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REVIEW

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Specification of midbrain territory

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Abstract The vertebrate neural plate is subdivided into four distinct territories comprising the presumptive forebrain, midbrain, hindbrain, and the spinal cord, shortly after gastrulation. Initially, this subdivision relies on a defined pattern of expression of distinct transcription and secreted factors within the newly formed neuroectoderm, even before morphological partitioning is evident. Subdivision of the neural plate into distinct territories is a complex process, which is also known as patterning or regionalisation, and involves both planar and vertical signals coming from within the neuroectoderm and from neighbouring non-neural tissues. During the course of embryogenesis, this gross subdivision of the neural plate is progressively refined by a variety of mechanisms, leading to the establishment of various subdomains that ultimately give rise to specific cell populations characteristic for the corresponding brain and spinal cord regions. Once again, a prominent feature of these later processes is the defined expression of specific genes within the developing neural tube. In the present review, we will concentrate on the genes active in the progressive refinement of the midbrain territory as a distinct subdivision of the brain. We will also give an outlook on genes that are active during early induction of the anterior neural plate and genetic mechanisms that control the generation of specific cell populations of the ventral midbrain, with special focus on the mesencephalic dopaminergic neurons.

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Introduction

Midbrain territory is first specified shortly after the induction of the neuroectoderm from the dorsal epiblast of the vertebrate gastrula. This specification relies mostly on the defined expression pattern of a set of transcription factors in the anterior neuroectoderm and is later refined by the establishment of signalling centres at the boundary of the midbrain and hindbrain and at the anterior neural ridge. Midbrain territory is therefore established in molecular terms even before morphological subdivision is apparent. Later, a clearly discernible midbrain vesicle, the mesencephalon, arises from the developing anterior neural tube. Morphologically, the mesencephalon is delimited at its anterior end by the diencephalic vesicle and at its posterior end by the isthmic constriction, which separates it from the hindbrain or rhombencephalon. Development of the midbrain territory and of the anterior hindbrain is tightly linked and dependent on the midbrain/hindbrain organiser (MHO).

In this review, we describe the mechanisms active in the early establishment of midbrain identity and the later refinement and maintenance of this territory relevant to the development of mesencephalic dopaminergic (mes-DA) neurons. Since some of these mechanisms have previously been extensively reviewed (Wurst and Bally-Cuif 2001; Liu and Joyner 2001a; Rhinn and Brand 2001), we focus on the advances that have been made on the subject during the last 3 years. For better comprehension, we confine our review mostly to mouse development; however, many of the mechanisms described here have been conserved throughout vertebrate evolution.

Otx2 is required for forebrain and midbrain development

The visceral endoderm, a derivative of the extra-embryonic primitive endoderm, underlies the epiblast prior to gastrulation. At the onset of gastrulation, vertical signals emanating from the visceral endoderm and the axial

mesendoderm induce the neural plate in the overlying epiblast. In the past few years, evidence has accumulated that the anterior visceral endoderm plays a crucial role in the induction of the anterior neuroectoderm, which will later give rise to the forebrain and midbrain (for a review, see Stern 2001). Cripto$^{-/-}$ mutant mice lack a membrane-anchored epidermal growth factor (EGF)-like protein in the proximal region of the epiblast and do not undergo normal gastrulation. In these mice, anterior neuroectoderm marker genes have been shown to be induced and maintained in the epiblast, even in the absence of the mesendoderm, albeit in the wrong position (Ding et al. 1998; Liguori et al. 2003). The earliest anterior neuroectoderm marker gene is *Otx2*, a homeodomain transcription factor that is expressed in the visceral endoderm and in the epiblast prior to the onset of gastrulation. During gastrulation, *Otx2* becomes progressively restricted to the anterior region of the mouse embryo in all three germ layers, including the prospective anterior neural plate. Conversely, another homeobox-containing transcription factor, *Gbx2*, is expressed throughout all germ layers in the posterior part of the embryo. The expression domains of *Otx2* and *Gbx2* in the anterior or posterior neural plate, respectively, subsequently approach each other and their posterior and anterior expression borders ultimately abut on what will be the midbrain/hindbrain boundary (MHB). Thus, the *Otx2* expression domain defines the prospective forebrain and midbrain territory, whereas the *Gbx2* expression field defines the prospective hindbrain region and spinal cord. Otx2 appears to be required in the anterior visceral endoderm for the induction of the rostral neural plate in the overlying epiblast and in epiblast-derived tissues for the specification and maintenance of anterior head structures, namely the forebrain and midbrain (Acampora et al. 1998; Rhinn et al. 1998; for a review, see Acampora et al. 2001). In the complete absence of Otx2 protein, no anterior head structures form and the entire brain rostral to rhombomere 3 is missing (Acampora et al. 1995; Ang et al. 1996; Matsuo et al. 1995). Conversely, genetic ablation of *Gbx2* leads to a rostralisation of the hindbrain, i.e. the anterior hindbrain corresponding to rhombomeres 1–3 does not form and the midbrain expands up to the rhombomere 3/4 boundary in these mutants (Wassarman et al. 1997).

