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Mutant LRRK2 enhances glutamatergic synapse activity and evokes excitotoxic dendrite degeneration

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ABSTRACT

Mutations in leucine rich repeat kinase 2 (LRRK2), which are associated with autosomal dominant Parkinson's disease, elicit progressive dendrite degeneration in neurons. We hypothesized that synaptic dysregulation contributes to mutant LRRK2-induced dendritic injury. We performed *in vitro* whole-cell voltage clamp studies of glutamatergic receptor agonist responses and glutamatergic synaptic activity in cultured rat cortical neurons expressing full-length wild-type and mutant forms of LRRK2. Expression of the pathogenic G2019S or R1441C LRRK2 mutants resulted in larger whole-cell current responses to direct application of AMPA and NMDA receptor agonists. In addition, mutant LRRK2-expressing neurons exhibited an increased frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) in conjunction with increased excitatory synapse density as assessed by immunofluorescence for PSD95 and VGLUT1. Mutant LRRK2-expressing neurons showed enhanced vulnerability to acute synaptic glutamate stress. Furthermore, treatment with the NMDA receptor antagonist memantine significantly protected against subsequent losses in dendrite length and branching complexity. These data demonstrate an early association between mutant LRRK2 and increased excitatory synapse activity, implicating an excitotoxic contribution to mutant LRRK2 induced dendrite degeneration.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by debilitating motor, and in many cases cognitive deficits. Efforts to understand the pathogenesis of PD have revealed genetic factors that increase the risk of developing PD. Leucine-rich repeat kinase 2 (LRRK2) mutations underlie *PARK8*-linked familial parkinsonism [1,2]. This protein is believed to play important roles in both familial and sporadic PD pathogenesis [3]. A deeper understanding of the pathologic cascade that leads to neurodegeneration downstream of mutant LRRK2 will likely render novel insights for the prevention and treatment of PD.

Neurite injury is a conspicuous feature of mutant LRRK2-associated neurodegeneration. Mutant LRRK2 expression in SH-SY5Y neuroblastoma cell line cultures and in mouse primary cortical neurons is associated with neurite degeneration [4] or reduced dendrite outgrowth [5,6]. Mutant LRRK2 expression in neurons *in vivo* or *in vitro* results in neurite injury that precedes cell death [7]. Functional neurotransmission abnormalities [8] and dystrophic neurite morphology have been reported in transgenic mutant LRRK2 mice [9,10]. Whereas numerous effector pathways, including autophagy [7,10–13], mitochondrial pathology [14–16], calcium toxicity [15], the ubiquitin proteasome [17], microtubule

Abbreviations: LRRK2, leucine rich repeat kinase 2; LRRK2-3HA, C-terminal 3×-hemagglutinin tagged LRRK2; cDNA, complementary deoxyribonucleic acid; mEPSC, miniature excitatory postsynaptic current; PD, Parkinson's disease; AMPA, (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid); NMDA, *N*-methyl-D-aspartate; VGLUT1, vesicular glutamate transporter 1; PSD-95, postsynaptic density protein-95; GFP, green fluorescent protein; WT, wild type; BAC, bacterial artificial chromosome; CNQX, (6-cyano-7-nitroquinoxaline-2,3-dione); APV, (2*R*)-amino-5-phosphonovaleric acid; CC3, cleaved caspase 3; DAPI, 4′,6-diamidino-2-phenylindole; SEM, standard error of the mean; DIV, days *in vitro*

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stability [18], growth cone dynamics [6,19], Fas-associated protein with death domain [20] and Rac1 [21], have been proposed, less is known about the possible upstream impacts of LRRK2 on synaptic function [22–25]. We hypothesize that synaptic dysregulation contributes to dendrite injury in mutant LRRK2 expressing neurons.

To test our hypothesis, we determined whether alterations in excitatory synapses underlie neurite retraction in neurons expressing PD-associated LRRK2 mutations. We found that mutant LRRK2-expressing neurons show evidence of increased glutamatergic synapses and increased vulnerability to synaptic glutamate stress, which occur well before the onset of neurite degeneration. Furthermore, the NMDA receptor antagonist memantine partially protected neurons from mutant LRRK2-induced dendrite degeneration. These findings suggest that mutant LRRK2 is associated with enhanced glutamatergic synapses and renders neurons more vulnerable to glutamate receptor toxicity.

2. Materials and methods

2.1. Neuronal cultures

Timed-pregnant female Sprague–Dawley rats (E16), obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA), were euthanized by CO_2 inhalation. This method of euthanasia is consistent with methods suggested by the Panel on Euthanasia of the American Veterinary Medical Association to minimize animal distress and was approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Embryos of either gender were harvested in ice-cold Hanks solution (Invitrogen). Cerebral cortices were dissected and dissociated *via* trypsinization and gentle pipette trituration. Cell suspensions were plated at a density of 100,000 cells/cm^2 onto glass cover slips (Carolina Biological) or plastic culture dishes coated with poly-D-lysine (0.1 mg/ml; Sigma) and laminin (5 μg/ml; Roche Diagnostics). Cultures were maintained at 37 °C with 5% ambient CO_2 in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) and 1% Glutamax-I (Invitrogen). Media refreshments were performed every other day. In some experiments, memantine, an NMDA receptor antagonist, was added to the culture media following neuronal transfection to maintain a concentration of 1 μM.

