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Msx1 is required for dorsal diencephalon patterning

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SUMMARY

The dorsal midline of the neural tube has recently emerged as a major signaling center for dorsoventral patterning. Msx genes are expressed at the dorsal midline, although their function at this site remains unknown. Using *Msx1*^{nlacZ} mutant mice, we show that the normal expression domain of *Msx1* is interrupted in the pretectum of mutant embryos. Morphological and gene expression data further indicate that a functional midline is not maintained along the whole prosomere 1 in *Msx1* mutant mice. This results in the downregulation of genes expressed laterally to the midline in prosomere 1, confirming the importance of the midline as a signaling center. *Wnt1* is essential for dorsoventral patterning of the neural tube. In the *Msx1* mutant, *Wnt1* is downregulated before the midline disappears, suggesting that its expression depends on *Msx1*. Furthermore, electroporation in the chick embryo demonstrates that *Msx1* can induce *Wnt1* expression in the diencephalon neuroepithelium and in the lateral ectoderm. In double *Msx1*/*Msx2* mutants, *Wnt1* expression is completely abolished at the dorsal midline of the diencephalon and rostral mesencephalon. This indicates that Msx genes may regulate *Wnt1* expression at the dorsal midline of the neural tube. Based on these results, we propose a model in which Msx genes are intermediary between Bmp and Wnt at this site.

Key words: Mouse Msx genes, Brain development, Subcommissural organ, Prosomeres, Roof plate, Dorsal midline, Wnt, Bmp, In ovo electroporation

INTRODUCTION

In recent years, a number of experiments have demonstrated that the dorsal midline of the vertebrate neural tube is required for the specification of dorsal cell fates in the central nervous system (reviewed by Lee and Jessell, 1999). Genetic ablation of the roof plate leads to the loss of dorsal interneurons in the spinal cord (Lee et al., 2000). This results from the elimination of non-autonomous signals provided by the roof plate. Proteins from the Bmp and Wnt families are good candidates to mediate dorsal neural tube organizing activity. Thus, the double *Wnt1/Wnt3a* mutation results in a pronounced reduction of dorsal interneuron progenitors in the spinal cord (Ikeya et al., 1997; Muroyama et al., 2002). Similarly, *Gdf7* inactivation leads to the selective loss of the D1A class of dorsal sensory interneurons (Lee et al., 1998). Furthermore, Bmp4 and Bmp7 can induce a dorsal fate in neural tube explants when added exogenously (Liem et al., 1997). Therefore, the roof plate constitutes an essential organizing center for dorsal patterning of the neural tube. Noticeably, most of these reports have dealt with the spinal cord, and little is known about the function of the dorsal midline in the more anterior part of the neural tube.

Msx genes encode homeodomain transcription factors that are expressed dorsally in the neural tube of all species throughout chordate evolution. This may therefore reflect an ancestral function for these genes in central nervous system (CNS) patterning. Indeed, the single Msx gene of *Amphioxus* is expressed exclusively at this site (Sharman et al., 1999). In *Drosophila*, *Msh*, the Msx ortholog, is required for the specification of lateral neuroblasts, which can be considered as the homologs of the dorsal neurons in vertebrates (Cornell and von Ohlen, 2000). In the mouse, the Msx gene family comprises three members (*Msx1*, *Msx2* and *Msx3*). All three genes are expressed in the dorsal aspect of the neural tube from early stages of neurogenesis (Robert et al., 1989; Catron et al., 1996; Shimeld et al., 1996; Wang et al., 1996). At the neural fold stage, *Msx1* is expressed in the future dorsal neural cells at the ectoderm/neuroectoderm boundary. Thereafter, at the neural tube closure stage, *Msx1* expression is restricted, in the CNS, to the dorsal midline along its entire length (Liem et al., 1995; Furuta et al., 1997) (A.B., Y.L. and B.R., unpublished).

In the midline, *Msx1* is co-expressed with Bmp and Wnt genes and might therefore play a role in signaling by these diffusible molecules. Whereas *Msx3* expression is restricted to the neural tube (Shimeld et al., 1996; Wang et al., 1996), *Msx1* and *Msx2* are expressed in a number of structures that are formed by inductive processes between ectoderm and mesoderm, such as limb buds, craniofacial processes and tooth buds. At these sites, functional inductive interactions are required for the expression of the two genes, and these interactions involve signaling molecules of the Bmp and Fgf families (reviewed in Davidson, 1995; Peters and Balling, 1999).

Msx1 homozygous mutants die at birth. They exhibit cleft palate, an arrest in tooth development, with defects in the craniofacial skeleton and inner ear (Satokata and Maas, 1994; Houzelstein et al., 1997). *Msx2* homozygotes are viable and fertile, but display pleiotropic defects, including abnormal teeth and loss of fur that can be related to inductive processes (Satokata et al., 2000) (M.-A.N. et al., unpublished). In addition, the cerebellar lobules are reduced in number and disorganized. Analysis of *Msx1*/*Msx2* compound mutants has further revealed functional redundancy between these genes in most of these processes because the absence of both Msx genes results in earlier and stronger phenotypes, particularly in skull bones and ectodermal organs (teeth, hair follicles and mammary glands).

The implication of Msx genes in ecto-mesodermal induction processes has been extensively studied. However, despite a number of data strongly indicative of a role for Msx genes in the development of the CNS, little is known about their role in this structure except for the implication of *Msx2* in cerebellum foliation. In this report, we demonstrate, using *Msx1*-null mutants that we had produced previously (Houzelstein et al., 1997), that this gene is necessary for the correct development of the dorsal midline of the diencephalon. In the *Msx1* mutant, the expression of genes normally expressed at the dorsal midline is extinguished specifically in prosomere 1 (P1), the most caudal subunit of the diencephalon. This leads to the downregulation of genes expressed laterally to the midline, confirming the importance of this structure for dorsal CNS patterning. Failure to form a functional dorsal midline in P1 precludes development of the subcommissural organ and leads to prenatal hydrocephalus. *Wnt1* downregulation is observed early and takes place before the disappearance of the midline. This observation, together with ectopic expression experiments in the chick embryo, indicate that *Wnt1* is a target of *Msx1*. In addition, analysis of *Msx1/Msx2* double homozygous mutants confirms that the Msx genes are required for expression of *Wnt1* at the pro- and mesencephalon dorsal midline. We propose a model in which Msx genes play a role in Bmp and Wnt signaling to form a functional dorsal midline. The latter acts as an organizing center to induce or maintain in neighboring tissues the expression of genes essential for dorsal brain patterning.

MATERIALS AND METHODS

Mouse embryos

Generation of null alleles of the mouse *Msx1* and *Msx2* homeobox genes by insertion of an *nlacZ* reporter gene and genotyping of the animals are described elsewhere (Houzelstein et al., 1997) (M.-A.N., A.B., C.R., T. Paquet, P .J. R. Barton and B.R., unpublished). *Msx1* mutants were isogenized on a C57BL/6J background. *Msx2* mutants were on a mixed 129/Sv×C57BL/6 genetic background.

In situ hybridization

Whole-mount in situ hybridization was performed as described previously (Houzelstein et al., 1997). DNA fragments used to generate RNA probes were the following: *Wnt3a*, 740 bp (3′ untranslated region obtained by PCR; F: GGA ATT CCA ATT TGG GCC GGA AGT CC; R: CGG GAG ATC TGA GTA TTA AGT GTC AGA GC); *Otx2*, 408 bp (coding for amino-acids 110 to 243); *Lim1*, 441 bp (coding for amino-acids 259 to 406); *Pax7*, *Pax6* and *Pax3* (complete cDNA kindly provided by Dr F. Relaix); *Wnt1*, 1100 bp (Parr et al., 1993) (kindly provided by Dr A. McMahon); *Bmp6*, 893 bp (EcoRI-SacI cDNA fragment kindly provided by Dr B. Hogan); and *Gbx2*, 620 bp (untranslated region; kindly provided by Dr G. Martin). In situ hybridization following staining for β-galactosidase activity was performed according to Tajbakhsh and Houzelstein (Tajbakhsh and Houzelstein, 1995).

Histology and histochemical staining

For histology, embryos or newborn animals were fixed overnight in Bouin's fixative, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax. Sections were cut at 15 μm and stained with Hematoxylin and Eosin. β-Galactosidase staining was performed as described by Houzelstein et al. (Houzelstein et al., 1997). Nile Blue staining was performed according to Anderson et al. (Anderson et al., 2002).

