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Title: Chronic and acute LRRK2 silencing has no long-term behavioral effects, whereas wild-type and mutant LRRK2 overexpression induce motor and cognitive deficits and altered regulation of dopamine release.

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Corresponding Author: Dr. Austen Milnerwood,

Corresponding Author's Institution:

First Author: Mattia Volta

Order of Authors: Mattia Volta; Austen Milnerwood; Stefano Cataldi; Dayne Beccano-Kelly; Lise Munsie; Igor Tatarnikov; Patrick Chou; Sabrina Bergeron; Emma Mitchell; Roscoe Lim; Alejandro Lloret; Holly Kordasiewicz; C. Frank Bennett; Carmela Paradiso; Michele Morari; Matthew Farrer

Abstract: INTRODUCTION: Germline silencing of the PD-related protein LRRK2 does not alter glutamate or dopamine release in adult mice, but some exploratory abnormalities have been reported with ageing. Contrastingly, high levels of human LRRK2 cause locomotor alterations and cognitive deficits accompanied by reduced striatal dopamine levels, with the latter also observed in G2019S mutant mice. Comparative behavioral testing of LRRK2 KO, overexpressor and mutant overexpressor mice has not previously been reported.
METHODS: Parallel, comparative behavioral characterization was performed assessing motor and cognitive abilities. Striatal ASO injections were conducted to investigate the effects of acute LRRK2 silencing on behavior and dopamine fiber density. Striatal synaptosomes were prepared from hG2019S mice to assess vesicular release of dopamine and inhibition by D2 stimulation.
RESULTS: Genetic ablation of LRRK2 has no long-term consequences on motor or cognitive function. Consistently, no effects on behavior or dopaminergic fiber density were observed following acute striatal silencing. Conversely, 12-month OE mice show persistent locomotor deficits and worsening of cognitive abilities; whereas, hG2019S mice display early hyperactivity and effective learning and memory that progresses to decreased motor and cognitive performance at older ages. The G2019S mutation does not affect vesicular dopamine release, but decreases sensitivity to D2-mediated inhibition of release.
CONCLUSION: LRRK2 silencing is well tolerated in mouse, arguing PD does not result from LRRK2 loss of function. High levels of WT and G2019S LRRK2 produce similar but temporally distinct phenotypes, potentially modeling different stages of disease progression. The data implicate gain of LRRK2 function in the pathogenesis of PD.

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***Cover Letter**

April, 2015

To whom it may concern;

TBD

Matthew J. Farrer PhD
Professor, Canada Excellence Research Chair,
Don Rix BC Leadership Chair in Genetic Medicine

Centre for Applied Neurogenetics
Djavad Mowafaghian Centre for Brain Health
2215 Wesbrook Mall, Vancouver, BC, V6T 1Z3
T. 604-827-1082 F. 604-822-0361 E. about@can.ubc.ca W. www.can.ubc.ca

Highlights

- Genetic deletion of LRRK2 is benign *in vivo*
- Acute knockdown of striatal LRRK2 does not affect behavior or dopamine terminals.
- Overexpression of WT-LRRK2 causes lasting behavioral deficits
- Overexpression of G2019S-LRRK2 leads to a progressive motor and cognitive phenotype
- G2019S-LRRK2 overexpression reduces sensitivity to D2-mediated inhibition of dopamine release

Chronic and acute LRRK2 silencing has no long-term behavioral effects, whereas wild-type and mutant LRRK2 overexpression induce motor and cognitive deficits and altered regulation of dopamine release.

Mattia Volta[a], Stefano Cataldi[a], Dayne Beccano-Kelly[a], Lise Munsie[a], Igor Tatarnikov[a], Patrick Chou[a], Sabrina Bergeron[a], Emma Mitchell[a], Roscoe Lim[a], Alejandro Lloret[c], Holly Kordasiewicz[c], C. Frank Bennett[c], Carmela Paradiso[d], Michele Morari[d], Matthew J Farrer[a*] and Austen J. Milnerwood[a,b*]

[a]*Centre for Applied Neurogenetics,*
[b]*Division of Neurology, University of British Columbia, 2215 Wesbrook Mall, Vancouver, V6T 1Z3, BC, Canada*
[c]*Isis Pharmaceuticals Inc, 2855 Gazelle Court, Carlsbad, CA, 92010, USA*
[d]*Dept. of Medical Sciences, Section of Pharmacology, University of Ferrara, Via Fossato di Mortara 17-19, 44121, Ferrara, Italy*

To be submitted to: *Parkinsonism and Related Disorders*

[*]To whom correspondence should be addressed: amilnerwood@gmail.com & mfarrer@can.ubc.ca

Abstract

INTRODUCTION: Germline silencing of the PD-related protein LRRK2 does not alter glutamate or dopamine release in adult mice, but some exploratory abnormalities have been reported with ageing. Contrastingly, high levels of human LRRK2 cause locomotor alterations and cognitive deficits accompanied by reduced striatal dopamine levels, with the latter also observed in G2019S mutant mice. Comparative behavioral testing of LRRK2 KO, overexpressor and mutant overexpressor mice has not previously been reported.

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Highlights

- Genetic deletion of LRRK2 is benign *in vivo*
- Acute knockdown of striatal LRRK2 does not affect behavior or dopamine terminals.
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Introduction

Mutations in leucine-rich repeat kinase 2 (*lrrk2*) are linked to clinically typical, familial Parkinson's disease (PD) [1]. The G2019S substitution alone accounts for up to 30% of PD in some populations [2]. LRRK2 is a large, multi-domain protein with GTPase and kinase activities [3]. Physiologically LRRK2 may regulate vesicle trafficking, endosome maturation, cytoskeletal dynamics [4] and synaptic transmission [5-9].

