**ABSTRACT**

The proper functioning of the dopaminergic system requires the coordinated formation of projections extending from dopaminergic neurons in the substantia nigra (SN), ventral tegmental area (VTA) and retrorubral field to a wide array of forebrain targets including the striatum, nucleus accumbens and prefrontal cortex. The mechanisms controlling the assembly of these distinct dopaminergic cell clusters are not well understood. Here, we have investigated in detail the migratory behavior of dopaminergic neurons giving rise to either the SN or the medial VTA using genetic inducible fate mapping, ultramicroscopy, time-lapse imaging, slice culture and analysis of mouse mutants. We demonstrate that neurons destined for the SN migrate first radially and then tangentially, whereas neurons destined for the medial VTA undergo primarily radial migration. We show that tangentially migrating dopaminergic neurons express the components of the reelin signaling pathway, whereas dopaminergic neurons in their initial, radial migration phase express CXC chemokine receptor 4 (CXCR4), the receptor for the chemokine CXC motif ligand 12 (CXCL12). Perturbation of reelin signaling interferes with the speed and orientation of tangentially, but not radially, migrating dopaminergic neurons and results in severe defects in the formation of the SN. By contrast, CXCR4/CXCL12 signaling modulates the initial migration of dopaminergic neurons. With this study, we provide the first molecular and functional characterization of the distinct migratory pathways taken by dopaminergic neurons destined for SN and VTA, and uncover mechanisms that regulate different migratory behaviors of dopaminergic neurons.

**KEY WORDS:** Dopaminergic neurons, Ventral midbrain, Tangential migration, Reelin, Chemokine

**INTRODUCTION**

Neuronal migration is one of the fundamental mechanisms establishing the complex organization of the brain. Migratory behavior and the underlying molecular mechanisms have been extensively studied in the dorsal brain, particularly in the cerebral and cerebellar cortex, where neurons undergo either radial or tangential migration to organize themselves into neuronal layers (Chédotal, 2010; Marin et al., 2010). By contrast, neurons in ventral brain areas are arranged into a complex array of neuronal clusters, so-called nuclei. Little is known about the migration events and the molecular mechanism that guide neurons to form these neuronal clusters.

Dopaminergic (DA) neurons in the ventral midbrain are organized into three major nuclei: the lateral substantia nigra pars compacta (SN), the medially located ventral tegmental area (VTA) and the posterolaterally located retrorubral field (RRF). The SN projects to the dorsal striatum and modulates motor behaviors. The VTA projects to corticollimbic targets and is important for reward behavior and the modulation of cognition, whereas the RRF projects to the ventrolateral striatum (Björklund and Dunnett, 2007; Ikemoto, 2007). DA neurons are of clinical interest as the degeneration of SN neurons underlies the major symptoms in Parkinson’s disease. Dopamine imbalances in the corticollimbic system, which receives inputs from the VTA, have been linked to attention deficit hyperactivity disorder, addiction and schizophrenia (Dailly et al., 2004; Sulzer, 2007; Winterer and Weinberger, 2004; Wise, 2009).

DA neurons arise from progenitors in the midbrain floor plate that express sonic hedgehog (SHH), forkhead box A1 and A2 (FOXA1/2) and LIM homeobox transcription factor 1 alpha and beta (LMX1A/B) (Ang, 2009; Blaess et al., 2011; Chung et al., 2009; Hayes et al., 2011; Joksimovic et al., 2009; Nakatani et al., 2010; Yan et al., 2011). The induction of these factors requires SHH signaling and, accordingly, DA progenitors express GLI1, a readout for SHH signaling, prior to the onset of SHH expression (Andersson et al., 2006; Blaess et al., 2011; Hayes et al., 2011). Moreover, a subset of DA progenitors is positive for WNT1 (wingless-related MMTV integration site 1) (Brown et al., 2011).

The first differentiated DA neurons appear between embryonic day (E) 10.5 and E11.5 (Bayer et al., 1995). DA neurons migrate from their ventral midline progenitor area to the mantle layer, where they settle in distinct clusters to form the SN, VTA or RRF. Two distinct migration models, based primarily on immunostaining for tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis and/or birthdating of ventral midbrain cells, have been put forward: (1) VTA and SN are primarily formed through radial migration (Hanaway et al., 1971), (2) DA neurons initially migrate radially towards the pial surface and then tangentially to form the VTA and SN (Kawano et al., 1995; Marchand and Poirier, 1983; Shults et al., 1990; Vasudevan et al., 2012). These studies could not unambiguously distinguish between different DA subpopulations during their migration phase nor did they monitor migratory behavior in real time. Therefore, it remains unclear which of these proposed migration paths are taken by DA neurons and whether DA neurons giving rise to the SN, VTA or RRF show distinct migratory behaviors. Markers that are expressed specifically in SN, VTA or RRF neurons throughout their development have not been identified, hampering the characterization of distinct DA migratory paths and...
the identification of the molecular mechanisms that regulate DA neuronal migration. Based on loss-of-function mouse mutants in which the formation of the SN and/or VTA is impaired, only the netrin receptor DCC (deleted in colorectal carcinoma), the extracellular glycoprotein reelin, and the cell adhesion molecule L1 (L1CAM – Mouse Genome Informatics) have been implicated in the regulation of DA neuronal migration (Ballmaier et al., 2002; Demyanenko et al., 2001; Kang et al., 2010; Nishikawa et al., 2003). Here, we show that cells destined for both the SN and the VTA migrate radially from the progenitor domain to the forming mantle layer, resulting in the intermingling of the two DA populations in the medial ventral midbrain at early differentiation stages. The separation of the distinct populations occurs through tangential migration of cells destined for the SN. We demonstrate that reelin signaling specifically regulates tangential migration of DA neurons whereas signaling through CXCR4, a receptor for the chemokine CXCL12, modulates the radial migration of DA neurons.

