The Pitx3-deficient aphakia mouse: a naturally occurring mouse model of dopamine deficiency
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Chapter 2

Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons

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Abstract

Mesencephalic dopaminergic (MesDA) neurons play critical roles in motor and behavioural processes; their loss in Parkinson’s disease (PD) results in striatal dopamine (DA) deficiency and a hypokinetic movement disorder. The Pitx3 homeobox gene is expressed in the MesDA system. We now show that only a subset of MesDA neurons express Pitx3 and that in Pitx3-deficient aphakia mice, this subset is progressively lost by apoptosis during fetal (substantia nigra, SN) and post-natal (ventral tegmental area) development, resulting in very low striatal DA and hypokinesia. Similar to human PD, dorsal SN neurons (Pitx3-negative) are spared in mutant mice. Thus, Pitx3 defines a pathway for survival of neurons that are implicated in PD and that are required for spontaneous locomotor activity.
Pitx3 is required for survival of MesDA neurons

Introduction

The physiological role and clinical relevance of mesencephalic dopaminergic (MesDA) neurons are well recognized in schizophrenia, addictive behavioural disorders, and Parkinson’s disease (PD). Rare cases of familial PD have been linked to mutations in α-synuclein and Parkin genes, but the cause of commonly encountered sporadic cases is unknown. Studies in twins and relatives of sporadic cases, however, suggest that susceptibility to the disorder might be predisposed, highlighting the importance of genes that control development and/or maintenance of MesDA neurons.

MesDA neurons are located in the ventral midbrain to form the substantia nigra (SN) and ventral tegmental area (VTA). Differentiation and anatomical localization of MesDA neurons are dependent on the action of various diffusible factors and transcription factors. MesDA neurons develop at sites where signals of sonic hedgehog (Shh) and FGF8 intersect, both being necessary and sufficient for the induction of DA neurons. Before expression of DA-specific markers, early ventral midbrain markers like En1/2, Lmx1b, Pax2/5 and Wnt1 are expressed in these cells. The appearance of the key enzyme in DA synthesis, tyrosine hydroxylase (TH), at embryonic day (E) 11.5 of mouse development, shortly follows expression of the orphan nuclear receptor Nurr1 (E10.5) and the homeobox transcription factor Pitx3 (E11). The expression of Nurr1 is not restricted to MesDA neurons and extends to large fields in the mesencephalon and diencephalon. Nurr1 null mutant mice fail to induce TH in MesDA progenitor neurons and die soon after birth. Whether these progenitors are lost during late fetal development or maintained post-natally is not entirely clear yet.

Pitx3 expression is confined to MesDA neurons and is maintained throughout adult life in both rodents and humans. Extraneural Pitx3 expression was shown in the eye, where it is present in the developing lens. In humans, mutations of the PITX3 gene were found in two families with inherited forms of cataracts and anterior segment mesenchymal dysgenesis. Similarly, abnormal eye lens development was observed in a naturally occurring mouse mutant, the aphakia (ak) mouse, which has two 5′ deletions in the Pitx3 gene, including one that deletes exon1.

Here we show that Pitx3 is only expressed in the ventral tier of the SN pars compacta (vSNc) and in about half of VTA DA neurons. In ak mice, we show undetectable midbrain Pitx3 expression, selective degeneration of vSNc DA neurons as well as of roughly half VTA neurons and greater than 90% decrease in dorsal striatal DA levels in association with marked reduction in spontaneous locomotor activity. The strong correlation between
Pitx3-expressing TH neurons and neuronal losses in ak mice and PD patients suggests that Pitx3 defines the neuronal population that is more susceptible to degeneration in PD. Ak mice thus represent a highly specific mouse model of neuronal loss in human PD.