Full recapitulation of PD cardinal features in LRRK2 models has proved challenging, although many exhibit disease-relevant phenotypes [10]. A "loss of function" effect of LRRK2 mutations causing PD seems unlikely, as LRRK2 knockout (KO) mice are grossly normal [7, 11, 12] and despite subtle ageing-associated thigmotaxis [13], there are no alterations to nigrostriatal [13] or corticostriatal neurotransmission [7]. Bacterial artificial chromosome (BAC)-transgenic mice have been engineered to express normal and mutant LRRK2 under the control of human regulatory elements [14]. We recently reported that young adult (6 months) BAC human wild-type LRRK2 overexpressing (OE) mice exhibit behavioral hypoactivity, cognitive impairment, reduced dopamine (DA) tone and altered D2 signaling [7]. While deficits in DA neurotransmission have also been reported in BAC human G2019S-LRRK2 overexpressing (hG2019S) mice [15, 16] only subtle motor differences were observed [16].

Here we compare KO, OE and hG2019S mice up to 12 months of age. Genetic deletion of LRRK2 did not cause behavioral effect. Furthermore acute silencing in adults by antisense oligonucleotide (ASO) treatment produced no significant effects on motor activity, cognition or striatal DA terminals. Conversely, behavioral deficits observed in young OE mice remained at 12 months, while cognition progressively declined. In hG2019S mice, early hyperactivity with no cognitive deficits transitioned to loss of hyperactivity and impaired cognition with age. Although vesicular release of DA from hG2019S striatal synaptosomes was similar to controls, D2 autoreceptor sensitivity was reduced. Dissimilarity between KO and hG2019S mice indicates that the mutation produces a behavioral "gain of function".

This comparative study demonstrates that progressive motor and cognitive decline occurs in hG2019S mice, which is associated with alterations to the nigrostriatal dopamine system. In this light, hG2019S mutant mice provide a useful model system in which to investigate etiology and mechanism-based treatment for familial, and potentially idiopathic, PD.

Materials and methods

Transgenic mice and behavior

Male C57BL/6J non-transgenic (WT), LRRK2 KO [6, 13], OE [7] and hG2019S [16] mice were maintained according to Canadian Council on Animal Care regulations. Behavioral experiments were as in [7], briefly, as follows:

Open Field: mice explored an arena (48cm x 48cm) for 15min. Phenotracker software (TSE Systems) was used to analyze videos post-hoc. Data is presented for initial 5 (when contextual habituation is minimal) and the entire 15-min experimental time. Animals that underwent surgery were tested only for 5min.

Cylinder test: mice were placed in a 1l beaker and videoed for 5min. The number of rearings and forelimb wall contacts were scored.

Novel object location: mice explored the open field containing two objects. After 24h mice were reintroduced (5min) and one of the objects had been moved to a novel (previously empty) location.

Novel object recognition: as above, with one object substituted for a novel object.

Drag test: mice were lifted by the tail, with forepaws bearing weight, and slowly dragged backwards (1m, 20cm/sec). The number of adjusting forepaw steps was scored.

Stereotactic surgery

Phosphate-buffered saline (PBS, 4µl) or 50µg ASO (4µl, Isis Pharmaceuticals, Carlsbad, USA) was stereotactically injected (1µl/min) into the right striatum (Bregma: AP=+0.2, ML=+2.0; VD=-3.2 below dura [17]) under isoflurane in KO and WT littermates. Animals were given lidocaine (30uL, s.q.), meloxicam (1mg/Kg, s.q.) and warm 0.9% saline (s.q.) for pre- and post-operative care. Injection needles were left in place for >5min, then removed prior to suture. Mice recovered for 3-4 weeks before behavioral testing.

Tissue preparation and western blotting

Mice were killed by rapid cervical dislocation and decapitation. Striata were rapidly (<5min) microdissected in buffer (ACSF, in mM: 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 5 $MgCl_2$, 2 $CaCl_2$, 10 glucose, pH 7.3–7.4) and flash frozen in liquid nitrogen [7]. Tissue was homogenized (200µl buffer, 1% NP-40, 20mM HEPES, 125mM NaCl, 50mM NaF and protease inhibitor

cocktail, Roche), kept on ice (1h) and supernatant collected following centrifugation (4,000g; 10mins). Protein (80ug) was resolved by SDS-PAGE [7]. Antibodies: rabbit anti-LRRK2 (1:1000, Abcam, ab133476), mouse anti-GAPDH (1:1000, Thermo Scientific, MA5-15738).

Immunohistochemistry and image analysis

Mice were deeply anesthetized (240mg/Kg sodium pentobarbital i.p.), then perfused with 4% paraformaldehyde (PFA), brains removed, submerged in 4% PFA (24h, 4°C) and stored (30% sucrose-0.8%NaN_3 PBS, 24h, 4°C). Cryostat sections (30µm) were collected, dehydrated and quenched (0.3% H_20_2 in MeOH, 30min RT, then 10mM sodium-citrate +0.05% Tween, 30min 37°C). Sections were rinsed (0.1% PBST 3× 10min), blocked (3% milk in 0.03% PBST 30min, RT then in 10%NGS in 0.03% PBST, 30min, RT), then incubated with primary antibodies (1:500 anti-LRRK2 Rabbit – Epitomics 3414-1; 1:1000 TH Rabbit –Abcam Ab112 in 5%NGS +0.01% NaN_3 in PBST overnight at 4°C). Sections were washed (PBST 3×10min), secondary antibodies applied (1:1000 α –Rabbit Biotin, Vector labs PK-6101; 1:1000 α –Mouse Biotin, Vector labs PK-6200, 120 min, RT) then washed (PBST 3×10min). Sections were exposed to Avidin-Biotin (1:500 in PBS. Vector labs PK-6101, 90min, RT) then washed (PBST, 3×10min). 3,3"-Diaminobenzidine Tetrahydrochloride (DAB, FisherSci 34065) staining was performed (~2.5min) then sections were slide mounted, dried overnight, cleared (Xylene) and coverslipped (Permount, Fisher Sci). Images were acquired on a Nikon E800 microscope and the % area of DAB immunosignal was measured with ImageJ (NIH, Bethesda, USA).

