Regulates Neurite Process Morphology

Introduction

Parkinson’s disease is the second most common neurodegenerative disease, typically presenting as a progressive movement disorder with slowness, rigidity, gait difficulty, and tremor at rest. The pathological hallmarks of PD include the loss of dopamine (DA) neurons in the substantia nigra (SN) of the ventral midbrain, as expected, but also surp{

Introduction

Parkinson’s disease is the second most common neurodegenerative disease, typically presenting as a progressive movement disorder with slowness, rigidity, gait difficulty, and tremor at rest. The pathological hallmarks of PD include the loss of dopamine (DA) neurons in the substantia nigra (SN) of the ventral midbrain, as expected, but also surprising heterogeneity regarding other pathological fea-

Summary

Mutations in LRRK2 underlie an autosomal-dominant, inherited form of Parkinson’s disease (PD) that mimics the clinical features of the common “sporadic” form of PD. The LRRK2 protein includes putative GTPase, protein kinase, WD40 repeat, and leucine-rich repeat (LRR) domains of unknown function. Here we show that PD-associated LRRK2 mutations display disinhibited kinase activity and induce a progressive reduction in neurite length and branching both in primary neuronal cultures and in the intact rodent CNS. In contrast, LRRK2 deficiency leads to increased neurite length and branching. Neurons that express PD-associated LRRK2 mutations additionally harbor prominent phospho-tau-positive inclusions with lysosomal characteristics and ultimately undergo apoptosis.

Results

LRRK2 Expression and Kinase Activity

To investigate the normal and pathological functions of LRRK2, we generated plasmid vectors for overexpression of wild-type or disease-associated mutant alleles of LRRK2 including G2019S, I2020T, Y1699C, and R1441G (Figures 1A and 1B). A V5/His-epitope tag was added to the amino terminus of each coding sequence to distinguish the plasmid-encoded protein from endogenous LRRK2. Plasmids were transiently transfected into COS7 cells, and cell lysates were analyzed by SDS-PAGE and Western blotting with antibodies specific for the V5 epitope tag or LRRK2. A single 280 kDa protein was observed in cytoplasmic lysates of cells transfected with either wild-type or mutant LRRK2 plas-

Discussion

The Familial Parkinsonism Gene LRRK2 Report

Regulates Neurite Process Morphology

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to disinhibited kinase activity relative to wild-type LRRK2 (Gloeckner et al., 2005; West et al., 2005).

Additionally, two short hairpin RNA (shRNA)-based plasmid vectors were generated to inhibit the expression of endogenous rodent LRRK2 by RNA interference (RNAi; see Experimental Procedures). Transfection of either of these plasmids into primary rat cortical cultures or rat C6 glioma cells led to a reduction in the level of LRRK2 mRNA and protein to less than 20% of baseline levels, as determined by real-time quantitative RT-PCR, Western blotting, and immunocytochemistry with polyclonal antibodies for LRRK2 (Figure 1C and Figures S1C and S1D). Immunocytochemical analysis of primary cortical cultures further revealed the presence of LRRK2 throughout the soma and neurite processes of neurons (Figure 1C). Immunocytochemistry with antibodies for the polyhistidine epitope tag or for LRRK2 indicated that plasmid-encoded wild-type and G2019S mutant LRRK2 localized similarly to the endogenous protein (Figure S2). The G2019S LRRK2 mutant protein was also present in distinctive spheroid-like inclusions within cellular processes and at intracellular membranous structures (see below).

LRRK2 Regulates Neuronal Process Morphology

Primary cortical cultures were transfected with plasmid vectors encoding wild-type or mutant forms of V5 epitope-tagged LRRK2 (or vector control), along with enhanced green fluorescent protein (eGFP) sequences to allow for the identification of transfected cells (~5% of neurons; data not shown).

