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

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Chapter 7

General discussion
The Pitx3-deficient *aphakia* mouse

In the previous chapters, we described our research findings regarding the midbrain and striatal phenotype in the *aphakia (ak)* mouse. The *ak* midbrain does not express Pitx3 and is developmentally depleted of ventral tier substantia nigra pars compacta (vSNc) dopamine (DA) neurons, with relative sparing of calbindin D$_{28K}$ (CB) positive dorsal tier SNc (dSNc) and ventral tegmental area (VTA) DA neurons (Chapters 2 and 3). In this context, we re-evaluated the expression of Pitx3 in the mesencephalic dopaminergic (MesDA) system of wild-type (wt) mice and found Pitx3 expression predominantly restricted to vSNc and about half of the VTA DA neurons (Chapters 2 and 3), i.e. the same MesDA neuronal subpopulations that are lost in *ak* mice. The *ak* MesDA neuronal depletion causes a dramatic reduction of DA levels in the neostriatum (Chapter 2), with DA terminals largely absent in the dorsolateral and medial neostriatum, and relatively preserved in the ventral neostriatum (Chapter 5). The reduced neostriatal DA levels are associated with 20% striatal atrophy (Chapter 5), increased expression levels of DA D$_2$ receptors and enkephalin (ENK), reduced expression levels of DA D$_3$ receptors and µ-opioid receptors (MOR) (Chapters 4 and 5), together with a marked reduction in spontaneous locomotor activity which can be reversed by the DA-precursor 3,4-dihydroxyphenylalanine (L-DOPA) (Chapters 2 and 4). The Pitx3-deficient *ak* mouse thus recapitulates cardinal characteristics of Parkinson’s disease (PD). It might provide further insight in its pathogenesis and serve as a valuable tool for testing new treatment strategies (Chapter 6).

**Pitx3-expression in wild-type MesDA neurons**

Our finding that the wt MesDA system is composed of two previously unrecognized DA neuronal subpopulations that are differentiated by expression of Pitx3 (Chapter 2-Fig. 1, Chapter 3-Fig. 4) is at variance with reports from other groups. Smidt et al. reported complete overlap of both Pitx3 message and Pitx3 protein with tyrosine hydroxylase (TH) protein and TH message in mouse MesDA neurons, using a self-generated anti-Pitx3 cRNA probe and polyclonal rabbit anti-Pitx3 antibody. Korotkova et al. reported 98% overlap of Pitx3 and TH protein in rat SN and VTA, using a commercial polyclonal rabbit anti-Pitx3 antibody. Zhao et al. generated a transgenic mouse with green fluorescence protein (GFP) targeted into the Pitx3-locus and reported Pitx3-GFP/TH co-expression in 98% of SN DA neurons and 95% of VTA DA neurons. How to reconcile these seemingly discrepant findings? The presence of transcripts of the Pitx3-locus in all MesDA neurons, as reported by Smidt et al. and Zhao et al., does not necessarily implicate Pitx3 protein production in all these neurons; mechanisms beyond transcriptional regulation, like phosphorylation of translation-initiation factors or ribosomal proteins, micro-RNA (miRNA)
and small-interfering RNA (siRNA) could prevent translation of Pitx3 messenger into protein in dSNc and half of VTA DA neurons.\textsuperscript{122} The immunohistochemical findings by Smidt et al. and Korotkova et al.\textsuperscript{203,321} however, clearly oppose our results. Whether our immunohistochemical protocols or the characteristics of our anti-Pitx3 antibodies are too stringent, or whether the results of Smidt et al. and Korotkova et al. are too unspecific,\textsuperscript{203,321} remains to be verified. Our polyclonal rabbit anti-Pitx3 antibody was generated with amino acids 9-45 of rat Pitx3,\textsuperscript{220} the antibody from Smidt et al. with amino acids 1-169 of rat Pitx3.\textsuperscript{321} Although both amino acid sequences are perfectly similar to mouse Pitx3, they differ considerably in their potential alignment with other homeodomain transcription factors that are also expressed in MesDA neurons. According to the protein database search program BLAST, amino acid sequence 9-45 of rat Pitx3 is 11% identical to mouse En1, 14% to mouse En2 and 0% to mouse Lmx1b.\textsuperscript{8,9} For amino acids 1-169, these percentages are 18%, 28% and 18%, respectively. Thus, chances of cross-reactivity and false-positive immunostaining seem to be higher for the antibody used by Smidt et al.\textsuperscript{321} The commercial polyclonal rabbit anti-Pitx3 antibody used by Korotkova et al. in rat was generated by Zymed\textsuperscript{\&} Laboratories (now part of the Invotrogen family),\textsuperscript{203} using the N-terminal region of the mouse Pitx3 protein.\textsuperscript{159} Unfortunately, no information on amino acid sequence can be retrieved from the manufacturer’s manual. Alternatively, our immunohistochemistry protocol may not have stained all MesDA neurons expressing Pitx3. The fact that our protocol used a 1:10 (Chapter 2) and 1:100 (Chapter 3) dilution of anti-Pitx3 antibody whereas Smidt et al. and Korotkova et al. used a 1:500 dilution,\textsuperscript{203,321} suggests our antibody is less sensitive than theirs. An additional analysis that compares the sensitivity and specificity of all available anti-Pitx3 antibodies with respect to Pitx3 immunostaining in wt MesDA neurons of different species might clarify the different observations.