Expression plasmid construction and electroporation in chick embryos

The complete coding sequence of a chick *Msx1* cDNA (Robert et al., 1991) was amplified by PCR (Forward primer: CGC CGG TCG ACT GCA TGG CCC CGG CT; Reverse primer: CGC CGC TCG AGG CGG CTC GGC CCT ATG TAA). These primers were designed to add *Sal*I and *Xho*I (underlined) restriction sites at the 5′ and 3′ of the *Msx1* sequence, respectively. The cDNA was inserted into the *Xho*I site of the pCIG plasmid (Megason and McMahon, 2002) (kindly provided by Dr A. McMahon). The resulting construct was electroporated into the diencephalon of chick embryos by a modification of the procedure described by Mathis et al. (Mathis et al., 2001). Plasmid DNA was prepared using Qiagen Maxiprep and resuspended in water at 3mg/ml of DNA and 0.01% fast green. Eggs were incubated at 37°C for 40 hours to reach the 8-16 somite stage. After removal of 3 ml of albumin, a window was made into the eggshell and Hank's solution (Sigma) was dropped onto the embryo. DNA solution was introduced in the neural tube or over the head lateral ectoderm using a pressure injection apparatus (Picospritzer, General Valve). Then, four square pulses (duration=50 ms) of 25 V were applied using curved platinum electrodes (5 mm separation) placed on either side of the embryo. Embryos were recovered in PBS 6-24 hours after electroporation, photographed for GFP expression profile, then fixed for 24 hours in PFA 4%, dehydrated progressively in methanol, and conserved at –20°C. In situ hybridization was performed with a chick *Wnt1* probe (Bally-Cuif and Wassef, 1994) as described above. Double in situ hybridization was performed using a modification of the protocol by Dietrich et al. (Dietrich et al., 1997). In brief, a chick *Msx1* probe (Robert et al., 1991) was labeled with fluorescein-UTP and the chick *Wnt1* probe with DIG-UTP (Roche). After hybridization, the fluorescein-labeled RNAs were detected using INT/BCIP (Roche) as substrate for the anti-fluorescein antibody-coupled alkaline phosphatase. Then, the alkaline phosphatase activity was inhibited by heating at 70°C for 30 minutes in PBS and the alkaline phosphatase-coupled anti-DIG-antibody was applied. Staining was performed with BM Purple AP substrate (Roche). The INT/BCIP red precipitate was washed out during processing of the second antibody.

RESULTS

The subcommissural organ is missing in *Msx1*$^{-/-}$ mutants

We have observed that some of the *Msx1* homozygous mutants display at birth a hydrocephalus, which is characterized by the dilatation of the third and fourth ventricles (A.B., Y.L. and B.R., unpublished). Anatomical analysis showed that the sub-commissural organ (SCO) is affected. The SCO is a circumventricular gland at the forebrain-midbrain boundary that originates from the neuroepithelial cells of the pretectal dorsal midline beneath the posterior commissure. Histological analysis on sagittal sections revealed that, at E15.5, the SCO was severely reduced or absent in all homozygous mutants and that the posterior commissure was disorganized (Fig. 1A,B). In addition, this commissure was colonized by numerous nucleated cells (as revealed by Hematoxylin staining) instead of being composed mainly of neural fibers (Fig. 1A,B). Morphological defects could be identified at this site from E11.5 in about one third of the homozygous mutant embryos, in the form of an indentation at the boundary between diencephalon and mesencephalon (Fig. 1C; see also Fig. 5H for E14.5).

By contrast, we did not detect any defect in the rest of the brain. In particular, the habenular recess and commissure, and the pineal gland situated anterior to the SCO region, were normal. Defects were found only between the pineal gland, which forms at the caudal boundary of prosomere 2, and the posterior commissure, which extends, caudally, to the diencephalon/mesencephalon boundary. On these anatomical criteria, all defects appear limited to structures that derive from prosomere 1 (P1) (Puelles and Rubenstein, 1993; Rubenstein and Puelles, 1994).

The *Msx1*^{nlacZ} expression domain disappears specifically from prosomere 1 in the *Msx1*$^{-/-}$ homozygous mutants

The fate of midline *Msx1*-expressing cells was analyzed at earlier stages in heterozygous and homozygous mutant embryos using the *nlacZ* reporter gene that we had targeted to the *Msx1* locus (Houzelstein et al., 1997). We first verified that the β-galactosidase activity in the CNS of mutant embryos matched *Msx1* transcript distribution. Similar results were obtained with X-gal staining and in situ hybridization at different stages of development, confirming that *Msx1* is expressed at the midline of the CNS from, rostrally, the optic stalk to, caudally, the tip of the tail (data not shown). Analysis of sections further confirmed that, at E12.5, *Msx1*^{nlacZ} is expressed in the ependymal layer at the dorsal midline of the brain over its entire length, particularly in the SCO cells underneath the posterior commissure (data not shown).

At E14.5, all homozygous mutant embryos tested (15/15) displayed a clear interruption of the β-galactosidase domain in the midline of the pretectum, between the pineal anlage and the di-mesencephalon boundary (Fig. 2A,B). These anatomical landmarks, together with gene expression data (see below) confirm that all defects are restricted to P1. In a few cases, a short remnant of the *nlacZ*-expressing domain could be detected just posterior to the pineal anlage, but the main region of the midline that includes the SCO anlage was not labeled.

At E12.5, the situation was much more variable. The pretectum midline aspect varied from a total absence of β-galactosidase activity (Fig. 2C,D) to an apparently normal appearance (not shown). At E9.5, in most embryos the midline looked normal. Between E10.5 and E11.5, the phenotype appeared progressively and differed from an embryo to the other (see Fig. 6B,D). Therefore, the disappearance of the *Msx1*^{nlacZ} expression domain is of variable onset.

The organization of the midline is affected in prosomere 1 of the *Msx1*$^{-/-}$ mutants

Analysis of the *Msx1* mutant had previously not detected any difference in *Msx1*^{nlacZ} expression between the homozygous mutant and the heterozygote (Houzelstein et al., 1997). This

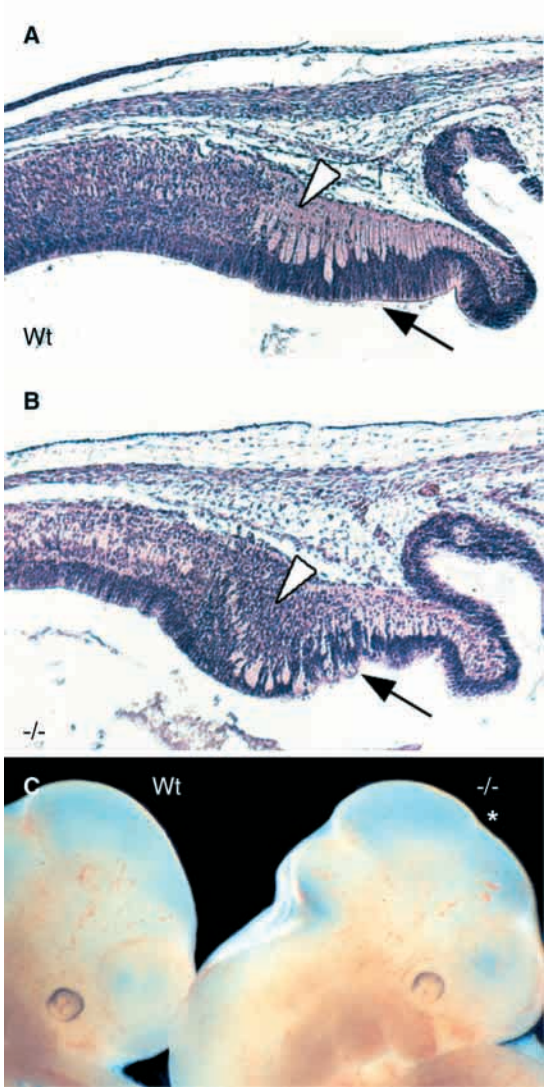


Fig. 1. Abnormalities of the pretectum in *Msx1*$^{-/-}$ embryos. (A,B) Sagittal sections at the level of the SCO of E15.5 wild-type (A) and homozygous mutant (B) embryos. Nuclei are stained in violet (Hematoxylin) and cytoplasm in pink (Eosin). In the homozygous mutant, the posterior commissure (B, arrowhead) exhibits an abnormal density of nuclei and a disorganization of fiber tracts, while the SCO is absent (arrow). (C) Comparison of *Msx1* homozygous mutant with wild type at E11.5. Note the indentation at the caudal diencephalon level (white asterisk). In all panels, anterior is towards the right; Wt, wild type; –/–, *Msx1*$^{-/-}$ homozygous mutant.

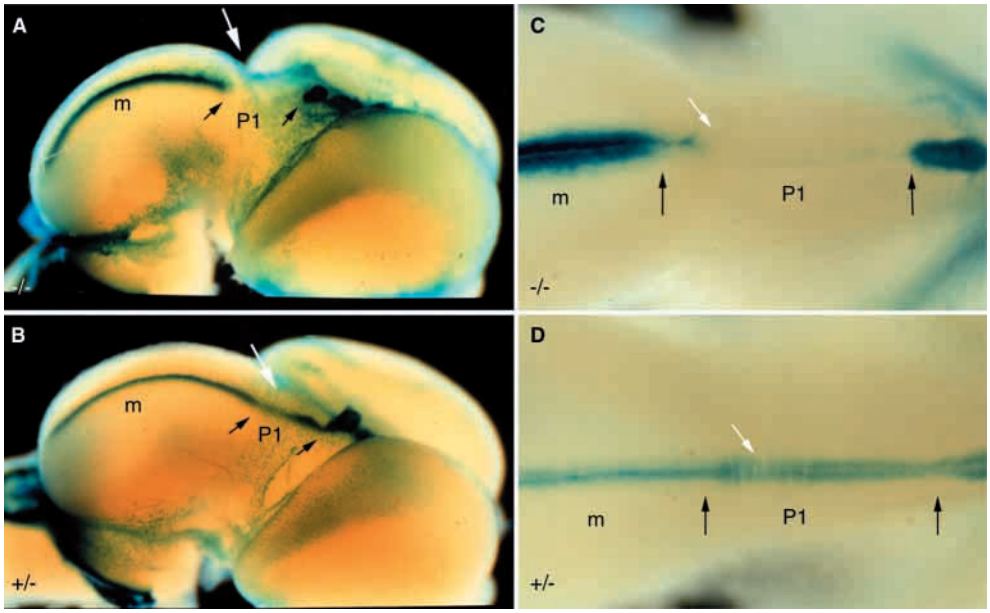


Fig. 2. Interruption of the *nlacZ* expression domain in *Msx1*$^{-/-}$ embryos. (A,B) Whole-mount X-Gal stained brains of E14.5 homozygous mutant (A) and heterozygous (B) embryos show that the absence of a functional *Msx1* leads to the loss of the *Msx1*-expressing domain in P1. (C,D) Dorsal views at a higher magnification of the P1 region of E12.5 homozygous mutant (C) and heterozygous (D) embryos after whole-mount X-Gal staining. Black arrows indicate the limits of P1 and white arrows the posterior commissure/SCO primordium region. In all panels, anterior is towards the right. m, mesencephalon; P1, prosomere 1; +/–, *Msx1*$^{+/-}$ heterozygote; –/–, *Msx1*$^{-/-}$ homozygous mutant.

implies that, at most sites, *Msx1* is not required for its own transcription. With a more extensive analysis, we now report that the *Msx1*^{nlacZ} expression at the dorsal midline of P1 is affected in the mutant. It is unlikely that the MSX1 protein should be necessary to maintain an MSX1-positive feedback loop specifically in P1. The most plausible explanation is that the structure of the pretectum dorsal midline is affected in the absence of *Msx1*.