Overexpression of either of two clinically associated missense mutant forms of LRRK2 within the kinase domain, G2019S and I2020T, led to a dramatic reduction in neurite length and branching evident with respect to both the longest neuronal processes, corresponding to

Figure 1. LRRK2 Regulates Neurite Process Morphology

(A) Schematic of the primary structure of LRRK2, including the leucine-rich repeat (LRR), Roc, COR, protein kinase (PK), and WD-repeat domains. Clinical mutations (in black) and the putative dominant-negative allele (in red) are shown.

(B) Upper panel displays an autoradiograph of the kinase assay with MBP substrate. Lower panel shows a Western blot for the V5 epitope tag, demonstrating equal expression of the LRRK2 alleles. V5 epitope-tagged wild-type or mutant alleles of LRRK2 were overexpressed in COS7 cells. Cell lysates were immunoprecipitated with an antibody to the V5 epitope-tag, and protein kinase assays performed with myosin light chain as a substrate.

(C) Knockdown of LRRK2 with transfection of shRNA or vector control in transfected primary rat P1 cortical cultures. Immunocytochemical analysis was performed with a polyclonal antibody to LRRK2. LRRK2 protein is found throughout the cytoplasm and neurites and is enriched at the cell cortex and at membrane structures. LRRK2 shRNA vector transfected cells display reduced LRRK2 staining relative to untransfected cells in the same culture. Insets are magnified (2.5 x) views of the transfected soma.

(D) Camera lucida drawings of LRRK2-transfected cortical neurons. Wild-type, G2019S, I2020T, R1441G, Y1699C, or K1906M LRRK2 alleles were transfected into cortical cultures at day 7 in vitro (7 DIV). At 2 weeks after transfection, cultures were imaged by confocal microscopy, and process length was quantified by an observer blind to the identity of the allele. Process complexity and length was reduced significantly in the G2019S and I2020T cultures relative to control vector or wild-type (wt) LRRK2, whereas the putative dominant-negative allele, K1906M, leads to an increase in process length. Knockdown of LRRK2 with an shRNA vector leads to increased process length relative to shRNA vector control.

(E) Quantification of soma diameter (lower panel) and total neurite process length (upper panel) as in (D). * p < 0.05. n = 55 cells in nine experiments for G2019S and wt LRRK2 vectors; n = 85 cells in 13 experiments for control vector; n = 25 in five experiments for LRRK2 shRNA; n = 20 cells in three experiments for K1906M and I2020T LRRK2; n = 15 cells in three experiments for all other sets.
axons, as well as total neurite outgrowth (Figures 1D and 1E and Figure S2 C). This was confirmed by antibody staining for axonal and dendritic markers (Figure S3A).

Neuronal polarity, as quantified by the ratio of axons to dendrites, appeared unaltered. Overexpression of a Parkinsonism-associated missense mutation in the ROC domain, R1441G, also led to a significant decrease in process length, whereas a mutation within the COR domain, Y1699C, induced a relatively modest decrease in process length that did not reach statistical significance (Figures 1D and 1E). Overexpression of wild-type \( LRRK2 \) did not alter neuronal morphology, and the soma size of neurons transfected with either wild-type (wt) or mutant \( LRRK2 \) allele cDNA appeared similar to vector control.

As Parkinsonism-associated \( LRRK2 \) alleles display disinhibited kinase activity and short processes, we hypothesized that neurons deficient in \( LRRK2 \) activity may demonstrate extended processes. First, we generated an additional mutant form of \( LRRK2 \), K1906M, that is predicted to lie within the ATP-binding segment of the kinase domain (Cobb and Goldsmith, 1995) and has been shown to generate a dominant-negative allele (Cobb and Goldsmith, 1995; Gloeckner et al., 2005).

Overexpression of the K1906M allele led to a significant increase in total process length, an effect that was particularly evident with respect to the length of the longest process (Figures 1D and 1E). In a second approach, \( LRRK2 \) accumulation was inhibited by RNAi. Cortical neurons transfected with either of two shRNA vectors specific for \( LRRK2 \) displayed a prominent increase in neurite process length (Figures 1D and 1E and data not shown).

