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DEVELOPMENT AND DISEASE

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

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SUMMARY

Mesencephalic dopaminergic (MesDA) neurons play crucial roles in motor and behavioral processes; their loss in Parkinson's disease (PD) results in striatal dopamine (DA) deficiency and 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 postnatal (ventral tegmental area) development, resulting in very low striatal DA and akinesia. Similar to human PD, dorsal SN neurons (which are 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.

Key words: Homeobox, Transcription factor, Pitx3 (Ptx3), Midbrain, Dopamine

INTRODUCTION

The physiological role and clinical relevance of mesencephalic dopaminergic (MesDA) neurons are well recognized in schizophrenia, addictive behavioral disorders (Egan and Weinberger, 1997; Swanson et al., 1998) and, most importantly, Parkinson's disease (PD). Rare cases of familial PD have been linked to mutations in the α-synuclein and Parkin genes (Polymeropoulos et al., 1997; Kitada et al., 1998), but the cause of the commonly encountered sporadic cases is unknown. Studies in twins and relatives of sporadic cases, however, suggest that susceptibility to the disorder might be predisposed prenatally (Tanner et al., 1999; Piccini et al., 1999; Montgomery et al., 1999; Sveinbjornsdottir et al., 2000), highlighting the importance of genes that control development and/or maintenance of MesDA neurons.

MesDA neurons are located in the ventral midbrain and 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 the signals of sonic hedgehog (Shh) and Fgf8 intersect, both being necessary and sufficient for induction of DA neurons (Ye et al., 1998). Before expression of DA-specific markers, early ventral midbrain markers like *En1/2*, *Lmx1b*, *Pax2/5* and *Wnt1* are expressed in these cells (Hynes and Rosenthal, 1999; Smidt et al., 2000). The appearance of the key enzyme in DA synthesis, tyrosine hydroxylase (TH) at embryonic day 11.5 (E11.5) of mouse development shortly follows expression of the orphan nuclear receptor Nurr1 (Nr4a2 – Mouse Genome Informatics) (E10.5) and of the homeobox gene *Pitx3* (E11). The expression of *Nurr1* is not restricted to MesDA neurons and extends in a large field in the mesencephalon and diencephalon (Zetterstrom et al., 1996). *Nurr1*-null mice fail to induce TH in MesDA progenitor neurons and die soon after birth (Zetterstrom et al., 1997). Whether these progenitors are lost during late fetal development or maintained postnatally is not entirely clear yet (Saucedo-Cardenas et al., 1998; Witta et al., 2000).

Pitx3 expression is, at the brain level, confined to MesDA neurons and is maintained throughout adult life in both rodents and humans (Smidt et al., 1997). Extraneural *Pitx3* expression was shown in the eye, where it is present in the developing lens (Semina et al., 1997). In humans, mutations of the *PITX3* gene

have been found in two families with inherited forms of cataracts and anterior segment mesenchymal dysgenesis (Semina et al., 1998). 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 (Rieger et al., 2001), including one that deletes exon 1.

We show that *Pitx3* is only expressed in the ventral tier of the SN pars compacta (vSNc) and in about half of the 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 or in individuals with PD 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

The *ak* mice originate from The Jackson Laboratories. The autosomal recessive *ak* mutation arose spontaneously in the 129/Sv-Sl^{J} strain (Varnum and Stevens, 1968) and was subsequently crossed into the C57BL/6 background (Semina et al., 2000). The 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 mice. For timed breeding experiments, mice were mated and the morning a vaginal plug was detected was considered to be E0.5.

Brain preparation and immunohistochemistry

Male P1, P21, P50 and P100 wild-type and *ak* mice were transcardially perfused with buffered 4% paraformaldehyde. Brains were collected, postfixed for 24 hours and embedded in paraffin (P50) or cryoprotected in 30% sucrose for an additional 48 hours (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. Rostrocaudal position of sections was assessed with the aid of the mouse brain atlas of Franklin and Paxinos (Franklin and Paxinos, 1997). For embryos, pregnant mothers were perfused transcardially with 4% paraformaldehyde. Embryos were dissected and their heads were postfixed for 24 hours and embedded in paraffin wax. 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 labeling method. Antibodies and dilutions used: anti-Pitx3 (Lebel et al., 2001), 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.

