Transcriptional control of early tract formation in the embryonic chick midbrain

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Accepted 1 February 2005

Development 132, 1785-1793
Published by The Company of Biologists 2005
doi:10.1242/dev.01731

Summary

The earliest step in establishing the complex neuronal networks in the vertebrate brain is the formation of a scaffold of axon tracts. How the formation of the early axon scaffold is controlled at the molecular level is unclear. Forming part of the scaffold, neurons located at the ventral midbrain-forebrain border (MFB) give rise to the medial longitudinal fascicle (mlf) and the posterior commissure (pc). We demonstrate that the homeobox genes *Sax1*, *Six3*, *Emx2* and *Pax6* are expressed in distinct domains in this area, suggesting that the specification of mlf and pc neurons might be controlled by the combinatorial activity of these transcription factors. We have tested this hypothesis by analysing the function of *Sax1* in the embryonic chick brain. Gain-of-function experiments with *Sax1* result in alterations to the early axon scaffold, most prominently an enlargement of the mlf at the expense of the pc. Ectopic expression of *Sax1* also affects the expression of other ventral homeobox genes, particularly *Six3* and *Emx2*. Our results indicate that the specification of neurons forming the early axon scaffold is governed by a homeobox code, thus resembling the mechanism of neuronal specification in the spinal cord.

Key words: *Sax1*, *Emx2*, *Six3*, *Pax6*, Mesencephalon, Tegmentum, Medial longitudinal fascicle, Posterior commissure, Early axon scaffold

Introduction

A characteristic feature of all vertebrates is the formation of a conserved set of longitudinal and commissural axon tracts during early brain development (Chedotal et al., 1995; Chitnis and Kuwada, 1990; Easter et al., 1993; Wilson et al., 1990). The early axon scaffold formed by these tracts is thought to act as a framework for the later, complex network of neuronal connections in the brain. We are interested in the molecular mechanisms that underlie the specification of the early differentiating neurons in the brain, which despite the detailed studies on early axon tract formation in a range of vertebrates have remained enigmatic.

The specification of neurons, however, has been studied in great detail in the spinal cord, where the initial dorsoventral patterning of the neural tube, resulting from the antagonistic action of ventralising and dorsalising signals is translated into spatially restricted expression of homeobox genes. The longitudinal, dorsoventrally restricted expression domains of the homeobox genes prefigure the longitudinal columns of neuronal subtypes in the spinal cord, as differentiating neurons adopt their distinct identities as a result of expressing a specific combination of homeodomain transcription factors (reviewed by Goulding and Lamar, 2000).

The organisation of the brain is more complex, and it harbours a greater diversity of neurons than does the spinal cord. Neurons are either organised into different layers, as in the tectum or the cerebral cortex, or into nuclei, as in the ventral midbrain. Nuclei occupy distinct positions along the rostrocaudal and dorsoventral axes, where their specification is likely to be controlled by coordinate patterning of the neural tube. Interestingly, parallels exist between the dorsoventral patterning of spinal cord and midbrain at the molecular level. As in the spinal cord, ventral patterning in the midbrain is governed by floor plate-derived Shh signalling: overexpression of sonic hedgehog (*Shh*) throughout the midbrain leads to an expansion of the basal plate-derived tegmentum territory at the expense of the dorsal tectum (Watanabe and Nakamura, 2000), while local misexpression induces the expression of ventrally expressed homeobox genes in a dose-dependent pattern (Agarwala et al., 2001). Several homeobox genes are normally expressed in the ventral midbrain in longitudinal domains – so-called arcs – in a similar arrangement to the homeobox gene expression domains in the spinal cord (Sanders et al., 2002). Up to five arcs, defined by the differential expression of homeobox genes, are established in response to a presumed gradient of Shh emanating from the floor plate. Ventral signalling, with resulting expression of homeobox genes, is crucial for correct development of the ventral midbrain. A direct requirement for *Shh* has been demonstrated for two groups of neurons in the tegmentum, the somatic motoneurones of the oculomotor nucleus (Chiang et al., 1996), and the dopaminergic neurons of substantia nigra and ventral tegmental area (Hynes et al., 1995). Consistent with an instructive function of homeobox genes in the specification of

ventral mesencephalic neurons, *Isl1* and *Phox2a* are essential for the formation of the oculomotor nucleus (Nakano et al., 2001; Pfaff et al., 1996), *Emx2* is required for proper development of the red nucleus (Agarwala and Ragsdale, 2002), and several homeobox genes including *Pitx3* and *Lmx1* are involved in the specification of dopaminergic neurons (reviewed by Smidt et al., 2003).

We are interested in whether homeobox genes similarly play a role in the formation of the early axon scaffold. The dominating longitudinal tract in the early scaffold is the medial longitudinal fascicle (mlf). Very little is known about the molecular mechanisms that underlie the formation of the mlf and its contributing nucleus – the interstitial nucleus of Cajal (INC). Tight genetic regulation seems particularly important for distinguishing the fate of mlf neurons from those forming the posterior commissure (pc), as neurons for both tracts are located principally in the same ventral cluster at the midbrain-forebrain border (MFB) (Tallafuss et al., 2003). Could homeobox genes play a role in specifying these early neuronal subtypes, and especially the mlf and pc cells? To answer this question, we first analysed the expression patterns of the homeobox genes *Sax1*, *Six3*, *Emx2* and *Pax6* in the ventral midbrain of chick embryos between HH15 and HH25, when the early axon scaffold is formed. These genes have previously been described to be expressed at the ventral MFB (e.g. Agarwala and Ragsdale, 2002; Bovolenta et al., 1998; Schubert et al., 1995), but their precise temporal and spatial patterns of expression have not been determined. Out of the expression analysis, *Sax1* emerged as a prime candidate for neuronal specification at the ventral MFB, as it is expressed predominantly in the ventral neuronal cluster, coincident with the INC, from the time the first neurons appear. *Sax1* is a member of the NK1 class of homeobox genes, which in vertebrates is usually represented by two closely related genes, *Sax1* and *Sax2* (Bae et al., 2004; Bober et al., 1994; Schubert et al., 1995; Simon and Lufkin, 2003; Spann et al., 1994), while additional, more divergent members have recently been described in *Xenopus* (Kurata and Ueno, 2003) and zebrafish (Bae et al., 2003). We have employed electroporation in the chick to study the function of *Sax1* in the specification of neurons at the ventral MFB. Misexpression of *Sax1* leads to an increase in the size of the mlf, and affects the expression of other ventral homeobox genes, suggesting that a homeobox gene code underlies the formation of the early axon scaffold, and that *Sax1* in particular regulates the formation of the mlf.

