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Diapocynin prevents early Parkinson's disease symptoms in the Leucine-Rich Repeat Kinase 2 (LRRK2^{R1441G}) transgenic mouse

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Abstract

The most prominent mechanism proposed for death of dopaminergic neurons in Parkinson's disease (PD) is elevated generation of reactive oxygen/nitrogen species (ROS/RNS). Recent studies suggest that ROS produced during PD pathogenesis may contribute to cytotoxicity in cell culture models of PD. We hypothesized that inhibition of ROS production would prevent PD symptoms in the LRRK2^{R1441G} transgenic (tg) mouse model of PD. These mice overexpress a mutant form of leucine-rich repeat kinase 2 (LRRK2) and are reported to develop PD-like symptoms at approximately 10 months of age. Despite similar expression of the transgene, our colony did not recapitulate the same type of motor dysfunction originally reported. However, tests of motor coordination (pole test, Rotor-Rod) revealed a significant defect in LRRK2^{R1441G} mice by 16 months of age. LRRK2^{R1441G} tg mice, or wild type littermates, were given diapocynin (200 mg/kg, a proposed NADPH oxidase inhibitor) three times per week by oral gavage starting at 12 weeks of age. Decreased performance on the pole test and Rotor-Rod in the LRRK2^{R1441G} mice was prevented with diapocynin treatment. No loss in open field movement or rearing was found. As expected, tyrosine hydroxylase staining was similar in both the substantia nigra and striatum in all treatment groups. Together these data demonstrate that diapocynin is a viable agent for protection of neurobehavioral function.

Keywords

Neurodegeneration; Parkinson's disease; neurobehavioral analysis

Introduction

In the last decade, Parkinson's disease patients with genetic mutations predisposing them to the disease have been identified [19, 24]. This finding catalyzed the development of multiple

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transgenic animal models for studying PD progression [8]. One gene, leucine-rich repeat kinase 2 (LRRK2) has been the subject of recent research, as there have been several mutations identified that lead to PD [16, 21]. Of these, mutation of arginine to glycine at position 1441 has been extensively studied. In general, the R1441G mutation is not thought to affect LRRK2 kinase activity [14]; however, it is located in the GTPase domain, and may impact protein function in that way. Mice overexpressing human LRRK2^{R1441G} were recently described [18]. These mice were reported to have no observable phenotype at 3 months, but had gross motor deficiencies by 10–12 months. Motor deficits were reversible with L-dopa treatment, but occurred in the absence of changes in total dopaminergic cell number as assessed by tyrosine hydroxylase (TH) staining.

In this study, we employed the LRRK2^{R1441G} tg mice as a Parkinson's disease model to study the impact of neuroinflammation on the progression of PD-like symptoms. Neuroinflammation is a well-known contributor to PD pathogenesis. Microglia isolated from brains of adult LRRK2^{R1441G} mice have higher pro-inflammatory cytokine secretion [12], while LRRK2 knockdown impairs transcription of pro-inflammatory genes in response to LPS [17]. We thus hypothesized that the LRRK2^{R1441G} mice would have chronic neuroinflammation similar to the human pathology.

Despite solid data supporting a neuroinflammatory mechanism in PD, therapeutic strategies to limit inflammation have yielded mixed results [10]. Indeed, prior non-steroidal anti-inflammatory drug (NSAID) use was reported to increase PD risk [6]. In the present study, LRRK2^{R1441G} or wild type littermates were treated with the anti-inflammatory compound diapocynin. Apocynin and its derivatives are reported to prevent translocation of p47phox from the cytosol to the membrane where NADPH oxidase (NOX2) is located, thus preventing assembly of the active enzyme [27]. NOX2 is the particular isoform which is known to be expressed in microglia, and produces superoxide radical anion ($O_2^{\cdot -}$) when activated [4]. Using a targeted strategy against NOX2 may thus be more efficacious in treating PD-specific neurobehavioral dysfunction.