Stereology and quantitative morphology

Unbiased estimates of midbrain DA neurons were obtained using the optical dissector method of West and Gundersen (West and Gundersen, 1990; West, 1993). The entire rostrocaudal 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 (Microbrightfiel). The SN and VTA were traced at low power (10×). TH cell counts were performed at 100× magnification (oil, NA 1.3) using a 60×60 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 wild-type and *ak* mice of ~115 days of age were maintained in standard animal housing conditions with a 12 hour light-dark cycle and lights on at 6 am. Tests were carried out between 4 pm and 3 pm the next day. At 3.30 pm, mice were placed in the 43×43 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 15×15 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 wild-type and *ak* mice of ~130 days of age were analyzed for postmortem tissue content of DA. After cervical dislocation, brains were cut into 1 mm sections on an 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 HPLC with electrochemical detection were done as described previously (Ste-Marie et al., 1999). Protein content was determined using the BCA assay in order to normalize dopamine 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 (postnatal day 50, P50) *ak* and wild-type mice. Cytoplasmic TH (Fig. 1A) and nuclear Pitx3 (Fig. 1C,E) immunostained the same midbrain region in wild-type 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 document these differences precisely, 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 the dorsal tier of the SNc (dSNc) where TH-positive cells are preserved (Fig. 1I,K). The VTA