Materials and Methods

Animals
Ak mice originate from The Jackson Laboratories. The autosomal recessive ak mutation arose spontaneously in the 129/Sv-S1 strain, and was subsequently crossed into the C57Bl/6 background. Ak mice used in this study were maintained in the C57Bl/6 background and provided to us by Dr. Jeff Murray, University of Iowa. C57Bl/6 mice were used as wild-type (wt) mice. For timed breeding experiments, mice were mated and the morning a vaginal plug was observed was considered E0.5.

Brain preparation and immunohistochemistry
Male post-natal day (P) 1, P21, P50 and P100 wt and ak mice were transcardially perfused with buffered 4% paraformaldehyde (PFA). Brains were collected, postfixed for 24 hours (h) and embedded in paraffin (P50) or cryoprotected in 30% sucrose for an additional 48 h (P1, P21 and P100). P50 midbrain-containing sections (5 μm) were mounted and immunostained for TH and Pitx3. P1, P21 and P100 brains were cut into 50-μm coronal sections encompassing the entire striatum and midbrain using a freezing microtome. Free-floating sections were collected for immunohistochemistry as separate sets so that each set contained every third serial section. One set of sections was immunostained for TH, another set was processed using 0.1% Cresyl Violet as a Nissl stain. Rostro-caudal position of sections was assessed with the aid of the mouse brain atlas of Franklin and Paxinos. For embryos, pregnant mothers were perfused transcardially with 4% PFA. Embryos were dissected and their heads were postfixed for 24 h and embedded in paraffin. Midbrain-containing sections (5 μm) were mounted and immunostained for TH.

Immunostaining was performed using an avidin-biotin-peroxidase complex (ABC) method and a fluorescein/rhodamine-fluorochrome labelling method. Antibodies and dilutions used: anti-Pitx3, 1:10; anti-TH (Chemicon polyclonal), 1:100; anti-TH (Immunostar monoclonal), 1:1000. Confocal microscopy was performed using a Zeiss LSM510 instrument. Apoptotic cells were identified using the Apoptag kit from Intergen according to the manufacturer’s recommendations. Percentage apoptotic cell was calculated relative to nuclei counted on Nissl-stained sections.
Pitx3 is required for survival of MesDA neurons

Stereology and quantitative morphology

Unbiased estimates of MesDA neurons were obtained using the optical dissector method of West and Gundersen. The entire rostro-caudal extent of the midbrain was examined in a 1:3 series of TH-stained coronal sections using an Olympus BX-40 microscope equipped with a motorized XYZ stage and StereoInvestigator software (Microbrightfield Inc.). The SN and VTA were traced at low power (10x). TH cell counts were performed at 100x magnification (oil, NA 1.3) using a 60 x 60 µm counting frame. A 10 µm dissector was placed 2 µm below the surface of the section at counting sites located at 150 µm intervals after a random start. Cell densities within SNc and VTA were determined in cresyl violet stained sections delineated according to adjacent TH-stained sections. Nissl-stained profiles greater than 7 µm in diameter were counted. Total profile counts were then divided by SNc or VTA surface area estimated with the StereoInvestigator software.

Locomotor activity measurements

Male wt and ak mice of approximately 115 days old were maintained in standard animal housing conditions with a 12 h light-dark cycle and lights on at 6 a.m. Tests were carried out between 4 p.m. and 3 p.m. the next day. At 3.30 p.m., mice were placed in the 43 X 43 cm Plexiglas arena of the Opto-Varimex-3 photocell-base monitor (Columbus Instruments) with water and food freely available, and recordings started 30 minutes later. The Opto-Varimex-3 animal activity monitor employs a 15 X 15 photocell beam grid to measure spontaneous ambulatory and stereotypic activities like grooming, scratching and other non-ambulatory activities (as well as the amount of time spent on these activities) by separating beam interruptions associated with ambulatory activity from total activity.

