Brief Communication

3,4-Dihydroxyphenylalanine Reverses the Motor Deficits in Pitx3-Deficient *Aphakia* Mice: Behavioral Characterization of a Novel Genetic Model of Parkinson's Disease

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Parkinson's disease (PD) is a neurodegenerative disease characterized by a loss of dopaminergic neurons in the substantia nigra. There is a need for genetic animal models of PD for screening and *in vivo* testing of novel restorative therapeutic agents. Although current genetic models of PD produce behavioral impairment and nigrostriatal dysfunction, they do not reproduce the loss of midbrain dopaminergic neurons and 3,4-dihydroxyphenylalanine (L-DOPA) reversible behavioral deficits. Here, we demonstrate that Pitx3-deficient *aphakia* (*ak*) mice, which have been shown previously to exhibit a major loss of substantia nigra dopaminergic neurons, display motor deficits that are reversed by L-DOPA and evidence of "dopaminergic supersensitivity" in the striatum. Thus, *ak* mice represent a novel genetic model exhibiting useful characteristics to test the efficacy of symptomatic therapies for PD and to study the functional changes in the striatum after dopamine depletion and L-DOPA treatment.

Key words: **Parkinson's disease; substantia nigra; *aphakia* mouse; Pitx3; dopaminergic neuron; animal model**

Introduction

Screening and *in vivo* testing of novel therapeutic agents for the improved treatment of Parkinson's disease (PD) is facilitated by animal models displaying defects of the nigrostriatal dopaminergic system and showing dopamine (DA)-dependent behavioral enhancement. Several genetic models based on genes (e.g., *α-synuclein* and *parkin*) implicated in the pathogenesis of familial PD have been developed. Although these animal models display important characteristics of PD, including dystrophic neurites, neuronal atrophy, and intracellular inclusions, no clear degeneration of the nigrostriatal DA system has been detected (Beal, 2001; Betarbet et al., 2002; Dawson et al., 2002; Dauer and Przedborski, 2003; Fernagut and Chesselet, 2004). Neurochemical animal models using selective neurotoxins can mimic the selective loss of substantia nigra neurons typical of PD and have been used to assess the efficacy of standard and novel therapeutic treatments (Beal, 2001; Betarbet et al., 2002; Dawson et al., 2002; Dauer and Przedborski, 2003). However, the labor-intensive toxin injections and the need to assess the degree of lesion for each animal hamper their wide use for high-throughput *in vivo* screening of drugs and the evaluation of novel therapeutic regimens for PD. These shortcomings support the need to establish genetic models of PD carrying hallmark features of PD: degeneration of the nigrostriatal DA system, sensorimotor impairment, and responsiveness to PD medications such as 3,4-dihydroxyphenylalanine (L-DOPA). Recent work from our laboratory, as well as others, has shown that Pitx3-deficient *aphakia* (*ak*) mice display significant loss of substantia nigra DA neurons and show defects of the nigrostriatal pathway, whereas DA neurons in the ventral tegmental area and other brain areas are less affected (Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004). The degree of DA loss in the dorsal striatum of *ak* mice is close to 90%, which is greater than the level at which adult animals and PD patients become symptomatic. Initial behavioral analyses of *ak* mice measuring general motor activity detected no deficits (Hwang et al., 2003; Nunes et al., 2003), statistically significant hypoactivity only during the dark cycle (no clear difference during the light cycle) (van den Munckhof et al., 2003), or a significant reduction in ambulatory activity when measured during the light cycle (Smidt et al., 2004). We speculate that these inconsistent reports of motor impairments in *ak* mice may be attributable to the measurement of gross motor activity rather than the use of tasks specifically sensitive to nigrostriatal dysfunction. Several nigrostriatal pathway-sensitive tests, such as the analysis of spontaneous exploratory activity in a cylinder, and tests for sensorimotor coordination on the challenging beam and the pole have been shown to provide sensitive measurements of altered function of the nigrostriatal dopamine system in mice

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(Ogawa et al., 1985; Goldberg et al., 2003; Fleming et al., 2004; Fleming and Chesselet, 2005). Here, we show that *ak* mice display significant sensorimotor deficits on these nigrostriatal pathway-sensitive tests and that these motor impairments are efficiently reversed by L-DOPA administration. Furthermore, we report evidence of "dopaminergic supersensitivity" in the striatum of *ak* mice, a prominent feature observed in animal models of PD, as well as in PD patients (Pifl et al., 1992).