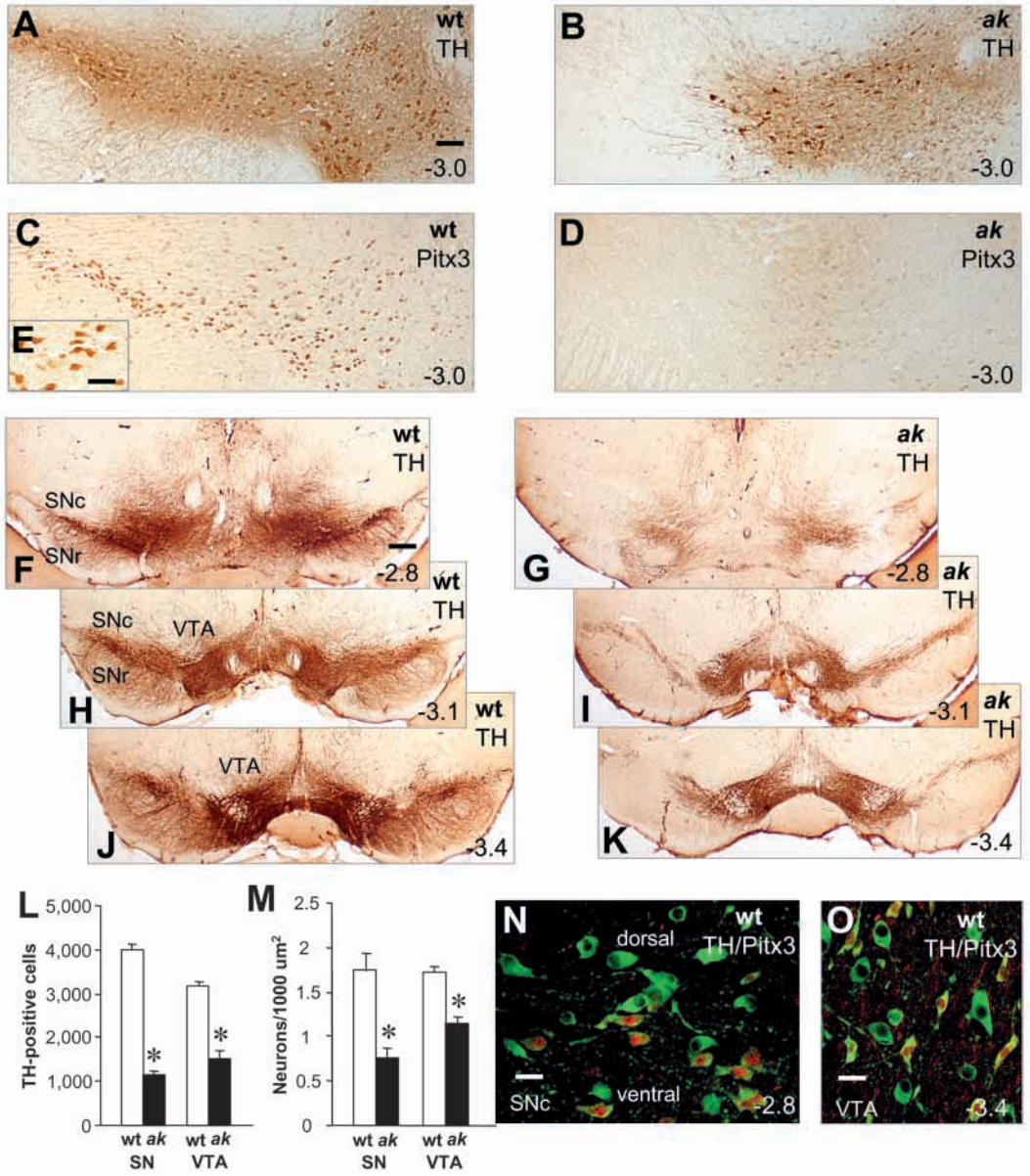

Fig. 1. The *aphakia* (*ak*) mice have no detectable Pitx3 and a markedly reduced midbrain dopaminergic system. (A-D) Adjacent coronal midbrain sections containing the SN in P50 wild-type (A,C) and *ak* (B,D) mice immunostained for TH (A,B) and Pitx3 (C,D). Rostrocaudal positions are indicated as millimeters relative to bregma in the lower right corner. (E) High-power view of the SN shown in C, highlighting the nuclear staining. By contrast, none of the weak background staining in D was nuclear. (F-K) Equivalent rostral-to-caudal coronal midbrain sections of P100 wild-type (F,H,J) and *ak* (G,I,K) mice immunostained for TH. (L) Stereological analysis of TH-positive cells of the left SN and VTA in wild-type and *ak* mice. The data are represented as the means±s.e.m. (n=4). (M) Density of Nissl-stained cell bodies in the left SN and VTA of wild-type and *ak* mice (n=4). A statistically significant decrease in TH-positive cell bodies and density of Nissl-stained cell bodies was detected in the SN and VTA of *ak* mice compared with controls (P<0.01, t-test). (N,O) Coronal sections through the 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.

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 wild-type and *ak* mice. Total TH-positive cells were reduced by 71% in the SN and by 52% in the VTA of *ak* mice compared with wild type (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 a 57% reduction of Nissl-stained neurons in the SNc and a 34% reduction in the 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 the dSNc of *ak* mice is provocative in the context of human PD, where relative sparing of these neurons also occurs

(Fearnley and Lees, 1991). 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 the ventral SNc (Fig. 1N) and for about half of the TH-positive neurons in the VTA, where both populations are intermingled (Fig. 1O). In the SN, Pitx3-positive DA neurons are essentially those of the ventral tier, whereas the dSNc 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. Indeed, expression of the *Gbf1* gene is not affected in *ak* mice and that of *Cig30* is reduced only by about 50% (Rieger et al., 2001); this latter gene is primarily expressed in liver and skin and codes for a protein that is implicated in long-chain fatty acid synthesis (Tvrdik et al., 1997).

