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The role of the homeobox transcription factor Pitx3 in the mesencephalic dopaminergic system

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Ce mémoire intitulé :

The role of the homeobox transcription factor Pitx3 in the mesencephalic dopaminergic system

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a été évalué par un jury composé des personnes suivantes :

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Résumé

Le rôle physiologique et l'importance clinique des neurones dopaminergiques mésencéphaliques (MesDA) sont bien connus dans des maladies telles que la schizophrénie, les comportements de dépendance et, de façon plus importante, chez les personnes atteintes de la maladie de Parkinson (PD). Les neurones MesDA sont localisés dans la partie ventrale du mésencéphale et forment la substantia nigra (SN) et le ventral tegmental area (VTA). L'expression du facteur de transcription à boîte homéo Pitx3 dans le cerveau est restreinte aux neurones MesDA et son expression est maintenue au cours de la vie adulte chez les rongeurs et les humains. *Pitx3* est aussi retrouvé hors du système nerveux, comme dans l'œil par exemple, où il est exprimé dans le cristallin en développement. Le développement anormal du cristallin a été observé chez la souris *aphakia* (*ak*), dans laquelle une mutation est survenue naturellement. Cette mutation, récemment identifiée, est causée par deux délétions dans le gène *Pitx3*, incluant une mutation qui délète l'exon1. La présente étude montre qu'un seul sous-groupe des neurones MesDA exprime *Pitx3* et que, dans la souris *aphakia* déficiente en *Pitx3* dans le cerveau, ce sous-groupe disparaît progressivement par apoptose durant le développement fétal (SN) et post-natal (VTA). Ceci résulte en un niveau très bas de dopamine dans le striatum et une akinésie. Similairement à la forme humaine de la maladie de Parkinson, les neurones dorsaux de la SN (négatifs pour *Pitx3*) sont épargnés chez la souris mutante. Ainsi, *Pitx3* définit une voie de survie pour les neurones impliqués dans la maladie de Parkinson et qui sont requis pour l'activité locomotrice spontanée.

Mots clés: boîte homéo, facteur de transcription, *Pitx3*, mésencéphale, dopamine, souris *aphakia*, maladie de Parkinson.

Abstract

The physiological role and clinical relevance of mesencephalic dopaminergic (MesDA) neurons are well recognized in schizophrenia, addictive behavioural disorders and, most importantly, Parkinson's disease (PD). MesDA neurons are located in the ventral midbrain to form the substantia nigra (SN) and ventral tegmental area (VTA). Expression of the homeobox transcription factor Pitx3 is, at the brain level, confined to MesDA neurons and its expression 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. Abnormal eye lens development was observed in the naturally occurring *aphakia* (*ak*) mouse mutant, which was recently found due to two 5' deletions in the *Pitx3* gene, including one that deletes exon1. Here, it is shown that only a subset of MesDA neurons express *Pitx3* and that in *aphakia* mice, which are *Pitx3*-deficient at the brain level, this subset is progressively lost by apoptosis during fetal (SN) and post-natal (VTA) development, resulting in very low striatal DA and akinesia. 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.

Key words: Homeobox, transcription factor, Pitx3, midbrain, dopamine, *aphakia* mouse, Parkinson's disease

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List of abbreviations

Ala	alanine
AADC	amino acid decarboxylase
ADCC	autosomal dominant congenital cataract
ADHD	attention-deficit hyperactivity disorder
ak	*aphakia*
A-P	anterior-posterior
ASMD	anterior segment mesenchymal dysgenesis
bp	basepair
CNS	central nervous system
DA	dopamine
DAT	dopamine membrane transporter
D-V	dorso-ventral
DZT	dizygotic twins
E	embryonic day
EGL	external germinal layer
Fgf8	fibroblast growth factor
GABA	gamma-aminobutyric acid
GPe	globus pallidus external segment
GPi	globus pallidus internal segment
HD	homeodomain
IsO	isthmic organizer
LDDC	3,4-dihydroxyphenylalanine decarboxylase
L-DOPA	3,4-dihydroxyphenylalanine
MesDA	mesencephalic dopaminergic
MHB	midbrain/hindbrain boundary
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MZT	monozygotic twins
6-OHDA	6-hydroxydopamine
P	postnatal day
PD	Parkinson's disease
PET	positron emission tomopgraphy
Pro	proline
PrP	prion protein promoter
RT-PCR	real-time polymerase chain reaction
Shh	sonic hedgehog
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
STN	subthalamic nucleus
TH	tyrosine hydroxylase
Thr	threonine
VTA	ventral tegmental area
wv	*weaver*

Dedication

To enthusiasm and those who encouraged mine.

I. INTRODUCTION

1. General introduction

The complexity of the mammalian brain depends for a great part on the diversity of differentiation and maturation pathways taken by neuronal cells. Several genes are known to define large domains of the central nervous system (CNS) during early development, acting on mitotic precursors. These genes pattern the neuroepithelium and specify the identity of neuronal precursors along the anterior-posterior (A-P) and dorso-ventral (D-V) axes [1]. Final differentiation occurs once precursors have exited the cell cycle and is characterized by expression of the enzymes or neurotransmitters required for neural function. Besides, many developing neural systems undergo a period of natural cell death, which determines the number of neurons that survive into maturity [2]. Few genes have been implicated in these late events of differentiation and maturation of specific neuronal populations.

2. Mesencephalic dopaminergic (MesDA) system

The catecholamine dopamine (DA) is an important regulator of many neural functions including motor regulation, neuroendocrine hormone release, cognition, emotive behaviour and reward [3]. Tyrosine hydroxylase (TH) catalyses the rate-limiting step for DA synthesis, hydroxylating tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), which is subsequently converted to DA by the

more abundantly expressed L-DOPA-decarboxylase (LDDC) or amino acid decarboxylase (AADC). Although these biosynthetic enzymes are present in all parts of DA neurons, the neurotransmitter is predominantly synthesized in the nerve terminals [4]. Upon release from the presynaptic terminal into the synaptic cleft, DA neurotransmission is terminated by reuptake into the presynaptic DA fibres by the DA membrane transporter (DAT), which is unique to DA neurons.

In mammals DA neurons are relatively few, when compared to the total number of brain neurons. They are mainly located in the ventral midbrain/mesencephalon to form the substantia nigra (SN) and the ventral tegmental area (VTA) [5]. The SN is composed of the dorsally situated pars compacta (SNc), that exhibits intense dopamine fluorescence [6], and the ventrally situated pars reticulata (SNr), that contains hardly any dopamine fluorescence but the neurotransmitter gamma-aminobutyric acid (GABA) instead [7]. In rodents, DA neurons arising from the SNc project to the striatum (corresponding to the caudate-putamen in primates) and receive innervation from multiple structures in the diencephalons and telencephalon. The ascending nigrostriatal pathway regulates motor control [4] and its degeneration in humans is associated with Parkinson's disease (PD) [8]. DA neurons from the VTA project to the limbic system and cortex, and are involved in emotional and reward behaviour and motivation [3]. Disturbances in the mesencephalic dopaminergic (MesDA) system have been associated with schizophrenia, addictive behavioural disorders and attention-deficit hyperactivity disorder (ADHD) [9, 10]. Since the physiological role and clinical relevance of DA neurons are well recognized, the mechanisms underlying their development have been the object of intense investigation.

2.1 Early MesDA neuron development

At embryonic day 11.5 (E11.5) of mouse development, the first TH-expressing neurons arise at the most ventral rim of the neuroepithelium lining up along the mesencephalic flexure of the ventral midbrain [11]. The precise anatomical localization and functional differentiation of DA neurons in the mammalian brain is achieved through the action and gradient disposition of various diffusible factors. Data from tissue transplantation and explant culture studies and biochemical and genetic experiments have demonstrated that DA neurons develop at sites where the signals of two distinct signalling molecules, sonic hedgehog (Shh) and fibroblast growth factor 8 (Fgf8), both necessary and sufficient for the induction of DA neurons, intersect [12]. These inductive molecules are thought to activate cascades of other signalling molecules and transcription factors, which lead to the final differentiation and maturation of DA neurons. Before the expression of DA-specific markers, (ventral) midbrain markers are present in these cells. Among the earliest markers of the region are *Otx2, Gbx2, Wnt1, Lmx1b, En1, En2, Pax2* and *Pax5*. The first specific signs of the birth of MesDA neurons shortly follow the induction of the orphan nuclear hormone receptor *Nurr1* (E10.5) [13] and the homeobox gene *Pitx3* (E11) [14], when expression of the key enzyme in DA synthesis TH is initiated and completed.

MesDA neurons are generated from proliferating cells facing the ventricle, migrate ventrally from the ventricular surface, and then laterally along the ventral pial surface to form the VTA and SNc [15]. Their generation in mammals occurs

according to a strict neurogenetic timetable that is linked to the neurogenetic gradients in the striatum [16]. Quantitative analysis of neuronal birth dates using pulse labelling with single injections of ^{3}H thymidine injections throughout mouse embryogenesis indicates that the neurons of both SN and VTA are generated on days E10-E14; most SN neurons are generated on days E11 and E12, most VTA neurons are generated on days E12 and E13 [17]. By E16, the distribution of TH neurons is basically similar to that seen in adult mice, in which the VTA and SNc are clearly distinguishable [15].

2.2 Late MesDA neuron development

During the course of development, most neuronal populations undergo a regressive event, termed natural, developmental, or physiological cell death [2]. This process eliminates more than half of the neurons initially present and seems to be highly regulated, often by the trophic support that neurons receive from their postsynaptic targets [18] as well as from afferent projections [19]. It has been postulated that the process of developmental cell death serves critical purposes, including the creation of appropriate relationships between the number of neurons and the size of the target so as to foster a structural harmony crucial to establishing a functional brain neurotransmission network [20].

MesDA neurons seem not to escape this sculpting process. Indeed, during rodent development, a large number of SNc and VTA neurons die by apoptosis [21, 22]. This process is essentially a postnatal event. In mice, the highest number of apoptotic neurons is seen at postnatal day 2 (P2) followed by a steep drop until P10.

Thereafter, the number of apoptotic neurons transiently increases, reaches a peak at P14 and drops again. By P32 and on into adulthood, apoptotic neurons are no longer seen [22]. Similar findings have been described in rat [21, 23].

Despite the occurrence of developmental apoptosis, however, there is no decrease in TH neurons numbers in SNc and VTA but, on the contrary, a progressive increase; the proportion of 'TH immunostainable' neurons relative to the total number of neurons present increases over time and parallels the increase in TH neuron numbers [22]. A substantial part of the MesDA neurons, which are all generated on days E10-E14, will thus not express TH until the first postnatal weeks Together with the finding that TH expression and dopamine levels do not reach adult levels before 4-6 weeks of age [24], this suggests that there is a process of 'phenotypic maturation' of MesDA neurons that extends into young adulthood.

