i>Hnf3β</i>	3		16	11.7±0.3	35	65	–	–
		4		8.9±0.4	–	12	–	88
<i>T</i>	4		9	9.3±0.9	–	100	–	–
		4		10.5±1.0	50	50	–	–
<i>Nkx2.1</i>	21		9	10.8±0.6	–	–	22	78
		9		8.0±1.1	–	100	–	–
<i>Nkx2.1</i>	21		9	12.2±0.3	–	89	11	–
			9	11.3±0.3	–	22	–	78
		21		9.0±0.2	–	100	–	–
		21		11.0±0.4	–	95	5	–
			17	8.6±0.3	–	–	–	100

### Compensatory gene activity occurs in the remaining AML and adjacent tissues after A1 ablation

Between the late-gastrula and early-neural-plate stages, *Gsc* gene activity is transiently detected in the presumptive prechordal plate (Filosa et al., 1997; Wakamiya et al., 1997; Belo et al., 1998). Although no expression of *Hnf3β* was detected in the healed wound 4–6 hours after ablation, when A1 embryos were examined for *Gsc* expression, more than 85% of them displayed de novo *Gsc* activity in the headfold, suggesting that some non-A1 tissues adopted prechordal characteristics (Table 1A; Fig. 6B). At the early-somite-stage, *Gsc* expression is restricted to the prechordal plate and the ventral diencephalon (Fig. 7A) and *Shh* is expressed in the axial mesendoderm along the entire length of the axis and in the ventral neural tube from the anterior forebrain to the anterior hindbrain (Fig. 7D,G). In the majority of the early-somite-stage A1 embryos, developed in vitro for 24–30 hours, *Gsc* (in 87% of embryo) and *Shh* (in all embryos) activities were expressed in the ventral tissues at the rostral end of the foreshortened neural axis (Table 2; Fig. 7B,E,H). Histological analysis demonstrated that in A1 embryos, *Shh* expression was enhanced in the ventral neuroectoderm (Fig. 7K compare 7J) and in the anterior mesendoderm (data not shown) of the truncated headfold. Normally, *Nkx2.1* expression is first detected at about the 3-somite stage in the ventral diencephalon (Fig. 7P; Shimamura and Rubenstein, 1997) and *Nkx2.1* activity is thought to be regulated by signals emanating from the prechordal plate (Shimamura et al., 1995; Kimura et al., 1996; Dale et al., 1997; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997; Qiu et al., 1998). In A1 embryos, *Nkx2.1* expression was detected in the ventral neural tube (Fig. 7Q and histological data not shown; Table 2). The upregulation of *Gsc* and *Shh* activity implies that tissues that show similar molecular activity as the anterior mesendoderm may have been reconstituted in response to the absence of A1 tissues. This is accompanied by the differentiation of brain tissues that have ventral forebrain characteristic as demonstrated by *Nkx2.1* activity.

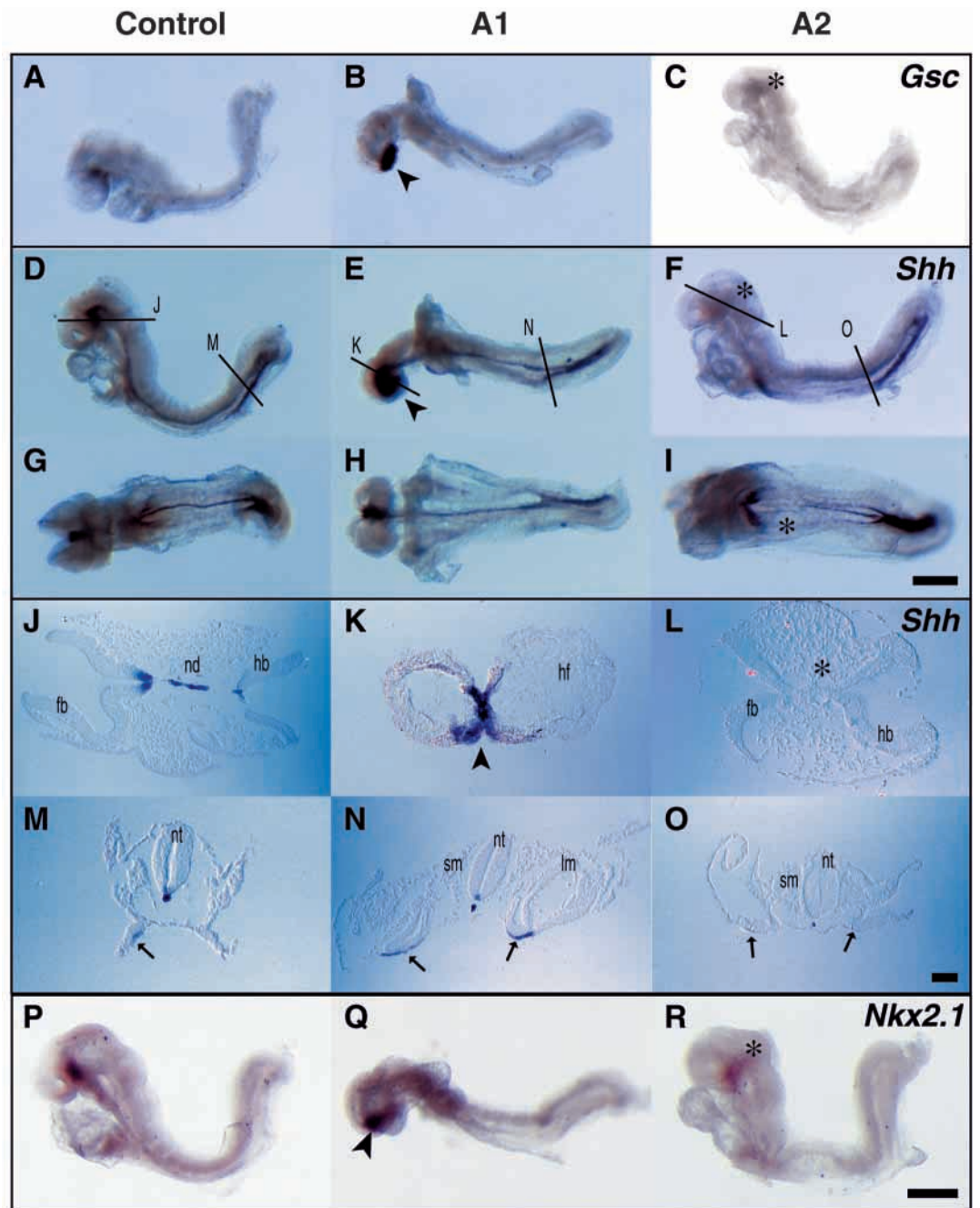
### *Gsc* activity is required for the regulation of *Nkx2.1* in the ventral neuroectoderm