Materials and Methods

Animals. ak mice used in this study were originally from The Jackson Laboratory (Bar Harbor, ME) (strain B6 × C57BLKS-*ak*; JR942), and homozygous *ak* mice were generated at the University of Iowa (Iowa City, IA; a gift from Dr. E. V. Semina). They were outcrossed several times to C57BL/6 mice and maintained in the C57BL/6 background. Several breeding pairs were transferred, expanded, and maintained at the Animal Care Facility at McLean Hospital. Wild-type (*wt*) C57BL/6 mice were obtained from The Jackson Laboratory and used as control. Mice homozygous for retinal degeneration 1 (*rd1* or *Pde6b*^{rd1}) mutation (*B6.C3-Pde6b*^{rd1}; The Jackson Laboratory) were used as a blinded mouse control. The *rd1* mice are also in the C57BL/6 background. Animal use was in accordance with Institutional Animal Care and Use Committee of McLean Hospital and followed National Institutes of Health guidelines.

Locomotor activity. Locomotor activity of adult male *wt* and *ak* mice were measured for 22 h using an infrared photobeam activity monitoring system (San Diego Instruments, San Diego, CA) connected to a microcomputer. For testing, animals were individually housed with *ad libitum* access to food and water in a novel environment (17 × 8 × 8 inch transparent plastic cages transected by a 4 × 8 horizontal infrared beam grid) maintained under a 12 h light/dark cycle with lights on from 7:00 A.M. to 7:00 P.M. Locomotor (ambulatory) activity, defined as a consecutive breaking of photobeams, was recorded at 10 min intervals between 1:00 P.M. and 11:00 A.M. on the subsequent day after a 2 h habituation period.

Benserazide and L-DOPA treatment. Eight- to 9-week-old *wt*, *rd1*, and *ak* mice were divided into two groups receiving either intraperitoneal injections of 12.5 mg/kg benserazide 20 min before receiving intraperitoneal injections of 25 mg/kg L-DOPA or two injections of saline separated by 20 min. Behavioral testing began 10 min after the L-DOPA injection. All of the subsequent behavioral tests were performed during the daytime.

Challenging beam traversal test. Motor performance was measured with a novel beam test adapted from traditional beam-walking tests (Goldberg et al., 2003; Fleming et al., 2004; Fleming and Chesselet, 2005). The detailed procedure was described previously (Fleming et al., 2004). Briefly, the beam (length, 1 m) started at a width of 3.5 cm and gradually narrowed to 0.5 cm in 1 cm increments. Animals were trained to traverse the length of the beam, starting at the widest section and ending at the narrowest section. Animals received 2 d of training before testing, and all of the training was performed without the mesh grid. On the day of the test, a mesh grid (1 cm square) of corresponding width was placed over the beam surface, leaving an ~1 cm space between the grid and the beam surface. Animals were then videotaped while traversing the grid-surfaced beam for a total of five trials. Videotapes were viewed and rated in slow motion for measuring the number of steps taken by each animal and time to traverse across five trials by an investigator blind to the mouse genotype and drug treatment. The animal groups used were *wt*/L-DOPA (n = 11), *wt*/saline (n = 11), *rd1*/L-DOPA (n = 7), *rd1*/saline (n = 5), *ak*/L-DOPA (n = 12), and *ak*/saline (n = 12).

Cylinder test. Spontaneous movement was measured by placing animals in a small transparent cylinder (height, 15.5 cm; diameter, 12.7 cm) (Fleming et al., 2004; Fleming and Chesselet, 2005). Spontaneous activity was videotaped for 3 min. The number of rears and hindlimb steps were measured for *wt*, *rd1*, and *ak* mice after benserazide/L-DOPA or saline treatment. Videotapes were viewed and rated in slow motion by an experimenter blind to the mouse genotype and drug treatment. A rear was counted when an animal made a vertical movement with both forelimbs removed from the ground. Hindlimb steps were counted when an animal moved both hindlimbs across the floor of the cylinder. The animal groups used were *wt*/L-DOPA (n = 11), *wt*/saline (n = 11), *rd1*/L-DOPA (n = 7), *rd1*/saline (n = 5), *ak*/L-DOPA (n = 12), and *ak*/saline (n = 12).