RESULTS

Fate-mapping strategy to follow the migration of DA neurons contributing to the SN or medial VTA

Using genetic inducible fate mapping, which heritably marks progenitors and their descendants, we and others have shown that SHH-expressing progenitors in the floor plate give rise to DA neurons. SHH is expressed in DA progenitors in the medial floor plate between E8.5 and E9.0, but at E11.5 it is excluded from the medial domain and restricted to lateral DA progenitors (Blaess et al., 2011; Hayes et al., 2011; Joksimovic et al., 2009). When genetic fate mapping of SHH-expressing cells is induced at E8.5, medial DA progenitors and their descendants are marked; these cells show a biased contribution to the SN (SN marking scheme). Induction at E11.5 marks lateral DA progenitors and their descendants; this population contributes preferentially to the medial VTA consisting of the paragniral and interfascicular nucleus (mVTA marking scheme) (Fig. 1). Progenitor populations labeled with the SN or mVTA marking scheme also give rise to neurons in the lateral VTA (parabrachial nucleus) and to a few cells in the mVTA or SN, respectively (Blaess et al., 2011; Hayes et al., 2011; Joksimovic et al., 2009). Nevertheless the two populations are sufficiently distinct to examine separately the migratory routes and behavior of DA neurons contributing to the SN or the mVTA (Fig. 1C). To achieve fluorescent labeling of fate-mapped cells, we used an EYFP reporter allele (R26F1/2F1) (Srinivas et al., 2001) (Fig. 1A).

Distribution of DA neurons destined for the SN shifts from medial to lateral during embryonic development

To identify fate-mapped, differentiated DA neurons, we performed immunostaining for TH and EYFP. We used this double-labeling approach because, in addition to DA progenitors and DA neurons, some non-DA neurons are labeled with the SN and mVTA marking scheme in the ventral midbrain (Blaess et al., 2011). Quantification of EYFP positive (EYFPpos) and THpos/EYFPpos cells at E13.5 and E14.5 showed that within the areas that contain differentiated DA neurons, >70% of EYFPpos cells were also THpos (supplementary material Fig. S1).

To investigate the migratory routes of DA neurons, we characterized the distribution of DA neurons labeled with the SN or mVTA marking scheme (THpos/EYFPpos) at three levels along the anteroposterior axis of the developing ventral midbrain (referred to as anterior, intermediate and posterior) between E11.5 and E16.5 (Figs 2, 3; supplementary material Fig. S2). From anterior to posterior, these areas correspond to the following DA nuclei in the ventral midbrain:...
adult brain: anterior SN; intermediate SN and anterior VTA; posterior SN, posterior VTA and RRF (Fig. 2P). Fig. 2 shows examples of the distribution of the fate-mapped DA-neurons at E13.5 and E15.5. To quantify the mediolateral position of the fate-mapped DA neurons, we determined the ventral midline in each section and divided the ventral midbrain into bins of 100 μm along the mediolateral axis (Fig. 3A-D). The number of fate-mapped DA neurons per bin was counted and expressed as percentage of the total number of fate-mapped DA neurons per section (Fig. 3E,F). At intermediate and posterior levels, DA neurons labeled with both marking schemes were found close to the midline (0-300 μm) between E11.5 and E13.5 (Fig. 2E-H; Fig. 3A,C,E,F; supplementary material Fig. S2B-D). At later stages (E15.5-E18.5), DA neurons labeled with the SN marking scheme were located in increasingly more lateral positions, whereas DA neurons labeled with the mVTA marking scheme maintained a relative medial position (0-400 μm) (Fig. 2K-N; Fig. 3B,D,F; supplementary material Fig. S2B,C). At anterior levels, DA neurons labeled with the SN marking scheme were lateral to the midline by E11.5 and were observed in increasingly lateral positions at subsequent stages (Fig. 2C-I; supplementary material Fig. S2A,D). The mVTA marking scheme labeled only a few DA neurons in anterior sections (Blaess et al., 2011) (Fig. 2D,J).

In summary, DA neurons destined for the SN and mVTA were intermingled in the medial mantle layer between E12.5 and E13.5 and only separated later (E14.5-E15.5) into two largely distinct clusters. These results suggest that all DA neurons initially migrate radially from the ventricular zone to the mantle layer, and that neurons forming the SN undergo active tangential migration to reach their final lateral position.

