Dopaminergic neurons modulate GABA neuron migration in the embryonic midbrain

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SUMMARY
Neuronal migration, a key event during brain development, remains largely unexplored in the mesencephalon, where dopaminergic (DA) and GABA neurons constitute two major neuronal populations. Here we study the migrational trajectories of DA and GABA neurons and show that they occupy ventral mesencephalic territory in a temporally and spatially specific manner. Our results from the Pitx3-deficient aphakia mouse suggest that pre-existing DA neurons modulate GABA neuronal migration to their final destination, providing novel insights and fresh perspectives concerning neuronal migration and connectivity in the mesencephalon in normal as well as diseased brains.

KEY WORDS: Midbrain, Neuronal migration, Parkinson’s disease

INTRODUCTION
Neuronal migration is a fundamental process in the development of the central nervous system because neurons eventually dwell in regions distinct from their origin. From ventricular zones, neurons and/or neuronal progenitors navigate along diverse courses, radially and tangentially, to their final destination and integrate into specific brain circuits (Corbin et al., 2001; Hatten, 2002; Marin and Rubenstein, 2001; Parnavelas, 2000). A concerted and/or sequentially regulated migration of both excitatory and inhibitory neurons is essential for the emergence of their proper connectivity and brain functions. Unlike in the telencephalon, where neuronal migration has been well elucidated, in the mesencephalon this vital event has been understudied and key factors remain to be defined. Dopaminergic (DA) neurons located in the three anatomically defined areas of the ventral mesencephalon (VM) – the substantia nigra (SN), ventral tegmental area (VTA) and retrorubral field (RRF) – are involved in controlling diverse brain functions, including motor control and cognition, emotion and reward behaviors (Björklund and Dunnett, 2007; Damier et al., 1999; Ding et al., 2011; Lennington et al., 2011; Schultz, 2001; Seeman et al., 1993; Smidt and Burbach, 2007). The migration routes of DA neurons are not well understood and the related literature is contradictory (Hanaway et al., 1971; Kawano et al., 1995), while the route of GABA neurons to the VM is unknown.

Here, we study the migratory trajectories of DA and GABA neurons and find that proper migration of GABA neurons to their final location in the VM is dependent on the pre-existing DA neuron palisade. These results provide several new concepts regarding functional interactions between DA and GABA neurons necessary for their proper migration and final connectivity that may translate into novel understanding of potential etiology as well as therapeutic development for many neurological diseases.

MATERIALS AND METHODS

Animals
Timed pregnant CD1 mice were purchased from Charles River Laboratories. Colonies of GAD65-GFP and ak/ak mice were maintained in our institutional animal facility. Day of plug discovery was designated embryonic day (E) 0. Animal experiments were in full compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the McLean Institutional Animal Care and Use Committee.

BrdU labeling and immunohistochemistry
A single BrdU injection (50 μg/g body weight) was administered to pregnant dams carrying E10 or E11 embryos. Embryonic brains were removed after 2 hours, at E13, E15, E17 and postnatal (P) day 0 stages, immersed in zinc fixative (BD Pharmingen) for 24 hours and processed for paraffin wax histology. BrdU immunohistochemistry was performed on 10-μm sections with a mouse monoclonal anti-BrdU antibody (1:75, BD Pharmingen). Other antibodies used were anti-TH (1:200, Millipore), anti-Otx2 (1:200, Neuronics), anti-GAD65/67 (Gad2/1 – Mouse Genome Informatics) (1:400, Millipore), anti-Lmx1b (1:100; Drs Carmen Birchmeier and Thomas Muller, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany), anti-Foxa2 (1:100, Santa Cruz), anti-Lmx1a (1:100, Millipore), anti-Klf6 (1:30, Sigma), anti-Pax6 (1:30, Sigma), anti-DAT (Slic6a3 – Mouse Genome Informatics) (1:200, Millipore), anti-Hetl (1:30, Sigma) and anti-calbindin (1:100, Swant). BrdU+ cells in the red nucleus and BrdU− GAD65/67− co-labeled cells in the VM were counted using ImageJ software (NIH).

Explant cultures
Basal plate (BP) and VM explants were dissected from mesencephalic slices of E15 wild-type (WT) and ak/ak embryos. Explants were plated in Matrigel (BD Biosciences) at a distance of 600 μm, overlaid with Neurobasal medium (1×, Invitrogen/Life Technologies) and co-cultured for 36 hours. Explants were fixed in 4% paraformaldehyde, stained with Hoechst (Sigma) and imaged. For quantification, BP explants were subdivided into proximal (P) and distal (D) quadrants. The areas occupied by migrating cells in each quadrant were determined using ImageJ. The P/D ratio was calculated and used as a measure of chemotaxis in each case.