Pole test. Animals were placed head upwards on top of a vertical wooden pole 50 cm in length (diameter, 1 cm) (Ogawa et al., 1985). The base of the pole was placed in the home cage. Once placed on the pole, animals oriented themselves downward and descended the length of the pole back into their home cage. All of the animals received 2 d of training that consisted of five trials for each session. On the test day, animals received five trials, and the time to orient downward was measured. The animal groups used were *wt*/L-DOPA (n = 9), *wt*/saline (n = 9), *rd1*/L-DOPA (n = 9), *rd1*/saline (n = 9), *ak*/L-DOPA (n = 10), and *ak*/saline (n = 9).

Statistics. Mean scores on challenging beam traversal, spontaneous activity, and the pole test were analyzed using a randomized 2 × 2 ANOVA comparing genotype (*wt* and *rd1* vs *ak* mice) and drug treatment (L-DOPA vs saline). Planned comparisons of mean scores for the behavioral measures were used to compare *wt*, *rd1*, and *ak* mice after L-DOPA treatment. All of the analyses were conducted with GB-STAT software (Dynamic Microsystems, Silver Spring, MD) for Macintosh. The level of significance was set at $p < 0.05$.

Immunohistochemistry. Mice were perfused transcardially with saline, followed by 4% formaldehyde in PBS. The brains were removed, post-fixed overnight, cryoprotected in glycerol (20% in PBS), sectioned (30 μm) on a freezing microtome, and processed further, as described previously (Hwang et al., 2003). Immunohistochemistry was performed using biotinylated anti-rabbit IgG (diluted 1:300; Vector Laboratories, Burlingame, CA), avidin–biotinylated peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories), and diaminobenzidine (Vector Laboratories) as the developing agent. Primary antibodies were rabbit anti-c-*fos* antibody (diluted 1:20,000; Calbiochem, La Jolla, CA) and rabbit anti-tyrosine hydroxylase (TH) antibody (diluted 1:200 Pel-Freez Biologicals, Rogers, AR).

Results

ak mice display no impairments in overall spontaneous locomotor activity

Despite the profound defect of the nigrostriatal dopamine system, behavioral impairments of *ak* mice have not been well documented. There have been inconsistent reports on the general motor activity of *ak* mice (Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004). Our preliminary examination of *ak* mice revealed no signs of abnormality in posture, weight, spontaneous behaviors, neurological reflexes, and sensorimotor responses, including righting, postural reflex, ear twitch reflex, startle, grip strength, and whisker orientation reflex (Hwang et al., 2003). We speculate that the inconsistent data regarding motor impairments in *ak* mice and the lack of motor deficits in our preliminary analyses may be attributable to the measurement of gross motor activity rather than the use of tasks specifically sensitive to nigrostriatal dysfunction. To address our hypothesis, we first measured the spontaneous ambulatory activity of *ak* and *wt* mice over 22 h in an automated photocell activity cage (Fig. 1*A*). *ak* mice showed higher ambulatory activity than *wt* mice during the "lights-on" period and lower activity during the "lights-off" period (Fig. 1*A*,*B*). This phenomenon may be, at least in part, attributable to a lack of the conventional diurnal rhythm in *ak* mice, resulting from blindness (Fig. 1*A*). When total horizontal movements during the 22 h period were compared, *ak* mice displayed slightly higher activity than *wt* mice (Fig. 1*B*). Therefore, our examination of the locomotor activity of *ak* mice did not reveal a PD-like behavioral deficit.

ak mice display motor deficits in nigrostriatal pathway-sensitive behavioral tests, which are reversed by L-DOPA

Because tests for gross motor activity did not expose any potential motor deficit in *ak* mice, we next adopted a battery of behavioral

