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Impaired dopaminergic neurotransmission and microtubule-associated protein tau alterations in human *LRRK2* transgenic mice

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ABSTRACT

Mutations in the *Leucine Rich Repeat Kinase 2* (*LRRK2*) gene, first described in 2004 have now emerged as the most important genetic finding in both autosomal dominant and sporadic Parkinson's disease (PD). While a formidable research effort has ensued since the initial gene discovery, little is known of either the normal or the pathological role of *LRRK2*. We have created lines of mice that express human wild-type (hWT) or G2019S Lrrk2 via bacterial artificial chromosome (BAC) transgenesis. *In vivo* analysis of the dopaminergic system revealed abnormal dopamine neurotransmission in both hWT and G2019S transgenic mice evidenced by a decrease in extra-cellular dopamine levels, which was detected without pharmacological manipulation. Immunopathological analysis revealed changes in localization and increased phosphorylation of microtubule binding protein tau in G2019S mice. Quantitative biochemical analysis confirmed the presence of differential phospho-tau species in G2019S mice but surprisingly, upon dephosphorylation the tau isoform banding pattern in G2019S mice remained altered. This suggests that other post-translational modifications of tau occur in G2019S mice. We hypothesize that Lrrk2 may impact on tau processing which subsequently leads to increased phosphorylation. Our models will be useful for further understanding of the mechanistic actions of *LRRK2* and future therapeutic screening.

Introduction

LRRK2 mutations represent a unique opportunity for the development of genetic model systems for Parkinson's disease (PD). *LRRK2* mutations are the most common form of familial parkinsonism and account for up to 40% of sporadic parkinsonism in certain populations (reviewed by Farrer, 2006). Clinically and pathologically, the features of *LRRK2*-associated parkinsonism are often indistinguishable from idiopathic PD; although pathologic variability exists even within PARK8-linked kindreds, ranging from nigral neuronal loss only, to neuronal loss with α-synuclein, ubiquitin or tau inclusions (reviewed by Whaley et al., 2006). To date, four different domains (ROC, C-terminal of ROC, kinase and WD40) of Lrrk2 are known to be affected by mutation/risk factors. While the ultimate downstream consequence of *LRRK2* mutation in humans is parkinsonism, it is still unclear if the variants share common pathogenic mechanisms or if individual variants exert specific effects.

Until recently, hypotheses about Lrrk2 dysfunction were based on data from lower model organisms and cellular systems. Several lines of evidence now point towards an important role of Lrrk2 in neuronal outgrowth and guidance (MacLeod et al., 2006; Plowey et al., 2008; Sakaguchi-Nakashima et al., 2007; van Egmond et al., 2008). Mechanistic studies have repeatedly observed increased kinase activity for the most common G2019S mutation, however the mode of action for the other mutations has remained conflicting (Greggio and Cookson, 2009). With the emergence of several rodent mutant *LRRK2* models, *in vivo* insight into the mechanistic actions is now forthcoming. ROC domain mutant human R1441G bacterial artificial chromosome (BAC) and R1441C knock-in mice both exhibit impaired

Abbreviations: *LRRK2*/Lrrk2, leucine rich repeat kinase 2; PD, Parkinson's Disease; hWT, human wild type; BAC, bacterial artificial chromosome; PCR, polymerase chain reaction; TH, tyrosine hydroxylase; NT, non transgenic; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; HPLC, high performance liquid chromatography; DAT, dopamine transporter; DAB, 3,3′-diaminobenzidine tetrahydrochloride; ANOVA, analysis of variance.

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dopamine release (Li et al., 2009; Tong et al., 2009), with additional behavioral and pathological abnormalities seen in the R1441G BAC mice. Mice over-expressing a murine *LRRK2* BAC containing a G2019S mutation have also been shown to have decreased dopamine release whereas mice over-expressing a murine wild-type *LRRK2* BAC have increased dopamine release and are hyperactive (Li et al., 2010). Inducible human G2019S and wild-type over-expressing mice, while absent of phenotype alone, both show synergistic effects when crossed with mutant A53T alpha-synuclein mice (Lin et al., 2009). Curiously, A53T mice on a murine *LRRK2* knockout background have an ameliorated phenotype (Lin et al., 2009) whereas the kidneys of *LRRK2* knockout mice accumulate alpha-synuclein (Tong et al., 2010). Interestingly, although extracellular dopamine release was not measured in *LRRK2* knockout mice, in two independent lines levels of striatal dopamine were normal and the knockout mice are without overt brain phenotype (Andres-Mateos et al., 2009; Tong et al., 2010).

In this study we report human wild type (hWT) and mutant G2019S mice that were generated via BAC transgenesis. We have performed a comprehensive analysis of the dopaminergic system in these mice as well as behavioral and pathological analysis. We show that human kinase domain G2019S over-expressing mutants, like ROC mutants and murine G2019S BAC mutants, have reduced extracellular dopamine levels, which can be detected without pharmacological intervention. Importantly, we demonstrate that BAC mice over-expressing human wild type Lrrk2 also exhibit decreased extracellular dopamine levels, lending support to gain of function mechanism. Furthermore, we show that G2019S mice exhibit a number of age related changes in tau protein, including mislocalization and increased tau phosphorylation. We present novel biochemical data that suggests that the tau banding pattern is altered in G2019S mice compared to age matched controls. Finally, we show that G2019S mice, but not hWT mice, display anxiety like behaviors. Our results both support and extend data recently obtained in other *LRRK2* models. The human G2019S and wild-type BAC mice will provide further insight into understanding basic mechanisms of *LRRK2* biology and aid future therapeutic design.