Heterochronic microtransplants
BP tissue obtained from E15 GAD65-GFP mesencephalic slices was inserted into ak/ak mesencephalon using fine tungsten needles under a high-magnification stereomicroscope. For some of these ak/ak slices (with transplanted BP), the VM was discarded and substituted by VM from WT
Mesencephalic neuronal migration

**RESULTS AND DISCUSSION**

**Neuronal migration silhouette in the mesencephalon**

BrdU birthdating studies have been widely used to study neuronal migration in the developing neocortex (López-Bendito et al., 2008; Mathis et al., 2010; Ori-McKenney and Vallee, 2011; Soriano and Del Rio, 1991; Supér et al., 2000; Wines-Samuelson et al., 2005). Because BrdU is integrated into the DNA of S-phase progenitor cells, it serves as a stable marker for cells born around...
the time of injection. First, we performed a thorough and systematic BrdU birthdating study to understand mesencephalic neuronal migration in CD1 mice. We labeled neuronal progenitors born at E10 with a single BrdU pulse and followed their migration in the mesencephalon until P0 (Fig. 1A-G). BrdU-labeled cells first spread out uniformly in the mesencephalon from E10-15 (Fig. 1A-C). However, a major change in neuronal migration was observed at E17 (Fig. 1D), by which time neurons had migrated both ventrally and perpendicular to the aqueduct to form the distinct anatomical architecture of the boat-shaped SN and VTA (Fig. 1D). The red nucleus area was significantly depleted of E10-labeled neuronal progenitors by this perpendicular migration. By P0, most of the neurons had segregated to both dorsal and ventral mesencephalon (Fig. 1E). The boat-shaped architecture was confirmed by tyrosine hydroxylase (TH) staining (Fig. 1F,G). E11-labeled neuronal progenitors followed the same route as E10-labeled neuronal progenitors and formed the distinct anatomical architecture of SN and VTA in VM (supplementary material Fig. S1A). Our results corroborate those of previous studies indicating that neurons of the SN and VTA in the mouse are generated on or before E12 (Bayer et al., 1995). E13-labeled neuronal progenitors migrated predominantly to the dorsal mesencephalon and E15-labeled progenitors gave rise to a limited number of lateral neurons (supplementary material Fig. S1B,C).

Although many BrdU+ neurons in SN and VTA at P0 were dopaminergic/TH+ (Fig. 1F,G), there were also many TH– neurons. Given that DA and GABA neurons constitute two major neuronal populations in the ventral midbrain, these neurons might be GABA neurons. Although GAD mRNA expression starting at E10.5 has been reported in BP, alar plate and dorsal mesencephalon (Guimerà et al., 2006; Katarova et al., 2000), and the number, frequency and topography of GABA neurons in SN and VTA regions of the adult brain have been characterized (Korotkova et al., 2004; Nair-Roberts et al., 2008; Olson and Nestler, 2007), it is not known when and how GABA neurons become admixed with ventral mesencephalic DA neurons during development. Are GABA neurons of the VM born elsewhere, then come to reside there to form the final connectivity? To address these fundamental questions we used the GAD65-GFP mouse model, in which GABA neurons can be clearly visualized (López-Bendito et al., 2004). Strikingly, we found that whereas at E13 the VM was completely devoid of GABA neurons (Fig. 1H-K), by E17 GABA neurons were substantially intermingled with DA neurons (Fig. 1L-Q). At E13, many BP GFP+ neurons were oriented...
ventrally (Fig. 1H,I) and by E17 a cohort of GFP+ neurons were positioned in stream-like routes to the VM (Fig. 1L,M,P,Q). Further, high-magnification images showed how GABA neurons of the VM are in close physical contact with DA neurons (Fig. 1N,P,Q). Birthdating experiments revealed that many E11-labeled neuronal progenitors migrated to contribute to GABA neurons of the VM by E17 (Fig. 1R-U).

Abnormal neuronal migration in the ak/ak mesencephalon

This novel finding that DA and GABA neurons occupy separate territories in the E13 mesencephalon (Fig. 1J) prompted us to hypothesize that the early-formed DA neuron palisade might have a role in GABA neuron migration into the VM at later developmental stages. In line with this possibility, the direction and entry of the GABA neuron stream were oriented towards VM territory (Fig. 1H,I), leading to intimate contact with DA neurons (Fig. 1L-Q). To address our hypothesis and to further investigate mesencephalic neuron migration, we postulated that the Pitx3-deficient aphakia (ak) mouse might provide an ideal animal model with a defective DA neuron architecture as Pitx3 is one of the crucial regulators of mesencephalic DA neuron development (Ding et al., 2011; Kim et al., 2007; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003) and there is selective and early loss of A9 DA neurons in the SN of ak/ak mice (Hwang et al., 2005; Smidt et al., 2004; van den Munckhof et al., 2003).

Since expression of both Pitx3 and Th begins at E11, we consistently studied the migration of E11-labeled neuronal progenitors in both WT and ak/ak mice (Fig. 2). Mesencephalic sections were analyzed at E17 with BrdU and TH markers (Fig. 2A-H). Strikingly, in the ak/ak mutant, BrdU+ cells were scattered aberrantly in BP regions and failed in their perpendicular migration to the SN. The distinct anatomical architecture of the boat-shaped SN and VTA outlined by BrdU+ cell migration did not form in the ak/ak mutant (Fig. 2D), in contrast to WT (Fig. 2A; supplementary material Fig. S1A). BrdU and TH double labeling revealed many E11-labeled cells displaying a dopaminergic phenotype after arriving at their final destination in both VTA and SN regions in WT mesencephalon (Fig. 2A-C,G), whereas in the ak/ak mutant the cells were unable to reach the SN and display their full dopaminergic phenotype (Fig. 2D-F,H). Instead, these E11-labeled cells appeared to be stuck or trailing in the middle of their migratory trajectory and distributed abnormally in the red nucleus area in the ak/ak mutant (Fig. 2F,H).