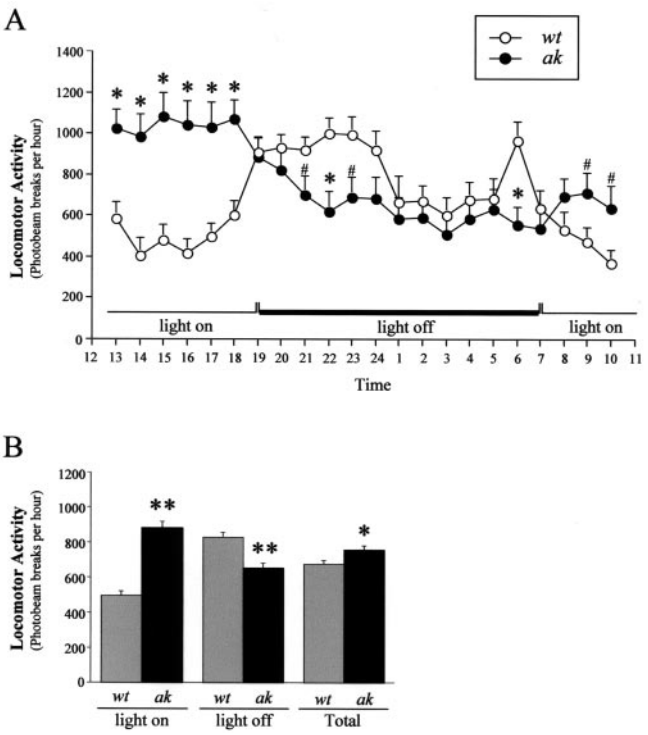

Figure 1. ***A***, Locomotor (ambulatory) activity of *wt* and *ak* mice, recorded as consecutive photobeam breaks per hour for 22 h. ***B***, Mean hourly locomotor activity of *wt* and *ak* mice during light hours, dark hours, and cumulatively. $n = 21$; $^{\#}p < 0.05$; $^{*}p < 0.01$; $^{**}p < 0.001$.

tests sensitive to nigrostriatal impairment, including spontaneous exploratory activity in a cylinder and sensorimotor coordination (challenging beam and pole test) (Fleming et al., 2004; Fleming and Chesselet, 2005). In addition, we tested whether L-DOPA treatment could reverse any motor deficits of *ak* mice. L-DOPA is currently one of the most effective therapeutic agents for the treatment of PD, and validation of PD animal models critically revolves around the observation that treatment with L-DOPA leads to behavioral enhancement/reversal of symptoms (Dawson et al., 2002). Mice homozygous for the retinal degeneration (*rd1* or *Pde6b*^{rd1}) mutation were included as a blinded control group. These *rd1* mice show an early-onset severe retinal degeneration because of a nonsense mutation in the *Pde6b* gene (Pittler and Baehr, 1991) encoding the β subunit of cGMP–phosphodiesterase. Degeneration becomes evident in the outer segments first as early as postnatal day 8, followed by the inner segments and photoreceptor cell bodies (Sanyal and Bal, 1973). Degeneration occurs so rapidly that only a thin layer of scattered rod photoreceptor cell bodies (lacking outer segments) remains at postnatal day 15, and the rod cell bodies disappear completely from the central retina by 36 d (Caley et al., 1972; Carter-Dawson et al., 1978), leading to blindness. Behavioral measurement to assess vision capabilities using the visual cliff task showed that *rd1* mice were no longer capable of pattern recognition shortly after 40 d of age (Nagy and Misanin, 1970).

On the challenging beam test, adult (8 to 9 weeks of age) *ak* mice treated with saline displayed much longer latencies to traverse the beam than both age-matched *wt* (more than twofold; $p < 0.01$) and *rd1* control (~1.7-fold; $p < 0.05$) mice (Fig. 2*A*). They also took ~25% more steps ($p < 0.01$) while traversing the challenging beam compared with *wt* controls (Fig. 2*B*). These results indicate that *ak* mice may mimic the slower movements and shorter steps observed in PD patients. L-DOPA administration significantly reduced both the beam traversal time ($p < 0.05$) and the number of steps taken ($p < 0.01$) by *ak* mice, almost to the levels of *wt* mice. In contrast, the performance of both *wt* and *rd1* mice on the challenging beam was unaffected by L-DOPA treatment (Fig. 2*A*,*B*).