Materials and methods

Chick embryos

Fertilised hens eggs were obtained from Winter Egg Farm (Royston). They were incubated at 38°C for the required stage. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Expression analysis

We employed RNA probes for chick *Sax1* (Spann et al., 1994), *Emx2* (Bell et al., 2001), *Isl1* (Tsuchida et al., 1994), *Pax6* (Goulding et al., 1993), *Phox2a* (Groves et al., 1995) and *Six3* (Chapman et al., 2002) for our analysis. For details of the whole-mount in situ hybridisation protocol, see Dietrich et al. (Dietrich et al., 1997). In double labelling experiments, digoxigenin- and fluorescein-labelled probes were consecutively detected with alkaline phosphatase-conjugated antibodies (Roche), using NBT/BCIP (Roche) and Vector Red (Vector Labs) as blue and red substrates.

To analyse the expression of *Sax1* in relation to mlf neurons, we retrograde labelled the mlf from the rostral hindbrain using fluorescein-labelled dextran (Molecular Probes). Embryos at HH23 were dissected in PBS, the ventral hindbrain was cut at the level of rhombomere 2, and a crystal of the dye applied. Embryos were incubated at 37°C in L15 medium (GibcoBRL) for 3 hours, and then fixed. Following in situ hybridisation for *Sax1*, the fluorescein label was detected with an alkaline phosphatase-conjugated anti-fluorescein antibody (Roche), as in double-labelling in situ hybridisation, using Vector Red as substrate (see also Agarwala and Ragsdale, 2002).

Retrograde labelling of mlf and pc

Specific axon tracts in the embryonic brain were retrograde labelled with lipophilic dyes. The mesenchyme was removed from day 5 chick embryos, and isolated, hemisected brains were fixed flat on black nitrocellulose membrane (Schleicher and Schuell). Crystalline DiI was applied on to the ventral part of rhombomere 2 to label the mlf, while DiO was used to label the pc from the roof plate of the caudal pretectum.

Immunohistochemistry

Neurons (cell bodies and axons) were detected with an antibody against Neurofilament-M (Zymed RMO270), visualised by a peroxidase-conjugated anti-mouse antibody (Jackson Laboratories) using Diaminobenzidine (Vector Labs) as substrate. When combined with in situ hybridisation, the primary antibody for immunohistochemical detection of neurofilament protein was applied after completing the colour reaction of the whole-mount in situ hybridisation procedure.

Electroporation

Two different expression constructs were used, both based on pCAβ-LINK-IRESeGFPm5-ClaI (Fig. 3I) (J. Gilthorpe, A. Hunter and A.L., unpublished), an expression vector in which a hybrid CMV/chick β-actin promoter (Miyazaki et al., 1989) drives the transcription of a polycistronic message encoding the gene of interest and – linked by an IRES element – enhanced green fluorescent protein (eGFP). pCAβ-*Sax1*-IRES-GFP was constructed by inserting the full coding region of the murine *Sax1* gene (Schubert et al., 1995) into the expression vector (Fig. 3L). For the assembly of pCAβ-*VP16Sax1*-IRES-GFP, first the eh1-like domain of the murine *Sax1* gene (Smith and Jaynes, 1996) was removed, and the transactivation domain of Herpes simplex VP16 (Triezenberg et al., 1988) was introduced in its place (Fig. 3L). The coding region for the hybrid *VP16Sax1* protein was again cloned into the base expression vector. The integrity of the expression constructs was confirmed by sequencing. The pCAβ-LINK-IRESeGFPm5-ClaI vector itself was used as control for non-specific effects of the electroporation.

Expression constructs were used at a concentration of 1 mg/ml, with Fast Green added to 0.1% to facilitate visualisation of the DNA solution. After cutting a window into the eggshell and opening the vitelline membrane, the DNA solution was injected into the neural tube at the level of the midbrain of HH11-13 chick embryos, using a PV820 Picopump (WPI). The anode, a 0.25 mm platinum wire, was placed lateral to the midbrain, and the cathode, a 0.125 mm flame-sharpened tungsten wire, was inserted into the neural tube. Two pulses of 12.5 V, 50 ms were applied, using a TSS10 stimulator (Intracel). The window was closed with Sellotape, and the eggs were then incubated for a further 1-3 days.

Sectioning and photography

Where required, embryos were sectioned after the whole-mount in situ hybridisation or immunohistochemistry procedures. Sections of 30 μm were cut on a Leica vibratome and mounted in 80% glycerol.

Photographs of whole embryos, dissected brain tissue or sections

were taken on a Zeiss Axiophot microscope with differential interference contrast, using a Zeiss Axiocam digital camera. Subsequent processing and assembly of the images was carried out with Adobe Photoshop.

Results

Expression domains of homeobox transcription factors subdivide the ventral midbrain

In all vertebrates, axon tracts in the early embryonic brain invariably form the early axon scaffold, a conserved array of longitudinal and commissural tracts. Particularly dense in early axon tracts is the ventral region around the MFB, through which the tract of the postoptic commissure (tpoc) passes, and from which the mlf and the pc originate. To unravel the molecular mechanisms that control the development of mlf and pc, we first studied the expression patterns of several homeobox genes around the ventral MFB. As a reference point for the subsequent expression studies, we employed *Isl1* (Pfaff et al., 1996) to mark the oculomotor nucleus located just caudal and ventral to the INC. *Sax1* (Schubert et al., 1995), *Six3* (Bovolenta et al., 1998; Tallafuss et al., 2003), *Emx2* (Agarwala and Ragsdale, 2002) and *Pax6* (Sanders et al., 2002; Schubert et al., 1995) have all been previously described to be expressed in the ventral midbrain, dorsal to the oculomotor nucleus. However, their precise expression patterns with respect to each other and to specific early midbrain nuclei such as the INC have not been determined.

Using whole-mount RNA in situ hybridisation, we detected the first expression of a homeobox gene in the ventral midbrain at HH15, when *Sax1* signals appeared at the ventral MFB. These were followed shortly by *Six3* and *Emx2* signals in the same region (not shown). In contrast, *Pax6* is not expressed in the midbrain until HH19. By HH20, distinct differences in the expression patterns of the different homeobox genes are evident (Fig. 1A-E). Signals for *Six3* and *Emx2* are split into ventral and dorsal stripes in the midbrain, separated by the emerging *Pax6* expression domain, and ventrally delimited by the *Isl1*-positive oculomotor nucleus (Fig. 1C-E). The *Sax1* signal spans the whole dorsoventral extent of the *Six3*/*Emx2* domains, including also the intervening *Pax6*-positive stripe (Fig. 1B). Interestingly, although all four genes are expressed in the mantle layer, the mRNA for *Sax1* is found exclusively in the outer margin of the mantle layer, aligning the marginal zone, while the *Six3* and *Emx2* signals are located further medially (Fig. 1B-D, the asterisk marks the medial limit of the *Sax1* signals).