**DA neurons giving rise to the SN have tangentially oriented leading processes**

On a cellular level, the process of migration consists of three steps: the extension of a leading process, the translocation of the nucleus into the leading process and the elimination of the trailing process.
The leading process selects the orientation of migrating neurons in response to chemotactic cues in the environment and its orientation can serve as an indicator for the direction of migration (Marín et al., 2010; Rakic, 1990). In midbrain sections, the morphology of TH pos/EYFP pos neurons labeled with the SN marking scheme indicated that the lateral displacement of DA neurons correlated with tangentially oriented leading processes, whereas cells labeled with the mVTA marking scheme had primarily radially oriented processes (supplementary material Fig. S3). To investigate the morphology of fate-mapped DA neurons (TH pos/EYFP pos) more precisely, we used ultramicroscopy. Ultramicroscopy allows fast multidimensional imaging of entire tissues with minimal photo-bleaching and good optical resolution. Cleared whole-mount brains immunostained for TH and EYFP were imaged (Fig. 4; supplementary material Fig. S4). Analysis of the acquired image stacks reconstructed in 3D showed that most DA neurons had a unipolar or bipolar morphology (Fig. 4B,D,F,H). DA neurons labeled with the SN marking scheme displayed radially oriented processes when located close to the ventricular zone, but tangentially oriented processes after reaching the mantle layer (Fig. 4A,D,G). By contrast, DA neurons labeled with the mVTA marking scheme had predominantly radially oriented processes and only few tangentially oriented processes (six out of 72 cells) (Fig. 4E-G). Classification of the processes into trailing processes (radially oriented towards ventricular zone or tangentially oriented towards midline) and leading processes (radially oriented towards pial surface or tangentially oriented away from midline) showed that neurons labeled with both marking schemes had leading and trailing processes (Fig. 4H). These results support a model in which DA neurons labeled with the SN marking scheme initially undergo radial migration to reach the mantle layer and subsequently switch to tangential migration to form the SN. By contrast, neurons giving rise to the mVTA have almost exclusively radial leading/trailing processes, consistent with a predominantly radial mode of migration.

Time-lapse imaging demonstrates distinct migratory routes for DA neurons destined for the SN or medial VTA

To examine the migratory behavior of neurons labeled with the SN or mVTA marking scheme in real time, we monitored the migration of fluorescently marked cells in organotypic slice cultures using time-lapse imaging (Fig. 5). E12.5 brain slices were cultured for one day and then subjected to time-lapse imaging for 8-15 hours (Fig. 5; supplementary material Table S1). Imaging of cells labeled with the SN or mVTA marking scheme in sagittal slices showed that they followed a radial migration path (Fig. 5D; supplementary material Movies 1, 2). Cells labeled with the SN marking scheme underwent...
tangential migration in horizontal slices (Fig. 5G; supplementary material Movie 3). During tangential migration, soma translocation followed the extension of a leading process (Fig. 5C; supplementary material Movie 3). Cells in which processes could be assessed in detail (n = 10) had transient trailing processes (5/10 cells) and made short backward movements (3/10 cells). In addition, some tangentially migrating neurons underwent a phase of active forward movement followed by a resting period (4/10 cells). By contrast, cells labeled with the mVTA marking scheme rarely underwent tangential migration in E12.5 horizontal slices (supplementary material Movie 4). Because mVTA neurons are born later than SN neurons, their lateral movement might occur later. To assess this possibility, we examined the migration of cells labeled with the mVTA marking scheme in E13.5 and E14.5 horizontal slices, but observed only very few tangentially migrating cells (supplementary material Movies 5, 6). These data demonstrate that neurons that contribute to the SN undergo radial and tangential migration, whereas neurons destined for the mVTA primarily follow a radial migration route.

Reelin signaling regulates tangential migration of DA neurons destined for the SN

Because DA neurons destined for the SN undergo tangential migration over long distances, molecular mechanisms specifically guiding laterally migrating DA neurons should exist. The reelin pathway could play a role in regulating tangential migration of DA neurons, as it has been reported that the SN does not form in Reeler mutants (Kang et al., 2010; Nishikawa et al., 2003; Trommsdorff et al., 1999). Reelin, a large extracellular glycoprotein, regulates migration in several brain areas and signals via the transmembrane receptors APOER2 (APOE receptor 2; LRP8 – Mouse Genome Informatics) and VLDLR (very low density lipoprotein receptor) and the downstream effector DAB1 (disabled 1) (D'Arcangelo et al., 1999; Rice et al., 1998).

We first examined whether components of the reelin signaling pathway are specifically expressed in DA neurons that undergo tangential migration. RNA in situ hybridization at E13.5 showed that Vldlr and Apoer2 were expressed at low levels throughout the region containing DA neurons (Fig. 6A,B; supplementary material Fig.
SSA,B). By contrast, Dab1 expression was largely restricted to the area where tangentially migrating DA neurons contributing to the SN are located (Fig. 6C; supplementary material Fig. S5C,E-J). Reelin was expressed dorsally to Dab1-expressing cells, in an area positive for the red nucleus marker POU4F1 (POU domain, class 4, transcription factor 1) (Fig. 6D; supplementary material Fig. S5D).

