Brief Communications

The Parkinson Disease Protein Leucine-Rich Repeat Kinase 2 Transduces Death Signals via Fas-Associated Protein with Death Domain and Caspase-8 in a Cellular Model of Neurodegeneration

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Neurodegenerative illnesses such as Parkinson and Alzheimer disease are an increasingly prevalent problem in aging societies, yet no therapies exist that retard or prevent neurodegeneration. Dominant missense mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common genetic cause of Parkinson disease (PD), but the mechanisms by which mutant forms of LRRK2 disrupt neuronal function and cause cell death remain poorly understood. We report that LRRK2 interacts with the death adaptor Fas-associated protein with death domain (FADD), and that in primary neuronal culture LRRK2-mediated neurodegeneration is prevented by the functional inhibition of FADD or depletion of caspase-8, two key elements of the extrinsic cell death pathway. This pathway is activated by disease-triggering mutations, which enhance the LRRK2-FADD association and the consequent recruitment and activation of caspase-8. These results establish a direct molecular link between a mutant PD gene and the activation of programmed cell death signaling, and suggest that FADD/caspase-8 signaling contributes to LRRK2-induced neuronal death.

Key words: Parkinson's disease; apoptosis; neuronal apoptosis; neuronal death; neuron death; caspase

Introduction

Parkinson disease (PD) is characterized by motor and cognitive dysfunction reflecting widespread neurodegeneration, particularly of midbrain dopaminergic neurons. PD most commonly presents as a sporadic illness, but over the past decade insights into the molecular mechanisms of PD-related neurodegeneration have emerged from the discovery of mutations underlying rare inherited forms of the disease (Cookson et al., 2005). Despite these advances, the key signaling events that cause neurodegeneration in PD remain poorly defined.

One set of signaling pathways that can elicit cell death in response to cellular insults such as an accumulation of misfolded proteins, or oxidative stress (both of which have been implicated in PD), are those of programmed cell death. The core of these pathways is composed of proteolytic caspases that, when activated, lead to a highly regulated process of cell death. Two broadly defined pathways can trigger programmed cell death: the intrinsic pathway, which is controlled by factors that are released by mitochondria and activate caspase-9, and the extrinsic pathway, which is typically initiated by cell surface "death receptors" such as tumor necrosis factor receptors (TNF-R) and Fas that lead to caspase-8 activation via the death adaptor Fas-associated protein with death domain (FADD). Whereas most studies have addressed the potential involvement of the intrinsic pathway in PD-related neurodegeneration, activated caspase-8 has been observed in postmortem PD brain tissue (Hartmann et al., 2001), and modulation of the extrinsic pathway can reduce dopamine neuron loss in the MPTP mouse model of PD (Dauer and Przedborski, 2003; Hayley et al., 2004; McCoy et al., 2006). Moreover, extrinsic pathway signals are crucial mediators of inflammation, which is also postulated to mediate neurotoxicity in PD (Hirsch et al., 2005).

Dominantly inherited missense mutations in leucine-rich-repeat kinase 2 (LRRK2) are the most common genetic cause of PD, causing a clinical syndrome that is clinically and pathologically indistinguishable from sporadic PD. LRRK2 contains both GTPase and kinase signaling domains, as well as LRR and WD40 protein-protein interaction domains (Fig. 1*A*). Many potentially pathogenic sequence alterations have been identified in LRRK2 (Goldwurm et al., 2005), but five missense mutations (Fig. 1*A*)

clearly segregate with PD in large family studies (Bonifati, 2007). Two of these mutations (R1441G, R1441C) are located in the GTPase domain (termed Ras of complex proteins, or "Roc" domain), a third (Y1699C) falls in a region between the GTPase and kinase domains (termed the C-terminal of Roc, or "COR" domain), and two other mutations (G2019S and I2020T) are in the kinase domain (Fig. 1*A*). Whereas these mutations may affect LRRK2 GTPase (Guo et al., 2007; Lewis et al., 2007; Li et al., 2007) and kinase (West et al., 2005; Greggio et al., 2006; Smith et al., 2006; West et al., 2007) activity, published studies differ as to whether other mutations in LRRK2 significantly alter its kinase function (West et al., 2005; Gloeckner et al., 2006; Greggio et al., 2006; MacLeod et al., 2006; Smith et al., 2006; West et al., 2007). LRRK2 induces apoptotic neuronal death (Iaccarino et al., 2007) that requires intact kinase function (Greggio et al., 2006; Smith et al., 2006), but physiologically relevant LRRK2 substrates or downstream effectors have yet to be identified.

