Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene *Pitx3*

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Summary

The mesencephalic dopamine (mesDA) system is involved in the control of movement and behavior. The expression of *Pitx3* in the brain is restricted to the mesDA system and the gene is induced relatively late, at E11.5, a time when tyrosine hydroxylase (*Th*) gene expression is initiated. We show here that, in the *Pitx3*-deficient aphakia (*ak*) mouse mutant, the mesDA system is malformed. Owing to the developmental failure of mesDA neurons in the lateral field of the midbrain, mesDA neurons are not found in the SNc and the projections to the caudate putamen are selectively lost. However, Pitx3 is expressed in all mesDA neurons in control animals. Therefore, mesDA neurons react specifically to the loss of Pitx3. Defects of motor control where not seen in the *ak* mice, suggesting that other neuronal systems compensate for the absence of the nigrostriatal pathway. However, an overall lower activity was observed. The results suggest that *Pitx3* is specifically required for the formation of the SNc subfield at the onset of dopaminergic neuron differentiation.

Key words: Mesencephalic, Dopaminergic, *Pitx3,* Substantia nigra

Introduction

The mesencephalic dopaminergic (mesDA) system is involved in the control of movement and behavior, as shown by the dramatic consequence of its degeneration in Parkinson's disease and its involvement in psychiatric and affective disorders (Grace et al., 1997). For this reason, the neurobiology, pharmacology and pathology of the mesDA system has been investigated extensively for many years. Although results suggest that dopaminergic neuron dysfunction may have a genetic component, little is known about the development of the mesDA system and the underlying genetic cascades. The early commitment of a neuronal cell population in the ventral midbrain to the development of the mesDA system largely depends on the proper specification of the isthmus (for a review, see Hynes and Rosenthal, 1999). Therefore, genes involved in boundary formation affect the emergence of mesDA neurons.

Two key molecules on the anterior side of the isthmus play an essential role in the commitment of progenitors to mesDA neuron development. The signaling molecule sonic hedgehog (Shh), which is generated in the neural plate, diffuses into the ventral mesencephalon and, together with FGF8, induces cells at a specific location in the ventral midbrain from which mesDA neurons later arise (Hynes et al., 1995; Hynes et al., 1997; Ye et al., 1998; Shamim et al., 1999; Hynes and Rosenthal., 1999). MesDA neurons, which express tyrosine hydroxylase (Th) and Pitx3 (Ptx3) (Smidt et al., 1997), first appear at the most ventral rim of the neuroepithelium, lining up along the mesencephalic flexure of the ventral mesencephalon. Ventral midbrain markers are present in this area prior to Pitx3 and Th. Among the earliest markers of the region are *En1* (Danielian et al., 1996; Wurst et al., 1994), *En2* (Hanks et al., 1995), *Wnt1* (Danielian et al., 1996), *Pax2* (Favor et al., 1996) and *Pax5* (Urbanek et al., 1997). *Th* expression is induced at E11.5, 1 day after the induction of the orphan nuclear hormone receptor *Nurr1* (*Nr4a2* – Mouse Genome Informatics) (Law et al., 1992). The expression of *Nurr1* is, however, not restricted to the ventral midbrain but is also expressed in the dorsal mesencephalon and diencephalon, where *Th* is not expressed at this developmental stage (Smidt et al., 2000). MesDA neurons in Nurr1 knockout mice do not express *Th*, *Dat* (*Slc6a3* – Mouse Genome Informatics) and *Vmat2* (*Slc18a2* – Mouse Genome Informatics) (Smits et al., 2003) and are lost after initial generation (Zetterström et al., 1997; Saucedo-Cardenas et al., 1998). Thus, *Nurr1* is part of a molecular cascade required for neurotransmitter synthesis and survival.

Additional cascades have been discovered that specify other properties of the mesDA neuronal phenotype. Recently, the homeobox genes *En1*, *En2* (Davidson et al., 1988; Simon et al., 2001) and *Lmx1b* (Johnson and Tabin, 1997; Dreyer et al., 1998a; Chen et al., 1998a; Chen et al., 1998b; Tucker et al., 1999) have been implicated in the development of the mesDA system (Smidt et al., 2000). *En1* and *En2* are not required for the induction of *Th*, but control the survival of mesDA neurons in a gene dose-dependent manner (Simon et al., 2001). Lmx1b is present in the premordial ventral mesencephalon and

diencephalon at E7.5, and continues to be expressed in the adult in a limited set of brain structures derived from these areas, including mesDA neurons (Smidt et al., 2000). Null mutation of *Lmx1b* showed that this gene is not required for the expression of *Nurr1* and is not essential for *Th* induction. Moreover, *Lmx1b*-null mutation impairs the proper development of mesDA neurons, such that the mesDA-specific homeodomain gene *Pitx3* is not expressed in *Th*-positive cells in the ventral midbrain (Smidt et al., 2000).

The *Pitx3* gene belongs to the *Pitx* (*Ptx*) subfamily of paired-like homeodomain proteins, of which two other members are known, *Pitx1* and *Pitx2*, which are involved in hindlimb patterning, mandible development (Lanctot et al., 1999), left-right asymmetry of the body (Lin et al., 1999), pituitary genesis (Suh et al., 2002) and pituitary hormone regulation (Quirk et al., 2001; Quentien et al., 2002). *Pitx3* is expressed in the eye and the brain (Semina et al., 1997; Smidt et al., 1997), where its expression is restricted to mesDA neurons in rodents and humans (Smidt et al., 1997). The *Pitx3* gene is induced at the same developmental stage as *Th* and continues to be expressed in adulthood (Smidt et al., 1997). Although in vitro studies have suggested that *Pitx3* is important for the induction of the *Th* gene (Lebel et al., 2001; Cazorla et al., 2000), analysis of the *Lmx1b*-null mutant indicated that *Pitx3* is not essential for *Th* expression (Smidt et al., 2000). Recent reports have shown that the *Pitx3* gene is inactivated in the aphakia (*ak*) mouse mutant (Semina et al., 2000; Rieger et al., 2001) and that the architecture of the midbrain DA system is compromised (Nunes et al., 2003; van den Munckhof et al., 2003; Hwang et al., 2003; Burbach et al., 2003). However, the early developmental fate of mesDA progenitors, and the role of *Pitx3* in this, are uncertain. We show that SNc DA neurons are absent in the *ak* mice from E12.5 onwards. *Pitx3* appears to be an essential part of a transcription factor cascade required for the developmental specification of the SNc subpopulation of mesDA neurons.

Materials and methods

Animals

The *Aphakia* (*ak*) strain used has been described previously (Semina et al., 2000; Rieger et al., 2001). C57Bl/6-Jico were used as control animals (Charles-River).

Surgery

Six- or 7-day-old C57Bl/6-Jico mice underwent surgery to remove the eyes. The mice were anesthetized with isoflurane (florane®) 2% and N_2O (carrier-gas) and O_2 (75:65% volume, respectively), and the eyelids were treated with lidocaine as local anesthetic. The animals were kept at 37°C with a heating mat. The eyelids were opened with a pair of scissors and the membrane covering the eye was removed with a micro hook. The eyeball was removed by cutting the nerve and blood vessel with a scalpel after they had been forced together by the use of a forceps. The eyelids were glued together with tissue glue (Histoacryl® (Braun)) applied with a micropipette. The animals recovered from surgery and did not appear to be affected by the procedure.

