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Motor Deficits and Altered Striatal Gene Expression in *aphakia(ak)* Mice

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Abstract

Like humans with Parkinsons disease (PD), the *ak* mouse lacks the majority of the substantia nigra pars compacta (SNc) and experiences striatal denervation. The purpose of this study was to test whether motor abnormalities in the *ak* mouse progress over time, and whether motor function could be associated with temporal alterations in the striatal transcriptome. *Ak* and *wt* mice (28 to 180 days old) were tested using paradigms sensitive to nigrostriatal dysfunction. Results were analyzed using a linear mixed model. *Ak* mice significantly underperformed *wt* controls in rotarod, balance beam, string test, pole test and cotton shred tests at all ages examined. Motor performance in *ak* mice remained constant over the first 6 months of life, with the exception of the cotton shred test, in which *ak* mice exhibited marginal decline in performance. Dorsal striatal semi-quantitative RT-PCR for 19 dopaminergic, cholinergic, glutaminergic and catabolic genes was performed in 1 and 6 month old groups of *ak* and *wt* mice. Preproenkephalin levels in *ak* mice were elevated in both age groups. *Drd1*, 3 and 4 levels declined over time, in contrast to increasing *Drd2* expression. Additional findings included decreased *Chrnα6* expression and elevated *VGluT1* expression at both time points in *ak* mice, and elevated *AchE* expression in young *ak* mice only. Results confirm that motor ability does not decline significantly for the first 6 months of life in *ak* mice. Their striatal gene expression patterns are consistent with dopaminergic denervation, and change over time, despite relatively unaltered motor performance.

Keywords

Aphakia; Denervation; Dopaminergic; Motor; Parkinson's; Striatum

1. Introduction

Chronic levodopa therapy in patients with Parkinson's disease (PD) frequently results in motor complications that limit the utility of the drug (Olanow et al., 2006; Linazasoro, 2007). These arise from the combined effects of progressive dopaminergic denervation and chronic levodopa therapy (Olanow et al., 2006). Both result in adaptive changes in striatal neurochemistry that, together, are thought to result in motor fluctuations and dyskinesias

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seen in treated patients (Jenner, 2000; Calon and Di Paolo 2002; Guigoni et al., 2005; Hurley and Jenner 2006; Linazasoro, 2007).

Striatal denervation in PD is accompanied by a host of secondary neurochemical alterations in dopaminergic receptors (Hurley and Jenner 2006) as well as in striatal glutaminergic (Kashani et al., 2007), serotonergic (Di Giovanni et al., 2006), cholinergic (Zhou et al., 2003) systems and neuropeptides (Backman et al., 2007). The *Pitx3* $-/-$ or aphakia (*ak*) mouse is a promising model of striatal denervation in PD as it exhibits the cell specificity of neurodegeneration observed in humans (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004), similar neuroadaptive phenomena at the level of the striatum (Smits et al., 2005; van den Munckhof et al., 2006), and locomotor deficits that are rescued by levodopa (Hwang et al., 2005; van den Munckhof et al., 2006). *Pitx3* is a member of the pituitary family of bicoid type homeobox transcription factors (Semina et al., 1997). It is expressed in midbrain dopaminergic neurons by E11.5 (Smidt et al., 1997), where it facilitates differentiation of the A9 cell group constituting the substantia nigra pars compacta (SNc; Chung et al., 2005). In homozygous *ak* mice, genomic deletions within the promoter and exon1 of the *Pitx3* gene (Rieger et al., 2001; Semina et al., 2000) result in incomplete lens development and aphakia. Undetectable expression of *Pitx3* protein in the midbrain is accompanied by failed development of approximately 90% of SNc neurons, and severe reduction of dorsal striatal dopaminergic innervation (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003). The *ak* mouse retains expression of *Pitx3* in skeletal muscle (L'Honoré et al., 2007). In addition, low levels of *Pitx3* expression in the eye can be detected by PCR (Rieger et al., 2001), indicating that the *ak* mouse represents a cell-specific hypomorph of *Pitx3*. Deletion of *Pitx3* using recombinant technology (L'Honoré et al., 2007) results in a similar ocular and midbrain phenotype.