Differences in the expression domains are also evident along the rostrocaudal axis, and become more pronounced as the brain develops (Fig. 1F-J). At HH25, the signals for *Six3*, *Emx2* and *Pax6* extend as curved, longitudinal domains from the MFB caudally, throughout the tegmentum (Fig. 1H-J,M-O). According to the nomenclature of Sanders et al. (Sanders et al., 2002), these correspond to arcs 2 and 3 (*Six3* and *Emx2*) and the intervening region (*Pax6*). Close to the MFB, the ventral

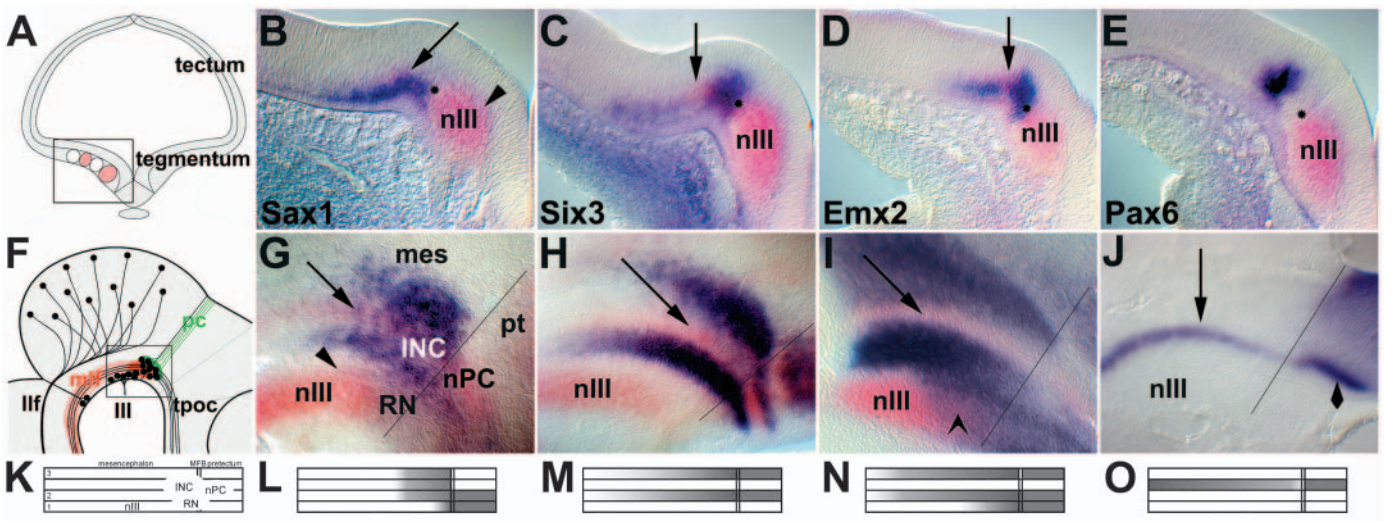

Fig. 1. Differential gene expression at the ventral MFB. (B-E) Cross-section through the mesencephalon of HH20 chick embryos, showing the area depicted in the schematic view (A). (F) Schematic lateral view of a HH25 chick brain, indicating the location of major tracts. Highlighted are the mlf in red and the pc in green. Black circles at the ventral MFB represent the respective nuclei. (G-J) Lateral view of the ventral mesencephalon and pretectum of HH25 chick embryos, focussing on the ventral MFB (see boxed area in F). The position of the MFB is marked by a line. Red staining in B-E,G-I indicates the expression of *Isl1* (arrowhead in B,G; ventral stripe, marking the oculomotor nucleus) and *Pax6* (arrow in B,G; dorsal stripe). (B,G) Blue staining indicates the expression of *Sax1* in a broad domain dorsal and rostral to the oculomotor nucleus. *Sax1* and *Pax6* signals overlap. (C,H) Expression of *Six3* around the ventral MFB. *Six3* staining in the ventral mesencephalon (blue) is split into two longitudinal domains by the intervening *Pax6* stripe (arrow). (D,I) The mRNA for *Emx2* (blue) is also largely localised in two longitudinal stripes in the ventral mesencephalon, divided by the *Pax6* signals (arrow). *Emx2* and *Isl1* signals partially overlap at the MFB (arrowhead). (E,J) *Pax6* is expressed in a single longitudinal stripe in the ventral mesencephalon (arrow in J), dorsal to the oculomotor nucleus (red *Isl1* signal in E). *Pax6* is also expressed in the pretectum, in a ventral patch of cells (arrowhead in J), and dorsally. (K-O) Schematic representation of nuclei position (K) and expression domains for *Sax1* (L), *Six3* (M), *Emx2* (N) and *Pax6* (O) in the ventral midbrain and pretectum. Numbers in K represent the midbrain arcs 1-3. III, oculomotor nerve; INC, interstitial nucleus of Cajal; llf, lateral longitudinal fascicle; mes, mesencephalon; mlf, medial longitudinal fascicle; nIII, oculomotor nucleus; nPC, nucleus of the posterior commissure (ventral part); pc, posterior commissure; pt, pretectum; RN, red nucleus; tpoc, tract of the postoptic commissure.

