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RING FINGER PROTEIN 11 (RNF11) MEDIATES NF-κB SIGNALING AND DOPAMINERGIC NEURODEGENERATION

By

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RING FINGER PROTEIN 11 (RNF11) MEDIATES NF-κB SIGNALING AND DOPAMINERGIC NEURODEGENERATION

By
Elaine L. Pranski
B.S., Washington College, 2007

Advisor: Ranjita S. Betarbet, Ph.D.

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ABSTRACT

RING Finger Protein 11 (RNF11) mediates NF-κB signaling and dopaminergic neurodegeneration

By: Elaine L. Pranski

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by selective loss of dopaminergic neurons in substantia nigra. A number of studies have highlighted chronic inflammation, through activation of NF-κB pathway, as a critical mediator of PD pathogenesis. The A20 complex is a key regulator of NF-κB signaling outside the central nervous system, however the existence as well as the contribution of A20 complex-mediated NF-κB signaling in the brain has not been investigated. Using immunohistochemistry and quantative real-time PCR, we demonstrated that essential components of the A20 complex are present in neurons in human brain. Further, we determined that RING finger protein 11 (RNF11) was the only component of the A20 complex with reduced expression in PD. Given that RNF11 is robustly expressed in neurons and co-localizes with a population of Lewy bodies in PD patients, it became imperative for us to investigate the function of neuronal RNF11 in NF-κB signaling pathway. Luciferase assays and p65 translocation analyses were conducted to assess NF-κB activity under knockdown of RNF11 in neurons, which suggested RNF11 acts as a negative regulator of canonical NF-κB signaling. The association of RNF11, NF-κB signaling, and PD, prompted us to investigate the role of RNF11-mediated NF-κB activation on survival of dopaminergic neurons in the 6-hydroxydopamine rat model of PD. Due to the endogenous expression of RNF11 in neurons, we employed targeted modulation of RNF11 expression in dopaminergic neurons using stereotaxic injections of adeno-associated viruses (AAV2) in the substantia nigra. RNF11 overexpression enhanced dopaminergic cell death while RNF11 knockdown substantially protected against 6-hydroxydopamine toxicity. Our results suggested that RNF11 in dopaminergic cells modulates susceptibility to 6-hydroxydopamine toxicity through NF-κB-mediated responses, including induction of antioxidants and anti-apoptotic factors. Together, our *in vivo* and *in vitro* studies provide compelling support that RNF11, a component of the A20 complex, can modulate NF-κB activation in dopaminergic neurons with critical influences on PD-associated neurodegeneration. Further investigation into RNF11-mediated NF-κB signaling in context of distinct cell types and a myriad of insults is critical for understanding the contribution of RNF11 to PD pathogenesis.

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This dissertation is dedicated in loving memory of Douglas Pranski

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Abbreviations used:

6-OHDA: 6-hydroxydopamine

AAV: adeno-associated virus

AD: Alzheimer's disease

ALS: Amyotrophic lateral sclerosis

AMP: anti-microbial peptide

ANOVA: analysis of variance

AP: anteroposterior

BST1: bone marrow stromal antigen 1

CD200: cluster of differentiation 200

CD200R: CD200 receptor

COMMD1: copper metabolism gene MURR1 domain-containing protein 1

COMT: catechol-o-methyltransferase

CYLD: cylindromatosis

DLB: dementia with Lewy bodies

DGKθ: diacylglycerol kinase, theta

DV: dorsoventral

EGFR: epidermal growth factor receptor

FTD: frontal temporal degeneration

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GWAS: genome-wide association studies

GUSB: beta-glucuronidase

HLA: human leukocyte antigen

IFN: interferon

IL: interleukin

IκB: inhibitors of κB

Imd: immune deficiency

IP: immunoprecipitation

JNK: c-Jun N-terminal kinase

LRRK2: leucine-rich repeat kinase 2

MAO-B: monoamine oxidase-B

MAPT: microtubule-associated protein tau

MCP-1: monocyte chemotactic protein 1

ML: mediolateral

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetreahydropyridine

Nedd4: neural precursor cell expressed developmentally down-regulated protein 4

NEMO: NF-κB essential modulator

NF-κB: nuclear factor κB

NSAID: non-steroidal anti-inflammatory drug

NURR1: nuclear receptor related 1 protein

qRT-PCR: quantitative real-time PCR

PD: Parkinson's disease

PDLIM2: PDZ and LIM domain protein 2

PGRP: peptidoglycan recognition proteins

PIAS: protein inhibitor of activated STAT

PINK1: PTEN induced kinase 1

Pirk: poor Imd response upon knock-in

PTEN: Phosphatase and tensin homologue

RGS10: regulator of G-protein signaling 10

RIP1: receptor-Interacting protein 1

RNF11: RING finger protein 11

SEM: standard error of the mean

shRNA: small hairpin RNA

Smad: SMAD family member

Smurf: SMAD specific E3 ubiquitin protein ligase

SN: substantia nigra

SNpc: SN pars compacta

SOCS1: suppressor of cytokine signaling 1

Su(dx): suppressor of deltex

TAX1BP1: Tax1 (human T-cell leukemia virus type I) binding protein 1

TGF: transforming growth factor

TH: tyrosine hydroxylase

TLR: Toll-like receptor

TNF: tumor necrosis factor

TNFAIP3: TNF-α-induced protein 3

TNFR: TNF receptor

Traf: tumor necrosis factor receptor-associated factor

UCH-L1: ubiquitin carboxyhydrolase L1

UPS: ubiquitin-proteasome system

VM: ventral midbrain

Chapter 1

Introduction and Background

1.1 History and clinical description of Parkinson's disease

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disease that was originally called the shaking palsy or *Paralysis agitans* because of the characteristic resting tremor associated with the disease. In 1817, James Parkinson originally described PD as "... Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured" (Parkinson, 1817). Jean-Martin Charcot was more descriptive in his accounts of PD patients over 50 years later; he noted that bradykinesia was also a separate but imperative feature of the disease, as well as that there were two forms of the disease, the tremorous

and the rigid form. He was the first to use the term Parkinson's disease, dismissing the original nomenclature because PD patients do not necessarily have tremor (Goetz, 1986). Nearly 100 years later after James Parkinson's original description, Friedrich Lewy observed cytoplasmic inclusions in PD brain. This was confirmed in work by Tretiakoff, Brissaud, and Foix and Nicolesco several years later, who also noted depigmentation, gliosis, and neuronal loss in the ventral mesencephalon substantia nigra (SN) of PD patients (Goetz, 2011). In 1960, work by Enhringer and Hornykiewicz showed depletion of striatal dopamine in PD patients (Fahn, 2003), leading to the primary current treatment today, levodopa, a natural precursor to dopamine.

PD is the second most common neurodegenerative disorder after Alzheimer's disease (AD) (Van Den Eeden et al., 2003; de Lau and Breteler, 2006). In 2005, there were more than 4 million PD patients (Dorsey et al., 2007). The disease affects approximately 1% of the population over the age of 65, more than 3% of those over age 75 years, and 0.3% of the general population in the US (de Lau and Breteler, 2006; Hirtz et al., 2007; Alves et al., 2008; Shulman et al., 2011). There is a higher prevalence among males (19.0 per 100,000 in males versus 9.9 per 100,000 in females) (Van Den Eeden et al., 2003). The majority of cases are sporadic, with only 10% reporting a family history (Thomas and Beal, 2007). Therefore, the lifetime risk for developing PD is approximately 1-2%, but first degree relatives of an affected individual are 2.7 to 3.5 times more likely to develop PD than those with no family history (Elbaz et al., 2002). Also, PD is less common among African-Americans (Van Den Eeden et al., 2003). Affected individuals can frequently survive two decades or longer (Elbaz et al., 2002; Fahn, 2003) and with the

rising aging population, the prevalence of PD is expected to rise radically causing increased importance to develop therapies to impede progression of the disease.

PD can be diagnosed with high accuracy by neurologists specializing in the diagnosis and management of movement disorders (Hughes et al., 2002). As one example of diagnostic criteria, the UK Parkinson's Disease Society's Brain Bank's clinical criteria for probable PD include bradykinesia and at least one of rigidity, tremor, or postural instability, with supportive features of chronic progressive disease course, unilateral onset and asymmetry during disease course, levodopa-responsive, and late levodopa-induced dyskinesias (Jankovic, 2008). Bradykinesia is the slowness of movements with progressive loss of amplitude or speed during attempted rapid alternating movements (Marsden, 1982; Edwards et al., 2008; Jankovic, 2008; Rodriguez-Oroz et al., 2009). In addition, the full arrest of movement is termed freezing. Other clinical displays of bradykinesia are hypomimia (decreased facial expression and blinking, also called a masked face), hypophonia (softer voice), micrographia (progressively smaller handwriting), and difficulty swallowing. Rigidity is increased muscle tone felt with passive movement in both flexor and extensor muscle groups (Edwards et al., 2008; Jankovic, 2008; Rodriguez-Oroz et al., 2009). The rigidity in PD patients is described as a cogwheel motion when it coexists with resting tremor because there is ratchet-like resistance to passive movements. The resting tremor is asymmetric/unilateral, typically beginning in the arm and appearing as a rhythmic oscillatory involuntary movement when the body part is relaxed (Deuschl et al., 1998; Bain, 2007; Edwards et al., 2008). It is most evident at rest and times of stress. With disease progression, this tremor becomes more

generalized, typically moving to the lower limbs, jaw, and tongue next. The loss of postural reflexes causes a stooped posture in PD patients and contributes to the propensity to fall (Edwards et al., 2008; Jankovic, 2008; Simuni and Sethi, 2008). PD gait is slow, with short shuffling steps, decreased arm swing, and slow turning. There is also akinesia (difficulty in initiating movements). In addition, there are a number of non-motor symptoms that cause deterioration in quality of life. These include fatigue, pain, apathy, anxiety, mood disorders including depression, hallucinations, memory issues or dementia, sleep disturbances, sensory issues (such as hyposmia, the loss of the ability to smell properly), and dysfunctions in the autonomic system (including constipation and orthostatic hypotension) (Emre et al., 2007; Ziemssen and Reichmann, 2007; Poewe, 2008; Silva et al., 2008; Castelo-Branco et al., 2009; Chaudhuri and Schapira, 2009; Lim et al., 2009a; Lim et al., 2009b; Gallagher et al., 2010; Doorn et al., 2012). The non-motor symptoms of PD are believed to precede the motor symptoms by as much as 20 years (Hawkes, 2008; Hawkes et al., 2010).

To confirm the clinical diagnosis of PD, the post-mortem brain is examined for (a) loss of dopaminergic cells within the SN of the ventral midbrain (Braak et al., 2004; Lew, 2007), and (b) presence of alpha-synuclein-positive cytoplasmic protein inclusions that are called Lewy bodies in the remaining neurons (Braak and Braak, 2000; Braak et al., 2007). This pathology has been extensively characterized throughout disease progression by Braak and colleagues (Braak et al., 2003; Braak et al., 2004) and organized into six stages indicating the progression and severity of disease. In Stage I and II (the pre-symptomatic stages), initial inclusions occur within the anterior olfactory nuclei in the olfactory

tubercle, the motor nuclei of the glossopharyngeal and vagus nerve located in the medulla oblongata, and adjoining portions of the pons. In Stage III, the pathology advances to the SN pars compacta (SNpc) but is not associated with widespread cell death. Pathology extends to the cortical areas (basal forebrain, amygdala, and medial temporal lobe structures) in Stage IV. In the end stages of disease (Stages V and VI), there is degeneration of the cells in the SNpc as seen by loss of depigmentation of the SN due to the melanin normally found in the dopaminergic cells, as well as extensive Lewy body and Lewy neurite pathology throughout the cortex (Braak et al., 2003; Braak et al., 2004). Additionally, alpha-synuclein pathology has been reported in the peripheral cutaneous nerves, autonomic nervous system, enteric nervous system, spinal cord, lower brainstem, and limbic structures prior to occurrence in the cortex (Braak and Del Tredici, 2008). This staging does not hold true for all PD patients (Uchikado et al., 2006; Fujishiro et al., 2008; Beach et al., 2009) and some neurologically normal individuals will have sparse to widespread Lewy body pathology (Parkkinen et al., 2005b; Parkkinen et al., 2005a; Zaccai et al., 2008; Frigerio et al., 2011; Halliday et al., 2011), so this staging strategy should be interpreted cautiously. However, it is interesting to note that using this schematic, non-motor features including impaired olfaction and gastrointestinal tract dysfunction would be predicted to be present before classical motor symptoms of the disease, which may prove useful for early diagnostic utility (Lim et al., 2009b; Savica et al., 2009; Tolosa et al., 2009; Hawkes et al., 2010; Savica et al., 2010; Schapira and Tolosa, 2010).

The primary symptoms of PD are motor-related because the disease is a disorder of the basal ganglia, a cluster of nuclei involved in the initiation and control of motor function (DeLong and Wichmann, 2007). The pathology most significantly affects the SNpc in the ventral midbrain, causing extensive degeneration of the dopaminergic neurons found in the nuclei. The degeneration causes a loss of the neurotransmitter dopamine in the striatum, the area of primary projection of the nigrostriatal nerve terminals and subsequent alterations in signaling of the two main efferent dopamine pathways. There is decreased activity in the direct pathway, while the indirect pathway experiences increased activity, which is associated with inhibition of thalamic projections to motor cortex, reducing the excitation of the motor cortex (Hamani and Lozano, 2003; DeLong and Wichmann, 2007). In order for overt clinical symptoms to appear, approximately 60% of dopaminergic SN cells and 80% of striatal dopamine must be lost (Ehringer and Hornykiewicz, 1960; Albin et al., 1989; Crossman, 1989; DeLong, 1990; Shulman et al., 2011).

The pathological hallmarks of PD are Lewy bodies and neurites. Lewy bodies can either be established and well-organized with a dense granular material and radiating filaments of misfolded proteins 10-15 nm in diameter, or have pale staining and be poorly circumscribed (Forno and Norville, 1976; Forno, 1987; Tiller-Borcich and Forno, 1988; Galloway et al.). The former type is more common in the SN and brainstem, while the latter is seen in the cortex (Ikeda et al., 1978). Both types of Lewy bodies are eosinophilic, intraneuronal inclusion bodies that are found in the perikarya of degenerating neurons (Forno and Norville, 1976; Forno, 1987; Braak et al., 1995).

Meanwhile, Lewy neurites are thread-like or cork-screw-like inclusions found in neuronal processes. Immunohistochemistry using antibodies against alpha-synuclein is the most sensitive method to identify Lewy bodies and neurites in tissue regardless of the etiology of the disease (Wakabayashi et al., 1993; Spillantini et al., 1997; Irizarry et al., 1998; Takeda et al., 1998; Trojanowski and Lee, 1998). Alpha-synuclein is normally localized to the presynaptic terminals and thus Lewy bodies represent aberrant cytological localization (Dickson, 2012). Additionally, alpha-synuclein immunoreactive glia can be detected in midbrain and basal ganglia in small numbers (Wakabayashi and Takahashi, 1996; Wakabayashi et al., 2000). The exact components of Lewy bodies are unknown but Lewy bodies have been shown to contain ubiquitin (Kuzuhara et al., 1988; Love et al., 1988; Lowe et al., 1988; Bancher et al., 1989), molecular chaperones (Auluck and Bonini, 2002), proteasome subunits (Kwak et al., 1991; Fergusson et al., 1996; Ii et al., 1997), and proteins linked to PD like parkin (Lowe et al., 1990; Spillantini et al., 1997; Schlossmacher et al., 2002; Bandopadhyay et al., 2005). It is unknown whether Lewy bodies represent a toxic component of the pathology of PD or a compensatory mechanism to sequester toxic by-products of the disease (Gispert-Sanchez and Auburger, 2006). It is also unknown how Lewy bodies arise, but aberrant phosphorylation, truncation, and oxidative damage to alpha-synuclein are implicated (Dickson, 2012).

Currently, the treatment of PD is limited to pharmacological replacement of dopamine through levodopa and carbidopa to lessen the symptoms of the disease. If a patient is not responsive to levodopa, dopamine agonists (selegiline) may be used, as well as inhibitors of catechol-o-methyltransferase (COMT) or monoamine oxidase-B (MAO-B) (Olanow

and Stocchi, 2004). Anticholinergics and amantidine may also be prescribed (Lang and Lozano, 1998; Hristova and Koller, 2000; Marjama-Lyons and Koller, 2001). If medication does not help, neurosurgical procedures such as pallidotomy, deep brain stimulation of the subthalamic nucleus, or fetal brain transplant to the caudate nucleus may help a subset of patients (Esselink et al., 2004). None of the current treatments treat the cause of the disease because it is still remains unknown, representing the prevailing obstacle in the development of PD therapies and therapeutics.

1.2 Etiology of Parkinson's disease

The exact mechanism for the death of dopaminergic neurons in the SNpc is unknown; however, there have been a number of genetic and environmental factors associated with risk of developing PD. While the observation that PD can associate with families has been noted for a number of years (Leroux, 1880; Allan, 1937), it is unknown whether these cases represent a true genetic contribution to the disease or are a by-product of similar environmental factors from families co-existing in the same place. Over the past ten years, mutations in a number of genes have been identified as causative factors of famial PD with a Mendelian pattern of inheritance (Farrer et al., 2006; Schapira, 2006; Bekris et al., 2010; Shulman et al., 2011). The frequency of these mutations is extremely rare representing between 3-5% of PD cases (Dauer and Przedborski, 2003; Klein and Westenberger, 2012; Wu et al., 2012). However, these mutations have allowed us to gain further insight into the pathological mechanisms underlying the disease, which, in the majority of PD cases, represent an interaction between genetic and environmental factors (Olanow and Tatton, 1999). Currently there are 18 specific chromosomal regions with a

confirmed or putative link to PD (Klein and Westenberger, 2012). The regions were identified by either genetic linkage analysis of large families, the known function of the protein product of the gene at that locus, or through genome-wide association studies (GWAS) on a population. Of the confirmed loci, the contributions of alpha-synuclein, Parkin, UCH-L1, PINK1, DJ-1, and LRRK2 to disease pathogenesis have been extensively studied.