Figure 2. \( LRRK2 \) G2019S Mutation Leads to a Progressive Neurite Loss and Decreased Survival
(A) Primary rat cortical P1 cultures were transfected with \( LRRK2 \) alleles at 7 DIV, as indicated, and process length and branching were quantified over time subsequently. Overexpression of mutant, but not wt, \( LRRK2 \) leads to a progressive decrease in process length over a 15 day period. In contrast, \( LRRK2 \) knockdown by shRNA leads to a significant progressive increase in neurite process length. \( \ast p < 0.05; n = 85 \) cells in 13 experiments for control vector; \( n = 45 \) cells in six experiments for wt \( LRRK2 \) vector; \( n = 37 \) cells in six experiments for \( G2019S \) \( LRRK2 \) vector; \( n = 25 \) cells in five experiments for \( LRRK2 \) shRNA; \( n = 20 \) cells in three experiments for \( I2020T \) \( LRRK2 \) vector.
(B) Overexpression of mutant \( LRRK2 \) alleles lead to a significant reduction in cell survival, as visualized by propidium iodide (PI) exclusion and absence of nuclear condensation. In (C), time course analyses of neuronal survival and the accumulation of intracellular inclusions are presented. \( n = 55 \) cells in nine independent experiments for control vector, \( G2019S \), and wt; \( n = 20 \) cells in three independent experiments for \( I2020T \). \( \ast p < 0.05. \)
(D) Rescue of the \( LRRK2 \) shRNA knockdown requires only the kinase domain of the protein. Primary neuronal cultures were cotransfected with \( LRRK2 \) shRNA along with full-length (FL) cDNA, a GTPase/COR/kinase fragment (deleted in amino- and carboxy-terminal domains), or the kinase domain alone. \( n = 31 \) cells in three experiments for shRNA + FL \( LRRK2 \); \( n = 15 \) cells in three independent experiments for all other groups.
(E) Deletion analyses of \( LRRK2 \) G2019S and wt alleles demonstrate a role for the kinase domain in the reduced neurite process length phenotype. The G2019S kinase domain alone is sufficient to reduce process length and leads to a more dramatic phenotype than the full-length protein or a truncated protein that harbors GTPase, COR, and kinase domains. The wt GTPase domain alone displays no activity. \( n = 15 \) cells in three independent experiments for each group.

\( LRRK2 \) Mutations and the Maintenance of Neuronal Processes
To distinguish between a role in the generation and the maintenance of process length, we performed a time course analysis of neuronal morphology in cortical cultures transfected with \( LRRK2 \) wild-type, \( G2019S \), or \( I2020T \) vectors, or with the \( LRRK2 \) knockdown shRNA vector. Individual cells were followed by fluorescence microscopy at 6, 9, 12, and 15 days subsequent to transfection. This time course analysis demonstrated that overexpression of PD-associated \( LRRK2 \) mutants \( G2019S \) or \( I2020T \), but not wild-type \( LRRK2 \), led to a progressive decline in the length and branching of neurite processes (Figure 2A and Figure S3B). In contrast, soma diameter was not significantly reduced in the mutant \( LRRK2 \) transfected neurons. Time course analysis...
of LRRK2 shRNA-mediated knockdown in cortical neuron cultures indicated a gradual and progressive increase in neurite process length (Figure 2A).

At late time points, decreased neuron survival was evident in neurons that express the Parkinsonism-associated LRRK2 mutant alleles. By day 15 posttransfection, survival was significantly reduced in mutant LRRK2 transfected cortical neurons in comparison to wild-type or vector-only transfected cells, as determined by exclusion of propidium iodide staining and lack of nuclear condensation (45% survival in the G2019S versus 90% in the wild-type or vector transfected cells; p < 0.05; Figures 2B and 2C). Immunocytochemistry for activated caspase-3 demonstrated that the mutant LRRK2 G2019S-transfected cells undergo an apoptotic mechanism of cell death (Figure S3C).