Synaptosome preparation and [^{3}H]-dopamine analysis

Synaptosomes were prepared from hG2019S and WT mice, as in [18]. Briefly, striatum was homogenized (ice-cold 0.32M sucrose, pH 7.4), centrifuged (10min, 2500g_{max} 4°C), supernatant removed and centrifuged (20min, 9500g_{max} 4°C). Synaptosome pellets were resuspended in Krebs' buffer, then incubated with 50nM [^{3}H]-dopamine ([^{3}H]-DA, 25min), then 1ml aliquots (~0.35mg protein) were injected into nylon filters (36.5°C) and superfused with Krebs' (0.4ml/min). Sampling (every 3min) began after equilibration (20min). Radioactivity was measured by liquid scintillation spectrophotometry. Data were presented as fractional release (tritium efflux/remaining tritium content) and stimulated (KCl, 10-20 mM) fractional release (stimulated tritium efflux/[tritium efflux/remaining tritium content]). The D2 agonist

pramipexole (PPX, 0.1-1nM) was applied from 6min before 10mM KCl pulses (120s) to the end of experiment.

Statistical Analysis

Analyses were performed with Prism6 (GraphPad, Inc.); direct comparisons by Student's t-test (two-tailed, herein t-test) or multiple comparisons by one-way ANOVA or two-way RM-ANOVA and post-tests as detailed.

Results

Constitutive or acute LRRK2 silencing produces no detectable behavioral phenotype

We recently reported that young adult male KO mice are behaviorally normal [7]. Previously, we detected subtle alterations to exploratory behavior in a cohort of aged mixed-sex KO mice from a separate colony [13]. Here we sought to clarify whether exploratory deficits occur in males from our colony at older age. In the open field (Fig.1), there were no differences between 12-month old KO and WT littermates in distance traveled (first 5min or total 15min, Fig.1.A.i&ii). No anxiety-like phenotype was detected (Fig.1.A.iii). In the cylinder test KO mice reared as frequently as WT (Fig.1.B) and similar performance was observed in the novel object recognition test (Fig.1.C). Genetic ablation of LRRK2 had no impact on motor function or long-term memory at this age.

The striatonigral system is not affected by constitutive deletion of LRRK2 [13], but kidney and lung phenotypes have been reported [12, 13]. To investigate acute silencing in the CNS we performed ASO injections in 3-6 month mice (Fig.2). Unilateral injections of PBS reduced rearing the cylinder test by ~60% at 3 weeks post-surgery, but did not alter limb preference or stepping activity in cylinder and drag tests (Fig.2.B.i-iii). There were no significant effects of ASO treatment in either genotype (Fig.2.B.i-iii).

To ensure the lack of behavioral effect was not due to ineffective silencing, LRRK2 levels were quantified; in the ASO injected hemisphere of WT mice LRRK2 DAB-immunopositive area was reduced by >90% (Fig.2.C.i&ii). Consistently there was a ~90% reduction in LRRK2 protein by Western blot (Fig.2.D.i&ii).

KO mice injected with ASO were also similar to their WT littermates and PBS-injected animals in all behavioral tests, indicating no off-target effects. We assessed the density of TH-

positive fibers in ASO-injected mice and saw no difference with respect to the non-injected hemisphere (Fig.2.E.i&ii). We also treated a separate cohort of WT mice with PBS or ASO and subjected them to open field and novel object recognition tests. LRRK2 silencing did not affect locomotion or induce thigmotaxis (Fig.2.F.i&ii) and long-term memory was preserved (Fig.2.G).

The data show that KO mice are behaviorally comparable to WT mice, up to 12 months of age. Moreover acute silencing of LRRK2 in adults had no detectable consequences for motor and cognitive behavior, or the integrity of the nigrostriatal DA system.

Related, temporally distinct phenotypes in OE and hG2019S mice

High levels of hWT-LRRK2 cause motor and cognitive deficits [7] and lower striatal DA tone [16] in young adult mice. We sought to extend our behavioral investigation of OE mice to 12 months. Hypolocomotion in the open field (first 5 min; Fig.3.A.i&Fig.S1.A.i) and reduced exploratory rearing in the cylinder test (Fig.3.B.i) were observed, consistent with our results in younger animals [7]. Motor defects still appeared to be independent of anxiety, since no differences in central area exploration were observed (Fig.S1.A.ii). The impairment in long-term recognition memory observed at 3-6 months was also present at 12 months (Fig. 3.C.i) but was additionally accompanied by a loss of long-term location memory (Fig.S1.B) [7]. The data demonstrate that motor deficits are maintained and that cognitive decline is progressive in OE mice.

The behavioral consequences of mutant LRRK2 overexpression were also investigated. At 3-6 months, hG2019S mice were hyperactive, relative to WT littermates. There was a strong trend to greater distance traveled in the first 5 minutes of open field (Fig.3.A.ii) and a significant increase in the total distance traveled over the complete 15-min session (Fig.S2.A.i). Rearing in the cylinder test was also significantly elevated (Fig.3.B.ii). Increased ambulation was independent of anxiety, as estimated by center area avoidance (Fig.S2.A.iii). In 3-month old hG2019S learning and memory were intact, as reflected by equivalent performance to WT littermates in the novel object recognition (Fig.3.C.ii) and novel object location tests (Fig.S2.C). By 12 months, early hyperactivity had declined, with no differences observed in distance traveled, time spent in the central area or rearing (Fig.3.A.iii&B.iii, Fig.S2.B.i&ii). However at this age, a specific cognitive deficit appeared, as reflected by impaired long-term recognition memory (Fig.3.C.iii), while spatial memory remained intact (Fig S2.D).