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 postnatal period (Bayer et al., 1995), the MesDA system is undergoing phenotypic maturation which includes developmental/programmed cell death (Jackson-Lewis et al., 2000). TH immunostaining throughout the E12.5 midbrain did not show significant differences between *ak* and wild-type mice (Fig. 2A-H), suggesting that the 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 (compare Fig. 2I with 2H), whereas the VTA is not affected (Fig. 2H-J). When counted, TH-positive cells were found to be reduced by 91% in the P1 SN, but not in the VTA (Fig. 2J). 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,

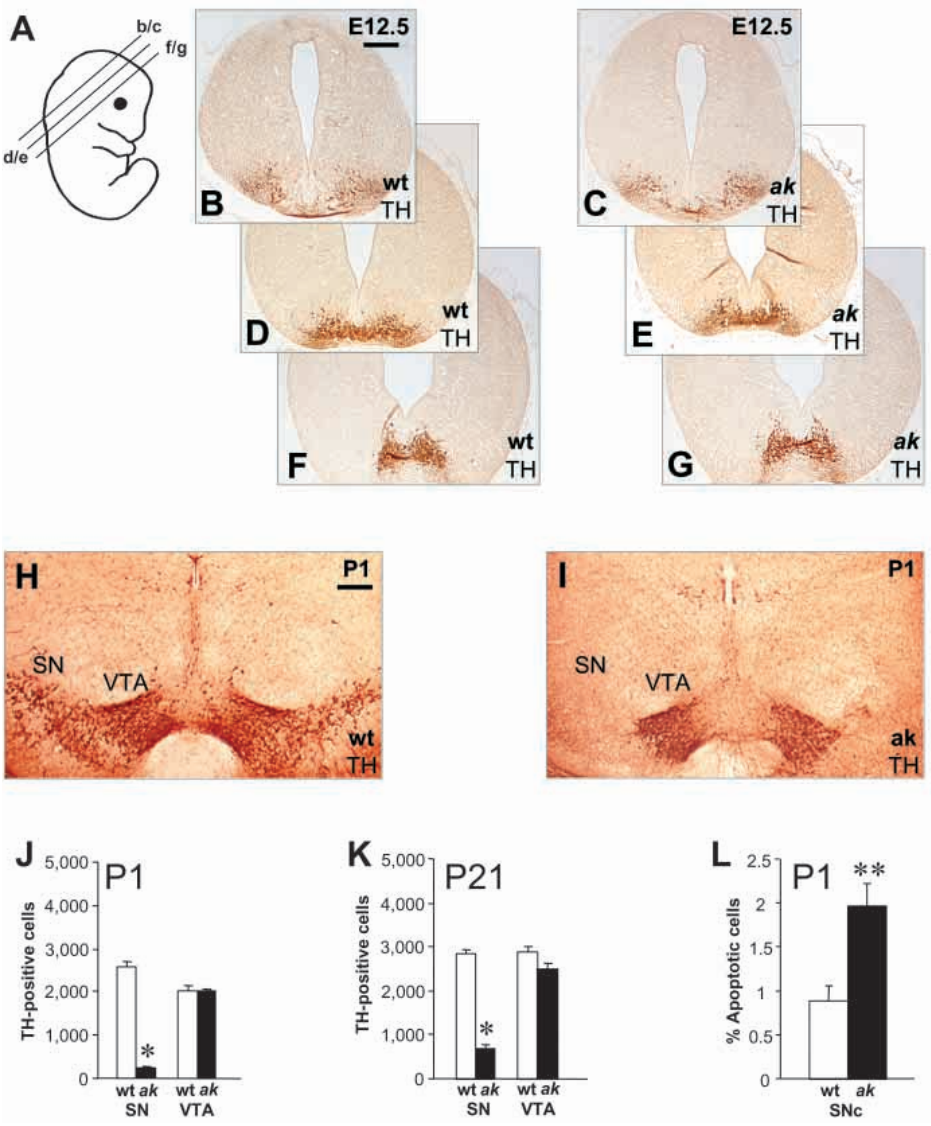

Fig. 2. TH-positive MesDA neurons are lost primarily during fetal period for the SN and during postnatal period for VTA. (A) Plane of sections for analysis of TH-positive neurons in brain of E12.5 embryos. (B-G) Equivalent rostral-to-caudal sections through the midbrain of E12.5 wild-type (B,D,F) and *ak* (C,E,G) embryos immunostained for TH. (H,I) Coronal midbrain sections containing the SN and VTA in P1 wild-type (H) and *ak* (I) mice immunostained for TH. (J) Stereological analysis of TH-positive cells of the left SN and VTA in P1 wild-type and *ak* mice. The data are represented as the mean±s.e.m. (n=4). (K) Stereological analysis of TH-positive cells of the left SN and VTA in P21 wild-type and *ak* mice (n=4). A statistically significant decrease in TH-positive cell bodies was detected in the SN of P1 and P21 *ak* mice compared with controls (P<0.01, t-test) with no difference in the VTA. (L) Frequency of apoptotic cells revealed by TUNEL assay in the left SNc of P1 wild-type and *ak* mice (n=4). A statistically significant increase in the frequency of apoptotic cells was detected in the SNc of P1 *ak* mice compared with controls (P<0.05, t-test).