We demonstrate *in vitro* that LRRK2 interacts with the death adaptor protein FADD, and that this interaction recruits and activates caspase-8-dependent neuronal death. Moreover, the potential role of the extrinsic cell death pathway in PD is supported by findings in postmortem brain tissue of patients with LRRK2-associated Parkinson disease.

Materials and Methods

Cloning of human LRRK2 cDNA. A human LRRK2 cDNA was amplified and fully sequenced from HEK cell cDNA and the translated amino acid sequence conformed to human LRRK2 AAI17181 in the NCBI database. All subsequent mutations were generated using site-directed mutagenesis and all mutant clones were resequenced to confirm their accuracy.

Plasmids. LRRK2 cDNA from HEK 293 cells was cloned in pcDNA-DEST53 (Invitrogen). Cytoplasmic domains of TNFR1, TNFR3, tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R) 1, TRAIL-R2, and Fas were cloned in pcDNA27, whereas FADD, TRADD, RIP1, and RAIDD cDNA were cloned in pcDNA3.1/nV5-DEST (Invitrogen). All subsequent mutants were generated using site-directed mutagenesis, and all mutant clones were resequenced to confirm their accuracy.

Cell lines and primary neuronal cultures. CAD cells were grown in DMEM/F12 (Invitrogen) supplemented with 8% fetal bovine serum. 293T cells were grown in DMEM (Invitrogen) with 10% serum. CAD cells were transfected with Lipofectamine/PLUS, whereas 293T cells were transfected with Lipofectamine 2000 or Lipofectamine LTX (Invitrogen). Cultures of cortical neurons from E16 mice were maintained in Neurobasal medium containing B-27 supplements (Invitrogen), and transfected with Lipofectamine 2000 4 d after being plated. Primary neurons
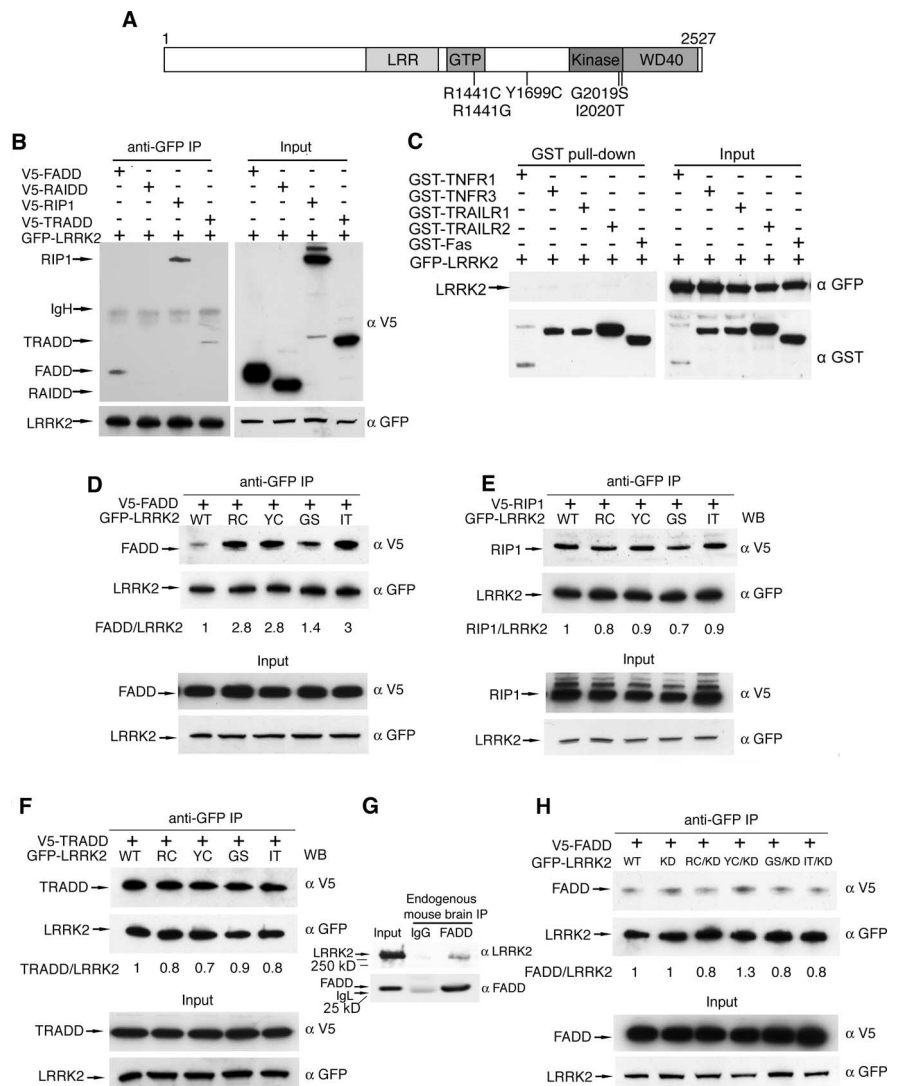

