The Pitx3-deficient aphakia mouse: a naturally occurring mouse model of dopamine deficiency
van den Munckhof, P.

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Chapter 3

Midbrain dopaminergic neurons expressing calbindin are selectively preserved in absence of Pitx3

Kelvin C Luk¹, Vladimir V Rymar¹, Pepijn van den Munckhof², Claude Steriade¹
Bifsha Panojot², Jacques Drouin², Abbas F Sadikot¹

¹Cone Laboratory, Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montréal, Québec, Canada
²Unité de recherches en génétique moléculaire, Institut de recherches cliniques de Montréal (IRCM), Montréal, Québec, Canada

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Abstract

The homeodomain transcription factor Pitx3 is required for survival of midbrain dopaminergic (MesDA) neurons. Pitx3-deficient mice exhibit severe but selective developmental loss of these neurons, with accompanying locomotor deficits resembling those seen in Parkinson's disease (PD). Here we demonstrate that Pitx3 is not indiscriminately required by all MesDA neurons for maintenance and that specific MesDA subpopulations are consistently spared in its absence. Virtually all surviving MesDA neurons in the substantia nigra pars compacta (SNc) and the majority of neurons in the adjacent ventral tegmental area (VTA) expressed calbindin D$_{28K}$ (CB), a protein previously associated with resistance to injury in PD and various animal models. Cell mapping studies in wild-type (wt) mice also revealed that Pitx3 was primarily expressed in the ventral SN, a region particularly susceptible to the neurotoxin 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP). Furthermore, Pitx3-expressing SN cells were preferentially lost following acute MPTP treatment. Thus, two distinct MesDA subpopulations are present in the wt SN: resistant Pitx3-independent neurons expressing CB and susceptible Pitx3-dependent neurons. Together, these data provide a link between Pitx3 function and the selective pattern of MesDA cell loss observed in PD.
Introduction

Parkinson’s disease (PD) is a progressive multi-system neurodegenerative disorder characterized primarily by locomotor deficits arising from massive loss of midbrain dopaminergic (MesDA) neurons in the substantia nigra pars compacta (SNc). Among the multiple nuclei which comprise the mammalian MesDA system, including the substantia nigra pars compacta (SNc), ventral tegmental area (VTA) and retrorubral field (RRF), neurons within the SNc appear to be particularly susceptible in PD. The precise factors contributing to this selective vulnerability are poorly understood.

Homeodomain transcription factors play critical roles in the specification and maintenance of MesDA neurons. We and others have previously shown that Pitx3, whose expression in the brain is restricted to MesDA neurons, plays a central role in their postmitotic survival. As in PD brains, aphakia (ak) mice harboring a functional deletion in the Pitx3 gene also display profound MesDA cell loss characterized by massive nigrostriatal degeneration alongside conspicuous sparing of mesolimbic projections. As a result, DA content in the dorsal striatum is reduced by over 90%, leading to locomotor and behavioural deficits which are partially reversible with administration of 3,4-dihydroxyphenylalanine (L-DOPA).

In both PD and the widely used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin model of the disease, MesDA cell loss is most pronounced in the ventral tier of SNc (vSNc) with sparing in the dorsal layers and adjacent VTA. While MesDA neurons in ak mice already undergo cell death in early development, similarities between the pattern of ak MesDA cell loss to PD prompted us to compare vulnerable and resistant MesDA subpopulations in ak mice with those in PD.

