Location and Size of Dopaminergic and Serotonergic Cell Populations Are Controlled by the Position of the Midbrain–Hindbrain Organizer

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Midbrain dopaminergic and hindbrain serotonergic neurons play an important role in the modulation of behavior and are involved in a series of neuropsychiatric disorders. Despite the importance of these cells, little is known about the molecular mechanisms governing their development. During embryogenesis, midbrain dopaminergic neurons are specified rostral to the midbrain–hindbrain organizer (MHO), and hindbrain serotonergic neurons are specified caudal to it. We report that in transgenic mice in which Otx2 and accordingly the MHO are shifted caudally, the midbrain dopaminergic neuronal population expands to the ectopically positioned MHO and is enlarged. Complementary, the extension of the hindbrain serotonergic cell group is decreased. These changes are preserved in adulthood, and the additional, ectopic dopaminergic neurons project to the striatum, which is a proper dopaminergic target area. In addition, in mutants in which Otx2 and the MHO are shifted rostrally, dopaminergic and serotonergic neurons are relocated at the newly positioned MHO. However, in these mice, the size ratio between these two cell populations is changed in favor of the serotonergic cell population. To investigate whether the position of the MHO during embryogenesis is also of functional relevance for adult behavior, we tested mice with a caudally shifted MHO and report that these mutants show a higher locomotor activity. Together, we provide evidence that the position of the MHO determines the location and size of midbrain dopaminergic and hindbrain serotonergic cell populations in vivo. In addition, our data suggest that the position of the MHO during embryogenesis can modulate adult locomotor activity.

Key words: development; substantia nigra; ventral tegmental area; raphe nuclei; isthmic organizer; hyperactivity

Introduction

Midbrain dopaminergic (mid-DA) neurons of the substantia nigra (SN), ventral tegmental area (VTA), and retrorubral field (RRF) project to the striatum, cortex, and nucleus accumbens (Björklund and Lindvall, 1984). They modulate a variety of functions, such as movement, rewarding, cognition, and feeding (Zhou and Palmiter, 1995; Cooper et al., 2001). A dysfunction of these cells is responsible for Parkinson’s disease and is thought to be involved in schizophrenia and attention-deficit hyperactivity disorder (ADHD) (Waldman et al., 1998; Barr et al., 2001; Cooper et al., 2001). Hindbrain serotonergic (5-HT) neurons are grouped in a rostral cluster (rost-5-HT) projecting to the midbrain and forebrain and a caudal cluster innervating the spinal cord (Tork, 1990). The rost-5-HT cells are implicated in a wide range of processes, such as mood, vegetative homeostasis (Coo-per et al., 2001), and movement control (Jacobs and Fornal, 1997). A dysregulation of these neurons is supposed to be associated with affective diseases and attention-deficit hyperactivity disorder (Quist et al., 2000; Cooper et al., 2001).

The molecular mechanisms directing the development of mid-DA and rost-5-HT neurons in vivo are still poorly understood (Goridis and Rohrer, 2002). The genetic inactivation of the orphan nuclear receptor Nurr1, which is widely expressed in the brain, leads to failure in the maturation of mid-DA neurons (Zetterström et al., 1997). Other transcription factors, such as Ptx3, which is also known as Ptx3, and Lmx1b, are expressed almost exclusively in mid-DA neurons in the brain, but they do not seem to be required for the induction of neurotransmitter identity (Smidt et al., 1997, 2000). Experiments using an explant culture system have demonstrated that the secreted factors fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH) are required for the induction of mid-DA neurons rostral to the midbrain–hindbrain organizer (MHO) and rost-5-HT cells caudal to it (Ye et al., 1998). Although these studies identified several genes necessary for the development of mid-DA and rost-5-HT neurons, it is still unclear how the location and size of these cell populations are determined in vivo.
During embryogenesis, the boundary between the midbrain and hindbrain acts as an organizing center (MHO), directing the patterning of the adjacent territories (Martinez and Alvarado-Mallart, 1990; Martinez et al., 1991; Marin and Puelles, 1994; Wassef and Joyner, 1997; Wurst and Bally-Cuif, 2001). At the onset of somitogenesis, the transcription factor *Otx2* is expressed directly rostral to the MHO in the midbrain and forebrain, whereas the transcription factor *Gbx2* is expressed directly caudal to it in the hindbrain and spinal cord. Shifting the expression domain of either gene (Broccoli et al., 1999; Millet et al., 1999) or reducing the level of OTX proteins below a critical threshold (Acampora et al., 1997; Suda et al., 1997) leads to a caudal or rostral shift of the organizer, indicating that the expression border between *Otx2* and *Gbx2* defines the position of the MHO (Hidalgo-Sanchez et al., 1999; Katahira et al., 2000; Simeone, 2000; Garda et al., 2001; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Wurst and Bally-Cuif, 2001). Furthermore, a number of other genes expressed at the MHO, such as *Fgf8*, *En1*, *En2*, *Wnt1*, *Pax2*, and *Pax5*, are involved in the maintenance of this territory (McMahon et al., 1992; Millen et al., 1994; Wurst et al., 1994; Favor et al., 1996; Schwarz et al., 1997; Meyers et al., 1998).