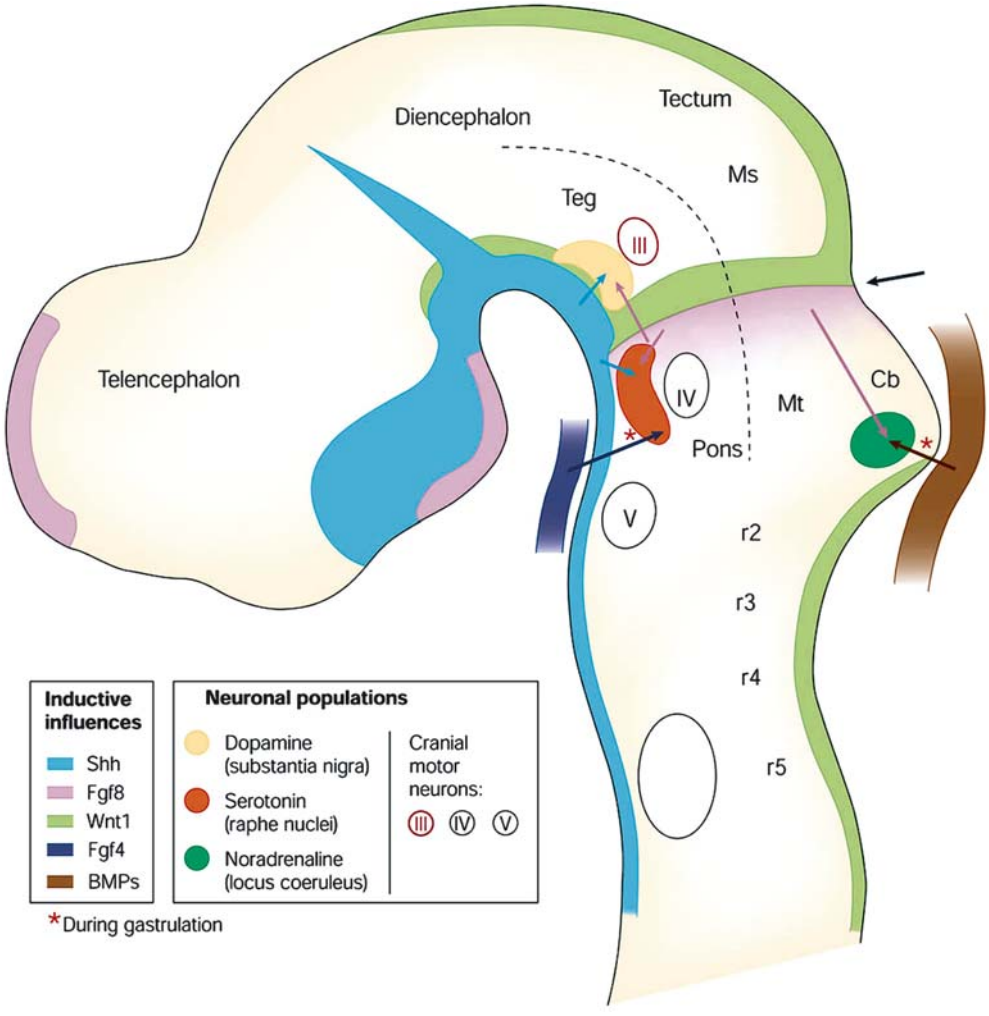

Fig. 1 Expression domains of some of the MHO genes in an E11 mouse embryo head and cell populations that are specified by their activity (*Cb* cerebellum, *Ms* mesencephalon, *mt* metencephalon, *r* rhombomeres, *Teg* tegmentum). Sagittal view of an E11 mouse embryo neural tube; anterior is *left*. Expression of the secreted factors fibroblast growth factor 8 (Fgf8), Wnt1 and sonic hedgehog (Shh) is depicted at the MHB (Fgf8 and Wnt1), in the anterior neural ridge and ventral diencephalon (Fgf8), in the dorsal midline of the midbrain, mesencephalic flexure and dorsal midline of the posterior hindbrain and spinal cord (Wnt1), and within the floor/basal plate of the spinal cord, hindbrain, midbrain and caudal forebrain (Shh). Mes-DA neurons are induced by a combination of Fgf8 and Shh (*arrows*). Rost-5HT neurons are specified by a combination of the same factors but they require an early inductive Fgf4 signal derived from the anterior mesoderm during gastrulation. Noradrenergic neurons of the locus coeruleus are induced by Fgf8 from the MHB (*pink and brown arrows*) and by bone morphogenetic proteins (BMPs) secreted from the adjacent non-neural dorsal ectoderm during gastrulation

Otx2 and *Gbx2* are required for the proper positioning but not for the induction of midbrain/hindbrain marker genes

After neural induction, *Otx2* is expressed in the anterior and *Gbx2* in the posterior part of the neuroectoderm. At the end of gastrulation, the expression domains of both genes approach each other so that they are transiently coexpressed in a narrow field (Garda et al. 2001). At the onset of somitogenesis, however, the posterior and anterior expression boundaries of *Otx2* and *Gbx2*, respectively, are refined to a mutually exclusive territory and abut each other at the 4–6 somite stage. The region at which the expression domains of both genes meet later becomes the MHB, as mentioned above. Long before this boundary becomes discernible as a morphological entity, the expression of a set of genes is initiated in this region, which thus acts as an important organising centre, the midbrain/hindbrain or isthmic organiser (MHO).

First, the expression of the paired-box transcription factor Pax2 is initiated in the presomitic head-fold stage around the *Otx2*/*Gbx2* boundary (Rowitch and McMahon 1995). In the 1-somite mouse embryo, expression of the homeodomain transcription factor engrailed 1 (En1) and of the secreted glycoprotein Wnt1 is then simultaneously initiated within the *Pax2* domain (Davis and Joyner 1988; Rowitch and McMahon 1995; McMahon et al. 1992). *En1* and *Wnt1* are initially coexpressed across the *Otx2*/*Gbx2* border in the prospective midbrain and hindbrain but, later, *Wnt1* expression is largely confined to the posterior *Otx2*-positive domain. Thereafter, at the 3–5 somite stage, transcription of the paired-box gene *Pax5* and of the homeobox gene engrailed 2 (*En2*) is initiated in a broad region overlapping the *Otx2*/*Gbx2* boundary (Davis et al. 1988; Asano and Gruss 1992). At the same stage, the secreted member of the fibroblast growth factor (Fgf) family, Fgf8, starts to be expressed in an initially broad domain confined to the rostral part of the *Gbx2*-positive territory (Crossley and Martin 1995). The expression domains of these genes subsequently are refined to a highly ordered pattern in the embryonic day (E) 9.5 mouse embryo, when the MHB becomes morphologically distinguishable as the isthmic constriction. Strikingly, *Wnt1* expression has mostly retracted and is now restricted to a narrow ring encircling the neural tube just rostral to the isthmic constriction and to the dorsal and ventral midline of the mesencephalon (Fig. 1; Wilkinson et al. 1987; Parr et al. 1993). In addition, *Wnt1* is expressed in the dorsal midline of the caudal diencephalon and along the dorsal midline of the caudal hindbrain and spinal cord. In contrast, the *Fgf8* domain is now restricted to a narrow ring in the rostral hindbrain just caudal to the isthmic constriction, thus abutting the *Wnt1* expression domain in the caudal midbrain (Crossley and Martin 1995). The expression domains of *En1* and *Pax2* have also been narrowed but, together with *En2* and *Pax5*, these genes continue to be transcribed in an overlapping region across the MHB. Two other members of the *Fgf* family closely related to *Fgf8*, viz. *Fgf17* and *Fgf18*, are also expressed across the MHB in a broader domain than that of *Fgf8* (Maruoka et al. 1998). However, these genes only start to be transcribed after *Fgf8* (Liu et al. 2003).