In situ hybridization

The brains of adult animals and E12.5 embryos of wild-type and *ak* mice were collected and immediately frozen on dry ice. Sagittal and coronal sections (16 μm) were cut on a cryostat and collected on SuperFrost Plus slides (Menzel Gläser). In situ hybridization with digoxigenin (DIG)-labeled probes was performed essentially according to Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers and Gerfin-Moser, 1993). Briefly, sections were fixed in 4% paraformaldehyde (PFA) for 10 minutes and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 minutes. Hybridization was carried out at 72°C in a hybridization solution containing 50% deionized formamide, 5×SSC, 5×Denhardt's solution, 250 μg/ml tRNA Baker's yeast and 500 μg/ml sonificated salmon sperm DNA. Post-hybridization washes were carried out in 0.2×SSC for 2 hours at 72°C. DIG was detected with an alkaline phosphatase-labeled antibody (Roche, Mannheim) using NBT/BCIP as a substrate. After DIG in situ hybridization, slides were dehydrated in ethanol, cleared in xylene and mounted using Entellan. DIG in situ hybridization was performed with the following probes: a *Bal*I/*Eco*RI (bp 915-1137) fragment of the rat *Th* cDNA (Grima et al., 1985), an *Eco*RI/*Pst*I fragment containing bp 1 to 285 of the rat *Pitx3* cDNA (Smidt et al., 1997), a *Nurr1* cRNA containing bp 1022 to the 3′ end of the full-length cDNA (U72354), an *En1* fragment containing bp 1-1842 of the full-length mouse cDNA sequence (L12703), an *En2* fragment (*Bgl*II/*Xba*I) containing bp 1351-2101 of the mouse cDNA sequence (L12705), a *Lmx1b* fragment (*Eco*RI) containing the full-length mouse cDNA sequence, a *Ret* fragment containing bp 1733-1281 of the mouse cDNA sequence (X67812), a dopamine transporter (*Dat)* fragment (*Apa*I/*Hind*III) containing bp 762-1127 of the rat cDNA sequence (m80570), an aromatic-L-amino acid decarboxylase (*Aadc*) fragment containing bp 22-488 of the coding region from the mouse cDNA, a vesicular monoamine transporter 2 (*Vmat2*) fragment containing bp 290-799 of the coding region from the mouse cDNA, a *Nli* fragment containing bp 651-1778 of the mouse cDNA (U69270),

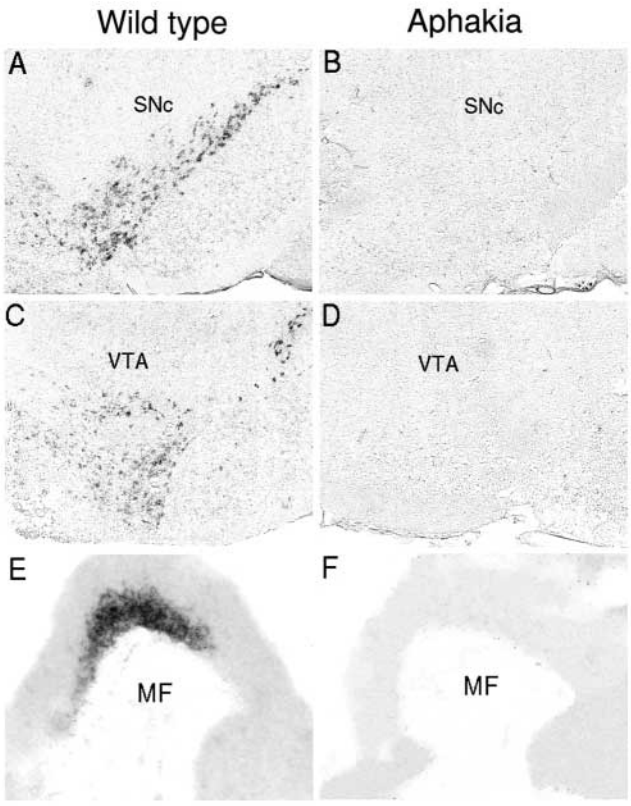


Fig. 1. Expression of *Pitx3* in wild-type (A,C,E) and *ak* mutants (B,D,F) in the adult brain (A-D) and at stage E12.5 of brain development (E,F). *Pitx3* was not expressed in the brains of *ak* mice. SNc, substantia nigra compacta; VTA, ventral tegmental area; MF, mesencephalic flexure.

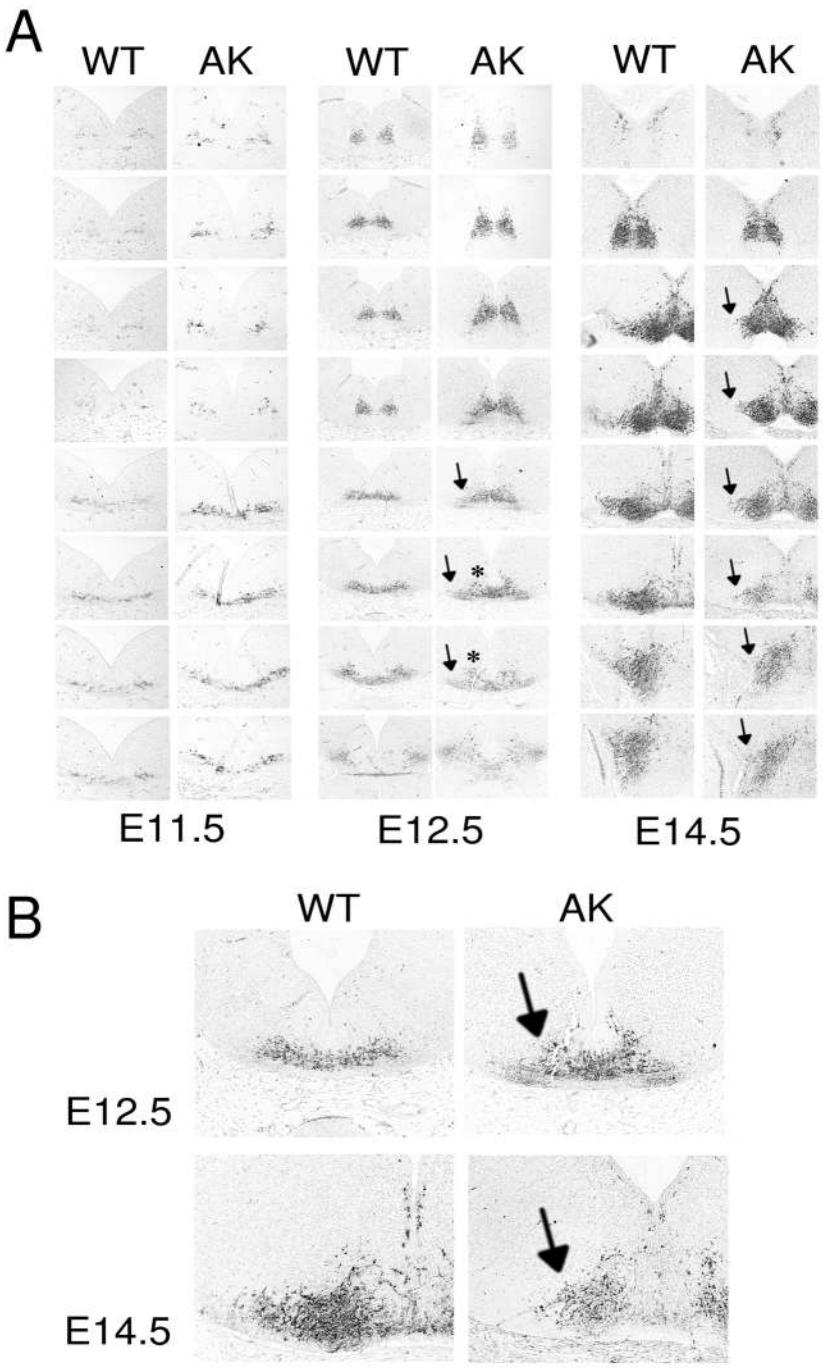


Fig. 2. Th-positive neurons in the mesencephalon at embryonic stages E11.5 to E14.5. (A) Coronal sections from the posterior to anterior mesencephalon. (B) Enlargement of sections displaying the aberrant development in the SNc region (arrow) at stage E12.5 and E14.5. The arrows indicate the anatomical positions where mesDA neurons are missing. The asterisk indicates the medial position where mesDA neurons are ectopically present in the *ak*.

a dopamine receptor 2 (*D2r*) fragment containing bp 342-1263 of the coding region from the mouse cDNA, a cholecystokinin (*Cck*) fragment containing the full cDNA of the rat (nm_012829), a neurotensin receptor 1 (*Ntr1*) fragment containing bp 426-931 of the coding region from the mouse cDNA, and an alpha-synuclein fragment containing bp 20-420 of the coding region from the mouse cDNA.