To investigate whether reelin signaling is indeed required for the tangential migration of SN neurons, we inhibited reelin function in slice cultures using the function-blocking antibody CR50 (Utsunomiya-Tate et al., 2000). We monitored the migratory behavior of cells fluorescently labeled with the SN marking scheme using time-lapse imaging (Fig. 6E-I; supplementary material Fig. S5K and Table S1). Inhibition of reelin signaling led to a significant reduction in the speed of tangentially migrating cells (Fig. 6F). This effect was specific, as treatment with reelin function-blocking antibody did not alter the speed of radially migrating cells labeled with the mVTA or SN marking scheme (Fig. 6G; supplementary material Fig. S5K and Table S1). Analysis of the trajectories of tangentially migrating EYFPpos cells (Fig. 6H,I) showed that reelin inhibition resulted in a significant deviation of migrating neurons \((n = 46)\) from the tangential trajectory compared with neurons in control slices \((n = 33)\) (Fig. 6I). As the leading process plays an important role in directing neuronal migration, we analyzed the morphology of THpos DA neurons in E13.5 wild-type and Reeler mutant brains. We found that in Reeler mutants, laterally positioned DA neurons lacked tangentially oriented processes, but formed processes that were perpendicular to the tangential plane (Fig. 6J,K).

To assess whether reelin signaling in DA neurons is indeed mediated by VLDLR/APOER2 and DAB1, we examined the midbrain phenotypes of Reeler mutant, Dab1 knockout (KO) or Apoer2/Vldlr double KO mice at postnatal day 25. In both Dab1 and Apoer2/Vldlr KO mice, the SN did not extend laterally at intermediate levels and DA neurons appeared to accumulate in the VTA region, a phenotype comparable to Reeler mutant mice (supplementary material Fig. S6A-D). At anterior levels, however, the SN formed, but was disorganized (supplementary material Fig. S6M-P). Immunostaining for GIRK2 (G protein-gated inwardly rectifying potassium channel 2; KCNJ6 – Mouse Genome Informatics) and calbindin which are predominantly expressed in either the SN or the VTA, respectively, showed that GIRK2pos DA neurons were located laterally to calbindinpos DA neurons at intermediate levels (supplementary material Fig. S6E-L) (Thompson et al., 2005).

These findings show that reelin regulates the speed, trajectories and polarization of tangentially migrating DA neurons through the VLDLR/APOER2 and DAB1 pathway. Reelin signaling is essential for the proper formation of the SN, but not for the separation of SN and VTA neurons.
CXCR4 and its ligand CXCL12 regulate the initial migration of DA neurons

The mechanisms that control the initial radial migration of DA neurons are unknown. Given that DA neurons migrate towards the ventral pial surface during their radially oriented movement, we hypothesized that an attractant expressed by the meningeal cell layer surrounding the pial surface could be important for this process. A potential candidate is the chemokine CXCL12. CXCL12 is a potent chemoattractant during neuronal migration in the forebrain and the hindbrain, where it is secreted from meningeal cells (fore- and hindbrain) or Cajal Retzius cells (forebrain). CXCL12 signals through the CXCR4 and CXCR7 (ACKR3 – Mouse Genome Informatics) receptors (Sánchez-Alcañiz et al., 2011; Wang et al., 2011).
We first analyzed the expression of *Cxcl12*, CXCR4 and *Cxcr7* in the ventral midbrain between E10.5 and E18.5 (Fig. 7; data not shown). CXCR4 was expressed in THpos DA neurons starting at E11.5 (Fig. 7A). At E13.5 and E15.5, CXCR4 was expressed in medially located THpos DA neurons, but also in THneg cells located below the ventricular zone (Fig. 7B,C). These THneg CXCR4pos cells were positive for NURR1 (nuclear receptor related 1 protein; NR4A2 – Mouse Genome Informatics), which is expressed in differentiating DA neurons before the onset of TH expression, and for LMX1A, a marker for DA progenitors and differentiated DA neurons (Fig. 7E,F) (Andersson et al., 2006; Zetterström et al., 1996). CXCR4 expression levels appeared to be slightly downregulated in cells located close to the pial surface (Fig. 7B,C,E,F). By E18.5, CXCR4 could no longer be detected in the ventral midbrain (Fig. 7D). By contrast, *Cxcr7* was only expressed in the ventricular zone (Fig. 7G). *Cxcl12* mRNA was expressed in the meningeal cell layer at the time of DA neuronal migration (Fig. 7H,I).