SNCA was the first gene reported with mutations that cause autosomal dominant PD (Polymeropoulos et al., 1997). *SNCA* encodes alpha-synuclein, a ubiquitously expressed 140 amino acid protein that negatively effects proteasome function and is localized to Lewy bodies in PD. Alpha-synuclein is hypothesized to have roles in learning, synaptic plasticity, vesicle dynamics, and dopamine transmission (Spillantini et al., 1997; Abeliovich et al., 2000; Lee et al., 2001; Zigmond et al., 2002). There are three missense mutations in *SNCA* that have been reported: A30P (Kruger et al., 1998), E46K (Zarranz et al., 2004), and A53T (Polymeropoulos et al., 1997; Athanassiadou et al., 1999; Spira et al., 2001; Zarranz et al., 2004; Ki et al., 2007; Puschmann et al., 2009). Additionally, over-expression of the wild-type form results in autosomal dominant PD, with reports of duplication and triplication events occurring in seventeen and three families, respectively (Chartier-Harlin et al.; Singleton et al., 2003; Farrer et al., 2004; Ibáñez et al.; Nishioka et al., 2006; Fuchs et al., 2007; Ahn et al., 2008; Brueggemann et al., 2008; Ikeuchi et al., 2008; Troiano et al., 2008; Uchiyama et al., 2008; Ibáñez et al., 2009). An increased number of *SNCA* copies has been associated with an earlier onset, more severe phenotype, and faster disease progression (Fuchs et al., 2007; Ross et al., 2008). These

mutations and multiplications increase the propensity of alpha-synuclein to aggregate into neurotoxic species (Bertoncini et al., 2005), which has been replicated with changes in oxidation and nitration (Ischiropoulos and Beckman, 2003), as well as phosphorylation (Fujiwara et al., 2002; Kahle et al., 2002). Accumulation of alpha-synuclein leads to a disruption of cellular homeostasis due to proteasome's inability to degrade it, and ultimately leads to cell death (Tofaris and Spillantini, 2005). It is believed that the Lewy body represents an attempt to rid the cell of toxic alpha-synuclein (Chen and Feany, 2005) and so the missense mutations are thought to cause PD through toxic gain of function (Bertoncini et al., 2005). Individuals with a mutation in *SNCA* have similar clinical and pathological features as those with idiopathic PD, including Lewy bodies and a response to levodopa. Interestingly, animals models using alpha-synuclein do not recapitulate all of the clinical and pathological features of PD (Masliah et al., 2000; Rochet et al., 2000; van der Putten et al., 2000; Giasson et al., 2002; Lee et al., 2002; Neumann et al., 2002; Fernagut and Chesselet, 2004; Chen and Feany, 2005; Dawson et al., 2010).

Parkin was the first gene identified causing an autosomal recessive form of the disease with onset occurring between 20 and 40 years (Kitada et al., 1998; Mata et al., 2002). *Parkin* mutations account for 10-20% of early-onset PD patients (Klein and Lohmann-Hedrich, 2007). Parkin is an E3 ligase with a number of substrates and interactors. Some mutations in *Parkin* decrease its ligase activity (Dawson, 2006) and are hypothesized to lead to a pathogenic accumulation of its substrates (Shimura et al., 2000; Shimura et al., 2001). A large number and wide spectrum of *Parkin* mutations have been detected

including point mutations or exon rearrangements causing partial or complete deletions and duplications (Kitada et al., 1998; Lucking et al., 1998; Abbas et al., 1999; Lücking et al., 2000; Hedrich et al., 2002; Kann et al., 2002; Nichols et al., 2002; West et al., 2002; Foroud et al., 2003; Mata et al., 2005). Interestingly, patients with complete *Parkin* deletion show no Lewy body or neurite pathology; however, there is gliosis and loss of dopaminergic neurons in the SNpc and locus coeruleus of these patients (Pramstaller et al., 2005), suggesting that parkin may be essential for Lewy body formation. Additionally, lack of understanding about the penetrance of *Parkin* exists due to reports of a single PARK2 mutation manifesting in an autosomal dominant manner (Klein et al., 2000; Farrer et al., 2001; Foroud et al., 2003; Sun et al., 2006). Wild-type parkin has been reported to be neuroprotective against alpha-synuclein toxicity, oxidative stress, and mitochondrial impairments (Oluwatosin-Chigbu et al., 2003; Moore, 2006). Additionally, oxidative stress is reported to cause the parkin protein to aggregate and disrupts its E3 ligase activity (Meng et al., 2011).

Ubiquitin carboxyhydrolase L1 (*UCH-L1*) is a neuronal protein that hydrolyzes ubiquitin to generate ubiquitin monomers that can be used for degradation (Doran et al., 1983; Wilkinson et al., 1989). One mutation (I93M) has been associated with autosomal dominant PD in only two cases (Healy et al., 2004a) and causes reduced enzyme activity, resulting in impaired protein clearance (Liu et al., 2002b). Molecular genetic testing has not revealed any other individuals with this mutation (or other mutations in *UCH-L1*) so it is suspected to be a coincidental polymorphism (Healy et al., 2004a; Healy et al., 2006). However, UCH-L1 has changes in sporadic PD as well. Not only is expression of

UCH-L1 decreased in PD, there are increases in the oxidized form (Lowe et al., 1990; Choi et al., 2004). Additionally, UCH-L1 is localized to Lewy bodies (Leroy et al., 1998). Given these intriguing findings, further investigation into the designation of *UCH-L1* as a familial PD-causing gene is merited.

Mutations in the *PINK1* gene are the second most common known cause of early-onset autosomal recessive PD (Ibáñez et al., 2006). Phosphatase and tensin homologue (PTEN) induced kinase 1 (PINK1) is a mitochondrial protein important for protein stability, localization, and kinase activity (Beilina et al., 2005; Petit et al., 2005) and is protective in neurons against mitochondrial dysfunction, oxidative stress, and stress-induced apoptosis (Deng et al., 2005; Pridgeon et al., 2007). There are 10 reported mutations in *PINK1*, with mutations being missense or nonsense and the most frequent mutation (40%) being Q456X (Pankratz et al., 2006). Two-thirds of the mutations in *PINK1* disrupt its kinase activity, impairing the clearance of damaged mitochondria (Youle and Narendra, 2011). Complete deletions or heterozygous whole-gene deletions exist as well but are rarer (Li et al., 2005b; Marongiu et al., 2007; Cazeneuve et al., 2009). Cases with *PINK1* mutations have varied features (Valente et al., 2001; Valente et al., 2002), representing approximately 1-9% of early onset cases depending upon the ethnic group examined (Hatano et al., 2004; Healy et al., 2004b; Rogaeva et al., 2004; Valente et al., 2004; Bonifati et al., 2005; Klein et al., 2005; Li et al., 2005b). Further identification of the substrates of PINK1 will be valuable to understand the role of mitochondria in the regulation of cell survival in PD.

DJ-1 was the third gene associated with autosomal recessive PD. Cases with *DJ-1* mutations are early-onset, representing approximately 1% of early onset cases (Dekker et al., 2003; Lockhart et al., 2004). DJ-1 is a ubiquitously expressed protein that is a molecular chaperone acting in the mitochondria to regulate transcription, protein quality, and coordination of oxidative stress responses (Tao and Tong, 2003; Canet-Avilés et al., 2004; Goldberg et al., 2005; Junn et al., 2005; Kim et al., 2005b; Kim et al., 2005a; Moore et al., 2005; Zhang et al., 2005a). Mutations in *DJ-1* can be homozygous deletions (Bonifati et al., 2003a), compound heterozygous deletions (Hague et al., 2003), or missense mutations (such as L166P) (Bonifati et al., 2003b; Olzmann et al., 2004). Most of the disease-causing mutations make DJ-1 less stable and promotes its degradation to low or absent (Bonifati et al., 2003a; Huai et al., 2003; Olzmann et al., 2004; Anderson and Daggett, 2008; Malgieri and Eliezer, 2008). Evidence suggests that DJ-1 is protective against reactive oxygen species (Thomas and Beal, 2007), but its mechanism of action is unknown.

Mutations in *LRRK2* are the most frequent known cause of late-onset autosomal dominant PD with a frequency of 2 -40% of famial PD cases in different populations (Di Fonzo et al., 2005; Gosal et al., 2005; Lesage et al., 2005b; Lesage et al., 2005a; Lesage et al., 2006; Ozelius et al., 2006; Ferreira et al., 2007). Leucine-rich repeat kinase 2 (LRRK2) is a ubiquitously expressed protein that is hypothesized to regulate signal transduction cascades (Zimprich et al., 2004). Patients with *LRRK2* mutations progress slowly and show inconsistent neuropathology, with patients having nigral degeneration with and without Lewy bodies (Giasson et al., 2006). There are more than 50 reported

missense and nonsense mutations, with at least 16 being pathogenic (Nuytemans et al., 2010) and the most common mutation being G2019S. This mutation is found in approximately 5-7% of familial PD cases (Di Fonzo et al., 2005; Gilks et al., 2005; Nichols et al., 2005).While some mutations in *LRRK2* have been reported to increase its kinase activity (MacLeod et al., 2006; Klein and Lohmann-Hedrich, 2007), not enough is known about the normal function of *LRRK2* to determine a pathogenic mechanism (Mata et al., 2006; Cookson, 2010).

Genes with a Mendelian pattern of inheritance have been primarily identified through usage of inheritance studies of genetic linkage in families. However, with diseases with complex phenotypes such as PD, the results of genetic linkage studies are not only difficult to reproduce but also do not fully explain the causes of disease. An alternative to linkage studies are genetic association studies, in which allelic variants are compared between those with the phenotype of interest and controls. This approach allows for detection of low-penetrance alleles (Hardy and Singleton, 2009). Five large PD GWAS studies were reported between 2009 and 2011, with each including several thousand PD and control subjects and yielding high statistical power (Satake et al., 2009; Simon-Sanchez et al., 2009; Hamza et al., 2010; Saad et al., 2011; Spencer et al., 2011). Among the loci listed as associated with PD in these reports are *LRRK2, MAPT, PARK16, SNCA, bone marrow stromal antigen 1 (BST1), Diacylglycerol kinase, theta (DGKθ), HLA* locus, and *GWA_12q24*. Futhermore, a meta-analysis of all reported PD GWAS was conducted in 2012, with nearly all of these loci showing genome-wide significant results in the meta-analyses (Lill et al., 2012). Interestingly, a number of the loci identified by

traditional linkage analysis were also identified with the more complex GWA analysis, connecting the etiology of sporadic and familial PD. Recently it was reported that the effect size for some of these loci (notably *BST1* and *MAPT*) differs greatly with ethnicity, while some (such as *SNCA* and *LRRK2*) have similar effects, indicating the role of population-specific heterogeneity in PD (Sharma et al., 2012). Two of these loci, *MAPT* and the *HLA* locus, will be discussed further.

The microtubule-associate protein Tau gene (*MAPT*) encodes a protein (tau) that forms filamentous inclusions in a number of neurodegenerative diseases, including AD, frontotemporal dementia (FTD), and other tauopathies (Hutton et al., 1998; Pittman et al., 2006; Ballatore et al., 2007). Recently, homozygosity at the H1 haplotype at the *MAPT* locus was found to increase risk of PD by nearly 50% (Zabetian et al., 2007). This association has been confirmed in several GWAS using subjects of European descent (Pankratz et al., 2009; Simon-Sanchez et al., 2009; Edwards et al., 2010), but has not been able to be replicated in Japanese subjects (Satake et al., 2009). While tau is essential for axonal transportation in neurons (Garcia and Cleveland, 2001), the only known link between tau and PD is the promotion of polymerization of alpha-synuclein by tau (Giasson et al., 2003).

The *human leukocyte antigen (HLA)* locus contains genes encoding the major-antigen-presenting proteins and other immune-related proteins. The HLA system, also called major histocompatibility complex in other vertebrates, is associated with a number of autoimmune disorders such as multiple sclerosis (Lincoln et al., 2009; Handel et al.,

2010) due to its role in immunosurveillance within the body (Ouchi et al., 2009). Specifically within PD, HLA-DR positive microglial cells contribute to the neuroinflammation in the SN of brains of PD patients (McGeer et al., 1988). The strong association between variants in the *HLA* locus and PD risk thus implies a role for the immune system in PD etiology (Hamza et al., 2010; Nalls et al., 2011; Simon-Sanchez et al., 2011). Initial fine-mapping within this locus has revealed that are four SNPs within the region that are not correlated with each other, suggesting multiple PD-*HLA* associations from either different alleles of the same gene or separate loci (Hill-Burns et al., 2011).

Despite the new putative PD genes described, approximately 40% of the risk for PD remains unexplainable (Lill et al., 2012). Implementation of GWAS for PD is not up to the same caliber as GWAS performed on other diseases that typically include tens of thousands of subjects. With additional subjects, variants with lower allele frequency can be identified as well as fewer false positives that would be advantageous for determining common and rare variants that could cause as well as modify disease risk. Additionally, many of the susceptibility loci uncovered by GWAS contain more than one putative underlying gene, indicating that additional work is necessary for a complete description of the risk factors for PD. An exciting next step in the study of the genetics of human disease will be the use of exome or genome sequencing, which is currently cost-prohibitive with the current large-scale studies needed.

Along with the known genetic factors, PD cases predominantly result from unknown causes and are thus deemed "idiopathic." In these cases, there may or may not be an identifiable environmental cause, as well as the contribution of the factor may be indeterminable. The environment was hypothesized to significantly contribute to PD even before genetic components of the disease were identified (Tanner and Langston, 1990). A permanent Parkinsonian syndrome with severe symptoms observed in a young group of intravenous drug users who had inadvertently injected the narcotic derivative and dopamine neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetreahydropyridine (MPTP) was the first strong implication of environmental contributions to PD (Langston et al., 1983). Furthermore, numerous epidemiological studies have associated exposure to classes of pesticides and environmental contaminants as major risk factors for PD, although with heterogeneous results (Ho et al., 1989; Tanner and Langston, 1990; Semchuk et al., 1991, 1992; Fleming et al., 1994; Tanner and Goldman, 1996; Hubble et al., 1998; Le Couteur et al., 1999; Corrigan et al., 2000; Priyadarshi et al., 2001; Brown et al., 2005; Li et al., 2005a; Ascherio et al., 2006; Steenland et al., 2006). These classes include organochlorines, rotenone, pyrethins/pyrethroids, organophosphates, paraquat, polychlorinated biphenyls, heptachlor, and dieldrin.

It can also be ambiguous if the underlying disease is truly PD or if it is a Parkinsonian-like disease. Other etiologies of Parkinsonism include vascular (vascular parkinsonism) (Critchley, 1929), drug-induced (anti-psychotic medications) (Montastruc et al., 1994), infectious (influenza A, lipopolysaccharide, and *Nocardia asteroides*) (Hudson and Rice, 1990; Casals et al., 1998; Takahashi and Yamada, 2001; Tam et al., 2002; Niehaus and

Lange, 2003), toxic (MPTP, manganese poisoning), and structural brain lesions (Massano and Bhatia, 2012). Finally, there are also Parkinson's plus and atypical forms of PD, such as corticobasal degeneration, progressive supranuclear palsy, and multiple systems atrophy, that have rapidly occurring neurodegeneration and distinguishable atypical symptoms from PD.

Hypotheses for several mechanisms that could contribute to the observed PD pathophysiology have been drawn from research using cellular and animal models of PD. Evidence suggests that PD has a multi-factorial pathogenesis with an elaborate interplay of genetic factors, modifying effects of susceptibility alleles, environmental exposures, and gene-environment interactions culminating in the dysfunction of several cellular processes including oxidative and nitrative stress, mitochondrial dysfunction, excitotoxicity, proteolytic impairment, and inflammation. In many cases, these factors cause interrelated events and it is difficult to determine which is the causative factor and the mediating factor leading to dopaminergic death through apoptosis (Tatton and Chalmers-Redman, 1998; Hirsch, 1999; Tatton, 2000; Jellinger, 2001). While production of free radicals occurs normally during synthesis of neurotransmitters and mitochondrial respiration, increased amounts of oxidative and nitrative stress markers are also observed in cases of cell loss and protein aggregation (Parker et al., 1989; Sofic et al., 1992; Owen et al., 1996; Alam et al., 1997; Jenner, 2003; Choi et al., 2006). Due to the excessive free radical generation from the enzymatic process of producing dopamine, dopaminergic neurons are especially sensitive to oxidative stress (Graham et al., 1978). Indeed, by-products of oxidative stress are higher in PD patients than in controls (Sofic et al., 1988;

Dexter et al., 1989). Mitochondrial dysfunction can lead to not only energy depletion, but production of additional oxidative stress in the cell (Schapira et al., 1992; Greenamyre et al., 2001; Schapira, 2008). It can also lead to enhanced susceptibility to the toxic effects of excitotoxicity (Rodriguez et al., 1998). Alterations in striatal dopamine in PD cause disinhibition of the subthalamic nucleus, resulting in excessive glutamergic input on the SNpc dopaminergic neurons. This leads to substantial calcium influx and cell death (Blandini et al., 1996; Greene and Greenamyre, 1996; Beal, 1998; Rodriguez et al., 1998). The ubiquitin-proteasome system (UPS) is responsible for the degradation of cytosolic proteins that are damaged or no longer needed by poly-ubiquitination as well as diverse signaling cascade generated by mono-ubiquitination of proteins (Ciechanover and Brundin, 2003; Hicke and Dunn, 2003). When the proteasome becomes impaired, there can be a toxic build-up of proteins in the cytosol, as well as aberrant signaling, which can both lead to cell death (Petrucelli and Dawson, 2004; McNaught et al., 2006; Olanow and McNaught, 2006). Lastly, inflammation that is not correctly regulated can lead to excessive production of cytokines, chemokines, and other inflammatory mediators that can be detrimental to cellular health and lead to increased free radical production (McGeer and McGeer, 1998; Liu and Hong, 2003; Wersinger and Sidhu, 2006; Stone et al., 2009; Kosloski et al., 2010).