Methods

Animals

All animal procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Generation of BAC transgenic mice

A BAC (RP-11 568G5) was identified *in silico* to contain the entire human *LRRK2* gene (NM_198578.3) and regulatory sequences, and was purchased from the Children's Hospital Oakland Research Institute (Oakland, CA). Library RP-11 genomic DNA originated from human male blood and was cloned into vector pBACe3.6 (Osoegawa et al., 2001). BAC mutagenesis was used to generate Lrrk2 c.6055 G to A transition that encodes the Lrrk2 G2019S amino acid substitution. BAC DNA was isolated using standard alkaline purification. Integrity was confirmed by restriction mapping and pulse field analysis. For oocyte injection the final DNA pellet was dissolved in Tris–EDTA and diluted in sterile injection buffer (5 mM Tris–HCl, pH 7.5, 0.05 mM EDTA, 50 mM NaCl and 0.1 mM polyamines (spermidine and spermine). Purified hWT and G2019S BAC DNA at concentrations ranging from 1.5 to 5 ng/μl was injected into FVB/N (Taconic, Germantown, NY) fertilized oocytes and transplanted into pseudo-pregnant ICR (Harlan, Indianapolis, IN) female mice. Subsequent offspring were genotyped by polymerase chain reaction (PCR) using two sets of primers designed within the vector arms and the human 5' and 3' insert, to identify founders. Transgenic founders were bred to FVB/N mice and transgenic F1 offspring analyzed for gene and protein expression.

Real time quantitative reverse transcriptase PCR

Mice were euthanized by cervical dislocation and brains were subsequently separated into different regions (olfactory bulb, hippocampus, striatum, cortex, mid-brain, brainstem, cerebellum) and frozen on dry ice. RNA was isolated using TRIzol® (Invitrogen, Carlsbad, CA,) according to manufacturer's instructions. cDNA was synthesized using Superscript II (Invitrogen). Real time PCR assays were performed in triplicate on a 384 well plate using an ABI 7900 detection system to assess the relative level of human *LRRK2* mRNA using TaqMan® probe Hs00417273_ml specific for human *LRRK2* (ABI, Foster City, CA, USA). In addition, expression analysis was performed with murine probes to *LRRK1* (Mm00713303_ml), *LRRK2* (Mm00481934_ml), *SNCA* (Mm00447333_ml) and *MAPT* (Mm00521988_ml). In all instances murine *GAPDH* (Mm99999915_ml) was used as the endogenous reference gene.

In situ hybridization

Cartridge purified oligonucleotides were synthesized by Sigma Genosys (St Louis, MO, USA). Murine *LRRK2* was detected with oligonucleotide designed to mouse exon 15 5′CTTGGTCATCTGGATACATCTGTAAGGTGTGGAGGACTGAGTCAACTGC'3 and human *LRRK2* with oligonucleotide 5′TTTAAGGCTTCCTAGCTGTGCTGTCATCATGACTCTG'3 designed to human exon 41. 100 ng of oligonucleotide was 3′ end labeled with ^{33}P α-dATP (Perkin Elmer, Boston, MA, USA) using Terminal Transferase (Roche, Indianapolis, IN, USA). Labeled oligonucleotides were purified using Nick columns (Amersham, Piscataway, NJ, USA) to remove unincorporated probe and the specific activity checked to ensure a minimum of 1×10^8 cpm/μg.

15 μm coronal frozen cryostat sections were fixed in cold 4% paraformaldehyde in Sörensen's phosphate buffer, dehydrated in ascending alcohols and incubated at 37 °C overnight in hybridization buffer (4× Standard sodium citrate, 50%(w/v) formamide, 10%(w/v) dextran sulfate, 200 mg/μl Herring Sperm DNA) containing ^{33}Pα-dATP labeled oligonucleotides. Competition hybridizations performed in the presence of excess unlabeled probe served as an additional control for each oligonucleotide. Slides were stringently washed three times in 1× SSC at 55 °C and exposed to Kodak MS high-resolution film (Eastman Kodak via Fisher Scientific, Rockford, IL) for 10–14 days.

Antibodies

Affinity purified rabbit polyclonal antibody PA0362 (C-terminal amino acid residues 2507-2527, Novus Biologicals, Littleton, CO, # 110-58771) was raised to synthetic human Lrrk2 peptides (Melrose et al., 2007). For immunohistochemistry on tissue sections tyrosine hydroxylase (TH) (1:200, Affinity Bioreagents/ ThermoFisher Scientific Rockford, IL) was used to visualize dopamine neurons and the dopamine transporter (DAT) was detected with a polyclonal antibody (1:500 Chemicon/ Millipore, Billerica, MA). Detection of α-synuclein was with mouse monoclonal α-synuclein (clone 42, 1:3500, BD Transduction Labs, San Jose, CA) and the α-synuclein phosphorylated at Ser129 antibody (1:1000) was a kind gift from Dr. Takeshi Iwatsubo, University of Tokyo. Activated microglia were detected by Iba-1 (1:2000, Wako Chemicals, Richmond, VA). Tau antibodies were CP-13 (1:1000 immunohistochemistry, 1/200 immunoblots), Tau-5 (1/ 500 immunoblots) and PHF-1 (1/500 Immunoblotting) all kind gifts from Dr. Peter Davies, Albert Einstein College of Medicine), 12E8 (1:10,000 immunohistochemistry) gift from Dr. Peter Seubert, Elan Pharmaceuticals) and Tau-1 (1/500 Immunoblots from Millipore).