Figure 1. Parkinson disease mutations enhance the interaction between LRRK2 and FADD. *A*, Domain structure and Parkinson disease mutations of LRRK2. LRR, leucine-rich repeat; Roc, Ras of complex GTPase; COR, C-terminal of Roc. Five dominantly inherited PD-causing missense mutations are indicated. *B*, LRRK2 interacts with death adaptor proteins of the extrinsic pathway. 293T cells coexpressing GFP-LRRK2 and V5-tagged death adaptor proteins were subjected to anti-GFP immunoprecipitation followed by anti-GFP and anti-V5 immunoblotting. *C*, LRRK2 does not associate with death receptors. 293T cells coexpressing GFP-LRRK2 with GST-tagged cytoplasmic domains of death receptors were subjected to GST pull-down followed by immunoblotting. *D*, Enhanced association of FADD with LRRK2 PD mutants. 293T cells were cotransfected with wild-type (WT) or PD mutant GFP-LRRK2 and V5-tagged FADD. Anti-GFP immunoprecipitates were analyzed by anti-V5 and anti-GFP immunoblots. Ratios indicate the binding of FADD to LRRK2 relative to WT-LRRK2. *E*, PD mutations fail to enhance the association of LRRK2 with RIP1. 293T cells were cotransfected with V5-tagged RIP1 and WT or PD mutant GFP-LRRK2 and were assessed as in *D*. *F*, PD mutations fail to enhance the association of LRRK2 with TRADD. 293T cells were cotransfected with V5-tagged TRADD and WT or PD mutant GFP-LRRK2 and were assessed as in *D*. *G*, Endogenous LRRK2-FADD complex formation in mouse brain. Whole-brain lysates from 1-year-old wild-type mice were subjected to immunoprecipitation with anti-FADD (clone 7A2). Copurified LRRK2 was determined with anti-LRRK2 immunoblotting. *H*, Blocking LRRK2 kinase function prevents the enhanced FADD association with LRRK2 disease mutants. 293T cells expressing V5-tagged FADD and GFP-tagged WT or kinase-dead (KD) LRRK2 were immunoprecipitated and immunoblotted as in *D*.

were transfected with LRRK2 expression constructs and pCMS-EGFP (Clontech) at 10:1 ratio. In cotransfection experiments, LRRK2 and FADD-DD or LZ-FADD-DD expression constructs were used at a 2:1 ratio. Each experiment was performed on coverslips in triplicate, at least three times, and >100 cells/coverslip were quantified. Apoptotic neurons were defined as cells having two or more condensed apoptotic nuclear bodies visualized using DAPI.