2.3 Genes in MesDA neuron development

2.3.1 Early genes

2.3.1.1 A-P patterning genes

Complexity within the CNS is generated in a stepwise manner. The formation of the mesencephalon (midbrain) and metencephalon (embryological precursor to adult anterior hindbrain structures including the cerebellum) is one of the best studied examples of sequential pattern formation. The initial A-P regionalization begins during gastrulation and is characterized by the broad induction of several genes in the anterior plate [25]. The presumptive fore-midbrain and the prospective rostral hindbrain are visualized at E7.75 of mouse development

by the expression of two transcription factors, *Otx2* and *Gbx2* respectively [26]. Their expression domains meet and are mutually exclusive at the future midbrain/hindbrain boundary (MHB) [27]. Gain-of-function studies have demonstrated that mutual antagonism between *Otx2* and *Gbx2* determines the position of the MHB [28]. *Otx2* knockout mice lack the forebrain, midbrain and rostral hindbrain [29-31], *Gbx2* knockout mice show abnormalities in the isthmus structure and caudal extension of the midbrain [32].

The second phase of A-P pattern formation begins when an organizer is established just caudal to the *Otx2/Gbx2* junction, the isthmic organizer (IsO), that develops in conjunction with the isthmus. Transplantation and explant studies have demonstrated that the IsO is responsible for patterning much of the midbrain and hindbrain along the rostrocaudal axis [33]. The proper establishment of the IsO is not just defined by the two transcription factors *Otx2* and *Gbx2* but rather by a complex cascade of genetic interactions. Soon after *Otx2* and *Gbx2*, the signalling molecule Wnt1 (encoded by a vertebrate homologue of the *Drosophila wingless* gene), *Fgf8* and the homeobox transcription factor En1 (encoded by a vertebrate homologue of the *Drosophila* segmentation *engrailed* gene) are activated in broad areas along the posterior midbrain and rostral hindbrain. Then their expression becomes more restricted and between E9 and E10, the restricted expression of these genes defines an isthmic molecular code: *Otx2* and *Wnt1* identify the midbrain side of the MHB, *Fgf8* and *Gbx2* identify the hindbrain side and *En1* identifies both sides [34]. Mutant mice that are homozygous null for *Wnt1* fail to form a normal MHB and, ultimately, lack most of the midbrain, the isthmic nuclei and the anterior cerebellum [35-40]. Partial loss-of-function mutations in *Fgf8* disrupt midbrain and

cerebellum development in mouse and fish [41, 42]. *En1*-mutants lack the cerebellum and inferior colliculus [43], a phenotype similar to, but milder than, that of *Wnt1* null mutants. The midbrain- and cerebellum-inducing properties of the IsO have so far only been demonstrated for Fgf8 [44, 45]; when implanted in the caudal diencephalon, Fgf8-soaked beads can induce an ectopic midbrain by modifying the fate of the host tissue surrounding the beads.

In addition to the genetic cascade mentioned above, expression studies from the past years have identified other early genes that get (transiently) expressed in the MHB of the developing embryo. *Pax2*, *Pax5*, *Lmx1b* and *En2* are expressed in overlapping bands of cells in the presumptive mid- and hindbrain and analyses of their gene mutants have provided an essential resource for further exploring the cellular events that control midbrain formation. The paired-box-containing transcription factor Pax2 (encoded by a vertebrate homologue of the *Drosophila paired* gene) is expressed between E7.5 and E11 and targeted inactivation results in exencephaly at the midbrain region [46]. *Pax5* shortly follows *Pax2* (E8.25) and reaches its maximal expression at E12.5 [47, 48]. The brain phenotype of homozygous *Pax5* mutant mice manifests as a change in the foliation pattern on the anterior cerebellum and as a reduction of the inferior colliculi near the midline that are first visible only at E16.5 [49, 50]. Interestingly, analysis of *Pax2/5* compound mutant embryos show a loss of midbrain/hindbrain anlagen and Fgf8/Wnt1/En1 expression, and a subsequent complete loss of the midbrain and cerebellum, together with fusion of the fore- and hindbrain [51]. The LIM homeobox gene *Lmx1b* is expressed at E7.5 in the future MHB region. Early developmental expression extends anterior into the ventral hypothalamic area and the ventral midbrain, and

posterior into the dorsal hindbrain and the dorsal part of the spinal cord [52]. Its expression continues into adulthood in MesDA neurons. In *Lmx1b* mutant mice, a small set of TH neurons develops in the ventral midbrain, but subsequently gets lost; after E16 no TH neurons are present in this region [52]. In addition, severe deficits were observed in other regions of the midbrain [52]. *En2* is expressed at E8.5, half a day later than *En1*, and has a similar expression profile to *En1* [53]. *En2* mutants show reduction in the size of the cerebellum and an abnormal cerebellar foliation pattern [54, 55]. Recently, it was shown that MesDA neurons express both engrailed proteins into adulthood [56]. The proteins are able to compensate for each other in the development of MesDA neurons and targeted deletion of both *En1* and *En2* genes results in complete loss of TH neurons by E14 [56].

2.3.1.2 D-V patterning genes

As stated before, the generation of a proper functional MesDA system requires a developmental programme that ensures that appropriate cell types are generated at the correct time, in specific positions and in the correct numbers. The first major insight into the understanding of these processes came with the discovery of Shh. Shh is produced by two ventral midline signalling centres: the notochord, the axial mesoderm that underlies the ventral neural plate; and the floor plate, a specialized population of cells at the ventral midline of the CNS [57]. Transplantation and explant studies had already demonstrated that these two signalling centres were responsible for ventralizing the neural tube [58], and Shh was shown to be present in these, precisely at the times when the centres were

known to have inducing capacities [57]. Subsequently, gain- and loss-of-function experiments demonstrated that Shh is both necessary and sufficient to induce ventral-neural-cell types [59, 60]. Shh was postulated and shown to function in a concentration-dependent way as a gradient morphogen, acting both directly and at long-range to pattern the ventral neural tube [61]. Signalling by a Shh gradient establishes distinct progenitor domains by regulating the expression of a set of homeobox transcription factors that comprises, at the spinal cord level, members of the Pax, Nkx, Dbx and Irx families [62-64]. These homeobox transcription factors have been subdivided into class I and class II proteins, based on their differential regulation by Shh signalling. Class I proteins are synthesized by neural progenitor cells in the absence of Shh, whereas production of class II proteins depends upon exposure to Shh [62-66]. By the induction or the repression of the levels of these transcription factors, Shh defines five progenitor domains in the ventral neural tube. In turn, the pairs of transcription factors that abut a common progenitor-domain boundary repress each other's expression, contributing to the definition of the neural progenitor domains and the positions at which postmitotic neurons are generated [67, 68]. It is possible that similar mechanisms are responsible for determining ventral cell fate in more anterior areas of the CNS. Recently, Shh-mediated induction of similar molecularly distinguished territories was described for the developing chick midbrain [69]. At E5, five midbrain territories can be identified: arc 1 (marked by PhoxA-expressing motorneurons), arc 2 (Gata2- and Foxa2-expressing neurons), a Pax6-expressing stripe, arc 3 (Gata2-expressing but Foxa2-negative neurons) and an Evx1 stripe [69]. It remains to be determined how these territories influence MesDA neuron development, but Shh has been clearly shown to

mediate their induction [12]: DA neurons develop at sites where the signals of the two molecules Shh and Fgf8 intersect. Besides, these two extracellular inducers are necessary and sufficient for the induction of DA neurons in multiple locations in the neural tube [12].

Recently, it was postulated that Fgf signalling, whose patterning activity along the A-P axis has been extensively studied, has also a role in D-V patterning of the MHB: the expression of Shh target genes was shown to be suppressed in a dose-dependent manner by a dominant-negative Fgf receptor [70], suggesting that apparently functional Fgf signalling is necessary for proper D-V patterning at the MHB.

2.3.2 Late genes

2.3.2.1 Nurr1

Nurr1 is a member of the orphan nuclear hormone receptor superfamily of transcription factors and is highly homologous to its other members *Nur77*, also called *NGFI-B* (nerve growth factor inducible-factor B) and *Nor1* [71]. *Nurr1* expression appears to be predominantly brain-specific, whereas *Nur77* and *Nor1* are found in many tissues [71]. In the adult rodent brain, the three genes are expressed in several unrelated regions of the CNS, in both distinct and overlapping patterns [72-74]. The most striking regional difference is the presence of Nurr1, but absence of Nur77 and Nor1, in the SN and VTA.

In the embryonic midbrain, *Nurr1* expression covers the entire ventral region. Only a small proportion of the *Nurr1*-expressing neurons overlaps with the

MesDA progenitor cells. Thus, its midbrain expression is not uniquely linked to MesDA neurons. *Nurr1* expression is confined to postmitotic progenitor cells and starts at E10.5 in the mouse, just before the induction of the dopaminergic markers Pitx3 (E11) and TH (E11.5) [13, 14, 75, 76]. The expression is maintained in the adult stage, albeit in a more limited pattern, but including the MesDA system [72, 73, 77].

The function of *Nurr1* has been addressed by the creation of null-mutant mice. *Nurr1* knockout mice develop until gestation but die soon after birth. Although the cause of death is not entirely clear, it appears to involve inability to suckle. Mutant mice display movement disturbances including severe difficulties in turning when placed on their backs and abnormal flexion-extension movements of the limbs [75]. Analysis of the brain of these animals showed that in newborn animals, no TH or AADC could be detected in the midbrain and no DA was detected at the level of the striatum [75, 78, 79]. The *Nurr1*-/- phenotype is remarkably midbrain-specific since hypothalamic dopaminergic neurons and neural crest-derived TH cells in the adrenal medulla, that both express *Nurr1*, are not affected by the ablation of *Nurr1* [78, 80, 81]. Possibly, these latter two cell populations remain unaffected due to their concomitant expression of Nur77 and Nor1 [72-74].

Although it was initially concluded that the absence of *Nurr1* causes "agenesis" of MesDA neurons [75], several additional studies demonstrated a more refined role of *Nurr1* in MesDA neurons. Midbrain progenitor cells in E12.5 *Nurr1* null mutants fail to induce TH, but express other MesDA markers like *En1/2, Lmx1b* and *Pitx3* [52, 76, 78]. Thus, it appears that the MesDA progenitor cells are initially generated and that the only marker that is missing is TH.

The perinatal survival of these *Nurr1*-/- MesDA progenitor cells is not entirely clear, since conflicting findings exist. Some studies showed increased apoptosis and decreased neuron numbers in the ventral midbrains of *Nurr1* mutant newborns [76, 78, 80], whereas others reported comparable number of apoptotic cells and no changes in cell number in the SN and VTA of wildtype mice and *Nurr1* mutants [82]. Another conflicting finding in *Nurr1* mutants is whether the MesDA progenitor cells migrate to their normal positions and extend projections toward the striatum. Wallén et al. [76] showed at E15.5 a marked reduction and mainly medial localization of MesDA markers, suggesting that the cells are unable to migrate laterally. In newborns, they showed no retrograde fluorogold tracer labeling in the ventral midbrain after striatal injection, suggesting that the too medially located MesDA progenitor cells were unable to innervate the striatum. Witta et al. [82], however, reported no apparent difference in topographic pattern of SN and VTA neurons in *Nurr1* mutant newborns and normal anterograde DiI tracer-labeling in the striatum after midbrain injection, suggesting that the nigrostriatal neuronal connections are well developed, even in the absence of DA. The reason for these discrepancies is unknown and could result from different genetic background of the *Nurr1* knockout mouse strains or differences in how the gene targeting of *Nurr1* was achieved.