Materials and Methods

Mice

A colony of LRRK2^{R1441G} (FVB/N-Tg(LRRK2*R1441G)135Cjli/J) and wild type litter mates was established from commercially available breeders [18]. Male mice were used for all experiments. Mice were housed on a 12 h light/dark cycle with ad libitum access to food and water. All experiments were performed in accordance with the Guide for Care and use of Laboratory Animals and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Genotype was determined using tail biopsy DNA amplified using the following primers: forward 5′-TGA TTC TCG TTG GCA CAC AT-3′ reverse 5′-GCC AAA GCA TCA GAT TCC TC-3′ (Figure 1A). Genotype was confirmed after 16 months using Sanger sequencing of a 261bp PCR product amplified from genomic DNA using the following primers: forward 5′-TGC CAC CTA AAA GGG TGA AG-3′ reverse 5′-CTT TGC GTT GCT TCT CAT CA-3′ (Figure 1B). Sequence analysis was performed using Sequencher 4.10.1 (Gene Codes Corporation; Ann Arbor, MI). Relative (LRRK2*R1441G)135Cjli transgene copy number was analyzed alongside samples from The Jackson Laboratory colony. Quantitative PCR was performed on genomic DNA isolated from tails of hemizygous, homozygous, and wild type mice using an ABI 7500 (Life Technologies; Grand Island, NY), (Figure 1C). Beginning at 12 weeks of age, and continuing for the duration of the experiment, mice were given 200 mg/kg diapocynin or saline, 3x/wk, via oral gavage. A similar treatment schedule and dosage was reported to yield detectable levels of diapocynin in brains of mice with compound identity confirmed by mass spectrometry [11, 29].

Diapocynin synthesis

Diapocynin (5,5′-dehydrodiacetovanillone, Figure 3A) was synthesized as previously described [9, 30]. Briefly, to a solution of 1.0 g of apocynin (6 mmol) in 200 ml of hot water, 75 mg of ferrous sulfate heptahydrate (0.3 mmol) and 714 mg (3.0 mmol) sodium persulfate were added and stirred for 30 min. The precipitated product was filtered and washed with cold water. The product was dissolved in 4 N sodium hydroxide (50 ml), filtered, and the brown solution was acidified with 4 N hydrochloric acid to pH 3.0. The precipitate was filtered, and the process was repeated twice more to yield 0.6 g (60%) of a white solid. Purity was confirmed by HPLC analysis on a Kinetex C18 column (Phenomenex, 100 mm × 4.6 mm, 2.6 μm) equilibrated with 0.1% TFA in water containing 10% acetonitrile. Diapocynin was eluted using a 10%–100% acetonitrile gradient in the mobile phase over 7 min, followed by 100% acetonitrile containing 0.1% TFA for an additional 2.5 min. Flow rate was 1.5 mL/min. Diapocynin eluted at 3.7 min (Figure 3B). Negative ion mode mass spectrometry (MS) confirmed a single product with an m/z of 329.0 ($M-H^+$), as expected (Figure 3C). This preparation of diapocynin inhibited superoxide production by retinoic acid-differentiated HL-60 cells stimulated with 1 μM phorbol-12-myristate-13-acetate (PMA) with an apparent IC50 of ca. 0.3 mM, as measured using HPLC-based monitoring of the conversion of hydroethidine into 2-hydroxyethidium [31].

Open field measurements

Ambulatory function was monitored using a 40 × 40 cm open field photobeam system (San Diego Instruments; San Diego, CA). Each mouse was placed in the open field apparatus and tracked for 20 min. Beam breaks were counted for total movement measurements. A second level of photobeams was used to concomitantly track rearing. Time-resolved data were used to determine the amount of time that each mouse paused at a particular coordinate. A heatmap of location within the open field apparatus was generated using crosstab analysis of each dataset in Microsoft Access 2010. Data were normalized to percentage of time spent at each coordinate for each mouse, and then averaged.

Motor coordination behavioral analysis

Coordination was assessed using the pole test and Rotor-Rod. Mice were placed face up on a 1 cm diameter ringstand pole, and must turn and descend to the base. On the Rotor-Rod (San Diego Instruments; San Diego, CA), mice were placed on a horizontal bar (3.175 cm diameter) which rotates. The speed of rotation was increased from 0 to 8 rpm in 10 sec, from 8 to 10 rpm in the next 5 sec, maintained at 10 rpm for 5 sec, and then increased from 10 to 11 rpm over the next 5 sec.

Gait analysis

Using different colored non-toxic paints, front and back paws of each mouse were painted, and the mice were allowed to run through a 7.5 × 61 cm channel. Footfalls were assigned, and the interstep distance was calculated. Stride count and interstep distance for each paw was averaged to yield the respective values for each mouse.