The restricted spatial and temporal expression of CXCR4 in medially located DA neurons suggested that CXCL12/CXCR4 signaling might play a specific role in regulating the initial radial migration of DA neurons. To investigate this, we analyzed the distribution of DA neurons at E14.5 and E16.5 in mice in which *Cxcr4* (*Cxcr4* KO) or *Cxcl12* (*Cxcl12* KO) were inactivated. Postnatal stages could not be analyzed because the mice die perinatally (Ma et al., 1998; Nagasawa et al., 1996). At intermediate and posterior levels, we observed ectopic THpos DA neurons dorsal to the DA neuronal field in the mutant embryos (Fig. 8A-C; data not shown). In addition, the morphology of the cells in this area was altered in posterior sections of *Cxcl12* and *Cxcr4* KO mutants at E16.5 (Fig. 8G-I). To quantify the change in cell distribution, ventral midbrain sections of *Cxcl12* KO, *Cxcr4* KO and wild-type embryos were divided into bins along the dorsoventral and mediolateral axis (Fig. 8D,J; see also Materials and Methods). The number of DA neurons was counted in each bin and normalized for the total number of counted DA neurons. This analysis confirmed the increased accumulation of DA neurons in the dorsal, off-midline area of the DA neuronal field in E14.5 *Cxcl12* KO and *Cxcr4* KO embryos and in E16.5 *Cxcr4* KO embryos (Fig. 8E,K). In *Cxcl12* KO mutants, the increase in dorsally located DA neurons was accompanied by a slight decrease in DA neurons in ventral, off-midline positions (Fig. 8F). By contrast, in *Cxcr4* KO embryos, DA neurons were decreased along the midline and increased in ventral, off-midline positions (Fig. 8E,F,L).

The altered distribution of DA neurons in *Cxcr4* or *Cxcl12* KO mice could be caused by changes in the speed and/or trajectories of migrating DA neurons, two aspects of migration that can be quantified in organotypic slice cultures. To this end, we inhibited CXCL12/CXCR4 signaling by adding the CXCR4 antagonist AMD3100 to the slice cultures and monitored the migration of cells labeled with the mVTA or SN marking scheme (supplementary material Fig. S7A-C and Table S1). To confirm that the antagonist abolishes CXCR4 signaling, we assessed the phosphorylation of CXCR4, which serves an indicator for CXCR4 activation (Busillo et al., 2010). We could show that phosphorylated CXCR4 is present in untreated, but not in antagonist-treated slice cultures, demonstrating that the antagonist inhibits CXCL12/CXCR4 signaling in our experimental setup (supplementary material Fig. S7D,E). However, neither the speed nor the trajectories of migrating cells was changed in cultures treated with the CXCR4 antagonist.
SUPPLEMENTARY MATERIAL

Fig. S7A-C; data not shown), suggesting that other aspects of migration are altered in the absence of CXCR4/CXCL12 signaling (see Discussion).

DISCUSSION

Migratory paths of DA neurons

Previous attempts to characterize the migratory routes of DA neurons using birthdating and/or TH immunostaining of ventral midbrain progenitors have produced contradictory results. Thus, several potential migratory paths have been proposed for neurons that form the SN (see Introduction) (Hanaway et al., 1971; Kawano et al., 1995; Shults et al., 1990; Vasudevan et al., 2012).

Our data show that DA neurons destined for the mVTA and SN undergo an initial radially oriented migration from the ventricular zone to the mantle layer. Radial migration of DA neurons probably occurs along densely packed radial glia fibers that extend from the ventricular zone to the mantle layer in the ventral midbrain. DA neurons are aligned along these radial processes and disruption of
radial glia results in impaired migration of DA neurons (Kawano et al., 1995; Shults et al., 1990; Tang et al., 2009).

Following this initial migration step, neurons contributing to the SN switch their migratory direction and move laterally. We demonstrate that future SN neurons undergo active tangential migration rather than being displaced laterally by newly generated medial cells or the overall growth of the tissue. Axonal processes originating from cell bodies in the ventrolateral midbrain and running along the mediolateral axis of the ventral midbrain could function as a scaffold for tangentially migrating neurons (Kawano et al., 1995).

The switch of SN neurons from radial to tangential migration is most obvious at intermediate anteroposterior levels of the developing DA neuronal field, where both SN and VTA are formed. DA neurons destined for the anterior SN assume relative lateral positions immediately after their radial migration, but our analysis of the mediolateral cell distribution shows that tangential migration also contributes to their final lateral position. At posterior levels, we only observed a few labeled DA neurons in lateral positions with the SN marking scheme. Therefore, we cannot draw any conclusion about the migratory behavior of cells giving rise to the RRF.

**Reelin regulates tangential migration of DA neurons**

Previous studies proposed that the SN does not form in *Reeler* mutants (Kang et al., 2010; Nishikawa et al., 2003). We show here that in *Reeler* as well as in *Dab1* KO and Apoer2/Vldlr double KO mutants, the lack of lateral dispersion of SN neurons is confined to intermediate levels, whereas the anterior SN is merely disorganized (see also Sharaf et al., 2013). We further demonstrate that reelin regulates the tangential migration of DA neurons by regulating their speed, trajectories and leading process orientation. These effects of reelin are consistent with some of its proposed functions during the migration of cortical projection neurons and cerebellar Purkinje cells (Britto et al., 2013; Miyata et al., 2010). Interestingly, in reelin pathway mutants, GIRK2pos (primarily SN) and calbindinpos (primarily VTA) neurons are still largely split into lateral and medial clusters, respectively, indicating that reelin is not required for separating the two cell populations. These data suggest that reelin is not important for the initial steps of tangential migration of SN neurons (including the switch from radial to tangential migration), but rather regulates the final tangential migration that allows SN neurons to reach their correct position in the ventrolateral midbrain (Fig. 9).