When spontaneous exploratory activity was measured in a transparent cylinder, *ak* mice displayed a significant decrease in rearing (~50%; $p < 0.05$) compared with *wt* mice, and this decrease was completely reversed by L-DOPA administration ($p < 0.01$) (Fig. 2*C*). The amount of hindlimb stepping, which was slightly decreased in *ak* mice, was robustly increased by L-DOPA treatment ($p < 0.01$) (Fig. 2*D*). In fact, L-DOPA-treated *ak* mice showed even higher spontaneous activity than *wt* mice in both rearing and hindlimb stepping tests. In sharp contrast, L-DOPA treatment did not affect rearing or hindlimb stepping in either *wt* or *rd1* control mice (Fig. 2*C*,*D*).

The pole test has been used to assess basal ganglia-related movement disorders in mice (Ogawa et al., 1985; Sedelis et al., 2001). When placed head upwards on top of a vertical pole, *ak* mice took much longer (~3.6-fold) to orient themselves downwards than both *wt* and *rd1* mice ($p < 0.01$). L-DOPA reduced the time it took *ak* mice to orient themselves downwards ($p < 0.01$) but had no effect on either *wt* or *rd1* mice (Fig. 2*E*).

Based on the above results, we conclude that *ak* mice perform worse than their age-matched *wt* and blinded mice controls on a battery of tests that are sensitive to defects of the nigrostriatal DA system, and their sensorimotor function can be significantly restored to the level of *wt* mice in most tests by treatment with L-DOPA. In addition, L-DOPA induced marked increases in some behaviors specifically in *ak* mice, suggesting an increased response to the behavioral effects of this drug.

ak mice display evidence of denervation supersensitivity of the striatum

To determine whether *ak* mice show other signs of PD-related pathology, we examined evidence of "DA denervation supersensitivity." DA denervation supersensitivity is a prominent feature observed in PD patients and in animal models of PD consisting of an altered responsiveness of striatal neurons to DA (Ungerstedt, 1971; Pifl et al., 1992). Changes in DA receptor density and in DA receptor-linked signal transduction pathways may contribute to this phenomenon (Lee et al., 1978; Zigmond et al., 1990). L-DOPA-mediated induction of the immediate early gene c-*fos* in the dopamine-depleted striatum has been suggested to reflect a supersensitized responsiveness of dopamine receptors (Robertson et al., 1989). To explore whether *ak* mice also retain similar striatal changes, we examined c-*fos* expression in the striatum of *ak* mice after L-DOPA administration. Our data show that c-*fos* gene expression is robustly induced by L-DOPA treatment in the striatum of *ak* mice (Fig. 3*D*,*F*). Interestingly, the profound induction of c-*fos* expression was limited to the dorsolateral area of the striatum of *ak* mice in which dopaminergic innervations were significantly diminished (Fig. 3*H*). On the contrary, c-*fos* induction was not detectable in the striatum of *wt* mice after L-DOPA administration (Fig. 3*C*,*E*) nor in the *ak* striatum after saline injection (Fig. 3*B*). These findings suggest that *ak* mice may retain striatal changes (DA receptor supersensitivity) typical of dopamine-depleted striata of patients with PD and seen in animal models of PD, further supporting our hypothesis that *ak* mice represent a valid animal model of PD.