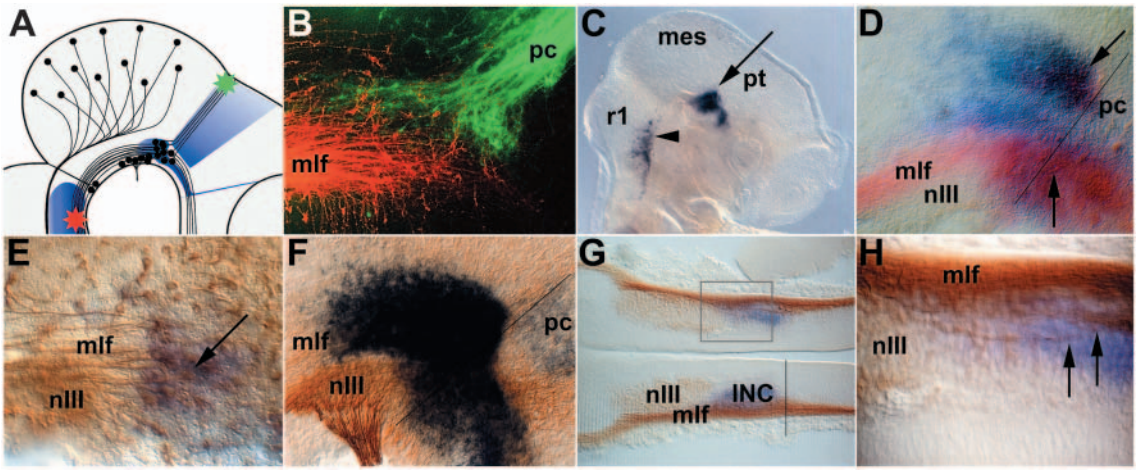

Fig. 2. Expression of *Sax1* in the nucleus of the mlf. (A) Schematic lateral view of the embryonic mesencephalon and pretectum, showing the expression domains of *Sax1* in blue, and indicating major axon tracts and the location of their cell bodies. The fully developed expression pattern of *Sax1* is depicted as seen in embryos from HH21. (B) Lateral view of the MFB of an HH27 chick embryo, with retrograde labelling of the mlf (DiI, red) from the rostral hindbrain and the pc (DiO, green) from the dorsal pretectum (see red and green asterisks, respectively, in A), showing the partial overlap of the corresponding nuclei for these axon tracts originating from the ventral MFB. (C) Lateral view of chick head at HH17, with the *Sax1* expression domains in rhombencephalon (arrowhead), mesencephalon (arrow) and pretectum in blue. (D) Lateral view of the ventral mesencephalon of an HH23 embryo. *Sax1* mRNA signals are in blue, and the red staining shows the mlf, retrograde-labelled with fluorescein-labelled dextran from the rostral hindbrain (see red asterisk in A). The nucleus of the mlf (arrows) overlaps with the *Sax1* expression domain. (E-H) Double-labelling for *Sax1* mRNA in blue and neurofilament protein in brown. Lateral views of the ventral mesencephalon of an HH17 (E) and an HH21 (F) embryo show that the *Sax1* domain overlaps with the cell bodies for the mlf (arrow in E). At HH21, *Sax1* staining is weakly visible in the dorsal pretectum. (G) A horizontal section through the ventral mesencephalon of an HH22 embryo (boxed area magnified in H) shows *Sax1*-expressing neurons projecting into the mlf (arrows). The MFB is marked by a line in D,F,G. INC, interstitial nucleus of Cajal; mes, mesencephalon; mlf, medial longitudinal fascicle; nIII, oculomotor nucleus; pc, posterior commissure; pt, pretectum; r1, rhombomere 1.

Emx2 domain stretches out ventrally into the *Isl1*-stained arc 1, outlining the prospective red nucleus (Fig. 1I,N) (Agarwala and Ragsdale, 2002). In contrast to the elongated expression domains of the former genes, the expression of *Sax1* remains more focussed in the rostral tegmentum and the MFB, where it overlays the domains of *Emx2*, *Six3* and *Pax6* (Fig. 1G,L, see also Fig. 2F). The signals for all genes except *Isl1* also continue into the ventral diencephalon. In the ventral pretectum, the *Pax6* mRNA is restricted to a small domain, ventrally bordered by the signals for *Sax1*, *Six3* and *Emx2*, and separate from the extensive *Pax6* expression domain in the dorsal pretectum by the dorsal stripe of *Six3* and *Emx2* expression (Fig. 1G-J,L-O).

Sax1 expression is closely associated with the mlf

The mlf is the earliest tract formed in the embryonic brain. In the chick, it can be detected from HH14, originating from the INC at the ventral MFB (Chedotal et al., 1995). By contrast, the pc develops several hours later (Chedotal et al., 1995). The nucleus of the pc (nPC) is dispersed over several locations in the MFB and pretectum (Chitnis and Kuwada, 1990). Simultaneous retrograde labelling of mlf and pc in an HH27 embryo demonstrates an overlap of INC and nPC in a cluster of neurons at the ventral MFB (Fig. 2B). This close association of mlf and pc neurons has previously been observed in zebrafish (Chitnis and Kuwada, 1990). The expression domain of *Sax1* corresponds to this ventral neuronal cluster (Fig. 2A,C), suggesting that *Sax1* might play a role in the specification of neurons in rostral midbrain and pretectum, particularly the INC.

To identify which neurons might express *Sax1*, we stained chick embryonic brains at different stages simultaneously for neurofilament protein and *Sax1* mRNA (Fig. 2E-H). At HH17, neurofilament staining detects the mlf as a bundle of longitudinal axons just dorsal to the floor plate. These can be traced to cell bodies at the ventral MFB, located within the *Sax1* expression domain at the MFB (Fig. 2E). The location of these neurons at the ventral MFB, immediately rostral and dorsal to the oculomotor nucleus, and their caudal ipsilateral projection identify them as mlf neurons. While at HH17 *Sax1* is expressed just in a small domain, the *Sax1* signals at HH21 are much stronger and mask the neurofilament staining. Still, the axons of the mlf are visible as they extend from the *Sax1* domain (Fig. 2F).

To confirm the conclusion that the mlf neurons express *Sax1*, we sectioned double-labelled embryos horizontally. Sections through the ventral mesencephalon show the *Sax1* expression domain located just rostral to the oculomotor nucleus (Fig. 2G). Higher magnifications revealed *Sax1*-expressing neurons projecting into the mlf (Fig. 2H). Furthermore, we combined retrograde labelling of the mlf with in situ hybridisation for *Sax1* mRNA. At HH23, cell bodies of mlf neurons were concentrated in two clusters around the MFB, a caudodorsal patch and a rostroventral area (see also Fig. 3J). Both areas overlap with the *Sax1* expression domains in ventral mesencephalon and pretectum (Fig. 2D). Our analysis shows that *Sax1* is expressed in the INC, the nucleus of the mlf.