Dopamine quantitation

Male wt and ak mice of approximately 130 days old were analyzed for postmortem tissue content of DA. After cervical dislocation, brains were cut into 1 mm sections on a ice-cold dissection plate; dorsal and ventral striatum were collected from two sections per brain with a biopsy punch (0.5 mm diameter). Homogenization of brain samples and DA quantitation by reverse-phase high pressure liquid chromatography (HPLC) with electrochemical detection were done as described previously (Ste-Marie et al. 1999). Protein content was determined using the bicinechonic acid (BCA) assay in order to normalize DA context.
Results and Discussion

Loss of Pitx3-positive TH-positive neurons in aphakia mice

To test whether ak mice are deficient in midbrain Pitx3 expression, we assessed Pitx3 levels using an antibody against Pitx3 in matched coronal sections through the midbrain of young adult (P50) ak and wt mice. Cytoplasmic TH (Fig. 1A) and nuclear Pitx3 (Fig. 1C, E) immunostained the same midbrain region in wt mice, whereas no Pitx3-immunoreactive cells were found in the midbrain of ak mice (Fig. 1D). A marked reduction of the MesDA system was also noted in ak mice (compare Fig. 1B with 1A). In order to precisely document these differences, serial midbrain sections of P100 mice were systematically analyzed for TH-immunoreactivity (Fig. 1F-K). The MesDA system includes the SN (Fig. 1F, H) and VTA (Fig. 1H, J). The SN is subdivided into pars reticulata (SNr) and compacta (SNC), with the latter containing the majority of TH-positive cell bodies (Fig. 1F). In ak mice, SNr and most of SNC are depleted of TH-positive fibers and cells, respectively (Fig. 1G), with the exception of dorsal tier SNC (dSNC) where TH-positive cells are preserved (Fig. 1I, K). The VTA is also affected, but to a lesser degree (Fig. 1I, K). To obtain an unbiased estimate of the number of TH-positive neurons in the SN and VTA, we performed stereological analysis on serial sections throughout the entire midbrain of wt and ak mice. Total TH-positive cells were reduced by 71% in SN and by 52% in VTA of ak mice compared to wt (Fig. 1L). To determine whether there is an actual loss of neurons or only of TH expression, total neuron densities were evaluated in Nissl-stained sections. This analysis showed 57% reduction of Nissl-stained neurons in SNC and 34% reduction in VTA (Fig. 1M). The strong correlation between numbers of TH-positive cells and neuronal densities (Nissl) indicates a net loss of MesDA neurons in ak mice. The maintenance of TH-positive neurons in dSNC of ak mice is provocative in the context of human PD where relative sparing of these neurons also occurs.

In this context, we re-evaluated the expression of Pitx3 in the MesDA system. This analysis revealed that Pitx3 and TH are co-expressed only in a subset of SN and VTA neurons. Pitx3-positive neurons account for most TH-positive neurons in vSNC (Fig. 1N) and for about half of TH-positive neurons in VTA, where both populations are intermingled (Fig. 1O). Dorsal tier SNC largely contains Pitx3-negative TH-positive neurons. These studies thus show that the MesDA system is composed of two previously unrecognized neuronal subpopulations that are differentiated by expression of Pitx3. The perfect correlation between Pitx3 expression and neuronal losses in ak mice strongly suggests that Pitx3 is required for development and/or maintenance of the Pitx3-expressing subset of neurons. This strong correlation is also consistent with the exclusion of other genes of the ak locus in the phenotype of ak mice.
Figure 1. Aphakia (ak) mice have no detectable Pitx3 and a markedly reduced midbrain dopaminergic system. (A-D) Adjacent coronal midbrain sections containing substantia nigra (SN) in post-natal day (P) 50 wild-type (wt) (A,C) and ak (B,D) mice immunostained for tyrosine hydroxylase (TH) (A,B) and Pitx3 (C,D). Rostrocaudal positions are indicated as millimeters relative to bregma in the lower right corner. (E) High-power view of SN shown in C, highlighting wt nuclear Pitx3 staining. In contrast, none of the weak ak background staining in D was nuclear. (F-K) Equivalent rostral-to-caudal coronal midbrain sections of P100 wt (F,H,J) and ak (G,I,K) mice immunostained for TH. (L) Stereological analysis of TH-positive cells of left SN and ventral tegmental area (VTA) in wt and ak mice. Data are represented as means ± standard error of the mean (SEM) (n=4). (M) Density of Nissl-stained cell bodies in left SN and VTA of wt and ak mice (n=4). A statistically significant decrease in TH-positive cell bodies and density of Nissl-stained cell bodies was detected in SN and VTA of ak mice compared with wt controls (p<0.01, t-test). (N,O) Coronal sections through right SN (N) and VTA (O) of a P50 wild-type mouse immunostained for TH (fluorescein-labeled, green) and Pitx3 (rhodamine-labeled, red) analyzed by confocal microscopy. Scale bars: in A, 125 μm for A-D; in F, 250 μm for F-K; in E, 30 μm for E; in N and O, 30 μm.
Indeed, expression of the Gbf1 gene is not affected in *ak* mice and that of Cig30 is only reduced by about 50%; this latter gene is primarily expressed in liver and skin and codes for a protein that is implicated in long-chain fatty acid recruitment.