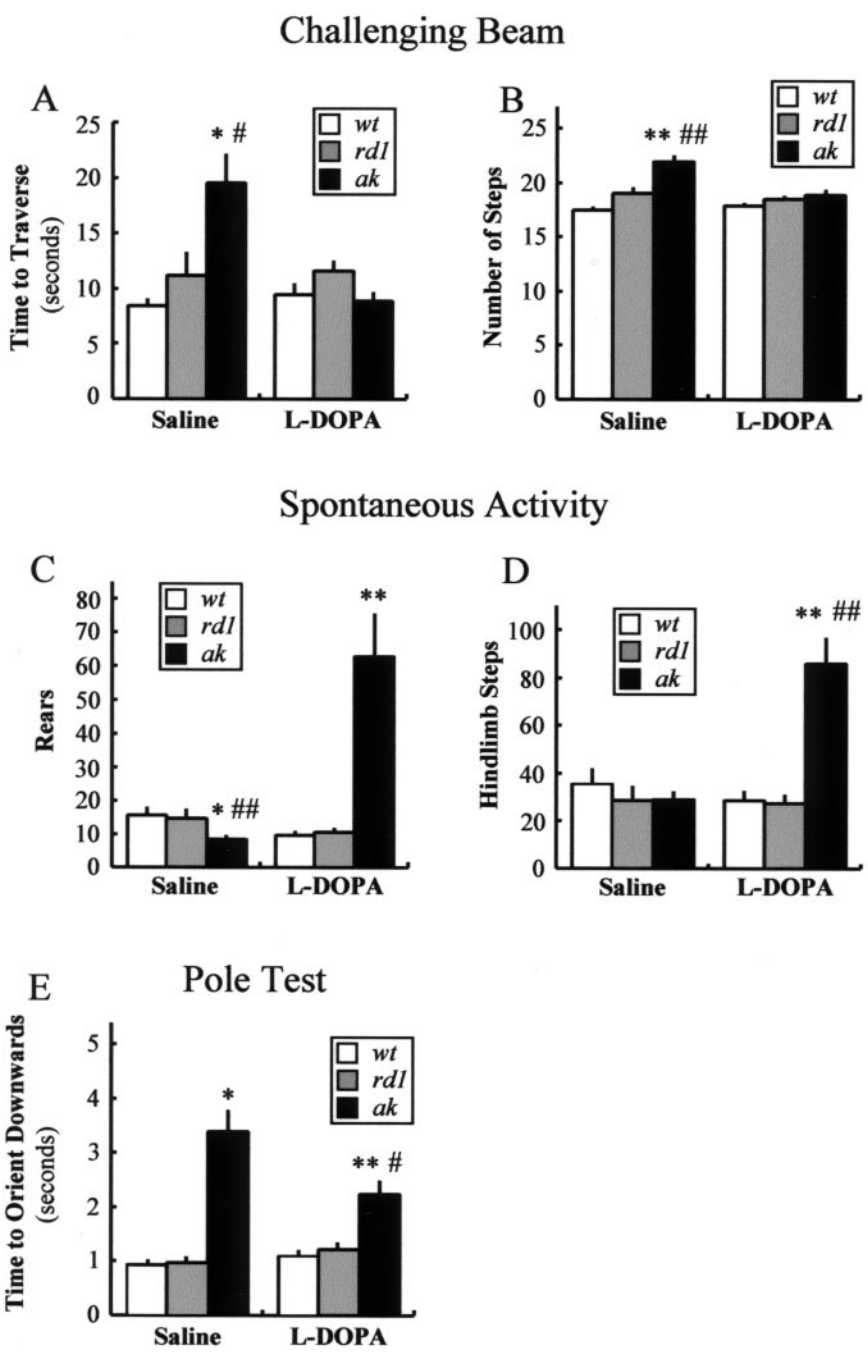

Figure 2. ***A***, The amount of time taken to traverse the challenging beam for *wt* (white bars), *rd1* (gray bars), and *ak* (black bars) mice. $^{\#}p < 0.05$ compared with *ak*/L-DOPA; $^{*}p < 0.01$ compared with *wt*/saline; $p < 0.05$ compared with *rd1*/saline. ***B***, Number of steps taken while traversing the challenging beam for *wt*, *rd1*, and *ak* mice. $^{\#\#}p < 0.01$ compared with *ak*/L-DOPA; $^{**}p < 0.01$ compared with both *wt*/saline and *rd1*/saline. ***C***, The number of rears made in the cylinder for *wt*, *rd1*, and *ak* mice. $^{\#\#}p < 0.01$ compared with *ak*/L-DOPA; $^{*}p < 0.05$ compared with *wt*/saline; $^{**}p < 0.01$ compared with both *wt*/L-DOPA and *rd1*/L-DOPA. ***D***, The number of hindlimb steps taken in the cylinder for *wt*, *rd1*, and *ak* mice. $^{\#\#}p < 0.01$ compared with *ak*/saline; $^{**}p < 0.01$ compared with both *wt*/L-DOPA and *rd1*/L-DOPA. ***E***, The amount of time taken to orient downwards on the pole for *wt*, *rd1*, and *ak* mice. $^{\#}p < 0.01$ compared with *ak*/saline; $^{*}p < 0.01$ compared with both *wt*/saline and *rd1*/saline; $^{**}p < 0.01$ compared with both *wt*/L-DOPA and *rd1*/L-DOPA.