**Pitx3-deficiency in aphakia MesDA neurons**

Our finding of absent Pitx3 immunostaining in the \(ak\) MesDA system (Chapter 2-Fig. 1) was confirmed by Hwang et al. and Smidt et al., who reported absent Pitx3 in situ hybridization and immunostaining in both embryos and adult \(ak\) animals.\textsuperscript{161,321} Its absence causes a dramatic reduction of SNc DA neurons (Chapter 2-Fig. 1, Chapter 3-Fig. 1 and 2).\textsuperscript{161,258,321,324} Our observation of relative sparing of a subset of SNc DA neurons in \(ak\) mice was also confirmed by other groups. Nunes et al. explicitly mentioned relative sparing of SN pars lateralis DA neurons. In addition, their figure on the distribution of TH-positive neurons in the midbrain of adult \(ak\) and wt mice shows a small bend of spared SNc DA neurons that corresponds with the area we labelled as dSNc (Chapter 2-Fig. 1).\textsuperscript{258} Similar figures by Hwang et al. and Smidt et al. also show sparing of SNc DA neurons.\textsuperscript{161,321} Thus,
although it remains unclear whether all SNC DA neurons express Pitx3 or not, its absence in ak mice causes a dramatic but not complete loss of these neurons. A subpopulation does not require Pitx3 for survival. Interestingly, 92% of this surviving SNC subpopulation was CB-positive, whereas in wt mice only 25% of SNC DA neurons showed CB-expression (Chapter 3-Fig. 2 and 3). Korotkova et al. showed in rat that these 25% are almost exclusively localized in dSNC, with only 2% of vSNC DA neurons expressing CB. This strengthens our delineation of a subpopulation of Pitx3-independent/CB-positive dSNC DA neurons that survives in ak mice (Chapter 3-Fig. 2 and 5).

The 33-52% reduction of VTA DA neurons in ak mice is smaller than the 71-81% reduction in the SNC (Chapter 2-Fig. 1, Chapter 3-Fig. 2 and 3). The loss of these VTA neurons is distributed evenly throughout the area and, most importantly, occurs much later and is fundamentally different in nature. The SNC DA neuron loss occurs during development and is clearly established in newborn ak mice (Chapter 2-Fig. 2). In fact, Smidt et al. showed in E12.5 ak mice that lateral TH-positive cells are never generated. In contrast, ak VTA DA neuron numbers are not different from those in wt animals until P35 (Chapter 3-Fig. 3) and then undergo reduction until P100 (Chapter 2-Fig. 1, Chapter 3-Fig. 3). Smits et al. reported a similar 45% reduction of VTA DA neurons in 3-4 months old animals. The apparently normal TH-immunostaining in the adult ak VTA as reported by Hwang et al. and Nunes et al. can be explained by the young age of their adult animals; P28 and P48-64, respectively. Thus, whereas the absence of Pitx3 in the SNC causes early developmental failure of DA neurons, VTA DA neurons are properly formed but do not survive during adult life without Pitx3.