Heterozygous *ak* mice are normal (Nunes et al., 2003). In homozygote *ak* mice, SNc cell loss (particularly in the ventral nigra) is almost complete by birth, whereas the ventral tegmental area (VTA) undergoes progressive postnatal cell loss (Nunes et al., 2003; van den Munckhof et al., 2003) so that approximately 50% of this region is lost by 100 days (van den Munckhof et al., 2003). *ak* mice exhibit relatively subtle behavioral and motor deficits (Hwang et al., 2003; 2005; van den Munckhof et al., 2003; 2006), and some conflicting data exist in this regard (Smidt et al., 2004; Hwang et al., 2005). It is not known whether motor signs progress with age in *ak* mice. Additionally, it is unclear how the striatal transcriptome responds to persistent dopaminergic denervation over time. Studies have reported increased, decreased or unchanged mRNA expression levels of dopamine receptors and neuropeptides in *ak* mice (Smidt et al., 2004; Smits et al., 2005; van den Munckhof et al., 2006). These inconsistencies may lie in differing methodologies used to assess striatal expression of these proteins or their transcripts.

The *ak* mouse appears to be a good model for exploring the effects of long-term levodopa or other treatments on motor function and adaptive mechanisms within the striatum. However, the effect of the primary gene defect on these phenomena in the *ak* mouse is incompletely described. The objective of this study was to perform a comprehensive study of motor function in *ak* mice using tests sensitive to nigrostriatal dysfunction, and to determine whether their motor impairments progress with age. In addition, we assessed whether striatal gene expression for markers of dopaminergic, cholinergic and glutaminergic systems were altered by dopaminergic denervation, and whether this pattern changed over time.

2. Results

2.1. Body Weight

ak mice were significantly ($p<0.0001$) lighter than *wt* mice (Table 1). This difference was significant in both genders, with female and male *ak* mice being 3.72 g and 6.51 g lighter on average respectively than their *wt* counterparts. In both genotypes, males were significantly heavier than females ($p<0.0001$). Therefore, to control for the effects of body weight and sex, these factors were included in all multivariable models.

2.2. Overall, *ak* mice underperform *wt* mice in all tests of motor function (Tables 1 & 2)

As only one previous report described the performance of *ak* mice in motor tests typically used to assess fine motor function and dexterity (Hwang et al., 2005), we tested motor performance of *ak* and *wt* mice on rotarod, balance beam, string test, pole test and cotton shred tests. *ak* mice significantly underperformed *wt* mice in all tests of motor function at all ages examined. *ak* mice were able to stay on the rotarod for significantly ($p<0.0001$) shorter times than *wt* mice. Similarly, *ak* mice also took significantly ($p<0.0001$) longer to complete the balance beam, string test and pole test. The amount of cotton shredded by *ak* mice was significantly ($p<0.0001$) less compared to that shredded by *wt* mice. Blind *rd 10* mice performed similar to *wt* mice in all of the above motor tests (results not shown), thus eliminating the possibility that blindness accounted for poor performance in *ak* mice.

2.3. Effect of interaction between age and genotype on motor performance (Table 3)

When the interactions between increasing age and genotype on motor performance were evaluated, a significant interaction of age and genotype was identified in the rotarod ($p=0.025$), balance beam ($p=0.004$), string test ($p=0.001$) and cotton shred tests ($p=0.035$). These data implied that with increasing age, a significant difference existed in the way *ak* and *wt* mice performed motor tests. Because of these findings, the effect of increasing age within each genotype was examined using repeated measures linear regression stratified by genotype (Table 3). In *wt* mice, there was significant improvement in performance on the balance beam test and statistically insignificant improvement noted for the remaining tests. With the exception of the cotton shred test, in which *ak* mice displayed declining ability (non-significant), *ak* mice also improved marginally in the remaining motor tests. Overall, results indicate that whole body motor performance in *ak* mice is comparable to that of *wt* controls in the first 6 months of age, but that fine motor skills (assessed by the cotton shred test) may decline slightly in *ak* mice.

2.4. Semi-quantitative RT-PCR

To assess the transcriptional consequences of dopaminergic denervation on striatal neurons, we examined mRNA expression of dopaminergic, cholinergic and glutaminergic genes in the dorsal striatum (Figure 1 and Table 4) in 6 young (28–35 days) and 6 mature (170–190 days) *ak* and *wt* mice. Expression of *Drd1a*, *Drd3* and *Drd4* was minimally affected in young *ak* mice. In mature *ak* mice, expression of all three genes decreased significantly compared to age-matched *wt* mice. *Drd2* levels were significantly lower in young *ak* mice compared to *wt* controls. In contrast to the other three *Drd* genes, expression of *Drd2* increased over time until it approximated that of *wt* mice. Therefore, it appears that over time, *Drd2* and *Drd1a*/*Drd3*/*Drd4* expression are oppositely regulated in *ak* mice. *Penk1* (preproenkephalin) mRNA expression was significantly elevated in both young and older *ak* mice. *Tac1* (Substance P) expression was not different in young *ak* mice. Its expression increased with age, however this change did not achieve statistical significance. With the exception of *Chrm1* and *Chrnα6*, striatal expression levels of nicotinic and muscarinic cholinergic receptors were not significantly altered at any age in *ak* mice. *Chrm1* was reduced in both

young and older *ak* mice, and this reduction was statistically significant in the young age group. *Chrnα6* was reduced in both age groups, and significantly reduced in older *ak* mice. *AchE* expression was significantly higher in young *ak* mice - this appeared to be transient, with downregulation in older mice. Expression of the other two catabolic enzymes examined, *Maoa* and *Comt*, was minimally altered in *ak* mice compared to *wt* mice. *VGLUT1* was elevated in both young and old *ak* mice, but was statistically significant only in the older age group. Although *VGLUT2* was also elevated (non-significant) at both time points in *ak* mice.