Here we show that vSNc neurons are selectively vulnerable in absence of normal Pitx3 function, with sparing of MesDA neurons in the dorsal tier of SNc (dSNc). Nearly all surviving nigral neurons in ak mice express the calcium-binding protein calbindin D28K (CB), a marker of resistant MesDA neurons in PD. Furthermore, Pitx3 and CB are largely complementary in their expression pattern with only a small group of MesDA cells expressing both markers. In wild-type (wt) animals, exposure to MPTP also led to preferential loss of Pitx3-expressing SNc neurons. Together, these data indicate that the pattern of MesDA cell loss observed in the SNc in ak mice and possibly in PD results from the presence of resistant Pitx3-independent neurons and vulnerable Pitx3-dependent neurons.
Methods and Materials

Animals and MPTP treatment
All animal procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research, as administered by the McGill University Animal Care Committee. Ak mice were backcrossed and maintained in the C57Bl/6 background while C57Bl/6 animals were used as wt. Male animals were transcardially perfused with 4% buffered paraformaldehyde (PFA) at postnatal days (P) 1, 21, 35, 100, or >700. Brains were then removed, postfixed for 24 hours (h), and immersed in phosphate-buffered sucrose (30%) for 48 h. Sectioning was performed using a freezing microtome (Microm, Walldorf, Germany) at 40 μm. Serial coronal sections were collected in phosphate-buffered saline (PBS) and immunostained for stereology. For MPTP studies, P35 mice received four intra-peritoneal injections (20mg/kg each) of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (5mg/mL in saline; Sigma, St Louis, MO). Control animals were injected four times with a similar volume of saline. Animals were sacrificed 10 days after treatment. Statistical analysis was performed using a two-way analysis of variance (ANOVA) procedure with Tukey’s HSD post-hoc test. Main and interaction effects were calculated using SAS software (v. 6.12, Cary, NC) or Datasim (v. 1.1, Drake Bradley, Bates College, ME).

Immunohistochemistry
Complete rostrocaudal coronal series representing every sixth section (i.e. 240 μm intervals) were immunostained for tyrosine hydroxylase (TH), CB or Pitx3 using an avidin-biotin-peroxidase complex method as previously described. Briefly, sections were incubated in anti-TH (1:1000, Immunostar), anti-CB (1:2000, Sigma) or anti-Pitx3 (1:100) antibody overnight after blocking. Sections were then washed, incubated for 1 h with biotinylated anti-mouse secondary antibody (1:200, Vector), followed by avidin-biotin complex (Vector, Burlingame, CA) as per manufacturer’s instructions. The final reaction was revealed by 3,3’-diaminobenzidine. Sections were counterstained lightly in 0.1% cresyl violet, dehydrated, and coverslipped. For colocalization experiments, double immunofluorescence for TH, CB, or Pitx3 was performed on 5 μm sections from paraffin embedded mouse brains as previously described. Confocal images (40X) were collected using a Zeiss LSM510 microscope.

Stereology and computer-assisted cell mapping
Neurons positive for either TH, CB or Pitx3 were quantified using the optical dissector method as previously described. The entire rostrocaudal extent of the midbrain was examined in TH-, CB- or Pitx3-stained coronal sections (1:6 series) using an Olympus BX-40
microscope equipped with a motorized XYZ stage and StereoInvestigator software (Microbrightfield, Williston, VT). In non-TH stained sections, SNc was defined ventrolaterally by SN pars reticulata (SNr) and medially by VTA or medial lemniscus. DA neurons of SNc and VTA were distinguished by their size and mediolateral orientation. After tracing SNc and VTA at low power, TH-, CB- or Pitx3-cell counts were performed at 100X magnification (oil, NA 1.3) by employing a 60 x 60 μm counting frame in conjunction with a 12 μm dissector placed 2 μm below the surface of the section. Counting sites were assigned using a randomly placed 125 x 125 μm grid.