This hypothesis is further substantiated by comparing the expression profiles of several genes normally expressed in the diencephalon dorsal midline, between *Msx1*$^{-/-}$ and wild-type or heterozygous embryos. In normal embryos, *Wnt1*, *Wnt3a* and *Bmp6* are expressed in the diencephalon dorsal midline at E11.5 (Parr et al., 1993; Furuta et al., 1997). In the *Msx1* homozygous mutants, the expression domains of these three markers were interrupted in the pretectum midline (Fig. 3A-D and data not shown). At E12.5, *Wnt3a* transcripts were no longer detectable at the pretectum midline (not shown), whereas expression of several genes that are excluded from the midline was observed at this site in the *Msx1* mutant. This is the case for *Pax6* which is normally expressed in two lateral domains adjacent to the midline (Fig. 3E; see also Fig. 5C). We further confirmed by in situ hybridization after staining for β-galactosidase in heterozygous embryos that there is no co-expression of *Pax6* and *Msx1*^{nlacZ} before E14.5 (data not shown), when *Pax6* begins to be expressed in the SCO layer (Estivill-Torrus et al., 2001). In the mutant, at E12.5, cells of the dorsal midline were observed to express *Pax6*, in such a way that the two lateral domains are no longer separated medially (Fig. 3F). At E14.5, similar results were obtained with *Pax7* and *Lim1* as well as *Pax6*. Expression of these genes in the mutant, in contrast to the normal situation, extends across the midline (arrows in Fig. 5B,D,F). These results suggest that in the absence of *Msx1*, dorsal midline cells are not maintained in P1.

Cell death is reduced in the dorsal midline of *Msx1*$^{-/-}$ homozygous embryos

The disappearance of the dorsal midline might be due to enhanced cell death in this structure. This was evaluated using Nile Blue sulfate on E10.5-E12.5 embryos. The dorsal midline of the diencephalon is a prominent site for cell death after neural tube closure (Schlüter, 1973). In normal embryos, we indeed observed that the rate of cell death is high at the dorsal midline of the P1 prosomere relatively to the lateral as well as more rostral or caudal territories. At E10.5, Nile Blue-staining cells formed a continuous line along prosomere P1 in most embryos (8/10) and were too numerous to be counted precisely (not shown). At E11.5, dead cells were still numerous, in the range of 20-40 per embryo (Fig. 4A). Unexpectedly, at these two stages homozygous littermate embryos displayed much fewer dead cells and heterozygotes had an intermediate phenotype (Fig. 4B; summarized in Table 1). No homozygous embryo was observed with a high or intermediate dead cell rate. After Nile Blue staining, embryos were labeled for β-galactosidase activity (Fig. 4C). Only embryos that displayed an uninterrupted midline were taken into account for cell death quantitative analysis (Table 1). These showed unambiguously that decrease in cell death preceded midline disappearance, indicating that cell death was not causative in this process. At E12.5, few cells were stained with Nile Blue sulfate and no significant difference was observed between normal and mutant embryos.

In conclusion, increased cell death does not account for the disappearance of the dorsal midline in *Msx1*$^{-/-}$ embryos. Together with the changes in gene expression at the midline (see above), these results suggest that, in the absence of Msx1, the dorsal midline changes identity to adopt more lateral territory properties, including a low rate of cell death.

The disruption of the dorsal midline affects the expression of dorsolateral markers specifically in prosomere 1

We further studied the expression pattern of several genes normally expressed in dorsolateral neural tissues of P1 such as *Pax6*, *Pax7* and *Lim1* (Fujii et al., 1994; Stoykova and Gruss, 1994). At E14.5, *Pax6* expression is downregulated in most mutant embryos and even undetectable in some, at the midline

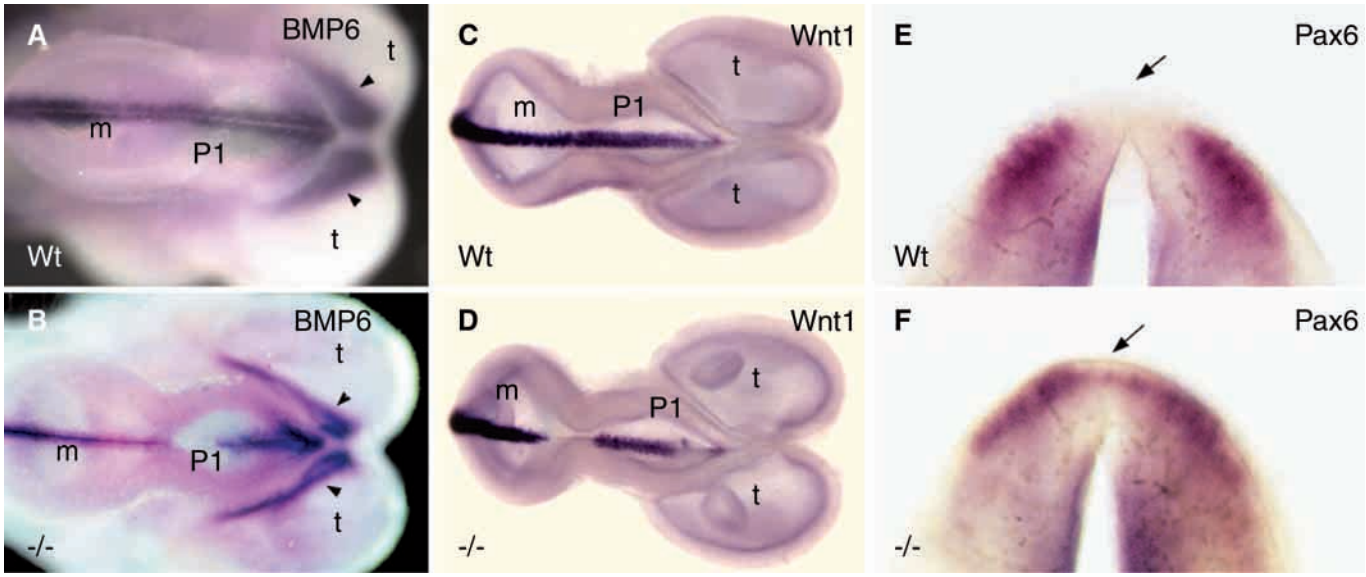
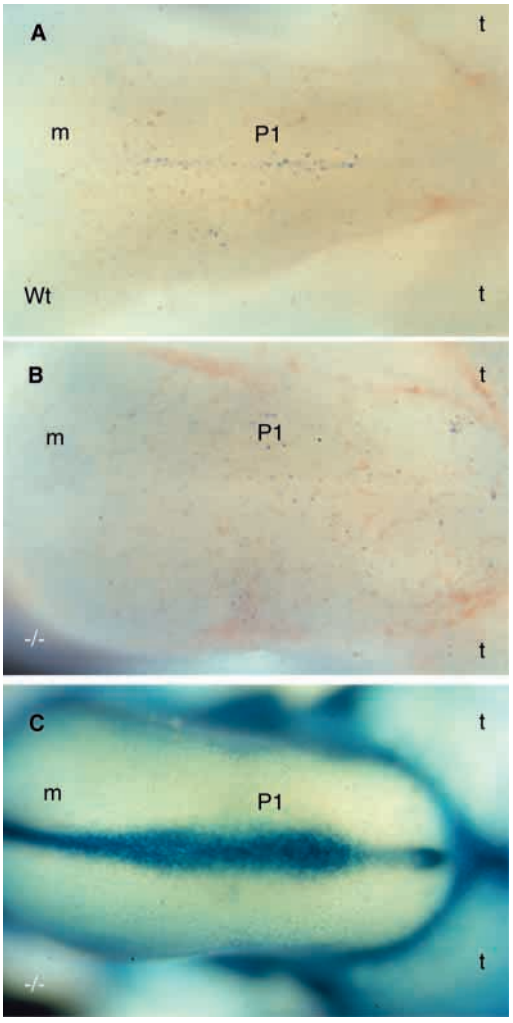