3. Discussion

Results of this study indicate that the *ak* mouse does not demonstrate significant worsening in motor performance in the first 6 months of life. *ak* mice experience altered striatal gene expression in a variety of neurochemical systems shown to be perturbed in PD and its animal models. These expression changes alter over time, despite no worsening in motor phenotype.

In contrast to previously described studies (Nunes et al., 2003), both male and female *ak* mice in this study were significantly lighter than age-matched *wt* mice at all ages examined. PD patients exhibit lower body weight in comparison to age-matched subjects (Chen et al., 2003). The cause is unknown, and various mechanisms of reduced food intake (Kunig et al., 2000; Muller et al., 2001; 2002) reduced intestinal motility (McDonald et al., 2003) or increased energy expenditure due to increased involuntary movements (Markus et al., 1992) are proposed. Limited feeding experiments in our laboratory reveal no difference in food intake between *ak* and *wt* mice (data not shown). Spontaneous activity in *ak* mice is reportedly decreased (van den Munckhof et al., 2003) or increased (Nunes et al., 2003). Co-ordinated evaluation of body weight, food intake and spontaneous activity may reveal a cause for reduced body weight in *ak* mice.

In this study, *ak* mice underperformed *wt* mice in tests of whole body coordination (rotarod, balance beam, pole and string tests) as well as fine motor skills (cotton shred test) at all ages examined. There was no difference noted between the genders in any of the tests performed. These results are consistent with previously reported results (Hwang et al., 2005) of beam traverse and pole tests in 8–9 week old *ak* mice. Our study is the first to evaluate motor deficits in *ak* mice in such wide range of tests and at different ages, and with such a large number of mice (50–60 animals per genotype). Apart from the cotton shred test, in which *ak* mice display marginal (non-significant) decline in performance, there is no decline in their performance in the remaining tests over the time period (up to age 184 d) examined. These findings are in accordance with similar spontaneous activity levels in 40 day and 100 day-old *ak* mice (van den Munckhof et al., 2006). These data imply that locomotor deficits in *ak* mice reside in their SNc lesion, and that this does not appear to be functionally progressive in the first 6 months of life. As no studies have examined old *ak* mice, the extent to which the *ak* phenotype mimics PD over the long term is unknown.

Increased striatal expression of *Drd2a* is commonly reported in human postmortem (Hurley and Jenner 2006; Joyce 1993; Piggott et al., 1999; Ryoo et al., 1998) and functional studies (Kim et al., 2002; Thobois et al., 2004) of PD, as well as in animal models (Morissette et al. 1998; Guillin et al. 2001). We did not identify an absolute increase in *Drd2* expression in our *ak* mice. Our study reveals progressive decline in striatal RNA expression of *Drd1a*, *Drd3* and *Drd4* in *ak* mice over time. In contrast, regulation of *Drd2* expression appears to be in the opposite direction, with initial decline followed by recovery. A consistent feature in our study, as well as both previously reported studies in *ak* mice (Smits et al., 2005; van den Munckhof et al., 2006) is that compared to *wt* mice, *Drd2* levels are elevated relative to

Drd1a levels. Receptor binding studies in 100–200 day old *ak* mice (van den Munckhof et al., 2006) demonstrated increased Drd2 receptor binding limited to the lateral Cpu. As we assessed the entire dorsal striatum, regional elevations in Drd2 expression may have been obscured. Denervation –induced striatal gene expression over time has not been examined in *ak* mice, but has been reported in the monkey (Bezard et al., 2001). In progressively MPTP-lesioned monkeys, Drd2 receptor binding is initially decreased, followed by later upregulation (Bezard et al., 2001). The biphasic pattern of Drd2 expression in that report and in our study may reflect paucity of presynaptic Drd2 receptors (Hurley and Jenner 2006) due to failed development of nigrostriatal projections (Nunes et al., 2003) followed by postsynaptic upregulation.