Reelin could regulate the speed and trajectories of laterally migrating DA neurons directly by acting as a chemoattractant for DA neurons. In the cortex, it has been shown that reelin, a large extracellular matrix molecule, is cleaved into smaller fragments that could potentially diffuse and build a chemoattractive gradient (Jossin et al., 2004; Lambert de Rouvroit et al., 1999). Whether reelin is similarly processed in the ventral midbrain remains to be examined.

Alternatively, reelin might be a permissive factor allowing tangentially migrating DA neurons to respond to other, still unknown, attractant molecules. Several lines of evidence support such a scenario. First, the *Reelin* mRNA expression domain, which overlaps with the red nucleus, is located dorsally rather than laterally to tangentially migrating, *Dab1*-expressing DA neurons. Second, it has been previously reported that reelin protein can be detected throughout the SN, where it might be secreted by incoming projections from striatal neurons (Nishikawa et al., 2003). Such a ubiquitous distribution of reelin would make it unlikely that reelin itself acts as a chemoattractive factor. Third, we show that reelin is important for the proper orientation of the leading processes of laterally migrating DA neurons. The dramatic misorientation of the leading processes of DA neurons in *Reeler* mutants could interfere with the ability of the DA neurons to respond to attractive cues in their environment.

**CXCL12/CXCR4 signaling modulates the initial migration step of DA neurons**

DA neurons that leave their progenitor domain and move radially towards the mantle layer express CXCR4, the receptor for the chemokine CXCL12. *Cxcl12* is expressed in the meninges surrounding the forming mantle layer. In both, *Cxcr4*+ and *Cxcl12*−deficient mice, we observed an accumulation of DA neurons in the dorsal, off-midline area of the DA neuronal field. The accompanying reduction in ventrally positioned cells in *Cxcl12* mutants as well as the lack of expression of CXCR4 in laterally positioned DA neurons suggest a role for CXCR4/CXCL12 signaling in radial migration (Fig. 9). These results are consistent with a recently published study (Yang et al., 2013). However, we observed fewer DA neurons along the ventral midline of *Cxcr4* mutants, indicating a more complicated effect of CXCR4 signaling on DA neuronal migration. Moreover, the phenotype in both KO mice appears to be transient, suggesting that CXCL12/CXCR4 signaling is not essential for DA neuronal migration, but rather regulates the efficiency of migration. As CXCL12 is the only known ligand for CXCR4, it is surprising that the *Cxcr4* and *Cxcl12* KOs have distinct phenotypes. CXCL12-independent functions of CXCR4 have not been demonstrated, but it has been recently shown that the neurotransmitter GABA directly binds CXCR4 and can act as an allosteric modulator (Guyon et al., 2013). Whether GABA or other potential modulators could influence CXCR4 function in the absence of CXCL12 in the ventral midbrain remains to be explored.

It is unclear which aspect of DA neuronal migration is regulated by CXCL12/CXCR4 signaling. Recently, it has been reported that the process orientation of MbDN is altered in *Cxcr4* KO mice (Yang et al., 2013). However, upon inhibiting CXCL12/CXCR4 signaling in our organotypic slice cultures neither the speed nor the trajectory of radially migrating cells was significantly changed. Other changes that might account for the ectopic accumulation of DA neurons in...
**RESEARCH ARTICLE**


Cxcl12- or Cxcr4-deficient mice, such as premature cessation or a delayed onset of radial migration, could not be assessed in the slice cultures. Premature cessation of migration upon CXCR4 inactivation has been reported for migrating sympathetic precursor cells in the chick (Kasemeier-Kulesa et al., 2010).

CXCL12 is thought to act as a paracrine signal for migrating neurons (Stumm and Höltt, 2007; Stumm et al., 2003). A paracrine action of CXCL12 seems unlikely in migrating DA neurons, because CXCL12 is expressed in the meninges whereas most CXCR4-expressing DA neurons are located within some distance of the pial surface. Alternatively, CXCL12 might act as a long-range attractive signal for DA neurons. Previous findings show that CXCL12 is an attractant for radially migrating granule cells in the dentate gyrus and can act as a chemotactic factor for dissociated neurons in vitro (Bagri et al., 2002; Hesselgesser et al., 1997; Klein et al., 2001).

**Conclusion**

Here we characterize in detail the migratory paths taken by DA neurons that ultimately give rise to the SN or the mVTA. We demonstrate that neurons destined for the mVTA undergo radial migration to reach their final position in the ventral midbrain. By contrast, the migration of neurons giving rise to the SN consists of several steps: an initial radial migration followed by active tangential migration resulting in the separation of SN and mVTA neurons and the formation of the SN in the lateral midbrain. We further show that reelin mediates the tangential migration that results in the final lateral position of SN. Finally, we provide evidence that the migration of DA neurons from their progenitor zone to the mantle layer is modulated by Cxcr4/CXCR4 signaling.