Using two transgenic mouse models in which the MHO is shifted either caudally or rostrally, we provide evidence that the activity of embryogenesis is of functional relevance for adult locomotor activity.

**Materials and Methods**

**Transgenic mice.** The generation of *En1<sup>+/Otx2<sup>-</sup></sup> mutants has been described previously (Broccoli et al., 1999). They are bred in a mixed C57BL/6, CD-1, and 129/SV genetic background.

*Otx1<sup>+/−</sup>;Otx2<sup>+/−</sup>* mice were generated by crossing *Otx1*<sup>+/−</sup>;*Otx2*<sup>+/−</sup> males with brother-sister breedings in a C57BL/6, 129/SV, DBA2 genetic background.

**Gene expression studies.** Brains of adult and embryonic mice were either transcardially perfused or immersion fixed with 4% paraformaldehyde in 0.1 M PBS. Shifting the expression domains was performed using the method of unbiased stereology, specifically the Cavalieri estimator of volume (Gundersen and Jensen, 1987) using an interactive computer system (KS400; Zeiss, Hallbergmoos, Germany). Whole-mount *in situ* hybridization was performed according to Broccoli et al. (1999).

**Retrograde tracing.** Six *En1<sup>+/Otx2<sup>-</sup></sup>* mutants and six matched wild-type littermates were used for this study. After deep anesthesia using a mixture of ketamine/rompun, animals were placed into a stereotaxic frame. Stereotoxic coordinates for injections were taken from the atlas of Franklin and Paxinos (1997). After drilling a hole into the skull directly over the injection site, 2–3 × 0.5 μl of a 2% fluorogold (FG) solution in water was pressure injected into the striatum using a Hamilton syringe. After injection, the injection cannula was left in place for at least 5 min to prevent spilling of the tracer along the injection tract. Surgery was finished by closing the skull with bone wax and sewing the scalp. At all times, animals were handled according to the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research as well as the European Communities Council Directive.

After a survival time of 5 d, the animals were again deeply anesthetized and perfused transcardially. Perfusion was performed for 2 min with 0.1 M PBS at room temperature, immediately followed by ice-cold 4% paraformaldehyde in 0.1 M PBS. After perfusion, the brains were removed from the skull, postfixed for 2 hr in 4% paraformaldehyde, and cryoprotected in 20% sucrose in 0.1 M PBS overnight. The brain was cut alternating into 1× 50-μm-thick and 2× 30-μm-thick horizontal frozen sections using a cryostat (Microm HM560). The 50-μm-thick sections were processed for visualization of TH and dopamine transporter (DAT) by immunohistochemistry. The 30 μm thick sections were processed for tracer visualization and simultaneous detection of tracer and TH as well as DAT.