In this study, Drd3 expression was minimally affected in young *ak* mice, but declined significantly with age. Drd3 receptor levels are reportedly decreased in the *ak* mouse (van den Munckhof et al., 2006) and other animal models of Parkinson's disease (Joyce et al., 2004; Quik et al., 2000; Wade et al., 2001). In animals, declining Drd3 receptors are associated with motor dysfunction, while increasing Drd3 levels accompany levodopa therapy (Quik et al., 2000) or functional recovery (Wade et al., 2001). Either increased or decreased D3 levels are reported in human patients (Hurley and Jenner 2006). Declining striatal expression of Drd1a and Drd4 in our *ak* mice are difficult to interpret in the light of published findings. Results regarding the striatal changes in Drd1-like receptors in Parkinson's disease are inconsistent (reviewed in Hurley and Jenner 2006). Similarly, rodent (Araki et al., 2001; Meissner et al., 2003) and monkey (Betarbet and Greenamyre 2004) models of striatal denervation fail to describe a consistent trend in alteration of Drd1 receptor expression. The status of Drd4 and Drd5 receptor expression in PD patients is unknown (Hurley and Jenner 2006).

In accordance with previous reports in similarly aged *ak* mice (van den Munckhof et al., 2006), preproenkephalin (Penk1) expression was increased in both young and mature *ak* striata in our study. Denervation increases preproenkephalin expression in various models of PD (Betarbet and Greenamyre 2004; Martorana et al., 2003; Meissner et al., 2003; Morissette et al., 1999; Quik et al., 2002). In our study, substance P (Tac1) levels were unchanged in young *ak* mice and insignificantly increased in older *ak* mice. This is in contrast to the autoradiographic findings of Smits et al., 2005, in which the expression of Tac1 was decreased in striatum of *ak* mice. These studies are difficult to compare as the ages of the mice examined in Smits et al., 2005 are not specified (adult). Our results indicate that gene expression in the striatum is a dynamic process, thus making interpretation of single time points difficult. This may also contribute to conflicting data in other studies. In other PD models, striatal substance P levels may increase (Betarbet and Greenamyre 2004; Martorana et al., 2003) or decrease (Bacci et al., 2004; Nisenbaum et al., 1996; Smits et al., 2005) after striatal denervation.

Acetylcholine-mediated neurotransmission plays a crucial role in modulating voluntary movement mediated by the striatum (Calabresi et al., 2000). Dopamine exerts a prominent inhibitory effect on Ach release in the striatum via Drd2 receptors located on striatal cholinergic cells (Bertorelli and Consolo 1990; DeBoer and Abercrombie 1996; Lehmann and Langer 1983; MacKenzie et al., 1989). Consequently, dopaminergic denervation in PD is thought to result in cholinergic hyperactivity with resultant abnormal striatal motor output (Barbeau 1962). AchE expression is markedly elevated in young *ak* mice, but declines over time, suggesting that striatal cholinergic hyperactivity may occur in young *ak* mice. This finding is consistent with other reports in animals. Basal efflux of Ach is higher in dopamine-depleted animals (Johnson and Bruno 1995) and inhibition of AchE exacerbates toxin-induced motor dysfunction (Ott and Lannon 1992; Salamone et al., 2001). Declining

AChE levels in mature *ak* mice suggest that adaptive mechanisms may reduce ACh release over time.

Striatal *Chrm1* expression is significantly reduced in young *ak* mice. The status of cholinergic muscarinic receptors in Parkinson's disease is not clear (Calabresi et al., 2006). Reduced striatal *Chrm1* is reported in humans with PD (Joyce 1993) but not in the striatally denervated rat (Kayadjanian et al., 1999). In contrast, *Chrn α 6*-containing nicotinic receptor function is consistently reduced in Parkinson's disease (Bordia et al., 2007), as well as in monkeys (Kulak et al., 2002; McCallum et al., 2005; Quik et al., 2005) and rodent models of PD (Bordia et al., 2007; Quik et al., 2003). In this study, we identified reduced *Chrn α 6* expression in *ak* striata. α 6- subunit containing nicotinic receptors are selectively expressed on dopaminergic terminals in the striatum (Wonnacott et al., 2000; Zoli et al., 2002). Therefore, it is possible that reduced *Chrn α 6* expression reflects a presynaptic striatal alteration due to the failure of dorsal striatonigral afferents to develop in these mice (Nunes et al., 2003).

Dopamine reduces glutamate release by acting on *Drd2* receptors located on cortico-striatal glutamatergic terminals (Calabresi et al., 2006). Consequently, an adaptive response to loss of striatal dopaminergic afferents is increased corticostriatal glutamate transmission (Calabresi et al., 1996; Lindefors and Ungerstedt 1990). Although only *VGluT1* achieved statistically significant overexpression in mature *ak* mice, both *VGluT1* and *VGluT2* expression levels were increased in *ak* mice compared to *wt* animals in both age groups. Our findings are in accordance with elevations of both transporters in the putamen of Parkinson's patients (Kashani et al., 2007). Animal studies are less consistent, with some studies identifying modest increases in *Vglut2* expression (Bacci et al., 2004) and other demonstrating no alterations (Robelet et al., 2004).