Discussion

Current genetic models of PD are useful to evaluate mechanisms leading to nigrostriatal dysfunction and protective therapies, but they do not reproduce the selective loss of midbrain dopaminergic neurons that leads to the characteristic neurological symptoms of PD. Therefore, novel genetic animal models with selective degeneration of the nigrostriatal dopamine system are necessary for the rapid and efficient screening of symptomatic treatments and the testing of novel restorative therapeutic regimens (e.g., stem cell transplantation and gene therapy approaches) developed to restore dopaminergic function. For these purposes, animal models should display nigrostriatal pathway-dependent sensorimotor deficits that can be reversed by L-DOPA treatment.

As a result of the selective lack of substantia nigra DA neurons, *ak* mice have a great potential to serve as a genetic model of PD. However, to date, there have been only contradictory reports on their behavioral deficits. One group reported that *ak* mice are less active during nighttime in an open field but as active as *wt* mice during daytime (van den Munckhof et al., 2003). Another group measured spontaneous ambulatory movement of *ak* mice in an open field during daytime (measured for 15 min between 10:00 A.M. and 3:00 P.M.) and observed great reduction of the locomotor activity compared with *wt* mice (Smidt et al., 2004). A third group described that general motor activity is even higher in *ak* mice than *wt* mice (Nunes et al., 2003). Our previous examination did not reveal any PD-like motor deficits in *ak* mice (Hwang et al., 2003). These conflicting reports, as well as our previous negative results, lead us to hypothesize that the potential nigrostriatal pathway-dependent motor deficits of *ak* mice may not be revealed by the measurement of gross motor activity but could be detected by measuring special tasks that are sensitive to defects of the nigrostriatal DA system. For example, it was shown previously in other studies that mice treated with mild doses of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) do not show motor deficits in the open field and the rotorod tests but display clear motor alterations in gait and the inverted grid test (Tillerson et al., 2002). Similarly, we have shown motor deficits in the challenging beam, cylinder, and pole tests in homozygous *parkin* knock-out mice and in mice overexpressing α-*synuclein*, two genetic models of PD exhibiting nigrostriatal dysfunction without nigrostriatal cell loss (Goldberg et al., 2003; Fleming et al., 2004). These nigrostriatal pathway-dependent tests have also provided sensitive measures of behavioral improvement by L-DOPA in the MPTP mouse model of PD (Tillerson et al., 2002). Indeed, our results described in this study demonstrate that *ak* mice display motor deficits that are efficiently reversed by L-DOPA in several nigrostriatal pathway-sensitive behavioral tests. A potential limitation of these studies is the blindness of *ak* mice resulting from Pitx3 deficiency. It has been shown that the Pitx3 gene is also expressed in the developing