Fig. 3. Expression of midline markers is affected in *Msx1*$^{-/-}$ mutant embryos. E11.5 wild-type (A,C) and mutant (B,D) embryos were whole-mount hybridized with a *Bmp6* (A,B) or a *Wnt1* (C,D) probe. Arrowheads in A,B indicate the *Bmp6* expression domains in the telencephalic choroid plexus. (E,F) Transverse sections across P1 of E12.5 embryos hybridized with a *Pax6* probe. In the mutant, the *Pax6* expression domain extends over the midline (F, arrow) from which it is normally excluded (E, arrow)). In A-D, anterior is towards the right. m, mesencephalon; P1, prosomere 1; t, telencephalon; Wt, wild type; –/–, *Msx1*$^{-/-}$ homozygous mutant.



and in the dorsolateral domain (Fig. 5C,D and data not shown). By contrast, *Pax6* expression is maintained in the epiphysis and in the telencephalon of *Msx1* mutants, in accordance with our observation that defects are limited to P1 (not shown). As *Msx1* and *Pax6* are not co-expressed in the diencephalon of *Msx1* mutants, downregulation of *Pax6* must be an indirect effect of the absence of *Msx1* in P1. *Lim1* is less strongly expressed laterally in the rostral part of P1 in the mutant at E14.5 (Fig. 5E,F). Similarly, *Pax7* expression is affected in the anterior part of P1 only, with a clear rostral boundary (Fig. 5A,B). This indicates that there are two functional domains in P1 seen here for *Pax7* and *Lim1* expression, one that is dependent on MSX1 activity and the other not. Analysis of the expression pattern of other genes expressed in the dorsolateral part of P1, such as *Otx2* and *Pax3*, confirms that a functional dorsal midline is necessary to specify neural tissues adjacent to the roof plate (not shown).

Strikingly, *Pax7* expression profile is not affected in the mesencephalon by the *Msx1* mutation (Fig. 5A,B). In situ hybridization with *Gbx2*, which is expressed dorsolaterally in prosomere 2, showed that the dorsolateral tissues of this prosomere are not affected either (Fig. 5G,H). Noticeably, expression which also takes place in the ventrolateral part of P1 was not affected in the *Msx1*$^{-/-}$ mutants, indicating a lateral

Fig. 4. Cell death is reduced in the diencephalon midline of *Msx1*$^{-/-}$ mutant embryos. Dorsal views of the diencephalon regions from E11.5 wild-type (A) or Msx1$^{-/-}$ (B,C) embryos. (A,B) Nile Blue staining. Dead cells are abundant (>20) in the midline of wild-type (A) but undetectable in that of *Msx1*$^{-/-}$ mutant (B) embryos. (C) β-Galactosidase staining of the same embryo as in B reveals that the diencephalon midline is still present. In all panels, anterior is towards the right. m, mesencephalon; P1, P1 prosomere; t, telencephalon; Wt, wild type; –/–, *Msx1*$^{-/-}$ homozygous mutant.

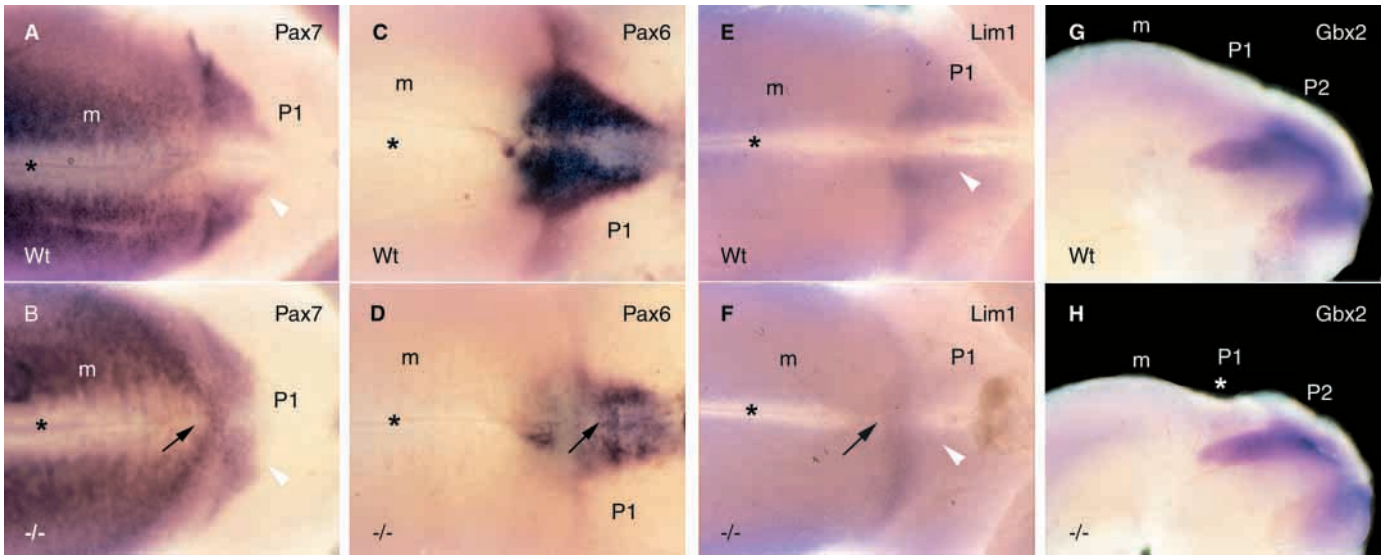


Fig. 5. Expression patterns of P1 and P2 markers in wild-type and homozygous mutant embryos at E14.5. In situ hybridization with a *Pax7* (A,B), *Pax6* (C,D), *Lim1* (E,F) or *Gbx2* (G,H) probe in wild-type (A,C,E,G) and in *Msx1*$^{-/-}$ homozygous (B,D,F,H) embryos. (A-F) Dorsal views. In *Msx1*$^{-/-}$ embryos, *Pax6*, *Pax7* and *Lim1* are expressed at the midline (B,D,F, black arrows), from which they are normally excluded (A,C,E). In addition, the dorsolateral domains of expression of these genes are reduced or absent in the entire P1 for *Pax6* and in its anterior part for *Lim1* and *Pax7* (white arrowheads). Note that *Pax7* and *Lim1* are not downregulated in the caudal part of P1, at the level of the posterior commissure. Black asterisks indicate the position of the dorsal midline (A-F). (G,H) Lateral views. *Gbx2* expression is normal in P2 and also in the lateral part of P1 in the *Msx1*$^{-/-}$ mutant (H). Note the dorsal depression at the level of P1 (H, white asterisk). The telencephalon has been removed to facilitate visualization of the entire diencephalon. In all panels, anterior is towards the right. m, mesencephalon; P1, prosomere 1; P2, prosomere 2; Wt, wild type; –/–, *Msx1*$^{-/-}$ homozygous mutant.

limit to the influence of the midline in this prosomere. These gene expression data further confirm that all defects are restricted to the dorsal part of P1 in the *Msx1* mutant, and demonstrate that the loss of *Msx1* function does not result in the transformation of P1 into a segment with more rostral or caudal identity.

Wnt1 is downregulated before the dorsal midline disappears in *Msx1*$^{-/-}$ embryos

Wnt1 has a dynamic pattern of expression with two major phases: at the neural plate stage, its expression takes place in a broad domain of the presumptive midbrain; from E9.5, expression is initiated at the dorsal midline of the neural tube, from the diencephalon through the spinal cord, with the exception of the r1 region in the metencephalon, and extends rostrally in the diencephalon up to the level of the P2/P3 boundary (Parr et al., 1993; Rowitch et al., 1998) (Fig. 8A). In this later phase, *Msx1* and *Wnt1* expression domains in the midline overlap in the spinal cord, the mesencephalon and the posterior diencephalon (Furuta et al., 1997) (A.B., Y.L. and B.R., unpublished). As mentioned previously, in *Msx1*$^{-/-}$ mutant embryos, at E11.5 *Wnt1* transcripts were diminished in the dorsal midline cells of P1, and *Msx1*^{nlacZ} expression was also reduced. To determine whether the downregulation of *Wnt1* precedes the disappearance of the *Msx1*^{nlacZ} expression domain, we performed in situ hybridization after staining for β-galactosidase activity at E10.5 and E9.5. At E10.5 in heterozygous embryos *Wnt1* and *Msx1*^{nlacZ} were co-expressed throughout the mesencephalon and the posterior diencephalon (Fig. 6C). In *Msx1*$^{-/-}$ embryos, *Wnt1* and *Msx1*^{nlacZ} were co-expressed in the mesencephalon. In some *Msx1*$^{-/-}$ embryos in which *Msx1*^{nlacZ} expression was weaker but still continuous in the P1 dorsal midline, the expression of *Wnt1* was reduced and limited to a few patches of cells (Fig. 6D). At E9.5 in *Msx1* mutant embryos, *Msx1*^{nlacZ} forms a continuous domain in P1 in most embryos. At this stage, *Wnt1* begins to be expressed at the dorsal midline of the diencephalon (Fig. 6A). In some mutant embryos, *Wnt1* expression was limited to a few scattered cells in P1 (Fig. 6B). These results demonstrate that, in all cases, *Wnt1* is downregulated before the *Msx1*^{nlacZ} expression domain has disappeared. This indicates that *Msx1* is required for the maintenance of *Wnt1* expression in the midline and may also be required for its initiation. Furthermore, it raises the possibility that the disappearance of a functional midline in the dorsal domain of P1 may be secondary to the downregulation of *Wnt1*.