**MATERIALS AND METHODS**

**Mouse lines and genetic inducible fate mapping**

Reeler mutants, Aporo2, Vldlr, Dabl, Cxcr4 and Cxcl12 knockout mice and genotyping protocols have been described previously (D’Arcangelo et al., 1995; Frykman et al., 1995; Howell et al., 1997; Ma et al., 1998; Nagasawa et al., 1996; Trommsdorff et al., 1999). ROSA26STOP-CreERT2 (R26	extsuperscript{ERT2}) mice were kindly provided by Dr Costantini (Srinivas et al., 2001); Shh	extsuperscript{CreER} mice were kindly provided by Dr Tabin (Harle et al., 2004). Genotyping protocols for the R26	extsuperscript{ERT2} allele and the CreER alleles were previously described (Kimmel et al., 2000; Soriano, 1999). Shh	extsuperscript{CreER} and R26	extsuperscript{ERT2} mice were maintained in an outbred CD1 background. Noon of the day a vaginal plug was detected was designated as E0.5. Tamoxifen (TM, 20 mg/ml) (Sigma-Aldrich) was dissolved in corn oil (Sigma-Aldrich). To reduce miscarriages, 5 mg/ml progesterone (Sigma-Aldrich) was added. Pregnant females were given 3-4 mg TM by oral gavage with animal feeding needles (Fine Science Tools) at 12 pm of E8.5 or E11.5. Animal studies were approved by the University of Bonn Animal Care and Use Committee.

**Organotypic slice cultures**

Organotypic slice cultures were prepared as described previously (Bodea and Blaess, 2012). After sectioning, slices were incubated for 24 hours before being imaged. Time-lapse imaging was performed at 100× magnification at 10-minute intervals for 8-15 hours on a Zeiss Axio-Observer inverted microscope fitted with an environmental chamber at 37°C, 5% CO2. Anti-reelin antibody (CR-50, 4 g/ml, MBL) in 50% glycerol/PBS (Utsunomiya-Tate et al., 2000) was added to inhibit reelin signaling; 50% glycerol/PBS alone was added as control. The CXCR4 antagonist AMD3100 was diluted in culture medium (120 μM, Sigma-Aldrich) (Lyso et al., 2011). The compounds were added to the slices right before imaging was started and kept in medium during the entire imaging time.

**Immunohistochemistry and in situ hybridization**

Heads or brains were dissected, fixed in 4% paraformaldehyde (PFA) for 30-120 minutes and either embedded in Tissue-Tek OCT (Sakura) for cryosectioning (12 μm sections) or processed for paraffin embedding (7 μm sections). For staining with anti-phosphorylated CXCR4, slice cultures were fixed in 4% PFA for 15 minutes and embedded in Tissue-Tek OCT (Sakura) for cryosectioning (12 μm sections). In situ hybridization and immunostaining were performed using standard procedures (Blaess et al., 2011). Plasmids containing reelin, Dabl, Vldlr and Aporo2 cDNA were kindly provided by Dr F. Tissir (Jossin et al., 2003). RNA probes for Cxcl12 and Cxcr7 have been described (Sánchez-Alcañiz et al., 2011; Stumm et al., 2003).

Primary antibodies used for immunostaining were: rabbit or mouse anti-

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<th>Primary Antibody</th>
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<tr>
<td>anti-TH (1:500)</td>
<td></td>
<td>UTSU053, Millipore</td>
</tr>
<tr>
<td>anti-NURRR1 (1:200)</td>
<td>Sc-990,</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti-POU4F1 (1:50)</td>
<td>Sc-8429,</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti-calbindin</td>
<td>(1:200,</td>
<td>Jackson ImmunoResearch</td>
</tr>
</tbody>
</table>
| anti-NURR1 (1:250) | (kindly provided by Dr Benovic (Busillo et al., 2010)) | Secondary antibodies were: donkey anti-rabbit IgG-Alexa 488 (1:500; Life Technologies), donkey anti-mouse or -rabbit Cy3 (1:200, Jackson ImmunoResearch), donkey anti-rat IgG-DyLight 488 or 674 (674, Jackson ImmunoResearch). For the detection of CXCR4 Cy3-labeled streptavidin was used (1:1000, Jackson ImmunoResearch). For combined RNA in situ hybridization and immunostaining, protocols were modified according to Eisenstat et al. (Eisenstat et al., 1999) and can be provided on request.

Imaging

Images were acquired using a Zeiss AxioObserver Z1 inverted microscope with an ApoTome set-up at 200× magnification or an Olympus IX81 confocal microscope at 400× magnification. To present the RNA in situ hybridization and immunofluorescence signal in one image, brightfield images of the RNA in situ hybridization signal were assigned to a RGB channel in Axiovision software; the channel was then inverted in Adobe Photoshop.

**Ultramicroscopy**

**Preparation of specimens and clearing**

E12.5 brains were fixed in 4% PFA for 30 minutes, stained with primary (48 hours) and secondary (24 hours) antibodies, dehydrated in a graded ethanol series, placed in hexane for 1 hour and then incubated in a clearing solution of 1:2 benzyalcohol:benzylbenzoat (Sigma-Aldrich) for 48 hours at room temperature [modified from Dodt et al. (Dodt et al. 2007)].

