Engrailed 1 shapes the dopaminergic and serotonergic landscape through proper isthmic organizer maintenance and function

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Engrailed 1 shapes the dopaminergic and serotonergic landscape through proper isthmic organizer maintenance and function

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ABSTRACT

The isthmic organizer (IsO) is a signaling center that specifies the correct and distinct embryonic development of the dopaminergic midbrain and serotonergic hindbrain. The IsO is a linear boundary between the two brain regions, emerging at around embryonic day 7-8 of murine embryonic development, that shapes its surroundings through the expression of instructive signals such as Wnt and growth factors. Homeobox transcription factor engrailed 1 (En1) is present in midbrain and rostral hindbrain (i.e. rhombomere 1, R1). Its expression spans the IsO, and it is known to be an important survival factor for both dopaminergic and serotonergic neurons. Erroneous composition of dopaminergic neurons in the midbrain or serotonergic neurons in the hindbrain is associated with severe pathologies such as Parkinson’s disease, depression or autism. Here we investigated the role of En1 in early mid-hindbrain development, using multiple En1-ablated mouse models as well as lineage-tracing techniques, and observed the appearance of ectopic dopaminergic neurons, indistinguishable from midbrain dopaminergic neurons based on molecular profile and intrinsic electrophysiological properties. We propose that this change is the direct result of a caudal relocation of the IsO as represented by ectopic presence of Fgf8, Otx2, Wnt1 and canonical Wnt-signalling. Our work suggests a newly-discovered role for En1: the repression of Otx2, Wnt1 and canonical Wnt-signalling in R1. Overall, our results suggest that En1 is essential for proper IsO maintenance and function.

KEY WORDS: mdDA, Engrailed 1, IsO, Mouse, Patterning

INTRODUCTION

The correct and distinct embryonic development of the midbrain and hindbrain is specified by a signaling center between these brain areas: the isthmic organizer (IsO). The mid- and hindbrain region harbor two essential neurotransmitter systems: mesodiencephalic dopaminergic (mdDA) neurons, which express a plethora of dopaminergic markers such as Th, Pitx3, Nurr1, Otx2, En1, En2, Dat, Lmx1a/b, Vmat2, Aadc, Pbx1, Pbx3, (Smidt and Burbach, 2007), and serotonergic (5-HT) neurons which express different markers such as Sert, Pet1 and Gata3 (Deneris and Wyler, 2012; Smidt and van Hooft, 2013). The correct function of the IsO is essential in determining the location and respective size of these neuronal systems.

The IsO is formed at around embryonic day (E)7-8 of murine embryonic development, at the exact position where the expression of mesencephalic Otx2 and metencephalic Gbx2 meet (Martinez-Barbera et al., 2001; Millet et al., 1999; Wassarman et al., 1997). Through the expression of fibroblast growth factor 8 (Fgf8) (Crossley and Martin, 1995) and secreted glycoprotein Wnt1 (Wilkinson et al., 1987), the IsO provides the surrounding areas with instructive signals that lead to the induction of mdDA neurons located in midbrain and 5HT neurons in the hindbrain (Brodski et al., 2003; Ye et al., 1998). Disruption of the Otx2/ Gbx2 boundary results in a positional shift of the IsO, and this is accompanied by an enlarged or reduced mid- or hindbrain (Broccoli et al., 1999; Martinez et al., 1999; Millet et al., 1999; Wassarman et al., 1997).