Antibodies. Mouse anti-glutathione *S*-transferase (GST) clone GST-2 and anti-FLAG M2 were purchased from Sigma. Mouse anti-green fluorescent protein (GFP) was from Roche. Rabbit anti-GFP was from Ab-
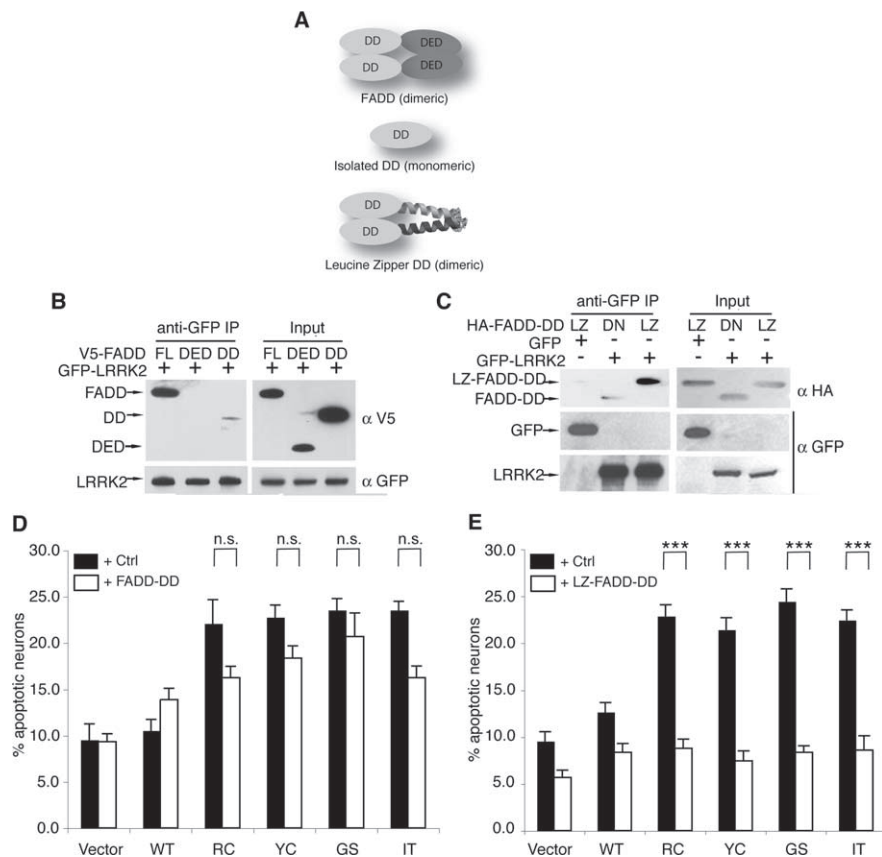
Figure 2. LRRK2-induced neuronal death requires FADD. **A**, A schematic depicts the domain structure of FADD, the isolated death domain (FADD-DD), and the leucine-zipper-DD (LZ-FADD-DD) in which the death domain is dimerized through the addition of a leucine zipper. **B**, FADD interacts with LRRK2 via its DD. GFP-LRRK2 was coexpressed with V5-tagged full-length FADD, FADD-DED, or FADD-DD in 293T cells, and was immunoprecipitated with anti-GFP. Copurified FADD or FADD domains were detected by anti-V5 immunoblotting. **C**, LRRK2-FADD interaction is enhanced by dimerization of FADD-DD. The interaction between GFP-LRRK2 and monomeric (DD) or dimeric (LZ) FADD-DD was assessed by anti-HA after immunoprecipitation with anti-GFP in 293T cells. **D**, FADD-DD is a poor inhibitor of LRRK2 neurotoxicity. Mouse cortical neurons were transfected with LRRK2 + lacZ (Ctrl) or LRRK2 + FADD-DD. A GFP reporter was cotransfected in each case. Transfected neurons displaying apoptotic nuclear morphology were counted 48 h after transfection using DAPI. Data are the mean ±SEM from three individual experiments of triplicate coverslips (n.s., nonsignificant; ANOVA with Tukey's *post hoc* test). **E**, Dimeric FADD-DD effectively blocks LRRK2 neurotoxicity. Mouse cortical neurons expressing LRRK + lacZ (Ctrl) or LRRK2 + LZ-FADD-DD were assessed as in **D** (***p < 0.001).