Ectopic expression of *Sax1* disrupts the early axon scaffold

The close association of *Sax1* expression and mlf neurons

raised the possibility that *Sax1* could be involved in establishing this early tract. To test this hypothesis, we used a gain-of-function approach where we expressed *Sax1* ectopically to study the effect on the morphology of the mlf (Fig. 3). We employed two different expression constructs, based on the pCAβ-LINK-IRESeGFPm5-ClaI vector (J. Gilthorpe, A. Hunter and A.L., unpublished) (Fig. 3L). pCAβ-*Sax1*-IRES-GFP contains the coding sequence for the mouse *Sax1* gene (Fig. 3O). Assuming that *Sax1* normally acts as a transrepressor, mediated through the binding of Groucho co-factors to its eh-1 like domain (Smith and Jaynes, 1996), this construct would repress the expression of *Sax1* target genes. We also designed a modified version, *VP16Sax1*, in which the transactivation domain of Herpes Simplex VP16 (Triezenberg et al., 1988) replaced the eh1-like transrepression domain (Fig. 3O). The pCAβ-*VP16Sax1*-IRES-GFP construct therefore encodes a protein that would transactivate *Sax1* target genes, thus acting as a dominant-negative regulator of Sax1 function.

When we introduced the *Sax1* expression constructs at HH10-13, we observed changes in the morphology of the early axon scaffold just 1 day after electroporation. While the mlf axons normally run close to the floor plate in a compact bundle (Fig. 3A), the fibres in the *Sax1*-expressing embryos stretch further dorsally, and their course is less regular (Fig. 3B). This phenotype appears even more pronounced after 2 days of ectopic *Sax1* expression, when immunohistochemical staining for neurofilament protein shows the irregular pattern of the longitudinal axon tract in the ventral midbrain (Fig. 3D-I). While in embryos expressing the control construct the axon scaffold appeared normal (Fig. 3D,G), following electroporation of pCAβ-*Sax1*-IRES-GFP the mlf expanded dorsally, occupying a larger region of the tegmentum (Fig. 3E,H). By contrast, the pc, although prominently stained in control embryos (Fig. 3D), was barely visible in the *Sax1*-expressing embryos (Fig. 3E). Ectopic expression of *VP16Sax1* did not result in such a strong phenotype, probably owing to lower levels of expression consistently achieved with the pCAβ-*VP16Sax1*-IRES-GFP construct. Still, *VP16Sax1* seems to have the opposite effect on the mlf, as the tract appeared less

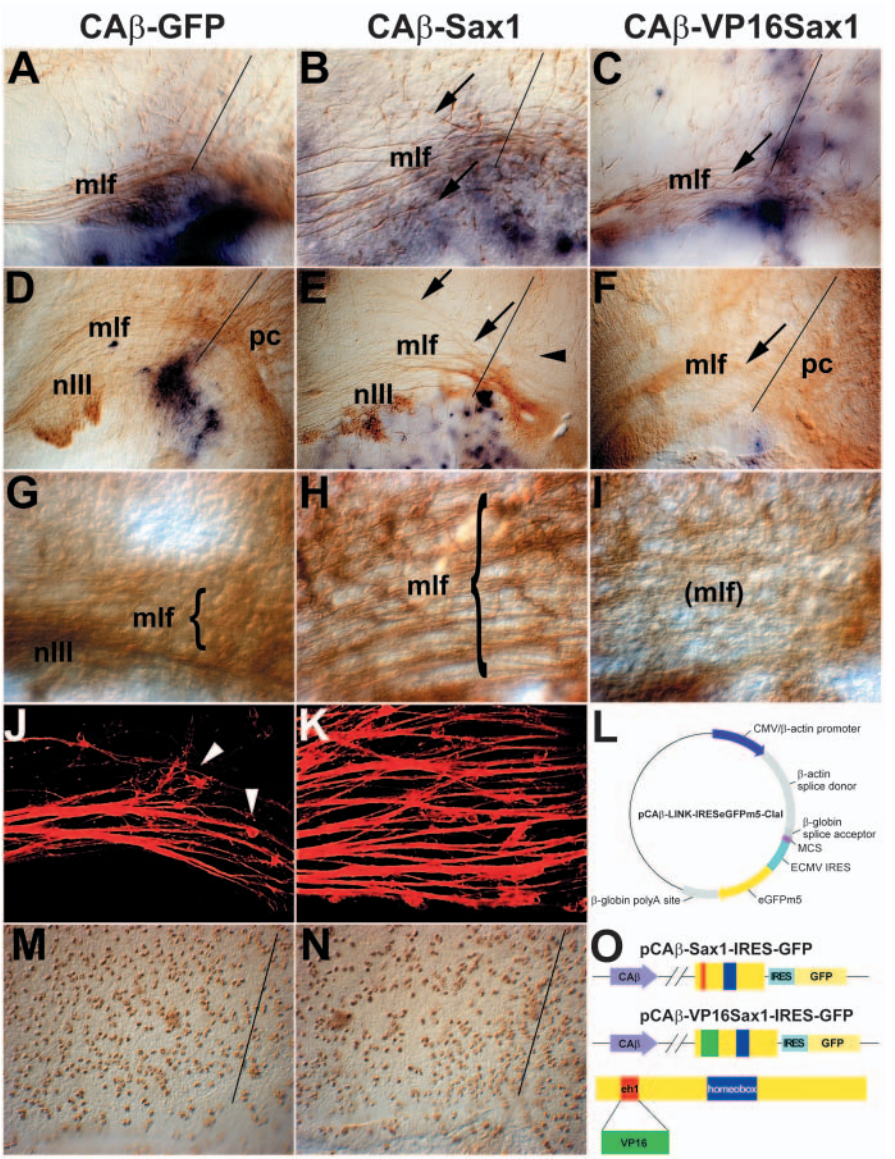

Fig. 3. Alterations to the mlf following ectopic expression of *Sax1*. (A-I) Lateral view the ventral mesencephalon of embryos, stained for neurofilament protein (brown) and eGFP mRNA (A-F, blue), analysed 1 day (A-C, HH18) or 2 days (D-F, HH21; G-I, HH19) after electroporation. The line marks the MFB. (A,D,G) After electroporation of the GFP-expressing control construct, the axon tracts appear normal. (B,E,H) Following ectopic expression of pCAβ-*Sax1*-IRES-GFP, the mlf is enlarged (arrows in B,E; also compare brackets in G and H), and the pc is no longer detectable (arrowhead in E). (C,F,I) Ectopic expression of the *VP16Sax1* constructs results in a diminished mlf. (J,K) The nucleus of the mlf revealed by retrograde labelling with DiI from the hindbrain, demonstrating enlargement of the mlf after ectopic *Sax1* expression (K) compared with an embryo expressing the control construct (J). The caudodorsal and rostroventral subnuclei of the mlf (arrowheads in J) are not distinct in *Sax1*-expressing embryos (K). (L) Map of the pCAβ-LINK-IRESeGFPm5-ClaI expression vector, outlining the CMV enhancer/chick β-actin promoter (CAβ), the multiple cloning site (MCS) into which genes of interest can be inserted and the coding region for GFP linked by the ECMV internal ribosome entry site (IRES). (M,N) Lateral view of the ventral mesencephalon stained for phospho-Histone H3 as proliferation marker. Embryos expressing the control construct (M) or the *Sax1* construct (N) show no obvious difference. (O) Schematic representation of the pCAβ-*Sax1*-IRES-GFP and pCAβ-*VP16Sax1*-IRES-GFP expression vectors that are based on pCAβ-LINK-IRESeGFPm5-*Cla*I. Also depicted is the domain swap where the eh1-like transrepression domain of *Sax1* (red) is replaced by the transactivation domain of Herpes simplex VP16 (green) to yield the constitutive transcriptional activator *VP16Sax1*. mlf, medial longitudinal fascicle; nIII, oculomotor nucleus; pc, posterior commissure.