Overall, striatal gene expression in the *ak* mouse is consistent with alterations in multiple neurotransmitter systems. These changes are consistent with those previously associated with dopaminergic denervation in human PD and animal models. These include relative dominance of indirect pathway markers (increase of *Drd2* relative to *Drd1a*, elevated preproenkephalin), cortico-striatal glutamatergic overactivity (elevated *VGluT1*), transient cholinergic hyperactivity (initially elevated AChE in young *ak* mice), and reduced *Chrn α 6* expression consistent with atrophic or reduced striatal dopaminergic terminals. The combination of nigral cell loss, dorsal striatal dopaminergic depletion and gene expression alterations make the *ak* mouse an excellent model in which to study striatal denervation. As *ak* mice underperform *wt* mice in a broad battery of tests designed to assess whole body and fine motor skill, they can be used to test the motor effects of novel therapies targeted to the striatum. Whether PD-like pathology progresses in the *ak* mouse is less clear. Progressive loss of dopaminergic neurons in the A10 region occurs in the *ak* mouse (van den Munckhof et al., 2006). In our study, striatal gene expression patterns also change over time, consistent with progressive adaptive responses. However, this was not accompanied by significant worsening in motor function. The reasons for this may be two-fold. First, we examined relatively young mice, and progressive motor defects may become evident in mice older than 6 months. Second, the functional consequences of altered expression of neurotransmitters and their receptors are difficult to predict, as some may be associated with motor dysfunction, whereas others may be compensatory mechanisms with variable or no effect on motor activity. Additional studies in older *ak* mice should clarify this issue.

4. Experimental Procedures

4.1. Animals

Three breeding pairs of *Pitx3^{ak}/2J (ak)* mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Homozygous males were bred with heterozygous females to generate wild-type (WT), heterozygous (Het), and homozygous (Hom) animals for experiments. The phenotype is not seen in hemizygous carriers, so a heterozygous mouse does not exhibit any phenotype. The WT and Het animals have normal phenotype and so are collectively referred to as *wt* in this article. The Hom or *Pitx3 -/-* mice are referred to as *ak* mice. As *ak* mice are blind, mice homozygous for retinal degeneration 10 mutation (B6.CXB1-Pde6brd10/J; The Jackson Laboratory) were used to control for the effects of blindness on motor tests (Chang et al., 2002). Animal care was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, and all procedures were approved by the Yale University Institutional Animal Care and Use Committee. The animals were on 12 hour light-dark cycle with lights on at 7 am. 60 *ak* (31 females, 29 males) and 54 *wt* (26 females, 28 males) mice with age ranging from 21–184 days (mean age: *ak*-66d, *wt*-64d) were tested in this study.

4.2. Body weight

Body weights (in grams) of *ak* (n=60) and *wt* (n=54) mice were measured before the beginning of motor tests.

4.3. Motor Tests

Different tests were performed to test motor ability. All tests were performed by the same person at the same time of day (between 1–3 pm) following 2–3 hrs habituation to the testing room, for 3 consecutive days.

4.3.1. Rotarod—The rotarod apparatus (Accurotor, Accuscan Instruments, Columbus, OH, USA) provides a sensitive means to measure deficits of motor coordination resulting from defects of the dopaminergic system (Baquet et al., 2004; Fetsko et al., 2005; Fowler et al., 2002; Iancu et al., 2005). Each mouse (n= *ak* -60, *wt* -54) was given three trials over 3 days on an accelerating protocol (0–40 rpm in 80 seconds). The latency to fall was measured in seconds (s). On the first day, mice were trained in three consecutive trials, the last of which was timed and constituted the day 1 measure of the three days. The same testing regimen was followed in all the following motor tests except the cotton shred test.

4.3.2. Balance Beam—The balance beam is a test of motor coordination (Cardozo-Pelaez et al., 1999; Cronise et al., 2005). The beam consisted of a 60 cm long horizontal wooden dowel (1cm diameter) placed at a height of 50 cm above a padded surface. Each mouse (n= *ak* -60, *wt* -54) was placed on the marked center of the dowel and the time taken to reach one of the platforms on either side was measured. Each platform on either side had a dark shelter to encourage the mouse to traverse towards it. We found that the mice readily traversed the beam after the initial training period.