**Immunohistochemistry.** Free-floating 50-μm-thick sections and dissected neural tubes [embryonic day 10.5 (E10.5)] were rinsed in 0.1 M PBS, preincubated for 1–2 hr in 10% fetal calf serum in 0.1 M PBS. Thereafter, the sections and neural tubes were incubated in primary antibody overnight at 4°C and constantly agitated (rabbit-anti-TH, 1:10,000; Pol-Freeze Biologicals, Rogers, AZ; rat-anti-DAT, 1:2000; Chemicon, Temecula, CA; mouse anti-Ilet-1, 1:1000; Developmental Hybridoma Bank, Iowa City, IA). Sections and neural tubes were then washed five times for 10 min in 0.1 M PBS; incubated for 45 min in biotinylated anti-rabbit IgG (Vectastain, 1:300; Vector Laboratories, Burlingame, CA), biotinylated anti-rat IgG (Vectastain, 1:300), and biotinylated antimouse IgG (Vectastain, 1:300), respectively; washed five times for 10 min in 0.1 M PBS, and incubated for 45 min in ABC solution (Vectastain, 1:300). After another 10 min wash in 0.1 M PBS, sections were transferred to 0.1 M Tris-HCl, pH 7.2, and immunohistochemistry was completed within 10–20 min using diaminobenzidine as a chromogen for visualization of the peroxidase reaction. Sections and flattened neural tubes were mounted on slides and embedded in ProTaq MountFluor (Quartett Immunodiagnostics, Berlin, Germany). Thirty-micrometer-thick sections were preincubated for 1–2 hr in 10% FCS in 0.1 M PBS. Thereafter, the sections were incubated in the dark in primary antibody overnight at 4°C (rabbit-anti-TH, 1:1000; Pol-Freeze Biologicals; rat-anti-DAT, 1:500; Chemicon). Sections were then washed five times for 10 min in 0.1 M PBS, incubated for 1 hr in Texas Red anti-rabbit IgG (Vectastain, 1:800) and Texas Red anti-rat IgG (Vectastain, 1:800), respectively, washed five times for 10 min in 0.1 M PBS, and mounted in ProTaq.

**NADPH–diaphorase histochemistry.** Free-floating 50-μm-thick sections were postfixed for 20 min in 4% PFA and washed three times for 5 min in 0.1 M PBS. For staining, they were incubated at 37°C in staining solution (1.2 μM β-NADPH, 0.1 mg/ml nitroleuro tetrazolium, 0.3% Triton X-100, and 1 mM MgCl₂ dissolved in 0.1 M PBS). Finally, sections were washed three times for 10 min in 0.1 M PBS, mounted, dehydrated, and mounted in ProTaq Mount Fluor.

**Transmitter measurement.** Brains of mice were dissected and shock frozen on dry ice and cut on a cryostat in 200-μm-thick sections. Striatal tissue was recovered by punching the region of interest. DA and 5-HT content was measured by HPLC according to Sillaber et al. (1998) and referred to tissue weight to obtain transmitter concentrations.
Stereological analysis. Three animals of each group and stage were analyzed. All volume measurements and cell counts were performed with the help of an interactive computer system (KS400; Zeiss). The volume of the SN [including the volume of the ectopic neurons in the paratemal area (PL)] was calculated using the method of Cavalieri. To determine the numerical density of TH-positive neurons, immunopositive neurons in this region were recorded using systematic random sampling and optical dissector methods. The total number of TH-positive neurons was calculated by referring their numerical density to the volume of the substantia nigra.

Activity measurements. All behavioral tests were performed between 9:00 A.M. and 1:00 P.M. To study motor activity, naive En1+/Otx2 and wild-type littermates were placed in an open field (30 × 30 cm; illumination, 30 lux), and locomotor activity was monitored by video-tracking for a period of 60 min.

Results

MHO positioning and mid-DA and rost-5-HT cell populations

First, we analyzed En1+/Otx2 mutants, in which one allele of the En1 coding sequence was replaced by an Otx2 minigene, leading to a caudal shift of the Otx2 expression domain in rhombomere 1 (rh1) (Broccoli et al., 1999). This shift, which is more pronounced dorsally, results in an enlargement of the inferior colliculi and a loss of the anterior vermis in the dorsal part of the midbrain–hindbrain region. However, no gross morphological alterations are detected ventrally (Broccoli et al., 1999).