To further investigate the abnormally migrating cells, we tested whether the stalled E11-labeled cells in the ak/ak red nucleus area contain DA progenitor cells. Many cells were positive for Otx2 (Fig. 2I), a marker for DA progenitors (Chung et al., 2009; Vernay et al., 2005), but never expressed the TH marker of DA neurons (Fig. 2E,F,H), indicating impaired differentiation. The stalled cells were also positive for the markers Lmx1b (Fig. 2J,K) and Foxa2 (Fig. 2L,M), confirming their DA progenitor identity. They were positive for the proliferating progenitor markers Lmx1a, Ki67 and...
The mean density of BrdU+ cells in the red nucleus of the ak/ak mutant (Fig. S2F), confirming the presence of abnormally proliferating progenitors (supplementary material Fig. S2A-E). In addition, Pax6 and negative for DAT, a marker of immature postmitotic DA neurons in the ak/ak mutant was significantly higher than in WT (Fig. 2N).

Taken together, our data provide strong evidence of the perpendicular migration of DA neurons to the VM and that this is significantly disturbed in the ak/ak mutant. Thus, in the absence of Pitx3, DA neuronal migration is impaired, contributing to severe loss of A9 DA neurons in the SN. Interestingly, we also found that migration of E13-labeled neuronal progenitors was similarly affected in the ak/ak mutant (supplementary material Fig. S3).

**Pre-existing DA neurons modulate GABA neuron migration to ventral mesencephalon**

GABA neuron development was also significantly affected in the ak/ak mesencephalon. By E17, in WT mouse embryos GABA neurons had settled together in close physical contact with TH neurons (Fig. 3A-C,G). This profile was substantially altered in the ak/ak mutant, leading to significantly limited contact of these neurons in the VM (Fig. 3D-F,H). GABA neurogenesis was unaffected in the ak/ak mutant (supplementary material Fig. S4). The stalled cells on the route of perpendicular migration or in the VM were not apoptotic (supplementary material Fig. S5), and so we examined whether the decreased GABA neuron profile in the ak/ak mutant by late embryonic stages was due to impaired migration. To search for cellular sources of guidance cues in the VM for migratory BP neurons, explants of VM were confronted with explants of BP from WT mice. BP neurons were markedly attracted towards VM (Fig. 3I,K,O). BP explants from WT mice, by contrast, showed no attraction to VM from the ak/ak mutant (Fig. 3L-O).

Birthdating studies indicated that E11-labeled neuronal progenitors contributed to GABA neurons of VM in WT embryos and many BrdU+ GAD65/67+ co-labeled cells were observed (Fig. 4A,C). In the ak/ak mutant, E11-labeled neuronal progenitors did not contribute significantly to GABA neurons of VM, as illustrated by the significant decrease in BrdU+ DA neuronal migration is impaired, contributing to severe loss of A9 DA neurons in the SN. Interestingly, we also found that migration of E13-labeled neuronal progenitors was similarly affected in the ak/ak mutant (supplementary material Fig. S3).

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with a VM from WT mouse, GFP+ cells exited the transplantation site and migrated robustly to integrate with DA neurons (Fig. 4L,N,O).

Together, these results strongly support our idea that the intact DA system of the VM guides the GABA neuron system to descend to VM and establish its connectivity with DA neurons. Furthermore, the reduction in GABA neurons observed in the ak/ak mesencephalon was reflected in adult (4 month old) mice as well (supplementary material Fig. S6).

Our data provide novel insights into neuronal migration in the embryonic mouse mesencephalon and its relevance for final ventral mesencephalic neuronal population and connectivity. First, our data support a model for DA and GABA neuron migration in the mesencephalon that depicts vertical migration of VTA precursors and perpendicular migration of both SN and VTA precursors (Fig. 4P). Second, our analysis of ak/ak mice indicates how A9 DA progenitor cells show blocked perpendicular migration and accumulate in the red nucleus area (Fig. 4Q). Perpendicular migration is therefore essential to set up the proper anatomical architecture of ventral mesencephalic structures. Third, we found that DA and GABA neurons occupy VM in a temporally sequential manner. Thus, at E13, the primary structure of DA neurons in the mesencephalon was reflected in adult (4 month old) mice as well. Furthermore, the reduction in GABA neurons observed in the ak/ak mice indicates how A9 DA neurons innervate the neurogenic subventricular zone in the midbrain of ak/ak mice. Functional diversity of ventral midbrain dopamine and GABAergic neurons. Mol. Cell. Neurosci. 29, 243-259.