4.3.3. Horizontal String Test—This is a test of strength and coordination. This test used the same apparatus as described for the balance beam, except that a taut string (5mm diameter and 60cm long) was placed between the vertical supports instead of a dowel. Each mouse (n= *ak* -59, *wt* -54) was placed on the center of the string and the time taken to reach the platform was measured.

4.3.4. Pole Test—The pole test has been used in mice previously to assess movement disorders due to striatal dopamine depletion or basal-ganglia abnormality (Fernagut et al., 2003; Fleming et al., 2004; Matsuura et al., 1997; Ogawa et al., 1985; Sedelis et al., 2001). Each mouse ($n = ak -60$, $wt -54$) was placed head upwards on top of an inclined (about 60°) wooden pole (1cm diameter and 50cm long) with a cardboard barrier to prevent upward traversal. The base of the pole was placed in the home cage. When placed on the pole, the mouse oriented downward and descended the length of the pole back into the home cage. The time was measured between the placement on pole and the return to home cage.

4.3.5. Cotton Shred—Both male and female mice shred cotton material to build nests, and analysis of shredding behavior has been used to assess nigrostriatal sensorimotor function in rodents (Fleming et al., 2004; Hofele et al., 2001; Sedelis et al., 2000; Szczypka et al., 2001; Upchurch and Schallert 1983). The behavior requires the use of orofacial and forelimb movements. A pre-weighed cotton nestlet was placed on the cage bedding of individually housed mouse ($n = ak -57$, $wt -43$) and the unshredded portion of the nestlet was weighed at 90 minutes, 3 hrs and 6 hrs time interval. The shredded cotton was calculated for each time interval and used for analysis.

4.4. RNA isolation and semi-quantitative RT-PCR assay

Following carbon-dioxide euthanasia and decapitation, the brain was removed and coronally sectioned under a dissecting microscope. The dorsal striatum only (dorsal to anterior commissure) was collected from six young (28–35d) and six old (170–190d) mice of both genotypes. Mice were equally split across gender. The dissected striatum was snap frozen using liquid nitrogen and then stored at -70°C . Total RNA was isolated from the striatum using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. Quantification of total RNA was assessed using a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, USA). First-strand cDNA was synthesized using 250ng of total RNA in a final volume of 20 μl according to manufacturer's protocol (SuperArray Bioscience Corporation, Frederick, MD, USA). Real-Time quantitative PCR was performed according to manufacturer's protocol to study the expression of different genes using SYBR Green (SuperArray Bioscience Corporation, Frederick, MD, USA) on Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, USA). Genes examined were: dopamine receptors (Drd1a, Drd2, Drd3, Drd4), preproenkephalin 1 (Penk1), tachykinin 1 (Tac1; otherwise known as Substance P), cholinergic muscarinic receptors (Chrm1, Chrm2, Chrm3, Chrm4, Chrm5), cholinergic nicotinic receptors (Chrn2, Chrn4, Chrn6), acetylcholinesterase (Ache), catechol-o-methyl transferase (Comt), monoamine oxidase A (Maoa), and vesicular glutamate transporters (VGluT1, VGluT2). GAPDH was used as a control gene, and was amplified in parallel with every other gene examined. Reaction conditions for PCR were 95°C for 15 min followed by cycling for 40 cycles of 95°C , 55°C , and 72°C for 30 seconds each. This was followed by a final annealing cycle at 72°C for 10 min. The melting curve was read every 2°C from 65°C to 95°C . 10 μl from all PCR reactions were run on an 1.5% agarose gel to ensure that single bands of the appropriate size were obtained (Supplemental Figure 1). Statistical analysis of gene expression is described in the next section.

4.5. Statistical analysis

The effects of genotypes on the five motor tests were estimated with generalized linear mixed models, which account for the correlation between repeated measurements within the same mouse. The time performance test outcomes for the rotarod, balance beam, string test and pole test were measured three times. The cotton shred outcome was the difference between time 0 and three time periods (90 min, 3 h and 6 h). Both fixed and random effects

models with different covariance structures were explored by comparing the goodness-of-fit of the different models: maximum likelihood methods to obtain the -2 residual log likelihood. We chose the fixed effect model with unstructured covariance to fit the data based on this goodness-of-fit statistic. The covariates included age (in days), sex, body weight, and test replicate number. For each outcome, the potential interactions between genotype and age were examined by including interaction terms, and if they were significant, stratified models were analyzed. A general linear model was used to examine the effect of genotype on mouse body weight adjusted for age and gender. All statistical tests were 2-tailed, and $P < 0.05$ was considered to indicate statistical significance. All analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

The relative expression ratios of each target gene in *ak* compared to *wt* mice in each of the two age groups were calculated according to the REST method (Pfaffl et al., 2002). We first calculated amplification efficiency (E) for each gene for each age group and genotype. The efficiency of every PCR reaction was calculated from the slope of its linear amplification region using at least 4 data points with LinRegPCR software (Ramakers et al., 2003; Supplemental Table 1). Efficiencies were either the same or the largest average difference was 0.02 (see Supplemental Table 1) with standard deviations of ≤ 0.02 , the standard stated by Schefe et al (2006). The correlations (R-square) for each individual efficiency was ≥ 0.998 , demonstrating a good fit.