Results

MesDA neurons expressing CB are Pitx3-independent

While ak mice show a dramatic reduction in total MesDA neuron number, we consistently observed in these animals persistent TH-expressing subpopulations (Figure 1). In agreement with previous reports, TH-cell loss was nonuniform and most pronounced within SNc, with loss of nigrostriatal projections to the neostriatum. In contrast, VTA and mesolimbic MesDA projections to the nucleus accumbens and olfactory tubercle were relatively spared. Remaining MesDA neurons in ak animals were located predominantly in dSNc and scattered throughout VTA, reminiscent of the pattern observed in human PD brains and in animal models using the dopaminergic toxins 6-hydroxydopamine (6-OHDA) or MPTP. We therefore examined whether this Pitx3-independent subpopulation corresponded to neurons expressing CB, which labels resistant MesDA neurons in PD patients. Double-immunostaining was performed to examine coexpression of CB in persisting TH-positive cells in ak mice at P100 (Fig. 2a-f). CB staining was observed in 92% (SNc) and 87% (VTA) of TH-positive cells (Fig. 2g-j). Colocalization was also relatively uniform, reaching as high as 97% at some coronal levels examined. Thus, the majority of surviving MesDA cells in ak mice are CB-positive. In agreement with this finding, stereological quantification of total number of TH-positive neurons in midbrains of 35 day-old ak and wt animals (Fig. 2k,l) revealed an 80 ± 2.5 % (mean ± standard deviation) reduction in SNc neurons of ak mice compared to wt mice. In contrast to TH-positive cells, no difference in total CB-positive cell number could be detected between the two groups (Fig. 2l). Moreover, the ratio of total CB-positive to TH-positive neurons in SNc of ak mice reached 95.5± 4.6% compared to only 24.5 ± 1.6% in wt animals (Fig. 2m), indicating that survival of CB-positive SNc cells is unaffected in absence of Pitx3. In comparison to wt mice, VTA of ak animals also showed a marked reduction (32 ± 1.9 %) in the number of TH-positive cells. In agreement with previous studies, TH-positive cell loss in VTA was less severe than in SNc.
Figure 1. Selective loss of ventral tier SNc (vSNc) neurons and corresponding dopaminergic projections in Pitx3-deficient aphakia (ak) mice. Midbrain sections of postnatal day (P) 35 wild-type (wt) (a-e) and ak (f-j) mice immunostained for tyrosine hydroxylase (TH), demonstrating marked loss of mesencephalic dopaminergic (MesDA) neurons in various dopaminergic nuclei. In contrast to the pronounced cell loss in SNc, there is relative sparing of TH-positive neurons in VTA. Note also the persistent MesDA populations present in ak mice, primarily within the medial and dorsal aspects of SNc (g,h). MesDA neuron loss is also observed in the retrorubral field (RRF) (e,j). TH-staining at the level of the neostriatum (k,l) reveals massive loss of DA terminals in neostriatal regions of ak mice, with relative sparing observed in ventral regions, including nucleus accumbens and olfactory tubercle. Approximate levels relative to bregma are indicated. Scale bars: in a and f, 400 µm for a-j; in k and l, 500 µm.
Interestingly, the ratio of CB-positive to TH-positive cells in VTA in wt mice was nearly three-fold that of SNc (Fig. 2m). However, in contrast to SNc where CB-positive cell number remained unchanged, CB-positive cells in VTA of ak mice were also significantly reduced (Fig. 2l), suggesting that CB expression alone does not protect VTA neurons from loss of Pitx3. Together these data confirm that Pitx3 is only necessary for survival of selective MesDA neuron subpopulations in SNc and VTA. Although CB is a robust marker of Pitx3-independent MesDA neurons in SNc, a significant number of CB-positive cells are lost from VTA in Pitx3-deficient ak mice.

To determine whether these MesDA subpopulations also fluctuated as a function of age, we compared ak and wt animals at various ages (P1, 21, 35, 100, and 700; Fig. 3). Within SNc, TH-positive cell numbers stabilized in both wt and ak animals after the fifth postnatal week with no significant changes in SNc MesDA cell counts at P35, 100, or 700; Fig. 3a). Importantly, the ratios of total CB-positive to TH-positive neurons were comparable between wt and ak mice even at P700, suggesting that CB-positive neurons persist with aging in the absence of Pitx3. These observations are concordant with the selective loss of TH-positive/CB-negative cells in SNc of ak mice. Whereas total VTA TH-positive cell numbers were similar in both genotypes at birth, this population gradually decreased after P21 before stabilizing at P100 in ak animals (Fig. 3b). This suggests that loss of Pitx3-dependent subpopulations in these two regions follow different temporal trajectories with loss in SNc preceding that of VTA.