How does Pitx3 exert its terminal differentiation role during development of vSNC DA neurons and its maintenance role during adult life in half of VTA DA neurons? Which genes are activated or inhibited by this homeobox-containing transcription factor? And which genes regulate Pitx3? Cazorla et al. and Lebel et al. identified a response element for Pitx3 in the TH gene promoter. However, Nurr1 knockout mice, that fail to generate TH-positive neurons in the mesencephalon, show normal Pitx3 expression at E11.5, indicating that Pitx3 expression is insufficient for proper MesDA neuron differentiation. Similarly, TH-positive but Pitx3-negative cells are generated in the ventral tegmentum of E12.5 Lmx1b knockout mice, indicating that Pitx3 is not necessary for TH expression. Jabobs et al. reported highly affected expression of aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1), also known as Ahd2, in ak mice. Ak offspring from Pitx3 heterozygote mice that were supplemented with retinoic acid from E10.75 to E13.75 showed a drastic reversal of MesDA neuron
Peng et al. demonstrated increased expression of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) following Pitx3 overexpression in cell lines and primary ventral mesencephalic cultures. Hwang et al. performed RNA microarray analysis on ventral midbrain TH-positive neurons of E12.5 wt and ak embryos, isolated by laser capture microdissection. They identified 243 independent genes that were downregulated twofold or more in ak DA neurons. Many of these genes were known to regulate cell cycle/growth, cell migration and transcriptional regulation. Among the highly affected genes were DA transporter (DAT), responsible for DA re-uptake into the nerve terminals, and vesicular monoamine transporter 2 (VMAT2), responsible for DA storage into synaptic vesicles. Jacobs et al. showed that the regulatory effect of Pitx3 on these latter genes is exerted through potentiation of Nurr1-mediated transcription: in the absence of Pitx3, Nurr1 is kept in a repressed state through interaction with the co-repressor SMRT (silencing mediator of retinoic acid and thyroid hormone receptor). BDNF, GDNF and, most strikingly, Ahd2 were not among the downregulated reported by Hwang et al. The biological relevance of their microarray results awaits therefore verification in knockout mouse models. The differences between SNc and VTA phenotype in ak mice have thus far not been extensively studied, probably because the VTA phenotype was not found in the young adult ak animals used by several groups. Korotkova et al. analyzed total cellular mRNA from 3-4 week old wt rat SN and VTA neurons and found ±6x higher Pitx3 expression levels in VTA versus SN neurons. Together with the higher ratio of CB-expressing DA neurons in the wt VTA compared to SNc (Chapter 3-Fig. 2 and 3), this might explain why VTA DA neurons are less susceptible to neurotoxins than SNc DA neurons (Chapter 3-Fig. 6). Reports on the regulation of Pitx3 are scarce. Smidt et al. identified Lmx1b, which' expression in the midbrain starts at E7.5 and is maintained in MesDA neurons throughout life, as a potential regulator of Pitx3 by showing absent Pitx3 expression in TH-positive ventral midbrain neurons of E12.5 Lmx1b knockout mice. However, they also showed a small group of Pitx3-positive (but TH-negative) cells just dorsal and posterior to these DA neurons, suggesting that Lmx1b is not the only regulator. Konstantoulas et al. showed that FoxP1, member of the hepatic nuclear factor-3/forkhead domain family of winged-helix transcription factors, initiates Pitx3 expression in embryonic stem cells and identified two high affinity binding sites for FoxP1 in the Pitx3 promoter. Previously, FoxP1 has been shown to control LIM-homeodomain transcription factor expression profiles during spinal motor neuron development. The regulation of Pitx3 might thus be sequentially orchestrated by the transcription factors FoxP1 and Lmx1B.
Striatal pathology in the *aphakia* mouse