The homeobox transcription factor engrailed 1 (En1) is involved in development and maintenance of the monoaminergic structures in the mid- and hindbrain (Deneris and Wyler, 2012; Fox and Deneris, 2012; Jensen et al., 2008; Lundell et al., 1996; Simon et al., 2005; Smidt and Burbach, 2007; Smidt and van Hooft, 2013; Wylie et al., 2010), and has been implicated to contribute to the maintenance of the IsO in zebrafish (Scholpp et al., 2003). Previous studies on the developmental role of En1 in the murine mid- and hindbrain region has long been hindered due to the assumption that absence of the En1 alleles results in the complete ablation of the cerebellum with perinatal lethality (Wurst et al., 1994). However, our group and others recently showed that this cerebellar ablation and perinatal lethality is caused by the genetic background of the mouse strain. This phenotype can be circumvented by back crossing the original 129/Sv line to a C57BL/6j background (Bilovocky et al., 2003; Veenvliet et al., 2013). Using this approach we showed that in the absence of En1, the expression of mdDA markers is diminished during embryogenesis. Paradoxically, ectopic mdDA markers can be detected in the metencephalon (Veenvliet et al., 2013). Furthermore, in a genome wide expression analysis of En1KO animals it was shown that besides the dopaminergic markers also several markers that are associated with the serotonergic system were down regulated, such as Gata3, Penk1 (Veenvliet et al., 2013). The ectopic expression of dopaminergic markers in the metencephalon and the deregulated serotonergic markers in the genome wide expression analysis suggest that En1 influences the cytoarchitecture of midbrain and the rostral hindbrain. In the present study we show that the dopaminergic neurons in the metencephalon are molecularly indistinguishable from correct positioned midbrain dopaminergic neurons. All critical dopaminergic markers that are present in the mdDA neurons of En1-ablated animals (Pitz3, Nurr1, Lmx1b, En2, Otx2, Th, Dat, Vmat2, Aadc, Pbx1 and Pbx3) are also present in the ectopic Th-expressing cells. Furthermore, electrophysiological recordings indicate that the ectopic Th-expressing cells fully resemble control mdDA neurons, both at prenatal and postnatal stages. In line with this observation, we show...
that the expansion of the DA neuronal field is accompanied by a diminished amount of 5-HT neurons in rhombomere 1 (R1). We propose that the appearance of ectopic dopaminergic (eDA) neurons is the direct result of an extension of midbrain patterning in R1, due to a caudal relocation of the IsO as represented by ectopic presence of Fgf8, Otx2, Wnt1 and canonical Wnt-signaling. In conclusion, En1 is essential for proper IsO maintenance and function.

RESULTS

Ectopic dopaminergic neurons arise in rostral hindbrain in absence of En1

To further substantiate our initial observations at E14.5 that in absence of En1 cells emerge in the metencephalon that possess a dopaminergic profile (expressing Pitx3, Nurr1, Th, Dat, Vmat2, Aadc; Fig. S1) (Veenvliet et al., 2013) we mapped the appearance of the ectopic DA neurons in time (Fig. 1A). Similarly we investigated the presence of essential transcription factors, expressed during early DA development (before E11) such as Lmx1b and En2 (Smidt and Burbach, 2007; Smidt et al., 2000a), and transcription factors Pbx1 and Pbx3, that are involved in DA subset specification (Veenvliet et al., 2013).

As early as E12.5 Th expression can be detected in an ectopic location caudal to the midbrain (Fig. 1A, arrowheads), indicating that at the first sign of dopaminergic development the ectopic DA neurons arise and these neurons can still be detected later in development at E16.5.

In En1KO animals, Lmx1b expression is enhanced in the ventral hindbrain area, which is especially apparent in more medial positions (Fig. 1B, middle panel, arrowheads). Furthermore, the En2 expression is extended into the hindbrain (Fig. 1B, right panel, arrowheads). Moreover, subset markers Pbx1 and Pbx3 are both ectopically expressed in the (ventral) hindbrain. Notably, the expression pattern of Pbx1 matches the ectopic expression of Th at E14.5 (Fig. 1C, arrowheads).

To assess whether these initial developmental defects are lasting towards adulthood we analyzed sagittal sections of neonatal and adult En1KO brains. Ectopic DA (eDA) neurons, identified by the expression of Th, are still found in (para)medial sections of the neonatal and adult En1KO brain at postnatal day (P)0 and P42 (Fig. 1D, E, arrowhead). Note however, that at P42 the density Th-positive neurons was somewhat diminished in SN and VTA as a consequence of the En1-ablation (Veenvliet et al., 2013). This similar

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Fig. 1. Ectopic dopaminergic neurons are found in the hindbrain of the En1KO as early as E12.5 and remain present into adulthood. (A) Th expression is found in the rostral hindbrain of En1 knockout embryos (En1KO) at prenatal stages embryonic day (E)12.5, E14.5 and E16.5 (arrowheads), as compared to wild-type embryos (En1WT). (B, C) Earlier dopaminergic markers, Lmx1b and En2 (B), and transcription factors that are involved in DA subset specification like Pbx1 and Pbx3 (C) are ectopically present in absence of En1 at E14.5 (arrowheads). (D, E) Sagittal sections of neonate and adult En1KO midbrain and hindbrain at postnatal day (P)0 and P42, reveal Th transcript is present in hindbrain (arrowheads). Both the size of the midbrain and ectopic Th-population seems to progressively diminish, as a consequence of the En1-ablation (Veenvliet et al., 2013). At 1 year it is difficult to differentiate between mdDA and eDA neurons. (Para)medial sections as shown in schematic: M, midbrain; R1, rhombomere 1; dotted line represents the position of the isthmus. Scale bars: 100 μm.
loss of cell density was observed for the Th-expressing cells in the
metencephalon (compare prenatal stages with P0 and P42, Fig. 1). At
1 year after birth only a few Th-positive cells are still present in the
En1KO, probably due to earlier described progressive degeneration in
the absence of En1 (Veenvliet et al., 2013). Consequently, while it is
no longer possible to clearly determine the exact location of the small
amount of remaining Th neurons, it suggests that the eDA neurons are
also characterized by a progressive cell loss possibly similar to mdDA
neurons in the absence of En1. Taken together, the analysis from
embryonic, neonatal and adult En1KO material indicates that eDA
neurons in En1KO mice are molecularly similar to mdDA neurons in
these mice.