**Pitx3 serves a maintenance function**

In order to address the origin of neuronal deficit in *ak* mice, we analyzed the developing MesDA system. At E12.5, most MesDA neurons have been formed and during the perinatal period, the MesDA system undergoes phenotypic maturation which includes developmental/programmed cell death. TH immunostaining throughout the E12.5 midbrain did not show significant differences between *ak* and wt mice (Fig. 2A-H), suggesting that early developmental processes are not affected in *ak* mice. At P1, however, the SN of *ak* mice is almost completely devoid of TH-positive cells (Fig. 2I compared to H), whereas the VTA is not affected (Fig. 2H, I, J). When counted, TH-positive cells were found to be reduced by 91% in P1 SN, but not in VTA (Fig. 2I). By P21, TH-positive cells are reduced by 82% in the SN and tend to be reduced in the VTA (Fig. 2K). Collectively, these data suggest that SN TH-positive neurons disappear during the fetal period, whereas VTA neurons are lost later with 52% reduction at P100 (Fig. 1L). To assess whether apoptosis contributes to the loss of MesDA cells in *ak* mice, we compared the frequency of TUNEL-positive cells in the SNc of P1 *ak* and wt mice. A significant increase in the frequency of apoptotic cells was observed in *ak* mice (Fig. 2L), in agreement with neuronal losses in SNc of P1 *ak* mice (Fig. 2J). These data indicate that early differentiation of MesDA neurons is not highly dependent on Pitx3, as shown in *ak* mice that carry a strongly hypomorphic (and possibly null) allele of this gene. However, survival of Pitx3-expressing MesDA neurons requires significant Pitx3 expression. Most sensitive are the vSNc neurons that are severely depleted by birth in *ak* mice, in contrast to those of VTA that are lost later.

**Striatal dopamine deficiency**

SN dopaminergic neurons project primarily to the dorsal striatum to regulate motor control, whereas VTA dopaminergic neurons project to the ventral striatum and modulate emotional behaviour. The impact of MesDA neuronal depletion in *ak* mice was assessed by immunohistochemical staining of striatal TH fibers (Fig. 3A, B) and HPLC measurement of striatal DA levels (Fig. 3C, D). A dramatic reduction of dopaminergic innervation was observed in the dorso-lateral striatum of *ak* mice, with relative sparing in the ventral striatum (Fig. 3B compared to A). Corresponding striatal DA levels were reduced by 93% in the dorsal striatum and by 69% in the ventral striatum (Fig. 3C, D). The severe depletion of dorsal striatal DA levels, which are supplied from vSNc neurons, correlates well with the pattern of DA deficiency observed in PD patients.
Pitx3 is required for survival of MesDA neurons

![Figure 2.](image)