The preferential loss of vSNc DA neurons in *ak* mice causes a dramatic reduction of DA levels in the neostriatum (Chapter 2-Fig. 3, Chapter 3-Fig. 1, Chapter 5-Fig. 1). This finding was confirmed by all other groups reporting on the *ak* mouse.\(^{30,88,161,187,258,321,324}\) In rat, vSNc and dSNc DA neurons have been shown to project to, respectively, neostriatal patches and matrix,\(^ {132}\) whereas medial parts of the SNc were shown to project to medial, and lateral parts to lateral regions of the neostriatum.\(^ {28,33}\) With preferential loss of vSNc DA neurons and relative sparing of dSNc, DA inputs to the neostriatum were expected to be absent in the patches and relatively spared in the matrix. However, DA inputs to the *ak* neostriatum followed a gradual decreasing gradient in both the ventral to dorsal and lateral to medial axes (Chapter 5-Fig. 1).\(^ {30}\) They were absent throughout the dorsal and medial neostriatum, without any residual input to the matrix, and relatively spared in the ventrolateral neostriatum and nucleus accumbens. Light patches of DA fibers with associated MOR-immunostaining were preserved within the relatively spared ventrolateral neostriatum, including the ventral part of the subcallosal region (Chapter 5-Fig. 1 and 2). In rat, this latter region, also known as the subcallosal streak, receives inputs from islands of DA neurons within the SNr.\(^ {132}\) How to explain the differences between the expected and observed pattern of DA denervation in the *ak* neostriatum? Do the strictly separated reciprocal connections between vSNc/SNr DA neurons and patches, and dSNc/VTA DA neurons and matrix in rat also apply to the *ak* mouse? The preservation of ventrolateral DA patches, including the ventral part of the subcallosal streak, would then imply sparing of subsets of vSNc/SNr DA neurons. And the observed absence of DA inputs to the dorsal and medial neostriatal matrix would then be caused by loss of dSNc DA neurons at the more rostral midbrain levels.\(^ {132}\) The relative sparing of SN pars lateralis DA neurons, reported by Nunes et al.\(^ {258}\) could explain why lateral parts of the neostriatum are less affected than medial parts. These authors also showed absent labelling in the *ak* SNc and unexpected labelling in the *ak* SNr following injections of the retrograde tracer Fluorogold in the striatum, and concluded that *ak* mice therefore have irregular circuitry between SN and neostriatum.\(^ {258}\) They did not, however, mention the exact site of the Fluorogold injection within the *ak* neostriatum. Besides, their Fluorogold labelled SNr cells might have been preserved islands of DA neurons within the SNr,\(^ {132}\) or alternatively non-dopaminergic nigrostriatal neurons.\(^ {150}\) Future experiments combining anterograde/retrograde axonal tracing and TH-immunostaining in the *ak* neostriatum and midbrain are needed to fully explain the observed pattern of DA denervation.

According to Graybiel and Gerfen et al., MesDA inputs to the neostriatum stimulate direct pathway neurons expressing γ-aminobutyric acid (GABA)/D₁/substance P (SP)/dynorphin...
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(DYN), and inhibit indirect pathway neurons expressing GABA/D2/ENK. The dramatic reduction of DA levels in the ak neostriatum would therefore be expected to cause downregulation of D1 receptors, SP and DYN, and upregulation of D2 receptors and ENK. However, because of the gradual decreasing gradient of neostriatal DA inputs along both ventral to dorsal and lateral to medial axes, expression levels of D1/D2 receptors and neuropeptides will vary greatly depending on the exact neostriatal location chosen for analysis. This might explain why we found normal levels of D1 receptors (Chapter 4, data not shown) whereas Smits et al. and Singh et al. reported reduced levels. In contrast, we showed increased levels of D2 receptors (Chapter 4-Fig. 5) whereas Smidt et al. and Singh et al. reported normal levels. The reported expression levels for DYN, ENK and SP similarly varied between research groups (Chapter 4-Fig. 7). Thus, our analysis of the medial versus lateral neostriatum, Smits’ analysis of the dorsal neostriatal area and Singh’s analysis of the neostriatum from dorsal border to anterior commissure might not be representative enough to account for the gradual decreasing gradient of DA inputs in the ak neostriatum. Future analysis of expression levels of D1/D2 receptors and neuropeptides in the most denervated dorsomedial versus the relatively spared ventrolateral ak neostriatum are needed to fully understand the consequences of DA reduction on direct and indirect pathway neurons.

The adult ak neostriatum contains approximately 1.35 million neurons and wt controls have similar neostriatal neuron numbers (Chapter 5-Fig. 3). Thus, the ak developmental loss of SNc DA neurons, which starts before E14.5 and is therefore thought to influence the DA content of the forebrain throughout the period of neostriatal neurogenesis, leaves neostriatal neuron numbers unaffected. No other group reporting on the ak mouse investigated neostriatal neuron numbers thus far. Our finding seems to oppose the results from Ohtani et al. and Ma and Zhou. Ohtani et al. reported DA-induced increased cellular output in E13 explanted cultures of the lateral ganglionic eminence (LGE), the transient embryonic neuroepithelial structure bordering the lateral ventricle in the basal telencephalon where neostriatal neurons are born. They showed that D1 receptor activation caused fewer cells to enter the S phase of the cell cycle and, consequently, a greater proportion of daughter cells to exit the cell cycle. D2 receptor activation caused opposite effects. Popolo et al. reported similar in vivo results in E15 embryos, although they did not perform stereological quantification of embryonic neostriatal neurons. Ma and Zhou reported DA-induced increased survival of neural precursor cells and differentiated cells in E14 striatal cell cultures, and showed that DA antagonized the generation of radical oxygen species by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Our finding of normal ak neostriatal neuron numbers suggests that there is not an important role to DA in neostriatal neurogenesis in this mouse mutant. Is
this result applicable to neostriatal neurogenesis in general? Reports on the in vivo role of dopamine on neostriatal neurogenesis are very scarce. Only Van der Kooy showed massive neostriatal shrinkage and more than 40% loss of neostriatal neurons in rat following mechanical lesions of the SN in the first postnatal week, but suggested that concomittant lesioning of striatonigral projections and retrograde neuronal death in the striatum might explain this observation.354 Since Ohtani et al. and Popolo et al. showed that the influence of DA is mediated via the opposing effects of D$_1$-like and D$_2$-like receptor activation,261,280 detailed expression-level analysis of D$_1$/D$_2$ receptors in the different subdivisions of the developing ak neostriatum is needed to better understand the observed normal neostriatal neuron numbers. In addition, quantification of ak neostriatal neurons during embryonic and postnatal development could determine whether compensatory plasticity from other afferent systems, like corticostriatal inputs, may allow for survival of the appropriate number of neostriatal neurons. Stereological quantification of neostriatal neurons in other genetic mouse models of developmental DA deficiency, like TH- and Nurr1-knockout mice, might therefore be instrumental to study whether the supposed DA-independency of neostriatal neurogenesis in ak mice is generally true.