Ectopic DA neurons are indistinguishable from mdDA
neurons based on their intrinsic electrophysiological
properties
To further proof that the observed eDA neurons are (functionally)
similar to mdDA neurons we investigated their electrophysiological
profile at different developmental stages. In order to specifically
visualize mdDA neurons in living slices we elected to use
Pitx3GFP/+ mice, in which GFP is uniquely expressed under the
control of Pitx3 (Maxwell et al., 2005). Since we showed that Pitx3 is
present in eDA neurons (Fig. S1), we inter crossed En1 mutant
animals with Pitx3GFP/+ animals, thus introducing GFP expression in mdDA
neurons and eDA neurons (Fig. 2A-C, Fig. S2).

Recordings from (a) midbrain Pitx3GFP/+ neurons, (b) En1KO;
Pitx3GFP/+ midbrain neurons and (c) eDA neurons in En1KO;
GFP/+ animals at E16.5 revealed a high input resistance and a
depolarized resting membrane potential within all three conditions,
and a general absence of spontaneous action potential firing
(Fig. 2D, Table 1). This is indicative of the immature state of these
neurons at E16.5, and based of the passive electrophysiological
properties, this state was not different between the three groups
(P>0.05 between the three conditions, for all properties, Table 1). At
P7 we were able to compare both passive and active properties
between the three groups. Though no difference were found between
the three conditions (P>0.05 between the three conditions, for all
properties, Table 1), all recorded neurons were capable of firing
action potentials. Furthermore, dopaminergic neurons in slice
preparation can be identified by characteristic hallmarks, such as
the presence of the h-currents (Chu and Zhen, 2010; Grace and
Onn, 1989). This current is reflected by the appearance of a
hyperpolarizing ‘sag’ in the patch clamp recordings, as is the case at
P7 in all three conditions (arrowheads, Fig. 2E). In addition, the
majority of neurons (85%) displayed spontaneous action potential
firing (Table 1). Thus, based on all included electrophysiological,
active and passive properties (Table 1), eDA neurons are not
dissimilar from control and En1KO mdDA neurons at E16.5 and P7.

The balance between dopaminergic and serotonergic
neuron has shifted in the absence of En1
It has previously been suggested that En1-derived cells make up
the entire midbrain and R1 area (as visualized by En1Cre;Wntl-
ΔMHB/+;R26dT+ model in (Yang et al., 2013). In order to
define the region that is under the control of En1 we lineage traced
En1 using En1Cre/+;R26RYFP/R26RYFP (En1CreWT;YFP) and
En1CreCre;R26RYFP/YFP (En1CreKO;YFP) animals. In double
labeling experiments at E12.5 we show that the region under the
control of En1 extends to the ventral diencephalon and caudally to
the presumed R1/R2 limit (Fig. 3A,C, indicated by the dashed
line). Moreover, confirming our previous data, eDA neurons were
generated in absence of En1 (En1CreKO;YFP) and are clearly
confined to the region in which En1’s influence is apparent (i.e.