cam. Mouse anti-V5 was from Invitrogen. Mouse anti-FADD was from BD transduction. Rat anti-FADD clone 7A2 was a gift from A. Strasser. Rabbit anti-mouse LRRK2 was a gift from Z. Yue (Li et al., 2007). Mouse anti-HA clone F-7 and rabbit anti-caspase-1 were from Santa Cruz Biotechnology. Mouse anti-caspase-8 clone 1C12 and rabbit anti-human caspase-9 were from Cell Signaling Technology. Mouse anti-caspase-8 clone C15 was from Alexis. Rabbit anti-caspase-8 and rabbit anti-caspase-9 were from MBL.

Immunofluorescent labeling. Forty-eight hours after transfection, formaldehyde-fixed neurons on coverslips were blocked in PBS containing 0.25% Triton X-100 and 5% normal donkey serum for 30 min. Coverslips were then incubated overnight at 4°C in rabbit anti-GFP antibodies diluted in block solution. The next day coverslips were washed, incubated with FITC-conjugated secondary antibodies, and washed in PBS before mounting using Vectashield Mounting Media with DAPI (Vector Laboratories). Immunostained neurons were then subjected to quantification for apoptosis.

GST-pulldown and coimmunoprecipitation analysis. 293T cells transfected with various expression constructs were Dounce homogenized in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1–0.5% NP-40, 2 mM EGTA, 2 mM MgCl2, 10% glycerol, 1 mM sodium orthovanadate, 10 mM NaF, 25 mM β-glycerophosphate, pH 7.2, and protease inhibitors). After centrifugation and preclearing, lysates were incubated with glutathione affinity gel (Sigma) or rabbit anti-GFP antibody with protein-A

agarose for 3 h to overnight. The immunocomplexes were washed five times with isotonic or hypertonic lysis buffer (250 mM NaCl) and released from beads by boiling in 1× Laemmli sample buffer for immunoblot analysis.

RNA interference. Penetratin1 (Pen1)-coupled small interfering RNA (siRNA) were generated as described previously (Davidson et al., 2004). The target sequences used were as follows: caspase-8 GCACAGAGAGAAGAAU-GAG; caspase-9 GGCACCCUGGCUUCA-CUCU. Three days after plating, primary neurons were treated with 350 nM Pen1-siRNA for 24 h. Cells were transfected and 48 h later, assessed for apoptotic death as described above.

Results

The kinase domain of LRRK2 is most closely related to that of LRRK1, followed by the receptor interacting protein (RIP) family of serine/threonine kinases (Manning et al., 2002), crucial regulators of cell survival and death (Meylan and Tschopp, 2005; Festjens et al., 2007). RIP1, the best studied member of this family, transduces signals downstream of death receptors (e.g., TNFα, Fas ligand, TRAIL) of the extrinsic cell death pathway. This led us to explore whether LRRK2 might also participate in extrinsic cell death signaling.

We first assessed whether, like RIP1, LRRK2 can interact with death adaptor proteins of the extrinsic cell death pathway. Coimmunoprecipitation (co-IP) experiments demonstrated that LRRK2 interacts with FADD and TRADD, two key death adaptor proteins. FADD, TRADD and RIP1 all contain death domains (DD), and LRRK2 also copurified with RIP1. However, LRRK2 did not bind the DD-containing protein RAIDD that is not implicated in extrinsic apoptotic signaling (Fig. 1*B*), nor did it bind any of the DD-containing death receptors (TNF-R1, TNF-R3, Fas, TRAIL-R1, TRAIL-R2) (Fig. 1*C*). These data suggest that LRRK2 specifically interacts with a subset of DD-containing proteins that transduce extrinsic cell death signals.