Both loss-of-function and gain-of-function experiments in mice have revealed that the *Otx2*/*Gbx2* expression boundary is established through the reciprocal repression of both genes and that this interface is required for the proper positioning of the MHO gene activity but not for its initial induction (Suda et al. 1997; Acampora et al. 1997; Wassarman et al. 1997; Broccoli et al. 1999; Millet et al. 1999; Martinez-Barbera et al. 2001; Li and Joyner 2001). Thus, in compound mutant mice that have only one functional *Otx* allele ($Otx1^{-/-}$; $Otx2^{+/-}$ mice; Acampora et al. 1997) and in mice ectopically expressing *Gbx2* in the caudal midbrain (*Wnt1–Gbx2* transgenic mice; Millet et al. 1999), a rostral shift of the midbrain/hindbrain genes *Fgf8*, *Gbx2*, *Wnt1* and *En1/2* is accompanied by a caudal repression of *Otx2* at early somite stages. These mice show at least a transient transformation of the midbrain and caudal forebrain to rostral hindbrain fates. Conversely, $Gbx2^{-/-}$ mice (Wassarman et al. 1997; Millet et al. 1999) and mice that ectopically express *Otx2* in the rostral hindbrain ($En1^{+/Otx2}$ mice; Broccoli et al. 1999) show a caudal shift of *Otx2*, *Wnt1* and *Fgf8* expression accompanied by, in the last-mentioned case, a rostral repression of *Gbx2*. These mice exhibit at least a partial transformation of rostral hindbrain to mesencephalic fates. Interestingly, in all these mutant mice, expression of the MHO genes *Fgf8*, *Wnt1*, *Pax2* and *En1/2* is relocated at the newly created *Otx2* expression boundary. Inactivation of *Otx2* and *Gbx2* by conditional mutagenesis at later embryonic stages in mice reveals the requirement of both genes for the maintenance of proper MHO activity, although the epistatic relationships of its contributing genes become much more complex (Li et al. 2002; Puelles et al. 2003, 2004).

Although these results indicate that neither *Otx2* nor *Gbx2* alone are required for the initial induction of the MHO genes, this issue has been addressed in a more recent set of experiments. In $Gbx2^{-/-}$; $Otx2^{hOtx1/hOtx1}$ double mutant mouse embryos, which lack both *Gbx2* and *Otx2* in the neuroectoderm, the transcription of *Pax2*, *Wnt1*, *En1* and *Fgf8* is correctly initiated at presomitic/early somite stages but all genes (including residual expression of *Gbx2* and *hOtx1*) are now coexpressed in the anterior neuroectoderm (Li and Joyner 2001; Martinez-Barbera et al. 2001; Wassarman et al. 1997; Acampora et al. 1998). This aberrant expression pattern is maintained in later embryonic stages. Notably, the initial positioning of *Otx2* expression in the anterior part and of *Gbx2* in the posterior part of the neuroectoderm in the gastrulating mouse embryo is not altered in these mutants. At E10.5, the time point at which the double mutants die, a partial rescue of anterior neural tissue becomes evident compared with the single mutant $Otx2^{hOtx1/hOtx1}$ embryo. The latter result may be interpreted as a less pronounced caudalisation of the anterior neural tube attributable to the lack of *Gbx2* in the double mutants. However, expression of forebrain- and midbrain-specific genes such as *Bf1* and *Atx*, respectively, is never initiated in the $Gbx2^{-/-}$;

Otx2$^{hOtx1/hOtx1}$ embryos, indicating that, in these mutants, the anterior neuroectoderm does not acquire proper regional identity (Martinez-Barbera et al. 2001). Taken together, the analysis of the *Gbx2*$^{-/-}$; *Otx2*$^{hOtx1/hOtx1}$ mutant mice shows that the *Otx2* and *Gbx2* expression domains in the neuroectoderm are established independently of each other but soon become interdependent. Furthermore, Otx2 and Gbx2 are not required for the initial induction of midbrain/hindbrain marker genes but for the establishment of the correct spatial relationships of their expression domains. Finally, a minimal Otx2 dosage is required for the proper regionalisation of the anterior neuroectoderm in forebrain and midbrain territories (instructive role), whereas Gbx2 may play a permissive role for anterior hindbrain development (Martinez-Barbera et al. 2001; Li and Joyner 2001; Li et al. 2002).

In conclusion, we currently know which genes are required for anterior–posterior (A/P) axis specification in the neuroectoderm and the subsequent establishment of regional identities determining the location of forebrain, midbrain and hindbrain in the anterior neural plate. An unresolved question to date, nevertheless, is the molecular identity and location of the signal(s) that are required for the initial induction of these genes. One hypothesis is that vertical signals coming from the underlying mesendoderm initiate expression of the midbrain/hindbrain genes. However, as mentioned above, this explanation has become less likely, as the expression of these genes is known to start normally and is even maintained in an in vitro culture system of *Cripto*$^{-/-}$ embryos, which do not undergo gastrulation and therefore lack mesendodermal tissue (Liguori et al. 2003). Thus, planar signals residing within the neuroectoderm itself may be responsible for the initiation of midbrain/hindbrain marker gene expression after gastrulation.