To test whether *Gsc* activity is critical for the regulation of *Shh* and *Nkx2.1* activity, we firstly examined the impact of the loss of *Gsc* in intact *Gsc*<sup>+/+</sup>, *Gsc*<sup>+/-</sup> and *Gsc*<sup>-/-</sup> early-somite-stage embryos. We found that the complete loss of *Gsc* activity was associated with the absence of *Nkx2.1* expression (Fig. 8) whereas *Shh* was not affected (Table 3). Embryos of the three genotypes were then subjected to A1 ablation to determine whether, in the absence of *Gsc* activity, there may be a different genetic response. All six *Gsc*<sup>-/-</sup> A1 embryos examined exhibited *Shh* activity in the notochord but only two of them expressed *Shh* activity in AML (Table 3; histological data not shown). Therefore, the lack of *Gsc* activity may compromise, but does not totally abrogate, the compensatory regulation of *Shh* activity observed in the wild-type A1 embryo. Four of the seven *Gsc*<sup>-/-</sup> A1 embryos showed no *Nkx2.1* activity (Table 3). Interestingly, the remaining three *Gsc*<sup>-/-</sup> A1 embryos did express *Nkx2.1* gene in the ventral neural tube (Fig. 8 and



**Fig. 6.** *Gsc* activity revealed by whole-mount in situ hybridisation and *lacZ* reporter expression. (A) *Gsc* gene expression marks the prechordal plate in the intact embryo (arrowhead). At 6 hours after ablation (B), A1 embryos display strong *Gsc* expression (arrowhead) whilst (C) A2 embryos show a loss of *Gsc* expression in the anterior region (asterisk). (D) *Gsc*<sup>+/lacZ</sup> A2-ablated embryo at 4–6 hours. The star marks the healed midline. β-galactosidase-positive cells are present in the remaining A1 segment (dashed line), although the transcription of the normal *Gsc* allele is no longer detectable by in situ hybridisation (see Fig. 4C). Embryos are orientated with anterior to the left. Scale bar, 25 μm for (A–C) and 15 μm for (D).





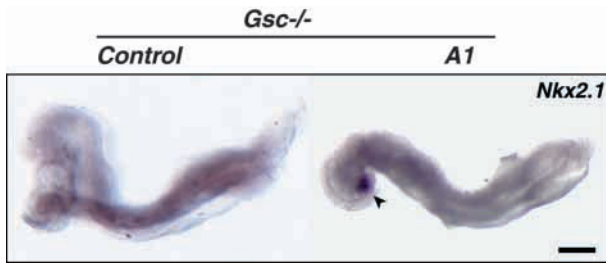
**Fig. 7.** Development of the midline tissues of the early-somite-stage embryo after A1 or A2 ablation. (A) *Gsc* expression is detected in the prechordal plate and ventral forebrain. (B) A1 embryo displays an enhanced *Gsc* activity in the anterior AML (arrowhead). (C) Asterisk marks the lack of *Gsc* expression in the anterior tissues of the A2 embryo. (D,G,J) *Shh* expression is detected in the anterior mesendoderm and ventral forebrain. (E,H) Enhanced *Shh* expression is found in the headfolds of A1 embryos (arrowhead). (K) Transverse section showing the expression of *Shh* in the ventral forebrain of A1 embryo. (F,I,L) A2 ablation abrogates the expression of *Shh* in the mesendoderm and in the ventral neural tube in regions anterior to the 4th somite. Asterisk marks regions that lack *Shh* activity. (M,N,O) At the level of the trunk, *Shh* is expressed in the gut endoderm (arrows) and notochord of (M) intact, (N) A1 and (O) A2 embryos. (P) *Nkx2.1* is expressed in the ventral forebrain of the control embryo. (Q) *Nkx2.1* activity is detected in A1 embryo (arrow head) but absent in (R) A2 embryos (asterisk marks the false positive signal created by the tissue shadow). Embryos are shown with anterior to the left, and either in lateral (A-F,P-R) or ventral (G-I) view. Abbreviations: fb, forebrain; hb, hindbrain; hf, headfold; lm, lateral plate mesoderm; nd, notochord; nt, neural tube; sm, somite. Scale bar, 100  $\mu$ m for (A-I), 125  $\mu$ m for (P-R) and 50  $\mu$ m for (J-O).

histology not shown; Table 3). This suggests that, although during normal development *Gsc* activity is required for *Nkx2.1* expression, in the course of the compensatory response to A1 ablation, a different but less effective mechanism results in the activation of *Nkx2.1* gene.

#### The loss of A2 segment results in the downregulation of gene activity in axial mesendoderm and ventral forebrain

In contrast to *Gsc* activation in A1 embryos, *Gsc* expression was downregulated (reduced in 33% and not detectable in 52%

of embryos) in A2 embryos 6 hours after ablation (Table 1A; Fig. 6C). Embryos that carry one *Gsc<sup>lacZ</sup>* allele (M. W. and R. R. B., unpublished data) were analysed for  $\beta$ -galactosidase activity to monitor the distribution of the *Gsc*-expressing cells (i.e. the presumptive prechordal plate). When the expression of the *lacZ* reporter gene was examined in heterozygous *Gsc<sup>+/lacZ</sup>* A2 embryos after 4-6 hours of in vitro development, *lacZ*-expressing cells were detected in these embryos ( $n=6$ ) demonstrating that A2 ablation has not removed the presumptive prechordal tissue (Fig. 6D). The lack of *Gsc* expression in A2 embryos is therefore likely due to the inability



**Fig. 8.** The expression of *Nkx2.1* in the ventral forebrain of the intact *Gsc*<sup>-/-</sup> embryo (left) and the anterior tissues of the A1-ablated *Gsc*<sup>-/-</sup> embryo (right). Arrowhead points to the *Nkx2.1*-expressing tissue. Scale bar, 100  $\mu$ m.

to maintain *Gsc* activity in the rostral AML beyond the first 4–6 hours after ablation rather than the removal of *Gsc*-expressing cells.