**Pitx3 expression is heterogeneous in SNc neurons**

The presence of a Pitx3-independent subpopulation led us to believe that Pitx3 may not be homogenously expressed among MesDA neurons as suggested by previous gene expression studies.

Therefore we directly compared TH and Pitx3 expression by double-immunostaining midbrain sections from wt mice. Confocal imaging revealed that the majority of TH-positive cells showed strong nuclear Pitx3 immunostaining (Fig. 4). Pitx3 was detected only within TH-positive cells in SNc, VTA, RRF and a small number of TH-positive neurons in the periaquaductal grey area (Fig. 4a-i and data not shown). Rostral-caudal mapping of sections revealed that TH-positive/Pitx3-negative neurons were intermingled with TH-positive/Pitx3-positive cells within VTA (Fig. 4c,j). In contrast to this arrangement, SNc showed a distinctive spatial segregation with TH-positive/Pitx3-negative cells located predominantly in dSNc, adjacent to TH-positive/Pitx3-positive cells that were localized in vSNc (Fig. 4f,k,m). Both markers were also detected in the so-called ventrally displaced DA neurons in the SNr.
Figure 2. Selective sparing of calbindin-expressing (CB) positive MesDA neurons in ak mice. TH and CB double-immunofluorescence of SNc (a-c) and VTA (d-f) in P100 ak mice. High power fields (inset) confirming that the majority of MesDA neurons co-express CB. Remaining MesDA neurons in ak mice were mapped from 40 µm-thick TH/CB double-immunostained sections. Representative rostral-to-caudal series of cell maps in ak mice are shown (g-j). Closed circles (red) denote TH-positive/CB-positive neurons, while TH-positive/CB-negative cells are shown as open triangles (blue). The majority of surviving MesDA neurons express both proteins, demonstrating that CB distinguishes Pitx3-dependent and Pitx3-independent MesDA neurons. Total TH-positive (k) and CB-positive (l) neuron numbers in SNc and VTA of P35 ak and wt mice quantified by stereology. The ratio of CB-positive to total TH-positive cell numbers (m) indicates relative sparing of CB-positive neurons in both MesDA regions of ak mice at P35 (* p<0.01; ** p<0.0001, t-test). Data are expressed as means ± standard deviation (SD) (n=4-5). Scale bars: in a and d, 100 µm for a-f.

Figure 3. Timetable of Pitx3-dependent MesDA neuron loss in ak mice. Total TH-positive neuron numbers in the SNc (a) and VTA (b) of wt (white bars) and ak (grey bars) mice were quantified using the optical dissector method. Counts were obtained from male animals at various ages ranging from P1 through P700. Total CB-positive neuron numbers in these two regions were also quantified independently in P35 and P700 animals (c,d) and expressed as a ratio to TH-positive cell numbers in a,b. In wt mice, CB-positive cells comprise a larger proportion of cells in VTA than in SNc. In ak mice, CB-positive cell numbers approach that of TH-positive SNc cells, suggesting no loss of this subpopulation within SNc in absence of Pitx3 expression. Data are expressed as means ± SD (n=4; **p<0.001, t-test compared to age-matched controls).
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Double-immunostaining for CB and Pitx3 (Fig. 5) was also used to confirm the chemical identity of dSNc TH-positive/Pitx3-negative cells, which possess conspicuous horizontally-oriented processes. CB-positive cells were distributed throughout VTA and RRF, but restricted in dSNc (Fig. 5a,d). The majority (89%) of Pitx3-positive neurons were not CB-immunoreactive. Furthermore, 96% of CB-positive cells lacked Pitx3-staining although a small proportion of Pitx3-positive neurons at the ventral/dorsal interface also expressed CB (Fig. 5a-d). Thus, two largely exclusive MesDA subpopulations marked by Pitx3 and CB (Pitx3-positive/CB-negative and Pitx3-negative/CB-positive, respectively) are present in SNc. As in SNc, the majority (81%) of CB-positive cells in VTA were also Pitx3-negative (Fig. 5b-d), although it is unclear whether Pitx3-positive/CB-negative cells represent the majority of MesDA neurons lost from this region in ak animals.