To explore the potential disease relevance of these LRRK2-interacting proteins, we next tested whether their interaction with LRRK2 was altered by PD-linked mutations. We found that all mutations tested enhanced the interaction between LRRK2 and FADD (Fig. 1*C*). In contrast, PD mutations had no effect on the association of LRRK2 with RIP1 or TRADD (Fig. 1*D,E*). These findings led us to determine whether these proteins interact in brain tissue, and LRRK2 did copurify with FADD in mouse brain lysates (Fig. 1*F*). Blocking LRRK2 kinase function prevents the death of primary neurons transfected with PD mutant forms of LRRK2 (Greggio et al., 2006; Smith et al., 2006; data not shown), so we next explored whether blocking kinase function also alters FADD binding. Indeed, the kinase-deficient LRRK2 mutation (K1906R) normalized the enhanced FADD binding by caused by PD mutations (Fig. 1*G*), consistent with a potential role for this death adaptor protein in LRRK2 neurotoxicity. These data led us to hypothesize that FADD may be recruited to LRRK2,

leading to the formation of a complex similar to the death inducing signaling complex (DISC) formed by Fas, FADD and other signaling proteins.

To further explore this notion, we characterized the interaction between LRRK2 and FADD. FADD transduces death signals by binding to ligand-activated Fas via its DD and recruiting and activating caspase-8 via its death-effector domain (DED) (Fig. 2A). FADD dimerizes on binding to Fas, a crucial event that greatly enhances both the FADD-Fas interaction and caspase-8 activation. Similarly, in co-IP studies we found that FADD binds to LRRK2 via its DD, yet the interaction was weak, despite the high expression level of this isolated domain (Fig. 2B). Mimicking the dimeric conformation of physiologically active FADD-DD by attaching a leucine-zipper domain (LZ; LZ-FADD-DD) restored its interaction with LRRK2 (Fig. 2C). LZ-FADD-DD and the isolated FADD-DD are dominant negative inhibitors of FADD signaling because they lack the DED necessary to recruit caspase-8. Thus, we used these molecules to ask whether blocking FADD function affects LRRK2-induced neuronal death. The monomeric FADD-DD failed to significantly suppress the neuronal death caused by LRRK2 PD mutants, although there was a trend for death suppression (Fig. 2D). In contrast, dimeric LZ-FADD-DD completely blocked LRRK2-mediated neurodegeneration (Fig. 2E). Thus, the strength of FADD-LRRK2 interaction (Fig. 2B,C) correlates with the ability of the dominant negative FADD mutant to block LRRK2-induced cell death (Fig. 2D,E), further supporting a functional relationship between these proteins.