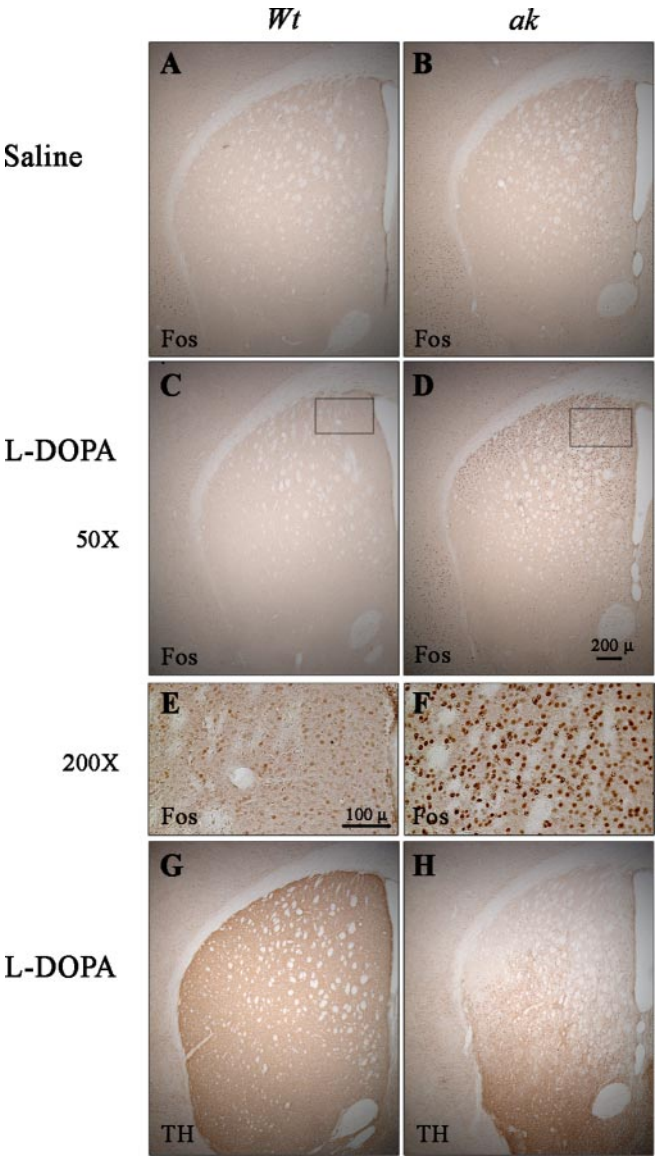

Figure 3. Photomicrographs showing c-*fos* (***A***–***F***) and TH (***G***, ***H***) immunoreactivities in the dorsomedial striatum of *wt* (***A***, ***C***, ***E***, ***G***) and *ak* mice (***B***, ***D***, ***F***, ***H***) after saline (***A***, ***B***) or L-DOPA/benserazide (***C***–***H***) administration. ***E***, ***F***, Higher magnifications (200×) of boxed areas indicated in ***C*** and ***D***, respectively.

eye of *wt* mice during the early embryonic stage and is critically involved in lens development (Semina et al., 1997). However, it is unlikely that the motor deficits of *ak* mice are artifacts of their blindness because (1) *ak* mice also show statistically significant behavioral deficits when compared with a blinded control group (*rd1* mice), and (2) L-DOPA reversed the motor deficits of *ak* mice without affecting the behaviors of both *wt* and *rd1* control mice.

In addition to the motor deficits observed on the nigrostriatal pathway-sensitive tests and the pharmacological reversal/enhancement of motor output by L-DOPA, *ak* mice displayed DA denervation supersensitivity, another feature prominent in neurochemical animal models of PD, as well as in PD patients. Furthermore, we observed that c-*fos* expression was greatly induced by L-DOPA (25 mg/kg, i.p.) in the dorsolateral striatum of *ak* mice in which dopaminergic denervation is most profound. This response is regarded as supersensitivity because the same dose of L-DOPA did not induce c-*fos* expression in the striatum of *wt* mice and the ventral region of *ak* mice, in which DA innervation is mostly preserved. Therefore, *ak* mice may provide valuable information on the functional and molecular changes in the striatum after dopamine depletion and subsequent L-DOPA treatment in PD. It will be of interest to see whether DA denervation supersensitivity is attributable to changes in the level of DA receptors and/or changes in signal transduction pathways. Of additional interest is the identification of the DA receptor subtypes involved in this process and the underlying signaling pathways and molecules. A comprehensive understanding of the striatal changes before and after L-DOPA administration may guide the development of novel therapeutic regimens to treat PD and L-DOPA-induced dyskinesia.

In summary, *ak* mice demonstrated motor deficits in nigrostriatal pathway-sensitive behavioral tests, were responsive to L-DOPA, and displayed evidence of striatal dopamine supersensitivity observed in animal models and PD patients. These data suggest that *ak* mice represent a novel genetic model that could be used to (1) characterize the functional changes of the striatum after DA depletion and subsequent L-DOPA treatment, (2) screen novel therapeutic compounds, and (3) evaluate the efficacy of restorative regimens developed for improved treatment of PD.

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