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 wild-type mice. A significant increase in the frequency of apoptotic cells was observed (Fig. 2L), in agreement with neuronal loss in SNc of P1 mice (Fig. 2J).

These data indicate that early differentiation of MesDA neurons is not highly dependent on the *Pitx3* gene, 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 the 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 behavior (Björklund and Lindvall, 1984). The impact of MesDA neuronal depletion in *ak* mice was assessed by immunohistochemical staining of striatal TH fibers (Fig. 3A,B) and high pressure liquid chromatography (HPLC) measurement of striatal DA levels (Fig. 3C,D). A dramatic reduction of dopamine-mediated innervation was observed in the dorsolateral striatum of *ak* mice, with relative sparing in the ventral striatum (compare Fig. 3B with 3A). 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 the vSNc correlates well with the pattern of DA deficiency observed in individuals with PD (Kish et al., 1988).

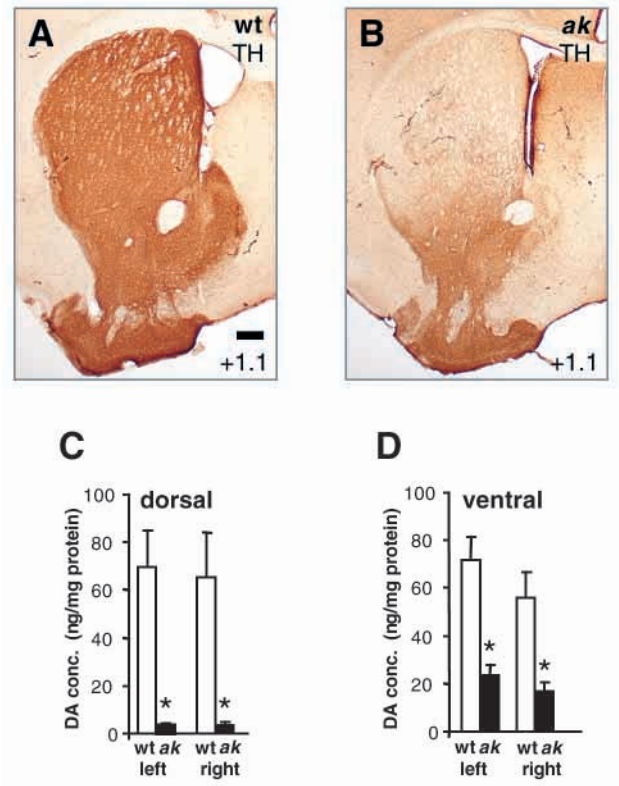

Fig. 3. The *ak* mice have deficient striatal dopaminergic innervation. (A,B) Coronal sections through the left striatum of P100 wild-type (A) and *ak* (B) mice immunostained for TH. Rostrocaudal positions are indicated as millimeters relative to bregma in the lower right corner. Scale bar: 250 μm. (C,D) DA concentration in dorsal (C) and ventral (D) striatum of P130 wild-type and *ak* mice. The data are represented as the mean±s.e.m. (n=4). A statistically significant decrease in DA concentration was detected in the dorsal and ventral striatum of *ak* mice compared with controls (P<0.01, t-test).