Table 1. Cell death in the P1 dorsal midline

Stage	Genotype	Number of embryos with Nile Blue-positive cells in P1: More than 20 cells	Ten to 20 cells	Three to five cells
E10.5	Msx1$^{+/+}$	8	1	1
E10.5	Msx1$^{+/-}$	5	6	1
E10.5	Msx1$^{-/-}$	0	0	8
E11.5	Msx1$^{+/+}$	5	1	0
E11.5	Msx1$^{+/-}$	2	7	0
E11.5	Msx1$^{-/-}$	0	0	5

Dead cells were counted on whole-mounts over the length of the P1 midline.

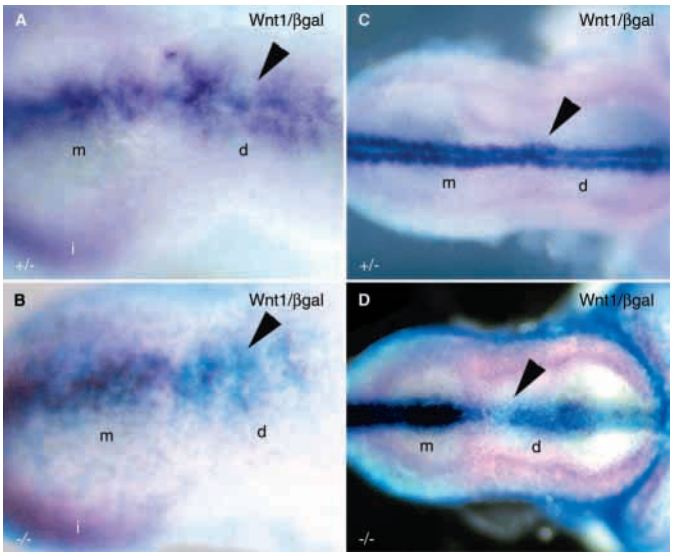


Fig. 6. *Wnt1* is downregulated before disappearance of the diencephalon midline in the *Msx1*$^{-/-}$ mutant. Embryos were stained for β-galactosidase activity (blue staining) before in situ hybridization for *Wnt1* (purple staining). At E9.5, some homozygous embryos (B) exhibit a reduced expression of *Wnt1* when compared with heterozygotes (A), while the *Msx1*^{nlacZ} domain is still present (arrowheads). (C,D) At E10.5, more embryos show this phenotype. This suggests that *Msx1* is required for the initiation (E9.5) and maintenance (E10.5) of *Wnt1* expression. Note that *Wnt1* expression in the isthmus (A, i), which derives from the first phase of expression, is not affected in the mutant (B, i). In all panels, anterior is towards the right. d, diencephalon; i, isthmus; m, mesencephalon; +/–, *Msx1*$^{+/-}$ heterozygote; –/–, *Msx1*$^{-/-}$ homozygous mutant.

Msx1 can induce *Wnt1* ectopically in the brain and in the lateral ectoderm

The striking overlap between *Msx1* and *Wnt1* expression domains in the CNS midline and the early downregulation of *Wnt1* in the *Msx1* mutant suggest that *Msx1* may genetically interact with *Wnt1*. Direct interactions between the two genes have been proposed, but no firm conclusion was reached (Shang et al., 1994; Iler et al., 1995). To investigate the possibility that *Msx1* might regulate *Wnt1* expression, we undertook electroporation of *Msx1* in chick embryo diencephalon and analyzed the expression of *Wnt1*. A chick *Msx1* cDNA (Robert et al., 1991) was inserted into the pCIG plasmid (Megason and McMahon, 2002). This plasmid contains an internal ribosome entry site followed by the coding sequence of a nuclear EGFP, downstream of the inserted sequence. This way, GFP fluorescence could be monitored after electroporation to identify cells that had incorporated plasmid DNA. Embryos were processed for in situ hybridization with a chick *Wnt1* probe. Fig. 7A,B shows that ectopic expression of *Msx1* in the lateral neuroepithelium of the diencephalon leads to the induction of *Wnt1*. Expression of *Wnt1* was further efficiently induced in the telencephalon and mesencephalon (Fig. 7A-D). *Wnt1* expression was always observed on the transfected side, associated with intense GFP fluorescence, and no signal was elicited by the pCIG plasmid itself (summarized in Table 2). More than 60% of treated embryos gave a positive response; the remaining ones had only a low level of GFP expression, suggesting that there is a threshold in the level of MSX1 protein that must be reached for the induction of *Wnt1* (data not shown).

We further electroporated pCIG-Msx1 DNA in lateral ectodermal cells of the head. In these cells too, *Msx1* induced expression of *Wnt1* (Fig. 7E,F). Therefore, induction of *Wnt1* expression is not restricted to the neural tube. In all cases, the *Wnt1* expression domain was included into the GFP-positive domain, suggesting that *Msx1* induces *Wnt1* expression by a cell-autonomous process. To investigate this observation more rigorously, we performed in situ hybridizations successively with a chick *Msx1* probe followed by a *Wnt1* probe on the same electroporated embryos. A limited number of cells were induced to express *Wnt1* in the *Msx1* expression domain, and each of these cells was expressing a high level of *Msx1* (Fig. 7E,F). This confirmed that induction of *Wnt1* requires a threshold level of *Msx1* expression. It further indicates that *Wnt1* induction is very likely to be cell autonomous.

To further investigate gene interactions, we performed a kinetics of *Wnt1* induction after electroporation of pCIG-Msx1 in the diencephalon (Table 2). Although GFP could be first detected 2 hours after electroporation (hae), no *Wnt1* induction was observed before 9 hae and it was still low at 12 hae Maximum induction was reached at 24 hae. Assuming that *Wnt1* induction is cell autonomous, this leaves enough time for an indirect, intracellular process of induction, in accordance with the known properties of MSX1 as a transcriptional repressor. However, conspicuous induction requires relatively high levels of MSX1 that might not be reached during the first hours. This leaves open the possibility of a direct activation of the *Wnt1* gene by MSX1.

Inactivation of both *Msx1* and *Msx2* leads to loss of *Wnt1* expression at the dorsal midline of the diencephalon

All three Msx genes in the mouse are expressed at the dorsal midline of the neural tube. *Msx1* and *Msx2* are expressed in the anterior part of the CNS at early stages, while *Msx3* expression does not extend rostrally beyond the isthmus (Shimeld et al., 1996; Wang et al., 1996). We reasoned that, if Msx genes are required for *Wnt1* expression, the pattern of expression of the latter should be more extensively affected in the double *Msx1/Msx2* mutants than in *Msx1* mutants. To test this hypothesis, we took advantage of an *Msx2* mutant strain constructed in our laboratory by substituting the *nlacZ* reporter gene to the coding sequence of the endogenous *Msx2* locus, using homologous recombination (M.-A.N., C.R., T. Paquet, P. J. R. Barton and B.R., unpublished).

Compound heterozygous animals were crossed to analyze the phenotype of double homozygous mutant embryos at E10.5 and E11.5. At these stages, a number of such embryos were resorbed. The others were all smaller than normal and most had a very abnormal morphology. In some embryos, an exencephaly encompassing the posterior mesencephalon and the rhombencephalon and a spina bifida were observed (data not shown). In all cases, the brain was small and abnormal. However, in some embryos the different brain regions were clearly visible and these were analyzed for *Wnt1* expression (Fig. 8B). In both E10.5 and E11.5 embryos, *Wnt1* expression was totally absent from the entire diencephalon and severely reduced in the mesencephalon, where only a small caudal

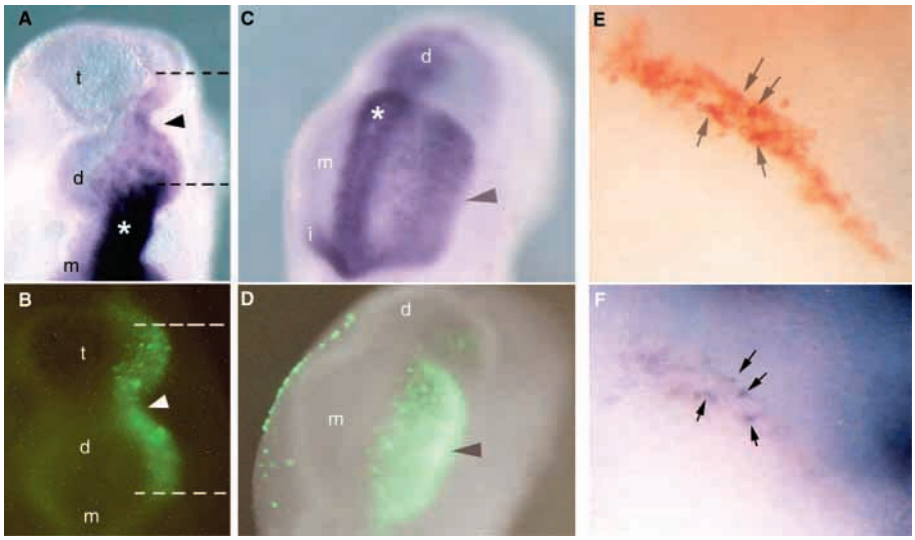


Fig. 7. Ectopic expression of *Msx1* induces expression of *Wnt1* in the chick brain and lateral ectoderm. (A-D) Dorsal views. *Msx1* cDNA in pCIG was electroporated into one side of the diencephalon of chick embryos. *Wnt1* is induced (A, arrowhead) in cells that have received pCIG-Msx1 DNA, visualized by GFP (B, arrowhead) that is expressed from the same plasmid. Broken lines delimit the domain in the neuroepithelium that has received plasmid DNA. (C,D) Another example of induction of *Wnt1* by *Msx1*. The mesencephalic neuroepithelial domain where DNA has been transfected (D, arrowhead, green fluorescence) expresses *Wnt1* (C, arrowhead). In A,C, the asterisk indicates the endogenous midline domain of *Wnt1* expression. (E,F) Lateral view of a chick embryo in which the *Msx1* cDNA has been electroporated in the lateral ectoderm, at the mesencephalon level. In E, *Msx1* expression is monitored by in situ hybridization (red signal). After washing out the red signal, the same embryos was further processed for in situ hybridization with *Wnt1* (F, purple signal). Note that *Wnt1* expression is always observed in cells that express *Msx1* at a higher level (arrows) and does not extend to neighboring cells. In E,F, anterior is towards the left. d, diencephalon; i, isthmus; m, mesencephalon; t, telencephalon.

domain remained (Fig. 8A,B and not shown). This remnant of expression may derive from the initial expression domain of *Wnt1* in the midbrain, which suggests that in the absence of functional *Msx1* and *Msx2*, *Wnt1* is not induced in the dorsal midline of the brain. In the rest of the central nervous system, where *Msx3* is also expressed, *Wnt1* expression looked normal.