Immunohistochemistry

Immunohistochemical staining for TH and the microglial marker Iba-1 (ionized calcium binding adaptor molecule 1) were performed essentially as described [11]. Briefly, mice were deeply anesthetized, and perfused with 4% paraformaldehyde. Brains were removed, fixed in 4% paraformaldehyde for 24 h, and then post-fixed in 30% sucrose for 48 h. Fixed brains were cut into 30 μm sections with a cryostat, mounted, and stained with anti-tyrosine hydroxylase (Calbiochem; Billerica, MA) or Iba-1 (Abcam; Cambridge, MA) antibodies. DAB-stained sections were imaged at multiple magnifications.

Statistical analysis

Motor function data were acquired using a San Diego Instruments Photobeam Activity System (PAS) Open Field apparatus or Rotor-Rod (San Diego, CA). Data were collected and analyzed using Microsoft Excel 2010 or Origin 8.5 (OriginLab, Northampton, MA). One-way ANOVA was used for significance tests unless otherwise specified in the legend. $p \leq 0.05$ was considered statistically significant.

Results

LRRK2^{R1441G} mice do not have deficient gross motor ambulatory function

Total ambulatory function was measured in an open field apparatus as described when mice were 16 months of age. During the 20 min tracking period, LRRK2^{R1441G} mice covered the same distance as wild type mice (Figure 2A). Beam coordinate pairs were used to determine the relative time each mouse spent at a point in the apparatus. As expected, wild type mice spent significant time exploring the corners of the open field apparatus. This behavior was not notably different in LRRK2^{R1441G} tg mice (Figure 2B). LRRK2^{R1441G} tg mice also did not differ from wild type in the number of dwells (defined as a stationary period ≥ 1 sec) or the duration of dwells (Figure 2C and D). Gait analysis was performed to ascertain differences in step behavior. LRRK2^{R1441G} tg and wild type mice had similar stride number and length (Figure 2E and F). All mice covered the same distance during this measurement (Figure 2G).

LRRK2^{R1441G} tg mice have deficient motor coordination which is reversed by diapocynin

After 16 months of treatment with diapocynin, mice were sacrificed and brain sections were stained for TH to indicate presence or absence of dopaminergic neurons. TH staining in the substantia nigra (Figure 3D–G) did not differ between wild type and LRRK2^{R1441G} tg mice as expected based on previous findings [18]. Long term treatment with diapocynin did not alter TH expression in either genotype. Diapocynin did not alter total open field movement in either genotype as expected based on the findings discussed in the above section (Figure 3H). Rearing in the open field apparatus was also measured, and showed no difference with diapocynin treatment in either genotype (Figure 3I).

Early symptoms of PD include decreases in motor coordination. Because we saw no changes in gross motor function, we next examined changes in two indices of motor coordination: the pole test and the Rotor-Rod. In the pole test (Video Still and Supplemental Video 1), LRRK2^{R1441G} tg mice performed markedly poorer than wild type mice. This is evident in both an increased time to turn and time to descend the pole. LRRK2^{R1441G} tg mice were also noted to have slower engagement of their tail on the pole, and frequently dragged their hind paws while descending. With diapocynin treatment, LRRK2^{R1441G} tg mice showed improvement on the pole test, matching the speed of wild type mice.

Mice were also assessed for their ability to balance on a moving Rotor-Rod apparatus. As shown in Figure 3J, LRRK2^{R1441G} tg mice had a markedly decreased latency to fall, indicating an inability to maintain balance on the rod. While long-term diapocynin treatment did not impact the function of wild type animals, the performance of LRRK2^{R1441G} mice returned to normal with this treatment.

Discussion

LRRK2^{R1441G} BAC tg mice have been reported to have dramatic deficiencies in gross motor function which are L-dopa responsive [18]. Within our colony, LRRK2^{R1441G} tg mice never exhibited a loss of gross motor function, but did exhibit other PD-like symptoms

suggestive of early stages of the disease. The lack of a motor function phenotype is consistent with more recent findings [2]. In this study, we found that mice at 16 months of age had normal open field behavior similar to wild type FVB mice, but exhibited loss of behavior associated with coordinated motor movement (e.g., balance). One possible explanation for this difference is that the original mice were bred onto an FVB/N strain from Taconic [The Jackson Laboratory, 1], whereas the commercially available strain, from which our colony is bred, are on an FVB/N sourced from The Jackson Laboratory. It is unclear what differences exist between these strains, and what effect this could have on motor function. Interestingly, $LRRK2^{R1441G}$ tg mice did not exhibit preference for the wall vs. middle of the enclosure, which would indicate a reliance on the wall for balance (Figure 2B). However, it is likely that this experiment may not be as sensitive as the Rotor-Rod and pole tests in delineating a balance deficit.