Reduced spontaneous locomotor activity

We then determined whether *ak* mice display altered locomotor behavior by measuring spontaneous ambulatory and stereotypic activities over 23 hour 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 wild type (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 with the effects of gene mutations that eliminate circadian rhythms, such as mutations of the *Clock* or *Per1* and *Per2* genes (King et al., 1997; Zheng et al., 2001). The latter result in loss of diurnal rythmicity, but not in reduction of total movement per 24 hour period as observed in *ak* mice. Moreover, the speed of spontaneous ambulatory movements was not different in *ak* compared with wild type (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

The *ak* mice thus recapitulate cardinal features of PD, in particular the akinetic subtype of PD. Indeed, the preferential loss of vSNc TH-positive neurons taken together with the severe depletion of dorsal striatal DA levels and the associated akinesia are very similar to the pathogenesis of PD (Kish et al., 1988; Fearnley and Lees, 1991; Jellinger, 2001). This close similarity raises the possibility that individuals with PD are preferentially susceptible to loss of Pitx3-positive rather than Pitx3-negative MesDA neurons (Fig. 5). This hypothesis is supported by previous observations (Smidt et al., 1997) 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 after birth (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998). Similarly, *Lmx1b*-deficient mice have complete loss of MesDA neurons from E16, major deficits throughout the midbrain and limb and kidney defects (Smidt et al., 2000; Chen et al., 1998). 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 individuals with PD, with ventral nigra being most affected (Dawson et al., 2002). By contrast, the *ak* mice are only deficient in this specific subset of MesDA neurons (Fig. 5) and they have normal 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.