Combined in situ hybridization-immunohistochemistry

Sections were treated as described above for DIG hybridization, except that after termination of the alkaline phosphatase reaction, the sections were washed twice for 5 minutes in TBS. Then they were incubated in 0.3% H_2O_2 in TBS for 30 minutes to reduce endogenous peroxidase activity, washed twice for 5 minutes in TBS, blocked with 4% fetal calf serum in TBS for 30 minutes, washed twice for 5 minutes in TBS, and incubated overnight at room temperature with either anti Th (Pel-Freez, Arkansas, USA; 1:1000) in TBST (0.5 M Tris-HCl at pH 7.4, 9% NaCl, 0.5% Triton) or anti Pitx3 (1:500) (Smidt et al., 2000) in TBST. The next day, sections were washed three times with TBS for 5 minutes, incubated for 1 hour with biotinylated goat anti-rabbit immunoglobulin in TBST (1:1000), washed three times with TBS for 5 minutes, incubated for 1 hour with avidin-biotin-peroxidase reagents (ABC elite kit, Vector Laboratories, 1:1000) in TBST and washed with TBS three times for 5 minutes. The slides were stained with DAB (3,3′-diamino-benzidine) until background was lightly stained. Slides were washed twice with demineralized water for 5 minutes, dehydrated with ethanol and mounted using Entellan.

Immunohistochemistry

PFA (4%)-fixed vibratome sections were used for direct immunohistochemistry, as described earlier (Smidt et al., 2000). Immunohistochemistry with paraffin wax sections was performed as described earlier for frozen sections (Smidt et al., 2000) with the following modifications. Sections were deparaffiniated through xylene and rehydrated through an ethanol series. Sections were boiled in 0.06 M sodium citrate (pH 6) for 9 minutes and then allowed to cool down to room temperature. The following antibodies were used: polyclonal rabbit anti-Th (1:1000; PelFreez) and a polyclonal rabbit anti-Pitx3 (1:500) (Smidt et al., 2000).

Nissl staining

Paraffin-embedded coronal midbrain sections (7 μm) obtained from adult wild-type and *ak* mice were mounted on SuperFrost plus slides (Menzel Gläser). Sections were deparaffinated, rinsed in water, stained for 10 minutes in 0.5% Cresyl Violet and briefly rinsed in an acetate buffer, pH 4. The sections were then differentiated in 96% ethanol for 30 seconds, dehydrated in 100% ethanol, cleared in xylene and mounted with Entellan.

Fluorogold retrograde tracing

A ‘David Kopf’ stereotact apparatus with mouse adaptor (Stoelting, USA) was used for injections in wild-type and *ak* mice at the following coordinates for the dorsal striatum: (bregma=0), 1.1 to anterior, 1.5 lateral. The skull surface was set at vertical position 0, and the injection depth was 3 mm. The animals were anesthetized with 2.5 μl/g Hypnorm (Janssen; 0.315 mg/ml fentanyl citrate, 10 mg/ml fluanisone, IP), additionally 0.8 μl/g dormicum (Roche; 5 mg/ml midazolam, IP) was given 5 minutes after the Hypnorm injection. During the operation ‘CAF-zalf’ (Apharmo) was applied to the eyes of the wild-type mice to prevent drying of the eyes. Body temperature was kept at 37°C by placing the mice on heating mats. The retrograde tracer Fluorogold

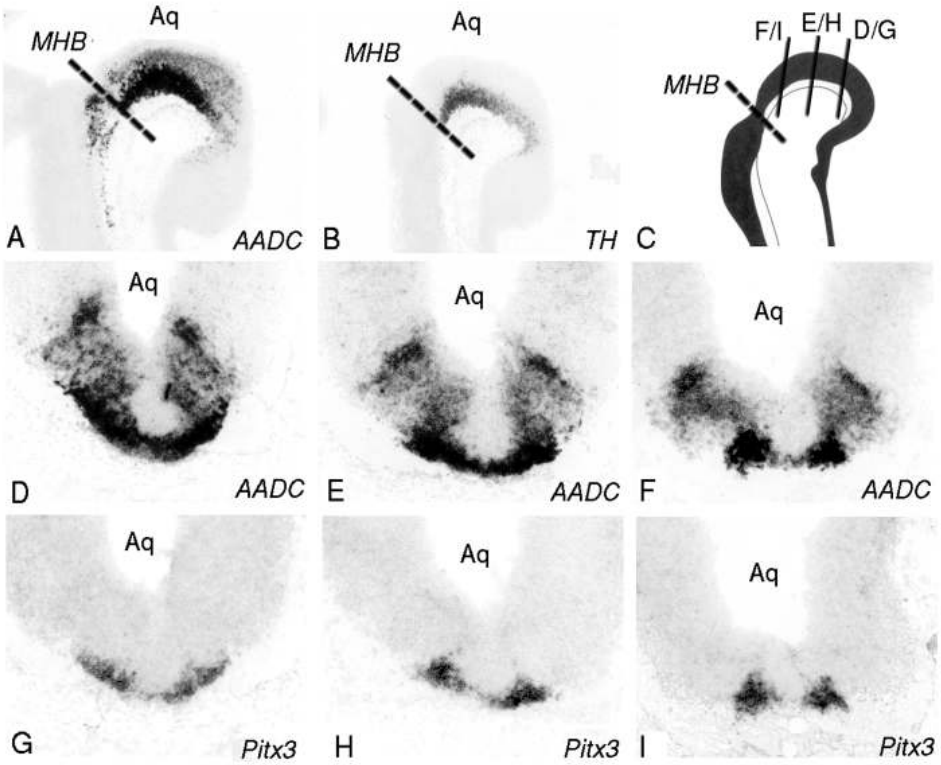


Fig. 3. *Aadc* and *Pitx3* expression in the mesencephalic neuronal field at E12.5. (A) *Aadc* expression in a sagittal section showing the mesencephalic dopaminergic (mesDA) field and additional positive neurons located dorsally in the midbrain and serotonergic neurons positioned caudal to the midbrain/hindbrain border (MHB). (B) Tyrosine hydroxylase (*Th*) expression in an adjacent section to A. (C) Schematic representation of a sagittal section showing the position of the coronal sections shown in D-I. (D-F) *Aadc* expression in coronal sections at three different positions as indicated in C. (G-H) *Pitx3* expression in adjacent sections to D-F. Aq, aqueduct.