1.3 Inflammatory signaling in Parkinson's disease

The brain exists in a relatively immunosuppressed state under normal conditions due to the protection of the blood brain barrier (Lassmann et al., 1991). However, inflammation is one of the first responses of the central nervous system to injury. Microglia are the first

cell type to respond to injury or immunological stimuli and act as the macrophages of the central nervous system (Ransohoff and Perry, 2009). In addition, there are two other types of glial cells- oligodendrocytes, specialized cells that form the myelin sheath around nerve fibers, and astrocytes, cells involved in the formation of the blood brain barrier, generation of metabolic substrates for neurons, and maintenance of neuronal homeostasis (Jessen, 2004; Ransohoff and Perry, 2009).

Microglia exist in two states, resting and activated. Resting microglia have a ramified morphology and extend their processes to survey the extracellular space in the central nervous system (Nimmerjahn et al., 2005). Upon activation directly by pathogens or foreign material or indirectly through pro-inflammatory signals secreted by other cells (Liu and Hong, 2003), microglia have an amoeboid morphology with nuclei hypertrophy and elongated processes. When activated, microglia provide protection to healthy cells by phagocytosing cellular debris, pathogens, and foreign material; secreting pro-inflammatory cytokines and reactive nitrogen and oxygen species; and up-regulating expression of surface membrane receptors, such as major histocompatibility class I and II (Nimmerjahn et al., 2005).

Similar to microglia, astrocytes play a protective role in the brain when activated by pro-inflammatory cytokines (Jessen, 2004). Astrocytes create a physical barrier between healthy and injured neurons and tissue through proteaglycan deposition and can also repair the blood brain barrier (Ransohoff and Perry, 2009; McGann et al.). Astrocytes can

also scavenge and neutralize compounds that may contribute to oxidative stress (Jessen, 2004; Molofsky et al., 2012).

While microglia are imperative for development and homeostasis of the central nervous system, chronic activation and dysregulation can injure neurons and is associated with neurodegenerative diseases (Block et al., 2007; Mena and García de Yébenes, 2008). Microglia release anti- and pro-inflammatory cytokines, immune-responsive peptides that include interleukins (IL), interferons (IFN), and tumor necrosis factor-alpha (TNF-α) (Allan and Rothwell, 2001; Rock et al., 2004; Perry, 2010), neurotrophic and neurotoxic factors, and neurotrophins (Perry, 2010). Additionally, they generate free radicals that can promote degeneration. In cases of chronic activation, the beneficial and protective effects of microglial secretion are negated and contribute to neurodegeneration (Rock et al., 2004). Excessive inflammation and increased expression of pro-inflammatory molecules have been detected in post-mortem brains of patients with neurodegenerative disease and are thought to contribute to the disease process. For example, increased activation of microglia and astrocytes is observed in the SN of post-mortem PD tissue (Akiyama et al., 2000; McGeer and McGeer, 2004, 2008). Additionally, increased expression of pro-inflammatory cytokines (i.e., TNF-α, IL-6) is observed in the SN and putamen of post-mortem PD brains (Sawada et al., 2006; McGeer and McGeer, 2008). Furthermore, non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to both decrease the risk of developing PD in epidemiological studies (Chen et al., 2005), and to decrease dopaminergic degeneration and mitochondrial dysfunction in a rat model (Hunter et al., 2007; Hunter et al., 2008). Moreover, in rats, TNF inhibitors protected

degeneration of dopaminergic neurons in two separate rat PD models (McCoy et al., 2006; McCoy et al., 2008).

The role of inflammation as both a precursor and secondary factor of neurological damage and disease has been well-documented (Ringheim and Conant, 2004; Rosenberg, 2005; Tzeng et al., 2005; Hoozemans et al., 2006; Kim and Joh, 2006; Zipp and Aktas, 2006; Teismann, 2012). Peripheral immune responses can trigger as well as exacerbate a number of neurological diseases, including PD (Hasegawa et al., 2000; Arai et al., 2006) due to the breakdown of the blood brain barrier. Neuronal damage can lead to secondary activation of microglia (reactive microgliosis) and produce additional toxic factors and damage in a self-propelling cycle (Liu and Hong, 2003; Block et al., 2007). Additional concerns about inflammation exist because of the low regenerative capacity of neurons due to the majority being post-mitotic (Wekerle et al., 1986). The nuclear factor κ B (NF-κB) pathway has been identified as a key mediator of inflammation and therefore warrants further investigation as an important target for drug development and discovery if activation of the pathway can be controlled in a cell-type specific manner (Barnes, 1997; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001; Aggarwal, 2004; Kumar et al., 2004).

NF-κB signaling

NF-κB is a family of transcription factors that play a role in many immunological processes (Baldwin, 1996; Townsend and Praticò, 2005), as well as neuronal development and synaptic signaling that underlies learning and memory (Hayden and

Ghosh, 2008; Sun and Ley, 2008). There are five subunits (p52/NF-κB_2, p50/NF-κB_1, RelB, c-Rel, and RelA/p65) that form heterodimers and homodimers. These transcription factors share an N-terminal Rel homology domain (Hayden and Ghosh, 2008). Differences in dimer interfaces determine binding partner preference (Hoffmann et al., 2006). The p50 and p65 heterodimers are the most prevalent activated form of NF-κB in mammalian cells (Karin and Ben-Neriah, 2000). They are found in virtually all cell types, including immune cells and the cells of the central nervous system (Baeuerle and Baltimore, 1996).

NF-κB is a fast-acting transcription factor because it is always present in an inactive state in the cell and can this respond quickly to tissue infection, injury, or trauma. Activators of NF-κB include bacterial products, viral proteins, pro-inflammatory cytokines, neurotransmitters (such as glutamate), and reactive oxygen and nitrogen species (Ghosh and Karin, 2002; Bonizzi and Karin, 2004). Different stimuli activate different signaling cascades for NF-κB activation, with the most common pathway being the canonical NF-κB pathway. In this pathway (see Fig. 1-1), activation of NF-κB transcription factors is dependent upon the associations with inhibitor of κB (IκB) proteins (Baeuerle and Henkel, 1994; (Baeuerle and Baltimore, 1996). Activation of IκB kinases [IκKα, IκKβ, and NF-κB essential modulator (NEMO)/ IκKγ] results in the phosphorylation of the inhibitory IκB (IκBα, IκBβ, and IκBε) proteins bound to NF-κB to sequester them in the cytoplasm (Karin and Ben-Neriah, 2000). This leads to K63-linked polyubiquitination and subsequent degradation of the IκB proteins. NF-κB is consequently released and translocates to the nucleus (due to their nuclear localization sequence) where it induces

the expression of many genes involved in cell proliferation and development, apoptosis, survival, and inflammatory responses (involved in both adaptive and innate immune responses such as cytokines, chemokines, adhesion molecules, and acute phase proteins) (Memet 2006, (Baeuerle and Baltimore, 1996). Differences in the solvent surface of these transcription factors mediate preferences in DNA sequence binding (Hoffmann et al., 2006).

While prompt activation of NF-κB signaling is necessary for an effective immune response, prolonged NF-κB activity must exist to prevent neuronal stress and ultimately cell death (Karin and Greten, 2005). Persistent NF-κB activation is associated with acute (i.e., stroke, seizures) and progressive (i.e., PD, AD, and amyotrophic lateral sclerosis (ALS)) neurodegenerative disease (Glass et al., 2010). For example, increased nuclear localization of p65 is observed in the SN of PD patients (Hunot et al., 1997). Additionally, dysregulation of NF-κB signaling and subsequent aberrations in spine density are hypothesized to cause synaptic pathology observed in PD and AD. Thus, regulation of the duration of NF-κB activation is under tight control and repressed in basal conditions (Verstrepen et al., 2010). There are several layers of regulation that overall either converge upon NF-κB transcription factor or receptor proximal events.

Regulation of NF-κB transcription factors is controlled by IκB proteins, copper metabolism gene MURR1 domain-containing protein 1(COMMD1), PDZ and LIM domain protein 2 (PDLIM2), and protein inhibitor of activated STAT (PIAS). As previously mentioned, the IκB proteins are responsible for retaining NF-κB dimers in the

cytoplasm. Additionally, IκBα is an NF-κB target gene. Newly synthesized IκBα enters the nucleus to promote the clearance of NF-κB dimers (Karin and Ben-Neriah, 2000). COMMD1 and PDLIM2 are also involved in the nuclear degradation of NF-κB dimers (Maine et al., 2007). As a component of the EC2S multi-subunit E3 ligase complex, COMMD1 is important for the recruitment of suppressor of cytokine signaling 1 (SOCS1) to the complex (Ryo et al., 2003; Natoli and Chiocca, 2008). SOCS1 is responsible for the sequence recognition of NF-κB dimers and is imperative for their degradation. PDLIM2 also works as an E3 ligase for p65 degradation (Tanaka et al., 2007) and is hypothesized to work in signal and cell-type specific manners from COMMD1(Vallabhapurapu and Karin, 2009). In addition to altering the degradation of nuclear p65, other negative regulators affect the function of the NF-κB transcription factors. PIAS binds to p65 to prevent its binding to DNA (Liu et al., 2005). PIAS is only responsible for negative regulation of a subset of NF-κB target genes (Tahk et al., 2007).

Regulation at the level of the receptors related to NF-κB signaling is conferred by cylindromatosis (CYLD) and TNF-α-induced protein 3 (TNFAIP3) or A20. CYLD is responsible for the deubiquitination of K63 linkages on NEMO and Traf2, crucial aspects of the c-Jun N-terminal kinase (JNK) and NF-κB signaling activation (Kovalenko et al., 2003; Trompouki et al., 2003). Thus, with absence of CYLD and TNF-α stimulation, increased ubiquitination of Traf2 and more rapid degradation of IκBα is observed, leading to enhanced NF-κB signaling (Massoumi et al., 2006). Interestingly, in addition, mice with dysfunctional CYLD express high levels of NF-κB transcription factor

precursors but, because the enzymatic proteolytic process is not altered, the actual level of expression of NF-κB transcription factors is not changed (Hovelmeyer et al., 2007).

The last reported negative regulator of canonical NF-κB signaling is A20, an NF-κB inducible gene that mediates activation of the pathway in a negative feedback loop (Krikos et al., 1992; Verstrepen et al., 2010) (see Fig. 1-2). A20 is both an E3 ligase and deubiquitinase. To mediate its inhibitory function, A20 first removes the K63-linked polyubiquitin linkages from receptor-Interacting protein 1 (RIP1) and then adds K48-ubiquitin linkages to RIP1 to target it for proteasomal degradation (Verstrepen et al., 2010). In order to fulfill its role, A20 forms a complex with three other proteins: Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BP1), and two other E3 ligases, Itch and RING finger protein 11 (RNF11). The interaction of A20 with these proteins is critical for its function (Shembade et al., 2007; Shembade et al., 2008; Shembade et al., 2009).

RNF11

RNF11 was first identified by the Saito group who were identifying novel RING finger proteins from a human neuroblastoma cDNA library in order to identify novel genes that were essential to the initiation and development of sporadic breast cancer (Burger et al., 1998; Seki et al., 1999; Kitching et al., 2003). Excluding one similar amino acid change, the cDNA for RNF11 encodes a 154-amino acid polypeptide with a consensus RING-H2 protein domain (Kitching et al., 2003) that is the same in human and mouse (Seki et al., 1999). Full-length homologues of the protein have also been identified in the DNA of

zebrafish (*Danio rerio*), the pufferfish (*Fugu rubripes*), the African clawed frog (*Xennopus laevis*), the Western clawed frog (*Silurana tropicalis*), and the fruitfly (*Drosophila melanogaster*) (Kitching et al., 2003). RNF11 contains an N-terminal PPxY motif that binds WW-domain containing proteins, such as Itch, neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4), and SMAD specific E3 ubiquitin protein ligase 1/2 (Smurf1/2) (Sudol et al., 1995; Kitching et al., 2003). In addition to its role in inactivation of the NF-κB signaling pathway (Shembade et al., 2009), RNF11 is important for the activation of the transforming growth factor (TGF)-β and epidermal growth factor receptor (EGFR) signaling pathways through associations with Smurf2 and SMAD family member 4 (Smad4) (Li and Seth, 2004; Azmi and Seth, 2005; Chen et al., 2008; Azmi and Seth, 2009). While RNF11 has confirmed up-regulation in breast cancer that is related to its role in the TGFβ and EGFR signaling pathways (Kitching et al., 2003; Subramaniam et al., 2003), the role of RNF11 in the brain is not as clearly defined. RNF11 is highly expressed throughout the brain in mainly neuronal populations (Anderson et al., 2007). In PD, RNF11 localizes to α-synuclein-positive Lewy bodies in the cortex and SN (Anderson et al., 2007). Additionally, *RNF11* is a candidate gene at the PARK10 locus, a region linked to increased incidence of sporadic PD in an Icelandic population (Hicks et al., 2002).

Due to the involvement of dysregulation of NF-κB signaling in PD pathology, additional work concerning the existence as well as the role of the A20 ubiquitin editing protein complex in regulation of NF-κB signaling in neurons, in normal and diseased brain, is warranted. Specifically, I was interested in the role of RNF11 due to the connections of

this protein with PD. This study could garnish the foundation for future work in developing novel therapeutics for PD.

1.4 Thesis Outline

In this thesis, I intend to identify the contribution of neuronal RNF11, as a member of the A20 ubiquitin-editing protein complex, to regulation of NF-κB signaling. I hypothesized that neuronal RNF11 would be a negative regulator of NF-κB signaling and that this function would be dependent upon proper localization of RNF11 within the cell. Furthermore, I tested the second hypothesis that manipulation of neuronal NF-κB signaling through RNF11 expression would modulate protection of dopaminergic cells in an *in vivo* model of PD. The results of these studies are laid out in four chapters (Chapters 3-6) that follow a common methods chapter (Chapter 2).

In Chapter 3, I determined the expression of RNF11 and other A20 ubiquitin-editing protein complex members in human brain as a step towards better understanding potential regulatory mechanisms of NF-κB signaling in the brain. I found that the essential components of the A20 ubiquitin-editing protein complex are present in human brain and mainly expressed in neurons. In addition, the A20 ubiquitin-editing protein complex members are differentially expressed throughout the brain. Lastly, in cases of PD, the expression of RNF11 is specifically reduced. This suggests a potential role that RNF11 could play in modifying disease progression in conditions with an inflammatory component.

In Chapter 4, I set out to identify the function of neuronal RNF11 in the NF-κB signaling pathway. I found that neuronal RNF11 negatively regulates canonical NF-κB signaling. Additionally, reduced or functionally compromised RNF11 could result in persistent NF-κB activation and chronic up-regulation of inflammatory responses. These results highlight the function of neuronal RNF11 and emphasize its potential role in neurodegenerative disease.

In Chapter 5, I tested whether CG32850, a gene with unknown function in *Drosophila melanogaster* and the *Drosophila* homologue of RNF11, functioned similarly to mammalian RNF11 as a negative regulator of immunity pathways of the fruit fly. CG32850 was found to be functionally homologous to mammalian RNF11. In addition, aberrations in susceptibility to death following the immunity paradigms were seen with knockdown of RNF11. These results identify RNF11 as a negative regulator of NF-κB signaling in a number of cell types, and suggest that RNF11 is necessary for proper signaling of cytoprotective processes.

In Chapter 6, I tested the hypothesis that activation of neuronal NF-κB signaling is important for protection of dopaminergic neurons in an *in vivo* model of PD. Using AAV2 to specifically target nigral neurons for manipulation of RNF11 expression, I looked at protection of dopaminergic neurons from the neurotoxin 6-hydroxydopamine (6-OHDA). Decreasing RNF11 expression *in vivo* was protective in the 6-OHDA model and also caused an up-regulation of NF-κB-induced genes. Conversely, over-expression of RNF11 *in vivo* enhanced death of dopaminergic neurons and dampened the

transcription of NF-κB associated genes. Lastly, through work in a cell culture model, I show that this protection is dependent on NF-κB activation of antioxidants and pro-survival factors.

Taken together, this thesis supports the characterization of RNF11 as a gene important for the pathogenesis of PD. In this dissertation I confirmed the *in vitro* role of neuronal RNF11, as well as the *in vivo* function of *Drosophila* RNF11 and mammalian RNF11, as a negative regulator of the NF-κB pathway. This work is the first to describe the A20 ubiquitin-editing protein complex in the normal and diseased brain, as well as the potentiating effect of neuronal NF-κB signaling through the A20 ubiquitin-editing protein complex on dopaminergic cell death in the 6-OHDA model. These results support further investigation of RNF11's role in NF-κB signaling, as well as other models of PD.