MHB is an organising centre that controls midbrain and rostral hindbrain development

The concept of the MHB as an important organising centre was originally derived from tissue transplantation and ablation experiments in chicken. Grafts of isthmic tissue into ectopic positions at the caudal forebrain, midbrain or hindbrain induced ectopic expression of midbrain/hindbrain marker genes and eventually an ectopic midbrain or cerebellum in the surrounding host tissue (Martinez et al. 1991; Martinez et al. 1995). Thereafter, functional inactivation of the genes expressed at the MHB (described above) in mice revealed their requirement for the establishment and/or maintenance of the midbrain and hindbrain compartment later in development. Since these aspects of MHO activity have previously been extensively described and discussed in previous reviews (Wurst and Bally-Cuif 2001; Liu and Joyner 2001a; Rhinn and Brand 2001), we will restrict ourselves here to the presentation of the most important and recent advances that have been made in the field.

Fgf8 is a key MHO gene and has midbrain/hindbrain patterning activity on its own

After the discovery of the organising activity in the MHB, it soon became clear that one of the key molecules mediating the patterning abilities of the grafted tissue is Fgf8. The primary *Fgf8* transcript is alternatively spliced in eight putative isoforms, two of which have been shown to be expressed in the MHB of the chick (Sato et al. 2001). These are the *Fgf8a* and *Fgf8b* isoforms. Transgenic mouse embryos in which ectopic expression of *Fgf8a* or *Fgf8b* in the midbrain is driven by a *Wnt1* enhancer (*Wnt1–Fgf8a/b* mice) have striking different phenotypes (Lee et al. 1997; Liu et al. 1999). *Wnt1–Fgf8a* mouse embryos show an enlarged midbrain and caudal diencephalon, because of overproliferation, but no alteration of MHO gene expression, indicating that the Fgf8a isoform does not possess patterning activity. In contrast, *Wnt1–Fgf8b* embryos show an early transformation of the midbrain and posterior forebrain into anterior hindbrain, concomitantly with an altered expression of *Otx2* (repressed) and *Gbx2* (induced) in the anterior neural plate. These results suggest that the Fgf8b isoform is the patterning molecule of the MHB, as has been confirmed by implantation of Fgf8b-coated beads into the caudal forebrain of chicken embryos (Martinez et al. 1999). Fgf8b-coated beads induce an ectopic midbrain and cerebellum in the caudal diencephalon after the previous induction of MHO genes such as *Wnt1*, *En1/2*, *Pax2/5* and *Gbx2* and the repression of *Otx2* at this ectopic location (Crossley et al. 1996; Martinez et al. 1999; Liu et al. 1999; Liu and Joyner 2001b).

The importance of Fgf8 for midbrain/hindbrain development has recently been shown by conditional inactivation of the *Fgf8* gene in mice (Chi et al. 2003). Deletion of *Fgf8* at the 10-somite stage, i.e. after the initial establishment of MHO activity, results in the complete absence of the entire midbrain and anterior hindbrain in both dorsal and ventral domains. This is attributable to massive cell death occurring in the midbrain/hindbrain area of these mutants after depletion of Fgf8. Furthermore, inactivation of *Fgf8* leads to the loss of *Wnt1*, *Gbx2*, *Fgf17* and *Fgf18* expression at the MHB prior to the onset of cell death. These results indicate that Fgf8 is required for the sustained growth of the midbrain/hindbrain region and the maintenance of the expression of some of the MHO genes, namely *Wnt1* in the posterior midbrain, *Gbx2* in the anterior hindbrain and *Fgf17*/*18* expression across the MHB.

Indeed, another recent report has shown that Fgf8 is engaged in a complex regulatory network, including the regulation of its own receptors and inhibitors (Liu et al. 2003). First, Fgf8b can induce the expression of *Fgf18* but not of *Fgf17*. Fgf18 alone does not appear to be required for midbrain/hindbrain development (Liu et al. 2002; Ohbayashi et al. 2002). Loss of *Fgf17* function in mice results in a truncation of the posterior midbrain and anterior cerebellum, a phenotype that is even more severe in *Fgf17*$^{-/-}$; *Fgf8*$^{+/-}$ double mutants, indicating a co-

operative effect of Fgf8 and Fgf17 on midbrain/hindbrain development (Xu et al. 2000). Such cooperation may well also apply to Fgf18 and Fgf8 but this has not yet been tested. However, neither Fgf17 nor Fgf18 appears to have the patterning activity of Fgf8b. Rather, they exert a similar effect on midbrain growth as Fgf8a and are incapable of altering MHO gene expression (Liu et al. 2003). Second, two intracellular inhibitors of receptor tyrosine kinase-signalling, including the Fgf-receptors Sprouty (Spry) 1 and 2, are expressed in a broad domain across the MHB (Minowada et al. 1999; Liu et al. 2003). Both *Spry1* and *Spry2* are directly induced by Fgf8b. Third, in vertebrates, four genes are known that encode Fgf receptors (Fgfr) of the tyrosine kinase family, *Fgfr1–4*, three of which are expressed in the developing neural tube (Walshe and Mason 2000). Only *Fgfr1* appears to be expressed across the MHB in the midbrain and hindbrain territory, whereas *Fgfr2* and *Fgfr3* appear to be excluded from the MHB (Trokovic et al. 2003; Liu et al. 2003). Indeed, Fgf8b can repress both *Fgfr2* and *Fgfr3* expression in neural tube explants. The conditional inactivation of the *Fgfr1* gene across the MHB or in the midbrain alone leads to a deletion of the inferior colliculi in the caudo-dorsal midbrain and of the cerebellar vermis in the rostro-dorsal hindbrain, whereas the ventral part of the midbrain/rostral hindbrain is not affected (Trokovic et al. 2003). These morphological alterations are preceded by changes in the expression patterns for *Pax2*, *Spry1*, *En1/2* and *Wnt1* at earlier embryonic stages, indicating that signalling through Fgfr1 is required for the sustained expression of these MHO genes. Furthermore, functional Fgfr1 appears to be required at the cellular level for the maintenance of a coherent MHB, probably by the direct regulation of cell adhesion (Trokovic et al. 2003). Nevertheless, since the conditional *Fgfr1* knock-out phenotype does not fully reproduce the conditional *Fgf8* knock-out phenotype, other Fgfrs must also be engaged in Fgf8 signal transduction at the MHB.