In a recent tissue culture study from Törnqvist et al. [83], TH neurons were found in dissected ventral mesencephalon from both E10.5 and E15.5 *Nurr1* mutants when cultured in the presence of serum medium. A similar finding was reported by Eells et al. [84], who detected TH neurons in dissociated ventral midbrain neurons from newborn *Nurr1* mutants that were grown on an astrocyte feeder layer. These

results strongly suggest that Nurr1 is not absolutely required for TH expression in ventral midbrain in vitro. Still, Nurr1 has been shown to activate transcription of the *TH* gene promoter [85-87], although others reported no effect on *TH* promoter activity [88]. Besides, Nurr1 has been reported to regulate *DAT* transcription [89]. Törnqvist et al. [83] also reported a clear difference in nerve fiber distribution in wildtype and *Nurr1* mutant cultures when co-cultured with striatal tissue. No well-defined nerve fiber bundles were found in knockout cultures suggesting that DA axon pathfinding does require Nurr1. It was noted, however, that these findings were derived from the same line of *Nurr1* knockout animals previously shown to lack nigrostriatal connections [76].

The relevance of the human homologue of the murine *Nurr1* gene, also known as the *NR4A2* or *NOT* gene [71]; [90] to the pathogenesis of diseases that affect the MesDA system has been addressed recently by several groups. Buervenich et al. [91] reported two different missense mutations in the third exon of the *NURR1* gene in two schizophrenic patients and another missense mutation in the same exon in an individual with manic-depressive disorder. All three mutations caused a similar 30-40% reduction of in vitro Nurr1 transcriptional activity. Chen et al. [92] identified a rare variant of the *NURR1* gene, with a nucleotide-deletion in the untranslated exon 1, in two out of one hundred and seventy-seven unrelated Han Chinese schizophrenic patients from Taiwan. Bannon et al. [93] reported a significant, more than 50%, reduction in *Nurr1* and *DAT* mRNA in postmortem MesDA neurons of cocaine abusers whereas the mRNA level of human vesicular monoamine transporter 2, a distinct transporter protein expressed in high abundance within MesDA neurons that mediates the intracellular storage of dopamine [94] but

is not known to be regulated by Nurr1, was unaltered. Xu et al. [95] found a significant correlation between homozygozity for the 7048G7049 polymorphism in intron 6 of the *NURR1* gene, that inserts a single nucleotide between nucleotide positions 7048 and 7049, and PD.

Taken together, it seems that Nurr1 is an essential factor in the differentiation program of MesDA progenitor cells and also fulfills a role in maintaining MesDA neuron phenotype within the mature CNS, by controlling TH and DAT expression in a direct or indirect way. It remains to be determined whether the absence of Nurr1 causes severe loss of MesDA neurons. Possibly, the *Nurr1*-/- MesDA progenitor cells cannot form or maintain the connections to their targets. The absence of TH alone is not sufficient for the absence of projections, since *TH* mutant mice still form projections to the striatum [96]. Alternatively, *Nurr1* may function parallel in a switch from proliferating stem cells to non-proliferating differentiating cells and be more fundamental in the control of the cell cycle; *Nurr1* expression starts at about the same time as neuronal differentiation becomes evident [76] and Nurr1 can induce cell cycle arrest and a highly differentiated cell morphology in the DA-synthesizing cell line MN9D [97].

2.3.2.2 Pitx3

2.3.3.2.1 The Pitx family

Pitx1, Pitx2 and Pitx3 are members of a paired family of homeodomain (HD) transcription factors [14, 98, 99]. HD transcription factor genes encode HD containing proteins, which play a key role in coordination of gene activity and

determination of cell fate in the development of organisms as diverse as yeast, plants, insects and mammals. The HD proteins share remarkable evolutionary conservation of both protein structure and function.

The first member of this paired family, Pitx1 (pituitary homeobox 1) was isolated as a transcription factor involved in pro-opiomelanocortin gene transcription in anterior pituitary corticotropes [99]. The gene is expressed in cell lines representing the five anterior pituitary neuroendocrine cell lineages [100]. Its expression defines the stomodeum and continues within stomodeal derivates, including the nasal pit and Rathke's pouch, the precursor to the anterior and intermediate lobes of the pituitary gland [101, 102]. *Pitx1* is also expressed in the first branchial arch and, more caudally, in the posterior lateral plate [102], where it was shown to be implicated in specification of hindlimb identity [103].

The second family member *Pitx2* is another early marker for the incipient Rathke's pouch and is expressed in all anterior pituitary neuroendocrine cell lineages except for the corticotrope lineage [100, 101]. It gets expressed in many tissues during development, including the left lateral plate mesoderm, derivates of the first branchial arch, the eye, brain, mandible, heart and limbs [101, 104-107]. During brain development, early *Pitx2* expression patterns within the prosencephalon and mesencephalon are consistent with the prosomeric model of fore- and midbrain development [104, 106, 108]. Subsequently, *Pitx2* expression becomes limited to discrete brain nuclei like the subthalamic nucleus (STN) and the zona incerta pars dorsalis of the thalamus [104, 106]. Recent studies on visceral organogenesis reported that manipulation of *Pitx2* expression is sufficient to reprogram the left/right body asymmetry in vertebrates and *Pitx2* appears to be the most

downstream gene in this laterality pathway that includes *Shh*, *activinβB*, *nodal* and *lefty-2* [109, 110].

Pitx3 is expressed from E11 in mouse mesencephalic progenitor cells and double-staining with *TH* probes showed that *Pitx3* expression completely overlapped with *TH*-positive cells, demonstrating that *Pitx3* is expressed in MesDA neurons [14]. This is the only site of *Pitx3* expression in the CNS and its expression is maintained throughout adult life in mice, rats and humans. Extraneural *Pitx3* expression was shown in the developing lens, nosecartilage, tongue, condensing mesenchyme around the sternum and vertebrae and in the head muscles [14, 111, 112].

2.3.3.2.2 Pitx3 in eye development

Mutations in the human homologue of *Pitx2*, *RIEG1*, play a well-known role in the Rieger syndrome (abnormalities of the anterior chamber of the eye, dental hypoplasia, craniofacial dysmorphism and umbilical abnormalities) and iris hypoplasia [105, 113]. Based on this role of *Pitx2* and the expression of *Pitx3* during lens development, *Pitx3* was suggested to be a strong candidate gene for eye disorders. Besides, *Pitx3* was mapped to the region of the *aphakia* (*ak*) mutation on mouse chromosome 19 [112]. *Ak* is a recessive mutation, described originally by Varnum and Stevens in 1968, that spontaneously occurred in the 129/Sv-Sl^{J} strain [114]. The *ak* mutation causes blindness due to microphthalmia with an absence of lens, iris and pupil. Retinal folds partially fill the vitreous chamber, lacrimal glands are enlarged and eyelids are closed in the adult. These anomalies are caused by an

arrest of lens development at the stage of the lens vesicle formation around E10.5-E11 [114, 115]. In the *ak/ak* mutants, there is persistence of the lens stalk at E12.5 interrupting the corneal mesenchyme and leading to a permanent close contact between the developing cornea and other ocular tissues without the formation of an anterior chamber [115, 116]. No other systemic abnormalities were reported in these mice. Recently, the *Pitx3* gene was shown zero recombination with the *ak* mutation and screening of the gene for mutations revealed a 652 basepair (bp) deletion in the 5' flanking region approximately 2500 bp from the transcription startsite and a 1423 bp deletion eliminating putative promoter sequences, the noncoding exon 1 and part of intron 1 [117, 118]. The levels of *Pitx3* transcripts are only about 5% of wildtype levels in *ak/ak* mice. These data strongly suggest that the ocular *ak* phenotype is caused by a dramatic reduction in *Pitx3* expression as a result of a double deletion in the gene. The relative contribution of the two deletions to the phenotype remains to be determined. The minor deletion contains sequences with some similarity to transcription binding sites and a large polymorphic GAA repeat [117, 118]. This repeat is approximately 150 bp larger in wildtype DBA and C57 mouse substrains than in the various wildtype 129 substrains and others tested [118], suggesting that large changes in this area do not affect eye development. The major deletion extends from the putative promoter into the first intron. Nonetheless, a low level of *Pitx3* transcripts is detectable. This means that a weak promoter activity must be present upstream, in between, or downstream of the deleted regions. Since the translation start site is located in exon 2, it is possible that some small amounts of *Pitx3* protein are made in *ak/ak* mice. However, since the residual portion of intron 1 cannot be spliced out due to the missing 5' splice site, translation of these transcripts may be

aberrantly initiated at any ATG triplet contained in the intron, possibly leading to no or aberrant protein formation [119].

The relevance of the human homologue *PITX3*, that is located in the q24-25 region of chromosome 10, was addressed for individuals with various eye anomalies [111]. Two mutations were identified in independent patients. A 17 bp insertion in the 3' end of the coding sequence, resulting in a frameshift, occurred in a patient with anterior segment mesenchymal dysgenesis (ASMD) and cataracts, and a guanine to adenine substitution, changing a codon for serine into a codon for asparagine, in the 5' end of the gene occurred in a patient with autosomal dominant congenital cataract (ADCC) [111]. The mutations affect regions outside of the HD in the N-terminal or C-terminal end of the protein and result in different phenotypes. These protein regions are thought to be involved in complex protein-protein interactions, imparting specificity and efficiency to HD protein function [120]. Functional studies are required to provide further insights into the mechanisms giving rise to the specific manifestations of these mutations. All the affected individuals from the ASMD family had cataracts of differing severity, suggesting that development of cataracts in ASMD patients might be a dominant feature associated with mutations in *PITX3* [111]. This is consistent with the strong expression of *Pitx3* during lens development.

2.3.3.2.3 Pitx3 in MesDA development

The neural expression pattern of *Pitx3* strongly suggests that it may be involved in development and/or maintenance of MesDA neurons and analysis of the

SN of PD patients revealed a reduced density of *Pitx3*-expressing neurons as compared to normal controls [14]. In recent cell culture studies, *Pitx3* was shown to activate the *TH* gene promoter through direct interaction [87, 88], suggesting that *Pitx3* contributes to the regulation of *TH* expression in MesDA neurons. *Nurr1* null mutant mice, that fail to generate *TH*-positive neurons in the mesencephalon, show at E11.5 a normal expression pattern of Pitx3, indicating that *Pitx3* expression is independent of *Nurr1* and insufficient for proper MesDA neuron differentiation [76, 78]. In E12.5 *Lmx1b* null mutant mice, TH-positive Pitx3-negative cells are generated in the ventral tegmentum indicating that *Lmx1b* and *Pitx3* are not necessary for TH expression [52]. In these embryos, only a small number of aberrantly located Pitx3-positive cells was identified, posterior and dorsal to the TH-positive cells. From E16 however, no TH-positive neurons were present in the region [52] and *Lmx1b* null mutants die as neonates (Michael et al. 1988). A molecular cascade involving *Lmx1b* and *Pitx3* thus seems to be tied to aspects of neuronal specification other than neurotransmitter phenotype, in particular survival of MesDA neurons.