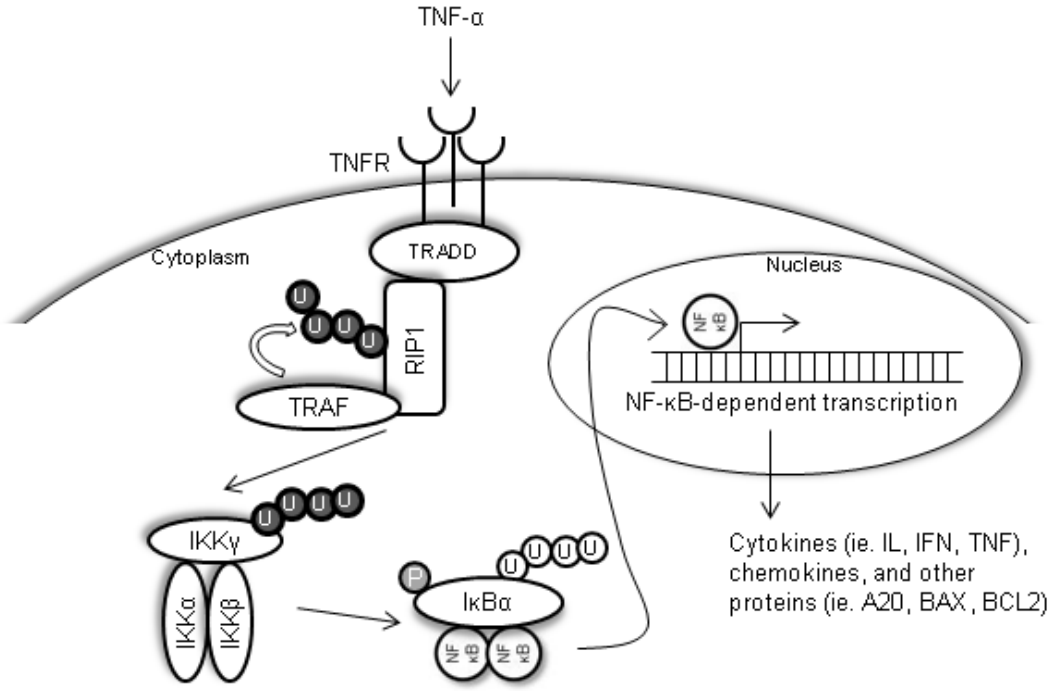


Figure 1-1. Stimulation of the canonical NF-κB signaling pathway by TNF-α

Stimulation of the canonical NF-κB signaling pathway begins with TNF-α binding with TNFR on the membrane of the cell. This binding causes the recruitment of TRADD and RIP1 to the cytoplasmic tail of TNFR. The TRAF family of proteins is responsible for interacting with this complex and causes the K63-linked polyubiquitination of RIP1. RIP1 then interacts with the IKK complex, comprised of IKKα, IKKβ, and IKKγ. IKKγ becomes K63-linked polyubiquitinated, which causes downstream interactions with IκBα. IκBα is an inhibitory protein that binds with NF-κB homodimers and heterodimers to keep them in an inactive state in the cytoplasm. IκBα becomes phosphorylated as well as K48-linked polyubiquitinated as a result of interactions with IKKγ. These K48-linked ubiquitin chains target IκBα to the proteasome for degradation, thus rendering the NF-κB homodimers and heterodimers that IκBα was bound to in a state where they can

translocate to the nucleus. Upon translocation, NF-κB subunits bind to specific NF-κB binding sites within DNA to influence transcription of genes that encode cytokines, chemokines, and other proteins important for immune reactions.

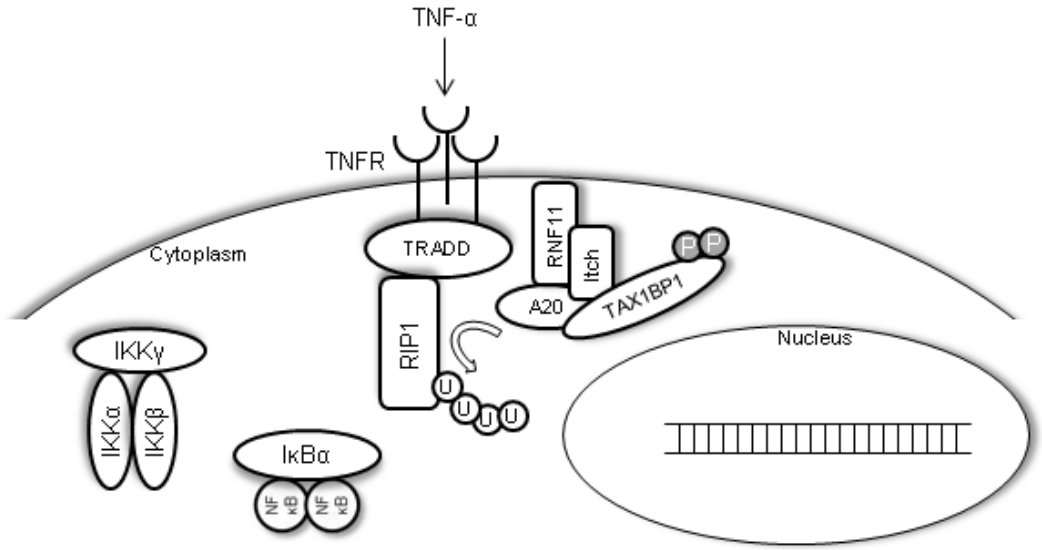


Figure 1-2. Inactivation of NF-κB signaling by the A20 ubiquitin-editing protein complex

Expression of A20 causes the formation of the A20 ubiquitin-editing protein complex within the cytoplasm of the cell. This complex is known to be comprised of A20, RNF11, Itch, and phosphorylated TAX1BP1. The complex interacts with RIP1 near the initiation of the signaling pathway cascade. With activation of the pathway, RIP1 is K63-linked pulyubiquinated. However, A20, when found as a member of the A20 ubiquitin-editing protein complex, acts as a deubiquitinase to remove the K63-linked ubiquitin chains on RIP1, terminating the activation of downstream signaling complexes (i.e. the IKK complex and IκBα/NF-κB complex). Additionally, A20 acts as an E3 ligase and attaches K48-linked polyubiquitin chains to RIP1, targeting it to the proteasome for degradation and eliminating the possibility of activation of the NF-κB signaling pathway.

Table 1. *PARK*-designated PD-related loci

Symbol	Locus	Disorder	Inheritance	Gene	Identification	Initial Study	Status	Remarks
PARK1	4q21-22	EO-PD	AD	*SNCA*	Linkage analysis	(Polymeropoulos et al., 1997)	Confirmed	Rare mutation, Lewy body pathology
PARK2	6q25.2-q27	EO-PD	AR	*Parkin*	Linkage analysis	(Matsumine et al., 1997)	Confirmed	Most common cause of AR-JP, represents 50% of AR cases, pleomorphic pathology (mostly Lewy body+)
PARK3	2p13	C-PD	AD	Unknown	Linkage analysis	(Gasser et al., 1998)	Unconfirmed	Gene not found since first described in 1998- risk factor?
PARK4	4q21-q23	EO-PD	AD	*SNCA*	Linkage analysis	(Farrer et al., 1999)	Erroneous-identical to *PARK1*	Rare, multiplication of *SNCA* gene
PARK5	4p13	C-PD	AD	*UCHL1*	Functional candidate gene approach	(Leroy et al., 1998)	Unconfirmed	Not replicated since 1998, rare mutation
PARK6	1p35-p36	EO-PD	AR	*PINK1*	Linkage analysis	(Valente et al., 2001)	Confirmed	Second most common cause of AR-JP
PARK7	1p36	EO-PD	AR	*DJ-1*	Linkage analysis	(van Duijn et al., 2001)	Confirmed	Rare mutation
PARK8	12q12	C-PD	AD	*LRRK2*	Linkage analysis	(Funayama et al., 2002)	Confirmed	Most common cause of AD-PD (2-7% of AD cases), pleomorphic pathology (Lewy body+, tau+, ubiquitin+)
PARK9	1p36	A-PD	AR	*ATP13A2*	Linkage analysis	(Hampshire et al., 2001)	Confirmed	Kufor-Rakeb syndrome with complex phenotype that would not be identified as classical Parkinsonism
PARK10	1p32	C-PD	Risk factor	Unknown	Linkage analysis	(Hicks et al., 2002)	Confirmed SL	Gene unknown since described in 2002
PARK11	2q36-27	LO-PD	AD	Unknown	Linkage analysis	(Pankratz et al., 2003a)	Not independently confirmed	Gene not found since described in 2002
PARK12	Xq21-q25	C-PD	Risk factor	Unknown	Linkage analysis	(Pankratz et al., 2003b)	Confirmed SL	Gene not found since described in 2003
PARK13	2p12	C-PD	AD or Risk factor	*HTRA2*	Candidate gene approach	(Strauss et al., 2005)	Unconfirmed	No cosegregation shown to support pathogenicity
PARK14	22q13.1	EO-p	AR	*PLA2G6*	Linkage analysis	(Gao et al., 2009)	Confirmed	Early-onset dystonia-parkinsonism
PARK15	22q12-q13	EO-p	AR	*FBX07*	Linkage analysis	(Shojaee et al., 2008)	Confirmed	Early-onset parkinsonian-pyramidal syndrome with a severe phenotype with spasticity and dementia
PARK16	1q32	C-PD	Risk factor	Unknown	GWAS	(Satake et al., 2009)	Confirmed SL	Has only been found in families of Asian descent
PARK17	12q11.2	C-PD	AD	*VPS35*	Exome sequencing	(Zimprich et al., 2011)	Confirmed	Other possible pathogenic variants in VPS35 have been identified
PARK18	3q27.1	C-PD	AD	*EIF4G1*	Linkage analysis	(Chartier-Harlin et al., 2011)	Unconfirmed	Implicates defects in mRNA translation initiation

Note: Table adapted from Klein and Westenberger, 2012. Abbreviations include: AD (autosomal dominant), A-PD (Atypical PD), AR (autosomal recessive), C-PD (Classical PD), EO-p (Early-onset Parkinsonism), EO-PD (Early-onset PD), GWAS (Genome-wide association studies), JP (Juvenile PD), LO-PD (Late-onset PD), SL (Susceptibility locus).

Chapter 2

Materials and Methods

2.1 Cell culture

Human embryonic kidney 293 (HEK293), neuroblastoma N2A, PC12, and SH-SY5Y cells were purchased from the American Type Culture Collection (Manassas, VA, USA). HEK293 cells were cultured in DMEM with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin (Bio Whittaker). SH-SY5Y cells were cultured in Eagle's minimal essential medium supplemented with Earle's balanced salt solution, L-glutamine, 10% FBS (Hyclone Laboratories, Logan, UT, USA), 1% nonessential amino acids (BioWhittaker, Inc, Walkersville, MD, USA) and 1% penicillin/streptomycin (BioWhittaker). N2A cells were cultured in DMEM /Ham's F-12 nutrient mixture supplemented with 10% fetal calf serum (Hyclone Laboratories), 1% nonessential amino acids and 1% penicillin/streptomycin. PC12 cells were cultured in Dulbecco's modified

Eagle medium (Mediatech, Manassas, VA, USA), 10% heat-inactivated horse serum (Gibco, Invitrogen, Grand Island, NY, USA), 5% fetal clone serum (Hyclone Laboratories, Logan, UT, USA), and 1% penicillin/streptomycin. Primary cortical neurons were prepared from wild-type C57BL/6 mice at embryonic day 18 as previously described (Davis et al., 2010). Cells were dissociated by trituration through a Pasteur pipette and plated on 0.25 mg/ml poly-L-lysine-coated dishes in neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing B-27 supplement (Invitrogen), 2 mM L-glutamine and 1% penicillin/streptomycin. Cytosine arabinoside was added at a final concentration of 5 μM on day 3 *in vitro* to control proliferation of non-neuronal cells. After 8 days *in vitro*, neuron-enriched cultures were used for experiments. Primary microglia and astrocytes were obtained from 1-day-old wild-type C57BL/6 mice as described elsewhere (Yepes et al., 2003; Zhang et al., 2009). Briefly, cells were dissociated into a suspension by trituration with a Pasteur pipette and plated onto six-well plates coated with 0.05 mg/ml poly-D-lysine and grown in DMEM (Invitrogen) supplemented with 25 mM glucose, 10% heat-inactivated horse serum, 10% heat-inactivated FBS, 2 μM glutamine and 1% penicillin/streptomycin. At the end of day 12 *in vitro*, floating microglia were separated from the stationary cultures and centrifuged at 80 *g* for 5 minutes to obtain a pellet of nearly pure microglia, which were then plated directly into poly-D-lysine-coated dishes. All cultures were maintained at 37°C in 5% CO_2.

2.2 Antibodies and reagents

Antibodies used were: A20 (ab13597; Abcam, Cambridge, MA, USA), A20 (NBP1-40684, Novus), A20 (550859, BD Pharmingen), A20 (sc-166692, Santa Cruz), β-actin (ab6276; Abcam), Flag (F1804; Sigma-Aldrich, St Louis, MO, USA), GFAP (MAB360, Millipore), GFP (Rockland, Gilbertsville, PA, USA), histone 1 (MAB052; Millipore, Billerica, MA, USA), Iba1 (ab5076, Abcam), Itch (611198; BD Transduction Laboratories, San Diego, CA, USA), MAP2 (M061755, BD Biosciences, San Diego, CA, USA), Olig2 (MABN50, Millipore), p65 (for immunocytochemistry, C22B4; Cell Signaling Technology, Beverly, MA, USA), p65 (for Western blotting, 3034; Cell Signaling Technology), rabbit polyclonal RNF11 (described in (Anderson et al., 2007)), TAX1BP1 (ab22049, Abcam), Traf6 (ab13853, Abcam), tyrosine hydroxylase (TH, Sigma-Aldrich, St. Louis, MO, USA), and V5 (MCA1360; AbD Serotec, Oxford, UK). Appropriate biotin or fluorescent conjugated secondary antibodies were purchased from Vector (Burlingame, CA, USA), Invitrogen (Carlsbad, CA, USA), Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and ThermoFisher Scientific (Waltham, MA, USA). The following reagents were utilized: 6-OHDA (Sigma-Aldrich), Bay 11-7085 (Santa Cruz Biotech, Santa Cruz, CA, USA), D-amphetamine (Sigma-Aldrich), and recombinant TNF-α (R&D Systems, Minneapolis, MN, USA).

2.3 Plasmids and transfections

Human RNF11 cDNA was originally subcloned into pcDNA3.1(+) (Invitrogen) using Kpn1and Not1 restriction sites as described previously (Anderson et al., 2007). Wild-type RNF11 was cut out of pcDNA and into pFUGW with BamHI and Asc1. A V5 sequence

was added to the N-terminus of the RNF11 sequence and was PCR-amplified into the plasmid. V5-RNF11, shScramble, and shRNF11 were subcloned into the pAAV vector using Age1 and Kpn1 sites. The NF-κB luciferase vector (pGL4.32[luc2P/NF-κB/Hygro]) and internal control *Renilla* vector (pGL4.74[hRluc/TK]) were purchased from Promega (Madison, WI, USA). The NF-κB luciferase vector contains a $(GGGAATTTCC)_5$ NF-κB response element protein promoter. Flag-A20, Flag-Itch, Flag-TAX1BP1, and Flag-TRAF6 were a kind gift from Dr Edward W Harhaj (Microbiology and Immunology, Miller School of Medicine, University of Miami, Miami, FL, USA). Transient transfections of SH-SY5Y, HEK293, PC12, and N2A cells were performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

2.4 RNA interference and virus production

Individual siRNA duplexes were purchased from Dharmacon Inc (Chicago, IL, USA) and tested for knockdown of RNF11 using quantitative RT-PCR (qRT-PCR) in SH-SY5Y cells (data not shown). The most effective sequence was cloned into pFH1UGW backbone by introducing Nhe1 and Pac1 overhangs at each end of the duplex. The sense sequence for RNF11 shRNA was 5′-GAT GAC TGG TTG ATG AGA T-3′, and the antisense sequence was 5′-ATC TCA TCA ACC AGT CAT C-3′. All constructs were verified by restriction enzyme digestion and sequencing. Lentiviruses for shRNA-RNF11 and shRNA-Scramble constructs were produced by the Emory University Viral Vector Core facility (Atlanta, GA, USA). Adeno-associated viruses (AAV) for V5-RNF11,

shRNF11, and shScramble constructs were also produced by the Emory University Viral Vector Core facility.

2.5 Site-directed mutagenesis

Site-directed mutagenesis of RNF11 (G2A, Y40A, H119/122A or H2, I101A, C99A and silent mutations at Q72/R73 to confer shRNA resistance) was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA) according to the manufacturer's instructions using N-terminal V5-tagged RNF11 as a template. The primers for site-directed mutagenesis are given in Table 2.1. The primers were utilized for PCR amplification, which was performed according to the company's protocol. All constructs were verified by restriction enzyme digestion and sequencing.

2.6 TNF-α stimulation and luciferase assays

All stimulations of cells with TNF-α were preceded by a 1-hour serum starvation period. Cells were stimulated with 10 ng/ml TNF-α at the indicated times. SH-SY5Y cells were transfected with the luciferase and *Renilla* plasmids for 24 hours. Cells were stimulated with TNF-α for 6 hours. Cell lysates were prepared in Passive Lysis 1× Buffer (Promega). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Firefly luciferase values were normalized according to *Renilla* luciferase values. Luciferase results are presented as fold changes relative to the untreated control sample. Control samples with luciferase, *Renilla* luciferase or vector transfection were assayed to confirm the specificity of luminescence.