To investigate whether the ventrally located mid-DA and rost-5-HT transmitter populations are affected by the subtle ventro-caudal shift of the MHO, we studied the expression domains of specific markers on consecutive sections of mutants and wild-type littermates at E12.5 by in situ hybridization. At this stage, DA and 5-HT markers are already robustly expressed. DA neurons were identified by probes for Dat, and the mid-DA neuron-specific marker Ptx3 (Smidt et al., 1997). 5-HT cells were marked by serotonin transporter (Sert) and Pet1 (Hendricks et al., 1999; Pfaar et al., 2002) expression.

In the wild-type littermates, the caudal edge of Dat (Fig. 1C) and Th (data not shown) expression extended closely up to the caudal expression border of Otx2 (Fig. 1B,C), with only a few cell layers spared. Also, in En1+/Otx2 mutants, in which the MHO is shifted caudally, the posterior limit of the Th and Dat expression was close to the caudal Otx2 expression domain. This results in a posterior shift of the caudal edge of the Th and Dat expression area. Because the rostral edge of these expression domains remains unaltered, the dopaminergic cell population is enlarged (Fig. 1F,G). Rost-5-HT neurons are located immediately caudal to the Otx2 expression domain (Fig. 1B,D). Shifting the Otx2 expression caudally leads to a reduction in the rostral-5-HT cell population (Fig. 1H).

Quantification of the Dat and Sert expression domains in the midbrain–hindbrain region of wild-type and En1+/Otx2 littermates revealed that the volume of the wild-type Dat expression domain was 12 × 10^6 μm^3 [average, ±0.14 × 10^6 (SEM)], whereas it was 35.76 × 10^6 μm^3 [average, ±0.69 × 10^6 (SEM)] in the En1+/Otx2 mutants. This difference was highly significant (t test; p = 0.003). On average, the volume of the Dat expression domain increased 2.98-fold [23.76 × 10^6 μm^3; average, ±0.55 × 10^6 (SEM)]. The volume of the Sert expression domain in wild-type littermates was 50.76 × 10^6 μm^3 [average, ±1.11 × 10^6 (SEM)], whereas it was 29.37 × 10^6 μm^3 [average, ±0.76 × 10^6 (SEM)] in En1+/Otx2 mutants. Again, this difference was highly significant (t test; p = 0.003). On average, the volume of the Sert expression domain decreased 0.58-fold [21.39 × 10^6 μm^3; average, ±0.55 × 10^6 (SEM)]. These results indicate that the caudal shift of the MHO leads to an increase in the Dat expression domain that is equivalent to the decrease in the Sert expression domain.

To determine whether a rostral shift of the MHO would lead to a complementary change of the location and size of mid-DA and rost-5-HT cell populations, we investigated Otx1+/−; Otx2+/− mutants. In these mutants, which have no functional Otx1 gene and only one intact Otx2 allele, the OTX protein levels are reduced under a critical threshold. As a consequence, the MHO is shifted rostrally close to the zona limitans intrathalamica within the diencephalon (Acampora et al., 1997; Suda et al., 1997). Otx1+/−;Otx2+/− mutants were compared with Otx2+/− littermates, which are phenotypically wild type. The MHO was defined by the caudal border of Otx2 and by Fgf8 expression (Fig. 2B,C,E,F). We observed that cells expressing Th were shifted rostrally into the diencephalic tissue immediately rostral to the ectopically positioned MHO (Fig. 2G,I). In addition, Ptx3, which is marking specific mesencephalic DA neurons at the exclusion of other DA neurons (Smidt et al., 1997), was also relocated rostrally (Fig. 2H,K). Complementary expression of the serotonergic marker Sert expanded up to the presumptive diencephalon posterior to the caudal Otx2 expression border. Because the caudal position of the 5-HT neurons was not altered, a considerable enlargement of this expression domain was observed (Fig. 2I,L).
tants in which the MHO was shifted caudally, the mid-DA population is still expanded at E15.5 (Fig. 3A,C) and the rost-5-HT cell population remained significantly reduced (Fig. 3B,D). The decrease in the 5-HT cell population primarily concerned the dorsal raphe (DR) nuclei (Fig. 3D), whereas the median raphe (MR) (Fig. 3D) was less affected. In Otx1−/−;Otx2−/− mutants, in which the MHO is shifted rostrally, a population of Dat-expressing cells can still be found at an ectopic position in the ventral diencephalon at E15.5, and the rost-5-HT cell population is expanded up to the caudal limit of the diencephalon (Fig. 3E–H). This indicates that the changes in the two transmitter populations induced by an aberrantly positioned MHO are maintained during embryogenesis.