MesDA neurons expressing Pitx3 are selectively vulnerable to MPTP

The observation that CB-positive MesDA neurons are spared following MPTP treatment or in the absence of Pitx3 suggests that Pitx3-dependent neurons in vSNc may be selectively vulnerable to various insults, in contrast to their Pitx3-independent CB-positive neighbors. To test this hypothesis, we quantified total number of TH-positive, CB-positive and Pitx3-positive neurons in wt mice following exposure to MPTP (Fig. 6). Mice treated with MPTP showed 25% reduction in total TH-positive cell number in SNc compared to saline treated animals (Fig. 6a,b,g). Interestingly, total Pitx3-positive cell number decreased by nearly 50% (Fig. 6c,d,h), indicating that they accounted to the bulk of SNc cell loss mediated by this neurotoxin. In contrast, the number of CB-positive neurons in both MPTP- and saline-treated animals was comparable (Fig. 6e,f,i), consistent with their previously reported resistance. Interestingly, VTA did not show a significant change in total TH-positive cell number with treatment, correlating with the higher proportion of Pitx3-independent cells present relative to SNc (Fig. 2l,m and 3d). Concordant with this, Pitx3-positive VTA neurons decreased by 23% after MPTP administration whereas the number of CB-positive cells were actually slightly elevated (Fig. 6h, i). Together, these results indicate that Pitx3-expressing neurons in vSNc and VTA display increased sensitivity in a common animal model of PD.
Figure 5. Pitx3 and CB midbrain populations are largely exclusive. Midbrain sections from wt P100 mice immunostained for Pitx3 and CB were mapped using Stereo Investigator and plotted according to their expression of both markers (a-f). Pitx3-negative/CB-positive (blue circles), Pitx3-positive/CB-positive (black diamonds) and Pitx3-positive/CB-negative (red triangles) are represented. Double-stained sections from SNc (g) and VTA (h) showing that neurons expressing either CB or Pitx3 alone are distributed almost exclusively within, respectively, dorsal tier SNc (dSNc) and vSNc, with only a small subset of Pitx3-positive/CB-positive cells at the interface of these two regions. In VTA, CB and Pitx3 expressing cells also remain largely separate but are scattered among each other. D: dorsal, L: lateral. Scale bar: in h, 50 µm for g and h.

Figure 6. Selective loss of Pitx3 neurons following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment. Representative midbrain coronal sections from wt mice treated with saline (a,c,e) or MPTP (b,d,f) immunostained for TH, Pitx3 or CB. MPTP treatment led to a reduction in MesDA neurons in SNc, but VTA remained relatively intact (a,b). Cells within SNc containing Pitx3 were also dramatically reduced (c,d), whereas CB-positive cells remained unchanged (e,f). High-power fields of indicated regions (boxes) are also shown (insets). Stereological quantification of total TH (g), Pitx3 (h) and CB (i) neurons in treated (white bars) and untreated (grey bars) animals. Data are expressed as means ± SD. ** p<0.01 vs. saline control, n=5. Scale bar: in a, 400 µm for a-f.