Despite normal neostriatal neuron numbers, we did find a 18% smaller neostriatal volume and 29% smaller neostriatal neuronal soma size in adult ak animals (Chapter 5-Fig. 3). No other studies reporting on the ak mouse investigated neostriatal volumes thus far. However, similar reductions in neostriatal volume were reported in TH-deficient mice and D$_1$ receptor knockout mice.90,385 Thus, DA signaling seems to exert a trophic effect on neostriatal neurons. Alternatively, the observed volume reduction could also solely be caused by the physical absence of large parts of the nigrostriatal and striatonigral pathways. Synaptic inputs to the neostriatum are located at spines on the dendritic trees of neostriatal neurons.193 Ingham et al. showed in rat that removal of nigrostriatal DA inputs by chemical lesioning caused a decreased density of these neostriatal spines.167 One could hypothesize that neostriatal neurons with less spines on their dendrites are smaller. Vice versa, ak neostriatal patch neurons lack most of their SNc projection neurons and might thus develop less axons. Detailed morphological analysis of dendrites and axons of ak neostriatal neurons is needed to unravel the cause of the observed smaller neuronal soma size. Because of the gradual decreasing gradient of DA inputs along both the ventral to dorsal and lateral to medial axes, future studies of neuron numbers, volumes and morphology in different neostriatal subterritories of the ak neostriatum might provide valuable data on the quantitative role of DA in neostriatal neuron development.
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**Locomotor deficits in the *aphakia* mouse**

Several research groups have compared locomotor behaviour between ak and C57Bl/6 control mice, but the results and their interpretation differed. We reported reduced ambulatory activity during the night hours of 23/24-hour registrations of spontaneous open-field activity in both P40 and >P100 ak mice (Chapter 2-Fig. 4 and Chapter 4-Fig. 1), and concluded that these animals display marked akinesia. In addition, we showed that administration of L-DOPA resulted in dose-dependent stimulation of spontaneous ambulatory activity (Chapter 4-Fig. 2). Hwang et al. mentioned normal spontaneous behaviours, neurological reflexes and sensorimotor responses, including righting, postural reflex, ear twitch reflex startle, grip strength and whisker orientation reflex in four week old ak animals.\(^{161}\) Nunes et al. mentioned higher levels of general motor activity in 6-8 week old ak mice.\(^{258}\) Smidt et al. reported increased activity on climbing tests and reduced ambulatory activity during daytime 15-minute registrations of spontaneous open-field activity in 3 month old animals, and concluded ak mice exhibit hyperactivation of the nucleus accumbens and hypoactivation of the caudate putamen.\(^{321}\) Hwang et al. reported increased ambulatory activity during day hours and reduced activity during night hours of 22 hour registrations of spontaneous open-field activity in 8-9 week old ak mice.\(^{162}\) However, the same animals displayed L-DOPA-responsive motor deficits on nigrostriatal pathway-sensitive tests like the transparent cylinder test that measures spontaneous exploratory activity, and the challenging beam and pole test that measures sensorimotor coordination.\(^{162}\) Kas et al. reported reduced ambulatory activity during the night phase and increased activity during the light phase of 49-hour registrations of spontaneous home cage behaviour in 3-4 month old ak mice.\(^{187}\) When studied during a 15-minute trial in a small novel arena, ak mice showed reduced ambulatory activity together with reduced frequencies, but increased durations of locomotor behaviours like rearing, vertical sitting, horizontal walking and grooming.\(^{187}\) However, when they subsequently repeated experiments with Pitx3 -/- mice and their +/+ littermate controls, generated from heterozygous breeding pairs instead of homozygous bred ak and C57Bl/6 animals, certain results were different: Pitx3 -/- mice did not show statistically significant reduced ambulatory activity during the dark phase of 49 hour home cage registration and the 15-minute novel arena trial, nor did they show less grooming. Kas et al. concluded therefore that Pitx3-deficiency is associated with increased spontaneous home cage activity during light-phase hours and increased consolidation of movement components such as rearing and horizontal walking, whereas the reduced activity of ak mice during night-phase hours should be not be attributed to Pitx3-deficiency but to breeding strategy.\(^{187}\) Similarly, Smits et al. reported increased ambulatory activity during day hours and normal activity during night hours of 25-hour
registrations of spontaneous home-cage activity in 3-4 month old Pitx3-/- animals.\textsuperscript{324} Finally, Beeler et al. compared locomotor behaviour between 50-160 day old Pitx3-/- and phenotypically normal littermate Pitx3 +/- mice during 1-hour registrations of spontaneous open-field activity.\textsuperscript{30} They reported increased activity in Pitx3-/- mice during the initial 5 minutes of registration, with no differences in the remaining 55 minutes. In addition, Pitx3-/- mice showed increased exploratory activity with age, whereas control mice showed the reverse.\textsuperscript{30}