**Figure 2.** TH-positive MesDA neurons are lost primarily during fetal period for SN and during post-natal period for VTA. (A) Plane of sections for analysis of TH-positive neurons in brains of embryonic day (E) 12.5 embryos. (B-G) Equivalent rostral-to-caudal sections through the midbrain of E12.5 wt (B,D,F) and ak (C,E,G) embryos immunostained for TH. (H,I) Coronal midbrain sections containing SN and VTA in P1 wt (H) and ak (I) mice immunostained for TH. (J) Stereological analysis of TH-positive cells of left SN and VTA in P1 wt and ak mice. Data are represented as means ± SEM (n=4). (K) Stereological analysis of TH-positive cells of left SN and VTA in P21 wt and ak mice (n=4). A statistically significant decrease in TH-positive cell bodies was detected in SN of P1 and P21 ak mice compared with wt controls (p<0.01, t-test) with no difference in VTA. (L) Frequency of apoptotic cells revealed by TUNEL assay in left SNC of P1 wt and ak mice (n=4). A statistically significant increase in the frequency of apoptotic cells was detected in SNC of P1 ak mice compared with wt controls (p<0.05, t-test).
Figure 3. Ak mice have deficient striatal dopaminergic innervation. (A,B) Coronal sections through left striatum of P100 wt (A) and ak (B) mice immunostained for TH. Rostrocaudal positions are indicated as millimeters relative to bregma in the lower right corner. Scale bar: in A, 250 µm for A and B. (C,D) DA concentration in dorsal (C) and ventral (D) striatum of P130 wt and ak mice. Data are represented as means ± SEM (n=4). A statistically significant decrease in DA concentration was detected in the dorsal and ventral striatum of ak mice compared with wt controls (p<0.01, t-test).

Reduced spontaneous locomotor activity
We then determined whether ak mice display altered locomotor behaviour by measuring spontaneous ambulatory and stereotypic activities over 23 h periods using a photocell grid counter. During the day, when mice are normally less active, no differences were observed between groups (Fig. 4A-E). However, ak mice showed a marked reduction in ambulatory (Fig. 4A) and stereotypic (Fig. 4D) activities during the night, as they walked 71% less than wt (Fig. 4B), spent 69% less time walking (Fig. 4C), made 53% less stereotypic movements (Fig. 4E) and spent 44% less time making stereotypic movements (Fig. 4F). Conversely, they spent 38% more time resting (Fig. 4G, H). In view of the ak mice eye defect, it is interesting to contrast the reduction of spontaneous movement in ak mice
Figure 4. Ak mice have impaired spontaneous locomotor activity. (A) Spontaneous ambulatory activity of mice recorded over 23 hours. The distance (cm) covered during each 1 hour period is shown for wt and ak mice. (B) Average distance covered per hour for wt and ak mice during daytime and night-time. (C) Average ambulatory time spent per hour for the recordings shown in A during daytime and nighttime. (D) Spontaneous stereotypic movements of mice recorded over 23 hours. The stereotypic movements during each 1 hour period are shown for wt and ak mice. (E) Average numbers of stereotypic movements per hour for wt and ak mice during daytime and nighttime. (F) Average time spent making stereotypic movements per hour for the recordings shown in D during daytime and nighttime. (G) Resting time of mice recorded over 23 hours. The resting time during each 1 hour period is shown for wt and ak mice. (H) Average resting time per hour for wt and ak mice during daytime and nighttime. (I) Average speed of spontaneous ambulatory movements for wt and ak mice during daytime and night-time. Data are represented as the means ± SEM (n=5). Locomotor activity scores for ak mice that are significantly different from wild-type scores are marked with an asterisk (p<0.01, t-test).
with the effects of gene mutations that eliminate circadian rhythms, such as mutations of the clock or Per1 and Per2 genes. The latter result in loss of diurnal rhythmicity, but not in reduction of total movement per 24 h period as observed in ak mice. Moreover, the speed of spontaneous ambulatory movements was not different in ak compared to wt mice (Fig. 4I), suggesting that the ak mutation and the associated blindness do not impair peripheral motor function. These results indicate that ak mice display marked akinesia.