The results indicate that hG2019S overexpression leads to an age-dependent, progressive phenotype that is similar but temporally distinct from the phenotype of OE mice. Early increases in motor activity and normal cognitive performance declined by 12 months of age.

D2 receptor autoregulation of DA release from striatal synaptosomes is diminished in hG2019S mice

Overexpression of G2019S-LRRK2 impairs the nigrostriatal DA system [15, 16]. We recently reported that OE mice display reduced striatal DA tone [7, 16], increased action of presynaptic D2 receptors and insensitivity to the hypoambulatory effect of the D2 agonist PPX [7]. In order to assay vesicular DA release and the effects of PPX, we prepared striatal synaptosomes from 6-month hG2019S and WT mice. Synaptosomes were loaded with [^{3}H]-DA, which is taken up into DAergic (DAT+ve) terminals. Spontaneous (Fig.4.A.i) and evoked (depolarization-induced by pulses of 10-20mM KCl, Fig.4.A.ii) efflux of [^{3}H]-DA was measured but there were no differences between genotypes. The data indicate that DA availability and depolarization-induced release is unaffected by overexpression of mutant LRRK2. To assess the function of D2 autoreceptors we pre-treated synaptosomes with PPX (0.1-1nM) prior to KCl pulses (10mM; Fig.4A.iii). At the lowest concentration (0.1nM), PPX significantly inhibited K^+-evoked [^{3}H]-DA release in WT, but had no effect in hG2019S DA release. Pretreatment with a higher concentration of PPX (1nM) significantly inhibited K^+-evoked release of [^{3}H]-DA in both genotypes.

The results indicate that overexpression of hG2019S-LRRK2 does not alter DA availability or stimulated vesicular release; however, the hG2019S mutation reduced the sensitivity of autoreceptor-mediated inhibition of release.

Discussion

LRRK2 silencing

Genetic deletion of LRRK2 did not result in behavioral abnormalities up to 12 months. This is consistent with an intact nigrostriatal DA system [13] and the absence of alterations to behavior and striatal glutamate transmission in younger mice [7]. A recent report showed decreased glutamate transmission and reduced dendritic spine density in striatal neurons of postnatal day 15 KO pups [5]; however, neither of these phenomena are apparent in adult KO mice [7, 13]. It may

be that LRRK2 regulates early neuronal development, but that this function is successfully compensated for during maturation. Consistent with this assumption, it has been shown that early alterations in neurite outgrowth in cultured KO neurons (over the first few days *in vitro*) are not sustained over longer periods (7-21 days *in vitro*) [19, 20]. The general lack of negative effects following germline ablation supports the notion that LRRK2 silencing may be tolerated *in vivo*.

Knockdown of LRRK2 by siRNA in cortical cultures increases evoked glutamate transmission at 14-16 days *in vitro* [9]; conversely, very subtle decreases in spontaneous release were found in KO neurons at 21 days *in vitro* [6]. Here targeted silencing (~90%) of striatal LRRK2 in adult mice had no effects upon motor activity, symmetry, cognition or striatal TH-positive fiber density. To the best of our knowledge this is the first investigation of LRRK2 silencing in adult mammalian central nervous system (CNS). Plasma levels of ASO following CNS administration are several orders of magnitude lower than with systemic administration [21]. Direct delivery of ASOs to the CNS that is required to achieve an effective concentration has a beneficial caveat; ASOs cross the blood brain barrier but the dilution restricts target selectivity to the CNS. This would avoid potential peripheral damage such as renal and pulmonary abnormalities observed in KO mice [11-13]. Together the data suggest that acute ablation of LRRK2 (in the murine brain) is also generally well tolerated and support the notion that ASOs may provide a therapeutic strategy against LRRK2 parkinsonism.

Mutant LRRK2 overexpression

High levels of hWT-LRRK2 in OE mice produce a robust, long-lasting impairment of motor function and cognitive deficits that worsened with age. At 3-6 months, OE mice have altered striatal D2-dependent short-term plasticity, hypodopaminergia and insensitivity to PPX-induced motor inhibition [7] in the absence of neurodegeneration [16]. Here we show that the motor phenotype is still present at 12 months, and now both hypolocomotion and cognitive deficits have been replicated in >5 separate cohorts of our OE mice. However, these findings are in contrast to previous reports concluding that overexpression of LRRK2 in mice either introduces no abnormalities or produces an increase in open field exploration [16, 22]. Our animals are relatively enriched in both the home cage environment and by the testing paradigm we employ, also the previous studies were conducted in different background strains of mice, grouped control animals from two lines, and the latter used a smaller (n<15/group) mixed cohort of males

and females. Any one or combination of these factors could explain the differences, but off-target effects due to BAC transgene insertion into the genome of different models also cannot be discounted. The expression levels of LRRK2 must also be taken into account, we observe ~3x endogenous LRRK2 protein expression in most brain tissues of our OE mice [7], whereas expression levels are much higher (5-6x endogenous) in other LRRK2 BAC OE mice [15, 22].

The data presented here benefit from the fact that all of our mice are bred from and with the same WT colony and large, all male cohorts are tested in standardized, parallel, comparative behavioral tests conducted under the same conditions by the same operators. This may go some way to reducing variability and potentially allowing detection of discrete phenotypes. Following this approach employed with KO and OE mice, modeling LRRK2 loss and gain of function respectively, we sought to investigate the behavioral effects of the hG2019S mutation.