It seems difficult to draw unequivocal conclusions from all these behavioural results and, for proper comparison, one should take into account several potential methodological confounding factors. First,\textit{ak} mice are blind and might rely more on nonvisual senses to survey an environment, especially in a novel environment. Smidt et al. and Hwang et al. therefore also used blind control mice beside the regular C57Bl/6 control mice.\textsuperscript{162,321} Smidt et al. reported increased activity on climbing tests for blind C57Bl/6 animals, but no reduced ambulatory activity during daytime 15-minute open-field registrations.\textsuperscript{321} The blind retinal degeneration 1 (\textit{rd1}) mice used by Hwang et al. performed numerically worse than C57Bl/6 controls on the nigrostriatal pathway-sensitive tests, but the differences did not reach statistical significance.\textsuperscript{162} The blindness of \textit{ak} mice might thus partially explain some of the observed behavioural disturbances. Future experiments with conditional Pitx3 knock-out mice are needed to elucidate the locomotor deficits that are specifically caused by the midbrain phenotype. Second, several of the above mentioned studies were done with relatively young \textit{ak} animals, in whom the VTA DA neuron loss had not yet fully developed. VTA DA neurons are involved in emotional, motivational and reward-related behaviours.\textsuperscript{305} Younger \textit{ak} animals with a relatively intact VTA might therefore behave differently than older \textit{ak} animals with 33-52\% less VTA DA neurons. Only in the experiments from our group and those from Beeler et al., both young and older \textit{ak} animals were used.\textsuperscript{30,350,351} Indeed, Beeler et al. reported increased exploratory activity with age in Pitx3-/- mice during 1-hour open-field activity registrations.\textsuperscript{30} Similarly, we showed relative hyperactivity in the first two hours of 23-24 hour open-field activity registrations in older but not in younger \textit{ak} mice (compare Chapter 2-Fig. 4 with Chapter 4-Fig. 1). This initial hyperactive reaction to a novel environment in older \textit{ak} animals might be attributable to compensatory activity in the nucleus accumbens in response to decreased DA inputs from the VTA.\textsuperscript{83,323,350} Third, as mentioned earlier, Kas et al. showed that the reduced activity of \textit{ak} mice during night hours, as reported by our group and Hwang et al.,\textsuperscript{162,350,351} should be attributed to homozygous breeding instead of Pitx3-deficiency.\textsuperscript{187} However, careful examination of the figures displaying multi-hour registrations of spontaneous home cage behaviour from Kas et al. and Smits et al., learns that also Pitx3-/- mice tend to exhibit reduced ambulatory activity during night
hours,^{187,324} albeit less obvious than in ak mice and without statistical significance. Fourth, Hwang et al. hypothesized, based on the seemingly conflicting behavioural results, that nigrostriatal pathway-dependent motor deficits of ak mice may not be accurately revealed by measuring spontaneous motor activity but should instead be evaluated with special tasks that have specific sensitivity to detect defects of the nigrostriatal DA system.\textsuperscript{162} However, all groups reporting multi-hour registrations of spontaneous motor activity in ak mice showed reduced ambulatory activity during night hours.\textsuperscript{162,187,350,351}

Taken together, it seems fair to conclude that adult ak mice show initial hyperactivity in a novel environment and hypoactivity during night hours of multi-hour spontaneous open-field activity registrations and nigrostriatal DA system specific tasks, with L-DOPA reversing the hypoactive phenotype.