Activation of microglia is documented in PD patients and animal models [20, 25], though whether this activation is causative for disease progression remains controversial [13]. Concomitant with microglial activation, NADPH oxidase activity is also increased in PD brains [28]. NOX2, the specific isoform of NADPH oxidase found in microglia, may then be a major source of reactive oxygen species (ROS) during PD progression along with deregulated mitochondria [3]. Thus, preventing this ROS by inhibition of NOX2 is attractive therapeutically. Recent work from our lab and others has demonstrated therapeutic efficacy of the putative NADPH oxidase inhibitors apocynin and diapocynin against the progression of neurodegeneration in various models [11, 26]. Intriguingly, the potential for NOX1 involvement in PD was recently described, and apocynin effectively prevented neuronal death, though the mechanism by which this protection occurred remains unclear [5]. Notably, apocynin did not improve behavioral changes in a transgenic mouse model of Alzheimer's disease, despite protection from oxidative protein damage [7].

In this study, we examined microglial activation by quantifying Iba-1 staining in the substantia nigra. Though gross motor function was not significantly impacted in $LRRK2^{R1441G}$ tg mice, we expected an increase in microglial activation due to the reversal of coordinated movement defects by diapocynin. Based on the absence of Iba-1 staining in $LRRK2^{R1441G}$ tg mice (data not shown), microglial activation in the substantia nigra does not appear to be a contributing factor in the motor deficits on Rotor-Rod and pole tests. Future studies are required to determine if there is inflammation occurring in other basal ganglia areas of the brain that may impact motor coordination. Alternatively, these mice may represent an early stage of the disease where ROS from activated microglia are not yet produced, or are produced at undetectable levels.

Despite the protective effect of diapocynin on early PD-like symptoms in the $LRRK2^{R1441G}$ mice, the mode of action of diapocynin in these mice is unknown. The high concentration of diapocynin required to inhibit hydroethidine oxidation to 2-hydroxyethidium (as a specific marker of $O_2^{\cdot -}$ production) suggests that direct inhibition of NOX2 is unlikely. Recently, a potential antioxidant capacity was ascribed to apocynin due to peroxidase-dependent hydrogen peroxide scavenging [15, 22]. Diapocynin is expected to participate in a similar reaction. In fact, it inhibits Amplex Red-derived fluorescence in the presence of H_2O_2 and peroxidase, but does not interfere with other non-peroxidase dependent detection methods (e.g. hydroethidine oxidation; data not shown). Furthermore, apocynin was recently shown to be a relatively poor direct radical scavenger [22]. Together, these data suggest that diapocynin is not working in this model directly as an antioxidant.

The future of PD patient care includes coupling an understanding of non-motor symptoms to new biomarkers to enable early disease detection [23]. Early detection of PD symptoms will allow for better patient care, specifically with regards to influencing the extended course of

the disease. In this respect, future studies utilizing these mice will examine the early non-motor symptoms of PD including hyposmia. Based on our findings, LRRK2^{R1441G} tg mice may offer better potential for the study of early PD symptoms than the gross motor defects expected later in PD progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

LRRK2	leucine-rich repeat kinase 2
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NOX2	NADPH oxidase isoform 2
NSAID	non-steroidal anti-inflammatory drug
PD	Parkinson’s disease
PMA	phorbol-12-myristate-13-acetate
ROS	reactive oxygen species
RNS	reactive nitrogen species
TH	tyrosine hydroxylase

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Highlights

- LRRK2^{R1441G} mice lose coordinated movement without gross motor deficits.
- Diapocynin prevents deficits in motor coordination in LRRK2^{R1441G} mice.
- LRRK2^{R1441G} mice may be useful for modeling non-motor PD symptoms.