To investigate whether the alterations in the location and number of mid-DA and rost-5-HT neurons observed during embryogenesis are still present in adulthood, we analyzed their location and extension in adult En1+/Otx2−/− mice, which are, in contrast to Otx1−/−;Otx2−/− mutants, viable and fertile. Consistent with the results obtained at embryonic stages, Th and Dat expression were expanded posteriorly (Fig. 4A–F), whereas the Sert and Pet1 expression domains were decreased in size. This decrease primarily affects the dorsal raphe nuclei (Fig. 4G–L).

To analyze the increased dopaminergic neuronal population in more detail, immunohistochemistry for TH and DAT was performed. A caudal extension of the dopaminergic cell group of the RrF could be observed, as well as the appearance of an ectopically located dopaminergic neuronal population in the PL as a caudal extension of the SN (Fig. 5A–D). Both ectopic cell populations stained for TH and DAT, indicating their dopaminergic identity (Fig. 5B,D). For quantification, we specifically determined the volume of the TH-positive neuronal population and their numerical density in the SN and its caudal extension. This was done because the SN is involved in the regulation of motor activity, which is increased in the mutants (see below). The volume of the SN marked by TH-positive cells in the wild-type mice was 724.70 ± 106 μm³ [average, ±9.58 × 10⁶ (SEM)], whereas it was of 977.49 ± 106 μm³ [average, ±45.61 × 10⁶ (SEM)] in the mutant mice. The difference in the volumes was significant (t test; p = 0.0114). The total number of TH-positive cells in the wild-type mice was 3590 [average, ±236(SEM)], whereas it was 6424 [average, ±102 (SEM)] in the mutants. This increase in cell number was significant as well (t test; p = 0.0304).

In addition, to determine whether the ectopically located DA neurons in the caudal extension of the SN innervate a proper DA target area, retrograde tracing studies were performed. A total of 1–1.5 μl of FG was injected in vivo into the striatum to visualize efferent projections of the mesencephalic dopaminergic cell groups. The FG injection involved major portions of the striatum and resulted in the retrograde labeling of many but not all DA neurons in the ipsilateral substantia nigra, the VTA, and the retrorubral field. In wild-type mice, no retrogradely labeled cells could be observed in the PL (data not shown). In contrast, in mutant mice, a substantial proportion of the ectopically located dopaminergic cells in this area were positive for FG, indicating that they do project to the striatum (Fig. 5G–I).

To investigate whether the changes in the size of mid-DA and rost-5-HT neuronal populations would lead to a change in the concentrations of neurotransmitters in a target area, we measured DA and 5-HT in the striatum of adult wild-type and En1+/Otx2−/− mutants by HPLC. The DA concentration was significantly increased (Wilcoxon matched-pairs test; n = 6; p = 0.028) by 15.3% (± SE 7.8%), whereas the 5-HT concentration was decreased to 24.5% (± SE 4.6%). This difference was significant as well (Wilcoxon matched-pairs test; n = 6; p = 0.028).

In summary, these results provide evidence that the position...
midbrain–hindbrain neuronal populations, we analyzed nuclei adjacent to these populations, such as the cranial nerves III, IV, and V and the nucleus pedunculopontinus.