**Pitx3-deficiency and Parkinson’s disease**

The loss of MesDA neurons in Pitx3-deficient ak mice suggests that these mice could serve as a model for PD. However, the neuropathological findings in PD are more extensive. Based on the distribution of α-synuclein-positive pathology in a patient cohort comprising both sporadic PD and incidental Lewy body disease, Braak et al. proposed a model for progression of Lewy-type α-synucleinopathy pathology, with a predictable caudo-rostral spread, beginning in the vagal dorsal motor nucleus (Stages 1 and 2), progressing to the MesDA system (Stages 3 and 4) and finally, to the basal forebrain and neocortex (Stages 5 and 6).\textsuperscript{50} Besides MesDA neuron loss, lower brain stem levels are also involved in PD. Indeed, Zarow et al. reported significant neuronal loss in the pontine locus coeruleus (LC) of PD patients.\textsuperscript{379} In ak mice, no α-synuclein/ubiquitin-positive Lewy bodies have thus far been reported, and LC neurons are unaffected.\textsuperscript{161,258} In addition, parkinsonian SNc DA neuron loss is a progressive neurodegenerative phenomenon throughout (late) adulthood,\textsuperscript{154} whereas in ak mice it occurs during early development without further progression (Chapter 2-Fig. 2 and Chapter 3-Fig. 3). Thus, at first sight, the value of Pitx3-deficient ak mice for better understanding the pathogenesis of PD seems limited. However, the pattern of MesDA neuron loss in PD patients and ak mice is provocatively similar. Fearnley and Lees quantitatively studied subregional melanin-pigmented neuron losses in the parkinsonian SNc, and compared them to normal age-matched individuals.\textsuperscript{101} Overall, PD patients exhibited 75% SNc cell loss, with vSNc (71-91%) affected to a much higher extent than dSNc (47-57%). SNc pars lateralis showed 61% cell loss. Damier et al. reported similar results in their analysis of subregional DA neuron losses in the parkinsonian SNc, except for the SNc pars lateralis that only showed 30% loss.\textsuperscript{76} In ak mice, MesDA neurons are affected in a comparable way; 71-81% overall DA neurons loss, with
vSNc affected much more than dSNc and SNc pars lateralis (Chapter 2-Fig. 1, Chapter 3-Fig. 1). Also for VTA, the 30-43% DA neuron loss in PD patients resembles the 33-52% loss in ak mice (Chapter 2-Fig. 1, Chapter 3-Fig. 2 and 3). In PD patients, CB-positive SNc DA neurons are relative spared from neurodegenerative cell death. In ak mice, the same neuron population also remains relatively intact (Chapter 3-Fig. 2). Moreover, our immunohistochemical analysis revealed that the SNc DA neuron subpopulations expressing Pitx3 and CB are largely exclusive (Chapter 3-Fig. 5). Thus, whereas CB confers protection to SNc DA neurons against pathology, Pitx3 seems necessary for survival of the MesDA neuronal subpopulation most susceptible to degeneration in PD.