2.7 Immunocytochemistry

SH-SY5Y cells were grown on coverslips coated with Matrigel (BD Biosciences, San Diego, CA, USA), and primary cortical neurons were grown on coverslips coated with poly-L-lysine (Sigma-Aldrich). After being manipulated, cells were fixed with 2% paraformaldehyde and immunostained as described previously (Anderson et al., 2007) with an antibody against p65. Cell nuclei were stained with Hoechst 333258 (Molecular Probes/Invitrogen). The fluorophore-conjugated secondary antibody used was goat anti-mouse Rhodamine Red-X (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For p65 analysis, coverslips were imaged at 40× magnification using an Olympus BX51 Fluorescence Microscope (Olympus America, Inc, Melville, NY, USA), and co-localization of p65 and 4′,6-diamidino-2-phenylindole in GFP-positive cells was quantified as described previously (Volpicelli et al., 2001). For each condition, at least 100 cells were quantified in the SH-SY5Y cell experiments and at least 50 cells were quantified in the primary neuron experiments. The percentage of p65-positive pixels above threshold intensity which overlapped with Hoechst staining was calculated for each cell. An area near the perimeter of the cell was used as background. For each experiment, the fold change in percentage of p65-positive pixels was normalized to unstimulated cells.

2.8 Immunoblotting

Immunoblotting was performed as previously described (Anderson et al., 2007). Cells were transfected for 24 hours with indicated plasmids and harvested in PBS with 50 mM Tris·HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 1% Nonidet P (NP)-40, Halt

Phosphatase Inhibitor Cocktail (Pierce Biotechnology, Rockford, IL, USA) and protease inhibitor cocktail (PIC) (Roche Diagnostics, Mannheim, Germany). Samples were spun at 14,000 rpm and protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were scanned using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA). β- actin was used as a loading control where appropriate.

2.9 Co-immunoprecipitation

Immunoprecipitation (IP) experiments were performed as described previously (Herskowitz et al., 2011). Equal amounts of protein were used for pull-down assays. Cell lysates were cleared with mouse immunoglobulin plus protein A Sepharose beads (Invitrogen) for 30 minutes at 4°C. IP experiments were then performed using antibodies against Flag, Itch, RNF11 or V5. Control IP experiments were performed with beads alone or with empty vector to demonstrate specificity. Immunoprecipitates were assessed by immunoblot analysis. Densitometric measurements for A20, Itch or RNF11 immunoreactivity in RNF11 immunoprecipitates were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Measurements were normalized to the amount of immunoreactivity of the same protein in the input samples. Unstimulated conditions were set at 100% for relative comparisons.

2.10 ELISA

Cells were serum-starved for 1 hour prior to stimulation with 10 ng/ml TNF-α. SH-SY5Y cells were stimulated for 4 hours before being rinsed, then replaced with fresh serum-free media to eliminate detection of TNF-α, which was used to stimulate cells. Media were collected 20 hours later from SH-SY5Y cells or 24 hours after stimulation of mouse primary cortical neurons. Media were briefly centrifuged at low speed after being collected. The manufacturer's instructions were followed for performing and analyzing the Human TNF-α ELISA Kit (Invitrogen) and the mouse MCP-1 Quantikine ELISA Kit (R&D Systems). Each sample was run in duplicate.

2.11 Nuclear fractionation

Fractionation was performed as described previously (Cheshire and Baldwin, 1997). Briefly, cells were rinsed in PBS, then harvested in cold extraction buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6, 60mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% NP-40, 1mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), Halt Phosphatase Inhibitor Cocktail and PIC). Cells were spun at 200 *g* for 5 minutes. The supernatant was collected as the cytoplasmic fraction. The pellet was rinsed in cold extraction buffer without NP-40. The pellet was resuspended in nuclear extraction buffer (20 mM Tris, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 25% glycerol, Halt Phosphatase Inhibitor Cocktail and PIC). The final salt concentration was adjusted to 400 mM before lysates were kept on ice for 10 minutes with occasional agitation. Lysates were spun at 16,000 *g* for 10 minutes, and the supernatant was collected as the nuclear fraction. Fractions were

examined using immunoblot analysis. Densitometric measurements for actin, histone 1, and p65 immunoreactivity in cytoplasmic and nuclear fractions were performed using ImageJ software. p65 measurements were normalized to the amount of immunoreactivity of the loading control. Unstimulated conditions were set at 100% to make relative comparisons.

2.12 Cell death assays

Cell viability was measured using the CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Absorbance was measured at 570 nM with a reference wavelength at 650 nM. After sample absorbance was corrected for background absorbance, cell death values were calculated as the percentage of control absorbance.

2.13 Human tissue

Post-mortem, control (pathologically clean) brains (n = 3) were obtained through Emory University's Brain Bank for analysis of expression of the A20 ubiquitin editing protein complex. The average age at death of the samples was 64 years. Blocks of formalin fixed tissue at the level of cerebral cortex, midbrain, pons, or inferior olive were sectioned on a freezing microtome. Free-floating sections (50 μm) were stored at -20°C in ethylene glycol cryopreservative. Human brain tissues used for qRT- PCR analysis of diseased tissue in this study were derived from 46 autopsy brains, which were pathologically evaluated for AD and PD, from the Emory University Brain Bank (Atlanta, GA, USA). The neuropathologic diagnosis of definite AD was made according to criteria of the

Consortium to Establish a Registry for Alzheimer's Disease (Mirra et al., 1991). The neuropathologic diagnosis of PD was based on the presence of nigral degeneration and Lewy bodies. Control cases had no clinical history or neuropathologic diagnosis of neurologic disease. Four groups were compared: 14 subjects aged 62 to 79 years (mean = 71) with clinically and pathologically confirmed PD, 17 subjects aged 58 to 84 years (mean = 70) with clinically and pathologically confirmed AD, 8 subjects aged 67 to 79 years (mean = 73) with a clinical diagnosis of dementia with Lewy bodies and neuropathologic findings of AD with concomitant Lewy pathology, and 17 control subjects aged 52 to 92 years (mean = 71). Human brain tissue used for immunohistochemical analysis of diseased tissue was derived from a subset of these cases with available paraformaldehyde fixed tissue.

2.14 Brightfield immunohistochemistry

50 μm thick coronal sections, through the frontal cortex from control and PD cases, were stained for Itch, RNF11, and TAX1BP1 as previously described (Betarbet et al., 2000). Briefly, sections were blocked with 10% normal goat serum and 10% Triton-X in TBS. Sections were incubated with primary antibodies for 72 hours at 4 deg C followed by appropriate biotinylated secondary antibody. The avidin-biotin complex method was used to detect the antigen signal (ABC elite kit, Vector). Peroxidase activity was developed using Sigma FAST 3,3`-diaminobenzadine. Tissue sections were dehydrated in a graded series of ethanols, immersed in histoclear, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA). Primary antibody was omitted for negative control reactions. Immunostained sections were examined using an Olympus BX51 Fluorescence

Microscope (Olympus America, Inc., Melville, NY, USA) and images were processed using Adobe Photoshop 7.0 software (San Jose, CA, USA).

2.15 Fluorescence immunohistochemistry of mammalian tissue

Brain sections were stained using a standard immunofluorescence protocol. Briefly, sections were blocked with 10% normal horse serum and 10% Triton-X in TBS. Sections were incubated with a combination of primary antibodies overnight at 4 deg C followed by appropriate fluorescent conjugated secondary antibodies. Sections were incubated with Hoechst 333258 to counter stain nuclei. Primary antibodies were omitted as a negative control. Images were captured using a Zeiss LSM 510 laser scanning confocal microscope. For final output, images were processed using Adobe Photoshop 7.0 software.

2.16 Extraction of RNA from cells or *Drosophila melanogaster* tissue

Total RNA was isolated from cells or *Drosophila melanogaster* using a standard TRIzol reagent (Invitrogen) extraction protocol. RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Ambion, Austin, TX, USA).

2.17 Extraction of RNA from tissue

Total RNA was isolated from approximately 100 mg of human or rodent brain tissue that was homogenized and sonicated before using an RNeasy Lipid Tissue kit (Qiagen, Valencia, CA, USA). cDNA was created using a High Capacity cDNA reverse transcription kit (Ambion).

2.18 Quantitative real-time PCR

Real-time PCR was performed with a 7500 Fast real time-PCR System (Applied Biosystems, Foster City, CA, USA) using 20ng/µl cDNA, TaqMan Universal PCR Master Mix II (Applied Biosystems), and gene-specific TaqMan probes (Applied Biosystems) described in Table 2.2. For each RNA sample, each primer set was run in triplicate. For rat tissue and cell culture data, results were normalized to the housekeeping gene GAPDH and evaluated by the $2^{\Delta\Delta Ct}$ method. RNA levels are expressed relative to the naive animal. For human tissue, results were normalized to the housekeeping gene GUSB and evaluated by comparative cycle threshold method.

2.19 *Drosophila* stocks and husbandry

Drosophila strains were reared in standard corn meal-dextrose-yeast containing food at 25 degrees C in controlled humidity incubators. OregonR flies were obtained from Drosophila Stock Collection (Bloomington, IN, USA), the C833-gal4 line was a kind gift from Dr. Todd Schlenke (Emory University) and is used to drive expression in the fat body (Hrdlicka et al., 2002), and the UAS-RNF11-RNAi line is from the Vienna Drosophila RNAi Collection (VDRC).

2.20 Immunohistochemistry of *Drosophila* tissue

Larval dissection, staining, and confocal microscopy were performed according to standard protocols (Franciscovich et al., 2008; Freeman et al., 2012). Larvae were dissected in calcium-free HL3 saline (Stewart et al., 1994), fixed in 4% paraformaldehyde, and stained overnight in blocking solution containing 0.1% Triton X-

100. RNF11 antibody was added at a concentration of 1:100 followed by appropriate fluorescent conjugated secondary antibodies. Images were captured using a Zeiss LSM 510 laser scanning confocal microscope. For final output, images were processed using Adobe Photoshop 7.0 software.

2.21 *Drosophila* immunity paradigm

Adult flies (5 days old, n = 10 per group) were pierced in the thoracic segments with a 0.1-mm-diameter stainless steel needle (Fine Science Tools) dipped in sterile Luria broth, *E. faecalis* gram-positive bacteria culture grown overnight and diluted to OD600 = 1.0, or *S. mercesens* gram-negative bacteria culture grown overnight and diluted to OD600 = 1.0. Following injury, flies were transferred to vials containing standard Drosophila medium without yeast. Immediately following injury and at 6 and 24 hours, flies were stored in liquid nitrogen for future processing.

2.22 *Drosophila* survival assays

10 young flies were placed in a food vial following infection. Each vial was kept at 25 deg C and changed every three days. Dead flies were counted every 3 hours for the first 72 hours and then every day thereafter. At least 100 flies were prepared for each genotype, and the experiments were carried out three times.

2.23 Animal studies

Young male adult Sprague Dawley rats (approximately 250 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in pathogen-free

climate-controlled facilities at the Animal Resources Center at Emory University. All animal studies were approved by the Institutional Animal Care and Use Committee at Emory University.

2.24 Stereotaxic injections

Sprague Dawley rats (n=20 per group) were anesthetized with 80 mg/kg ketamine and 8 mg/kg xylazene and placed in stereotaxic frame. Their eyes were covered in ophthalmic ointment. The scalp was sterilized before the skull was exposed and incised. We followed a previously published protocol to target the AAV to the nigra (Bartus et al., 2011). A unilateral injection of AAV (2 µl of 1.6 x 10^9 vg/µl) was given at a rate of 1 µl/2.5 min into the nigra of the right hemisphere (anteroposterior (AP), -5.3 mm from bregma; mediolateral (ML), +2.3 mm from bregma; and dorsoventral (DV), -7.2 mm from below the surface of dura). The intrastriatal 6-OHDA injection was performed 2 weeks after intranigral AAV injection. We followed a previously published protocol with a decreased concentration of 6-OHDA to induce a moderate retrograde lesion in the nigrostriatal pathway (Kirik et al., 1998). A unilateral injection of 10 µg of 6-OHDA (2 µl of 5 µg/µl) was given at a rate of 1 µl/2.5 min into the striatum of the right hemisphere (AP, -1.2 mm from bregma; ML, +3.4 mm from bregma; and DV, -5.0 mm from below the surface of dura).

2.25 Microdissection and harvest of tissue for qRT-PCR

At 4 weeks after 6-OHDA lesion, animals were deeply anesthetized with isofluorane and decapitated. The brain was rapidly harvested and the ventral mesencephalic region was

microdissected from both hemispheres on an ice-cold Petri dish. Samples were flash-frozen in liquid nitrogen and stored at -80 degrees C until processed for RNA extraction.

2.26 Rotational behavior analysis

At 3 weeks after 6-OHDA lesion, amphetamine-induced rotational behavior was monitored as published previously (Kirik et al., 1998). Animals received 5 mg/kg D-amphetamine (Sigma-Aldrich) intraperitoneally, and rotational asymmetry was scored as a complete rotation toward the lesion (ipsilateral). The net rotational asymmetry was expressed as rotations/minute.

2.27 Perfusion and tissue processing for histology

At 4 weeks after 6-OHDA lesion, animals were deeply anesthetized with isofluorane and intracardially perfused with 4% paraformaldehyde in PBS, pH 7.4. Brains were post-fixed for 48 hours in the same paraformaldehyde solution and cryoprotected in 10% sucrose for 24 hours and 30% sucrose for 24 hours. Serial coronal sections (40 μm thickness) through the entire striatum and substantia nigra were collected and stored in anti-freeze solution for further analysis.

2.28 Stereological analysis of nigral dopaminergic neurons

The optical fractionator probe of Stereoinvestigator software (MicroBrightField, Version 10.04, MBF Bioscience, Williston, VA, USA) was used to obtain an unbiased estimate of

TH-immunoreactive neurons in the SNpc as described previously (Hutson et al., 2011). Every 4th nigral sections were processed for TH immunohistochemistry as previously described (Betarbet et al., 2000). The boundary of the SNpc was defined according to previous demarcations in the rat (German and Manaye, 1993). Cells were counted blinded with a 40 × objective using a Zeiss Imager.M2 microscope (Thornwood, CO, USA) through the extent of the SNpc. Stereologic parameters were as follows: counting frame, 50 × 50 μm; optical dissector, 18 μm; grid size, 140 × 140 μm. Every fourth section was stained for TH and yielded a target coefficient of error (Gundersen's m = 1) of < 0.10. A dopaminergic neuron was defined as a TH-immunoreactive cell body with a clearly visible unstained nucleus.

2.29 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.03 software (GraphPad Software, Inc, La Jolla, CA, USA). Statistical significance was set at $P < 0.05$. All experimental results are presented as means ± SEM for at least three independent experiments. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ unless otherwise noted.

In **Chapter 3**, differences among means were analyzed using one-way analysis of variance (ANOVA). When ANOVA showed significant differences, comparisons between means were tested by either Dunnett's multiple comparison or Bonferroni selected multiple comparison post hoc tests.

In **Chapter 4**, one-way ANOVA was performed for analysis of qRT-PCR for RNF11 levels and luciferase assays. Tukey's post-test was used for the luciferase assay with mutant RNF11 constructs, and Bonferroni post-tests were used for the remaining ANOVAs. Two-way ANOVAs with repeated measures were performed for analysis of p65 translocation, and standard two-way ANOVA was used to analyze the mRNA and protein expression of inflammatory markers and the densitometric analysis of p65 fractions. Bonferroni post-tests were performed for all two-way ANOVAs. Densitometric analysis of co-IPs was performed using paired *t*-tests.

In **Chapter 5**, differences between endogenous fly lines were assessed using paired two-tailed *t*-tests. Differences between fly lines in the fold change induction following septic injury were analyzed using repeated measures two-way ANOVAs with Bonferroni post-tests. Differences in survival curves were assessed by the log-rank (Mantel-Cox) test.

In **Chapter 6**, differences among means were analyzed using one-way ANOVA (neuron mRNA, rat mRNA, rotational analysis, and stereology) or two-way ANOVA (p65 analysis, PC12 cell mRNA, and cell death assays). When ANOVA showed significant differences, comparisons between means were tested by either Dunnett's multiple comparison or Bonferroni selected multiple comparison post hoc tests.