Fig. 2. eDA neurons are electrophysiologically identical to control
mdDA neurons. (A) Bright field image of a
medial plane of a hemitube at E16.5,
including a demarcation of mdDA area by a
green dotted line. P1, prosomere 1; M,
midbrain; R1, rhombomere 1; MF,
mesencephalic flexure. White arrowhead
shows the location of the retroflexus. The
white dotted line marks the outline of the
mesencephalic tissue. (B,C) Visualization of
mdDA area by endogenous Pitx3GFP
signal in control (B) and En1KO;Pitx3GFP/+ (C)
animals, including eDA neurons (green
arrowheads) in hemitubes at E16.5. Schematic
electrodes in A-C indicate locations of recordings. (D,E) Traces at
E16.5 (D) and P7 (E) exemplify the absence of
dissimilarities between control mdDA
neurons, En1KO;GFP/+ mdDA neurons and
En1KO;GFP/+ eDA neurons. The presence of
the hyperpolarizing sag reveals the
dopaminergic characteristic of the h-current
in all three conditions at P7 (arrowhead).
The observed changed organization in the mid-hindbrain region suggests that the IsO may be defective as a consequence of En1 ablation, in addition to possible direct transcriptional defects in programming of dopaminergic and serotonergic neurons. During murine brain development the IsO (as defined by Fgfl8 expression) is set at the juxtaposition of Otx2/Gbx2 expression at the rostral and caudal edge respectively (Crossley and Martin, 1995; Millet et al., 1999). This border is established at ~E8 and continues to shape its surroundings until E12.5. In a conditionally En1-lineage tracing experiment using En1<sup>Cre-ERT<sup>−/−;R26R<sup>F/F</sup></sup> animals we investigated the distribution of YFP-positive cells, when induced with Tamoxifen at E10.5. In the presence of En1 the Cre-induced YFP expression was restricted to the En1 positive cells at the IsO, forming a well-organized YFP-positive triangular area (Sgaier et al., 2005) surrounding the IsO at E14.5 (Fig. S3A,B). In contrast, the YFP-positive cells within the En1<sup>Cre-E<sup>ERT</sup>/++;R26R<sup>F/F</sup></sup> animals were sparsely found and not restricted to one location (Fig. S3C’). This is indicative of critical changes in the cytoarchitectural organization of the border between the midbrain and R1 in the absence of En1.

In order to understand which specific changes occur at the IsO in absence of En1, we investigated the expression of the pivotal isthmic determinants: Otx2, Gbx2, Wnt1 and Fgfl8. At E12.5 Otx2 expression is normally restricted to the ventral midbrain (and more anterior brain regions) terminating at the IsO (Fig. 5A,B). Importantly in En1KO animals this expression is extended, far into the ventral hindbrain in a mosaic manner (Fig. 5C,D, arrowheads). The expression of Wnt1 is equally extended into the ventral hindbrain compared to controls (Fig. 5E-H, arrowheads). Interestingly, the extended pattern of Otx2 and Wnt1 expression is present in multiple stripes and this striate expression is overlapping (as suggested by the pseudo-overlay of adjacent sections in Fig. 5ii). In order to confirm whether the changes in Otx2 expression in En1 mutants were restricted to the En1-derived ventral R1-area, we analyzed Otx2 expression in En1<sup>Cre-WT</sup>;YFP and En1<sup>Cre-KO</sup>;YFP reporter animals. As expected, the ectopic expression of Otx2 (and Th as a reference) at E12.5 is confined to the En1-expression domain (Fig. 5I,J,M,N). As described above, while in control sections Otx2 expression is restricted at the IsO, the posterior limit in En1<sup>Cre-KO</sup>;YFP sections is shifted more caudally and now overlaps with the caudal border of the En1 expression domain (Fig. 5K,L, arrowheads).