In the REST method, relative expression ratios of target genes relative to the reference genes are computed based on the real-time PCR efficiencies (E) and the crossing point (CP) difference (Δ) of an unknown sample versus a control (Δ CP control – sample). This method incorporates efficiencies of the target and reference gene into the calculation, thus providing a more refined measurement of relative expression ratios. Variation was expressed as confidence intervals - gene expression values whose confidence intervals do not include 1 (i.e. equivalent expression between *ak* and *wt* mice) were considered significant at $p < 0.05$.”

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ache	acetylcholinesterase
ak	aphakia
DA	dopamine
Drd	dopamine receptor
Chrm	cholinergic muscarinic
Chrn	cholinergic nicotinic
Comt	catechol-o-methyl transferase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Maoa	monoamine oxidase A
MPTP-1	methyl 4-phenyl 1,2,3,6 tetrahydropyridine
PD	Parkinson’s disease
Penk1	preproenkephalin 1
SNe	substantia nigra compacta
Tac1	tachykinin 1
VgluT	vesicular glutamate
VTA	ventral tegmental area

Striatal expression profile in *ak* compared to WT mice **Figure 1. Striatal gene expression in young and mature *ak* mice**

Expression levels of *Drd1a*, *Drd3* and *Drd4* are significantly reduced in mature *ak* mice. *Drd2* expression appears to be oppositely regulated, and is significantly reduced in young *ak* mice, but not in older *ak* mice. *Penk1* is significantly increased in *ak* mice at both ages. *Chrm1* and *Chrm6* are significantly reduced in young and mature *ak* mice respectively. *VGLUT1* is overexpressed in *ak* mice at both ages – this achieves statistical significance only in mature mice. *AChE* expression is increased significantly in young *ak* mice, but declines thereafter. Bars indicate the level of expression in young (gray bars) and mature (black bars) *ak* mice compared to *wt* mice, where equivalent expression is equal to 1. $n=6$ in each group. * indicate ratios of expression levels whose 95% confidence intervals do not include 1.0 and thus are statistically significant at $p < 0.05$.

Table 1

Descriptive statistics in *ak* and *wt* mice.

Test	<i>ak</i>			<i>wt</i>		
	n	Mean	SE	n	Mean	SE
Rotarod	60	31.5	0.99	54	43.7	1.39
Balance beam	60	3.4	0.16	54	2.8	.011
Pole test	59	16.5	1.21	54	12.1	0.61
String test	60	26.0	1.23	54	12.7	0.59
Cotton shred	57	0.36	0.05	43	0.59	0.06
Body weight	60	18.92	0.97	54	24.23	0.99

Means of balance beam, pole test and string test are given as time (seconds) taken to complete the task. Rotarod results are reported as the mean time (seconds) on the rod before falling. Cotton shred results describe the mean percentage of cotton shredded after 6 hours. Body weight means are given in grams. Data was collected from *ak* mice (mean age: 66 days) and *wt* mice (mean age: 64 days).

Table 2

Effect of genotype on motor performance comparing *ak* to *wt* (reference) mice.

Test	Coefficient	SE	P value
Rotarod	−18.76	1.62	<0.0001
Balance beam	0.96	0.21	<0.0001
Pole test	16.76	1.5	<0.0001
String test	9.45	1.3	<0.0001
Cotton shred	−0.81	0.15	<0.0001

Data was analyzed using a repeated measures linear regression model, adjusted for age, sex and body weight. The coefficients reflect the performance of *ak* mice compared to *wt* mice. The negative values of coefficients reflect shorter time (seconds) on the rotarod or fewer (grams) cotton shredded by *ak* mice, and positive coefficients reflect longer times (seconds) taken by *ak* mice to traverse the balance beam or complete the pole and string tests. The unit of measurement in all the tests is seconds (except grams for cotton shred).

Table 3

Effect of age on motor tests, stratified by genotype.