The role of *Pitx3* in MesDA development has not yet been addressed by detailed neurological analysis of humans heterozygous for a mutation in the gene or by creation of *Pitx3* null-mutant mice. No apparent neurological abnormalities were reported in the ASMD and ADCC patients mentioned above [111]. Whether the spontaneously occurring *ak* mouse mutant is also, as in the eye, a severely affected hypomorphic or null allele for *Pitx3* at the brain level remains to be determined. If so, this mutant might be a very useful model to study the role of *Pitx3* in MesDA development. The characteristics of neural *Pitx3* expression are remarkable and the

data from *Lmx1b* null mutant mice suggest that *Pitx3* might be implicated in the survival of MesDA neurons. Degeneration of these neurons is a hallmark of PD [8]. If the *ak* mouse mutant turns out to have severely affected *Pitx3* expression in MesDA neurons, it might provide a useful model for further research on (genetic) causes of PD.

3. Parkinson's disease

3.1 General introduction

More than 180 years ago, James Parkinson first described the disorder that still bears his name [121]. The classic triad of major signs of PD is made up of slowly progressive resting tremor, rigidity and bradykinesia/akinesia and the diagnosis is made on the basis of these clinical criteria. The disease is an important public health problem with a prevalence of ~1% at 65 years of age, increasing to 4-5% by the age of 85, affecting millions of people worldwide [122, 123]. The impact of the disease is indicated by the fact that mortality is two to five times as high among affected persons as among age-matched controls, resulting in a marked reduction in life expectancy [124-126]. Thus, PD greatly shortens life as well as causing debility during life.

Although there are rare familial forms of PD, the majority of PD is sporadic and its specific etiology is unknown. There is, however, evidence for a role of both genetic and environmental factors, for example exposure to pesticides, farming, well water and rural living [127].

PD is characterized by the progressive death of selected but heterogeneous populations of neurons, most importantly the neuromelanin-laden dopaminergic neurons of the SNc and, although less affected, the VTA. Other affected regions are selected aminergic brainstem nuclei (both catecholaminergic and serotoninergic), the cholinergic nucleus basalis of Meynert, hypothalamic neurons and small cortical neurons (particularly in the cingulated gyrus and entorhinal cortex), as well as the olfactory bulb, sympathetic ganglia and parasympathetic neurons in the gut. Within the SNc, neuronal loss tends to be greatest in the ventrolateral tier (loss is estimated to be 60-70% at the onset of symptoms) followed by the medial ventral tier, with the dorsal tier relatively spared [8, 128]. This pattern of cell loss is specific to PD; it is opposite of that seen in normal aging and differs from patterns found in striatonigral degeneration and progressive supranuclear palsy. Recently, it was shown that Calbindin D_{28K}, a well known member of the class of calcium-binding proteins, specifically marks the dorsal tier of the SNc, i.e. those nigral compartments that are the least affected by the PD cell loss [129, 130]. There is thus the recognition that the regions most affected in PD form discrete and identifiable units. This raises the possibility that (i) variable amounts of exogenous or endogenous toxins throughout the SNc, (ii) variable defense mechanisms against these, (iii) variable expression of genes implicated in the disease process or combinations of these form the basis of the stereotypic cell loss in PD.

The selective neuronal loss results in regional loss of striatal DA, most prominently in the dorsal and intermediate subdivisions of the putamen [131]. This DA-deficient state is associated with increased activity of the inhibitory GABA-employing (GABAergic) output nuclei in the basal ganglia, i.e. the internal segment

of the globus pallidus (GPi) and the SNr. The heightened action of the GPi and SNr is thought to arise from at least two mechanisms: reduced inhibition by a "direct" GABAergic connection from the striatum (caudate nucleus and putamen) and excessive excitation through an "indirect" pathway that contains two inhibitory neuronal connections, the first from the striatum to the external segment of the globus pallidus (GPe), and the second from that segment to the STN. The STN excites the GPi and the SNr by means of the neurotransmitter glutamate. Because the GPi and SNr use the inhibitory neurotransmitter GABA, the increased output of the basal ganglia leads to excessive inhibition and, effectively, to a shutdown of the thalamic and brainstem nuclei that receive their outflow. The excessive thalamic inhibition leads to suppression of the cortical motor system, possibly resulting in akinesia, rigidity and tremor, whereas the inhibitory descending projection to brainstem locomotor areas may contribute to abnormalities of gait and posture [127, 132-134].

Another important pathological feature is the presence of degenerating ubiquitin-positive neuronal processes in cell bodies and neurites, Lewy bodies; these eosinophilic hyaline inclusions that contain an accumulation of filaments and granular material with a dense core and loose radiating peripheral filaments are consistently observed in the selectively vulnerable neuronal populations [135]. The mechanism of Lewy-body formation, the importance of Lewy bodies to the pathogenesis of PD and their role in the neurodegenerative process are largely unknown [136].

The mechanisms responsible for cell death in PD are also largely unknown. In addition to the presence of Lewy bodies, postmortem examination of affected

brains of sporadic PD patients showed increased oxidative stress such as glutathione depletion, iron deposition, increased markers of lipid peroxidation, oxidative DNA change and protein oxidation [137]. Another key postmortem finding is decreased expression and activity of mitochondrial complex I in the midbrain. This defect is specific, as it does not occur in other brain areas and it has not been observed in other neurodegenerative diseases. Other components of the mitochondrial respiratory chain are unaffected in PD. Thus, in sporadic PD, oxidative stress and mitochondrial dysfunction appear to play prominent roles in the death of DA neurons in the SNc [137]. Increasing evidence suggests that this death may be apoptotic [138], but this notion is not universally accepted [139]. A critical question remains why specific neurons are selectively vulnerable in PD. Besides the already mentioned mitochondrial dysfunction, a possible answer may lie in their ability to take up both endogenous and extrinsic toxic components through selective carrier mechanisms, such as the DAT. Other possible explanations include their increased metabolic stress, high physiologic rates of protein oxidation, selective generation of potential toxins or failure to detoxify or dispose them and specific requirements for neurotrophic support.

3.2 Genetic causes of Parkinson's disease

There is increasing evidence that genetic factors have an important role in PD, even in the most common sporadic cases of the disease. Earlier studies of twins were originally believed to have excluded an important genetic contribution to PD, since they failed to show a higher concordance among monozygotic twins (MZT)

than among dizygotic twins (DZT) [140]. However, several lines of evidence have suggested the need for a reconsideration of this issue. A recent large study, for example, found high rates of concordance among MZT when one twin had young-onset disease, i.e. before 40 years of age [141]. A study using ^{18}F-Dopa and positron emission tomography (PET) to study dopaminergic function in twin pairs at baseline clinically discordant for PD, showed that concordance levels for dopaminergic dysfunction were significantly higher in MZT than in DZT with both no apparent history of familial aggregation of the disease [142]. Furthermore, the level of concordance in MZT increased with time and 33% of MZ cotwins with abnormal baseline scans subsequently became concordant for clinical PD, supporting the assumption that the nigrostriatal dopaminergic dysfunction identified by ^{18}F-Dopa PET represents subclinical manifestation of the disease [142]. Epidemiological studies have found that, apart from age, a family history of PD is the strongest predictor of an increased risk of the disease [143], although the role of shared environmental exposure in some families must be considered. A recent population-based study on Iceland showed that patients with both young- and late-onset (i.e. onset >50 years of age) PD were significantly more related to each other than matched groups of controls [144].

Until recently, evaluations of candidate genes involved in the DA system in families with multiple PD patients and in patients with apparently sporadic PD were generally unrewarding. A major breakthrough in this field recently came with the identification of two distinct mutations in the *α-synuclein* gene (*SNCA*), located on chromosome 4q, in several families with autosomal dominant forms of PD. One mutation, Alanine53Threonine (Ala53Thr), was reported in a single large Italian

family with very high penetrance (roughly 90 percent) and three smaller Greek families that may be very distantly related [145], and the other, Alanine30Proline (Ala30Pro), was reported in a family of German origin [146]. α-Synuclein is a highly conserved, abundant 140-amino-acid protein of unknown function that is expressed mainly in presynaptic nerve terminals in the brain [147]. Lewy bodies are consistently stained with antibodies to α-synuclein [148]. Several other studies have, however, failed to detect mutations in *SNCA* in a large number of other families [146, 149, 150], and in sporadic cases [146], suggesting that PD is only rarely caused by such mutations. Another breakthrough was the identification of two mutations in the *parkin* gene, located on chromosome 6q, in four unrelated Japanese families with autosomal recessive juvenile parkinsonism. In one family, five out of twelve exons were deleted, the three other families had a deletion affecting exon 4 alone [151]. Parkin is similar to the ubiquitin families of proteins, which are involved in the pathogenesis of several neurodegenerative diseases and are a component of the paired helical filaments in Alzheimer's disease [152-154] and of Lewy bodies in PD [155-157]. Recent investigations suggest that missense mutations in *parkin* can also cause PD that appears indistinguishable from sporadic PD [158]. These observations promise to provide important insights into the pathogenesis of nigral degeneration and Lewy-body pathology.

Although the debate whether PD is caused by environmental agents, hereditary factors, or a combination of both has not finished, studies from the recent past have shown overwhelming evidence for a role of genetic factors in susceptibility to PD, in both the familial and sporadic cases of the disease. The

general goal of current genetic studies in the area of PD is to find putative susceptibility genes.

3.3 Experimental and animal models of Parkinson's disease

In many neurodegenerative disorders, animal models have provided tremendous insight and clues into the pathogenesis of these disorders. For direct relevance to human PD, an ideal animal model for this disease should have the following characteristics: first, a "clinically" normal complement of DA neurons at birth with selective and gradual loss of DA neurons commencing in (early) adulthood. The losses should exceed 50% and be readily detectable using biochemistry and neuropathology. Second, the model should have easily detectable motor deficits, including the cardinal features of PD, which are bradykinesia/akinesia, rigidity and resting tremor. Third, the model should show the development of characteristic Lewy bodies. Fourth, if the model is genetic, it should be based on a single mutation to allow robust propagation of the mutation, as well as crossing with enhancer or suppressor strains. Last, it should have a relatively short disease course of a few months, allowing rapid and less costly screening of therapeutic agents.

The first agent used to model PD was 6-hydroxydopamine (6-OHDA). After injection into the striatum or SN, it selectively accumulates in DA neurons and kills them owing to toxicity that is thought to involve the generation of free radicals. 6-OHDA is an effective toxin in rats, mice, cats and primates that has been predominantly used to produce unilateral lesions. In rats, the extent of DA depletion

can then be assessed by examining rotatory behaviour in response to amphetamine and apomorphine [159]. It has therefore proved to be useful for pharmacological screening of agents that have effects on DA and its receptors. 6-OHDA lesions do not result in Lewy bodies and can produce non-specific damage to other neurons.