**Fluorescence**

Optically sectioned volumetric imaging was performed using a light-sheet microscope based on an inverted microscope (TI-Eclipse, Nikon Instruments). Cleared specimens were placed in a custom-made sample cuvette (Helima) filled with clearing solution and illuminated by a thin light-sheet (thickness w0=2-6 μm, width 700 μm) (Ritter et al., 2010). Fluorescence was collected orthogonal to the excitation light-sheet by either a 10× or 20× air objective (CFI Plan Fluor, Nikon) and imaged onto a scientific grade CMOS camera (Oresa Flash 4.0, Hamamatsu) using either a standard or additional 1.5× magnification tube lens. 3D image stacks were acquired by moving the sample through the light-sheet in steps of 1 μm with a motorized sample stage (Physik Instrumente). The detection objective was placed on a piezo-driven mount (Physik Instrumente) to compensate for focal shift due to the refractive index mismatch between clearing solution (nBABB=1.56) and immersion medium (nair=1.00). The displacement of the objective was coupled linearly to the motion of the sample stage such that ΔzStage=[(1–nair/nBABB) *ΔzFOC=(1–nair/nBABB) * ΔzStage [similar to equation 1 in Silvestri et al. (Silvestri et al., 2012)].

**Image processing**

When a thin light-sheet was used, the Rayleigh length was smaller than the image field of μ0=2-6 μm) (Ritter et al., 2010). Fluorescence was collected orthogonal to the excitation light-sheet by either a 10× or 20× air objective (CFI Plan Fluor, Nikon) and imaged onto a scientific grade CMOS camera (Oresa Flash 4.0, Hamamatsu) using either a standard or additional 1.5× magnification tube lens. 3D image stacks were acquired by moving the sample through the light-sheet in steps of 1 μm with a motorized sample stage (Physik Instrumente). The detection objective was placed on a piezo-driven mount (Physik Instrumente) to compensate for focal shift due to the refractive index mismatch between clearing solution (nBABB=1.56) and immersion medium (nair=1.00). The displacement of the objective was coupled linearly to the motion of the sample stage such that ΔzStage=[(1–nair/nBABB) *ΔzFOC=(1–nair/nBABB) * ΔzStage [similar to equation 1 in Silvestri et al. (Silvestri et al., 2012)].

ΔzFOC=[1–nair/nBABB] * ΔzStage [similar to equation 1 in Silvestri et al. (Silvestri et al., 2012)].

ΔzStage=[(1–nair/nBABB) *ΔzFOC=(1–nair/nBABB) * ΔzStage [similar to equation 1 in Silvestri et al. (Silvestri et al., 2012)].
projection of all background-subtracted frames acquired in the same sample plane. Image processing was performed using MATLAB (The Mathworks) functions written for this purpose.

**Quantification**

**Fate-mapped DA neurons**

The distribution of DA neurons was quantified at three anteroposterior levels (n≥3 embryos for each time point). Sections were imaged on a Zeiss Axios Observer using the AxioVision Mosaic software. Images were acquired using a 20× LD A-Plan objective and an ApoTome set-up to assess double labeling (Zeiss). For each level, the mediolateral distribution of fate-mapped DA neurons (double-positive for TH and EYFP, TH<sup>+</sup>/EYFP<sup>+</sup>) was determined by dividing the ventral midbrain into bins of 100 μm thickness along the mediolateral axis. Statistical significance was determined using two-way ANOVA, with Bonferroni post-hoc multiple comparison test (Prism 5, GraphPad).

**Migration**

The velocity of moving particles (total distance traveled per time) in slice cultures was assessed using Fiji MTrack2 plugin (Schindelin et al., 2012). The average migration speed of migrating cells was 25 μm/hour, but ranged between 2 μm/hour and 40 μm/hour. Cells were divided in three speed categories, so that each category contained approximately one-third for the total number of TH<sup>+</sup> cells (expressed as percentage) (supplementary material Table S1). Statistical significance was analyzed with one-way ANOVA and LSD post-hoc test (n=3-5 animals). Migration trajectories of EYFP<sup>+</sup> cells were manually tracked with Fiji MtrackJ plugin (Meijering et al., 2012). The midline of a horizontal slice was assigned as x-axis. The best-fit tangential path was considered as being perpendicular to the x-axis and was assigned as y-axis. The trajectory in reference to the x- and y-axes was obtained by tracking somal coordinates through the imaging sequence. Deviations from the best-fit trajectory (θ) were represented in degrees (0°-90°) (supplementary material Table S1).

**Cxcr12 and Cxcr4 knockouts**

The area containing TH<sup>+</sup> DA neurons was divided into two bins along the dorsoventral axis (from the ventricle to the ventral pial surface) and into eight (E14.5) or ten (E16.5) bins along the mediolateral axis (from the midline to the lateral pial surface, on either side of the midline). For each side, the number of TH<sup>+</sup> cells was determined in each bin and normalized for the total number of TH<sup>+</sup> cells (expressed as percentage) (supplementary material Table S1). To take into account TH<sup>+</sup> neurons on both sides of the midline, an average was formed between the left and the right side. Statistical significance was analyzed with unpaired Student’s t-test (n=3). Migration trajectories of Cxcr12<sup>−/−</sup> and Cxcr4<sup>−/−</sup> knockout embryonic brains. A.S.A. and A.A.-P. performed the Reeler mutant, Apoe2/β2/β1 and Dab1 knockout embryonic brains. S.B. designed the study, participated in the data analysis and helped to draft and write the manuscript. All authors read and approved the manuscript.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099937/-/DC1

**References**