In *Msx2*^{nlacZ} heterozygous animals, at E10.5, the *Msx2*^{nlacZ} expression domain extends over the mesencephalon and the diencephalon where it overlaps with that of *Msx1*^{nlacZ}, but the intensity of the signal was much weaker for *Msx2* than for *Msx1*, especially in P1 (not shown). At E12.5, *Msx2*^{nlacZ} was further downregulated and almost completely undetectable in P1, while *Msx1* was strongly expressed at this site (Fig. 8C,D). On the contrary, the pineal gland displayed a strong staining for the two genes (Fig. 8C,D arrowheads). The transient expression of *Msx2* in P1 may explain the variation in the timing and extent of *Wnt1* and *Msx1*^{nlacz} downregulation. Our results suggest that *Msx1* and *Msx2* have similar functions in dorsal CNS patterning and furthermore that *Msx3* may be sufficient to compensate for the loss of *Msx1* and *Msx2* in the spinal cord and part of the rhombencephalon.

DISCUSSION

Msx1 disruption leads to dorsal midline defects

We have analyzed the effects of *Msx1* loss of function in the developing central nervous system. In the mouse, all three Msx genes (*Msx1*, *Msx2* and *Msx3*) are expressed in the dorsal midline of the neural tube (Robert et al., 1989; Catron et al., 1996; Shimeld et al., 1996; Wang et al., 1996). A normal roof plate fails to form in the caudal diencephalon of *Msx1* mutant mice, as shown by the downregulation of genes expressed at the dorsal midline (*Msx1*^{nlacz}, *Bmp6*, *Wnt1*, *Wnt3a*), which can be observed from E9.5, and the apposition of expression domains normally restricted to dorsolateral regions (*Pax6*, *Pax7*, *Lim1*). The rate of cell death is not enhanced in the mutant midline, but instead corresponds to that observed in more lateral territories. Altogether, these results suggest that it is not midline cell survival that is affected by the mutation, but cell identity. These defects interfere with the development of structures derived from the dorsal neuroepithelium, such as the posterior commissure and the subcommissural organ, leading to hydrocephalus.

All defects are restricted to prosomere 1

According to the neuromeric model, the diencephalon is subdivided into three anatomical subunits called the prosomeres. Prosomere 1 (P1) constitutes the most caudal subunit of the diencephalon and is located between the pineal gland and the mesencephalon. Prosomeres 2 and 3 form the dorsal and ventral thalamus, respectively. Gene expression studies have shown that prosomeres have distinct genetic identities provided by the expression of combinatory sets of genes (Puelles and Rubenstein, 1993; Rubenstein et al.,

Table 2. Kinetics of *Wnt1* induction by *Msx1*

Transfected plasmid	*Wnt1*-expressing embryos/total			
	6 hours after electroporation	9 hours after electroporation	12 hours after electroporation	24 hours after electroporation
pCIG-*Msx1*	0/13	2/8	2/6	33/53*
pCIG (control)	–	–	–	0/17*

*Induction rate by *Msx1* relative to control is highly significant statistically (probability that the two distributions are equivalent $P=2.02\times10^{-6}$ according to Fisher's exact test).

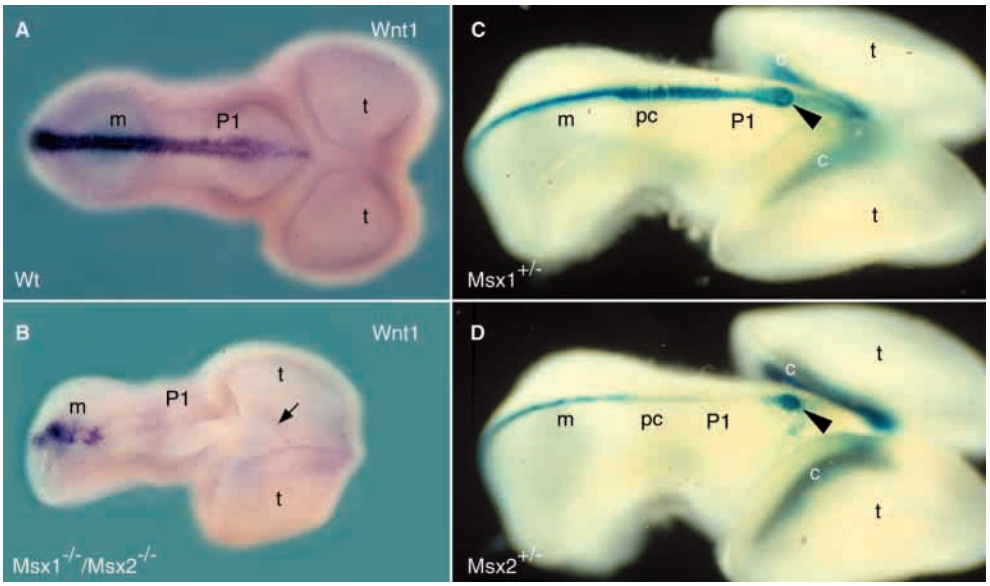


Fig. 8. Msx genes are necessary for *Wnt1* expression. (A,B) Comparison of *Wnt1* expression patterns revealed by in situ hybridization between a wild-type (A) and a double homozygous (*Msx1*$^{-/-}$/*Msx2*$^{-/-}$) mutant embryo (B) at E10.5. Expression is absent in the diencephalon and observed only caudally in the mesencephalon. Note that the midline area of the anterior diencephalon and telencephalon appears abnormal (black arrow). (C,D) Dissected brains from E12.5 *Msx1*$^{+/-}$ (C) and *Msx2*$^{+/-}$ (D) embryos stained for β-galactosidase. *Msx1* and *Msx2* are co-expressed in the mesencephalon, but the *Msx2* expression level is very low in P1. Note that both genes are expressed in the pineal gland (arrowheads). Expression of both genes is prominent in the telencephalic choroid plexus. In all panels, anterior is towards the right. c, telencephalic choroid plexus, m, mesencephalon; pc, posterior commissure; P1, prosomere 1; t, telencephalon; Wt, wild-type.

1994). Furthermore, in the *Pax6*$^{sey/sey}$ mutant, P1 is partially transformed into a mesencephalic structure (Mastick et al., 1997). This provides functional evidence in favor of the prosomeric model.

In the *Msx1* mutant, abnormalities are restricted to the territory between the pineal gland and the mesencephalon, which are landmarks for the P1 territory. Interruption of the *Msx1*^{nlacZ} midline expression domain also coincides exactly with P1. Therefore, both morphological and genetic evidence indicates that the *Msx1*$^{-/-}$ mutation affects only P1, confirming that this prosomere constitutes a genetic entity. *Msx2* is expressed in the diencephalon at E10.5, but more weakly than *Msx1*, particularly in P1, and is further downregulated at E12.5. Therefore, *Msx2* may partially compensate for the absence of *Msx1* in P1 at early stages. The low residual expression of *Msx2* is probably responsible for the variability in the time of onset of midline defects that we observed.

In a slightly different neuromeric model, Figdor and Stern (Figdor and Stern, 1993) have proposed that P1 is subdivided into two neuromeres: a caudal and a rostral one. In the *Msx1* mutant, *Pax7* and *Lim1* expression in P1 is downregulated only in the more rostral region of P1. This indicates that there are, relative to the expression of these genes, two functional domains in P1, one that requires *Msx1*, and the other that is independent of it.

The SCO is missing and this leads to hydrocephalus

The SCO is a specialized part of the ependyma at the diencephalon dorsal midline, which develops in close association with the posterior commissure from E11. In the *Msx1* mutant, the SCO is absent and the posterior commissure is affected at late stages. As *Msx1* is expressed by the SCO-forming cells throughout development (P. Fernandez-Llebrez and B.R., unpublished), further investigation is needed to determine whether *Msx1* has a direct effect on the development of the SCO or whether abnormalities in the developing SCO are the consequence of more general dorsal midline defects.

Msx1 mutant mice display prenatal hydrocephalus with an incomplete penetrance (data not shown). Although the function of the SCO has not been completely characterized, prenatal hydrocephalus is correlated with failure of SCO differentiation in mouse and rat spontaneous mutants (Takeuchi et al., 1988; Perez-Figares et al., 1998). In the swaying (*Wnt1*$^{sw/sw}$) and small eye (*Pax6*$^{sey/sey}$) mutants also, the SCO fails to form and hydrocephalus is observed (Louvi and Wassef, 2000; Estivill-Torrus et al., 2001). The absence of the SCO is therefore the most likely cause of hydrocephalus in the *Msx1* mutant. Furthermore, in this mutant, *Wnt1* and *Pax6* are downregulated and therefore crosstalk between these genes may be involved in the proper development of the SCO.