Betarbet and colleagues produced a model of PD by infusing rats intravenously with rotenone [160], a naturally occurring compound from the roots of certain plant species, which has been used as an insecticide and to kill or sample fish populations in lakes or reservoirs. It is known to be a high-affinity specific inhibitor of mitochondrial complex I. The rats developed a progressive degeneration of nigrostriatal neurons with a loss of TH, DAT and vesicular monoamine transporter immunoreactivity. Furthermore, the SN neurons showed cytoplasmic inclusions that were reminiscent of Lewy bodies. The rats showed bradykinesia, postural instability, unsteady gait and some evidence of tremor, and the deficits improved after treatment with the DA receptor agonist apomorphine. These findings are remarkable because they show that an inhibitor of mitochondrial complex I, which acts throughout the brain, can produce selective degeneration of nigrostriatal neurons, therefore indicating that SN neurons are particularly vulnerable to these inhibitors. This model meets most of the criteria needed for an excellent animal model of PD. However, it suffers from much variability in the susceptibility of individual rats to the toxin. In the study of Betarbet and colleagues, only 12 out of 25 rats treated with standardized doses of rotenone developed lesions [160] and precludes therefore the usefulness of the model for testing neuroprotective agents. It could be, however, valuable in examining cell replacement strategies such as transplantation of fetal nigral neurons

or stem cells, and it might lead to novel insights into the pathophysiology of the death of SN neurons in PD.

The best-characterized model of PD has been developed by using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [161]. The discovery of MPTP occurred in 1982 when a group of drug addicts in California developed subacute onset of severe parkinsonism. Investigation revealed that the syndrome was caused by self-administration of a synthetic heroin analogue that had been contaminated by a byproduct, MPTP, during manufacture. MPTP administration was subsequently shown to model PD in both mice and primates. MPTP toxicity in primates replicates all the clinical signs of PD, including resting tremor, rigidity, akinesia and postural instability [161, 162], and has been very useful for studying the striatal circuitry involved in PD pathophysiology. Primates treated with MPTP show an excellent response to L-DOPA and dopamine receptor agonists. As in PD, the SNc neurons are particularly vulnerable, whereas VTA neurons are more resistant. The toxin also causes a loss of locus coeruleus neurons, cells that are also lost in PD [163]. It is not entirely clear how MPTP administration leads to neuronal death. Intracellular 1-methyl-4-phenylpyridinium (MPP^+), MPTP's active metabolite, inhibits mitochondrial complex I [164] and increases the production of free radicals [165]. There is experimental evidence that this results in cell death by apoptosis [166-169], but also a possible role for inflammation in the pathogenesis of MPTP toxicity has been suggested [170-172]. The DA neuron selectivity of MPTP neurotoxicity is thought to stem in part from the conversion of MPTP by monoamine oxidase B, also implicated in DA catabolism, and from avid affinity of MPP^+ for the DAT. In aged MPTP-treated primates, there were neuronal

inclusions that partially resembled Lewy bodies [173], but typical electronmicroscopic features were not observed [174]. The main difficulty with MPTP toxicity as a model of PD, however, is that it is an acute or subacute process, whereas PD is a slowly progressive illness that evolves over years. With this in mind, chronic administration of MPTP in primates using several dosing regimes has been shown to produce slow evolution of a parkinsonian syndrome, with uneven striatal DA fibre loss and more selective depletion of SN neurons [163, 175-177]. The MPTP animal model has been extremely valuable in testing neuroprotective and neurorestorative strategies [178-183], and many of these discoveries are currently in various stages of therapeutic development [137, 184]. The most striking difference with PD is the lack of Lewy body formation, but it seems that more chronic administration of MPTP could produce Lewy bodies, similarly to chronic administration of rotenone [185].

The spontaneously occurring *weaver* (*wv*) mouse mutant suffers from severe ataxia that is obvious by about the second post-natal week [186]. *Wv/wv* mutant mice are characterized by a cerebellum that is drastically reduced in size due to depletion of the major cell type of the cerebellum, the granule cell neuron [187-189]. The precursors to granule cell neurons in the cerebellar external germinal layer (EGL) are present at birth and are apparently normal [189]. However, rather than leaving the EGL during the first post-natal week, the precursors die *in situ*. The *wv* locus is on mouse chromosome 16 and was recently shown to have a single base-pair substitution mutation in the *Girk2* gene, a potassium channel gene that is expressed in the brain [190, 191]. The mutation affects a highly conserved domain of the voltage-gated, calcium dependent and inward rectifying families of potassium

channels [192], thereby eliminating channel conductance or altering ion selectivity [193-197]. Beside the cerebellar phenotype, *weaver* mice also have a number of other defects. Interestingly, dopaminergic input to the striatum is lost during the first few weeks after birth due to the death of DA neurons in the SN [198]. In addition, male homozygous mice are sterile [199] and both heterozygous and homozygous animals undergo sporadic tonic-clonic seizures [200]. At birth, *wv/wv* mice have normal numbers of MesDA neurons, but at P3 some deficit in dopaminergic uptake occurs [201, 202], indicating that these normally generated and migrated neurons already have functional deficits. By P7, abnormalities in the neurites of TH-positive neurons are evident in the *wv/wv* SN [203]. By P20, approximately 42% of the TH-positive neurons in the *wv/wv* SN have degenerated, with all the SN subdivisions involved, whereas the VTA is then still unaffected [204]. By P90, the SN cell loss is 69% and the VTA cell loss 26% [204]. ^{3}H Thymidine autoradiography of MesDA neurogenesis showed that late-generated MesDA neurons, i.e. generated on E12 and E13, are more vulnerable to *weaver* gene action than those that are generated earlier [202]. Taken together, these data suggest that the *weaver* potassium channel mutation may have an effect on neurite function and formation that precedes the later observed cell death of postmitotic MesDA neurons and may even influence its occurrence. *Wv/wv* mice represent thus far the only naturally occurring animal model of genetically determined nigrostriatal deficiency. Research on this mouse mutant has revealed important information, especially on MesDA neurogenesis. However, the concomitant severe cerebellar pathology with associated locomotor dysfunction, makes this mutant not very suitable as animal model for PD, a disease that finds most of its etiology in the midbrain.

As mentioned earlier, mutations in *α-synuclein* are related to PD in some pedigrees. A novel model of PD was recently produced by expressing mutant and normal forms of α-synuclein in *Drosophila* [205]. The flies showed an age-dependent loss of dorsomedial neurons that stained for TH, with no difference in the toxicity of wildtype and mutant α-synuclein. Other subsets of DA neurons, however, showed no abnormalities, consistent with the differential vulnerability of DA neurons in human PD, and cortical neurons and serotonin neurons were spared. In addition, α-synuclein-stained inclusions were observed in neurons, which resembled Lewy bodies. The flies developed locomotor dysfunction with age, but it was not determined whether these are due to dysfunction of DA neurons, nor if these deficits could be reversed by the use of DA receptor agonists. There was also retinal degeneration, which is not a typical feature of PD, but this could be a unique feature of the *Drosophila* model. The well-characterized genetics of *Drosophila* will allow the rapid characterization of enhancer and suppressor mutations and it is without doubt that this model will teach us much about the pathogenesis of α-synuclein-induced neurodegeneration in flies. However, the challenge will be to verify potential neuroprotective agents and genetic modifiers in human PD.

A number of transgenic mice models overexpressing human α-synuclein have been developed. While none of these mice accurately model PD, a number of the models exhibit significant synucleinopathy-induced neurodegeneration, thus modelling characteristics of human α-synuclein pathology. Expression of human wildtype or Ala53Thr α-synuclein using the Thy1 promoter that directs neuron-specific expression but no expression in SN neurons, resulted in increased α-synuclein staining in ±80% of motor neurons (some also showing Lewy-body-like

pathology) with significant degeneration of neuromuscular junctions and axonal degeneration [206]. The mice exhibited a progressive loss of motor function, but it was not determined whether there was loss of DA neurons nor whether the motor deficit was responsive to DA receptor agonists. Mice overexpressing wildtype α-synuclein, using the platelet-derived growth factor promoter, develop a progressive accumulation of α-synuclein- and ubiquitin-immunoreactive inclusions in the neocortex, hippocampus and SN [207]. However, there were both cytoplasmic and nuclear inclusions, and the latter are not a typical feature of PD. Besides, no fibrillar aggregates that are characteristic of Lewy bodies were seen. There was no loss of DA neurons within the SN, but there was a loss of DA terminals in the transgenic line with the highest expression level. There was also a decrease in TH protein levels and activity, although DA levels were not measured. The mice suffered postural instability at one year, but it was not determined if these deficits were reversible with DA receptor agonists. Mice expressing human Ala53Thr mutant α-synuclein, but not wildtype or Ala30Pro mutant, under the control of the mouse prion protein promoter (PrP) develop an adult-onset neurodegenerative disease with progressive motor dysfunction leading to death [208, 209]. The neurological dysfunction was associated with age-dependent intracytoplasmic neuronal accumulation of α-synuclein and pathological neuronal accumulations of ubiquitin. Besides, the α-synuclein inclusions contained 10-16 nm wide fibrils similar to human Lewy bodies [209]. However, it remains to be determined whether the mouse PrP human Ala53Thr α-synuclein transgenic mice exhibit DA neuron loss and whether the neurological deficits can be reversed by the use of DA receptor agonists. Although overall, the results from the various α-synuclein transgenic mice studies indicate that

formation of α-synuclein aggregates is associated with some neurodegenerative characteristics, none of the models have yet shown to have significant abnormalities in DA neurons. It is conceivable that rodent DA neurons are resistant to α-synuclein toxicity and that higher levels are needed to achieve α-synucleinopathy-induced PD. Consistent with this notion is the observation that stable, virally mediated overexpression of human wildtype and Ala53Thr mutant α-synuclein with adeno-associated virus in SNc neurons of rats leads to progressive age-dependent loss of DA neurons, motor impairment and α-synuclein-positive cytoplasmic inclusions [210].

As it is likely that PD will prove to be heterogeneous in its aetiology, with genetic factors predominating in some cases, and environmental factors in others, it is as yet unclear whether toxic or genetic models will prove to be most useful as animal model of PD. None of the currently available models recapitulate all of the key clinical and neuropathological features of PD. Because each of the models does recapitulate significant features of PD and represents parts of the same puzzle, combinatorial study of multiple models are warranted to provide a more fully developed view of PD pathogenesis. An important question that remains to be answered is why PD ravages the SNc in such a stereotypic manner with the ventral tier most affected and the dorsal tier relatively spared. None of the currently available animal models has accurately copied this pattern of SNc cell loss and beside the observation of Calbindin D_{28K} specifically marking the dorsal tier of the SNc in humans, no genetic markers that define specific subsets of SNc neurons have yet been identified.