At the early-somite-stage, in contrast to A1 ablation findings, the AML tissue left behind after A2 ablation did not display any *Gsc* and *Shh* activity even though the ablation had not removed the tissues that normally express these two genes (Table 2; Fig. 7C,F,I). The loss of *Shh* and *Gsc* activity in the ventral forebrain and axial mesendoderm was accompanied by the lack of *Nkx2.1* expression (Table 2; Fig. 7R). In addition, *T* and *Hnf3 $\beta$*  were not detected in the midline anterior to the fourth somite (Table 2). Consistent with the lack of expression of the markers, histological examination of A2 embryos revealed that tissues that morphologically resemble the floor plate and the axial mesoderm were absent in the head region (Fig. 7L). Ablation of midline tissues posterior to A2 produced different defects of the axial mesendoderm. Eight of ten node-ablated embryos showed normal *Gsc* expression in the AML (data not shown) and only the notochord and the floor plate in the trunk were affected (Davidson et al., 1999). Ablation of the anterior third of the PS had no effect on the formation of midline structures as demonstrated by the appropriate expression of *Hnf3 $\beta$*  ( $n=10$ ; data not shown). Thus, the maintenance of rostral axial mesendoderm seems to specifically require an intact A2 segment and not other more posterior midline structures.

### Defective development of the forebrain and midbrain in the absence of AML

When examined after 24–30 hours of *in vitro* development, A1 embryos displayed variable deficiencies of anterior structures, ranging from absence of the optic eminence and small headfold to a severe truncation of the anterior neural axis (Figs 7, 9). Nevertheless, appropriate region-specific neural markers were still expressed in the truncated neural axis, although the expression domains were often altered. The expression domain

of *En1*, a marker for the midbrain (Davis and Joyner, 1988), was shifted rostrally suggesting that the dorsal and rostral forebrain domains were significantly reduced (Fig. 9N,M). In some A1 embryos, *Hesx1* and *Wnt1* expression domains (Parr et al., 1993) were juxtaposed, indicating that the neural tissue normally found between these brain segments was either absent or severely reduced (Fig. 10). Moreover, the *Hesx1* domain itself was reduced or absent in A1 embryos (Table 4; Fig. 9G,H) while the expression of *Six3* (Oliver et al., 1995) and *Fgf8* (Crossley and Martin, 1995) was either markedly reduced or missing in more than 89% of A1 embryos (Table 4; Fig. 9A,B,D,E). This specific loss of *Six3* and *Fgf8* expression suggests that the differentiation of the rostral forebrain is impaired in the A1 embryos.

*Hesx1* expression was substantially reduced or extinguished in the forebrain of the A2 embryos examined after 24–30 hours of *in vitro* development (Table 4; Fig. 9I). Moreover, *Fgf8* activity in the forebrain was absent ( $n=4$ ) or was expressed unilaterally ( $n=4$ ) in some A2 embryos (for a total of 11 embryos examined; Table 4; Fig. 9C). *Fgf8* expression was also reduced or absent at the midbrain-hindbrain junction ( $n=9$ ; Fig. 9C) but *Wnt1* expression was normal (data not shown). Despite these defects in neural patterning, morphogenesis of the neural tube was not overtly affected in A2 embryos (Table 4). The results suggest that the maintenance of *Hesx1* and *Fgf8* activities in the brain is dependent on the functional integrity of the AML, which may be disrupted just by A2 ablation. When *Gsc*<sup>-/-</sup> mutant embryos were analysed for *Hesx1* activity, we found no difference in expression among embryos of the three genotypes (*Gsc*<sup>+/+</sup>  $n=9$ ; *Gsc*<sup>+/-</sup>  $n=24$ ; *Gsc*<sup>-/-</sup>  $n=4$ , data not shown). This result indicates that the loss of *Gsc* activity in the rostral AML could not account for the downregulation of the expression of the forebrain marker *Hesx1* in A2-ablated embryos.

### AML can induce ectopic neural gene expression

Transplantation experiments were performed to test the ability of the AML tissues to induce region-specific neural gene activity. A1 segment, containing the prechordal plate, and the A2 segment, containing the notochord, were grafted separately to the lateral region of host embryos (Fig. 1B).