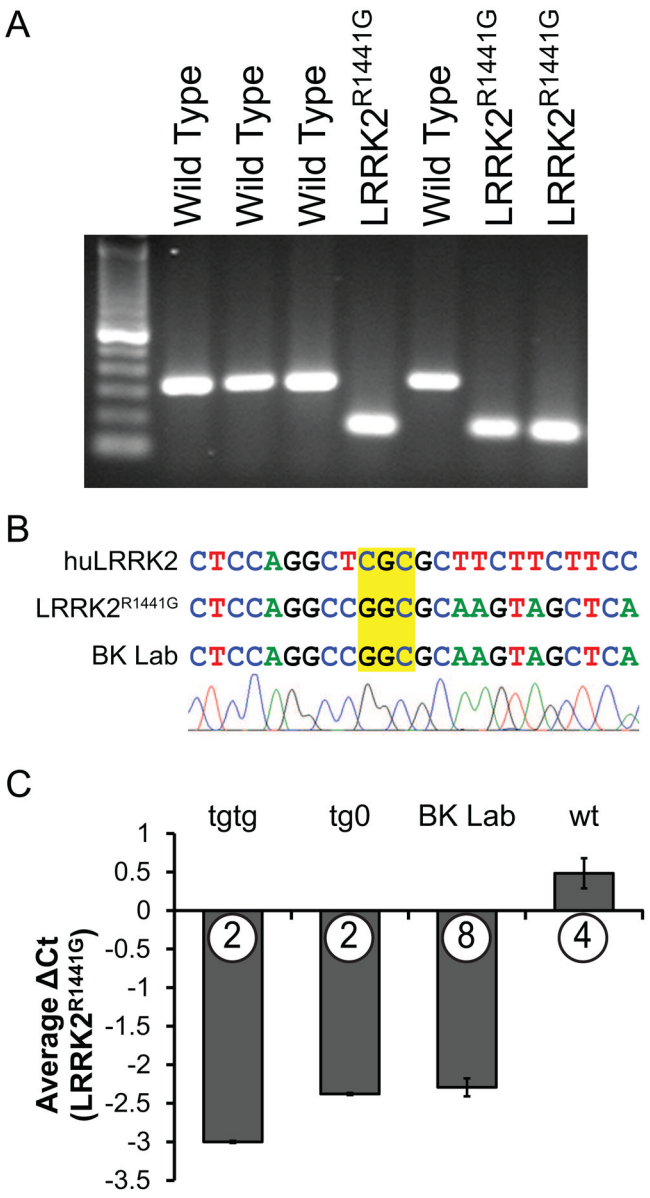

Figure 1. LRRK2^{R1441G} tg mice genotyping

Wild type FVB and LRRK2^{R1441G} tg genotype was confirmed using PCR (A) and Sanger sequencing (B). Transgene copy number was compared to animals with known genotype to confirm hemizygous expression of LRRK2^{R1441G} (C). Samples from each mouse were run in triplicate. Data shown are means ± s.d. n≥2 mice per group as indicated.