### Table 1. Overview of recorded electrophysiological parameters at E16.5 and P7

<table>
<thead>
<tr>
<th></th>
<th>I&lt;sub&gt;Na&lt;/sub&gt; (nA)</th>
<th>I&lt;sub&gt;K&lt;/sub&gt; (nA)</th>
<th>V&lt;sub&gt;rest&lt;/sub&gt; (mV)</th>
<th>sag (mV)</th>
<th>R&lt;sub&gt;m&lt;/sub&gt; (GΩ)</th>
<th>τ&lt;sub&gt;m&lt;/sub&gt; (ms)</th>
<th>C&lt;sub&gt;m&lt;/sub&gt; (pF)</th>
<th>AP amplitude (mV)</th>
<th>AP half width (ms)</th>
<th>AP threshold (mV)</th>
<th>AHP amplitude (mV)</th>
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<tr>
<td><strong>E16.5</strong></td>
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<tr>
<td>WT mdDA</td>
<td>0.30±0.08</td>
<td>1.0±0.15</td>
<td>−28.3±4.8</td>
<td>n.d.</td>
<td>2.76±0.32</td>
<td>53.0±4.9</td>
<td>10.1±1.0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>KO mdDA</td>
<td>0.45±0.10</td>
<td>0.88±0.11</td>
<td>−30.9±2.3</td>
<td>n.d.</td>
<td>2.63±0.67</td>
<td>47.8±11.6</td>
<td>8.8±1.07</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>KO eDA</td>
<td>0.37±0.05</td>
<td>0.64±0.11</td>
<td>−31.1±2.1</td>
<td>n.d.</td>
<td>2.51±0.51</td>
<td>45.0±4.8</td>
<td>10.5±1.5</td>
<td>n.d.</td>
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<td><strong>P7</strong></td>
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<tr>
<td>WT mdDA</td>
<td>3.74±0.67</td>
<td>4.62±0.65</td>
<td>−49.6±2.7</td>
<td>24.4±3.3</td>
<td>0.56±0.10</td>
<td>75.9±12.9</td>
<td>72.2±13.4</td>
<td>41.0±4.6</td>
<td>5.50±0.80</td>
<td>−31.2±1.9</td>
<td>18.6±1.5</td>
<td>1.29±0.48</td>
</tr>
<tr>
<td>KO mdDA</td>
<td>3.70±0.59</td>
<td>3.85±0.88</td>
<td>−46.2±2.0</td>
<td>34.6±4.0</td>
<td>0.73±0.13</td>
<td>71.4±7.4</td>
<td>56.8±5.7</td>
<td>49.5±3.8</td>
<td>5.05±0.55</td>
<td>−30.1±1.5</td>
<td>18.9±1.7</td>
<td>1.18±0.40</td>
</tr>
<tr>
<td>KO eDA</td>
<td>3.92±0.51</td>
<td>5.22±1.07</td>
<td>−51.1±2.3</td>
<td>27.1±4.7</td>
<td>0.55±0.10</td>
<td>78.5±8.8</td>
<td>81.6±11.6</td>
<td>52.7±2.9</td>
<td>4.59±0.90</td>
<td>−30.8±2.0</td>
<td>14.9±3.5</td>
<td>1.55±0.54</td>
</tr>
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</table>

I<sub>Na</sub>: peak Na<sup>+</sup> current; I<sub>K</sub>: peak K<sup>+</sup> current; V<sub>rest</sub>: resting membrane potential; R<sub>m</sub>: input resistance; τ<sub>m</sub>: membrane time constant; C<sub>m</sub>: capacitance; AP: action potential; AHP: after hyperpolarization

**Th**-expressing neurons are positioned in the YFP-positive area; Fig. 3B).

### Absence of En1 leads to a disorganized IsO

The observed changed organization in the mid-hindbrain region suggests that the IsO may be defective as a consequence of En1 ablation, in addition to possible direct transcriptional defects in...
section whereas two separate bands of Fgf8 expression (of which the latter was positioned more caudally) were observed in the mutant (Fig. 5T,U). Adjacent sections analyzed for Wnt1 expression reveal that the appearance of multiple bands of Fgf8 expression are accompanied by a mosaic expression pattern of Wnt1 (Fig. 5V-Y). Markedly, the pseudo-overlay of Fgf8 and Wnt1 reveals a mutual exclusive expression pattern (Fig. 5vi). Since Fgf8 is considered to be present in the hindbrain-derived part of the IsO, whilst Wnt1 is only present in midbrain-derived tissue (Martinez et al., 1999), their mutual exclusive stripped pattern suggests that the IsO is fragmented in the En1-ablated mice. Notably, we investigated the expression of Gbx2 to determine if the observed distorted IsO was influenced by aberrant expression of Gbx2. However, similar to control, the Gbx2 expression was absent at E12.5 in En1KO animals, while adjacent sections with Otx2 expression reveal a clear posterior shift (Fig. S4).

Since we observed over-expression of Wnt1 around the IsO in the En1KO (Fig. 5G,H,X,Y) we examined if the absence of En1 also resulted in changes in (canonical) Wnt-signaling. To do so, we generated En1WT;BatGal+ and En1KO;BatGal+ animals which serve as reporters for possible changed canonical Wnt signaling (Maretto et al., 2003; Mesman et al., 2014). In absence of En1 we find a strong upregulation of β-galactosidase activity, indicative for an upregulation of canonical Wnt signaling (Fig. 5BB,CC; for a complete data overview, including Wnt1 and Th expression as reference see Fig. S5). Thus, in contrast to control animals (Fig. 5Z,AA), in the absence of En1, both Wnt1 expression (Fig. 5DD-GG) and canonical Wnt-signaling are markedly upregulated at E14.5. To conclude, our data indicate that En1 is essential for the (direct or indirect) repression of Wnt1 and Otx2 in R1, and establishment or maintenance of appropriate Fgf8 expression, which together may form the molecular basis of a disturbed IsO in the absence of En1.