These data suggest that the LRRK2-FADD interaction may serve to recruit and activate caspase-8. In the absence of exogenous FADD, only a trace amount of caspase-8 copurified with LRRK2 (Fig. 3A). The expression of exogenous FADD significantly increased the amount of caspase-8 that copurified with LRRK2, suggesting that LRRK2, FADD, and caspase-8 are components of a multiprotein complex (Fig. 3A). To evaluate the function of caspase-8 in transducing the LRRK2-induced death signal, we reduced endogenous caspase-8 levels in neurons by RNA interference (RNAi). The efficiency and the specificity of this caspase-8 siRNA have been previously established in primary neuronal cultures (Davidson et al., 2004) and is effective against Fas-mediated neuronal death (Carol Troy, personal communication). Knocking down caspase-8 significantly reduced LRRK2-induced neurodegeneration (Fig. 3B). In contrast, RNAi knockdown of caspase-9, which transduces signals in the intrinsic apoptotic pathway, did not significantly attenuate LRRK2-induced neuronal death (Fig. 3C) (this siRNA is effective against HNE-induced neuronal death; Carol Troy, personal communication).
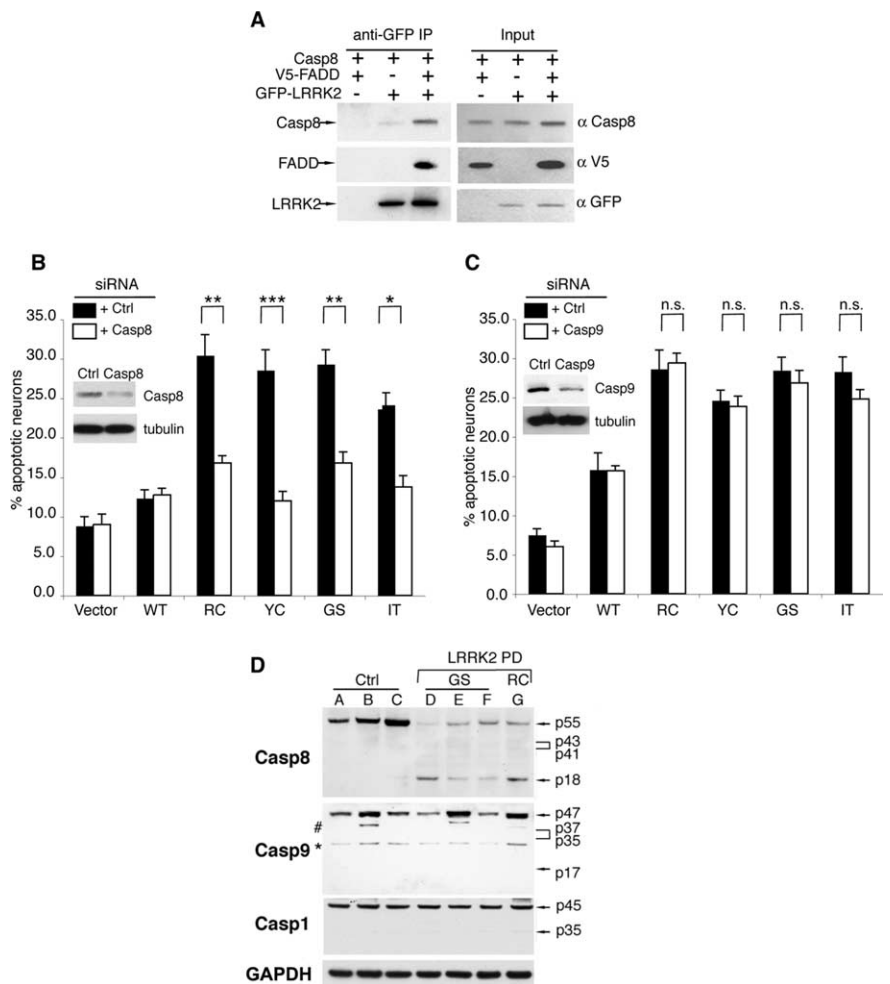

Figure 3. LRRK2-induced neuronal death is caspase-8 (Casp8) dependent. **A**, FADD recruits caspase-8 to LRRK2. 293T cells were transfected with GFP-LRRK2, V5-FADD, and caspase-8 inactive mutant (C360S), as indicated. GFP-LRRK2 was immunoprecipitated, and copurified FADD and caspase-8 were detected by V5 and caspase-8 antibodies, respectively. **B**, Knockdown of caspase-8 blocks LRRK2-induced neurotoxicity. Mouse cortical neurons were incubated with Penetratin1-linked scrambled control (Ctrl) or caspase-8 siRNA 24 h before transfection with GFP-tagged wild-type (WT) or mutant LRRK2. Data are the mean ± SEM from three individual experiments of triplicate coverslips (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). A representative immunoblot of caspase-8 levels after treatment with Penetratin1-linked siRNA is shown (inset). **C**, Knockdown of caspase-9 (Casp9) fails to prevent LRRK2-induced neuronal death. Caspase-9 RNAi, transfection, and neuronal death were performed and assessed as in **B**. **D**, Caspase-8 is selectively activated in brain tissue from patients with LRRK2 PD. Striatal lysates were analyzed by caspase-8 (clone 1C12), caspase-9, and caspase-1 (clone A19) immunoblotting. Caspase-2 was undetectable using three commercial antibodies (data not shown). The locations of the pro-caspase isoforms and their corresponding cleavage products are indicated (*nonspecific immunoreactive bands; #potential cleavage products with higher than expected mass: 37 and 35 kDa).