prominent than in control embryos (compare Fig. 3C,F,I with Fig. 3A,D,G).

The results of the immunohistochemical analysis are mirrored by retrograde labelling of the ventral longitudinal tract from the ventral hindbrain (Fig. 3J,K). Again, the mlf was enlarged in the *Sax1*-expressing embryos (Fig. 3K) compared with embryos just expressing the control construct (Fig. 3J). In addition, while in the control embryo cell bodies were organised into two subclusters, located caudodorsally and rostroventrally (Fig. 3J, arrowheads), mlf neurons in the *Sax1*-expressing embryos were scattered throughout the ventral MFB, following no apparent pattern (Fig. 3K).

The expansion of the mlf after ectopic *Sax1* expression could be the result of increased proliferation of mlf precursors, or of mis-specification of neurons normally destined for a different fate. Using an antibody against the mitosis marker phospho-Histone H3 (PH3), we analysed the electroporated embryos for differences in cell proliferation in the tegmentum. We found no obvious differences in the PH3 staining between embryos electroporated with either pCAβ-LINK-IRESeGFPm5-*Cla*I (Fig. 3M) or pCAβ-*Sax1*-IRES-GFP (Fig. 3N) that could explain the expansion of the mlf, suggesting that *Sax1* misexpression leads to the mis-specification of neurons at the ventral MFB towards mlf neuron fate.

Ectopic expression of *Sax1* disrupts the homeobox gene code in the tegmentum

In the spinal cord, homeobox genes regulate their expression by mutual cross repression (Muhr et al., 2001). This mechanism ensures that sharp expression boundaries are formed, translating into distinct neuronal fate decisions. If similar mechanisms act in the midbrain, *Sax1* misexpression should affect the expression of other homeobox genes in the ventral midbrain. To test this hypothesis, we studied the expression patterns of *Six3*, *Emx2*, *Pax6* and *Phox2a* in electroporated embryos (Fig. 4). Normally, *Six3* and *Emx2* are expressed in a subdomain of the *Sax1*-expressing region, albeit in cells located more medially (Fig. 1). This pattern is unchanged in embryos expressing the GFP expression construct (Fig. 4A,D). By contrast, 1 day after electroporation of pCAβ-*Sax1*-IRES-GFP, signals for both genes are reduced or lost, depending on the level of ectopic *Sax1* expression (Fig. 4B,E). Misexpression of *VP16Sax1* has a profound effect on *Emx2* expression, leading to the upregulation of *Emx2* ventrally, and even to ectopic transcription of *Emx2* in the dorsal midbrain (Fig. 4C). *Six3* expression, by contrast, is not altered by *VP16Sax1* (Fig. 4F).

Phox2a expression was analysed two days after electroporation, when it labels the motoneurones in the oculomotor nucleus. Following *Sax1* misexpression, the *Phox2a* expression domain extends further rostrally (Fig. 4H), while electroporation of pCAβ-*VP16Sax1*-IRES-GFP reduces or even abolishes the expression of *Phox2a* in the oculomotor and trochlear nuclei (Fig. 4I). Similar results were obtained with other markers for the oculomotor nucleus, such as *Isl1*, *BEN* and *GAP43* (not shown). Corresponding to the expanded oculomotor marker expression in *Sax1* overexpressing embryos, the oculomotor nucleus was enlarged, and neurofilament staining frequently showed the presence of two or three nerves emerging from it (not shown). In contrast to the