Table 2.1. Primers for site-directed mutagenesis

Mutagenesis Construct	*Forward Primer*	*Reverse Primer*
Myristoylation mutant (G2A)	5′-CTC GAT TCT ACG ACC GGT ATG GCG AAA TGC CTC AAA TCC CCC ACC-3′	5′-GGT GGG GGA TTT GAG GCA GTT CGC CAT ACC GGT CGT AGA ATC GAG-3′
PPPY domain mutant (Y40A)	5′-GCC GCC GCC GCC AGC TCA GGA ACA AGT TCC AG-3′,	5′-CTG GAA CTT GTT CCT GAG CTG GCG GCG GCG GC-3′
RING domain mutant (H2)	5′-CGA TTT CTG CCG TGC ATG GCC ATC TAT GC-3′	5′-CTA TAC AGT CCA GGG CAT AGA TGG CCA TG-3′
RING domain mutant (I101A)	5′-GAT CCG GGA GTG TGT GGC CTG TAT GAT GGA CTT TG-3′	5′-CAA AGT CCA TCA TAC AGG CCA CAC ACT CCC GGA TC-3′
RING domain mutant (C99A)	5′-GAT CCG GGA GGC TGT GAT CTG TAT GAT GGA C-3′	5′-GTC CAT CAT ACA GAT CAC AGC CTC CCG GAT C-3′
shRNA-RNF11 resistance	5′-CAG CTG ACT GAA GAG GAA CAA ATT AGG ATA GCT CAG AGG ATA GGT C-3′	5′-GAC CTA TCC TCT GAG CTA TCC TAA TTT GTT CCT CTT CAG TCA GCT G-3′

Table 2.2. Primers for quantitative real-time PCR

Gene	*Mouse*	*Rat*	*Human*	*Drosophila*
A20	-	-	Hs00234712_m1	-
BCL2	-	Rn00586772_m1	-	-
BDNF	-	Rn02531967_s1	-	-
Cactus	-	-	-	Dm01807756_m1
Diptericin B	-	-	-	Dm01821557_g1
Drosomycin	-	-	-	Dm01822006_s1
GAPDH	ABI 4308313	ABI 4308313	-	-
GSS	-	Rn00564188_m1	-	-
GUSB	-	-	Hs99999908_m1	-
MCP-1	Mm00441242_m1	-		-
NF-κB 1	-	Rn01399583_m1	-	-
Relish	-	-	-	Dm02134843_g1
RNF11	Mn00450014_m1	Rn01417031_m1	Hs00702517_s1	Dm01820594_m1
Rpl32	-	-	-	Dm02151827_g1
SOD1	-	Rn00566938_m1	-	-
TH	-	Rn00562500_m1	-	-
TNF-α	-	Rn99999017_m1	Hs00174128_m1	-

Chapter 3

RNF11 expression is reduced in Parkinson's disease

Portions of the work presented in this chapter have been published:

Pranski, EL, Van Sanford, CD, Dalal, NV, Orr, AL, Karmali, D, Cooper, DS, Costa, N, Heilman, CJ, Gearing, M, Lah, JJ, Levey, AI, Betarbet, RS. *Comparative distribution of protein components of the A20 ubiquitin-editing complex in normal human brain*. 2012. Neuroscience Letters. 27 June;520(1):104-9.

Portions of the work presented in this chapter have been prepared for publication:

Pranski, EL, Dalal, NV, Van Sanford, CD, Herskowitz, JH, Gearing, M, Lazo, C, Miller, GW, Lah, JJ, Levey, AI, Betarbet, RS. *RING finger protein 11 (RNF11) modulates susceptibility to 6-OHDA-induced nigral degeneration and behavioral deficits through NF-κB signaling in dopaminergic cells.* Submitted to Journal of Neuroscience.

3.1 Introduction

The NF-κB signaling pathway is well known for its ubiquitous roles in inflammation, immune responses, and control of cell division and apoptosis (Mattson and Camandola, 2001). These roles of NF-κB signaling are apparent in the central nervous system where they can range from neuronal development, synaptic signaling that underlies learning and memory and coordination of immune responses to toxic stimuli (Ea et al., 2004; Imielski et al., 2012). Activation of NF-κB is normally transient, and persistent NF-κB activation is associated with several autoimmune diseases and cancer in the peripheral system (Takeuchi and Akira, 2010) and in both acute (i.e., stroke, seizures) and progressive (PD, AD, ALS) neurodegenerative disease (Glass et al., 2010). However, inhibition of NF-κB signaling can prevent adult neurogenesis in the dentate gyrus (Imielski et al., 2012). Dysregulation of NF-κB signaling has been cited as a potential source of synaptic pathology in AD and PD due to the importance of NF-κB transcriptional regulation of spine density in mature neurons (Boersma et al., 2011). Thus duration of NF-κB activation is tightly regulated causing genes that play key roles in amplification and effector functions to be actively repressed under basal conditions (Verstrepen et al., 2010). One such regulator of NF-κB activation is A20, an ubiquitin-editing protein, which regulates NF-κB activation in a negative feedback loop (Verstrepen et al., 2010). Specifically, A20 contains several NF-κB binding sites within the promoter of the human gene to induce its expression in response to NF-κB signaling (Krikos et al., 1992). Recent experiments have established that A20 mediates its inhibitory function in a complex with three other proteins, TAX1BP1, and two E3 ubiquitin ligases, Itch (also known as AIP3) and RNF11 (Shembade et al., 2009). The inhibitory role of A20 is critically dependent on

its interaction with other complex members, TAX1BP1, Itch and RNF11. TRAF6, a member of the tumor necrosis factor receptor-associated factor (TRAF) family, and a known substrate of A20 is also an E3 ligase (Shembade et al., 2009). To better understand the role of A20 ubiquitin-editing protein complex in the central nervous system we determined the presence and distribution of RNF11, TAX1BP1, Itch and TRAF6 proteins in normal human brain. Since A20 is an inducible protein (Beyaert et al., 2000), we have determined A20 mRNA expression levels in various normal human brain regions. Furthermore, due to the involvement of dysregulation of NF-κB signaling in neurodegenerative disease, the expression of the A20 ubiquitin editing protein complex members in normal and diseased brain was also investigated.

3.2 Differential expression of A20 and RNF11 in normal human brain

To determine specificity of the antibodies used for immunohistochemistry, we looked at endogenous expression of complex members and over-expression of FLAG-tagged constructs in HEK293 cells (Fig. 3-1). We tried numerous commercially available antibodies against A20 but they either did not label human brain tissue or the specificity could be confirmed by pre-adsorption. However, in HEK293 cells, endogenous levels of A20 were detectable at the predicted molecular weight for this protein using immunoblotting (Fig. 3-1A). Endogenous expression levels of Itch, TAX1BP1, and Traf6 were also detectable with a band near their predicted molecular weight (Fig. 3-1B, 3-1C, 3-1D, 3-1E). With confirmation that our antibodies were specific for the different A20 ubiquitin-editing protein complex members, we set out to explore the distribution of the complex in different regions of the human brain.

Distribution of A20 mRNA in various brain regions from control cases was analyzed by qRT-PCR due to the technical issues with the available A20 antibodies. A20 mRNA was differentially expressed in various brain regions examined (Fig. 3-2A). In comparison to frontal cortex, A20 expression was increased in striatum, hippocampus, pons, and medulla (2.3, 2.7, 2.0, and 2.9 fold, respectively). Unlike A20, large differences in mRNA expression of RNF11 were not detected (Fig. 3-2B). The detection of A20 mRNA expression in the different regions suggested that an A20 ubiquitin-editing complex exists in these regions and thus, expression of the individual complex members was studied in human tissue.

3.3 Differential expression of RNF11, Itch, TAX1BP1, and Traf6 in normal human brain

Immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was abundantly detected in cerebral cortex of human brain (Fig. 3-3A, 3-3B, negative control showed in Fig. 3-4). For this study we chose frontal and cingulate cortices for evaluation since they have the characteristic six cortical layers. Expression levels for all four proteins were detected in neurons in all the six isocortical layers. In particular, cell bodies of neurons in layers III, IV and V had the densest immunoreactivity. Small and large pyramidal and multipolar neurons and their processes were immunolabeled for all proteins. Furthermore, while immunoreactivity for RNF11 and Itch was detected mainly in neuronal cytoplasm, TAX1BP1 and TRAF6 immunoreactivity was detected in both neuronal cytoplasm and nuclei (Fig. 3-3A). Immunoreactivity for all proteins was observed in glia-like cells in white matter underlying the cortical tissue (Fig. 3-3B) as evident from location and cell

morphology. Double-labeling with Iba1 confirmed microglial expression (Fig. 3-5). Minimal co-localization was observed with GFAP (astrocytes) or Olig2 (oligodendrocytes) antibodies and A20 ubiquitin-editing protein complex components (data not shown).

Immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was detected in various regions of the hippocampal formation, consisting of dentate gyrus, hippocampus, subiculum, and entorhinal cortex. Immunoreactivity for all proteins was observed specifically in the homogenous pyramidal cell layer of CA1-3 fields of hippocampus (Fig. 3-3C) and granule cell layer of dentate gyrus. Similar to cortical tissue, immunoreactivity for all proteins was mainly detected in neuronal cells.

Immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was detected in striatal medium-sized (mainly medium spiny neurons) and their processes (Fig. 3-3D). Additionally, immunoreactivity was observed in striatal large neurons (cholinergic neurons) and their processes. Limited immunoreactivity for the different proteins was detected in the fiber bundles crossing the striatum.

Immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was detected in large neurons of substantia nigra pars compacta, which have characteristic neuromelanin pigment (Fig. 3-3E). Immunoreactivity was observed in the smaller neurons of substantia nigra pars reticulate, which are typically GABAergic cells.

Immunoreactivity for RNF11, TAX1BP1, TRAF6 and Itch was detected in various brainstem regions (Fig. 3-6A). Similar to other regions, immunoreactivity for all proteins was mainly detected in neurons. Neurons of the dorsal motor nucleus of vagal nerve and reticular formation were immunoreactive for all proteins (Fig. 3-6B). Similarly, neuronal immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was detected in the convoluted bands of cells in the inferior olivary nucleus, positioned dorsal to the pyramids. Immunoreactivity for all proteins was detected in glial-like cells of the pyramids.

3.4 Reduction of RNF11 mRNA expression in Parkinson's disease

Given that A20 ubiquitin-editing protein complex is a crucial regulator of the canonical NF-κB pathway (Verstrepen et al., 2010) and that chronic activation of NF-κB is associated with PD neurodegeneration (Hunot et al., 1997; Mattson and Meffert, 2006; Glass et al., 2010), we hypothesized that for aberrant NF-κB pathway, expression of one or more of the A20 ubiquitin-editing protein complex components would be altered. Therefore we first examined the distribution of the various components of the A20 ubiquitin-editing protein complex in PD brain tissue.

Previously, we demonstrated that RNF11, Itch and TAX1BP1, components of the A20 ubiquitin-editing protein complex, are constitutively and highly expressed in neurons as compared to glial cells in various regions of normal human brain (Fig. 3-3, 3-6). Moreover, mRNA expression of A20, a NF-κB-inducible protein, was also detected in all the brain regions examined (Fig. 3-2). Given the vulnerability of cortical neurons and

spread of pathology to neocortical regions during the later stages of PD (Braak et al., 2006), we chose to examine neocortical tissue from diseased brain along with age-matched controls. To determine if alterations in expression were specific to PD we also examined samples from AD and dementia with Lewy bodies (DLB) cases. We first examined the expression of A20 and RNF11 mRNA by qRT-PCR and observed that the mRNA level of RNF11 was significantly decreased in DLB ($P < 0.01$) and PD ($P < 0.001$) patients compared to control cases (control: 4.696, AD: 3.313, DLB: 2.125, PD: 1.643 mean relative cycle threshold) (Fig. 3-7A). In contrast, the mRNA level of A20 did not significantly differ between PD, AD and DLB groups (Fig. 3-7B).

3.5 Reduction of RNF11 protein expression in Parkinson's disease

In order to confirm these results with an orthogonal approach, we examined the protein expression of components of the A20 ubiquitin-editing protein complex by immunohistochemistry in PD and matched control cases. As previously shown, detection of A20 by immunohistochemistry is not possible with the available commercial antibodies. Therefore, we examined the expression of the other crucial members of the A20 ubiquitin-editing protein complex: RNF11, Itch, and TAX1BP1. A striking difference in intensity of RNF11 immunoreactive neurons in the frontal cortex was observed between control and PD tissue (Fig. 3-7C). The staining for RNF11 in PD tissue was substantially diminished in comparison to control cases, although faint RNF11 immunoreactivity could be observed in a portion of neurons in PD tissue. However, no obvious difference in Itch (Fig. 3-7D) or TAX1BP1 (data not shown) immunoreactivity was observed in cortical tissue between control and PD samples.

3.6 Discussion

In this study we determined the expression and distribution of A20 ubiquitin-editing complex components including A20, RNF11, TAXIBP1, Itch and TRAF6 in normal human brain in order to demonstrate the existence of this crucial regulator of NF-κB signaling pathway in central nervous system. Prior to immunohistochemical analysis, we examined specificity of commercial antibodies available for proteins in the A20 ubiquitin-editing protein complex. In absence of peptides to perform pre-absorption experiments, we induced over-expression of proteins of interest in HEK293 cells and demonstrated that our antibodies were able to detect endogenous and over-expressed proteins. A similar protocol has been used to determine the specificity of commercial antibodies in past studies (Levey et al., 1997).

Our investigation demonstrated presence of TAX1BP1, Itch, RNF11 and TRAF6 in normal human brain. Furthermore, we showed that TAX1BP1, Itch, RNF11 and TRAF6 are differentially expressed in all brain regions examined, are mainly localized in neurons, and follow region-specific neuronal cytoarchitecture. A20 is an inducible, cytoplasmic protein, which is constitutively expressed in thymocytes and peripheral T cells with minimal expression elsewhere at steady state (Wu et al., 2009; Uhlen et al., 2010). We were unable to detect specific A20 protein expression in the brain despite using four different commercially available antibodies. This prompted us to demonstrate the presence of A20 mRNA in various brain regions using qRT-PCR analysis of homogenized brain tissue containing both neuronal and glial cell populations. Our studies suggest that A20 protein has minimal expression in normal brain with the potential to be

induced in response to appropriate stimuli, perhaps as observed following MPTP-treatment (Liu et al., 2008) or TNF-α stimulation in mice (Lee et al., 2000). However, we do not see significant differences in A20 expression between control, AD, DLB, or PD tissue.

RNF11 is a putative E3 ubiquitin ligase with a RING-H2 domain. In humans, RNF11 expression is up-regulated in various cancers (Burger et al., 2006). Additionally, *RNF11* is a candidate gene for late onset PD at the PARK10 locus (Hicks et al., 2002) and RNF11 co-localizes with α-synuclein-positive Lewy bodies (Anderson et al., 2007). There are no other reports of RNF11 expression in the central nervous system and the Protein Atlas has yet to complete its analysis of RNF11 protein expression (Uhlen et al., 2010). Our studies suggest that the expression of RNF11 is specifically reduced in PD in comparison to control tissue, highlighting RNF11's potential role in modifying disease progression.

TAX1BP1 is an anti-apoptotic protein that is constitutively expressed in human cells and binds to TAX protein of the human T-cell leukemia virus type 1. TAX protein is localized in the nucleus and cytoplasm of infected cells. There it interacts with different host proteins such as TAX1BP1 in order to regulate viral and cellular transcription, signal transduction pathways, and apoptosis (Ulrich et al., 2007). This would explain the cytoplasmic and nuclear localization of TAX1BP1 in neurons (Fig. 3-3, 3-6). However there is no report as yet of TAX1BP1 expression and distribution in central nervous system, though the Protein Atlas does confirm the cytoplasmic and nuclear localization of

TAX1BP1 in neurons and cells (Uhlen et al., 2010). We did not observe significant changes in TAX1BP1 expression in disease tissue.

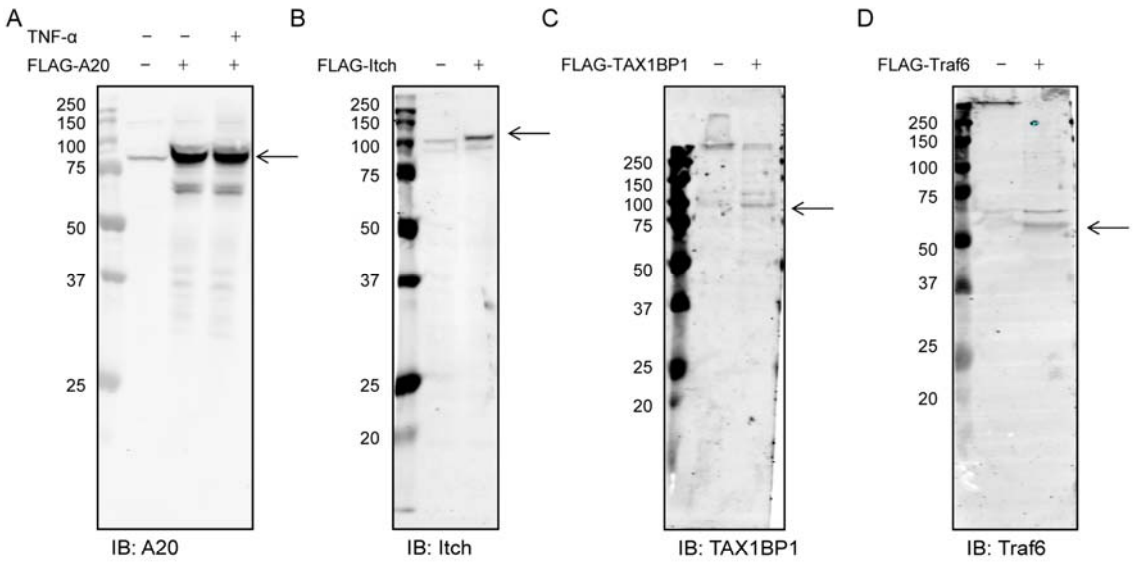
Itch, a member of the NEDD4 family of E3 ubiquitin ligases, has four WW domains for specific interactions with PY motif-containing substrates. Itch knockout mice, similar to A20 and TAX1BP1 knockout mice, display severe immunological disorders associated in every organ in the body (Baumann et al., 2010; Lohr et al., 2010). In humans, Itch is strongly expressed in the gastrointestinal tract, pancreas, neuronal cells and lymphoid tissues (Lohr et al., 2010). According to the Protein Atlas, Itch immunoreactivity is mainly detected in cytoplasm of cells (Uhlen et al., 2010), similar to our observations (Fig. 3-3, 3-6). In neurodegenerative disease tissue, we did not observe significant changes in Itch expression.