Interestingly, introduction of the mutation led to an age-dependent phenotype that was temporally distinct from OE mice. Hyperactivity was observed in 6-month hG2019S mice, in agreement with our previous observations [16], and the mice were successful in the learning and memory paradigms. Ageing resulted in diminished exploratory and locomotor performance in hG2019S, to the point at which they were similar to their littermates. At this advanced age hG2019S mice also exhibited deficits in long-term recognition memory. Of note spatial memory, which is impaired in 12 month-old OE mice, remained unaffected in 12 month-old hG2019S, suggesting a less aggressive onset of phenotype in the hG2019S mice. Other G2019S mouse models exhibit PD-like phenotypes including neurodegeneration, but it would appear that very high levels of overexpression and/or viral-mediated gene delivery are required (see [10]). Conversely, in a G2019S knock-in mouse (with LRRK2 expression at endogenous levels) no degeneration is seen but a long-lasting hyperactive phenotype is observed [23]. With higher levels of mutant LRRK2 in the BAC hG2019S early hyperactivity diminishes with age, whereas even higher levels of LRRK2 in OE mice produce a consistently hypoactive phenotype. Together the data suggest that subtle and aggressive mouse models, with respect to LRRK2 levels, recapitulate different stages of disease progression.

Alterations to the DA system without neurodegeneration are reported in other BAC LRRK2 models [7, 15, 16], and here we show that striatal DA terminals from hG2019S mice are less sensitive to D2-mediated inhibition. This is consistent with observations in younger OE [7] and R1441C knock in mice [24]. A higher dose of PPX (1nM) eventually impaired [^{3}H]-DA

outflow, indicating impairments to D2 signaling that can be overridden. As this occurs at an age-point when hG2019S mice are hyperactive, it could be argued that reduced autoinhibition of DA release contributes to increased locomotion. At this age, both OE and hG2019S mice exhibit reduced DA tone [7, 16] yet display hypoactivity and hyperactivity, respectively. In this light it seems likely that the motor phenotype is independent of DA tone and that the mutation must engender dysfunction beyond a simplistic gain of function.

Future comparisons of this data with other LRRK2 models, including G2019S knock-in mice undergoing the same standardized behavioral paradigms, will ideally provide the phenotypic outcome measures needed for the development and testing of therapeutic interventions. Moreover, by focusing future investigations upon the underlying neurophysiological correlates of behavioral phenotypes, such as the regulation of DA transmission, we may shed light upon the biological substrates and etiology of LRRK2 parkinsonism and potentially idiopathic PD.

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Figure Legends

Figure 1. Motor and cognitive abilities are normal in 12-month old KO mice. **A)** Open field exploration. The distance traveled in the initial 5min (A.i) and across the entire 15min session (A.ii) was not different between KO mice and WT littermates. Time spent in the central area of the arena was not changed in KO mice (A.iii). **B)** Spontaneous vertical exploration in the cylinder test was similar in KO and WT mice. **C)** In the Novel Object Recognition paradigm, both KO and WT mice demonstrated a clear preference for the novel object. ANOVA followed by Bonferroni post test (# $p<0.05$).

Figure 2. Acute knockdown of striatal LRRK2 does not affect motor or cognitive function and striatal DA terminals area preserved. **A)** Schematic representing the surgical administration of PBS/ASO into the right striatum (top) and the experimental timeline (bottom). **B)** LRRK2 ASO did not induce differences in vertical exploration (B.i) and symmetry in the cylinder test (B.ii). Symmetry and balance were also intact when assessed in the drag test (B.iii). **C)** LRRK2 immunohistochemistry (C.i) showed significant reduction of LRRK2-positive area in the injected striatum as compared to the contralateral side (C.ii). **D)** Western blot analysis (D.i) showed significant reduction of LRRK2

protein levels in ASO-injected striata of WT animals when compared to PBS-injected WT animals (D.ii). **E)** TH immunohistochemistry (E.i) revealed no differences in DA nerve endings in the ASO-injected striata of WT animals, when compared to the contralateral side (E.ii). **F)** Distance traveled (F.i) and time spent in the central area (F.ii) of the open field were not different from ASO- and PBS-treated WT mice. **G)** During cognitive testing, both PBS- and ASO-treated WT mice successfully recognized the novel object. ANOVA followed by Bonferroni post test (##p<0.01); t-test (**p <0.01).

Figure 3. Behavioral deficits in 12-month old OE mice and age-dependent phenotype of hG2019S mice. **A)** The distance traveled in the initial 5min of the open field was significantly reduced in OE mice at 12 months (A.i). Conversely, hG2019S mice showed a strong trend for increased locomotion at 6 months (A.ii) that became comparable to WT mice at 12 months (A.iii). **B)** Vertical exploration in the cylinder test was significantly decreased in 12-month old OE mice, when compared to WT (B.i). The number of rearings was significantly increased in 6-month old hG2019S mice (B.ii) and decreased to similar levels a WT mice at 12 months (B.iii). **C)** Recognition memory deficits were observed in OE mice at 12 months, as they equally explored the familiar and novel object (C.i). Cognitive abilities were intact in hG2019S mice at young (3 months) age (C.ii) but failure in recognizing the novel object was observed with ageing, at 12 months (C.iii). ANOVA followed by Bonferroni post test (##p<0.01); t-test (*p <0.05).

Figure 4. Striatal nerve endings from hG2019S mice display reduced sensitivity to D2-mediated inhibition of DA release as measured in a synaptosomal preparation. **A)** Spontaneous [^{3}H]-DA efflux (A.i) was comparable between hG2019S mice and WT littermates. Quasi-physiological [^{3}H]-DA release induced by a K^+ pulse was not significantly different in hG2019S mice with both 10mM and 20mM KCl (A.ii). Pre-application of the D2 receptor agonist PPX significantly inhibited K^+-induced overflow in WT mice, at both 0.1 and 1nM. Conversely, K^+-mediated [^{3}H]-DA release from hG2019S synaptosomes was not affected by pre-treatment with 0.1nM PPX. The higher concentration of PPX (1nM) significantly reduced [^{3}H]-DA overflow in hG2019S mice (A.iii). ANOVA followed by Bonferroni post test (*p<0.05, **p<0.01).