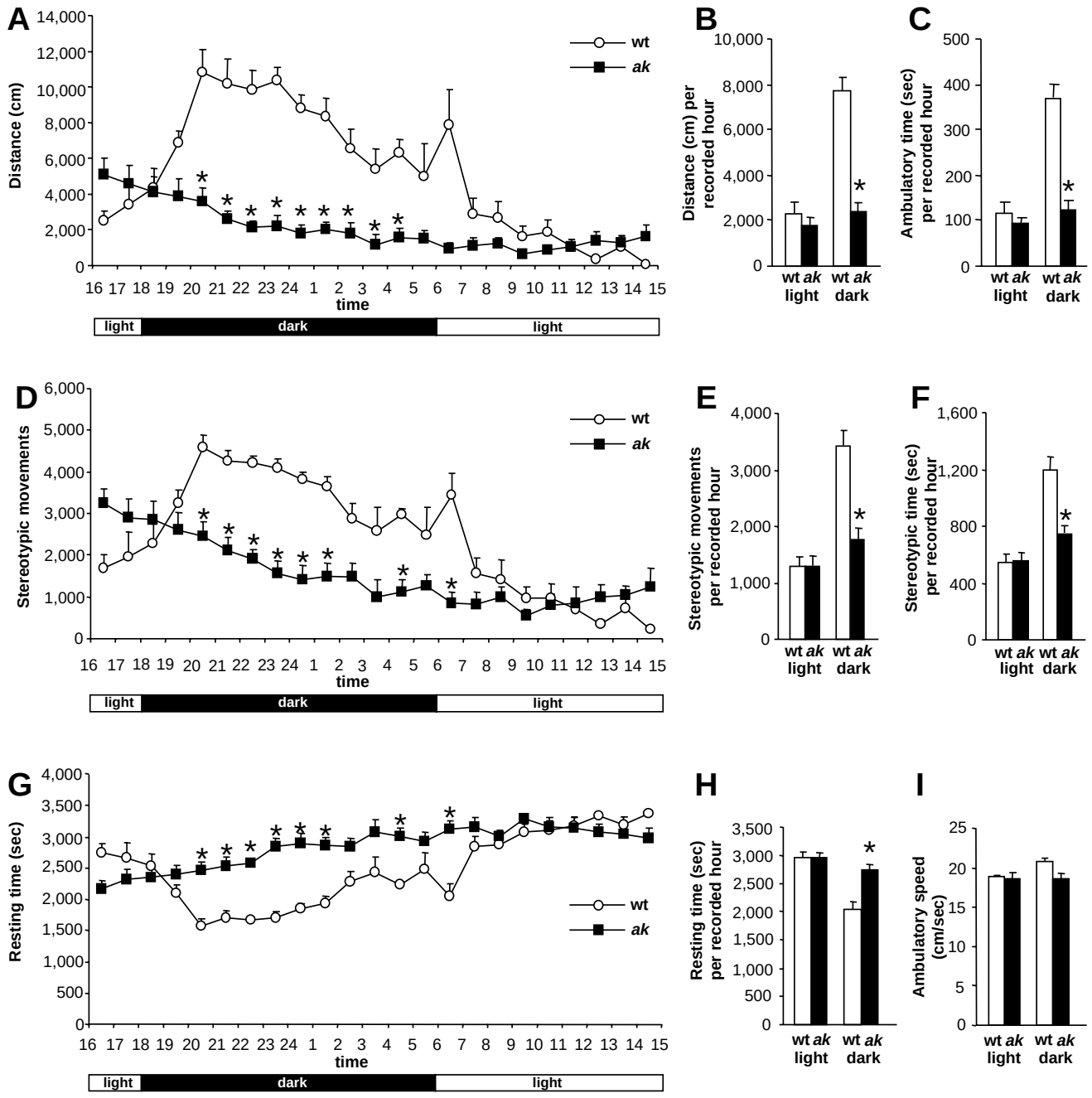

Fig. 4. The *ak* mice have impaired spontaneous locomotor activity. (A) Spontaneous ambulatory activity of mice recorded over 23 hours. The distance (cm) covered during each 1 hour period is shown for wild-type and *ak* mice. (B) Average distance covered per hour for wild-type and *ak* mice during daytime and night-time. (C) Average ambulatory time spent per hour for the recordings shown in E during daytime and night-time. (D) Spontaneous stereotypic movements of mice recorded over 23 hours. The stereotypic movements during each 1 hour period are shown for wild-type and *ak* mice. (E) Average numbers of stereotypic movements per hour for wild-type and *ak* mice during daytime and night-time. (F) Average time spent making stereotypic movements per hour for the recordings shown in H during daytime and night-time. (G) Resting time of mice recorded over 23 hours. The resting time during each 1 hour period is shown for wild-type and *ak* mice. (H) Average resting time per hour for wild-type and *ak* mice during daytime and night-time. (I) Average speed of spontaneous ambulatory movements for wild-type and *ak* mice during daytime and night-time. All locomotor activity data are represented as the mean ± s.e.m. (n=5). Locomotor activity scores for *ak* mice that are significantly different from wild-type scores are marked with a asterisk (P<0.01, t-test).