TRAF6 is a member of the TRAF family of proteins that are involved in regulating cell death, survival and cellular responses to stress (Arch et al., 1998; Chung et al., 2002; Ea et al., 2004). TRAF6 has been implicated in PD and traumatic brain injury (Chen et al., 2010; Zucchelli et al., 2010). As demonstrated in our studies, TRAF6 expression levels were detected in human, nigral dopaminergic neurons (Zucchelli et al., 2010) while its cytoplasmic and nuclear localization has been identified in other human brain regions (Uhlen et al., 2010). Neuronal TRAF6 expression has also been reported in normal, adult rat brain (Chen et al., 2010). Like TAX1BP1 and Itch, alterations in TRAF6 expression were not observed in disease tissue.

As mentioned above, components of the A20 ubiquitin-editing protein complex are expressed predominantly in lymphoid tissues that are actively involved in inflammatory responses outside of central nervous system. However RNF11, TAX1BP1, Itch and TRAF6 are constitutively and highly expressed in neurons as compared to glial cells, suggesting that NF-κB activation is tightly regulated in neurons, the post-mitotic cells of the central nervous system, as an inherent protective strategy. Indeed, Kaltschmidt et. al. found increased NF-κB activation in cells around plaques in normal and AD patients (Kaltschmidt et al., 1999). However, they also observed a down-regulation in NF-κB activity between early and late plaque stages, which was suggested to make these cells more sensitive to insults and degeneration. We do see expression of A20 ubiquitin-editing protein complex components in microglial cells, the immune cells of the central nervous system, possibly due to their important role in inflammatory responses and initiation of NF-κB signaling pathways (McCoy et al., 2008; Conductier et al., 2010).

Our investigations, for the first time, describe and demonstrate that the essential components of the A20 ubiquitin-editing complex are present and mainly expressed in neurons. This study provides useful information about region specific expression of the A20 ubiquitin-editing protein complex components that will be invaluable while determining the role of NF-κB signaling pathway, in normal and diseased brain. This will be specially important since signaling molecules that are involved in the activation and regulation of NF-κB -dependent gene expression have drawn much interest as potential targets for treatment of several autoimmune and neurodegenerative diseases.

Additionally, our studies show differential expression of RNF11 in control and disease tissue, while other members of the A20 ubiquitin-editing protein complex do not display such changes. This highlights RNF11's potential role in modifying progression of PD. Through manipulation of RNF11 expression, activity of the A20 ubiquitin-editing protein complex would also be altered, setting forth a potential target for controlling the observed upregulation of NF-κB signaling in PD.



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Protein	Predicted Molecular Weight
A20	80 kD
Itch	113 kD
TAX1BP1	86 kD
Traf6	54 KD

Figure 3-1. Antibody characterization of A20 complex components

HEK293 cells were transiently transfected with FLAG-A20, FLAG-Itch, FLAG-TAX1BP1, FLAG-Traf6, or vector and then separated by SDS-PAGE. Endogenous A20 and Itch immunoreactivity is detected at approximately the correct molecular weight, and this immunoreactivity is increased upon FLAG-tagged construct overexpression. While endogenous TAX1BP1 and Traf6 immunoreactivity is not detected, a band at the correct molecular weight is detected with FLAG-tagged construct overexpression.

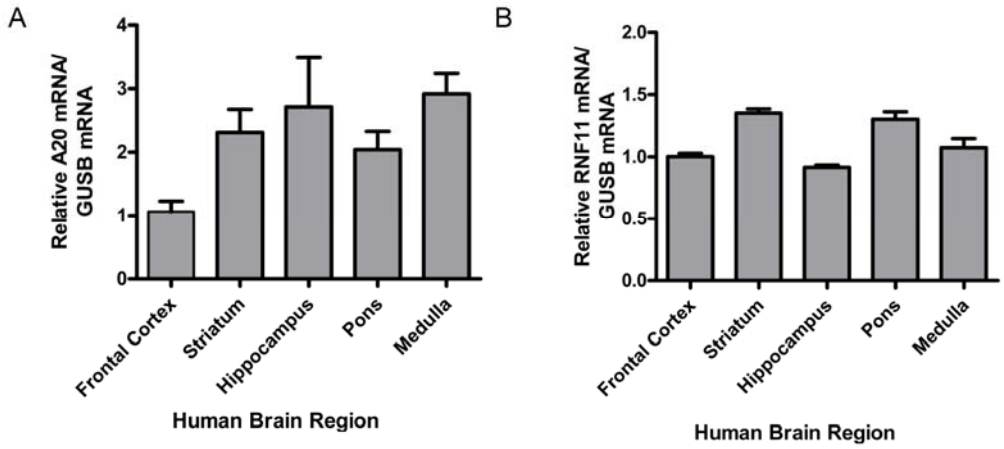

Figure 3-2. A20 is differentially expressed throughout human brain

A, B. RNA from control human cases were analyzed by qRT-PCR for A20 and RNF11 expression, relative to GUSB. A20 and RNF11 are detectable in all brain regions examined and levels are not significantly different by one-way ANOVA.

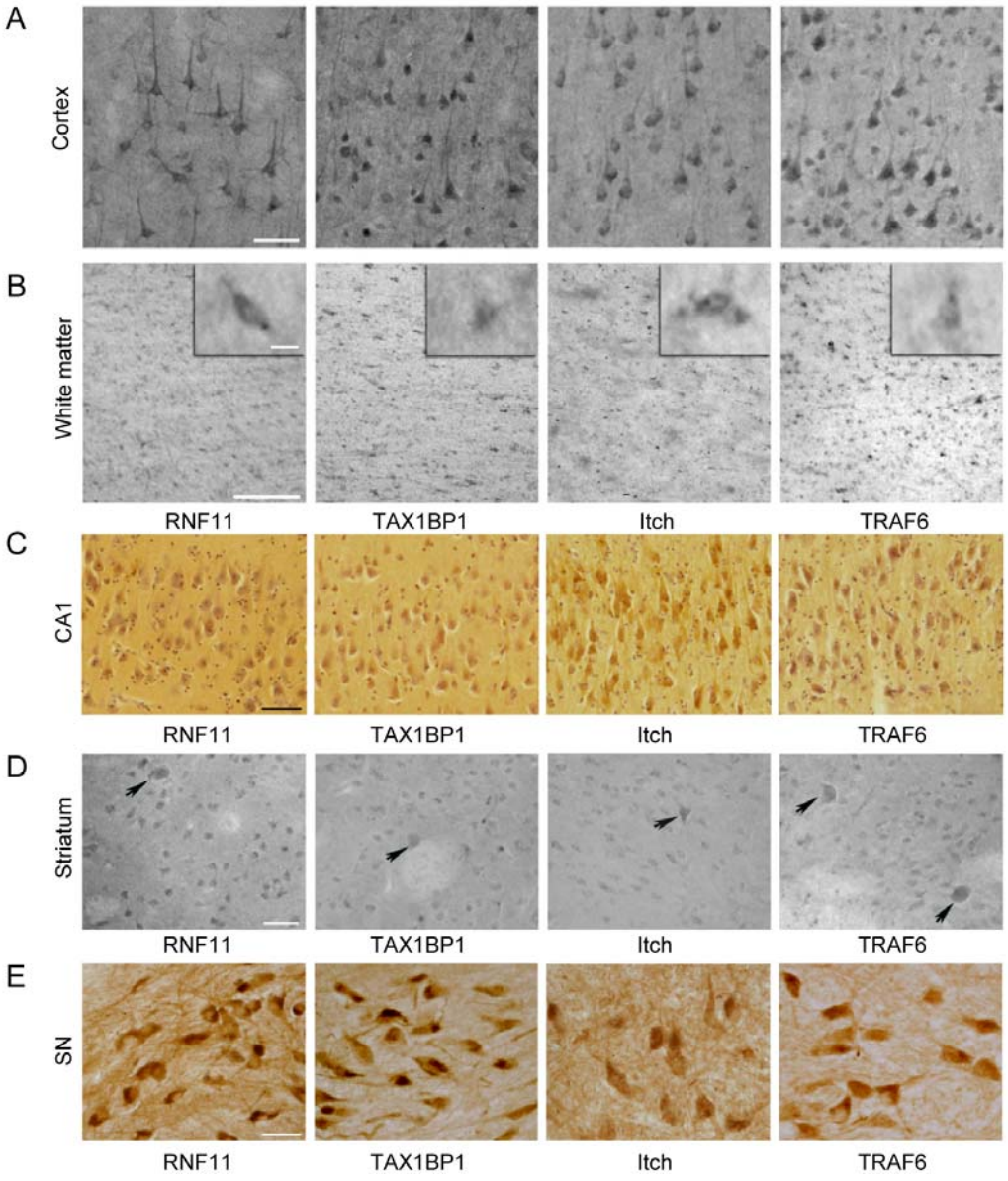

Figure 3-3. Expression of A20 complex components in normal human brain

A. RNF11, TAX1BP1, Itch and TRAF6 have abundant neuronal expression throughout cerebral cortex. RNF11 and Itch immunoreactivity was detected mainly in the cytoplasm while TAX1BP1 and TRAF6 immunoreactivity was detected in both cytoplasm and nucleus. B. In white matter underlying the cortex, immunoreactivity for all proteins was detected in glia-like cells (inset shows magnification of individual cells). C.

Immunoreactivity for all proteins (brown precipitate) was detected in all pyramidal cells of CA1 in hippocampus (haematoxylin counterstain for nuclei shown in purple). D. Immunoreactivity for all proteins was detected in the striatal medium-sized spiny neurons and large neurons (arrowhead). Limited immunoreactivity for different proteins was detected in the fiber bundles of the striatum. E. Immunoreactivity for all proteins (brown precipitate) was detected in large substantia nigra (SN) neurons with neuromelanin pigment (dense dark brown granules). Scale Bar- 50 μm for all except 3-3B- 100 μm, inset -10 μm.

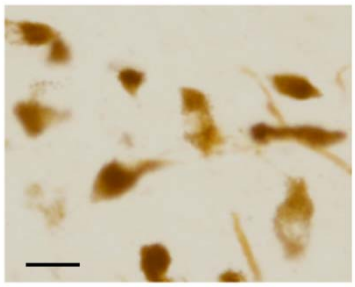

Figure 3-4. Negative control for A20 complex component staining

A section through the substantia nigra was processed without primary antibody. This image demonstrates neuromelanin pigmentation in dopaminergic neurons but they lack brown immunoreactivity for the various A20 ubiquitin-editing protein complex components. Scale Bar- 50 μm

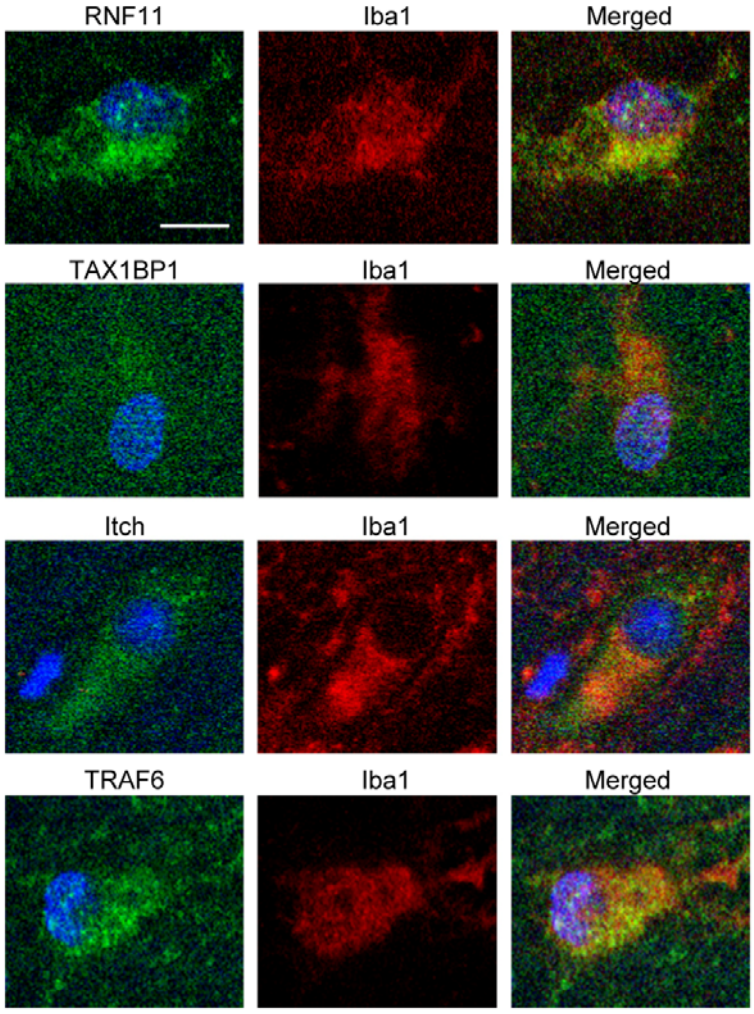

Figure 3-5. Expression of A20 complex components in microglial cells in the cortex

Sections from cerebral cortex were double-stained for each of the A20 ubiquitin-editing protein complex components, RNF11, TAX1BP1, Itch and TRAF6 with a microglial marker Iba-1 and imaged on a confocal microscope. Note that RNF11, TAX1BP1, Itch and TRAF6 (green) immunoreactivity co-localized with Iba1-positive (red) cells within the underlying white matter in cortical sections. Scale bar- 10 µm.

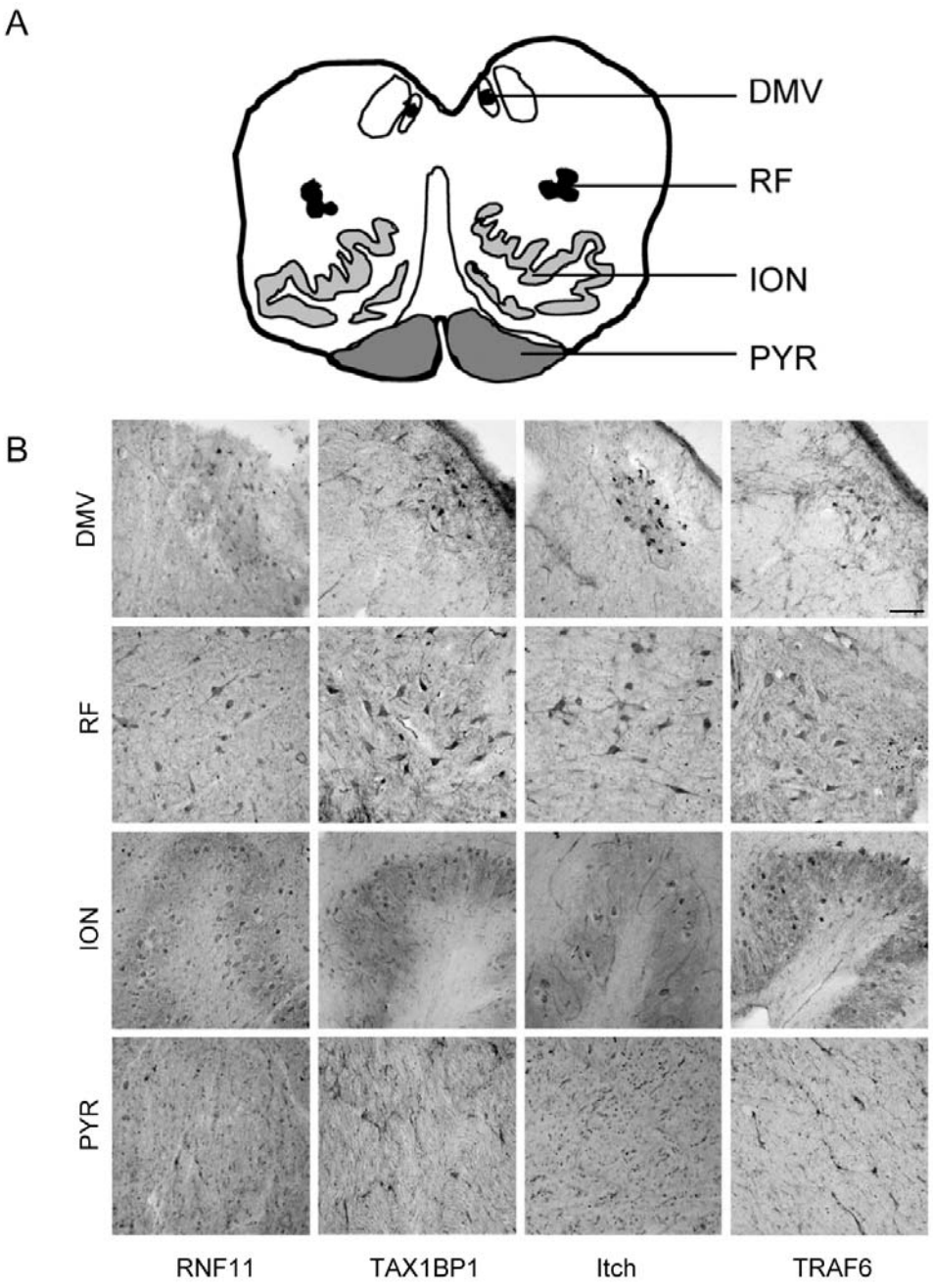

Figure 3-6. Expression of A20 complex components in normal human brainstem region

A. Schematic of the human medulla to illustrate various nuclei examined. B. Neurons of the Dorsal Motor Nucleus of Vagal nerve (DMV) and Reticular Formation (RF) were

immunoreactive for RNF11, TAX1BP1, Itch and TRAF6. Similarly, neuronal immunoreactivity for all proteins was detected in the convoluted bands of cells in the inferior olivary nucleus (ION). Immunoreactivity for RNF11, TAX1BP1, TRAF6 and Itch was detected in glia in the pyramids (PYR). Scale Bar- 100 μm.