Structure/Function Analysis of LRRK2 Reveals a Critical Role for the Kinase Domain

To establish a structure/function relationship of LRRK2 domains, we sought to “rescue” the shRNA knockdown allele phenotype by overexpression of LRRK2 cDNA sequences. Transfection of the LRRK2 shRNA vector along with overexpression of wild-type LRRK2 sequences effectively rescues the elongated process morphology phenotype (Figure 2D and Figure S3D). Interestingly, the kinase domain alone is sufficient for functional rescue of the knockdown shRNA phenotype. Deletion analysis revealed that the kinase domain alone is also sufficient for the shortened neurite phenotype in the context of Parkinsonism-associated LRRK2 G2019S allele expression (Figure 2E). Finally, analysis of the phenotype of primary midbrain cultures showed that LRRK2 expression similarly regulates neurite process morphology in dopamine neurons (Figure S4).

LRRK2 Mutations Induce tau-Positive Spheroid Axonal Inclusions In Vitro

Prominent spheroid-like aggregates were observed within the neuronal processes in all of the G2019S (37 out of 37) and I2020T (20 out of 20) transfected cortical neurons, but only rarely in vector alone (3 out of 85) or wild-type LRRK2 (4 out of 45) transfected cells, as determined by fluorescence microscopy for the eGFP marker (Figures 3A and 3B). The inclusions stained positively with a monoclonal antibody for the His epitope-tagged G2019S LRRK2 protein. tau protein phosphorylated at serine 202 (phospho-tau), visualized by immunostaining with a phospho-specific antibody, appeared to accumulate specifically in the spheroidal inclusions, but these structures did not stain positively with an antibody for αSyn (Figure 3A and Figure S5).

Ultrastructural analyses by electron microscopy revealed that neurons expressing the LRRK2 G2019S mutant allele, but not control vector, display abnormal accumulation of abundant electrondense structures suggestive of swollen lysosomes (Figures 3C and 3D). Additionally, multivesicular bodies (MVBs), distended

Figure 3. tau-Positive Spheroidal Inclusions in Neurons that Overexpress Mutant G2019S LRRK2

(A) Confocal microscopy for GFP marker or immunohistochemistry for the His-tag epitope on LRRK2 G2019S demonstrate the presence of spheroid inclusions within processes of all transfected cells (identical results were obtained with the I2020T mutation; data not shown). LRRK2 protein does not appear to be enriched in the inclusions relative to the GFP marker. The inclusions additionally stain with antibodies for tau phosphorylated at serine-202 (P-tau). Arrows point to inclusions.

(B) Quantification of aggregates as in (A). Aggregates are infrequent with wt LRRK2 overexpression or vector alone. Aggregates are significantly more abundant in cells expressing mutant LRRK2 than in cells expressing wt LRRK2 or vector alone (p < 0.006). wt LRRK2 expression does lead to an increase in aggregate formation relative to vector alone (p < 0.05; n = 85 cells in 13 experiments for control vector; n = 45 cells in six experiments for wt LRRK2; n = 37 cells in six experiments for G2019S LRRK2; n = 20 cells in three experiments for I2020T.

(C and D) Ultrastructural analysis of primary cortical neurons expressing LRRK2 G2019S mutant allele or control vector by electron microscopy. LRRK2-expressing cells harbor abundant electrondense structures that are suggestive of swollen lysosomes (arrow), as well as multivesicular bodies (asterisk) and distended mitochondria associated with vacuoles (arrowhead). These are absent from control vector transfected cells (data not shown). At highest magnification (D), membranes appear to surround the inclusions.

(E) Primary rat cortical cultures overexpressing the G2019S LRRK2 allele were immunostained with an antibody to LAMP1, a membrane marker for acidic organelles including lysosomes and late endosomes. Staining is apparent at neurite inclusions (arrow) and colocalizes with LRRK2.
mitochondria associated with vacuoles, and disrupted cytoskeletal structures are observed. Consistent with these findings, immunohistochemical analysis and confocal microscopy of neurons expressing the G2019S LRRK2 allele revealed prominent membranous structures that stain with antibodies for the lysosomal markers LAMP1 (Figure 4E) and cathepsin D (data not shown). A time course analysis revealed that the inclusions appear by day 6 posttransfection, coincident with the neurite process phenotype (Figure S3D).