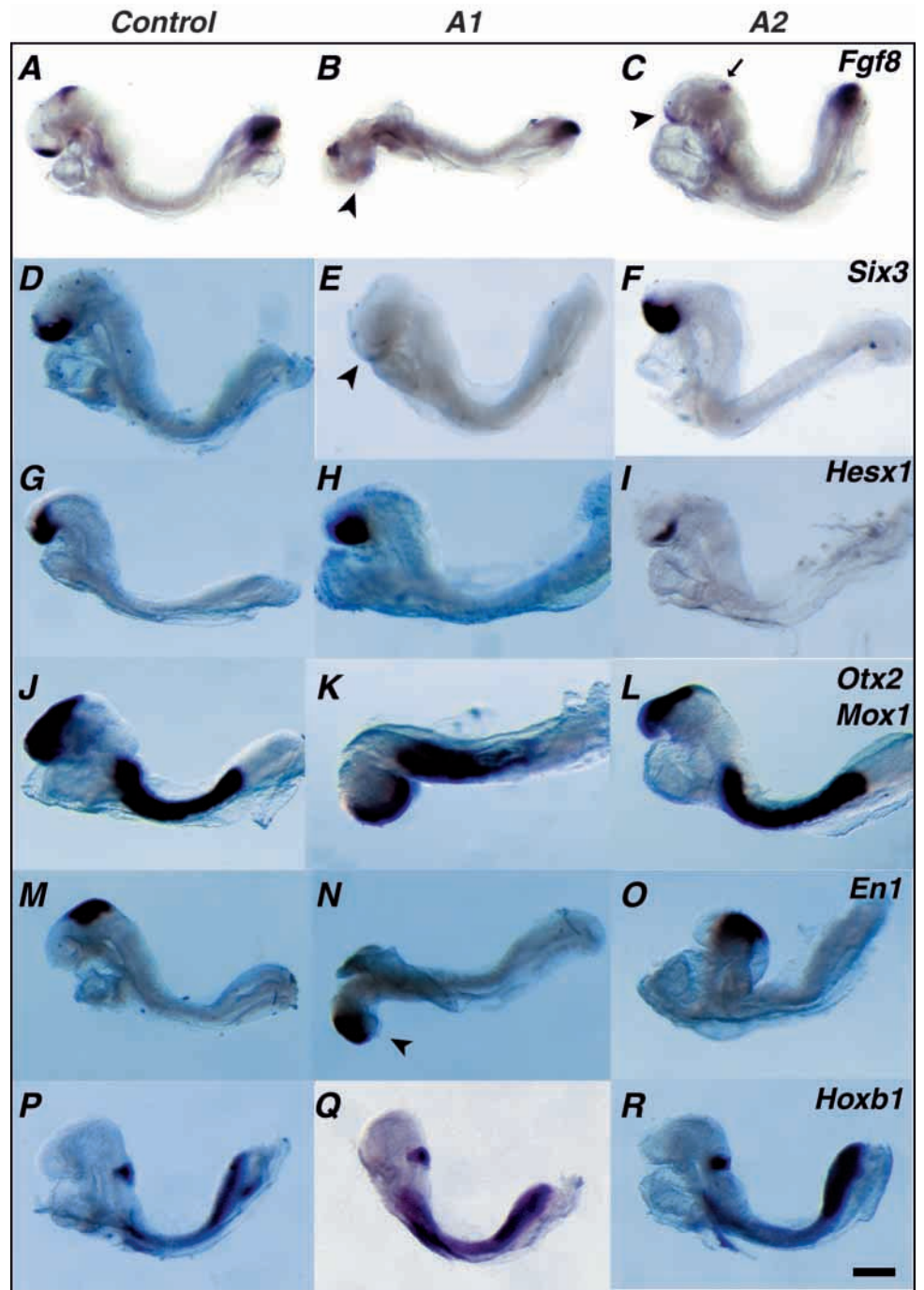
To determine whether the grafted segments retain their regional characteristics in ectopic sites, A1 and A2 grafts were assessed for *Hnf3 $\beta$*  and *Gsc* activity 6 hours after transplantation. Both A1 and A2 tissues expressed *Hnf3 $\beta$*  activity normally associated with midline tissues ( $n=2$ , data not shown). Only 3 of 8 A1 grafts contained a weak *Gsc* activity, while all A2 grafts strongly expressed *Gsc* (Table 1B; Fig. 11A–C). Therefore A1 tissues, when isolated from other AML, failed to maintain the prechordal property, a finding consistent

**Table 3.** Expression of *Shh* and *Nkx2.1* in anterior mesendoderm and ventral neural tube in intact and A1 embryos of different *Gsc* genotypes

	Gsc embryos					
	[+/+]		[+/-]		[-/-]	
	Intact	A1	Intact	A1	Intact	A1
<i>Shh</i> expression in anterior neural tube	8/8	10/11	12/12	8/8	7/7	2/6*#
<i>Nkx2.1</i> expression in ventral forebrain	14/14	12/16	8/8	9/12	1/10#	3/7

Significant difference from \* intact *Gsc*<sup>-/-</sup> group and # intact *Gsc*<sup>+/+</sup> and *Gsc*<sup>+/-</sup> groups by  $\chi^2$  2 $\times$ 2 test at  $P<0.01$ .

**Fig. 9.** Effect of A1 and A2 ablations on the expression of brain-segment-specific markers at the early somite stage. (A) *Fgf8* expression is normally expressed in the rostral forebrain, the mid/hindbrain boundary, the foregut and the PS. (B) A1 embryo showing the loss of *Fgf8* in the anterior forebrain (arrowhead) but expression in other tissues is unaffected. (C) A2 embryo displays a diminished *Fgf8* activity in both forebrain (arrowhead) and mid/hindbrain (arrow) tissues. (D) *Six3* activity is found in the rostral forebrain in the intact embryo. (E) *Six3* expression is greatly reduced in A1 embryo (arrowhead) but (F) is normal in A2 embryo. (G) *Hesx1* expression domain is localized to the ventral forebrain. (H) A1 embryo expresses *Hesx1* whilst (I) A2 embryo displays reduced expression in the forebrain. (J-L) Expression of *Otx2* in the anterior neural tube and *Mox1* in the somites is not affected by A1 or A2 ablation. (M) *En1* expression marks the midbrain in the intact embryo. (N) *En1* expression is extended to the rostral part of the neural tube in A1 embryo. The arrowhead marks the truncated anterior region that does not express *En1*. (O) A2 ablation results also in a rostral shift of *En1* expression, indicating a reduction of forebrain tissues. (P-R) *Hoxb1* activity in the third rhombomere and the posterior neural tube is not affected by the ablation. Embryos are shown in lateral view with anterior to the left. Scale bar, 100  $\mu$ m



with the loss of *Gsc* expression in the remaining A1 tissues after removing A2 tissues. The upregulation of *Gsc* activity in the transplanted A2 tissue is reminiscent of the compensatory upregulation of *Gsc* observed in the remaining AML tissues after A1 ablation (Table 1A).

After transplantation, more than 87% of the A1 and all A2 fragments were incorporated in the brain region of the early-somite-stage host embryo. When fragments of lateral germ layer (LG) tissues of the late gastrula embryo were transplanted, only 49% of the grafts were found in the host neural tube. The A1, A2 and LG grafts were examined for the expression of the pan-neural marker *Sox2* (Wood and

Episkopou, 1999) and regional markers such as *Hesx1*, *Otx2* and *En1* (Table 5). *Sox2* expression was found in most A1 and A2 grafted tissues (about 66%) and in 71% of LG grafts. However, only 13% of LG grafts expressed *Otx2* whereas this activity was detected in 72% of A1 and A2 grafts. Both A1 and A2 grafts also expressed *En1* but only A1 grafts expressed *Hesx1*. *En1* or *Hesx1* activity was never detected within LG grafts. These findings indicate that, upon transplantation, the AML tissues retain their original segment-specific neural characteristics whereas LG tissues, although expressing *Sox2*, did not display any consistent regional neural gene activity.