(Fluorochrome, USA) was iontophoretically delivered through a glass micropipette (internal diameter 10-15 μm) filled with 2% Fluorogold in 0.1 M cacodylate buffer (pH 7.3). The tracer was applied using a midgard 51413 precision current source instrument [polarity –, 5 μA for 10 minutes (pulse setting 7 seconds on, 7 seconds off) red pen(+) at the ear of the mice, black pen(–) in Fluorogold solution]. The glass capillaries (1.5 mm OD, 0.86 mm ID, Borosilicate glass, standard wall with inner filament, Clark electromedical instruments, Reading, UK) were prepared on a micro-pipette-puller (Getra, Munchen, Germany) with the following settings: Heating: 7.5-8, Magnet: 3. The animals were killed 48 hours after the infusions, and the brains were isolated and fixed for 24 hours in 4% PFA (fresh) at 4°C. Sections (50 μm) were cut with a vibratome (Leica) and examined under a fluorescence microscope or used for additional immunohistochemical experiments.

Climbing test

The test was performed as described (Costall et al., 1978a; Costall et al., 1978b) except that climbing was scored every 5 minutes for 90 minutes (0=all paws on the floor, 1=2 paws on the cage, 2=all paws on the cage). None of the animals was injected with drugs.

Medium open field test

The open field consisted of a Plexiglas open cylinder (diameter 20 cm, height 30 cm) placed on a white plastic board. Locomotor activity was monitored for 15 minutes using a fully automated observation system (1.6 frames per second, Ethovision, Noldus Information Technology, The Netherlands). The animals were used at 3 months of age. The experiment was performed under normal lighting conditions between 10 am and 3 pm.

Automated quantitative gait analysis

The gait of wild-type and *ak* mice was analyzed as described previously (Hamers et al., 2001).

Results and discussion

ak mice are Pitx3 deficient

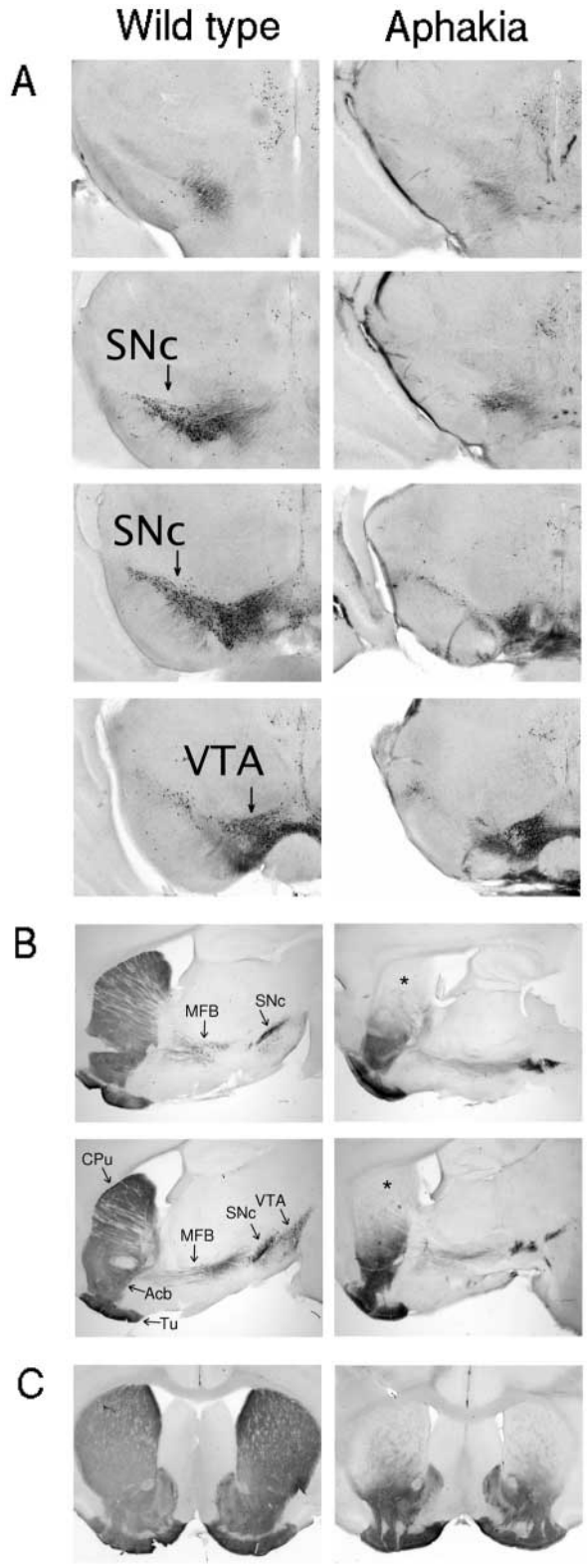
The *Pitx3* gene is expressed solely in mesDA neurons in the brain (Smidt et al., 1997). Aphakia (*ak*) mutant mice which suffer from a eye defect (Varnum and Stevens, 1968), have a double genomic deletion within the *Pitx3* locus (Semina et al., 2000; Rieger et al., 2001). These deletions are present in the upstream enhancer region, the promoter area, exon 1 and part of intron 1. The *ak* mouse may therefore be considered a null-mutant for the *Pitx3* gene. Indeed, *Pitx3* expression was not detectable in mesDA neurons of adult *ak* mice (Fig. 1A-D) and E12.5 *ak* embryos (Fig. 1E,F). This extends earlier findings of the lack of expression of *Pitx3* in the lens (Semina et al., 2000), showing that the *Pitx3* gene is not expressed at all. This provides the means to study the development of the mesDA system in the absence of the unique homeodomain gene *Pitx3*.

Early development of mesDA neurons in ak mice

In order to examine the genesis and developmental fate of mesDA neurons in the absence of Pitx3, the expression of Th throughout the entire midbrain was analyzed from E11.5 onwards (Fig. 2). On E11.5, when Th was expressed for the first time, the distribution of Th-positive neurons in the ventral midbrain was indistinguishable between wild-type and *ak* mice. A thin rim of Th-positive cells was observed in the ventral midbrain. These cells are thought to originate from the neuroepithelium lining the ventricular space and to migrate ventrally by interacting with radial glia cells (Hanaway et al., 1971; Marchand and Poirier, 1983; Shults et al., 1990; Kawano et al., 1995; Hall et al., 2003). In line with this, we observed

Th-positive neurons in close contact with fibrillous structures in paraffin wax sections (data not shown).

The first differences in neuroanatomy were seen on E12.5, in the rostral part of the Th domain. At this position, the lateral Th-positive neurons were absent in *ak* mice. In a more medial position, Th-positive cells accumulated in a more dorsal field (Fig. 2 asterisks). In wild-type mice, Th-positive neurons accumulated in a more lateral position which forms the SNc. This distinct cell group was absent in E12.5 *ak* embryos and onward (Fig. 2, arrows).



As mesDA progenitor neurons start to express *Th* after they have migrated to a ventral position in the midbrain, we sought to determine their fate by analyzing *Aadc* (*Ddc* – Mouse Genome Informatics) expression. *Aadc* is expressed solely by catecholaminergic and serotonergic neurons (Cooper et al., 1977) and is first expressed 2 days earlier than *Th* (Teitelman et al., 1983). Analysis of sagittal sections of E12.5 wild-type mice showed that *Aadc* was expressed in serotonergic neurons located caudal to the mid-hindbrain border (MHB) and in midbrain dopaminergic neurons located rostral to the MHB (Fig. 3A), being expressed over a larger area than Th (Fig. 3B). In coronal sections from three different levels containing dopaminergic neurons (Fig. 3C), *Aadc*-positive neurons were seen immediately below the neuroepithelium (Fig. 3D-F). This suggests that the *Aadc* gene is activated early in the differentiation of neuroepithelial cells.