Figure S1. Motor and cognitive deficits in 12-month old OE mice are not related to increased anxiety. **A)** The total distance traveled in the open field was reduced in OE mice during the initial 5min (A.i) while no significant change in the time spent in the central area was detected across the entire 15-min session (A.ii). **B)** Spatial memory was also impaired at 12 months of age in OE mice as they equally explored the object moved to the novel location and the object that remained in the familiar one. ANOVA followed by Bonferroni post test (#p<0.05); t-test (**p<0.01).

Figure S2. Hyperactivity in 6-month old hG2019S mice disappears at 12 months of age, while spatial memory is preserved in young and old age. **A)** The total distance traveled across the 15-min open field session was significantly increased in hG2019S mice when compared to WT, at 6 months of age (A.i). A significant effect of genotype was observed when data were subdivided in 5-min bins (A.ii). Anxiety levels were not changed as no significant

difference between hG2019S mice and WT littermates were detected at 6 months (A.iii). **B)** Locomotor activity in the open field decreased in 12-month old hG2019S mice at levels comparable to WT littermates (B.i) with central area exploration remaining unchanged (B.ii). **C)** Location memory is intact in young (3 months) hG2019S mice. **D)** Ability to recognize the novel location is not affected by ageing in hG2019S mice. ANOVA followed by Bonferroni post test (#p<0.05, ##p<0.01).

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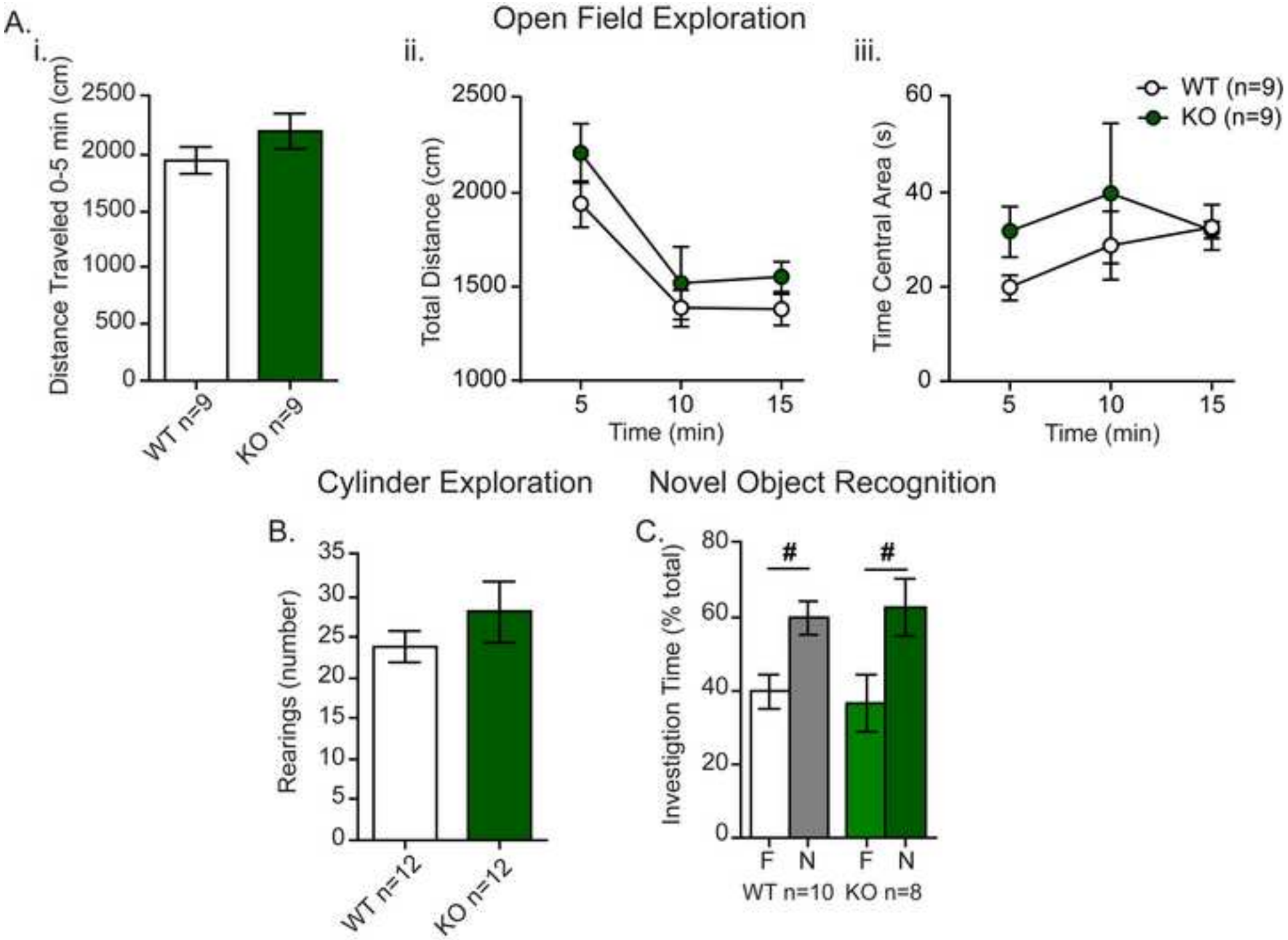