**Table 4. The expression of markers in the forebrain of A1- and A2-ablated embryos**

	Control	Ablated embryos		Somites	Morphology		Gene expression: % of embryos		
		A1	A2		Eye eminence	Anterior truncation	Normal	Reduced	Absent
		<i>Fgf8</i>	9				11.8±0.7	9	0
Forebrain*		14		11.6±0.4	1	13	7	43	50
			11	10.0±0.7	11	0	73	–	27
<i>Six3</i>	19			9.5±0.3	19	0	100	–	–
		28		10.7±0.3	3	25	11	61	28
			8	7.6±0.6	7	1	100	–	–
<i>Hesx1</i>	20			9.7±0.4	20	0	100	–	–
		27		10.6±0.3	2	25	4	81	15
			17	9.9±0.3	16	1	11	30	59
<i>Otx2</i>	16			10.2±0.5	16	0	100	–	–
		13		10.1±0.5	3	10	69	8	23
			14	10.1±0.5	14	0	86	14	–
<i>En1</i>	6			9.8±0.2	6	0	100	–	–
		14		11.5±0.5	0	14	100	–	–
			4	nd	4	0	100	–	–
<i>Fgf8</i>	9			11.8±0.7	9	0	100	–	–
Midbrain*		14		11.6±0.4	1	13	86	14	–
			11	10.0±0.7	11	0	18	64	18
<i>Hoxb1</i>	4			11.0±0.6	4	0	100	–	–
		5		11.0±0.3	0	5	100	–	–
			7	9.6±0.3	7	0	100	–	–

\*Embryos analysed for both forebrain and midbrain *Fgf8* expression. nd, not determined.

**Table 5. Expression neural markers in the grafts of anterior midline (AML) fragments and the host tissues**

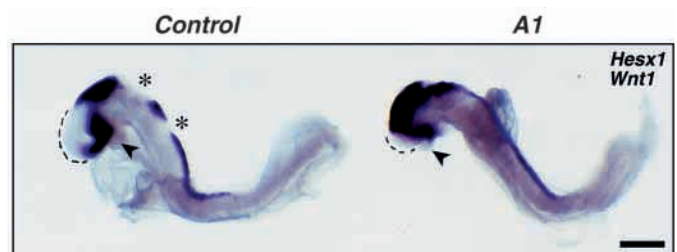
Transplantation	Total	Gene expression: no of specimens										
		<i>Sox2</i>		Total	<i>En1</i>		Total	<i>Otx2</i>		Total	<i>Hesx1</i>	
		Graft	Host		Graft	Host		Graft	Host		Graft	Host
A1	6	2	3	13	3	2	15	12	4	6	4	0
A2	6	6	1	13	3	10	14	9	5	6	0	0
LG	14	10	0	10	0	0	16	2	0	3	0	0

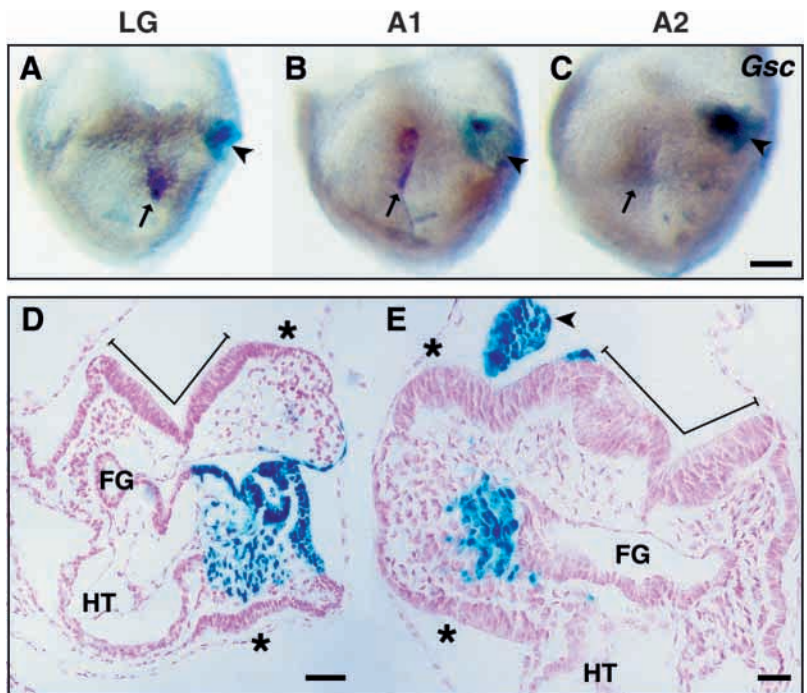
A1, rostral AML fragment; A2, caudal AML fragment; LG, lateral germ layers; see Fig. 1. Numbers in the “Total” column indicate the number of grafts conducted with each tissue fragment, “Graft” indicates the number of grafted fragments expressing the marker whereas the “host” indicates the number of specimen showing expression in the host tissues.