Test	ak			WT/het		
	Coefficient	SE	P value	Coefficient	SE	P value
Rotarod	0.038	0.037	0.297	0.013	0.063	0.842
Balance beam	-0.01	0.006	0.128	-0.016	0.005	0.003 *
Pole test	-0.054	0.048	0.264	-0.015	0.028	0.597
String test	-0.045	0.039	0.251	-0.052	0.03	0.084
Cotton shred	-0.001	0.001	0.355	0.004	0.002	0.073

The coefficients reflect the age-related performance of *ak* and *wt* mice within genotype. Both *ak* and *wt* mice stay on the rotarod longer, and take shorter times to complete the balance beam, string and pole tests. In *wt* mice, improvement on the balance beam is significant. With age, *ak* mice shred less cotton, whereas *wt* mice shred more. Positive coefficients reflect longer times (sec) taken to complete motor tasks on the motor tests or more grams of cotton shredded, and negative coefficients indicate shorter times or fewer grams of cotton shredded. The unit of measurement in all the tests is seconds (except grams for cotton shred).

* indicate significant difference.

Table 4

Striatal gene expression in *ak* mice compared to *wt* mice.

Gene	28–35 days			170–190 days		
	<i>ak</i> (mean,SD)	<i>wt</i> (mean,SD)	<i>ak/wt</i> (rER, CI)	<i>ak</i> (mean,SD)	<i>wt</i> (mean,SD)	<i>ak/wt</i> (rER, CI)
Drd1a	23.09±1.10	22.29±0.72	0.93(0.70–1.24)	23.51±0.77	23.04±1.13	0.82(0.67–0.94)*
Drd2	24.25±0.95	23.42±0.33	0.85(0.73–0.99)*	23.67±0.99	23.54±0.79	0.96(0.86–1.10)
Drd3	28.09±0.88	27.69±0.58	1.06(0.93–1.15)	28.81±0.68	28.37±0.71	0.87(0.69–0.92)*
Drd4	30.39±0.84	30.06±0.46	0.98(0.85–1.32)	30.90±0.68	30.50±0.98	0.89(0.69–0.94)*
Penk1	20.02±1.08	20.68±0.87	1.27(1.09–1.46)*	20.83±1.40	21.80±0.92	1.29(1.09–1.56)*
Tac1	22.37±0.57	22.14±0.51	1.02(0.91–1.10)	23.05±1.26	23.51±0.97	1.13(0.99–1.27)
Chrm1	23.62±0.63	22.72±0.44	0.92(0.77–0.95)*	23.37±1.21	23.46±0.12	0.98(0.89–1.01)
Chrm2	27.45±0.66	27.42±0.28	1.11(0.96–1.18)	27.26±0.94	27.62±0.64	1.02(0.95–1.15)
Chrm3	26.80±0.79	26.60±0.76	1.00(0.93–1.12)	27.75±1.35	27.96±0.36	1.02(0.87–1.17)
Chrm4	23.37±0.74	22.85±0.70	0.94(0.82–1.22)	24.25±1.16	24.63±0.86	1.08(0.95–1.17)
Chrm5	28.10±1.15	28.45±0.83	1.25(0.98–1.23)	28.44±0.82	28.64±0.45	1.15(0.82–1.19)
Chrna4	27.80±0.81	27.55±0.42	1.06(0.96–1.20)	27.13±0.58	27.47±0.73	1.02(0.82–1.26)
Chrna6	31.94±0.57	31.06±0.58	0.94(0.86–1.02)	31.44±1.02	30.91±0.86	0.81(0.74–0.93)*
Chrb2	24.92±0.73	25.15±0.45	1.20(1.0–1.14)	24.28±0.81	25.00±0.30	1.22(0.97–1.29)
Vglut1	22.28±1.47	23.11±1.32	1.11(0.99–1.76)	23.02±1.15	24.23±0.74	1.38(1.05–1.49)*
Vglut2	26.46±2.43	26.76±1.10	1.21(0.73–1.84)	26.31±1.26	26.91±1.09	1.12(0.77–1.44)
Maoa	25.41±0.53	25.07±0.67	1.16(0.96–1.26)	25.07±0.70	25.45±0.35	1.05(0.95–1.06)
Comt	23.79±0.86	23.33±0.88	1.15(0.94–1.37)	24.25±0.89	24.73±0.64	1.08(0.94–1.12)
Ache	23.60±1.03	23.83±0.34	1.36(1.13–1.39)*	24.23±0.52	24.90±0.67	1.15(0.97–1.19)

Mean cycle numbers with standard deviations (SD) for each gene are reported for *ak* and *wt* mice in each age group (n=6 per group; total 24 mice) in columns 2, 3, 5 and 6. The relative expression ratio (rER) of gene expression with 95 % confidence intervals (CI) between the genotypes in young animals and mature animals are reported in columns 4 and 7. These values were controlled for efficiency (Ramakers et al. 2003) and normalized to expression of GAPDH according to Pfaffl et al (2002).

* indicate ratios of expression levels whose 95% confidence intervals do not include 1.0 and thus are statistically significant at $p < 0.05$.