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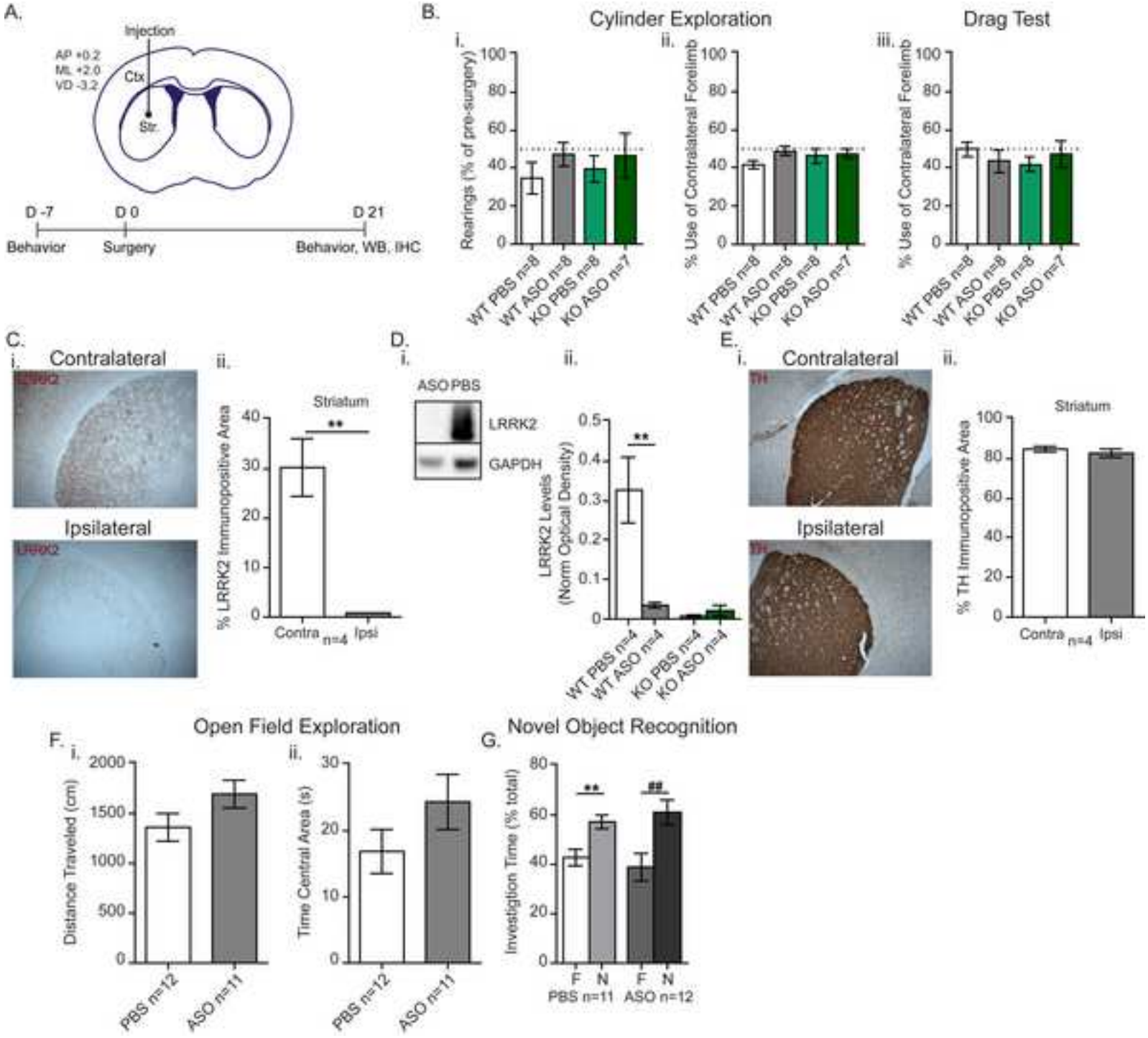