**DISCUSSION**

**Engrailed 1 is essential for proper IsO development and function**

During normal murine brain development the IsO (as defined by Fgf8 expression) is set at the juxtaposition of Otx2/Gbx2 expression at ~E7-8 (Crossley and Martin, 1995; Martinez et al., 1999; Millet et al., 1999). The requirements for proper IsO development change dynamically over time; the classic removal of Gbx2 results in an expanded expression of Otx2 and a caudal relocation of the IsO.
(Wassarman et al., 1997), whereas the IsO region develops normally, when Gbx2 is conditionally removed after \( \sim E9 \) (Li et al., 2002). Furthermore, at E12.5 the linear expression of Fgf8 marks the caudal limit of Otx2 expression as a boundary between midbrain and R1, although Gbx2 is no longer present in R1 (Fig. S4). Consequently, it has been suggested that a Gbx2-independent route is required to repress Otx2 in the metencephalon after \( \sim E9 \) (Li et al., 2002).

In the full En1KO we observed a (mosaic) expansion of Otx2 expression at E12.5, which resulted in a fragmentation of the IsO as is marked by ectopic Fgf8 expression (Fig. 5). Strikingly, the altered expression of Otx2 in the En1KO was mimicked by the identical expression of Wnt1 (Fig. 5ii), whilst fragmented Fgf8 expression revealed to be mutually exclusive to the Wnt1 expression (Fig. 5vi). These data are in line with previous research on overexpression of Otx2 and Wnt1 in midbrain and R1, although Gbx2 is no longer present in R1 (Li et al., 2002).

In support of our current work that reveals that En1 is essential for proper IsO maintenance and function, other groups have shown that En1 fulfills a similar role in limb development. En1KO (Fig. 4), R1-derived serotonergic system severely changed in absence of En1. (A-R) At E14.5 all serotonergic markers investigated showed a severely changed expression pattern in absence of En1. Pet1 (A-F), Sert (G-L) and Gata3 (M-R) lost expression in DRN in medial section (asterisks). In paramedial sections the cytoarchitecture is changed (arrowheads) (B′; H′, N′, E′, K′, Q′). (S-Z) At P42 Sert expression is strongly diminished (asterisks) and the Sert expressing neurons that were still present, are differently organized in En1KO brains compared to control (arrowheads). (Para)medial sections as shown in schematic, area shown by red box in schematic. Scale bars: 100 \( \mu m \).

To recapitulate, these studies and our data suggest that the changed dynamics of the IsO in absence of En1 are primarily due to misexpression of Otx2 and Wnt1, resulting in the appearance of eDA neurons at the expense of 5HT neurons as a secondary consequence. In this sense, En1 might fulfill a role that is very similar to that of Gbx2, i.e. the repression of Otx2. Evidently, Gbx2 represses Otx2 earlier in development, however after \( \sim E9 \) Gbx2 is no longer required (Li et al., 2002), which just coincides with the time point of the expression of En1 in the region. This notion is supported by the observation that the phenotype of the En1KO highly resembles the Gbx2KO (Wassarman et al., 1997). We propose that En1 is essential for continued repression of Otx2 thereby enabling a 5-HT phenotype in R1.

**A general role for En1 in boundary formation?**

In support of our current work that reveals that En1 is essential for proper IsO maintenance and function, other groups have shown that En1 fulfills a similar role in limb development. En1KO
animals display limb malformations such as polydactyly and a double-dorsal paw (i.e. the ventral paw becomes hairy and pigmented), independent of the genetic background of the mutant line (Adamska et al., 2004; Bilovocky et al., 2003; Wurst et al., 1994). This phenotype is explained by an ectopic or secondary apical ectodermal ridge (AER), a signaling center similar to the IsO. The AER forms an Fgf8-expressing border between ventral ectodermal tissue and dorsal ectodermal tissue during limb formation (Moon and Capecchi, 2000). These changes in AER development are accompanied by the ectopic expression of Wnt7a and Wnt-signaling (Adamska et al., 2004; Cygan et al., 1997; Loomis et al., 1998). To recapitulate, in En1-ablated animals both the AER and the IsO are characterized by ectopic expression of Fgf8, Wnts and canonical Wnt-signaling, resulting in the expansion of dorsal ectoderm and midbrain, which ultimately leads to polydactyly as well as dopaminergic neurons in the metencephalon.

How does En1 support a dopaminergic cell fate in midbrain but suppress it in R1?