In the presence of either A1 and A2 grafts, an extra headfold or broadened host neural plate was often observed in the host embryo (Fig. 11D,E), whereas none of the LG grafts had elicited the differentiation of extra neural tissue. Analyses of the host tissues in the vicinity of the grafts revealed that *Sox2* expression was detected in 50% and 17% of embryos receiving A1 and A2 graft respectively (Table 5; Fig. 12B,C). An expansion of *Otx2* expression was observed in the host neural tube in 27% of A1 grafts and 36% of A2 grafts (Table 5, Fig. 12E,F). However, no de novo *Hesx1* activity was induced in

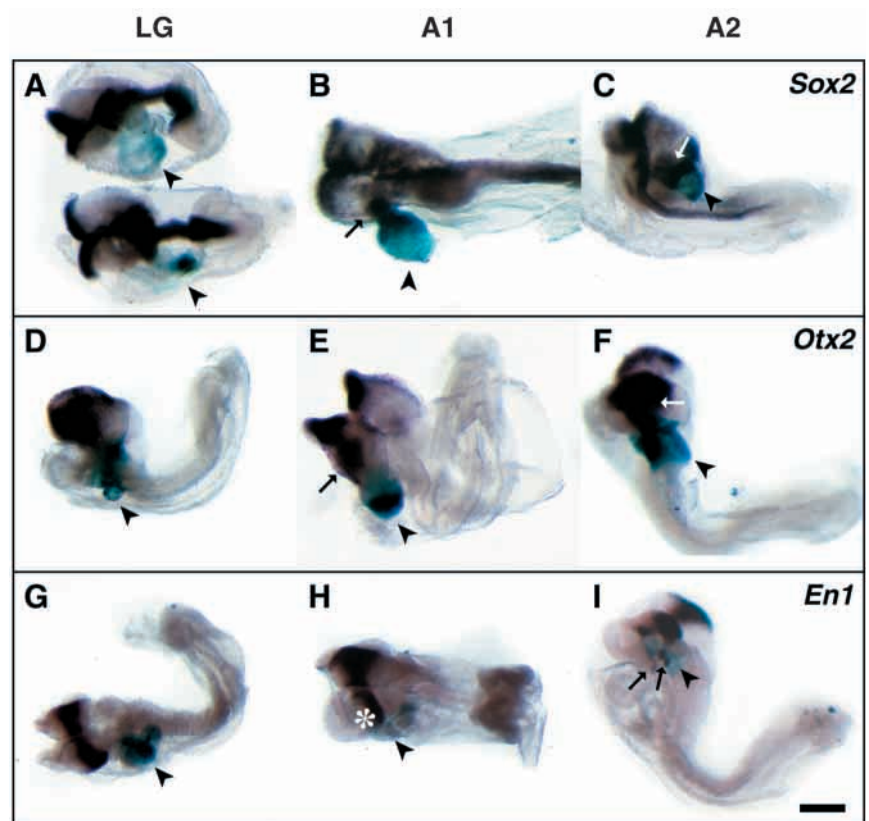
the host tissues by either A1 or A2 grafts (Table 5). A2 grafts elicited ectopic expression of *En1* activity more frequently (77%; Fig. 12I) than A1 grafts (15%). Interestingly, in 61% of the cases where the A1 graft had been incorporated into the host midbrain, *En1* expression in the host tissue was suppressed (Fig. 12H). Control LG grafts never induced de novo expression of segment-specific neural genes in the host tissue (Table 5; Fig. 12A,D,G). Our results show that, although both A1 and A2 grafts can elicit de novo expression of particular neural genes, they are unable to impart any patterning activity on the host neural axis under these experimental conditions.

**Fig. 10.** Regionalisation of the neural tube of A1 embryos assessed by double in situ hybridisation of *Hesx1* and *Wnt1*. *Hesx1* is normally expressed in the ventral forebrain and *Wnt1* is expressed in the midbrain, upper hindbrain, the dorsal region of the lower hindbrain and spinal cord (left panel). A1 ablation (right panel) results in a rostral expansion of the *Hesx1* domain of expression leaving a markedly reduced amount of *Hesx1*-free tissue (dashed line). The expression domain of the *Wnt1* gene shifts rostrally and the normally separate expression domains in the dorsal neural tube (marked with an asterisk in the intact embryo) merge together. Arrowhead marks the first branchial arch. Scale bar, 100 µm.





**Fig. 11.** (A-C) Analysis of *Gsc* activity in transplanted AML tissues after 6 hours of development. The blue X-gal staining marks the graft-derived tissues and the purple coloration shows the in situ hybridisation signal. (A) LG control, *Gsc* expression is present in the prechordal plate (arrow) of the host embryo but not in the LG graft (arrowhead). (B) Both the host prechordal plate (arrow) and the A1 graft (arrowhead) express *Gsc* although the signal is much weaker in the graft. (C) A2 graft (arrowhead) displays strong *Gsc* expression whereas the host prechordal plate shows a light signal (arrow). (D,E) Transverse sections of the head region of the host embryo receiving AML (A1+A2) grafts showing the formation of extra neural tissues (asterisk) in the host neural plate (demarcated by the solid lines). (E) Arrowhead points to a piece of graft-derived tissue that protrudes into the amniotic cavity. FG, foregut; HT, Heart. Scale bar, 30  $\mu\text{m}$  for (A-C) and 60  $\mu\text{m}$  for (D,E).