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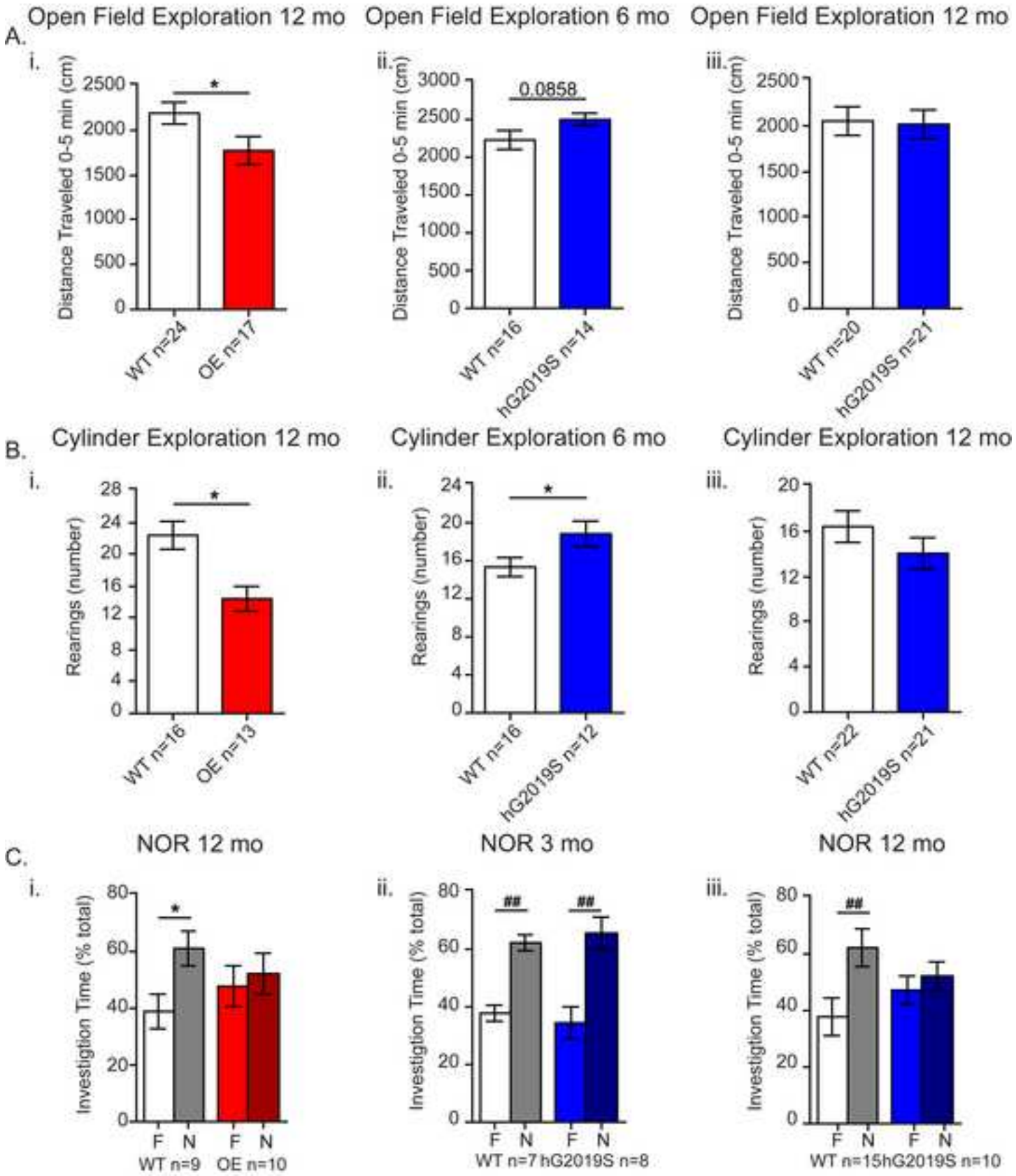

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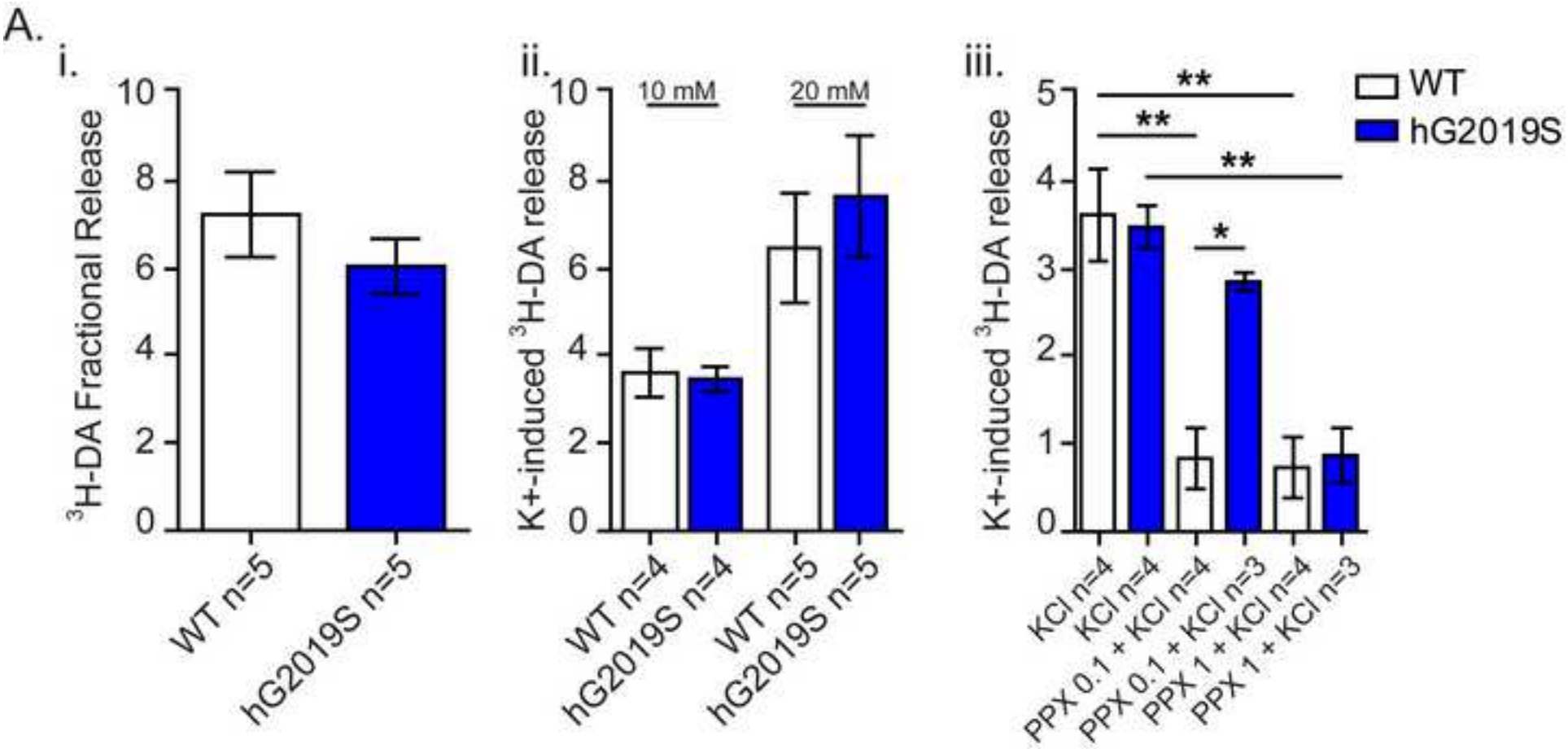

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