Wnt1 is necessary but not sufficient for midbrain/hindbrain development

Targeted inactivation of the *Wnt1* gene in mice leads to an early deletion of the midbrain and subsequently of the rostral hindbrain (rhombomere 1) as a result of extensive cell death in this region (McMahon and Bradley 1990; Thomas and Capecchi 1990; Mastick et al. 1996; Chi et al. 2003). Morphological deletion of the midbrain/hindbrain region in $Wnt1^{-/-}$ mutants is preceded by an early loss of *Fgf8* expression in the rostral hindbrain, followed by the loss of *En1* expression in the mid-/hindbrain region (Lee et al. 1997; McMahon et al. 1992). This observation has suggested that Wnt1 is directly required for the maintenance of *Fgf8* expression in rhombomere 1 and directly or indirectly maintains *En1* expression across the MHB. Indeed, expression of *En1* driven by the *Wnt1* enhancer in $Wnt1^{-/-}$ mice rescues most of the $Wnt1^{-/-}$ mutant phenotype (Danielian and McMahon 1996). Thus, it remains unclear whether Wnt1 plays a more direct role in midbrain/hindbrain development. So far, no evidence exists for *Wnt1* having a patterning activity in the midbrain/hindbrain region, although it is the only gene that, during early somitogenesis, demarcates the prospective midbrain territory within the neural plate. Ectopic expression of *Wnt1* from the *En1* locus by using a knock-in strategy in mice does not impair the positioning of the MHO or the expression of MHO genes (Panhuysen et al. 2004). Instead, overexpression of *Wnt1* in the caudal midbrain leads to a dramatic increase in size of the inferior colliculi (a caudo-dorsal midbrain derivative) in adult mice because of enhanced cell proliferation (Panhuysen et al. 2004). Therefore, Wnt1 may rather be required for sustaining the growth of the posterior midbrain/anterior hindbrain during development, by acting in a similar manner to the Fgf8a isoform.

En and *Pax* genes are required for proper mid-/hindbrain development

Both *En* and *Pax* genes are expressed in the prospective midbrain and rostral hindbrain region of the neural plate from early stages of somitogenesis on, thus suggesting that they also play a crucial role in the proper development of this region. $En1^{-/-}$ single mutant mice have a deletion of the dorsal and ventral parts of the midbrain and rostral hindbrain (Wurst et al. 1994). In contrast, $En2^{-/-}$ mutant mice are viable and show only minor cerebellar defects (Millen et al. 1994). Unlike the *En* single mutants, $En1^{-/-}$; $En2^{-/-}$ double mutant mice have a complete deletion of the midbrain/hindbrain region; this is reflected in an early loss of *Wnt1*, *Fgf8* and *Pax5* expression at the MHB (V. Blanquet et al. unpublished; Liu and Joyner 2001b). Thus, *En* genes are involved in the maintenance of a functional MHO.

Pax2 is expressed at presomitic stages even before all other MHO genes. A critical role of Pax2 in the induction of *Fgf8* transcription has been shown in $Pax2^{-/-}$ mice, in which *Fgf8* expression at the MHB is never initiated (Ye et al. 2001). Although the expression of other genes of the MHO is correctly initiated in the $Pax2^{-/-}$ mutant mouse, further development of the midbrain/hindbrain region is not sustained in this mouse type leading to a deletion of this region later in embryogenesis (Bouchard et al. 2000). $Pax5^{-/-}$ mutant mice show a partial deletion of the inferior colliculi and a slightly enlarged cerebellum (Urbanek et al. 1994). Again, $Pax2^{-/-}$; $Pax5^{-/-}$ double mutant mice have a more severe phenotype lacking most of the midbrain and cerebellum (Schwarz et al. 1997). Thus, like the *En* genes, the *Pax* genes cooperate in a dose-dependent manner for the establishment and maintenance of the MHO.

Interestingly, a caudal shift of the *Pax6* expression domain in the forebrain is seen at early somite stages in both *En1/2* and *Pax2/5* double mutants (Liu and Joyner 2001b; Schwarz et al. 1999); therefore, *En1/2* and *Pax2/5* may demarcate the anterior end of the midbrain compartment by repressing forebrain-specific genes such as *Pax6*.

Other factors/mechanisms must act in the maintenance of the midbrain/forebrain boundary, however, since there is a gap between the *En1/2* and *Pax2/5* expression domains in the midbrain and *Pax6* expression in the diencephalon at later embryonic stages.

Sonic hedgehog controls growth but not dorso-ventral patterning of the midbrain

All the aspects described so far ultimately result in the establishment of the A/P polarity and the pattern of the midbrain and hindbrain. How are the dorso-ventral (D/V) compartments in the midbrain and anterior hindbrain established, especially as we know that, morphologically, there are differences between the dorsal and ventral derivatives of the midbrain and hindbrain. The dorsal midbrain gives rise to the superior and inferior colliculi (together known as the tectum), whereas the ventral midbrain generates the tegmentum with its characteristic motor nuclei. The rostro-dorsal hindbrain, on the other hand, develops into the cerebellum, whereas the rostro-ventral hindbrain generates the pons and associated nuclei. Furthermore, some of the mouse mutants described above clearly show a stronger dorsal compared with ventral phenotype.