To determine whether the ectopic dopaminergic neurons in the adult are indeed located in the rostral hindbrain rather than in a transformed midbrain, we studied the identity of the surrounding tissue in the PL of En1+/−/Otx2−/− mutants. For this purpose, we examined the presence of the pedunculopontine nucleus, which is located in the PL. NADPH−diaphorase histochemistry revealed the preserved identity of this nucleus (Fig. 5D,F). Thus the ectopic DA neurons are located in the rostral hindbrain.

The nucleus occulomotorius (cranial nerve III) develops immediately rostral to the MHO adjacent to the mid-DA neurons, whereas the nuclei trochlearis and trigeminalis (cranial nerves IV and V) develop caudal to it at the same anteroposterior level as the rost-5-HT neurons. The analysis of Isl1-1 staining in En1+/−/Otx2−/− mutants at E10.5 shows that the nucleus occulomotorius is not expanded to the caudal Otx2 border and enlarged, as is the case for the dopaminergic neuronal population (Fig. 5j); compare Fig. 1IC,G). In addition, the nuclei trochlearis and trigeminalis are not altered, corresponding to the change in the rost-5-HT cell group (Fig. 5f; compare Fig. 1D,H). In Otx1+/−; Otx2+/− mutants at E12.5, the nuclei trochlearis and trigeminalis were relocated at the newly positioned MHO but did not change in size (data not shown).

Together, our results show that other cell populations in the ventral midbrain–hindbrain region are not affected similarly to the mid-DA and 5-HT neurons, suggesting that the position of the MHO has different effects on distinct cell populations.

MHO positioning and adult behavior

Mid-DA and rost-5-HT neurons modulate different aspects of adult behavior (Cooper et al., 2001), such as motor activity (Zhou and Palmiter, 1995; Jacobs and Fornal, 1997). Because the position of the MHO defines the size ratio between these populations, we hypothesized that an aberrant position of the MHO in En1+/−/Otx2−/− mutants would lead to a change in their motor activity. This would indicate that the position of the MHO during embryogenesis is of functional relevance for adult behavior.

Locomotor activity of En1+/−/Otx2−/− mutants and wild-type mice was studied in an open field by video-tracking. A highly significant enhancement of locomotion in En1+/−/Otx2−/− mice compared with their wild-type littermates (F(1,47) = 22.95; p < 0.00001) independent of gender (genotype × gender interaction: F(1,47) = 0.32; p = 0.57) was revealed by multivariate ANOVA (Fig. 6).

Atactic behavior of En1+/−/Otx2−/− mutants has been described pre-
viously (Broccoli et al., 1999) and was attributed to the cerebellar defect. Therefore, to investigate whether there is a correlation between the ataxia and the locomotor activity, we tested the animals of the open-field study by means of the rotarod. However, no correlation between ataxic behavior and locomotor activity was observed (data not shown).

Discussion
MHO positioning and development of mid-DA and rost-5-HT neuronal populations
The position of the MHO is determined during embryogenesis by the interaction of Otx2 and Gbx2 (Broccoli et al., 1999; Millet et al., 1999). Our results suggest that Otx2 is an important upstream signal for mid-DA specification and Gbx2 is important for rost-5-HT development. Although we cannot exclude the possibility that these two genes are directly involved in the development of these cell populations, it is more likely that signals generated at the MHO, such as Fgf8, are mediating these effects (Ye et al., 1998). In addition, Shh is necessary for the induction of mid-DA and rost-5-HT neurons in an explant culture system (Ye et al., 1998). However, both transmitter populations are equally dependent on Fgf8 and Shh, suggesting that there must be additional signals specifying DA versus 5-HT identity. In En1<sup>+/Otx2</sup> mutants, the shift of the MHO leading to a change in the location and extension of mid-DA and rost-5-HT neuronal populations takes place after the onset of somitogenesis (E8). This indicates that these additional signals are generated within the neural epithelium and are most likely emitted by the MHO after this time point. The results presented here also suggest that genes expressed at the MHO, such as En1, En2, Wnt1, Pax2, Pax5, Pax8, Fgf17, Fgf18, Lmx1b, and Ptx3 (McMahon et al., 1992; Millen et al., 1994; Wurst et al., 1994; Schwarz et al., 1997; Smidt et al., 1997, 2000), might be involved in the specification of DA versus 5-HT neurons. It has been shown that En1 and En2 are necessary for the maintenance but not for the specification of mid-DA neurons in a gene dose-dependent manner (Simon et al., 2001).