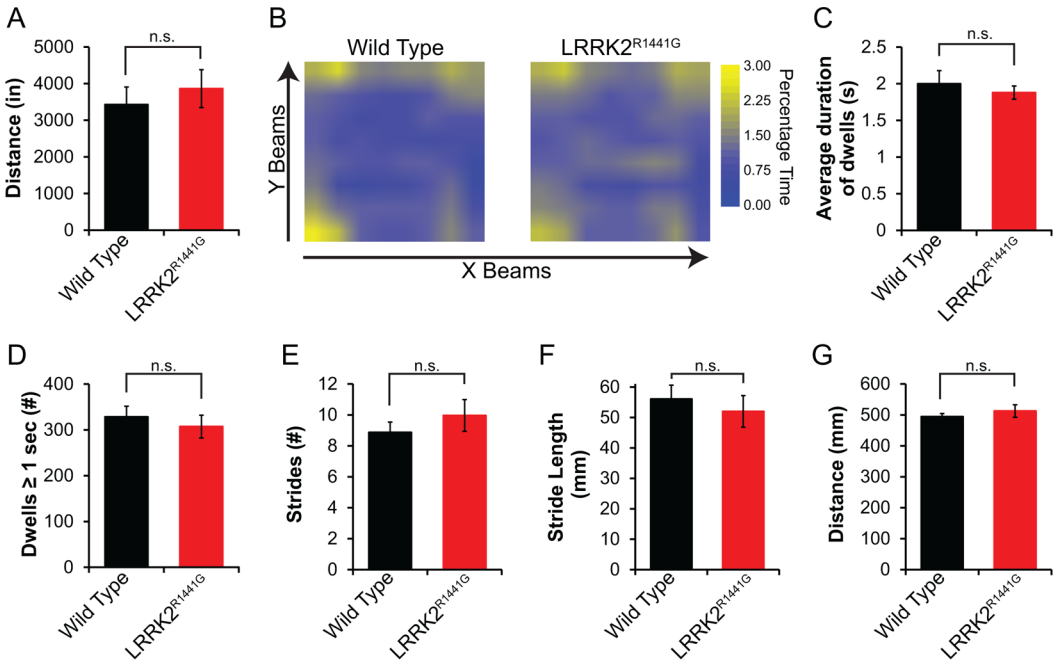

Figure 2. Open field movement is not decreased in LRRK2^{R1441G} tg mice

From open field photobeam tracking experiments, the total distance travelled was calculated (A). Location in the open field apparatus was tracked as percentage time occupying each coordinate for each animal. Averages were then plotted as a heat map (B). The average duration of each dwell (C) and total number of dwells ≥ 1 s (D) were calculated. Gait analysis was used to monitor stride count (E) and length (F) over a constant distance (G). Data shown are means ± s.d. Significance was assessed by two-tailed t-test, and p values are shown for each measurement. n≥4 mice per group.

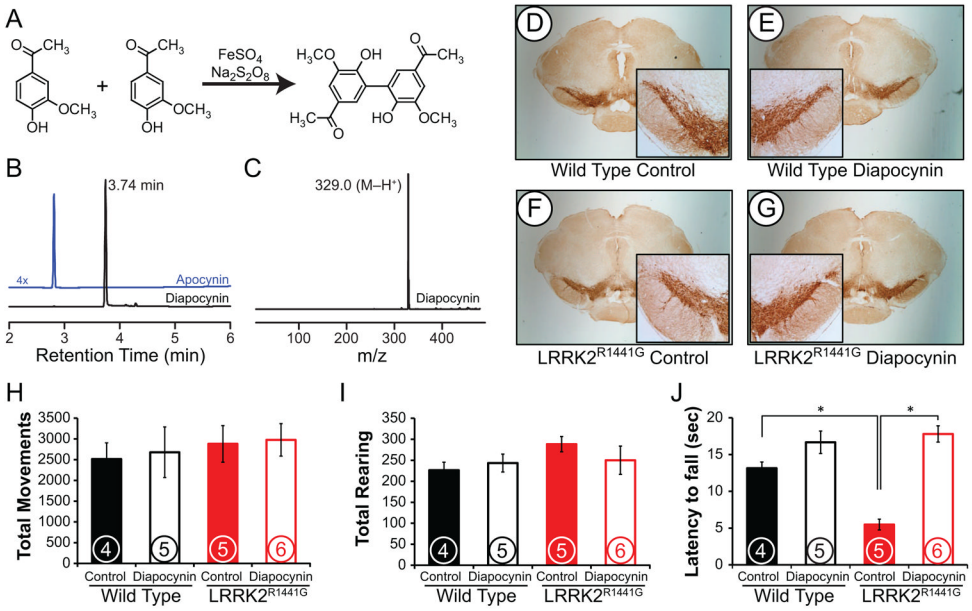

Figure 3. Diapocynin corrects loss of motor coordination without affecting gross motor function

Diapocynin was synthesized through the reaction of two apocynin monomers in the presence of ferrous sulfate and sodium persulfate (A). Purity of the isolated compound was confirmed by HPLC (B) and MS analysis (C). An apocynin standard was run in parallel to confirm purity of the diapocynin solution. At 16 mo, brains were formaldehyde fixed, sliced, and slices containing the nigral region were stained for TH (D-G). Images shown are representative with 4x magnification in insets. Total movement (H) and rearing (I) were assessed over 20 min in an open field using a photobeam system. Latency to fall from a Rotor-Rod apparatus was also measured (J). Data are the means ± s.d. *, $p \leq 0.05$ as determined by one-way ANOVA with Bonferroni post-hoc. Encircled numbers represent the number of animals in each group.