Our results suggest that caspase-8 activation may be a pathogenic event in PD patients with LRRK2 mutations. To further test this notion, we measured whether caspase-8 is activated in brain lysates from control subjects and PD patients with LRRK2 mutations. Caspase-8 is activated by homodimerization (Boatright et al., 2003), which also leads to autoproteolytic processing of the enzyme into multiple smaller species (43, 41, 18, and 10 kDa; the 10 kDa fragment is not detected by the commercial antibodies 1C12 or C15). The presence of one or more of these cleavage products was found in all patients with LRRK2 mutations (Fig. 3D, supplemental Table 1, available at www.jneurosci.org as supplemental material). One of two patients tested with idiopathic disease (i.e., no LRRK2 mutation) also showed low level caspase-8 activation (data not shown); more cases will be required to determine the fraction of idiopathic PD that may show activation of the extrinsic pathway. Processing of caspase-8 can

lead to decreased levels of the full-length enzyme (Yang et al., 1998), and we also found markedly decreased levels of pro-caspase-8 in the PD patients with LRRK2 mutations. In contrast, we did not observe a clear pattern of activation for the apical caspases that control the intrinsic pathway (caspase-9) or inflammation-related death signaling (caspase-1) (Fig. 3D).

Discussion

Our findings link a PD-causing gene directly to the activation of a cell death signaling pathway, and we provide direct support for this notion with evidence from human postmortem tissue from PD patients bearing two different LRRK2 mutations. We demonstrate that PD-linked mutations enhance the interaction of LRRK2 with FADD, leading to the recruitment and activation of caspase-8. Our data also show that blocking LRRK2 kinase function eliminates the increased FADD binding caused by PD mutations, providing a potential mechanism for how this mutation blocks LRRK2-mediated neuronal death.

Multiple lines of evidence support the notion that apoptotic machinery contributes to neurodegeneration in PD. For example, apoptotic nuclei have been identified in DA neurons of the substantia nigra, and many reports have documented altered levels or activation of key apoptotic molecules in PD postmortem tissue (Vila and Przedborski, 2003). Nevertheless, a longstanding question about such data is whether they indicate a key role for cell death signaling pathways, or reflect a late downstream consequence of PD-related cellular demise. Our data suggest that, at least in patients with LRRK2 mutations, activation of the apoptotic cascade may be an important early event in disease pathogenesis. The significance of these findings is highlighted by the fact that LRRK2 is the most common genetic cause of PD, but future work is necessary to determine the relevance of our findings to idiopathic PD, in which a role for LRRK2 is less clearly defined.

Two studies have implicated LRRK2 in the control of neurite morphology (MacLeod et al., 2006; Plowey et al., 2008) and, interestingly, many reports document the presence of cell death signaling molecules in neurites and synaptic terminals (Mattson et al., 1998; Chung et al., 2003; Cowan and Roskams, 2004; Carson et al., 2005). These studies indicate that caspase activation can occur locally within neuronal processes, leading to process degeneration. Particularly relevant to our findings is the study of Carson et al. (2005), who reported that caspase-8 is activated in presynaptic terminals in response to a deafferentated lesion, and is subsequently retrogradely transported to the cell body where it eventually triggers apoptotic cell death. Moreover, a number of extrinsic pathway-related molecules are concentrated within neuronal processes, and this pathway can modulate neuronal process morphology and synaptic function (Meffert et al., 2003; Boulanger and Shatz, 2004; Ma et al., 2006; Stellwagen and Malenka, 2006; Heckscher et al., 2007). Thus, our data raise the possibility that FADD/caspase-8 signaling may contribute to the neuritic pathology associated with LRRK2, and that this process leads to retrograde signaling that ultimately culminates in cell body death. Indeed, such a scenario would be consistent with studies of human PD, suggest that the midbrain dopaminergic neuron degeneration begins in striatal terminal projections and is later followed by cell body death in the substantia nigra (Scherman et al., 1989; Fearnley and Lees, 1991; Lee et al., 2000).

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