LRRK2 Mutation in Adult Nigral Dopamine Neurons

We further analyzed LRRK2 function in adult rat substantia nigra dopamine neurons (DNs) using an adeno-associated virus-2 (AAV-2)-mediated gene transduction model. As the kinase domain of LRRK2 is sufficient to induce the G2019S-associated cellular phenotypes in vitro (Figure 2D), and because of the genome size limitation of viral vectors, we overexpressed the kinase domain alone of either wild-type or G2019S mutant LRRK2. AAV-2 vectors were stereotactically injected into the substantia nigra pars compacta within the ventral midbrain of young adult rats along with GFP vector to allow visualization of transduced cells. After 1 month, rats were sacrificed, and histological examination of the brain was performed. All analyses were performed by an observer blinded to the animal genotype. In the context of G2019S overexpression, and with wild-type LRRK2 overexpression to a lesser extent, dopaminergic axonal processes extending into the striatum displayed prominent abnormal morphology and inclusions (as identified by GFP expression and TH immunostaining; Figures 4A and 4B, n = 8 for each group), consistent with the in vitro phenotype. Immunohistochemical analysis revealed that the inclusions stained positively for phospho-tau (at serine 404) but not for aSyn (Figure 4A and Figure S6). Dopamine neurons in the substantia nigra appeared grossly normal in the context of G2019S LRRK2 expression, but apoptosis was significantly increased, as quantified by nuclear morphology and immunohistochemistry for activated caspase-3 (Figures 4A and 4B and Figure S6). Overexpression of the

Figure 4. Overexpression of LRRK2 G2019S Kinase Domain Leads to Phospho-tau-Positive Inclusions, Increased Apoptosis, and Altered Process Morphology in Rat Nigral Dopamine Neurons

(A) Adult rats were transduced unilaterally into the substantia nigra with AAV2 vectors harboring the G2019S or wild-type LRRK2 kinase domain, or empty vector, along with AAV2-GFP. Histological examination was performed at 1 month. G2019S or wild-type LRRK2 kinase domain transduction leads to the appearance of inclusions enriched in phospho-tau (at serine 404) as well as structural defects within TH-positive axons. Scale bar, 20 μm.

(B) Activated caspase-3 staining in the nucleus of TH-positive infected neurons (indicated by white arrows) was seen to increase in G2019S LRRK2 kinase domain transduced cells. Similar levels of transduction were observed in the substantia nigra for all vectors (Figure S6B); Scale bar, 100 μm in upper panels, 10 μm in lower panels. LRRK2 G2019S or wt kinase domain transduction leads to a significant accumulation of inclusions relative to control vector alone. Inclusions in the striatum (>5 μm in diameter) were quantified. LRRK2 G2019S kinase domain transduction also resulted in a significant increase in activated caspase-3 staining localized in the nucleus. n = 8 animals in each group. **p < 0.05.

(C) To examine the role of LRRK2 on brain development, cDNA for wt or mutant alleles of LRRK2, or LRRK2 shRNA, were introduced along with a GFP reporter into neural progenitor cells in E16 rat neocortex by in utero electroporation or lentiviral transduction. GFP-positive cells were examined by confocal microscopy 4 days later. Electroporation of G2019S or I2020T alleles of LRRK2 led to a significant reduction in longest process length relative to wt LRRK2 or control vector plasmid. Branch point number was reduced with overexpression of either wt or mutant LRRK2 alleles relative to the vector control. In contrast, knockdown of LRRK2 by shRNA lentiviral transduction resulted in a significant increase in process branching relative to lentiviral vector control (which is not significantly different from the plasmid vector control; data not shown). LRRK2 knockdown resulted in a small increase in the length of the longest process, but the effect was not significant (p = 0.104). Branch points are indicated by white arrows. Scale bar, 20 μm. n > 25 cells in >3 independent experiments for each group.