Discussion

The non-uniform patterns of MesDA neuron losses observed in various animal models of PD and in human disease imply existence of multiple subpopulations with distinct levels of susceptibility. Identifying these individual neuronal subpopulations and factors which
contribute to their differential vulnerability should significantly further our understanding of mechanisms governing MesDA degeneration in PD and related disorders. One possible group of candidates include homeodomain transcription factors whose expression are largely restricted to MesDA neurons, namely Engrailed 1/2, Lmx1b, and Pitx3. Disruption of any of these genes leads to prominent and selective cell loss in the mesencephalon, indicating their role as critical regulators in the development and maintenance of MesDA neurons.

Our findings provide clear evidence for discrete MesDA subpopulations distinguished by their dependence on Pitx3 for survival. Importantly, the preservation of specific TH-positive neuron groups in ak mice demonstrates that not all MesDA neurons require this transcription factor for survival. On the other hand, Pitx3 appears to be critical for survival of vSNc neurons. Indeed, this same Pitx3-dependent subpopulation is also particularly susceptible to degeneration following MPTP treatment in wt animals, strongly implicating Pitx3 as a marker of vulnerable MesDA subpopulations in this region. In agreement with our initial report, cell-mapping experiments of immunostained sections showed that Pitx3 expression is predominantly restricted to these same ventral tier neurons, and represented as scattered subpopulations in VTA in normal midbrains. This finding indicates Pitx3-expressing MesDA neurons as the main population lost in ak mice. Interestingly, previous reports examining localization of Pitx3 message suggest Pitx3 message is present in all MesDA neurons. A plausible explanation would be that Pitx3-expression depends on mechanisms beyond transcriptional regulation alone, although additional studies are needed to verify this.

CB-expressing cells on the other hand, which comprise the majority of MesDA neurons in dSNc, appear to be Pitx3-independent. Previous reports have shown this group of MesDA neurons to be relatively resistant to injury in PD. Similarly, CB-expressing MesDA neurons are also spared in rodents following exposure to neurotoxins such as MPTP and 6-OHDA. Calcium-binding proteins such as CB may provide neuroprotection by reducing intracellular calcium levels which generate reactive oxygen. The fact that another calcium-binding protein, calretinin, also colocalizes to resistant MesDA subpopulations, indicates that calcium homeostasis may be a critical contributing factor. In agreement with this, it has recently been shown that the unique voltage gated L-type calcium channels expressed by SNc neurons render them susceptible to both MPTP and 6-OHDA exposure while blockade with isradipine, a common calcium channel antagonist, protects these neurons. Interestingly, CB-positive MesDA neurons in dSNc and VTA are also overwhelmingly spared in weaver mutant mice. On the other hand, previous susceptibility studies in CB-null mice failed to demonstrate a significant role for CB in
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MesDA neuronal survival, suggesting that additional factors such as differences in target innervations and electrophysiology may also play a major role. Moreover, differences between SNc and VTA observed in this study suggest that CB alone cannot account for increased resistance in MesDA neurons.

The striking resemblance between the pattern of MesDA neuron loss seen in ak mice, toxin-induced DA neurodegeneration, and human PD suggests that the Pitx3 pathway underlies the common vulnerability of these MesDA subpopulations. Recent data indicate that early survival of MesDA neurons is influenced by Pitx3 via transcriptional regulation of the enzyme aldehyde dehydrogenase 2 (Ahd2), an enzyme responsible for retinoid production. Furthermore, Pitx3 appears to regulate key components of the DA metabolic pathway such as vesicular monoamine transporter 2 (VMAT2) and vasoactive intestinal polypeptide by forming a transcriptional complex with Nurr1, another MesDA transcription factor enriched in MesDA populations. Whether Pitx3 function directly influences survival of mature MesDA neurons remains largely unexplored.

In summary, our findings show a close link between Pitx3 and MesDA neurons most susceptible to degeneration. Massive loss of Pitx3 neurons has previously been reported in human PD. The recent discovery in PD patients of dysfunctional elements regulated by Pitx3, as well as identification of polymorphisms in the PITX3 promoter region that associate with increased risk of sporadic PD, highlights the need for additional studies on the role of this transcription factor in both normal midbrain physiology and disease.