**Pitx3 and neurodegeneration in Parkinson’s disease**

Ak mice thus recapitulate cardinal features of PD, in particular the akinetic subtype of PD. Indeed, the preferential loss of vSNc TH-positive neurons together with severe depletion of dorsal striatal DA levels and associated hypokinesia are very similar to the pathogenesis of PD. This close similarity raises the possibility that PD patients are preferentially susceptible to loss of Pitx3-positive rather than Pitx3-negative MesDA neurons (Fig. 5). This hypothesis is supported by previous observations, but will demand further investigation.

Previously reported models of MesDA neuronal deficiency may not be as selective or as similar to PD. Indeed, Nurr1-deficient mice have complete agenesis of MesDA neurons and die soon after birth. Similarly, Lmx1b-deficient mice have complete loss of MesDA neurons from E16, major deficits throughout the midbrain and limb and kidney defects. Furthermore, currently available animal models for PD, whether induced by neurotoxins or by overexpression of different forms of α-synuclein or Parkin, have not been able to explain the highly specific and stereotypic pattern of MesDA cell loss in human PD, with vSNc being most affected. In contrast, ak mice are deficient in this specific subset of MesDA neurons and have normal other midbrain structures. They may thus provide a useful model to test therapies (drugs, cellular or gene therapy) for PD and to define a molecular mechanism explaining the selective sensitivity of Pitx3-expressing MesDA neurons to degeneration.

Finally, the dependence on Pitx3 for survival of Pitx3-positive TH neurons, and the sensitivity of Pitx3-positive MesDA cells to degenerate in PD, suggest that Pitx3-dependent function(s) may relate to the pathogenesis of human PD. Such function or downstream target gene(s) may contribute to control cell survival/death in development and/or in pathogenesis of the MesDA system. Pitx3 gene mutations may be involved in the etiology of diseases that affect the MesDA system. Thus far, two PITX3 mutations have been identified in families with autosomal-dominant cataracts and autosomal-dominant anterior segment mesenchymal dysgenesis.
Neuronal cell loss in *aphakia* mice and Parkinson’s disease

![Diagram](https://example.com/diagram.png)

**Figure 5.** Similar distribution of MesDA neuronal losses in *aphakia* mice and individuals with Parkinson’s disease (PD). (A) The right midbrain of a normal mouse showing the distribution of TH-positive/Pitx3-positive (green with red core) and TH-positive/Pitx3-negative neurons (green) in SNc and VTA. Most vSNc TH-positive neurons are Pitx3 positive, whereas dSNc largely contains Pitx3-negative TH-positive neurons. About half of the VTA TH-positive neurons are Pitx3 positive, and both populations are intermingled. (B) In *aphakia* mice, SNc Pitx3-positive neurons are lost between E12.5 and P1, whereas VTA cells are lost postnatally. (C) Outline of the right MesDA system of a normal human showing the distribution of TH-positive neurons in SNc and VTA. (D) Individuals with PD typically have most severe cell depletion in vSNc, followed by dSNc and VTA (modified, with permission, from Jellinger). A decrease of PITX3-positive neurons was shown in samples from individuals with PD, the regional distribution of human PITX3-positive neurons remains to be established.
These patients are not known to have parkinsonian symptoms. It is noteworthy that both mutations have dominant effects in patients that still have an intact PITX3 allele. Since a midbrain phenotype may not be expected in hemizygous carriers as heterozygous ak mice do not exhibit any phenotype (data not shown),\(^{358}\), it is likely that these human mutations cause a dominant effect that may for example, impair protein-protein interactions.\(^{307}\) This would be consistent with the position in the N- or C- termini of Pitx3 rather than in the homeodomain that has been implicated in many loss-of-function mutations in the related PITX2 gene.\(^{12}\) Thus, it would be worthwhile to investigate whether PITX3 allelic polymorphism can be detected in families with PD.

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