Together, the results suggest that Otx2 determines the position of a genetic cascade involving Fgf8, En1, En2, and other unidentified genes expressed at the MHO together with Shh to control the localization, number, and survival of mid-DA neurons. In contrast, Gbx2 positions together with Otx2, a molecular network including Fgf8, and yet undefined signals from the MHO that regulate together with Shh the development of rost-5-HT neurons.

An unexpected finding of this study was that the mid-DA and rost-5-HT cell populations were not only relocated in mutants in which the MHO was shifted, but also changed in size, depending on the position of the MHO (Fig. 7). This suggests that the MHO is not only an anatomical hallmark but could actively influence the function of these two neuronal populations by defining their size through its location.

Although the MHO is shifted caudally in the En1<sup>+/Otx2</sup> mice and the size ratio between mid-DA and rost-5-HT cell populations is altered, the rostral limit of the mid-DA and the caudal border of the rost-5-HT neurons remains unaltered. This suggests that there must be additional signals cooperating with those
generated by the MHO to define the most anterior location of the mid-DA neurons as well as the most posterior location of the rost-5-HT neurons.

In Otx1\(^{+/H11002}/H11002\);Otx2\(^{+/H11001}/H11002\) mutants, the MHO is shifted rostrally, in contrast to the En1\(^{+/H11001}/Otx2\) mice, and to a greater extent beyond the original anterior limit of the mid-DA cell population. The mid-DA cell group is relocated rostral to the newly positioned MHO at an ectopic position in the diencephalon, without changing in size, but still expressing the midbrain DA-specific marker Ptx3. This suggests that the MHO is not dependent on other signals to induce mid-DA neurons. Consistent with the observations in the En1\(^{+/H11001}/Otx2\) mutants, the caudal limit of the rost-5-HT cell population remains fixed. This indicates that here also, additional signals (in this case from the posterior hindbrain) determine the posterior extension of the rost-5-HT cell population.

An obvious difference between the mutants is that in Otx1\(^{+/H11002}/H11002\);Otx2\(^{+/H11001}/H11002\) mice, the newly positioned mid-DA cell population does not change in size, whereas in En1\(^{+/H11001}/Otx2\) mice, the rost-5-HT cell population is decreased. This suggests that the anterior part of the neuroepithelium is permissive for the induction of mid-DA neurons, whereas its posterior part is not entirely permissive for signals of the MHO inducing the development of rost-5-HT neurons.

Together, this suggests that the position of the MHO determines the size ratio between the mid-DA and rost-5-HT cell populations and is sufficient to induce at least the mid-DA neurons in the anterior neural epithelium. In addition, as yet unidentified signals emitted from the forebrain, possibly from the zona limi-

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Figure 5. Ventral midbrain–hindbrain structures and projections of ectopic dopaminergic neurons in En1\(^{+/H11002}\) mutants. A, B, TH immunohistochemistry on horizontal sections. Shown here is the left half of the ventral midbrain–hindbrain region of adult wild-type (WT) mice (A) and the right half of the ventral midbrain–hindbrain region of adult En1\(^{+/H11002}\) mice (B). Ectopic TH-positive neurons are located caudally to the Rf (arrowhead) and SN (arrows). The latter are located in the PL. C, D, DAT immunohistochemistry marking dopaminergic neurons. E, F, NADPH–diaphorase histochemistry identifying the pedunculopontine nucleus (PN) on horizontal sections. Shown here are the right halves of the ventral midbrain–hindbrain region of adult mice. D, F, Adjacent sections of DAT and NADPH–diaphorase (NADPH-D) staining demonstrating the presence of the pedunculopontine nucleus around the ectopic DA neurons and indicating the preserved hindbrain identity of the tissue surrounding the ectopic cells. G–I, Cells in the PL of En1\(^{+/H11002}\) mutants. G, TH-positive neurons in the PL. H, After an injection of FG into the striatum, retrograde labeled cells are found in the PL. I, Overlay image showing that most TH-positive cells are retrogradely labeled (arrows). However, some TH-positive neurons were not (arrowhead). J, Dorsally opened and flattened anterior neural tube of E10.5 embryos. Double labeling with Otx2 whole-mount in situ hybridization and Islet-1 immunohistochemistry, marking the cranial nerves III, IV, and V. The nucleus oculomotorius (III) is not expanded to the caudal Otx2 border and enlarged, as is the case for the dopaminergic neuronal population (compare Fig. 1C, G). In addition the nuclei trochlearis (IV) and trigeminalis (V) are not altered, corresponding to the change in the rost-5-HT cell population (compare Fig. 1D, H).
adequately repositioned. Alternatively, it suggests that different cell populations in the midbrain–hindbrain region depend on different genetic signals emitted by the MHO or are, however less likely, independent of it. Additional analysis will reveal which signals are implicated in the development of different neuronal populations.