Pitx3 was expressed in *Aadc*-positive neurons located in the most ventral position in the midbrain, in a pattern similar to *Th* expression, but not in *Aadc*-positive neurons located just below the neuroepithelium (Fig. 3G-I). These data indicate that Pitx3 affects the terminal differentiation of mesDA neurons and that it is not involved in either the proliferation or the migration of neurons from the neuroepithelium to the ventral midbrain.

Architecture of mesDA neurons of adult ak mice

The anatomical architecture of mesDA neurons was analyzed by comparing the distribution of Th-immunoreactive neurons in the midbrain of *ak* and wild-type mice (Fig. 4). The morphology of the mesDA system of *ak* mice was dramatically altered in two aspects: the field of dopaminergic bodies in the ventral midbrain was smaller (Fig. 4A) and there were fewer projections to the striatum. (Fig. 4B,C). These findings are consistent with other findings (Nunes et al., 2003; van den Munckhof et al., 2003; Hwang et al., 2003). In *ak* mice, Th-positive neurons were not detected in the SNc. The region of the VTA was less affected, although in the region where the SNc and VTA are overlapping, Th positive neurons were also absent (Fig. 4A). The non-*Pitx3* expressing dopamine systems including the periglomerular dopamine neurons (A16) located in the olfactory bulb and the tuberohypophycial/incertohypothalamic dopamine neurons (A11-A14) located in the hypothalamus were not affected, as analyzed by Th staining

Fig. 4. Level of tyrosine hydroxylase (Th) protein measured by immunohistochemistry in wild-type and *ak* brain sections. (A) Coronal section of the mesencephalic dopaminergic (mesDA) neuronal region. The normal architecture of the system is indicated in the wild-type (arrows). Similar sections from the *ak* brain are paired to match the wild-type level. (B) The left two panels indicate the neurons of the mesDA system and their architecture, the projecting axons and the target area in the striatum in sagittal sections. The right two panels show similar sections in the *ak* brain with the altered mesDA neuron architecture and the missing projection area in the caudate putamen indicated by the asterisk. (C) Th expression in coronal sections of the striatal area. CPu, caudate putamen; MFB, main forebrain bundle; VTA, ventral tegmental area; SNc, substantia nigra compacta; Acb, nucleus accumbens; Tu, tubercle olfactorium.

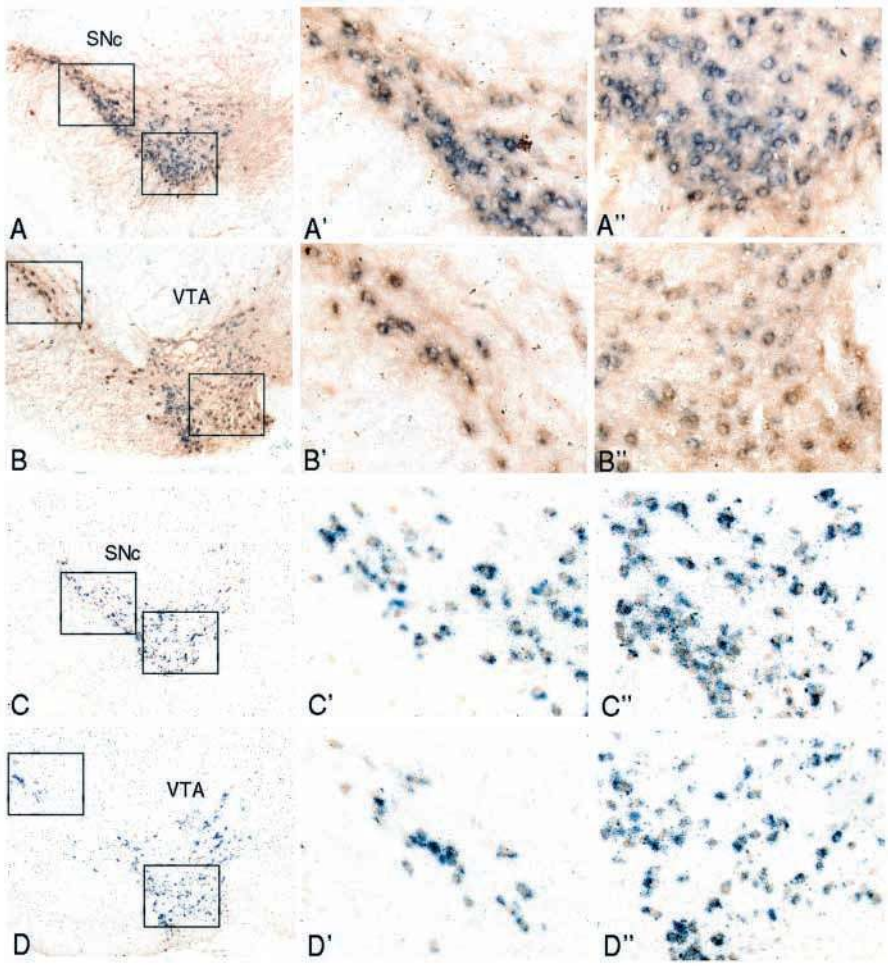


Fig. 5. Colocalization of Pitx3 and tyrosine hydroxylase (Th) in adult coronal sections in the mesencephalic dopaminergic (mesDA) neuronal field. Colocalization of *Pitx3* mRNA (blue) and Th protein (brown) in adult SNc (A-A″) and VTA (B-B″) neurons of wild-type mice. Colocalization of *Th* mRNA (blue) and Pitx3 protein (brown) in adult SNc (C-C″) and VTA (D-D″) neurons of wild-type mice. The boxed areas indicated in A-D are given in a higher magnification in the panels on the right. SNc, substantia nigra compacta; VTA, ventral tegmental area.

in coronal sections through the brain (data not shown). This was expected as *Pitx3*, in contrast to *Lmx1b* and *Nurr1*, is expressed only in mesDA neurons in the brain (Smidt et al., 1997). Furthermore, the defect of Pitx3 seems to be restricted to a subset of mesDA neurons. To address the reportedly limited expression of Pitx3 in a subset of DA neurons and the compromised survival of these neurons (van den Munckhof et al., 2003), the overlap in expression of Th and Pitx3 was investigated in detail by combined in situ hybridization/ immunohistochemistry for transcripts and protein of both Th and Pitx3 (Fig. 5).