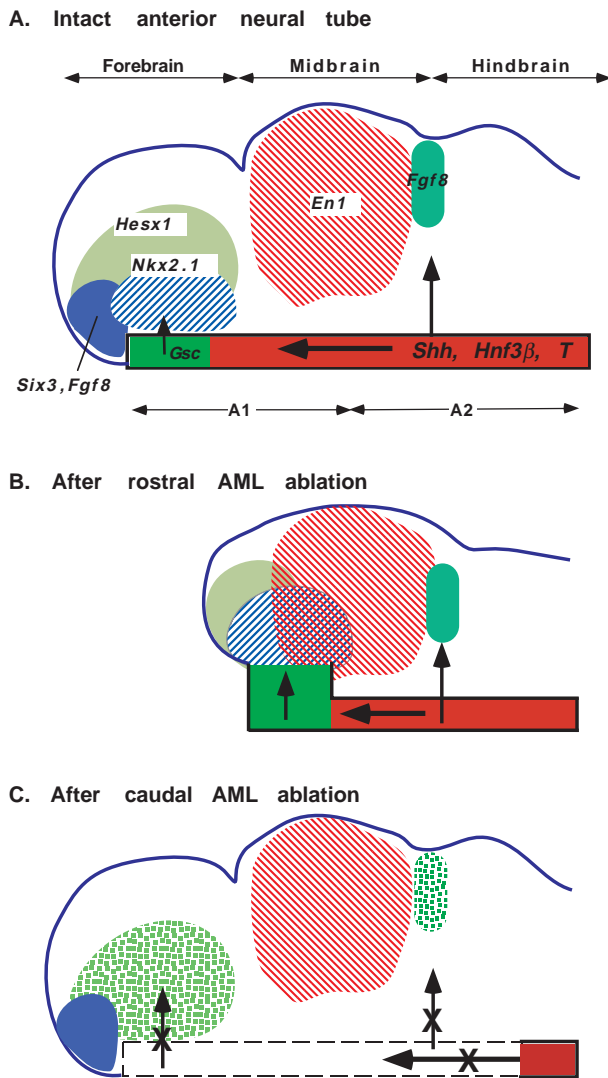


**Fig. 12.** Induction of general (*Sox2*) and regional (*Otx2*, *En1*) neural markers after transplantation of A1, A2 or LG tissues. *lacZ*-expressing graft-derived tissues are marked by the arrowhead. (A,D,G) Control LG grafts display expression of *Sox2* but not *Otx2* and *En1*. No signal is detected in the host tissues next to the LG grafts. (B,E) A1 grafts induce the expression of *Sox2* and *Otx2* (arrows) in the adjacent host tissues. (H) A1 graft suppresses the expression of *En1* gene in the host midbrain on the side of the graft (asterisk). (C,F,I) A2 grafts induce *Sox2*, *Otx2* and *En1* expression in the host (black or white arrows). Embryos are shown with anterior to the left; (C,D,F,I) lateral view, (A,B,E,G,H) dorsal view. Scale bar, 125  $\mu\text{m}$ .

## DISCUSSION

Whether the correct patterning and morphogenesis of the anterior neural plate of the mouse embryo are dependent on the inductive interaction with the anterior mesendodermal tissues has been a contentious issue (Bally-Cuif and Boncinelli, 1997).

In this study, we have investigated the impact of removing either the AML, the node or the anterior PS of the early neural plate stage mouse embryo and the subsequent effects on anterior development. We have shown that the removal of the AML leads to defective morphogenesis of the anterior neural tube during neurulation. Furthermore, the ablation of specific



segments of the AML has differential effects on the regionalisation of the neural tube and the differentiation of the midline tissues (Fig. 13). The ablation of the rostral AML disrupts morphogenesis and regionalisation of the forebrain. However, a compensatory molecular mechanism leads to the upregulation of genes specifically expressed in the rostral AML and the specification of ventral forebrain tissues takes place. We further showed that *Gsc* is required for the morphogenetic activity of the rostral AML. The ablation of the caudal AML does not cause the truncation of the neural axis but leads to the failure of the differentiation of the axial mesoderm, ventral diencephalon and floor plate of the head region. The functional requirement of this caudal segment for the maintenance of the prechordal plate and the ventral forebrain development represents a previously unexpected role. Finally, transplantation experiments have shown that both segments of the AML can induce neural gene activity but exhibit limited patterning activity.

#### Development of the forebrain and midbrain require AML activity

The expression analysis of region-specific markers has shown that A1 ablation leads to a reduction or absence of expression of

**Fig. 13.** Summary of the effects of the ablation of specific segments of the AML on gene expression and morphogenesis of the brain and midline tissues of the early-somite-stage embryo. (A) Gene expression domains in the anterior neural tube and axial mesoderm (the green and red painted boxes delineate the prechordal plate and the notochord, respectively). (B) Rostral AML (A1) ablation results in the truncation of the head, accompanied by the loss or reduction of *Six3*, *Fgf8* and *Hesx1* activity in the rostral forebrain. Following A1 ablation, the caudal AML tissues upregulate *Gsc* and *Shh* activity (enlarged green box) which is accompanied by the expression of *Nkx2.1* activity in the brain tissues. (C) Caudal AML (A2) ablation results in the downregulation or loss of the forebrain marker *Hesx1* (dotted light green), the lack of expression of *Nkx2.1* in the ventral diencephalon and the reduction of *Fgf8* expression in the tissues at the mid/hindbrain boundary (dotted dark green). A2 embryos display a complete loss of the axial mesoderm, ventral diencephalon and floor plate in the anterior neural tube (dashed empty box) indicating a critical requirement of the caudal AML for the maintenance of the rostral AML tissues. Arrows are showing putative planar and vertical signals emanating from the mesoderm that may be responsible for the maintenance and regionalisation of the mesoderm and its patterning activity on the neural tube.

forebrain markers, *Hesx1*, *Six3* and *Fgf8*. These findings indicate a requirement of the rostral AML for the patterning of the forebrain neural precursors during neurulation. The ablation of the rostral segment of the AML may also have removed the anterior neural ridge (ANR) precursor cells at the junction of the anterior neural plate and the non-neural ectoderm. Specific signals emanating from the ANR have been shown to play a role in growth and regional specification within the forebrain (Shimamura and Rubenstein, 1997; Houart et al., 1998) and their removal may affect the growth of the forebrain tissues and result in truncation of the cephalic neural tube. Interestingly, in A1 embryos, the *En1*- and *Wnt1*-expressing midbrain tissues seem to merge with the *Hesx1*-expressing forebrain tissues. The loss of rostral midline tissues therefore seems to cause a deficiency of forebrain tissues. The deficiency in forebrain tissues may reflect a predominant differentiation of the neural precursors into midbrain tissues in the absence of rostral AML at the early neural plate stage. A potential role for the AML that contains the prechordal plate is the maintenance of an anteriorising signal to counteract the posteriorising signals imposed on the forebrain (Ang et al., 1994; Bally-Cuif and Boncinelli, 1997; Bang et al., 1997; Foley et al., 1997; Rowan et al., 1999).

Ablation of the caudal segment of the AML also leads to significant changes in the patterning of the forebrain. The anterior marker *Hesx1* is downregulated or absent in the early-somite-stage A2 embryos. Moreover, A2 ablation results in the loss of *Gsc* and absence of *Nkx2.1* activity in the forebrain. However, only *Nkx2.1* expression is affected in *Gsc*-deficient embryos while *Hesx1* expression is unaffected. These findings strongly suggest a requirement for *Gsc* activity in the specification of the ventral forebrain. In contrast, *Gsc* activity is not essential for the morphogenetic activity of the AML responsible for maintenance of the early anterior regional neural marker *Hesx1* (Thomas and Beddington, 1996).