Not much is known about the molecular events underlying early D/V patterning of the midbrain and rostral hindbrain (rhombomere 1). Compelling evidence has accumulated that the secreted glycoprotein sonic hedgehog (Shh) is a key signal in the specification of ventral cell identities along the entire vertebrate neural tube (for a review, see Jessell 2000). *Shh* is first expressed in the axial mesoderm, also called the notochord, underlying the developing neural plate. Shh secreted from the notochord then induces *Shh* expression in the ventral most cell layer, the floor plate, of the neural tube. Whereas *Shh* expression in the prospective spinal cord and caudal hindbrain is restricted to the narrow stripe of the floor plate, its expression broadens considerably at the level of the MHB and covers almost the entire basal plate of the midbrain and caudal forebrain. Two recent publications have begun to unravel the role of Shh in midbrain and caudal forebrain (diencephalon) development (Ishibashi and McMahon 2002; Britto et al. 2002). Shh appears to be required for the normal growth of the diencephalic and mesencephalic primordia by sustaining proliferation and survival of both ventral and dorsal precursors. Although *Shh* is not expressed and Shh signalling is not active in the dorsal parts of the neural tube, there may be a Shh-dependent signalling relay to the dorsal midbrain/diencephalon. This relay mechanism includes another member of the Fgf family, *Fgf15*, which is expressed in the dorsal and lateral neural tube adjacent to the *Shh* domain in an Shh-dependent manner (Ishibashi and McMahon 2002). Fgf15 in turn regulates the expression of a member of the Wnt signalling pathway, *Tcf4*, and Tcf4 may be required for the expression of a Wnt target gene, *cyclin D1*. Shh, however, does not appear to be required for the normal patterning of the midbrain and caudal forebrain as judged by the normal expression of MHO and forebrain marker genes and normal development of ventral midbrain structures after ablation of *Shh* expression in the notochord and floor plate of the midbrain (Britto et al. 2002) or in *Shh* mutant mice (Ishibashi and McMahon 2002). Surprisingly, genes that show a strict Shh-dependence in the caudal hindbrain and spinal cord are not affected by the removal of *Shh* expression from the notochord and floor plate in the midbrain (Britto et al. 2002). In addition, a clear dorsalisation of the caudal forebrain and midbrain is not evident in *Shh* mutant mice, in contrast to evidence known from the spinal cord (Ishibashi and McMahon 2002). Taken together, these results suggest that, in the midbrain and caudal forebrain, Shh acts rather as a growth factor promoting cell proliferation and survival than as a patterning molecule.

Activity of the MHO controls the generation of specific cell populations in the ventral midbrain and rostral hindbrain

So far, we have described processes that mostly take place during the early specification of the midbrain territory within the neural tube, prior to or at the onset of neurogenesis in the brain (E9.5–E10.5 in the mouse). The MHO, however, is still functional for at least 4 more days in mouse embryonic development. As demonstrated in four recent publications (Brodski et al. 2003; Puelles et al. 2003, 2004; Li et al. 2002), this has lasting consequences for the generation of specific cell populations in the ventral midbrain and rostral hindbrain. First, the position of the MHO in early development defines the location and size of the mesencephalic dopaminergic (mes-DA) and rostral hindbrain serotonergic (rost-5HT) cell populations (Fig. 2; Brodski et al. 2003). Shifting the MHO to a more caudal position by ectopically expressing *Otx2* in the rostral hindbrain ($En1^{+/Otx2}$ mice; Broccoli et al. 1999) enlarges the mes-DA population to the same caudal extent and at the expense of the rost-5HT population (Fig. 3). Conversely, shifting the MHO to a rostral position by lowering Otx dosage in the anterior neural tube ($Otx1^{-/-}$; $Otx2^{+/-}$ mice; Acampora et al. 1997) relocates the mes-DA and rost-5HT neurons to this ectopic position, whereby the size of the rost-5HT population is increased at the expense of the mes-DA population. These changes persist into adulthood and the additional or ectopic neurons are functionally integrated into the mature brain, as judged by their projection fields and neurotransmitter release.

Second, conditional inactivation of the *Otx2* gene in the lateral midbrain at E10.5 ($Otx1^{cre/+}$; $Otx2^{flox/-}$ mice; Puelles et al. 2003) results in the anterior expansion of *Fgf8*, *Pax2* and *En1*, whereas all the other MHO genes are not or only mildly affected. At the same time, expression of *Shh* and of several of its positively regulated target genes in the midbrain floor/basal plate is considerably expanded dorsally, whereas negatively regulated targets of

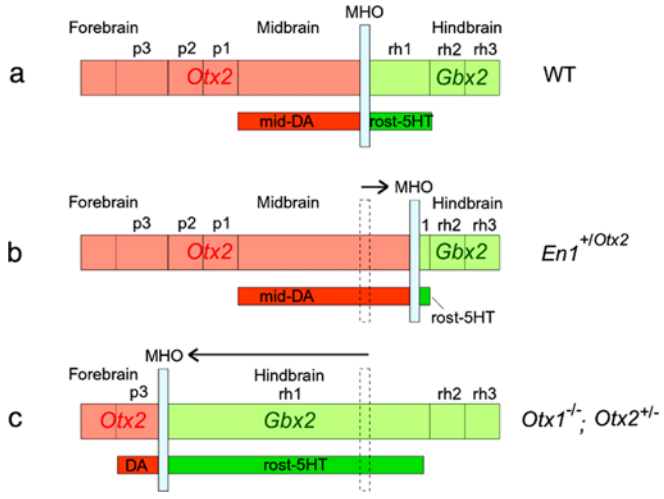

Fig. 2a–c The position of the MHO controls the size and location of the midbrain dopaminergic (*mid-DA*) and rost-5HT cell (*rost-5HT*) populations (*p* prosomere, *rh* rhombomere). **a** In the wildtype (*WT*), mid-DA neurons are generated in the ventral midbrain rostral to the MHO within the *Otx2* expression domain, whereas rost-5HT neurons develop in the ventro-rostral hindbrain (rhombomere 1, rh1) caudal to the MHO within a *Gbx2*-positive territory. **b** In $En1^{+/Otx2}$ mice, the *Otx2* expression domain and subsequently the MHO are shifted caudally into rh1 (*arrow*). As a consequence, the size of the mid-DA cell population is expanded at the expense of the rost-5HT cell population. **c** Because of the reduced Otx dosage in $Otx1^{-/-}$; $Otx2^{+/-}$ mice, the MHO is repositioned at the p2/3 boundary in the forebrain (*arrow*). In this mouse mutant, mid-DA neurons are induced rostral to the ectopic position of the MHO in smaller numbers, whereas the rost-5HT cell population is expanded to the new caudal border of the MHO. This indicates that activity of the MHO is sufficient to induce these two cell populations along the A/P axis and that the position of the MHO controls their location and size within the rostral neural tube. Abbreviations: *mid-DA* midbrain dopaminergic neurons; *p* prosomere; *rh* rhombomere; *rost-5HT* rostral hindbrain serotonergic neurons. Reprinted with permission after Brodski et al. (2003)