(D) Quantification of neuron process length and branching; *p < 0.05; **p < 0.005.
wild-type kinase domain alone led to the induction of some axonal inclusions, but to a lesser extent than the G2019S mutant.

**LRRK2 Regulates Neurite Process Morphology in the CNS**

Axonal processes appeared to be reduced in complexity in the context of G2019S expression relative to vector control in the adult rat gene transduction model (Figure S6C), but this was difficult to quantify due to the high density of neuronal processes in the intact CNS. To circumvent this, we used a technique that allows for the marking of individual genetically altered neurons within an otherwise normal CNS environment: in utero intracerebral gene transduction of rat embryos by vector injection into the lateral ventricles. Genetic manipulation of neuronal progenitors within the periventricular cell layer of E16 rat embryos can be achieved by either plasmid vector electroporation or lentiviral transduction (Tsai et al., 2005). After a 5 day period in utero, the embryos (E21) are sacrificed, and brain sections are visualized by confocal fluorescence microscopy. Electroporation of vector alone labels neuronal nuclei throughout layers 1 and 2 of the cerebral cortex that appear morphologically as neurons and are immunostained with a neuronal marker, TuJ1 (Figure S6D). Overexpression of PD-associated mutant LRRK2 alleles, G2019S or I2020T, dramatically reduced both the length and the branching of neuronal processes, relative to control vector (Figures 4C and 4D), consistent with the phenotype observed in neuronal primary cultures. In contrast, cortical neurons transduced with LRRK2 shRNA display a significant increase in the number of branch points relative to control vector-transduced cells (Figures 4C and 4D). Also, total axon length appeared to be increased in the knockdown cells, although this did not reach statistical significance. In summary, LRRK2 appears to regulate neuronal process morphology in the intact CNS, consistent with the primary culture analysis.

**Discussion**

A common feature of neurodegenerative disorders of aging is the loss of neuronal processes early in the disease course. In Parkinson's disease, loss of dopaminergic axons and terminals that project from the substantia nigra to the striatum precedes mDN cell death. Our data suggest a role for LRRK2 in the maintenance of neuronal process length and complexity in the mammalian brain. Autosomal-dominant mutations in LRRK2 lead to disinhibited kinase activity, a reduction in neuron process length and complexity, and the accumulation of tau-positive inclusions with lysosomal characteristics. Ultimately, neurons that overexpress mutant alleles of LRRK2 undergo apoptosis. Suppression of LRRK2 with shRNAs or a dominant inhibitory allele leads to an opposite phenotype of increased neurite process length and complexity.

A prominent early feature of LRRK2 disease-associated mutant neurons is the presence of inclusions that stain for phospho-tau and display abnormal lysosomal features. It is possible that the observed inclusions are not causally linked to the altered neurite process phenotype, as the role of inclusions in neurodegenerative disorders remains unresolved. However, we hypothesize that pathological lysosome inclusions may represent a proximal event in LRRK2 G2019S action given the observed time course of their appearance and the presence of LRRK2 in these inclusions. A lysosomal nidus of function would offer a potential cellular mechanism for the altered neurite morphology, as prior studies have shown that lysosomal regulatory proteins, such as VAMP7 and synaptotagmin VII, play parallel regulatory roles in neurite morphology (Arantes and Andrews, 2006; Martinez-Arca et al., 2001). Additional investigations are necessary to determine the relationship of inclusions with respect to the altered neurite morphology phenotype.

A hypothetical molecular target for LRRK2 kinase activity would be tau, as the two proteins colocalize in inclusions. Of note, tau protein is also implicated in the regulation of neurite process morphology as well as in neurodegeneration. However, we have thus far failed to detect evidence of direct phosphorylation of tau by LRRK2 in vitro (data not shown). We favor a model in which LRRK2 indirectly modifies tau through modulation of the activity of other kinases previously implicated in tau phosphorylation, such as GSK3β. Additional studies will be necessary to pinpoint the action of LRRK2 in these signaling cascades.