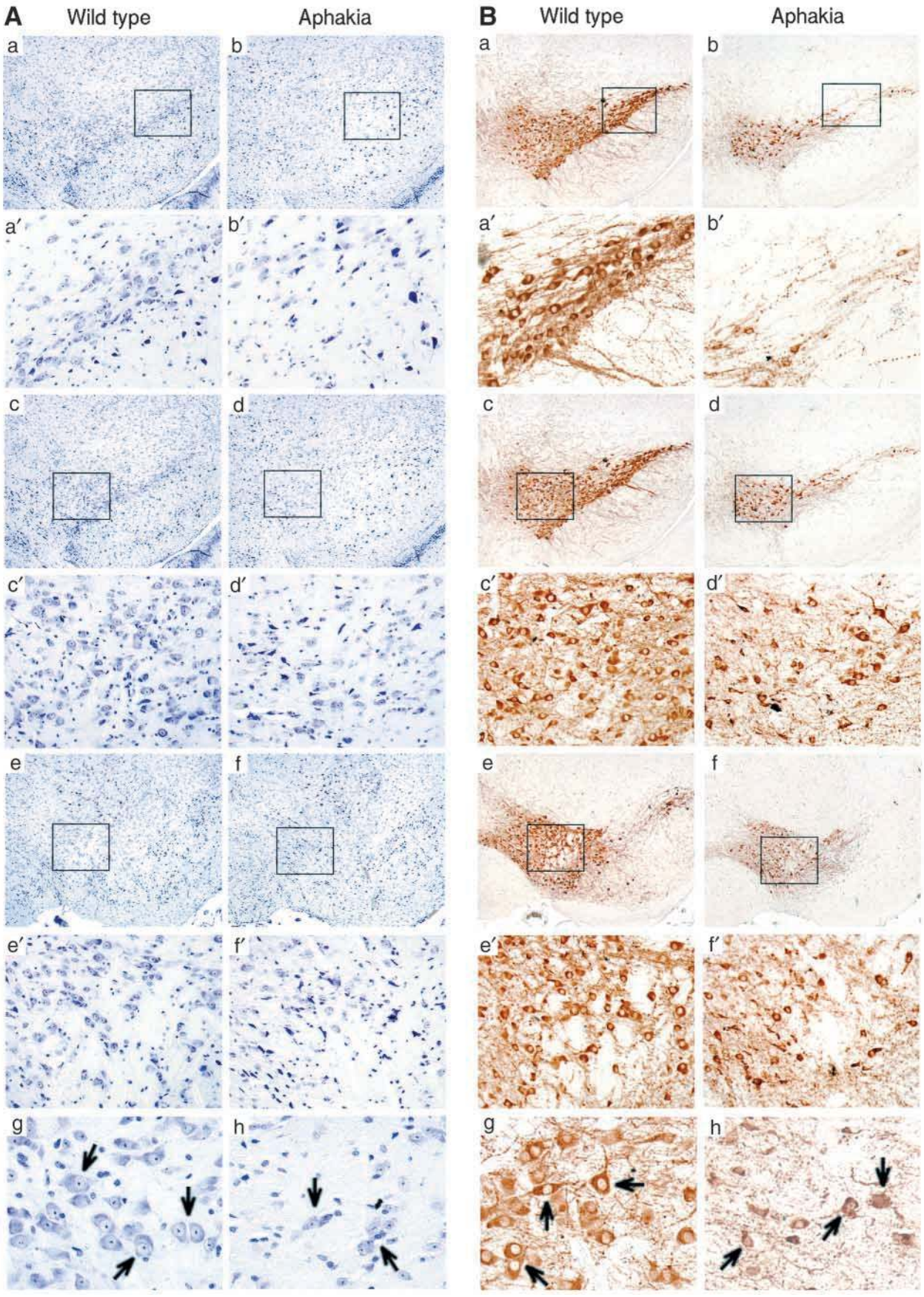
In neurons of the SNc (Fig. 5A) and VTA (Fig. 5B) the expression of *Pitx3* message and Th protein overlapped completely, as did the expression of Pitx3 protein and *Th* message (Fig. 5C,D). Thus, Th and Pitx3 are co-expressed in mesDA neurons in control animals. In conclusion, mesDA neurons are selectively affected by *Pitx3* deficiency and the most severely affected neurons are those that normally form the SNc and a subpopulation of the VTA.

Restricted connectivity of mesDA neurons and forebrain regions in ak mice

Analysis of sagittal sections immunostained for Th revealed dramatic changes in the connectivity of the mesDA cell bodies with main target areas, specifically the caudate putamen (Fig. 4B). In the striatal projection area, Th-positive fibers were mainly lost in the dorsal caudate putamen. Parts of the medial forebrain bundle were still visible. These fibers innervated ventral striatal areas, particularly the nucleus accumbens area and the olfactory tubercle. The caudate putamen was devoid of innervating Th-positive axons. This loss of mesDA projections was seen throughout the caudate putamen (Fig. 4C).

In order to determine whether mesDA neurons in the SNc were absent in *ak* mice or no longer expressed Th, Nissl staining was performed (Fig. 6A). Sections were compared to adjacent sections stained for Th (Fig. 6B). In *ak* mice, particularly DA neurons in the lateral part of the SNc were absent (Fig. 6A, parts a,b,a′,b′) and the density of DA cells in the medial part of the SNc (Fig 6A, parts c,d,c′,d′) was reduced. Taken together, the data indicate that a null mutation of the *Pitx3* gene results in the absence of mesDA neurons in the SNc. Moreover, when present the mesDA neurons in the VTA of *ak* mice had an altered

Fig. 6. Coronal sections showing Nissl-staining (A) of the wild-type and *ak* adult mesDA neuronal field compared to adjacent sections stained for Th (B). Coronal sections of lateral SNc neurons (a,a′,b,b′), medial SNc neurons (c,c′,d,d′) and neurons of the VTA (e,e′,f,f′) are depicted. Boxed areas shown in A-F are shown at a higher magnification in the figures below (a′-f′). (g,h) High magnification of mesDA neurons in the VTA area, showing the altered morphology (arrows) in the *ak* compared with the wild-type mice.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morphology (Fig. 6A,B, parts e-h; indicated by arrows in g,h), consistent with data from a recenlty published study (Hwang et al., 2003). This indicates that mesDA neurons of the SNc and the VTA, differ in their dependence on *Pitx3* expression. The loss of neurons in the *ak* brain results in altered mesDA projections to the caudate putamen.

To determine whether projections between the dorsal caudate putamen and the midbrain were still present in *ak* mice, retrograde tracing studies with fluorogold were performed in the dorsal caudate putamen. In wild-type mice, fluorogold-labeled neurons were detected in the SNc and the VTA (Fig. 7A). These neurons were positive for Pitx3, confirming that these were mesDA neurons. In the *ak* mice, such neurons were not detected in the mesencephalic field: of all sections, only one neuron containing fluorogold was found (Fig. 7B). This shows that these projections were absent in the *ak* mice.

Gene expression in mesDA neurons of ak mice

Nurr1 is involved in establishing the DA phenotype of mesDA neurons (Zetterström et al., 1997; Saucedo-Cardenas et al., 1998), and experiments with *Nurr1* knockout mice showed that the expression of *Th* in mesDA neurons requires Nurr1. In contrast to *Nurr1* knockout mice, in *ak* mice *Th* was expressed in mesDA neurons when present, suggesting that they retain their neurotransmitter phenotype. To determine whether other genes required for the neurotransmitter phenotype were affected by the loss of Pitx3, the expression of *Dat*, *Vmat2*, *Aadc* and *D2r* (*Drd2* – Mouse Genome Informatics) was analyzed in neurons of SNc (Fig. 8A) and VTA (Fig. 8B). The transcripts were present in the few mesDA neurons detected in the *ak* brain. In addition, expression of other mesDA associated markers including cholecystokinin (Cck), the neurotensin receptor 1 (Ntr1), alpha synuclein, Nurr1, Lmx1b, En1, En2, Ret and Nli was analyzed (Fig. 8 and not shown) in *ak* and wild-type mesDA neurons. These results indicated that all the genes involved in the DA synthesis, release pathway and developmental pathway are retained in the VTA-neurons in the *ak* mice. This demonstrates that the SNc neurons are lost and that the detected mesDA neurons probably display the normal DA phenotype.