2.2. Molecular constructs and culture transfection

Full-length wild-type (WT) and mutant LRRK2 cDNAs (pathogenic PD mutation G2019S or R1441C; kinase impaired K1906M) with C-terminal triple-hemagglutinin tags (LRRK2-3HA) were expressed *via* the pcDNA3.1 vector [11]. Neuronal cultures were co-transfected with pRK7-eGFP and either empty pcDNA3.1 vector or mutant LRRK2 cDNA constructs with 0.1% Lipofectamine 2000 reagent (Invitrogen) on days *in vitro* (DIV) 12–15. A molar ratio of 1:2 (eGFP:LRRK2-3HA) was employed in electrophysiology experiments, and ratios of 1:2 and 1:9 were used in immunofluorescence experiments. A mouse anti-HA Tag IgG (Covance, Clone 16B12) was used to confirm LRRK2-3HA protein expression in neuroblastoma cells *via* western blot (1:1000 primary antibody dilution) and in cultured cortical neurons *via* immunocytochemistry (1:100 primary antibody dilution). RT-PCR was performed on neuronal cultures with primers spanning the junction of the C-terminus and the 3HA Tag of the LRRK2 cDNAs (LRRK2-3HA primer sequences (403 base pair product): LRRK2-7179-Forward: 5′-AAGGGAGG TAATGGTAAAAGAAA-3′; LRRK2-3HA-Reverse: 5′-CCGCCCTCAACAGA TGTTCG-3′; eGFP primer sequences (402 base pair product): eGFP-Forward: 5′-GAGCTGGACGGCGACGTAAACGG-3′; eGFP-Reverse: R: 5′-GACGTTGTGGCTGTTGTAGTTG-3′). The transfection efficiency, determined by GFP fluorescence and HA Tag immunofluorescence, was less than 1% (40–75 neurons per cover slip). There was typically one transfected neuron in a medium-power (100×) microscopic field, allowing us to focus our analysis on the postsynaptic effects of mutant LRRK2 expression in individual neurons in the context of non-transfected presynaptic terminals from surrounding neurons.

2.3. Electrophysiological recordings

Coverslips containing rat primary cortical neuron cultures were placed in a recording chamber containing Ringer solution (140 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose; pH 7.3) with tetrodotoxin (2 μM) and strychnine (1 μM). Transfected cells expressing GFP were visualized *via* epifluorescence microscopy. Whole-cell voltage clamp recordings (Axopatch 200 Amplifier) were obtained through glass micropipettes (tip resistance 3–5 MΩ) filled with intracellular solution (130 mM CsCl, 10 mM HEPES, 10 mM EGTA for glutamate receptor agonist responses and 118 mM Cs Methane Sulfonate, 12 mM CsCl, 10 mM HEPES, 10 mM EGTA for mEPSC recordings). The mean ± SEM series resistance was 16.1 ± 0.8 MΩ and was compensated at 80% in all experiments. Holding potentials were −55 mV for agonist responses and −60 mV in mEPSC recordings (corrected for liquid junction potentials of 5 and −10 mV, respectively). Cells were exposed to ionotropic glutamate receptor agonists (100 μM AMPA or 10 μM NMDA/10 μM glycine for 20 second periods) through a gravity-driven, multi-barrel fast perfusion system [26]. NMDA receptor currents were inhibited during applications of AMPA with 1 mM $MgCl_2$. Data were digitized (Digidata 1200) and recorded to a PC running pClamp 9.1 software suite. Whole-cell current responses were quantified as the mean steady state current during the final 5 s of agonist applications. The mEPSC mean amplitudes and frequencies were analyzed using the event detection module of pClamp 9.1.

2.4. Synaptic protein immunocytochemistry

Cover slips were fixed with 4% paraformaldehyde with 4% sucrose followed by 100% methanol, permeabilized with 0.1% Triton-X and blocked with Superblock Buffer (Thermo Scientific). Primary and secondary antibody concentrations employed were as follows: mouse anti-PSD-95 IgG (1:200; Neuromab 75-028, UC Davis), mouse anti-VGLUT1 IgG (1:200; Neuromab 75-066, UC Davis), rabbit anti-LRRK2 antibody (1:50; MJFF2 [c41-2], Abcam ab133474), Alexa Fluor 594-conjugated donkey-anti-mouse and goat-anti-rabbit IgGs (1:1000; Jackson ImmunoResearch Laboratories). Nuclei were counterstained with DAPI (1:500; Invitrogen). Cover slips were mounted on glass slides with gelvatol and imaged on an Olympus Fluoview 1000 confocal microscope. Proximal dendrites of 25–30 that consecutively encountered transfected neurons for each LRRK2 construct were imaged with a 63× magnification, 1.40 NA apochromatic lens oil immersion objective. Identical excitation and image acquisition parameters were employed for all constructs. Images were analyzed for synapse protein immunofluorescence puncta with NIH ImageJ [27]. Immunoreactive puncta were identified on 50–250 μm GFP-positive dendrite segments from each neuron with the NIH ImageJ intensity threshold function and quantified with the Analyze Particles module of NIH ImageJ with respect to puncta density (per micron of analyzed dendrite length) and puncta area. The threshold intensity for each antigen was set as the mean plus 3 standard deviations of background dendritic cytoplasm signal sampled from 5 neurons. Identical background measurements and intensity thresholds were employed in all transfection groups.

2.5. Synaptosome preparations and western blots

DIV15-17 rat cortical neurons were scraped and homogenized with a 25G needle in HEPES-buffered sucrose [0.32 M sucrose, 5 mM HEPES, pH 7.4] supplemented with protease inhibitor cocktail (Sigma, P8340). Homogenized extracts were spun at 1000 *g* for 10 min at 4 °C. Supernatant (S1) was centrifuged again at 1000 *g* for 10 min at 4 °C and saved (S1′, whole cell lysate). S1′ was centrifuged at 10,000 *g* for 15 min for the cytosolic (S2) fraction. The pellet was washed one time with 1 ml

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