Shh signalling are repressed in their dorsal expression domains. Proliferation in the ventral midbrain (tegmentum) of these mice is remarkably increased, probably because of the mitogenic effect of the expanded *Shh* domain. As a consequence, the mes-DA population is considerably enlarged, apparently at the expense of other cell populations in the ventral midbrain of these mutants. Conditional inactivation of the *Otx2* gene in the entire caudal midbrain at E9.5 ($En1^{cre/+}$; $Otx2^{flox/flox}$ mice; Puelles et al. 2004), in contrast, results in the opposite phenotype. The motor nuclei of the ventral midbrain are either strongly reduced or missing in these mice, whereas in the dorsal midbrain/hindbrain region, the cerebellum is greatly expanded at the expense of the inferior colliculi. At the molecular level, these mutant mice show a rostral shift of the MHO only in its dorsal part, whereas the ventral position of the MHO remains unchanged. This is probably a result of the repressive action of *Otx1*, since in $En1^{cre/+}$; $Otx2^{flox/flox}$; $Otx1^{-/-}$ triple mutants, the ventral *Fgf8* domain is shifted anteriorly. Surprisingly, *Gbx2* is not shifted rostrally in these triple mutant mice but rather is absent from the new caudal border of the MHB. As a consequence, despite the more anterior expression of *Fgf8*, the *Gbx2*-negative territory retains midbrain identity. $En1^{cre/+}$; $Otx2^{flox/flox}$ mice show a notable dorsal expansion of the ventral *Shh* domain in the caudal midbrain. Unlike the $Otx1^{cre/+}$; $Otx2^{flox/-}$ mice, this dorsal *Shh* expansion is not paralleled by a uniform dorsal expansion of *Shh*-responsive genes. Instead, some expression domains of these genes are completely lost and others show a ventral shift in $En1^{cre/+}$; $Otx2^{flox/flox}$ mutants, reflecting a distinct requirement of Otx2 for the regulation of these genes. The molecular changes in the floor plate result in a severe reduction of mes-DA cells, the ectopic generation of rost-5HT neurons and the complete absence of the red nucleus in the ventral midbrain of these mice.

Third, conditional inactivation of the *Gbx2* gene in the rostral hindbrain at E9.0 ($En1^{cre/+}$; $Gbx2^{flox/-}$ mice; Li et al. 2002) leads to a cerebellar phenotype (reduction of the medial part or vermis and aberrant foliation) and a slight enlargement of the inferior colliculi. In these mutants, both *Wnt1* and *Fgf8* are expanded caudally into the dorsorostral hindbrain, being coexpressed in this region. Surprisingly, and similar to that observed for *Gbx2* expression in the $En1^{cre/+}$; $Otx2^{flox/flox}$; $Otx1^{-/-}$ triple mutant mice, *Otx2* expression is expanded only slightly posteriorly around the dorsal midline in rhombomere 1 of $En1^{cre/+}$; $Gbx2^{flox/-}$ mice. Therefore, the *Otx2*/*Gbx2*-negative territory in these mutants may be assumed to retain a rostral hindbrain identity.

Taken together, the ectopic positioning of the MHO and conditional inactivation of *Otx2* and *Gbx2* at later stages of embryonic development demonstrate a distinct requirement of MHO genes for the proper determination of different progenitor domains along the A/P and D/V axes of the midbrain and rostral hindbrain; these domains will generate specific cell populations in this region. Thus, MHO activity extends beyond the "mere" demarcation of the midbrain and rostral hindbrain territory from the rest of the neural tube. Instead, MHO activity progressively defines more restricted compartments within this territory; these compartments eventually give rise to the complex cellular organisation of the mature brain. In addition, the experiments reveal that the epistatic relationships among the genes comprising the MHO change over time. Thus, originally interdependent genes become independent of each other and new repressive or activating interactions are established with so far unknown factors.

MHO activity is maintained by preventing premature neuronal differentiation in the MHB

MHO gene activity clearly has to be maintained over a period of several days during embryonic development. This is achieved on the one hand through the regulatory networks among the MHO genes described above. On the other hand, as reported by Hirata et al. (2001), premature differentiation of the neuroectodermal precursor cells into neurons has to be prevented in the region of the MHB to sustain MHO activity. Two members of the basic helix-loop-helix (bHLH) family of transcription factors, viz.