Behavioral characteristics of ak mice

To investigate the *ak* mice for deficiencies in the dopaminergic neuron-associated behaviors we compared *ak* mice with wild-type and blinded wild-type mice, made blind on postnatal day 6-7 (P6-7). Despite the absence of dopamine-mediated innervation of the caudate putamen, no altered posture, or walking pattern or tremor was detected in the *ak* mice. This suggested that motor control itself is not affected in *ak* mice. However, *ak* mice showed aberrant spontaneous behavior in a 'climbing test', developed to evaluate dopaminergic neuron activity in the striatum (Costall et al., 1978a; Costall et al., 1978b). Both blind-mice and *ak* mutants had increased climbing behavior compared with controls (Fig. 9) The *ak* mutants had higher activity scores than the blind controls, but the difference was not statistically significant; however, the *ak* mice displayed lower activity than the blinded controls and normal controls in an open field (Fig. 9B). Together, these data suggest that the altered organization and connectivity in the *ak* mesDA system causes hyperactivation of the nucleus accumbens area, resulting in increased climbing behavior, and hypoactivation of the caudate putamen, resulting in lower overall activity levels. Moreover, it was striking that

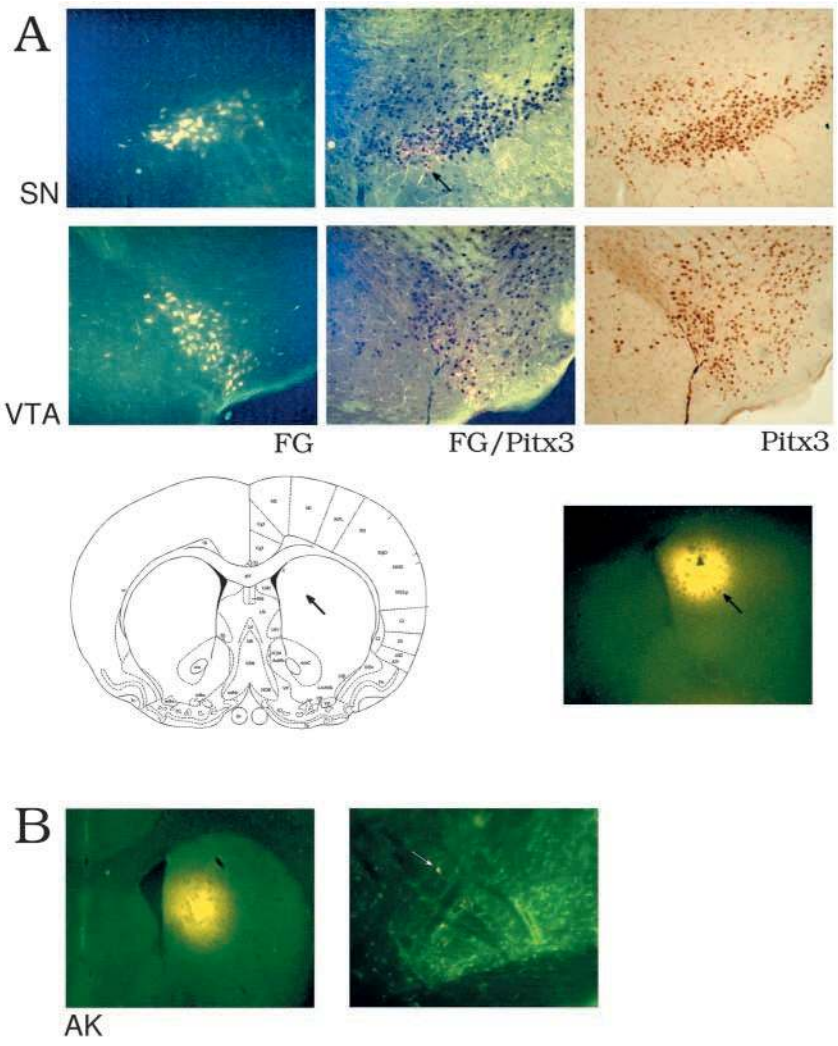

Fig. 7. Retrograde tracing in the mesencephalic dopaminergic (mesDA) system and double labeling by Pitx3 immunohistochemistry. (A) Retrograde tracing in the control brain. The left two panels (FG) show the fluorogold label in the traced neurons in the mesDA system. The middle two panels (FG/Pitx3) show the double labeling. The arrow indicates one of the double labeled mesDA neurons. The right two panels (Pitx3) show the labeling by Pitx3 alone. The lower two panels show the injection site (right) and a schematic representation indicating the injection site (arrows). (B) Retrograde tracing in the *ak* mutant brain. The left panel represents the injection position. The right panel shows a neuron (arrow) in the VTA region that was traced by injection in the dorsal striatum.

only the motor output and not the motor skills were affected. Thus, the neuroanatomical aberrations in the *ak*-mutant are reflected by behavioral changes. Interestingly, the *ak* mice had lower overall motor output, consistent with data from other mesDA-lesion animal models. The hyperactivation of the nucleus accumbens in relation to the hypoactivity of the caudate putamen seems a unique feature of the *ak* animal, suggesting that the *ak* mice can be regarded as a new interesting model of mesDA dysfunction.

In conclusion, in this study we demonstrated that the *ak* mouse can be considered as a Pitx3 knockout. Specific changes occur in the mesDA system as a result of the loss of Pitx3 expression, resulting in fewer mesDA neurons in the lateral SNc, fewer projections to the caudate putamen and an altered morphology of VTA neurons. These results suggest that differences in Pitx3-dependence exists. Different models for cell genesis and migration in the midbrain have been described (Fig. 10A). The first model (Kawano et al., 1995) (Fig. 10A, model 1) states that mesDA precursors are generated in the neuroepithelium of the medial part of the midbrain and migrate to the ventral side, where some of these neurons migrate laterally to form the SNc. In this model, Pitx3 could play a role in the lateral migration of neurons that form the SNc as Pitx3 is expressed in the ventral field of the midbrain. This is, however, unlikely because we found that on E11.5 the complete primordial mesDA neuronal field was present in the wild-type and in the *ak* mice, indicating that according to this model Pitx3 cannot be involved in either lateral or ventral migration events. In addition, Aadc-positive neurons were present in lateral positions in the dorsal midbrain field, just below the neuroepithelium. If Aadc-positive neurons in this position function as mesDA precursors, then precursors would have to be generated in lateral midbrain positions. Therefore, our data suggest that this model is unlikely. In the second model, precursors are formed in the dorsal parts of the midbrain, just below the ventricular zone where cell division takes place (Hanaway et al., 1971) (Fig. 10A, model 2). The VTA neurons and the medial SN are generated from the medial ventral one-third of the basal plate and migrate ventrally in an 'inverted fountain' shape pattern. The neurons forming the more lateral SN are generated from a more lateral neuroepithelial patch and migrate perpendicular to the ventricle. The precursors start to display the full dopaminergic phenotype when they arrive in the ventral field; at this point *Th*