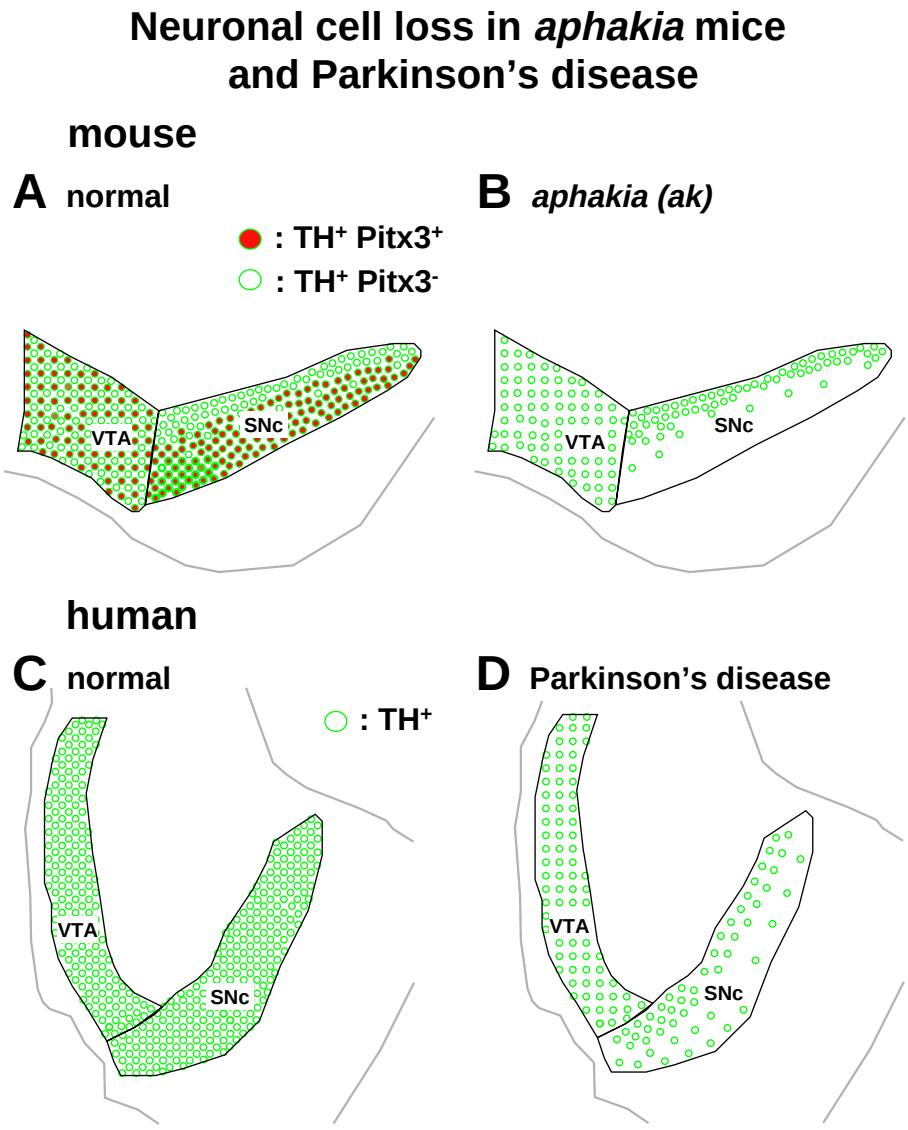

Fig. 5. Similar distribution of MesDA neuronal losses in *ak* mice and in individuals with Parkinson's disease (PD). (A) The right midbrain of a normal mouse showing the distribution of TH-positive Pitx3-positive (green with red core) and TH-positive Pitx3-negative neurons (green) in SNc and VTA. Most ventral SNc TH-positive neurons are Pitx3 positive, whereas the dorsal SNc 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 *ak* 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 the most severe cell depletion in ventral SNc, followed by dorsal SNc and VTA [modified, with permission, from Jellinger (Jellinger, 2001)]. Although a decrease of PITX3-positive neurons was shown in samples from individuals with PD (Smidt et al., 1997), the regional distribution of human PITX3-positive neurons remains to be established.

Finally, the dependence on Pitx3 for survival of Pitx3-positive TH neurons and the sensitivity of Pitx3-positive MesDA cells to degenerate in PD (Smidt et al., 1997) 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. Further investigation of developmental defects resulting from Pitx3 deficiency may provide novel insight into disease pathways involved in PD.

Pitx3 gene mutations may be involved in the etiology of diseases that affect the MesDA system. So far, two PITX3 mutations have been identified in families with autosomal-dominant cataracts and autosomal-dominant anterior segment mesenchymal dysgenesis (Semina et al., 1998). These individuals are not known to have parkinsonian symptoms. It is noteworthy, however, that both mutations have dominant effects in individuals that still have an intact PITX3 allele. Because a midbrain phenotype may not be expected in hemizygous carriers, as heterozygous *ak* mice do not exhibit any phenotype (Varnum and Stevens, 1968) (data not shown), it is likely that these human mutations cause a dominant effect that may for example, impair eye-specific protein:protein interactions (Semina et al., 1998). This would be consistent with their 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 (Amendt et al., 1998). Thus, it would be worthwhile to investigate whether PITX3 allelic polymorphism can be detected in families with Parkinson's disease.

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