Up to recently, information on the role of Pitx3 in the human central nervous system was very scarce. Nelander et al. showed PITX3-expression in postmitotic DA neurons of the human embryonic ventral mesencephalon. Smidt et al. reported reduced density of PITX3-expressing neurons in the SNc of PD patients. In the last two years, however, several groups have identified PITX3 polymorphisms in individuals with PD. In Germany, Fuchs et al. performed a genetic association study in more than a thousand sporadic PD patients and healthy controls, and reported a strong association of the single-nucleotide polymorphism (SNP) rs3758549 C/T substitution in the PITX3 promoter with PD. The C allele of this polymorphism appeared to be a recessive risk allele with an estimated population frequency of 83%. In Austria, Haubenberger et al. confirmed this strong association of the PITX3 promoter SNP rs3758549 C/T substitution with PD in their analysis of 365 familial and sporadic PD patients and 418 controls. The association was strongest for the sporadic patients, and subanalysis did not show influence of onset age of PD. However, in their cohort the recessive T allele appeared to act as the risk factor. In China, Yu et al. analyzed 316 sporadic PD patients and 305 controls, and also found a significant association between the PITX3 promoter SNP rs3758549 C/T substitution with PD. Again, the T allele appeared to be the risk modifier for PD; its frequency was significantly higher among early-onset (onset age ≤50 years) but not late-onset patients. In the United States of America (USA), Le et al. analyzed DNA samples from 265 familial and sporadic PD patients and 210 age-matched controls, and reported significantly more SNP rs2281983 C/T substitution in exon 3 of the PITX3 coding sequence in PD patients. The C allele was associated with early-onset but not late-onset PD, and showed significantly higher frequency among familial PD. The frequency among sporadic PD patients was moderately higher, albeit not statistically significant. They also tested for SNP rs4919621 A/T substitution in intron 1 of the PITX3 noncoding sequence, and found a significantly higher occurrence of the A allele among early-onset, late-onset and familial but not sporadic PD. In their analysis of 361 sporadic PD patients and 333 controls in Sweden, Bergman et al. confirmed the significant higher frequency of this intron 1 SNP rs4919621 A allele among
Discussion

They did, however, not replicate the association of the PITX3 promoter SNP rs3758549 C/T substitution with PD. Taken together, PITX3 SNPs carry a significant and reproducible association with PD throughout the world. These are the first preliminary results that show the potential value of our knowledge on Pitx3-deficient ak mice for better understanding PD. Especially the PITX3 promoter SNP rs3758549 C/T substitution is of interest, since ak mouse also carry two deletions in the 5' flanking region of the Pitx3 gene, eliminating putative promoter sequences.

Do identical genetic mechanisms apply to PITX3-related PD patients and Pitx3-deficient ak mice? And does the higher frequency of risk alleles in early-onset versus late-onset PD share similarities with the developmental ak phenotype in mice? The clinical signs of PD arise when 60-70% of SNc neurons are estimated to be lost. The variability of the age of onset and severity of parkinsonian symptoms could of course be solely determined by the speed and progression of the Lewy-type α-synucleinopathy pathology. An alternative hypothesis, however, would be that genetic factors within MesDA neurons determine how vulnerable, or how resistant, these cells are to α-synucleinopathy-induced neurodegeneration. A perhaps even more unconventional thought would be to suggest that genetic factors during early MesDA neuron development determine the number of SNc and VTA DA neurons people are born with, and people with low MesDA neuron numbers from the start might then be more at risk for PD later in life, when Lewy bodies gradually accumulate. Indeed, it is generally accepted that between 16 and 30% of neurologically-normal elderly people have incidental Lewy body disease. Perhaps it is not the accumulation of Lewy bodies in the lower brain stem and midbrain as such which causes pathology, but the genetically determined vulnerability of MesDA neurons which defines whether, at what age and how severe an individual human being develops PD. Based on the neuropathological observations in ak mice and the above mentioned genetic association with PD throughout the world population, Pitx3 might very well be an important factor in human MesDA neuron resistance against pathology.

Conclusion

Pitx3-deficiency in the ak MesDA system causes 71-81% loss of mainly vSNc DA neurons during early development and 33-52% loss of VTA DA neurons during adult life. Although the underlying molecular mechanisms are still far from being elucidated, a sequentially orchestrated genetic cascade involving FoxP1, Lmx1b, Pitx3 and Ahd2, necessary for adequate production of the neuronal patterning and differentiation factor retinoic acid from retinol, seems crucial. Preliminary data suggests that the regulatory effect of Pitx3
might occur through potentiation of Nurr1-mediated transcription. The pattern of MesDA neuron loss and concommittant DA reduction in the neostriatum in ak mice are strikingly similar to those observed in PD patients. Notwithstanding the neuropathological differences between the highly selective midbrain phenotype in ak mice and the extensive Lewy-type α-synucleinopathy pathology in PD, Pitx3 seems necessary for survival of the MesDA neuronal subpopulation most susceptible to degeneration in PD. Indeed, polymorphisms of the PITX3 gene put human beings throughout the world at risk for developing PD. Future research is needed to further elucidate how this important MesDA neuron transcription factor regulates vulnerability to neurodegeneration. In addition, it might proof to be a valuable target for new treatment strategies against PD. Finally, more detailed analysis of the remaining nigrostriatal en striatonigral projections in ak mice will clarify the fundamental role of DA in neostriatal development.