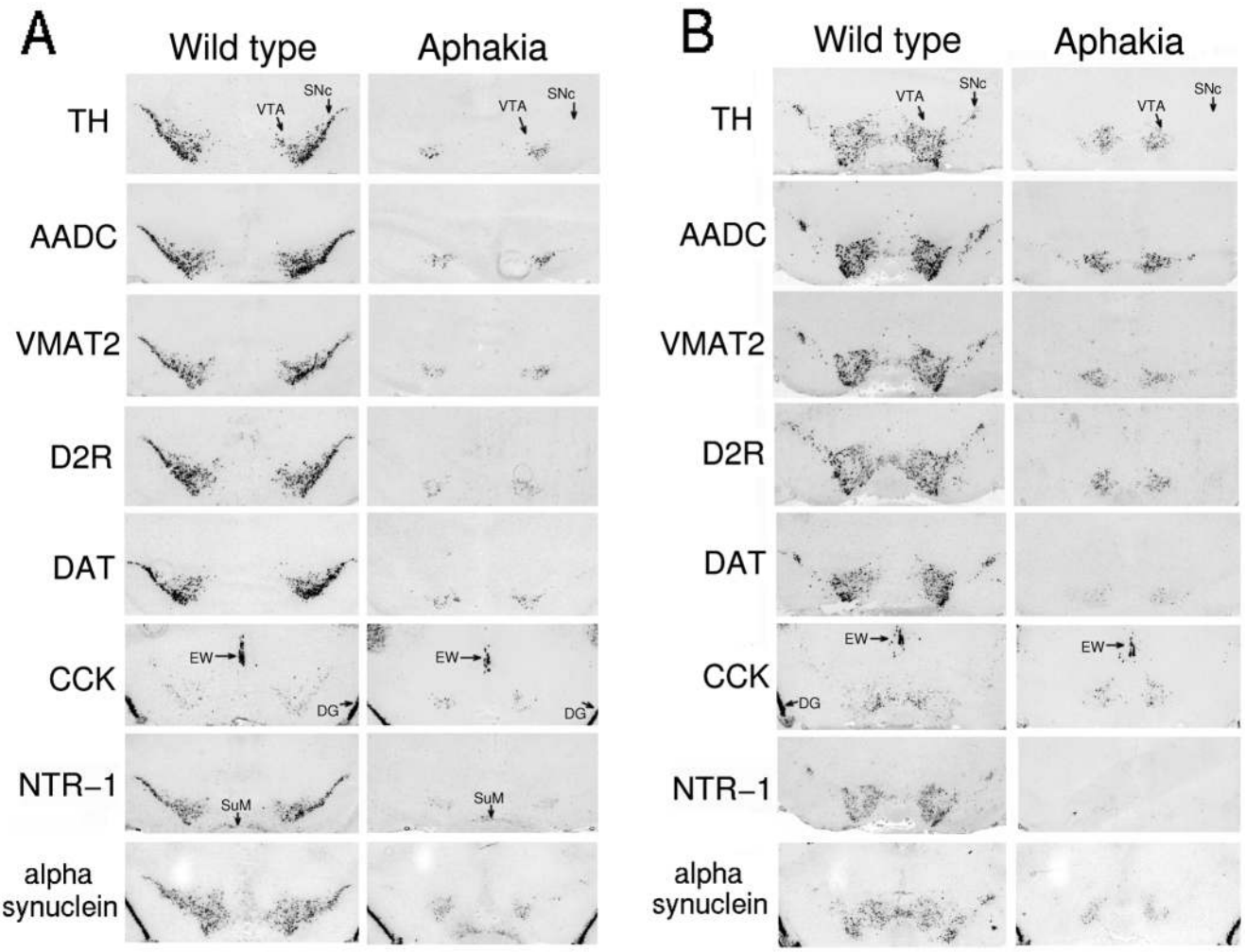

Fig. 8. Analysis of mesencephalic dopaminergic (mesDA)-associated gene expression in SNc (A) and VTA (B) of *ak* mice compared with wild-type mice. Coronal sections of *Th*, *Aadc*, *Vmat2*, *D2r*, *Dat*, *Cck*, *Ntr1* and alpha-synuclein expression in mesDA neurons are depicted. SNc, substantia nigra compacta; VTA, ventral tegmental area; EW, Edinger-Westphal nucleus; DG, dentate gyrus; SuM, supra mammilary nucleus.

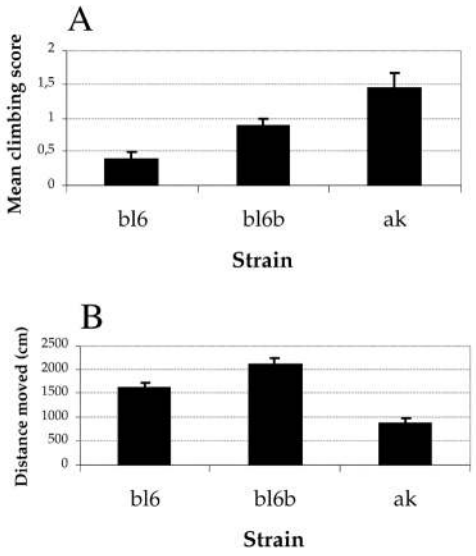

Fig. 9. Behavioral analysis of wild-type (bl6), blind wild-type (bl6b) and *ak* mice. (A) Level of climbing behavior (n=10, all males). The y-axis represents the average score of 10 animals in each group. The error bars indicate the standard error of the mean within the groups. The groups were significantly different from each other (Krukal-Wallis test, P<0.01). Wild-type mice/blind mice (P<0.01); wild-type mice/*ak* (P<0.01); blind mice/*ak* (P=0.124). B) Horizontal movement in an open field. Movement was recorded by automatic analysis software (Ethovision). All groups were significantly different from each other (One-way ANOVA P<0.01), as well as within groups (P<0.01, for all combinations).

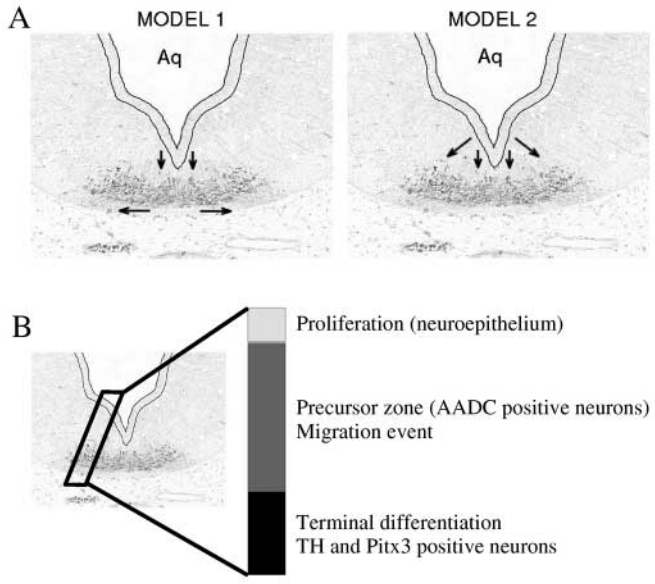

Fig. 10. Graphical representation of the pattern of cell genesis and migration in the midbrain. (A) Reported models for development of the mesencephalic dopaminergic (mesDA) neuronal field combined with tyrosine hydroxylase (Th) staining in coronal sections of E12.5 midbrain. The neuroepithelium is marked by a double line. Model 1 represents vertical migration followed by lateral migration. Model 2 represents perpendicular migration of SN precursors and ventral migration of VTA precursors. The direction of the migration in both models is indicated by arrows. (B) Schematic drawing representing the molecular events of mesDA development, in line with model 2. Aq, aqueduct.

and *Pitx3* expression can be detected. Our data for Th neurons present on E11.5 and *Aadc* expression are in line with these observations. In this model, Pitx3 does not play a role in the initial formation of mesDA neurons because it is not expressed in the assumed precursor cells. This suggests that Pitx3 is essential in the final step of differentiation and that loss of *Pitx3* is lethal to the neurons that should develop into the SNc, because we observed neuron loss already on E12.5 (Fig. 10B).

The *ak* mutant can serve as a model for developmental disorders related to the mesDA system in humans. Moreover, it will provide tools to unravel how mesDA neurons acquire the specific characteristics and connectivity that distinguish this neuronal system from other dopaminergic neurons in the central nervous system. Knowledge about these processes may pinpoint candidate genes of interest to screen in human populations with disorders of dopaminergic neuron function. Finally, the behavioral characteristics of these mice may open new avenues to delineate novel aspects of the behavioral pharmacology of dopamine.

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