II RESULTS

1. Project description

There is no data in the literature exploring the in vivo role of *Pitx3* in MesDA development. The only developmental data on neural *Pitx3* currently available is descriptive, showing expression in the MesDA system [14] and analysing *Pitx3* expression pattern in MesDA progenitor cells in *Nurr1* and *Lmx1b* knockout mice [52, 76, 78]. These data indicate that neural *Pitx3* expression is confined to MesDA neurons, independent of *Nurr1*, dependent and possibly downstream of *Lmx1b*, insufficient for proper MesDA neuron differentiation but possibly implicated in aspects of neuronal specification other than neurotransmitter phenotype, in particular survival of MesDA neurons [52, 76, 78]. The role of *Pitx3* in MesDA development has not yet been addressed by creation and analysis of *Pitx3* null-mutant mice. The spontaneously occurring *ak* mouse mutant has recently been identified as a severely affected hypomorphic or null allele for *Pitx3* during eye development. During my Master's studies, I have analysed neural *Pitx3* expression in *ak* mice and, while using this mouse mutant as a hypomorphic or null allele for *Pitx3* at the brain level, analysed the in vivo role of *Pitx3* in MesDA development.

2. Article

PITX3 IS REQUIRED FOR MOTOR ACTIVITY AND FOR SURVIVAL OF A SUBSET OF MIDBRAIN DOPAMINERGIC NEURONS

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Résumé

Les neurones dopaminergiques mésencéphaliques (MesDA) jouent un rôle critique dans les processus moteurs et comportementaux. Dans la maladie de Parkinson, leur perte résulte en une déficience en dopamine au niveau du striatum et un trouble locomoteur hypokinésique. Le gène à boîte homéo *Pitx3* est exprimé dans les neurones dopaminergiques mésencéphaliques. Nous montrons maintenant qu'un seul sous-groupe de ces neurones exprime *Pitx3* et que dans la souris *aphakia* déficiente en *Pitx3*, ce sous-groupe disparaît progressivement par apoptose durant le développement fétal (substantia nigra) et post-natal (ventral tegmental area), résultant un niveau très bas de dopamine dans le striatum et une hypokinésie. De façon semblable à la forme de maladie de Parkinson humaine, les neurones dorsaux de la substantia nigra (négatifs pour *Pitx3*) sont épargnés chez la souris mutante. Ainsi, *Pitx3* définit une voie de survie pour les neurones qui sont impliqués dans la maladie de Parkinson et qui sont requis pour l'activité locomotrice spontanée.

Abstract

Mesencephalic dopaminergic (MesDA) neurons play critical 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 post-natal (ventral tegmental area) development, resulting in very low striatal DA and hypokinesia. 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.

Intoduction

The physiological role and clinical relevance of mesencephalic dopaminergic (MesDA) neurons are well recognized in schizophrenia, addictive behavioural 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, Jr. 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 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 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 mutant 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 post-natally 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 were 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 exon1.

We now 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 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

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 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. Rostrocaudal position of sections was assessed with the aid of the mouse brain atlas of Franklin and Paxinos (Franklin and Paxinos G., 1997). For embryos, pregnant mothers were perfused transcardially with 4% paraformaldehyde. 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 (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 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 wild-type 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 wildtype 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 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 wildtype 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 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 to 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 only reduced 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 rythms (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 (Bayer et al., 1995) and during the post-natal period, 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, 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 dopaminergic 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 PD patients (Kish et al., 1988).

Reduced spontaneous locomotor activity

We then determined whether *ak* mice display altered locomotor behavior 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 24 h 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 h period as observed in *ak* mice. Moreover, the speed of spontaneous ambulatory movements was not different in *ak* compared to 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 hypokinesia 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 PD patients are preferentially susceptible to loss of Pitx3-positive rather than Pitx3-negative MesDA neurons. 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 different forms of alpha-synuclein or parkin, have not been able to explain the highly specific and stereotypic pattern of MesDA cell loss in human PD, with ventral nigra being most affected (Dawson et al., 2002). In 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.

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 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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Legends to Figures

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 to 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.

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 to 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 to controls ($P<0.05$, *t*-test).

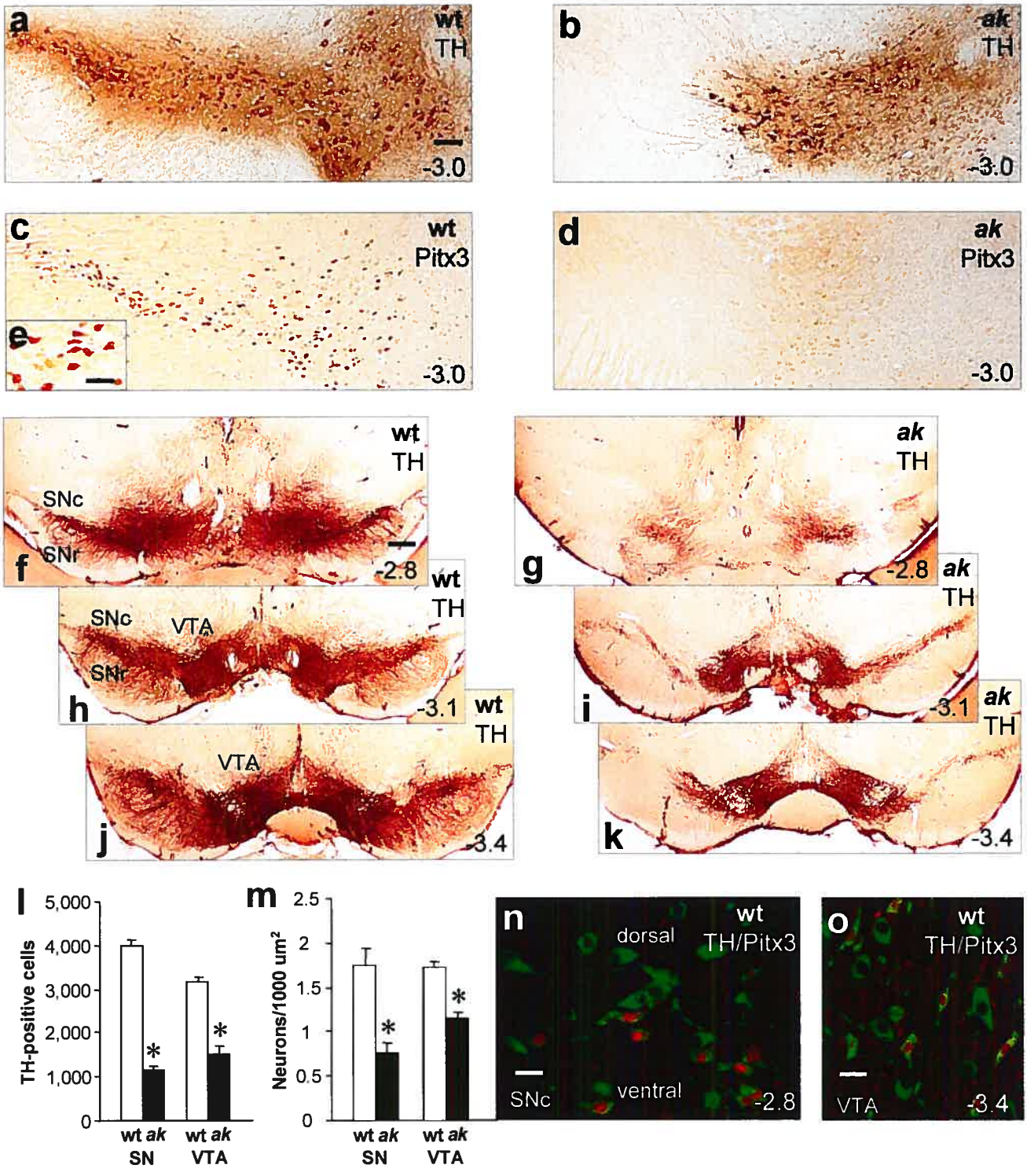
Fig. 3. The *ak* mice have deficient striatal dopaminergic innervation. (A,B) Coronal sections through the left striatum of P100 wild-type (A) and *ak* (B) mice immunostained for TH. Rostrocaudal positions are indicated as millimeters relative to bregma in the lower right corner. Scale bar: 250 μm. (C,D) 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 to controls ($P<0.01$, *t*-test).

Fig. 4. The *ak* mice have impaired spontaneous locomotor activity. (A) Spontaneous ambulatory activity of mice recorded over 23h. The distance (cm) covered during each 1h 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 A during

daytime and night-time. (D) Spontaneous stereotypic movements of mice recorded over 23 h. The stereotypic movements during each 1h 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 (D) during daytime and night-time. (G) Resting time of mice recorded over 23h. The resting time during each 1h 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 (green) neurons 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.