#### Caudal AML activity is required for the maintenance of the prechordal mesoderm and can compensate for the loss of rostral AML tissues

The loss of *Gsc*, *Shh*, *Hnf3β* and *T* activity in the axial

mesendoderm and floor plate in the head region in the A2 embryo (Fig. 13) suggest that the caudal AML is required for the maintenance of the prechordal plate and the ventral neuroectoderm of the anterior neural tube. In this study, since the ablation removes the axial mesendoderm and neuroectoderm precursors, we were not able to discriminate the relative role of the two tissue components in the maintenance of the rostral AML. The disruption of the specification of ventral neural fates and the absence of *Nkx2.1* activity in the forebrain can be related to the loss of anterior mesendoderm in A2 embryos. This role of the AML on dorsoventral patterning is consistent with the notion that axial mesendoderm provides inductive signals for the establishment of dorsoventral polarity and the specification of neuronal types in the neural tube (Ruiz i Altaba, 1994; Tanabe and Jessell, 1996; Dale et al., 1997, 1999; Shimamura and Rubenstein, 1997).

In contrast to A2 embryos, A1 embryos express *Shh*, *Hnf3 $\beta$*  and *T* in the midline and are able to reconstitute the *Gsc* activity after the ablation of the *Gsc*-expressing rostral AML (Fig. 13). Moreover, the presence of *Nkx2.1*-expressing cells in A1 embryos suggests that the reconstituted AML has the ability to differentiate into ventral forebrain tissue. Consistent with the response of the caudal AML in the embryo following A1 ablation, the same tissue fragment expresses *Gsc* strongly after its separation from the rostral AML and transplantation to ectopic site. This raises the intriguing possibility of the existence of morphogenetic interactions between the two segments of the AML, which serve to maintain their segment-specific genetic activity and tissue potency.

Collectively, these results suggest that the rostral segment is dependent on signals emanating from the caudal segment to maintain its function. In return, the rostral AML may act to inhibit the caudal AML from adopting a prechordal fate. When the caudal AML is released from this constraint it will activate de novo genetic activity to restore the patterning role that normally resides within the rostral AML.

### **Goosecoid is essential for the specification of the ventral forebrain and is involved in the regulatory mechanisms leading to the reconstitution of rostral AML activity**

Several studies have supported a role for *Shh* acting alone (Roelink et al., 1994; Ericson et al., 1995, 1996; Shimamura and Rubenstein, 1997) or with *Bmp7* (Dale et al., 1997, 1999) in the regulation of the *Nkx2.1* activity. In this study, a molecular analysis performed on early-somite-stage *Gsc*-deficient embryos demonstrates an absence of *Nkx2.1* expression within the ventral neural tube. Since *Shh* expression appears normal in these *Gsc*<sup>-/-</sup> embryos, we conclude that *Shh* activity is not sufficient and that *Gsc* activity is required for *Nkx2.1* expression in the ventral diencephalon.

In wild-type embryos, both *Shh* and *Gsc* expressions are enhanced in the AML following A1 ablation. Interestingly, the same ablation performed on *Gsc*<sup>-/-</sup> embryos provides evidence that these embryos are compromised in their ability to upregulate *Shh* activity in the anterior neural tube, although expression in the mesendoderm appears unaffected. This finding supports a requirement for *Gsc* in the mechanism leading to the reconstitution of the AML activity. Surprisingly, we found that some *Gsc*<sup>-/-</sup> embryos display *Nkx2.1* expression within the ventral neural tube following A1 ablation. An initial

interpretation of these observations may be that a parallel molecular mechanism is promoted for the reconstitution of the AML and the specification of ventral diencephalon fate within *Gsc*<sup>-/-</sup> A1 embryos. An alternative interpretation may be that the compensatory expression of *Shh* activity that occurs in some *Gsc*<sup>-/-</sup> A1 embryos is sufficient to elicit *Nkx2.1* expression.

### **Does the mouse AML possess neural-inducing and regionalising properties?**

Results of our transplantation experiments show that both rostral and caudal AML possess neural-inducing ability (formation of extra neuroectoderm and induction of *Sox2*) and patterning activity (ectopic induction of regional markers such as *Otx2* and *En1*). This finding is reminiscent of those of other studies in which the anterior mesendoderm (including some axial, paraxial and lateral tissues) from neural-fold-stage mouse embryos can induce the expression of *Otx2* and *En1* in the early gastrula epiblast (Ang and Rossant, 1993; Ang et al., 1994). In our experiments, we found that the lateral germ layers, which contain neuroectoderm precursors but lack axial mesendoderm, display no inductive activity. This strongly suggests that the neural inductive activity is provided by the axial mesendoderm in the AML fragment and is not brought about by homeogenetic induction by the neural precursors in the graft.

Contrasting results on the anteriorizing and ventralising activity of the prechordal plate have been reported for the mouse and the rat embryo (Dale et al., 1997; Shimamura and Rubenstein, 1997). In our study, an inhibition of *En1* expression is found adjacent to rostral AML grafts in the host midbrain. However, we found that neither segment of the AML has the ability to impart anterior forebrain identity to the host neural plate (no ectopic induction of the anterior marker *Hesx1*). The suppression of *En1* but not induction of *Hesx1* in the host midbrain by A1 grafts suggest that rostral AML may modify but cannot impart novel anterior regional characteristics to more posterior levels of the host neural tube. This finding may reflect a restriction in the competence of the neural plate to respond to inducing signals. It has been demonstrated that the germ layers of the late primitive streak embryo can respond to inducing signals by expressing region-specific neural markers, *Otx2*, *En1* and *Krox20* (Tam and Steiner, 1999), and that later the headfold stage ectoderm retains the ability to respond to *En2*-inducing signals in explant-recombination assays (Ang and Rossant, 1993). Nevertheless, the window of time during development when the neuroectoderm remains competent to respond to specific inducing signals is not yet established in the mouse.

Results of our study point to a role of the AML in the maintenance of the segmental characteristics of the neural axis and subsequent regionalisation of the developing forebrain. Furthermore, our findings provide evidence that continuous interactions between the rostral and caudal segments of the AML are essential for the regionalisation and patterning activity of the anterior mesendoderm.

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