LRRK2 G2019S overexpression in rodent adult dopamine neurons leads to loss of nigrostriatal processes, tau-positive inclusions, and apoptosis and is thus a useful animal model for early LRRK2-associated disease. Additional studies in nonhuman primates are now feasible using the AAV-2-based vectors we describe. These cellular and animal models may promote the discovery of effective therapeutics for LRRK2-associated disease.

**Experimental Procedures**

**Vectors and In Vitro Assays**

LRRK2 cDNA constructs were generated that harbor mouse LRRK2 sequences at the 5′ terminus (NM_025730; bp 1–3738 of the coding sequence) and human LRRK2 sequences downstream (AY_792511; bp 3734–7584 of the coding sequence) and therefore would not be subject to shRNA silencing by a rodent-specific vector. The knockdown shRNA vector targets a region of the rodent LRRK2 gene (NM_025730; bases 4789–4809) that is conserved in rodents but divergent in human LRRK2 cDNA. G2019S, I2020T, K1906M, Y1699C, and R1441G LRRK2 were generated by PCR-mediated mutagenesis.

Immunoprecipitation and Western blot analysis were performed essentially as described (Staropoli et al., 2003).

For analysis of LRRK2 kinase activity toward myelin basic protein, immunoprecipitated immune complexes were incubated in kinase buffer (30 mM Tris [pH 7.5], 20 mM MgCl2, 2 mM MnCl2) in the presence of 10 μM [γ-32P]ATP, 10 mM cold ATP, and 1 μg myelin basic protein or myosin light chain protein (Sigma). Phosphorylated myelin basic protein was detected by SDS-PAGE and autoradiography.

**Cell Culture and CNS Transduction Assays**

Sprague-Dawley P1 rat primary dissociated cortical cultures were prepared and transduced essentially as described in Xia et al. (1996) with modified culture media explained below. Immunofluorescence and microscopy were performed as described (Martin et al., 2006). The following antibodies and dilutions in staining solution (1% NDS, 0.1% Triton X-100 in PBS) were then used for primary immunostaining: mouse anti-phospho-tau AT8 clone ( Fitzgerald Industries), 1:100; mouse anti-tau1 (Chemicon MAB361), 1:200; rabbit anti-cleaved caspase 3 (Cell Signaling), 1:500; mouse
anti-α-synuclein (Transduction Labs) 1:200; anti-Tetra-His (Qiagen), 1:500. Primary staining incubation times were 2 hr for phospho-his and his staining, and otherwise overnight. LAMP1 rabbit polyclonal antibody (GeneTex), 1:100; LRRK2 rabbit polyclonal antibody (Chemicon), 1:200; mouse monoclonal anti tau-1 (Chemicon), 1:100; rabbit α-P-tau (Santa Cruz; 1:200); rabbit polyclonal anti tau phosphoserine-404 (Santa Cruz), 1:200; rabbit polyclonal anti-VMAT2 (Chemicon), 1:200; sheep α-TH (Pelfreeze; 1:500); rabbit polyclonal anti-cleaved caspase-3 (Cell Signaling), 1:250. Lyso-Tracker Red DND-99 and MitoTracker Red CMXRos (Invitrogen) were used in culture medium at concentrations of 100 nM and 500 nM, respectively, for live imaging of cells on a Zeiss LSM510 Meta Confocal Microscope. Electron microscopy was performed as in Troy et al. (1992). Images were analyzed using Image-Pro Plus (Mediacybernetics) software version 5.1.0.20. Statistical analysis was performed using Statview software version 5.0. p values were obtained using Fisher’s post hoc ANOVA.

In utero gene transduction was performed essentially as in Tsai et al. (2005), and AAV-2-mediated transduction into the adult rat SN essentially as in Kirik et al. (2002). Details of the stereotactic injection procedures are described in Supplemental Experimental Procedures.

Supplemental Data
The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/52/4/587/DC1/.

Acknowledgments

References