corresponding author: J. Drouin

figure 1

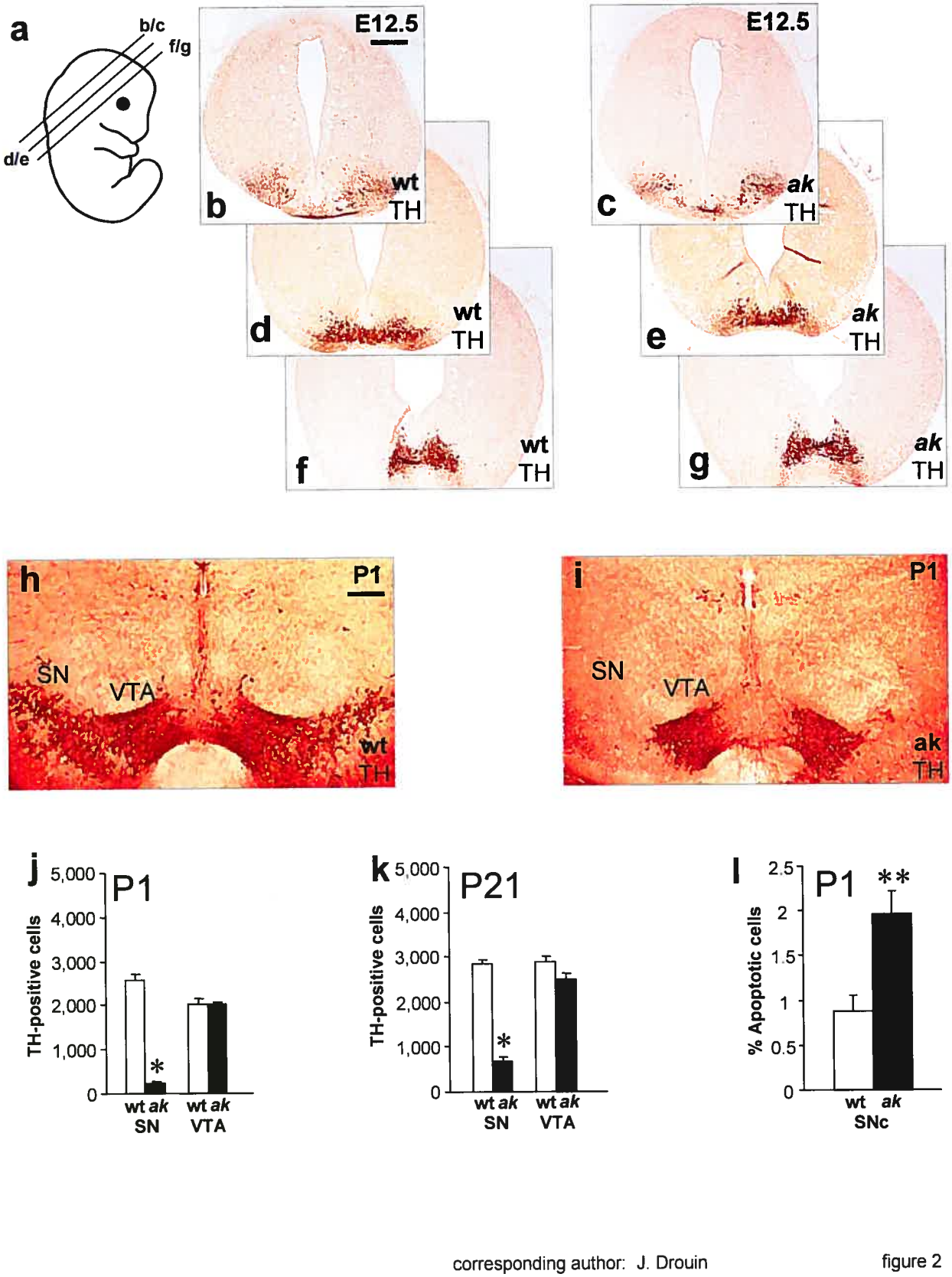


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figure 2

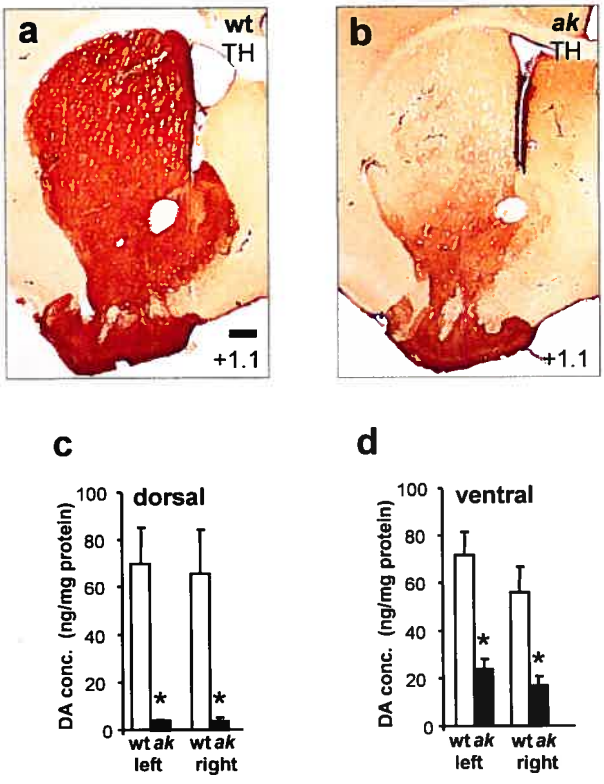

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figure 3

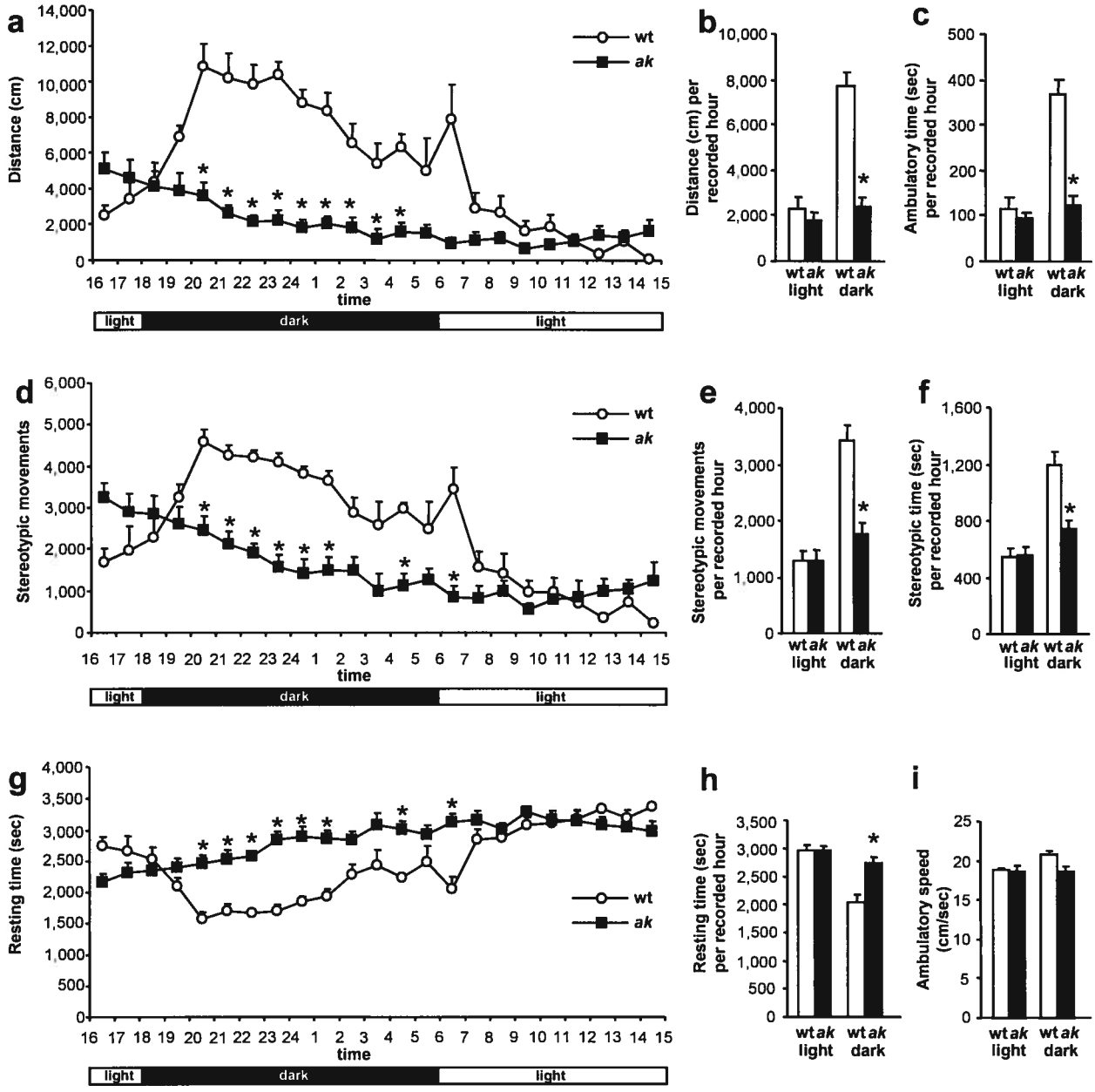

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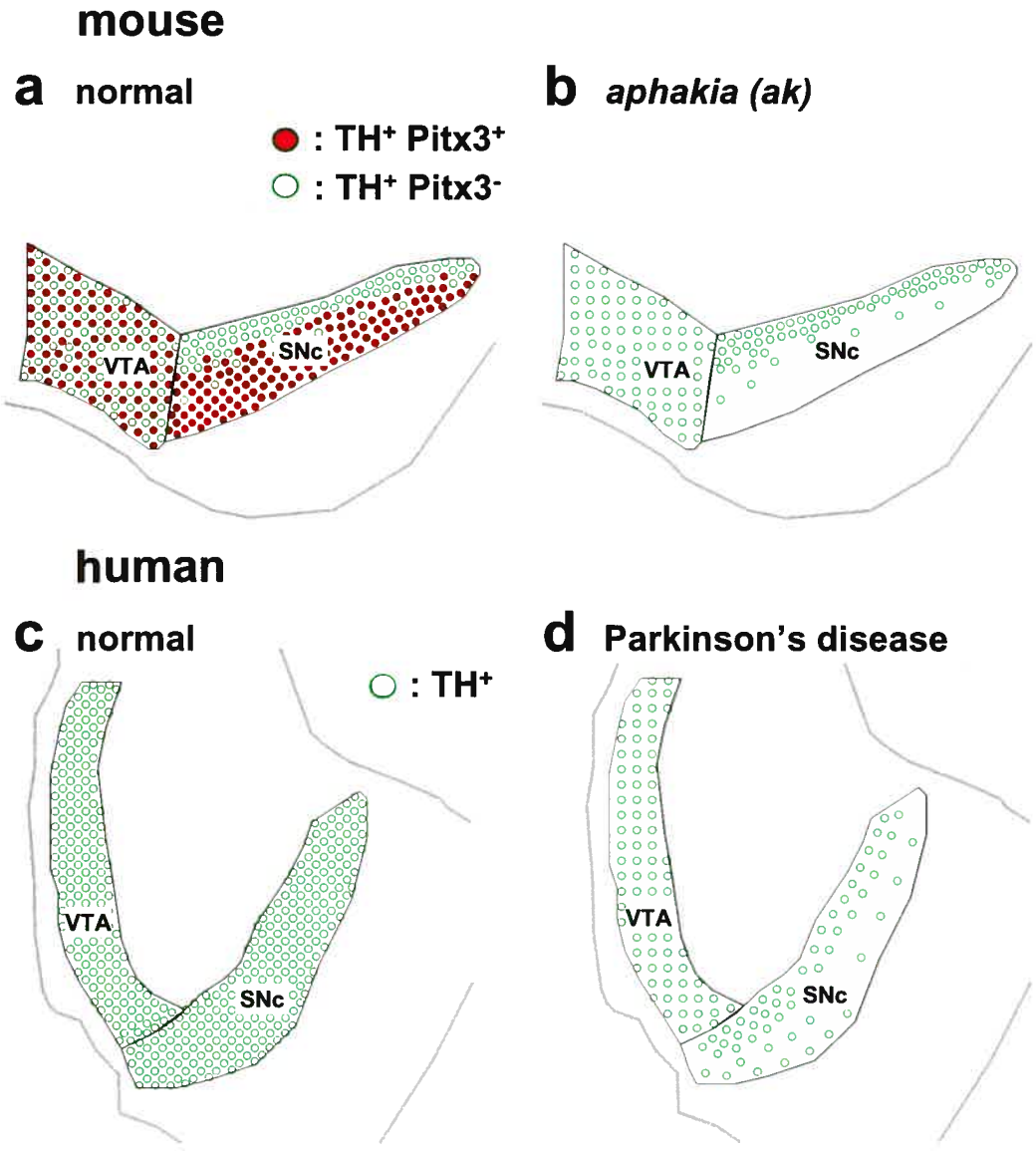
figure 4

Neuronal cell loss in *aphakia* mice and Parkinson's disease



Corresponding author: J. Drouin figure 5

III. DISCUSSION AND PERSPECTIVES

The experimental data presented in this mémoire shed a new light on *Pitx3* expression in the MesDA system and the in vivo role of the protein in this highly complex midbrain structure. Until today, *Pitx3* was believed to be expressed throughout the MesDA system and to fulfill a hitherto unknown function in all MesDA neurons. We now show that in adult mice, *Pitx3* is expressed in most TH-positive neurons in the ventral tier of the SNc and about half of the TH-positive neurons in the VTA, whereas the dorsal tier of the SNc contains largely Pitx3-negative TH-positive neurons. Pitx3 is therefore, together with Calbindin D_{28K}, the only protein thus far known to specifically mark a discrete and identifiable region within the SNc. Even more interestingly, whereas Calbindin D_{28K} specifically marks the dorsal tier of the SNc, i.e. the nigral compartment least affected by PD, our results indicate that *Pitx3* is specifically expressed in the ventral tier of the SNc, i.e. the nigral compartment most affected by this disease. *Pitx3* is the first gene identified having specific expression in those SNc neurons that are most vulnerable for the PD process. It remains to be determined if these conclusions can be extrapolated to the human situation, but since the mouse midbrain architecture is highly similar to that in humans, there is reason to believe that *Pitx3* might be a unique marker for these SNc neurons throughout mammalian species. In the VTA, Pitx3-positive and Pitx3-negative cells appear to be intermingled, with no discrete anatomical distribution. Even here though, the gene might mark those neurons that

are lost in human PD, since VTA cell losses in PD have no discrete distribution neither [8].