En1 is present in midbrain and R1, its expression spans the IsO. This introduces the question via which mechanism En1 induces dopaminergic neurons rostrally and serotonergic neurons caudally of the IsO? In drosophila, En exerts a repressive effect on its targets in the absence of Exd, whilst in the presence of Exd, En activates its targets (Serrano and Maschat, 1998). The murine orthologue of Exd is Pbx1, and if its relationship to En1 in mouse is similar to the relationship between Exd and En in drosophila, the differential presence of Pbx1 in midbrain (but not R1) might play a decisive role in determining the activator role of En1 on its targets in midbrain (but not R1). Research on the Pbx1KO reveal that mdDA neurons develop a normal molecular profile (though neurons display disrupted axon guidance) (Sgadò et al., 2006). However, the possibly compensatory presence of Pbx3, might cloak the true regulatory role of Pbx1 on En1 function. Secondly, in an impressive double publication Kurokawa and colleagues show a repressive regulator mechanism controlling Otx2 expression (Inoue et al., 2012; Kurokawa et al., 2004a,b). Otx2 contains an enhancer region 115 kb 3’ downstream (the X29 sequence) which induces Otx2 expression activities in midbrain from E8.5 onwards. The authors propose that the TAATTA sequence within X29 is recognized by Brn1/2/4 and Oct6 in midbrain leading to activation of Otx2, whereas Gbx2 competes for binding to the same sequence in hindbrain and successfully represses Otx2. Interestingly, En1 is similarly known to recognize TAATTA (Draganescu and Tullius, 1998), and thus the repressive
Concluding remarks
In the current manuscript we investigated the role of En1 in the viable En1-null mouse (the \( En1^{tm1Alj/+} \) animals back-crossed to the C57BL/6J line; Bilovocky et al., 2003; Veenvliet et al., 2013) in order to link developmental mechanisms of mid-hindbrain formation to the adult landscape of dopaminergic and serotonergic neurons. Our work suggests that En1 controls the repression of Otx2, Wnt1 and canonical Wnt-signaling in ventral R1. Ablation of En1 changes the patterning around the IS0 and induces properly coded and functional eDA neurons at the expense of serotonin neurons. This suggests that En1 is pivotal to IS0 maintenance and function.

MATERIALS AND METHODS

Animals
Embryos from several mouse lines were isolated at embryonic day (E)12.5, E14.5, and E16.5, considering the morning of detection of the vaginal plug as E0.5. Tissue was isolated at postnatal day (P)0 (day of birth), P7, P42 and one year after birth.

Several mutant mouse lines were used during this study; all of them were back-crossed to the C57BL/6J line. First, \( En1^{tm1Alj/+} \) animals were back-crossed to the C57BL/6J line generating \( En1^{+/+} \) (WT), \( En1^{tm1Alj/tm1Alj} \) (Het) and viable \( En1^{tm1Alj/+} \) (KO) offspring (previously described in Bilovocky et al., 2003; Veenvliet et al., 2013). Second, \( En1^{tm1Alj/+} \) animals were inter-crossed with \( Pitx3^{GFP/+} \) in which the Pitx3 gene is substituted by a GFP allele (Jacobs et al., 2011) in order to breed \( En1^{tm1Alj/+};R26RYFP/R26RYFP \). Third, \( En1^{tm1Alj/+} \) animals were inter-crossed with the transgenic mouse line \( B6.Cg-Tg(BAT-lacZ)3Picc/J \) (BAT-GAL) (Maretto et al., 2003), in order to generate \( En1^{tm1Alj/+};BatGal/+ \). Fourth, \( En1^{tm1Alj/+} \) animals were inter-crossed with \( En1^{CreERT/+};R26RYFP/R26RYFP \) (Sgaier et al., 2005) to generate \( En1^{CreERT/+};R26RYFP/+ \) animals. We back-crossed these animals in order to study the following genotypes: \( En1^{CreERT/+};R26RYFP/R26RYFP \) (\( En1^{CreERT/+};R26RYFP \) and \( En1^{CreERT/+};R26RYFP \)) and \( En1^{CreERT/+};R26RYFP/R26RYFP \) and \( En1^{CreERT/+};R26RYFP \) (Sgaier et al., 2005) were also back-crossed with \( En1^{tm1Alj/+} \) animals to enable En1- lineage tracing by induction of Cre-ERT, oral administration (using a gavage) of Tamoxifen (Sigma) 20 mg/ml in corn oil (Sigma), at different time points (E10.5). When Tamoxifen was administered at E10.5 embryos were isolated at E14.5.

All animals were genotyped by PCR using specific primers (Table 2). \( Pitx3^{tm1Alj/+} \) animals were recognized by the shape of the lens, which is malformed in all Pitx3-deficient animals. All procedures and experiments were performed according to the guidelines and with the approval of the Dutch Ethical Committee of the University of Amsterdam.