Relation between *Msx1* and *Wnt1* expression in the diencephalon

Wnt1$^{-/-}$ mutant mice present a deletion of the mesencephalon and rhombomere 1 territories, leading to the apposition of prosomere 1 and rhombomere 2, and defects in ventral prosomere 2 (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Mastick et al., 1996). The ventral part of P1 is not affected in the *Wnt1*$^{-/-}$ mutant. However, the dorsal midline seems to depend on *Wnt1* for its patterning, as E-cadherin, which is normally excluded from the diencephalon midline and expressed in two bands lateral to it, forms a single medial band in the *Wnt1*$^{-/-}$ mutant (Shimamura et al., 1994). Furthermore, as previously mentioned, the subcommissural organ, a P1 midline derivative, is abnormal in the *Wnt1*$^{sw/sw}$ mutant (Louvi and Wassef, 2000). We have shown that in the *Msx1* mutant, there is a downregulation of *Wnt1* and *Wnt3a* at E11.5. Therefore the absence of *Wnt* signaling may explain the disappearance of the dorsal midline.

Our data show that in *Msx1* mutants, *Wnt1* may be downregulated as early as E9.5, before any morphological alteration is detectable. This is the stage when the second *Wnt1* expression domain, at the dorsal midline, starts to form (Parr et al., 1993; Echelard et al., 1994). Furthermore, *Wnt1*

downregulation just precedes the disappearance of *Msx1*^{nlacZ}-expressing cells. These observations suggest that MSX1 is required for the maintenance of *Wnt1* expression in the diencephalon dorsal midline, and may be required for the activation of the second phase of *Wnt1* expression. This is further supported by the analysis of the *Msx1/Msx2* double mutants. At E10.5 in doubly homozygous embryos, *Wnt1* expression is absent from the diencephalon and reduced in the mesencephalon. A remnant of expression caudally in the mesencephalon suggests that the early phase of *Wnt1* expression has been initiated, but the second phase does not seem to take place in the absence of any functional Msx.

Msx1 has been proposed to be directly implicated in the regulation of *Wnt1* expression (Echelard et al., 1994; Shang et al., 1994; Iler et al., 1995; Rowitch et al., 1998). Indeed, forced ectopic expression of *Msx1* in the chick brain demonstrates that *Msx1* is an inducer of *Wnt1* in the neural tissue. It has been shown that a 110 bp element, which is conserved between the pufferfish *Fugu rubripes* and the mouse, within a 1.1 kb enhancer, is sufficient for the activation of *Wnt1* expression in the mid- and forebrain (Rowitch et al., 1998). This element contains at least two homeobox-binding sites that are capable of binding MSX1 in vitro and in vivo with a high affinity, suggesting that MSX1 may interact directly with the *Wnt1* promoter. However, mutation of the main binding site for MSX1 does not prevent transgene expression in the diencephalon (Iler et al., 1995). Furthermore, MSX1 has been characterized as a transcriptional repressor, and as such is unlikely to activate *Wnt1* directly (reviewed by Bendall and Abate-Shen, 2000). *Msx1* might be involved indirectly in the regulation of *Wnt1* expression, either by inducing a paracrine signaling loop or, intracellularly, by repressing a transcriptional inhibitor of *Wnt1*. The former hypothesis is unlikely, as expression of *Wnt1* in electroporated embryos is always contained within *Msx1* expression domain and strictly limited to *Msx1*-expressing cells. The kinetics of induction is compatible with repression of a *Wnt1* repressor, as no activation of *Wnt1* is observed before 9 hae. As a point of comparison, expression of *Pax6*, which by several criteria is a likely direct target of *En2*, is affected as early as 3 hae of an *En2*-expressing vector (Araki and Nakamura,1999).

Msx1 and signaling in induction processes

Expression of *Msx1* may be induced by several Bmps, such as Bmp2, Bmp4 and Bmp*7*, in different organs during embryogenesis (reviewed by Davidson, 1995). Reciprocally, Msx gene products may regulate *Bmp* expression. The most compelling evidence for interplay between Bmp and Msx genes is provided by the tooth germ, where Bmp4 induces *Msx1* expression in the mesenchyme, which in turn is required for induction and maintenance of *Bmp4* expression at this site (Chen et al., 1996). Both Bmp4 and Bmp7 have the capacity to induce roof plate cells in spinal neural plate explants, which also leads to *Msx1* expression, while Bmp4 can induce ectopic *Msx1* expression in lateral explants of the telencephalon (Liem et al., 1995; Furuta et al., 1997; Lee and Jessell, 1999). Accordingly, application of noggin, a Bmp2-Bmp4 and Bmp7 inhibitor, to the dorsal neural plate downregulates *Msx1* (Muroyama et al., 2002). At the neural plate stage, *Msx1* gene expression is restricted to the lateral, prospective dorsal, region of the neural folds (Robert et al., 1989) (A.B., Y.L. and B.R., unpublished) (Liem et al., 1995), in a manner consistent with a lateral induction coming from the ectoderm which expresses *Bmp4* and *Bmp7*. After neural tube closure, *Bmp7* expression is maintained at a high level in the epidermal ectoderm of the forebrain while *Bmp4* is expressed in the roof plate (Liem et al., 1995; Furuta et al., 1997). A hierarchical relationship between Bmp and Wnt genes in the dorsal neural tube has been suggested, but not definitely established. Application of Bmp4 on forebrain explants only led to a slight extension in the *Wnt1* expression domain (Golden et al., 1999). On the contrary, ectopic expression of a constitutively active Bmp receptor (*caBmpr1a*) under the control of nestin regulatory sequences led to an expansion of the *Wnt1* expression domain, to include the entire alar domain of the neural tube (Panchision et al., 2001). This suggests that Bmp signaling may induce *Wnt1* expression.

On the other hand, roof plate ablation has demonstrated that the dorsal midline is an essential patterning center for the dorsal neural tube (Lee et al., 2000). Roof plate cells produce signaling molecules such as Wnts or Bmps that are essential for the specification and proliferation of dorsolateral neural cells (Liem et al., 1995; Liem et al., 1997; Furuta et al., 1997; Lee and Jessell, 1999; Megason and McMahon, 2002). Mutations in the genes for these signaling factors lead to patterning defects of the dorsal CNS (Ikeya et al., 1997; Lee et al., 1998; Kim et al., 2001; Muroyama et al., 2002). In *Msx1*$^{-/-}$ mice, several of these genes are downregulated in the dorsal midline of the diencephalon, and this is the most likely explanation for the downregulation of genes normally expressed in the dorsolateral domains.

Based on these data, a model for dorsal midline formation is proposed, in which a Bmp signal coming from the lateral/dorsal ectoderm or mesoderm induces midline expression of *Msx1* at early stages (Fig. 9). This in turn is essential to maintain a functional midline that produces the diffusible signals Bmp6, Wnt1 and Wnt3a, leading to

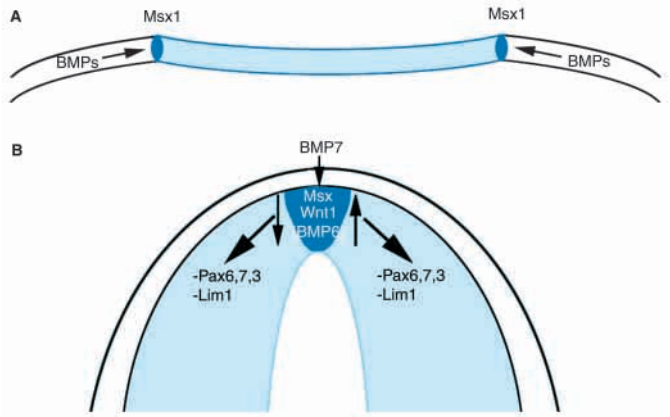


Fig. 9. A model for the induction and function of Msx genes in the dorsal midline of the diencephalon. (A) Initial induction of Msx expression in the neural folds is the consequence of a Bmp signal from the lateral ectoderm (Liem et al., 1995; Lee and Jessell, 1999). (B) After closure of the neural tube, Msx genes are expressed at the dorsal midline. They are necessary to induce the expression of *Wnt1* in the dorsal midline and maintain the integrity of the dorsal midline cells which are in turn required to provide inductive signals to the dorsolateral domains, characterized by the expression of genes such as *Pax3*, *Pax6*, *Pax7* and *Lim1* in the posterior diencephalon.

patterning of adjacent dorsolateral neural tissues. We propose that, in the case of the dorsal midline in the diencephalon, Msx genes are intermediaries between Bmp and Wnt and that this is essential for the maintenance of a functional dorsal midline.

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REFERENCES

Anderson, R. M., Lawrence, A. R., Stottmann, R. W., Bachiller, D. and Klingensmith, J. (2002). Chordin and noggin promote organizing centers of forebrain development in the mouse. *Development* **129**, 4975-4987.

Araki, I. and Nakamura, H. (1999). Engrailed defines the position of dorsal di-mesencephalic boundary by repressing diencephalic fate. *Development* **126**, 5127-5135.