**Influence of MHO positioning on adulthood**

The main focus of this study was to investigate the role of the position of the MHO for the embryonic development of mid-DA and rost-5-HT neurons. In contrast to *Otx1<sup>+/−</sup>;*Otx2<sup>+/−</sup> mutants, *En1<sup>+/Otx2</sup>* mice are viable and fertile. Therefore, they also offer the possibility of analyzing the influence of the location of the MHO on adult mid-DA and rost-5-HT neurons as well as on behavior.

Here we report that in *En1<sup>+/Otx2</sup>* mutants, the increase in the size of the mid-DA and the reduction in the rost-5-HT cell populations persist into adulthood. This suggests that the location of the MHO could be a way of modulating the function of these transmitter systems in adulthood by regulating their location and size during development. If this hypothesis is true, the additional neurons should innervate a proper DA target area and the mutants should show an altered behavior. Indeed, we found that additional DA neurons project to the striatum, and that mutants with a caudally shifted MHO are hyperactive.

To investigate whether *En1<sup>+/Otx2</sup>* mutants show a change in behavior, we decided to study the motor activity of these mice as it is influenced by the DA and 5-HT system (Zhou and Palmiter, 1995; Jacobs and Fornal, 1997). The observed hyperactivity is in accordance with previous studies. Pharmacological experiments have demonstrated that dopamine receptor stimulation is correlated with motor activity (Clark and White, 1987). These findings were supported by genetic experiments (Zhou and Palmiter, 1995; Giros et al., 1996; Gainetdinov et al., 1999). The inactivation of the dopamine transporter gene (*Dat*) leads to an increased synaptic dopamine concentration causing hyperactivity (Giros et al., 1996; Gainetdinov et al., 1999) and is regarded as a model for ADHD (Gainetdinov et al., 1999). The hyperlocomotion of these mutants can be antagonized by serotonergic activity, indicating that an imbalance between these two transmitter systems is responsible for this phenotype (Gainetdinov et al., 1999). *En1<sup>+/Otx2</sup>* mutants have other alterations in addition to changed mid-DA and rost-5-HT neuronal populations, such as an enlarged inferior colliculi and a loss of the vermis (Broccoli et al., 1999). In humans, hypoplasia of the posterior vermis has been implicated in ADHD (Castellanos and Tannock, 2002), which makes it conceivable that the missing vermis in the *En1<sup>+/Otx2</sup>* mice may account for the hyperactivity. However, the performed rota-rod test suggests that there is no correlation between atactic behavior, which would rather be attributed to a cerebellar defect, and the observed hyperactivity. Therefore, it seems unlikely that the cerebellar defect in the *En1<sup>+/Otx2</sup>* mice is associated with their hyperactivity. Although we cannot exclude the possibility that other changes in these mutants contribute to the altered locomotor activity, it seems most likely that this altered behavior is caused by increased DA and decreased 5-HT activity.

Together, we provide evidence that the position of the MHO, which is determined during embryogenesis, regulates the location and size of mid-DA and rost-5-HT neuronal populations. In addition, we suggest that it is of functional relevance for adult behavior.
References