In situ hybridization (ISH)

In situ hybridization was performed as described previously (Smits et al., 2003). Digoxigenin-labeled probes for \( Th, En2, Vmna2, Dat, Nur1, AADC, Lmx1b, Pitx3, Phx1, Phx3 \) and \( Wnt1 \) were used as previously described (Grima et al., 1985; Hoekstra et al., 2013; 2012; Mesman et al., 2014; Smits et al., 2003a). Additional probes: Fgfl (500-1003 bp, NM_010205), Otx2 (NM_144841.2, bp 592-1165), Sert (bp 1827-2326, NM_000091.1), Gapd (bp 1312-1685, NM000091.1), Per1 (bp 885-1444, NM_153111.2), Gusb (bp 777-1199, NM_012062).

Fluorescence immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA) in PBS, cryoprotected in 30% sucrose in PBS and subsequently stored at −80°C. Sagittal sections (16 µm) were cut on a cryostat, after which they were washed with TBS and blocked in 4% fetal calf serum (FCS) in THZT (50 mM Tris-HCl pH 7.6, 0.5 M NaCl, 0.5% Triton X-100). After another wash treatment with TBS, sections were incubated overnight at 4°C with primary antibody in THZT. Sections were washed three times (TBS) the following morning and incubated for minimally 2 h at room temperature (RT) with secondary antibody in TBS, followed by wash treatment with PBS. Finally sections were embedded with Fluorsave (Biochemical). Primary antibodies that were used: Rabbit α-Th (Pel-Freeze, 1:1000), Rabbit α-serotonin (ImmunoStar, 1:500), Rabbit α-γtubulin (Abcam, 1:1000). Secondary antibodies that were used: Goat α-Rb Alexa Fluor 555 (1:1000), Goat α-Rb Alexa Fluor 488 (1:1000), Goat α-Chicken Alexa Fluor 488 (1:1000), all from Invitrogen.

X-galactosidase staining protocol

Fresh frozen sections were defrosted and post-fixed with 4% PFA for 45 min. Sections were washed with PBS three times, and once with staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2 in PBS). Sections were incubated at RT with staining solution, complemented with 1 mg/ml X-galactosidase (X-gal) for several hours, protected from light, until staining was optimal. Sections were then rinsed in PBS, dehydrated and embedded in Entellan (Merck).

Electrophysiology recordings

For recordings at E16.5, pregnant dams were sacrificed by cervical dislocation and embryos were isolated from the uterus. Neural tubes were micro-dissected on ice in 5% FCS Leibovitz-15 (Sigma), the telencephalon was removed and the remaining neural tube was cut along the medial axis. The resulting ‘hemitubes’ were subsequently used for recordings at the medial plane. The eDA neurons are discriminated from mdDA neurons in the En1-mutant based on their ectopic (caudal) location in the hindbrain.

For recordings at P7, brains were isolated in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (120), KCl (3.5), CaCl2 (2.5), MgSO4 (1.3), NaH2PO4 (1.25), NaHCO3 (25), glucose (25), continuously bubbled with 95% O2/5% CO2 (pH 7.4) and 250 µm-thick sagittal slices were cut on a vibroslicer (Leica VT1000S). Hemitubes and slices were transferred to a recording chamber and continuously superfused with ACSF. Patch pipettes were pulled from borosilicate glass and had a resistance of 4-6 MΩ when filled with internal solution containing (in mM): potassium gluconate (105), KCl (30), EGTA (5), CaCl2 (0.5), HEPES (10), and Mg-ATP (5)(pH 7.3 with KOH). GFP-positive neurons were visualized using differential interference contrast microscopy on a Zeiss FS2 microscope equipped with standard epifluorescence. Whole-cell recordings were made using an EPC9 patch-clamp amplifier and PULSE.
software (HEKA Electronik, Lambrecht, Germany). Cells were voltage clamped at −70 mV (corrected for liquid junction potential) and series resistance was compensated for at least 70%. Signals were filtered at 1-5 kHz, sampled at 10 kHz, and off-line analysis was performed using Igor Pro (Wavemetrics, Lake Oswego, OR, USA).

**Statistical analysis**

Values of electrophysiological measurements are expressed as means± standard error of the mean (s.e.m.). Comparisons were made using two-tailed Student’s t-test. P<0.05 was considered significant, and indicated using an asterisk (*).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

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**References**


RESEARCH ARTICLE