Bally-Cuif, L. and Wassef, M. (1994). Ectopic induction and reorganization of Wnt-1 expression in quail/chick chimeras. *Development* **120**, 3379-3394.

Bendall, A. J. and Abate-Shen, C. (2000). Roles for Msx and Dlx homeoproteins in vertebrate development. *Gene* **247**, 17-31.

Catron, K. M., Wang, H., Hu, G., Shen, M. M. and Abate-Shen, C. (1996). Comparison of MSX-1 and MSX-2 suggests a molecular basis for functional redundancy. *Mech. Dev.* **55**, 185-199.

Chen, Y., Bei, M., Woo, I., Satokata, I. and Maas, R. (1996). Msx1 controls inductive signaling in mammalian tooth morphogenesis. *Development* **122**, 3035-3044.

Cornell, R. A. and von Ohlen, T. (2000). Vnd/nkx, ind/gsh, and msh/msx: conserved regulators of dorsoventral neural patterning? *Curr. Opin. Neurobiol.* **10**, 63-71.

Davidson, D. (1995). The function and evolution of Msx genes: pointers and paradoxes. *Trends Genet.* **11**, 405-411.

Dietrich, S., Schubert, F. R. and Lumsden, A. (1997). Control of dorsoventral pattern in the chick paraxial mesoderm. *Development* **124**, 3895-3908.

Echelard, Y., Vassileva, G. and McMahon, A. P. (1994). Cis-acting regulatory sequences governing Wnt-1 expression in the developing mouse CNS. *Development* **120**, 2213-2224.

Estivill-Torrus, G., Vitalis, T., Fernandez-Llebrez, P. and Price, D. J. (2001). The transcription factor Pax6 is required for development of the diencephalic dorsal midline secretory radial glia that form the subcommissural organ. *Mech. Dev.* **109**, 215-224.

Figdor, M. C. and Stern, C. D. (1993). Segmental organization of embryonic diencephalon. *Nature* **363**, 630-634.

Fujii, T., Pichel, J. G., Taira, M., Toyama, R., Dawid, I. B. and Westphal, H. (1994). Expression patterns of the murine LIM class homeobox gene lim1 in the developing brain and excretory system. *Dev. Dyn.* **199**, 73-83.

Furuta, Y., Piston, D. W. and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124**, 2203-2212.

Golden, J. A., Bracilovic, A., McFadden, K. A., Beesley, J. S., Rubenstein, J. L. and Grinspan, J. B. (1999). Ectopic bone morphogenetic proteins 5 and 4 in the chicken forebrain lead to cyclopia and holoprosencephaly. *Proc. Natl. Acad. Sci. USA* **96**, 2439-2444.

Houzelstein, D., Cohen, A., Buckingham, M. E. and Robert, B. (1997). Insertional mutation of the mouse Msx1 homeobox gene by an nlacZ reporter gene. *Mech. Dev.* **65**, 123-133.

Ikeya, M., Lee, S. M., Johnson, J. E., McMahon, A. P. and Takada, S. (1997). Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* **389**, 966-970.

Iler, N., Rowitch, D. H., Echelard, Y., McMahon, A. P. and Abate-Shen, C. (1995). A single homeodomain binding site restricts spatial expression of Wnt-1 in the developing brain. *Mech. Dev.* **53**, 87-96.

Kim, R. Y., Robertson, E. J. and Solloway, M. J. (2001). Bmp6 and Bmp7 are required for cushion formation and septation in the developing mouse heart. *Dev. Biol.* **235**, 449-466.

Lee, K. J., Dietrich, P. and Jessell, T. M. (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. *Nature* **403**, 734-740.

Lee, K. J. and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu. Rev. Neurosci.* **22**, 261-294.

Lee, K. J., Mendelsohn, M. and Jessell, T. M. (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev.* **12**, 3394-3407.

Liem, K. F. J., Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91**, 127-138.

Liem, K. F. J., Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Louvi, A. and Wassef, M. (2000). Ectopic engrailed 1 expression in the dorsal midline causes cell death, abnormal differentiation of circumventricular organs and errors in axonal pathfinding. *Development* **127**, 4061-4071.

Mastick, G. S., Davis, N. M., Andrew, G. L. and Easter, S. S. J. (1997). Pax-6 functions in boundary formation and axon guidance in the embryonic mouse forebrain. *Development* **124**, 1985-1997.

Mastick, G. S., Fan, C. M., Tessier-Lavigne, M., Serbedzija, G. N., McMahon, A. P. and Easter, S. S., Jr (1996). Early deletion of neuromeres in Wnt-1–/– mutant mice: evaluation by morphological and molecular markers. *J. Comp. Neurol.* **374**, 246-258.

Mathis, L., Kulesa, P. M. and Fraser, S. E. (2001). FGF receptor signalling is required to maintain neural progenitors during Hensen's node progression. *Nat. Cell Biol.* **3**, 559-566.

McMahon, A. P. and Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.

Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-2098.

Muroyama, Y., Fujihara, M., Ikeya, M., Kondoh, H. and Takada, S. (2002). Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. *Genes Dev.* **16**, 548-553.

Panchision, D. M., Pickel, J. M., Studer, L., Lee, S. H., Turner, P. A., Hazel, T. G. and McKay, R. D. (2001). Sequential actions of BMP receptors control neural precursor cell production and fate. *Genes Dev.* **15**, 2094-2110.

Parr, B. A., Shea, M. J., Vassileva, G. and McMahon, A. P. (1993). Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119**, 247-261.

Perez-Figares, J. M., Jimenez, A. J., Perez-Martin, M., Fernandez-Llebrez, P., Cifuentes, M., Riera, P., Rodriguez, S. and Rodriguez, E. M. (1998). Spontaneous congenital hydrocephalus in the mutant mouse hyh. Changes in the ventricular system and the subcommissural organ. *J. Neuropathol. Exp. Neurol.* **57**, 188-202.

Peters, H. and Balling, R. (1999). Teeth. Where and how to make them. *Trends Genet.* **15**, 59-65.

Puelles, L. and Rubenstein, J. L. (1993). Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. *Trends Neurosci.* **16**, 472-479.

Robert, B., Lyons, G., Simandl, B. K., Kuroiwa, A. and Buckingham, M. (1991). The apical ectodermal ridge regulates *Hox-7* and *Hox-8* gene expression in developing chick limb buds. *Genes Dev.* **5**, 2363-2374.

Robert, B., Sassoon, D., Jacq, B., Gehring, W. and Buckingham, M. (1989). Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis. *EMBO J.* **8**, 91-100.

Rowitch, D. H., Echelard, Y., Danielian, P. S., Gellner, K., Brenner, S. and McMahon, A. P. (1998). Identification of an evolutionarily conserved 110 base-pair cis-acting regulatory sequence that governs Wnt-1 expression in the murine neural plate. *Development* **125**, 2735-2746.

Rubenstein, J. L., Martinez, S., Shimamura, K. and Puelles, L. (1994). The embryonic vertebrate forebrain: the prosomeric model. *Science* **266**, 578-580.

Rubenstein, J. L. and Puelles, L. (1994). Homeobox gene expression during development of the vertebrate brain. *Curr. Top. Dev. Biol.* **29**, 1-63.

Satokata, I. and Maas, R. (1994). Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat. Genet.* **6**, 348-356.

Satokata, I., Ma, L., Ohshima, H., Bei, M., Woo, I., Nishizawa, K., Maeda, T., Takano, Y., Uchiyama, M., Heaney, S. et al. (2000). Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat. Genet.* **24**, 391-395.

Schlüter, G. (1973). Ultrastructural observations on cell necrosis during formation of the neural tube in mouse embryos. *Z. Anat Entwicklungsgesch* **141**, 251-264.

Shang, Z., Ebright, Y. W., Iler, N., Pendergrast, P. S., Echelard, Y., McMahon, A. P., Ebright, R. H. and Abate, C. (1994). DNA affinity cleaving analysis of homeodomain-DNA interaction: identification of homeodomain consensus sites in genomic DNA. *Proc. Natl. Acad. Sci. USA* **91**, 118-122.

Sharman, A. C., Shimeld, S. M. and Holland, P. W. (1999). An amphioxus Msx gene expressed predominantly in the dorsal neural tube. *Dev. Genes Evol.* **209**, 260-263.

Shimamura, K., Hirano, S., McMahon, A. P. and Takeichi, M. (1994). Wnt-1-dependent regulation of local E-cadherin and alpha N-catenin expression in the embryonic mouse brain. *Development* **120**, 2225-2234.

Shimeld, S. M., McKay, I. J. and Sharpe, P. T. (1996). The murine homeobox gene Msx-3 shows highly restricted expression in the developing neural tube. *Mech. Dev.* **55**, 201-210.

Stoykova, A. and Gruss, P. (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J. Neurosci.* **14**, 1395-1412.

Tajbakhsh, S. and Houzelstein, D. (1995). In situ hybridization and beta-galactosidase: a powerful combination for analysing transgenic mice. *Trends Genet.* **11**, 42.

Takeuchi, I. K., Kimura, R., Shoji, R. and Matsuda, M. (1988). Dysplasia of subcommissural organ in congenital hydrocephalus spontaneously occurring in CWS/Idr rats. *Experientia* **44**, 338-340.

Thomas, K. R. and Capecchi, M. R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346**, 847-850.

Wang, W., Chen, X., Xu, H. and Lufkin, T. (1996). Msx3: a novel murine homologue of the Drosophila msh homeobox gene restricted to the dorsal embryonic central nervous system. *Mech. Dev.* **58**, 203-215.