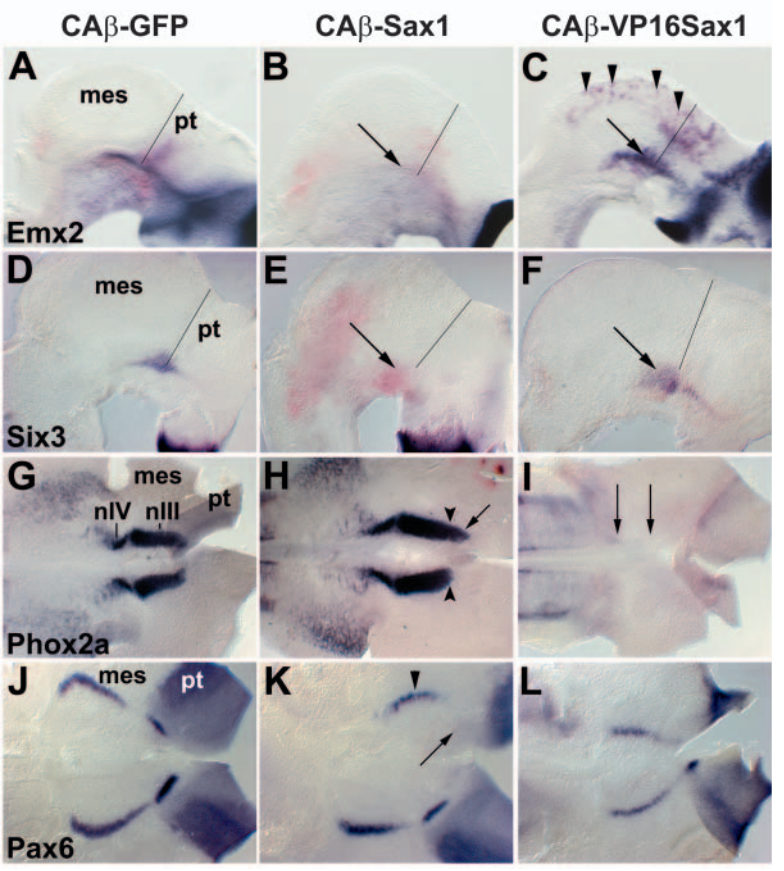

Fig. 4. Regulation of other homeobox genes by *Sax1*. (A-C) Lateral view of brains at HH18, stained for *Emx2* mRNA in blue and *GFP* mRNA in red. After electroporation of the control construct (A), the single *Emx2* stripe in the ventral mesencephalon (the dorsal stripe only appears at HH20) is evident. Ectopic expression of *Sax1* represses *Emx2* expression in the mesencephalon (B, arrow), while expression of *VP16Sax1* leads to an enlargement of the ventral *Emx2* stripe (C, arrow), and also induces ectopic spots of *Emx2* in the dorsal mesencephalon and diencephalon (arrowheads). (D-F) Expression of *Six3* in blue and *GFP* in red, following electroporation. The pattern of *Six3* is normal after electroporation of the control construct (D), while ectopic expression of *Sax1* abolishes the expression of *Six3* in the mesencephalon (E, arrow). *VP16Sax1* has no effect on *Six3* expression (F, arrow). (G-I) *Phox2a* expression in an HH21 embryo, 2 days after electroporation. In a control embryo, *Phox2a* signals label the trochlear and oculomotor nuclei (G). Unilateral ectopic expression of *Sax1* leads to a rostral expansion of the *Phox2a* expression in the oculomotor nucleus (H, arrow) compared with the control side (arrowheads marks the normal rostral limit of *Phox2a* expression), while *VP16Sax1* represses *Phox2a* expression in the oculomotor and trochlear nuclei (I, arrows). (J-L) *Pax6* expression in an HH21 embryo, 2 days after electroporation. The *Pax6* stripe in the ventral mesencephalon is largely unaffected by *Sax1* (K, arrowhead) or *VP16Sax1* (L) expression. By contrast, the ventral *Pax6* domain in the pretectum, prominent in embryos expressing the control construct (J), is lost following ectopic expression of *Sax1* (K, arrow). mes, mesencephalon; nIII, oculomotor nucleus; nIV, trochlear nucleus; pt, pretectum.

former genes, the ventral stripe of *Pax6* expression in the mesencephalon was largely unaffected by the expression of *Sax1* or *VP16Sax1* (Fig. 4J-L), although expression of *Sax1* did block the expression of *Pax6* in the ventral pretectum (Fig. 4K).

These results show that – like the homeobox genes in the spinal cord – the homeobox genes in the ventral midbrain can apparently crossregulate each other. *Sax1* in particular has a profound effect on the expression of *Emx2* and *Six3*.

Discussion

The formation of an axon scaffold during early brain development is a conserved feature of all vertebrates. Despite the abundance of morphological studies on the early axon tracts, the molecular mechanisms governing the development of this scaffold are largely unknown. However, recent studies in chick have suggested that homeobox genes in the ventral midbrain could play a similar role in specifying neuronal fate as they do in the spinal cord. Our experiments provide evidence that the differential expression of homeobox genes is indeed instructive to the fate of neurons in the ventral midbrain, and that, in particular, *Sax1* regulates the specification of mlf and pc neurons from the ventrocaudal cluster of neurons at the ventral MFB.

Expression domains for homeobox genes subdivide the ventral midbrain and pretectum

We have found that a number of homeobox genes are expressed in distinct domains in the ventral midbrain and pretectum during early brain development in the chick. Among these, *Emx2* and *Pax6* have been described previously as part of the arcuate plan that suggests the organisation of the ventral midbrain into longitudinal domains, called arcs (Agarwala and Ragsdale, 2002; Sanders et al., 2002). The arcs can be visualised by the expression of homeobox genes such as *Phox2a* for arc 1 or *Pax6* dividing arcs 2 and 3 (Sanders et al., 2002). In our analysis, we have included two further genes labelling arcs 2 and 3, *Six3* (Bovolenta et al., 1998) and *Emx2* (Bell et al., 2001). The expression patterns of both genes in the ventral midbrain are largely overlapping. However, only the *Emx2* signal also extends ventrally into the rostral part of arc 1, where it labels the prospective red nucleus (Agarwala and Ragsdale, 2002). All of these genes are expressed throughout most of the midbrain, stretching from the MFB almost to the isthmus. This suggests that they may form part of a general patterning machinery for the whole ventral midbrain. By contrast, *Sax1* is expressed predominantly around the MFB, abutting the oculomotor nucleus dorsally and rostrally. This *Sax1* expression pattern in the chick is similar to its orthologue *Sax1* (Schubert et al., 1995) and its paralogue *Sax2* (Simon and Lufkin, 2003) in mouse. Likewise, the zebrafish *sax2* gene is expressed in the ventrocaudal cluster at the MFB (Bae et al., 2004). In double labelling experiments for *Sax1* mRNA and either neurofilament protein or retrograde labelling to visualise the mlf, we have demonstrated that the *Sax1* expression domain overlays the INC, hinting at a specific function of *Sax1* in the specification of neurons at the MFB, particularly those forming the mlf.

Sax1 regulates the formation of the mlf

What is the role of *Sax1* in the formation of the mlf? Our misexpression experiments demonstrate that the expression of *Sax1* has to be tightly regulated to ensure the normal development of the mlf: ectopic expression of *Sax1* interferes with the patterning at the ventral MFB, and leads to an expansion of the mlf. This result suggests that *Sax1* is involved in the formation of the mlf, possibly by specifying mlf fate in differentiating neurons. However, in the converse experiment, *VP16Sax1* expression reduces the size of the mlf, but does not completely abolish its formation. This might be explained by incomplete penetrance of the constitutively activating construct against the background of endogenous *Sax1* and *Sax2* expression. *Sax2* expression in the mouse midbrain overlaps the *Sax1* expression domain, and mice lacking *Sax2* do not show an apparent midbrain phenotype (Simon and Lufkin, 2003), arguing for possible compensation by its paralogue.

The same cluster of neurons that includes the INC also harbours the ventral part of the nucleus of the pc. The pc is formed well after the mlf, with the first axons extending dorsally visible at HH17. In embryos expressing *Sax1* ectopically, the number of pc neurons is reduced so that the pc is barely visible. It is unclear how neurons in the ventral cluster are specified to mlf or pc fate, but ectopic expression of *Sax1* seems to interfere with this process. There is a possibility that fate specification is influenced by the birth date of individual neurons, as in the case of oculomotor and red nucleus neurons developing successively from arc 1 (Agarwala and Ragsdale, 2002). In such a scenario, early birth would supports mlf fate, while later birth would favour pc neurons. *Sax1* would be thus linked to the timing of neurogenesis. Indeed, in the early embryo *Sax1* is transiently expressed alongside *Cash4* in the caudal neural plate, preceding neurogenesis (e.g. Henrique et al., 1997). Although the role of *Sax1* in the caudal neural plate is unknown, it may be involved in neurogenesis in the caudal CNS, a role also recently assigned to the distantly related NK1-class homeobox gene *Pnx* (Bae et al., 2003). In the brain, *Sax1* is normally only expressed in postmitotic neurons, while with the electroporation method we introduce *Sax1* into neural progenitors, which may influence the time point when neural cells leave the cell cycle.