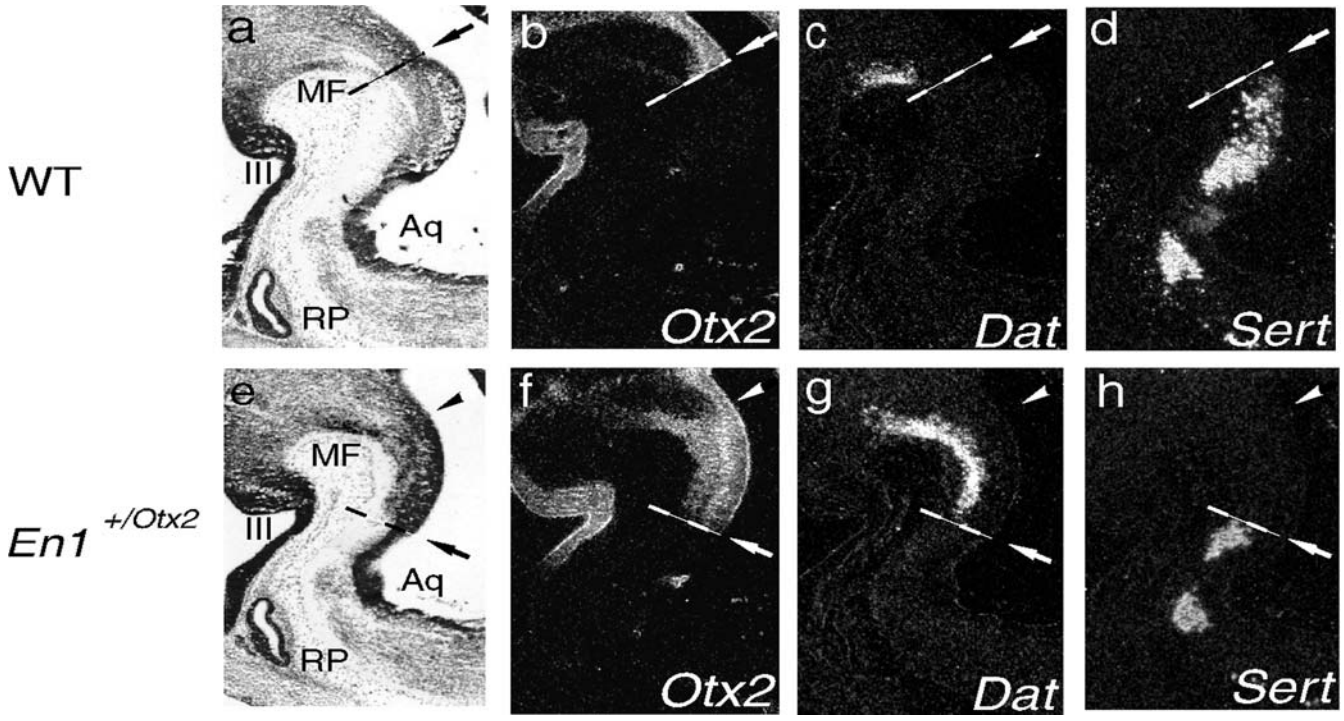

Fig. 3a–g A caudal shift of the MHO in $En1^{+/Otx2}$ mutant mice leads to an enlargement of the mid-DA neuronal population and a decrease of the rost-5HT cell group (*arrowheads* normal position of the MHO, *MF* mesencephalic flexure, *III* third ventricle, *Aq* aqueduct, *RP* Rathke's pouch). **a**, **e** Bright field images. **b**–**d**, **f**–**h** Dark field images of adjacent sagittal sections from E12.5 wild-type (*WT*, **a**–**d**) and $En1^{+/Otx2}$ embryos (**e**–**h**) after mRNA in situ hybridisation for *Otx2*, dopamine transporter (*Dat*) and serotonin-transporter (*Sert*). **b**, **f** The caudal *Otx2* expression border marks the position of the MHO (*dashed lines and arrows*). **c**, **d** In WT embryos, mid-DA cells identified by *Dat* expression are located in the mesencephalic flexure rostral to the MHO (**c**), whereas rost-5HT neurons (marked by *Sert* expression) are located caudal to the MHO (**d**). **f**–**h** Shifting the MHO caudally through ectopic expression of *Otx2* in the rostral hindbrain (**f**) leads to an enlargement of the mid-DA neuronal population to the same caudal extent (**g**), and to a complementary reduction of the rost-5HT cell group (**h**) in $En1^{+/Otx2}$ embryos. Reprinted after permission from Brodski et al. 2003

Hes1 and *Hes3*, are expressed in the midbrain/hindbrain region of the mouse embryo from early somite stages onwards (Allen and Lobe 1999; Hirata et al. 2001). *Hes3* expression is later restricted exclusively to the MHB (Hirata et al. 2001). *Hes* and related genes have been shown, in several species, to act as inhibitors of neuronal differentiation by maintaining neural precursor cells in a proliferative undifferentiated state. Indeed, lack of both *Hes1* and *Hes3* genes in mice results in severe patterning defects at the MHB: the midbrain and anterior hindbrain are missing (Hirata et al. 2001). As has been shown by Hirata et al. (2001), this phenotype is attributable to a premature termination of MHO gene activity, even at E10.5 in the double mutant embryos, and premature differentiation of the proliferating neural precursor cells at the MHB into neurons. $Hes1^{-/-}$ or $Hes3^{-/-}$ homozygous single mutants do not show these neural tube defects, indicating that both genes are functionally redundant. Although Hirata et al. (2001) have not experimentally addressed this issue, both *Hes* genes are probably integrated into the MHO regulatory network, acting downstream or at the same level of the MHO genes described above. Therefore, as long as the midbrain and anterior hindbrain compartments are specified and refined by the action of some of the MHO genes, the region in which these genes are expressed (namely the MHB) has to be kept in an undifferentiated proliferative state by the action of other MHO genes.

Concluding remarks

The specification and patterning of the midbrain territory within the neural tube during embryonic development is a highly complex and, so far, not completely understood process. Over the past few years, however, it has become clear that this is accomplished through the spatiotemporally coordinated interaction of signalling centres located along the A/P and D/V axes of the developing mouse embryo and of transcription factors expressed within the neuroectoderm. Signalling molecules and nuclear effectors together establish and control a complex genetic network that ultimately generates the cellular diversity and ordered structure of the mature brain.

Future research will focus on the events downstream of the patterning activities described in this review, activities that ultimately lead to the generation of all the various cell populations in the vertebrate midbrain and rostral hindbrain. Of special interest is the identification of genes and genetic networks that integrate the positional cues in the midbrain/rostral hindbrain territory and commit neural precursor cells to a specific cell fate. Given our current

insights from other regions of the vertebrate neural tube, these are probably other members of the homeobox and bHLH families of transcription factors, acting in concert with signal transduction cascades that have yet to be identified.

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