The analysis of the *ak* mouse mutant revealed that it is in fact a severe hypomorphic or even null allele for *Pitx3* at the brain level. Rieger et al. performed quantitative real-time polymerase chain reaction (RT-PCR) of *Pitx3* with total RNA from heads of *ak* mutant and wildtype embryos and newborns and measured residual expression of approximately 5% in mutants compared to wildtype [118]. We found no Pitx3-immunoreactive cells in the MesDA system of P100 *ak* mice. The antibody against Pitx3 we used during our experiments was raised against amino acids 9-45 of rat Pitx3 [88], i.e. against amino acids encoded by DNA sequences that are unaffected by the double-deletion mutation in the *Pitx3* gene in *ak* mutant mice. Thus, at the brain level of P100 *ak* mice, the low level of *Pitx3* transcripts does not seem to get translated in functional protein; a situation that can be well explained by the fact that intron 1 cannot be spliced out of the aberrantly formed transcript due to the deleted 5' splice site. P100 *ak* mice are thus very likely a null allele for *Pitx3* at the brain level. Pitx3-immunohistochemistry throughout embryogenesis and early postnatal life needs to determine whether the *ak* mutation is also a null allele for *Pitx3* at younger developmental ages, but the RT-PCR data from Rieger et al. strongly suggests this [118].

Our systematic analysis of serial midbrain sections in *ak* and wildtype P100 mice revealed that Pitx3-deficiency results in a severe depletion of MesDA neurons. This depletion affects the different compartments of the MesDA sytem in a different way and follows the expression pattern of *Pitx3*; the ventral tier of the SNc is almost completely depleted of TH-positive neurons, the VTA lacks about half of its TH-

positive neurons, whereas the TH-positive neurons in the dorsal tier of the SNc are largely preserved. The early development of the MesDA does not seem to be affected in *ak* mice, but the SN neurons disappear during the fetal period and the VTA neurons during the first postnatal months. Increased apoptosis seems to contribute to this cell loss. Thus, *Pitx3* does not only mark those MesDA neurons that are most vulnerable for the PD process, it is also required for their survival. The observation that the decrease in TH-positive cells in the SN is 91% at P1, 82% at P21 and 71% at P100 probably finds its explanation in the phenomenon of 'phenotypic maturation' of MesDA neurons that extends into young adulthood. A substantial part of the MesDA neurons, which are all generated on days E10-E14, will not express TH until the first postnatal weeks and the proportion of 'TH immunostainable' neurons relative to the total number of neurons present increases over time and parallels the increase in TH neuron numbers in the SNc and VTA in the first postnatal weeks [22]. The relative 'increase' of the number of TH-positive cells in the SN of *ak* mice suggests that those SN neurons that 'phenotypically mature' during fetal life are more affected by the Pitx3 deficienty than those that 'phenotypically mature' later, during the first postnatal weeks. Together with the anatomical distribution of these affected cells, one might speculate that the MesDA neurons that are most vulnerable for PD-related degeneration are those that 'phenotypically mature' during early MesDA development and that are Pitx3-positive. In the VTA of *ak* mice, TH-positive cell numbers are not different at P1, tend to be lower at P21 and are reduced by 52% at P100, indicating that these VTA neurons are lost later than the affected SN neurons. This may suggest that, although this subpopulation of VTA neurons requires Pitx3 for their longterm survival, they

are relatively more resistant to Pitx3-deficiency than their SN counterparts. Alternatively, VTA TH-positive neurons that 'phenotypically mature' during fetal life could be less affected by the Pitx3 deficiency than those that 'phenotypically mature' later, during the first postnatal weeks. One way or another, Pitx3 deficiency clearly does not affect the SN and VTA in the same way, underlining the different properties of these neuronal populations.

The consequences of the *ak* midbrain phenotype are dramatic; DA levels are reduced by 93% in the dorsal striatum, by 69% in the ventral striatum and the *ak* mice have severe locomotor deficits. The preferential DA depletion in the dorsal striatum correlates well with the preferential loss of ventral tier SNc neurons, which project to this brain structure. The DA depletion results in a marked reduction in spontaneous ambulatory and stereotypic activities and a*k* mice are therefore severely akinetic. Again, both these characteristics of *ak* mice are very similar to the pathogenesis of human PD [131, 211]. From the observation that the speed of spontaneous ambulatory movements was not reduced in *ak* mice, one could conclude that *ak* mice are not bradykinetic, an important characteristic of PD [121]. However, during our studies we only measured spontaneous movements and it remains to be determined whether *ak* mice reach normal ambulatory speed when they are forced to run or undergo a rotarod test. Besides, bradykinesia is defined as both slowing of and difficulty in initiating movements and the observation that *ak* mice spent significantly less time walking and less time making stereotypic movements may indicate a difficulty in initiating movements. It remains to be determined whether the DA depletion in the ventral striatum, which correlates well

with the loss of VTA neurons, modulates the emotional behaviour of *ak* mice and whether this influences the observed locomotor deficits.

The *ak* mice thus recapitulate cardinal features of PD, in particular the akinetic subtype of PD [211]. They therefore provide a useful animal model of PD; MesDA neuronal losses in *ak* mice exceed 50% and can be readily detected using biochemistry and neuropathology, the mice have easily detectable akinesia and the model is based on a single mutation to allow robust propagation of the mutation as well as crossing with enhancer or suppressor strains. However, not all characteristics of an ideal model of this disease have yet been investigated in *ak* mice. Besides, not all of them may be met. First, although MesDA neuronal losses in *ak* mice exceed 50%, the mice are already born with a severely affected complement of MesDA neurons and it remains to be determined whether there is progressive loss of these neurons throughout adult life, as in human PD. On the other hand, the *ak* disease process seems short and would allow rapid screening of neuroprotective agents during the fetal period (SNc) and the the first postnatal months (VTA). Second, it remains to be determined whether Lewy bodies develop in *ak* mice. Third, although *ak* mice are akinetic, other cardinal symptoms of PD, as rigidity and resting tremor, are still to be measured. No obvious rigidity and tremor were noted during routine handling of the *ak* mice, but previous experience with pharmacological depletion of DA or DA-receptor antagonist suggests that mice do not develop these symptoms [184]. Fourth, it remains to be determined what postsynaptic effects the *ak* midbrain phenotype with associated severe depletion of dorsal striatal DA levels has on the development and function of striatal DA receptors, and whether the *ak* akinetic

motor deficit is responsive to treatment with DA receptor agonists. Taken together, it is too preliminary to state that the *ak* mouse mutant is an ideal animal model of PD, recapitulating all of the key clinical and neuropathological features of PD. It is the first known animal model, however, that accurately copies the stereotypic pattern of SNc cell loss as seen in the human disease. Therefore, *ak* mice provide a very useful animal model, not only to test therapies (drugs, gene or gene therapy) for PD, but also to define a molecular mechanism explaining the selective sensitivity of Pitx3-expressing MesDA neurons to degeneration.

Indeed, the requirement of the homeobox transcription factor Pitx3 for survival of Pitx3-expressing MesDA neurons suggests that Pitx3-dependent functions may relate to the pathogenesis of human PD. Such function or downstream target genes, thus far unidentified, may contribute to control of cell survival/death in development and/or in pathogenesis of the MesDA system. Thus, *Pitx3* gene mutations may be involved in the etiology of diseases that affect the MesDA system. Thus far, two mutations in the coding sequence of this gene have been identified in families with ADCC and ASMD [111], diseases that show similarities with the ocular phenotype of *ak* mice. Locomotor performance has not been reported in these heterozygous patients and it is noteworthy that both *PITX3* mutations identified did not affect the homeodomain but rather amino acids in the flanking sequences that have been proposed to participate in protein:protein interactions. It is possible that the residues mutated in these alleles do not exert their dominant effect in MesDA neurons as they appear to do for lens development. Thus, it would be worthwhile to determine whether *PITX3* alleles or mutated downstream target genes may predispose to PD.

Because of the implications in neurological and mental disorders, DA belongs to the most intensively studied neurotransmitters of the brain and DA systems have been the main target of neuropharmacology for more than 40 years. The dramatic neurological consequences of degeneration of MesDA neurons in PD highlight the functions of this DA system in the control of motor behavior at the level of the striatum. The experimental data presented in this mémoire identify the murine homeobox transcription factor Pitx3 as crucial for the survival of a highly specific subset of MesDA neurons, which shows close resemblance to the MesDA neurons most vulnerable in PD. The *Pitx3* gene therefore exerts an essential role in the regulation of movement control. Interestingly, expression of the second member of the *Pitx* gene family, *Pitx2*, is at the brain level confined to discrete nuclei of the basal ganglia like the STN and the zona incerta pars dorsalis of the thalamus [104, 106]. The STN excites the output nuclei in the basal ganglia, the GPi and SNr, by the means of the neurotransmitter glutamate [132]. In PD, reduced inhibtion of the STN by the "indirect" GABAergic connection from the striatum, due to the DA depletion in the dorsal striatum, causes increased output of the basal ganglia that excessively inhibits the thalamic and brainstem nuclei, resulting in resting tremor, rigidity and bradykinesia/akinesia [127]. The STN thus plays an essential role in the downstream effects of the midbrain pathology in PD, and inhibition of STN excitation by bilateral deep-brain stimulation has striking benefits in terms of all aspects of parkinsonism [212]. However, the STN does not only operate downstream of MesDA neurons. It is clearly established that STN glutamatergic neurons influence both dopaminergic and non-dopaminergic neurons in the SN [134, 213], and increases in glutamate may induce inhibition and degeneration of SN neurons

[214, 215]. Recent studies in neurotoxin-induced animal models of PD show that STN inhibition increases striatal dopamine levels and exerts neuroprotective effects on SN DA neurons [216, 217]. The STN thus clearly influences SN function. Since the role and clinical relevance of the STN are gradually elucidated and seem crucial to both proper midbrain and basal ganglia physiology, it is important to understand the development of this brain nucleus. The characteristic brain expression of the *Pitx2* gene herein is remarkable and the crucial role of *Pitx3* in the development of MesDA neurons, stresses the need for further research on the role of the *Pitx2* gene in the STN. *Pitx1* is, beside the pituitary gland, not expressed at the brain level. The other two members of the *Pitx* gene family, however, might both be crucial in the genetic pathways controlling midbrain and basal ganglia development.

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