Alternatively, the specification of mlf and pc could be the result of intrinsic differences, possibly the differential expression of homeobox genes. Again, our results are consistent with this mechanism. The mlf enlargement after ectopic expression of *Sax1* is not linked to any apparent change in cell proliferation. Although formally it is also possible that ectopic *Sax1* expression changes axon guidance cues to misroute pc neurons onto a caudal path, our findings argue for a change of cell fate in the affected cells as the most likely cause of the observed phenotype. In addition, the repression of *Emx2* and *Six3* indicates an altered spatial patterning of the ventral MFB. In zebrafish, *Six3* labels pc neurons as well as the INC (Tallafuss et al., 2003). Possibly, the specification of pc or mlf neurons depends on the balance of homeobox gene expression at the ventral MFB. Loss of *Six3* and *Emx2* expression – together with increased or ectopic *Sax1* expression – might shift this balance in favour of mlf specification.

It is possible that both mechanisms, temporal and molecular difference, work hand in hand, as *Sax1* is expressed closer to the marginal surface of the neural tube than *Six3* and *Emx2*. This not only explains how the latter escape transcriptional

repression by Sax1, but may also reflect a link between the time of neuronal differentiation and the expression of specific homeobox genes, thus adding a temporal dimension to the spatial pattern of differential gene expression.

A genetic network governing nuclei formation at the ventral MFB

Recently, several studies have described the molecular patterning of the ventral midbrain, and – together with the data presented in our study – we can begin to assemble the genetic network that controls the formation of ventral midbrain nuclei. Patterning of the ventral midbrain along the two main axes occurs under the influence of neighbouring tissues: Fgf8 from the isthmus sets up the caudorostral polarity of the midbrain (e.g. Crossley et al., 1996), and Shh derived from notochord and floor plate constitutes the ventralising signal (Watanabe and Nakamura, 2000). In response to both signals, homeobox genes are expressed in distinct patterns in the ventral midbrain (Agarwala et al., 2001; Sanders et al., 2002). The midbrain arcs largely subdivide the tegmentum into distinct domains along the dorsoventral axis, but they also display distinct rostrocaudal features. Thus, *Emx2* is expressed in arcs 2 and 3 in the entire midbrain, but only rostrally extends into arc 1. *Sax1* expression in arcs 2 and 3 and the intervening region is restricted to the rostral midbrain, close to the MFB.

Several studies have now implicated homeobox genes with the formation of particular nuclei in the ventral midbrain, indicating the importance of proper patterning for the correct development of tegmental neurons. Our study demonstrates that the homeobox 'code' is already crucial for the specification of neurons that form the early axon scaffold. We also show for the first time that a homeobox gene expressed around the ventral MFB can directly or indirectly regulate the expression of other homeobox genes, providing a possible patterning mechanism. The regulatory activity of Sax1 appears to be highly specific, as ectopic expression of *Sax1* abolishes the expression of *Emx2* and *Six3*, but not *Pax6* in the ventral midbrain. However, *Pax6* expression in the adjacent ventral pretectum, where *Pax6* and *Sax1* are expressed exclusively, is lost after ectopic expression of *Sax1*. At the same time, the *Isl1* expression domain extends rostrally into the area where normally *Emx2* would be expressed. Quite possibly, the rostral extension of the *Isl1* domain (and the oculomotor nucleus) is an indirect effect of *Sax1*, reflecting the loss of *Emx2* expression. Likewise, the loss of the oculomotor nucleus following expression of *VP16Sax1* could be an indirect effect of the ectopic *Emx2* expression induced by the dominant-negative variant of Sax1. In this scenario, Emx2 would repress the expression *Isl1* in the rostral arc 1. Although such an effect has yet to be investigated, it is a conceivable mechanism by which *Emx2* may specify differentiating neurons towards red nucleus rather than oculomotor nucleus fate (Agarwala and Ragsdale, 2002). This would resemble the mechanism suggested by our own data, with *Sax1* crucially influencing the fate decision between mlf and pc.

These lines of evidence point to a possible recurring theme for nucleogenesis in the ventral midbrain: differentiating neurons at a given position face binary decisions of cell fate, and their choice is influenced by their relative birth date and is controlled by the differential expression of homeobox genes. An important characteristic of this possible mechanism is the mutual repression of the fate-determining transcription factors to avoid ambiguity in the cell fate, a strategy also employed in other examples of cell fate selection from a common precursor pool such as the specification of neuronal fate in the vertebrate spinal cord (Briscoe et al., 2000) and of muscle cell identity in the *Drosophila* embryo (Jagla et al., 2002). Interestingly, NK1 class genes are involved in both processes: the fly homologue of *Sax1*, *slouch*, is a muscle identity gene (Knirr et al., 1999); and *Sax1* itself is expressed in a subset of interneurones in the spinal cord (Schubert et al., 1995).

Conclusion

We propose that, as a result of broad rostrocaudal regionalisation mediated by the isthmic organiser, by local interactions at the MFB, and by dorsoventral patterning by floor plate and roof plate, homeobox genes are expressed in distinct domains in the ventral midbrain and pretectum. Their expression domains may become sharpened by reciprocal repressive interaction between the homeobox genes. We show that perturbing this intricate pattern by overexpressing the ventrally expressed homeobox gene *Sax1* ectopically leads to disturbed dorsoventral patterning of the midbrain and affects the organisation of the early axon scaffold. We conclude that the (combinatorial) expression of specific homeodomain transcription factors determines neuronal cell fate in the tegmentum of midbrain and pretectum.

We are grateful to A. Fainsod, C. Goridis, P. Gruss and T. Jessell for providing probes for in situ hybridisations, and to J. Gilthorpe and A. Hunter for the pCAβ-LINK-IRESeGFPm5 expression vector. We express our special thanks to S. Dietrich, K. Riley and C. Sharpe for their helpful comments during the preparation of the manuscript. The project was generously supported by a Project Grant from The Wellcome Trust (number 059207).

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