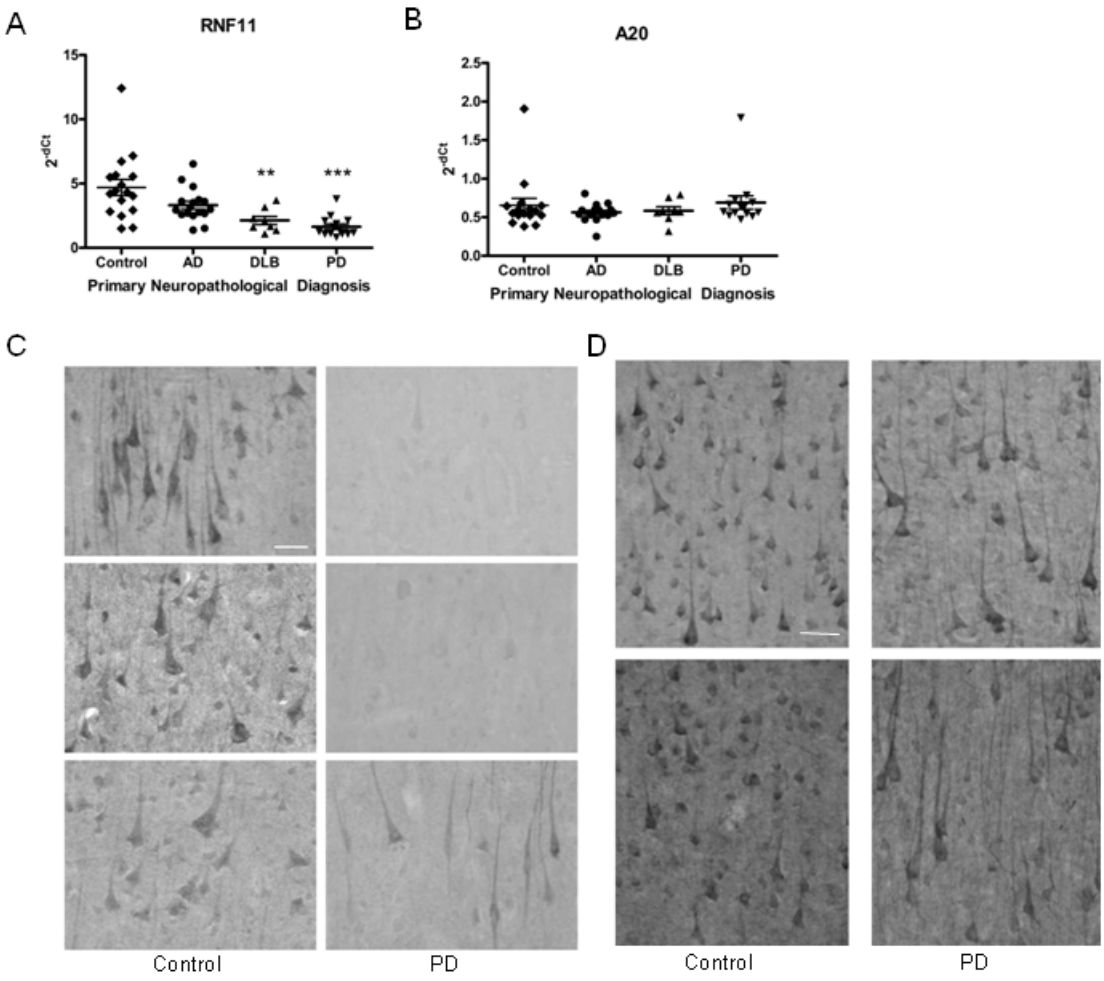

Figure 3-7. Down-regulation of RNF11 mRNA and protein expression in Parkinson's disease brain tissue

A, B, Total RNA was extracted from neocortical tissue of control, Alzheimer's disease (AD), dementia with Lewy bodies (DLB), and Parkinson's disease (PD) cases and examined by real-time PCR for expression of RNF11 and A20 mRNA. Values expressed are mean comparative cycle threshold ± SEM with GUSB levels as an internal control. **, $P < 0.01$; ***, $P < 0.001$. Frontal cortex from control and PD cases were analyzed by immunohistochemistry using antibodies against RNF11 (C) and Itch (D). Scale bar: 50 μM

Chapter 4

Neuronal RNF11 is a negative regulator of NF-κB signaling

Portions of the work presented in this chapter have been published:

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4.1 Introduction

The NF-κB transcription factor has important roles in the regulation of programmed cell death, cell proliferation and differentiation, innate and adaptive immune responses, and inflammation (Perkins, 2007). Defects in NF-κB signaling, such as persistent activation, can contribute to the pathology of several cancers and inflammatory diseases (Perkins,

2007). NF-κB also has notable ramifications in the vertebrate nervous system, where the absence of NF-κB activity during development can result in abnormal neurite branching and loss of learning and memory. Notably, persistent activation of NF-κB signaling is associated with chronic neuroinflammation and is implicated in the progression of neurodegenerative diseases (Mattson and Camandola, 2001; Hunot and Hirsch, 2003; Zhang et al., 2005b; Ghosh et al., 2007; Liang et al., 2007a; Tran et al., 2008; Kaltschmidt and Kaltschmidt, 2009; Glass et al., 2010; Perry, 2010; Bonini et al., 2011). To ensure regulated transient activity, NF-κB signaling is tightly controlled with several layers of modulation. Several NF-κB target genes can function as NF-κB inhibitors, such as A20, IκBs and CYLD (Sun, 2008; Harhaj and Dixit, 2011). The ubiquitin-editing protein A20 is a key negative regulator of NF-κB signaling and functions downstream of innate immune receptors such as TNF receptor (TNFR) and Toll-like receptors (TLRs) (Jacque and Ley, 2009). A20-deficient mice have widespread inflammation in all organ systems (Boone et al., 2004), and recent studies have indicated that the regulatory function of A20 is dependent upon interactions with the E3 ligase Itch, TAX1BP1 and RNF11 (Shembade et al., 2009; Shembade and Harhaj, 2010; Shembade et al., 2010; Parvatiyar and Harhaj, 2011). RNF11 is a 154-amino acid protein with differential expression in cancer, including breast, pancreatic and colon, as well as in PD (Seki et al., 1999; Kitching et al., 2003; Subramaniam et al., 2003; Anderson et al., 2007). RNF11 contains a RING H2 finger domain in its C-terminus, which is a hallmark characteristic of an E3 ubiquitin ligase (Lorick et al., 1999). RNF11 enhances TGF-β signaling through interactions with Smad4 and Smurf2 (Subramaniam et al., 2003; Colland and Daviet, 2004; Li and Seth, 2004; Azmi and Seth, 2005). It is also an essential component of the

A20 ubiquitin-editing complex in the periphery and can negatively regulate NF-κB signaling in human monocytic cell lines (Shembade et al., 2009). We have previously demonstrated that RNF11 is differentially expressed throughout the brain and that neurons express higher levels of RNF11 compared to glial cells in tissue (Anderson et al., 2007) (Fig. 4-1); however, the function of RNF11 in the nervous system remains to be determined. On the basis of the characterization of RNF11 in monocytic cell lines (Shembade et al., 2009), we hypothesized that neuronal RNF11 modulates NF-κB activity by interacting with the A20 ubiquitin-editing complex. To establish the effects of neuronal RNF11 on NF-κB signaling, targeted knockdown of endogenous RNF11 was employed in human neuroblastoma cells and primary cortical neurons. Reduced RNF11 expression resulted in persistent NF-κB signaling, and association of RNF11 with the A20 ubiquitin-editing protein complex was demonstrated in neuroblastoma cells and primary cortical neuron cultures. Site-directed mutagenesis of RNF11 functional motifs revealed that the myristoylation domain was necessary for RNF11's association with the A20 ubiquitin-editing protein complex as well as RNF11's regulation of canonical NF-κB signaling. Furthermore, depletion of RNF11 resulted in aberrant regulation of inflammatory signaling in neuroblastoma cells and primary cortical neurons. Taken together, these studies support a role for RNF11 in regulated activation of canonical NF-κB signaling in neurons and neuroinflammation.

4.2 RNF11-mediated regulation of NF-κB signaling

To examine the effects of RNF11 on neuronal NF-κB signaling, we transduced SH-SY5Y cells with lentivirus expressing shRNA targeted against RNF11 (shRNA-RNF11 cells) or scramble shRNA sequence (shRNA-Scramble cells). qRT-PCR was used to measure the

efficiency of RNF11 knockdown. The endogenous RNF11 mRNA level was reduced by 75% in shRNA-RNF11 cells compared to untransduced cells ($P < 0.05$) and shRNA-Scramble cells ($P < 0.05$) (Fig. 4-2A). To measure the activity of NF-κB signaling in these cell lines, shRNA-RNF11 cells and shRNA-Scramble cells were transiently co-transfected with plasmids containing an NF-κB response element driving expression of firefly luciferase or the T7 promoter driving expression of *Renilla* luciferase. TNF-α specifically binds and cross-links TNFR-1 and TNFR-2, which are members of the TNFR superfamily and are constitutively expressed in the brain; moreover, binding of TNF-α to its receptors causes downstream activation of NF-κB signaling in neurons [36]. Cells were exposed to 0 or 10 ng/ml of TNF-α for 6 hours. NF-κB-dependent firefly luciferase activity levels were normalized to control *Renilla* luciferase activity, and the level of NF-κB activity was calculated as the fold change between stimulated and unstimulated samples. Stimulation with TNF-α caused a 10-fold increase in NF-κB activity in the shRNA-RNF11 cells, which was significantly different from the observed 3-fold increase in NF-κB activity in untransduced cells ($P < 0.001$) and shRNAScramble cells ($P < 0.001$) (Fig. 4-2B). No difference was observed between shRNAScramble cells and untransduced cells. These results indicate that reduced expression of RNF11 increases NF-κB activation in neuroblastoma cells.

Canonical NF-κB signaling involves phosphorylation of IκBα by the IκB kinase (IKK) complex as a result of upstream receptor and associated complex activation. IκBα is a cytoplasmic protein that binds NF-κB transcription factors, including p65, to inhibit their activity. Phosphorylation of IκBα releases p65 and other transcription factors from the

complex and allows them to translocate to the nucleus and drive NF-κB-dependent transcription (Liu and Chen, 2011). To investigate whether changes in RNF11 expression affect p65 translocation to the nucleus, shRNA-RNF11 and shRNA-Scramble cells were immunostained with p65 and Hoechst 333258, a nuclear stain, and analyzed for p65 co-localization with Hoechst 333258 following stimulation with TNF-α for 0, 30, 60, or 120 minutes. The fold change in co-localization at steady state for each cell line was calculated. After 30 minutes of stimulation, an increase of p65 immunoreactivity in the nucleus was observed in all cell lines (representative images shown in Fig. 4-3A, quantification shown in Fig. 4-3B). p65 overlap with Hoechst 333258 decreased at 60 minutes, as well at 120 minutes to the levels observed at 0 minutes. The overlap between p65 and Hoechst 333258 in both untransduced and shRNA-Scramble cells was similar to levels observed at 0 minutes. Conversely, the amount of p65 overlap with Hoechst 333258 at 120 minutes was nearly 60% higher in shRNA-RNF11 cells compared to untransduced cells ($P < 0.01$) or shRNA-Scramble cells ($P < 0.01$). No significant differences between untransduced and shRNA-Scramble cells were observed at any time point.

To validate RNF11's effects on NF-κB signaling in primary cells, analyses for p65 translocation were performed in murine cortical neurons. Neurons were transduced with lentivirus driving expression of shRNA targeted against RNF11 or a scramble shRNA sequence, and qRT-PCR analyses revealed approximately 85% knockdown of endogenous RNF11 in shRNA-RNF11 neurons compared to untransduced neurons ($P < 0.001$) and shRNA-Scramble neurons ($P < 0.001$) (Fig. 4-3C). No significant difference

in RNF11 expression was observed between untransduced and shRNA-Scramble neurons. Neurons were analyzed for co-localization of p65 and Hoechst 333258 following stimulation with TNF-α for 0, 30 and 120 minutes. After 30 minutes of TNF-α stimulation, an approximately 2-fold increase in p65 immunoreactivity in the nucleus was observed under both conditions (Fig. 4-3D). At 120 minutes post-stimulation, p65 overlap with Hoechst 333258 in shRNAScramble cells was similar to levels observed at 0 minutes, whereas co-localization between p65 and Hoechst 333258 remained elevated in shRNA-RNF11 cells ($P < 0.01$) (Fig. 4-3D). The results of these experiments reveal that reduced RNF11 expression leads to persistent p65 localization to the nucleus. Coupled with the NF-κB reporter analyses described above, these results strongly suggest that RNF11 is a negative regulator of neuronal NF-κB signaling.

To support the p65 immunofluorescence data, SDS-PAGE was performed to examine p65 levels in cytoplasmic and nuclear fractions from SH-SY5Y shRNA-Scramble and shRNARNF11 cells that were isolated after 0, 30 or 120 minutes of TNF-α stimulation. Western blot analyses revealed that p65 immunoreactivity decreased in the cytoplasmic fraction by 78% but increased more than sevenfold in the nuclear fraction after 30 minutes of TNF-α stimulation in shRNA-Scramble cells (Fig. 4-3E, quantification in Fig. 4-3F, 4-3G). After 120 minutes of TNF-α exposure, p65 immunoreactivity decreased in the nuclear fraction and increased in the cytoplasmic fraction to approximately steady-state levels in the shRNA-Scramble samples. In contrast, levels of p65 immunoreactivity from shRNA-RNF11 samples after 30 and 120 minutes of stimulation in the cytoplasmic and nuclear fractions did not significantly change. We observed an approximately 70%

decrease from steady state at both time points in the cytoplasmic fractions and more than 6.5-fold and 5-fold increases at 30 and 120 minutes, respectively, from steady state in the nuclear fractions ($P < 0.001$). These results are consistent with our hypothesis that RNF11 is a negative regulator of TNF-α- induced canonical NF-κB signaling in neurons.

4.3 Association of neuronal RNF11 with the A20 ubiquitin editing protein complex

The results of previous studies in a monocyte cell line suggest that united members of the A20 ubiquitin-editing protein complex are required for mitigation of the canonical NF-κB signaling pathway (Shembade et al., 2008). The essential members of the complex are the deubiquitinase and E3 ligase A20, the E3 ligase Itch, TAX1BP1 and RNF11 (Shembade et al., 2009; Parvatiyar and Harhaj, 2011). The A20 ubiquitin-editing protein complex is responsible for deubiquitination of K63 ubiquitin linkage on RIP1 and attachment of K48 ubiquitin linkage to RIP1, which thereby facilitates proteasomal degradation of RIP1 and termination of NF-κB signaling (Liu and Chen, 2011). To determine whether RNF11 associates with the A20 ubiquitin-editing protein complex in a neuronal system, we performed coimmunoprecipitation (co-IP) experiments in N2A cells. Because of low endogenous expression of RNF11, N2A cells were transduced with lentivirus expressing wild-type RNF11 tagged with a V5 epitope (N2A V5-RNF11). Additionally, owing to the low level of endogenous A20, N2A V5-RNF11 cells were transiently transfected with plasmid expressing FLAG-tagged A20 (FLAG-A20). V5-RNF11 immunoprecipitates were enriched with A20 immunoreactivity, and control immunoprecipitates with V5 antibody omitted were absent of A20 (Fig. 4-4A). Reciprocal co-IPs revealed that RNF11 was enriched in FLAG pull-down assays for FLAG-A20 (Fig. 4-4B), indicating that

RNF11 and A20 associate in neuroblastoma cells. In parallel, co-IPs were performed to examine RNF11's association with Itch. Pull-down assays with V5 antibody from N2A V5-RNF11 cell lysates were enriched with Itch immunoreactivity, whereas control co-IPs with V5 antibody omitted were absent for Itch (Fig. 4-4C). Reciprocal co-IPs showed V5-RNF11 enrichment in endogenous Itch immunoprecipitates (Fig. 4-4D). These findings indicate that RNF11 associates with A20 and Itch in neuronal cell lines and suggests that at least a portion of the A20 ubiquitin-editing protein complex exists in neuronal systems.

To determine whether TNF-α stimulation affects RNF11's association with the A20 ubiquitin-editing protein complex, co-IP experiments were performed in primary neurons following stimulation with TNF-α for 0 or 30 minutes. RNF11 immunoprecipitates were enriched with A20 immunoreactivity at steady state and following TNF-α stimulation (Fig. 4-4E), and a significant increase in A20 immunoreactivity was observed following TNF-α stimulation for 30 minutes in RNF11 immunoprecipitates (0 minutes: 100%, 30 minutes: 128.7%; $P < 0.05$) (Fig. 4-4F). Additionally, parallel co-IP experiments were conducted to examine the association between RNF11 and Itch following TNF-α stimulation. RNF11 immunoprecipitates were enriched with Itch immunoreactivity under both steady-state and TNF-α stimulation conditions (Fig. 4-4H), and, like A20, a significant increase in Itch immunoreactivity was observed in RNF11 immunoprecipitates following stimulation with TNF-α (0 minutes: 100%, 30 minutes: 165.8%; $P < 0.05$) (Fig. 4-4I). Importantly, RNF11 levels were similar for all immunoprecipitates (Fig. 4-4E, 4-4H, quantification shown in Fig. 4-4G, 4-4J). These

results demonstrate that RNF11 can exist in complexes with A20 and Itch in neurons and that TNF-α stimulation enhances the association of A20 and Itch with RNF11.

4.4 Myristoylation domain is required for the function of RNF11

The myristoylation domain of RNF11 is necessary for proper recruitment to early endosomes, whereas the PPxY motif is important for RNF11's associations with Itch-, Smurf2- and Nedd4-like proteins (Subramaniam et al., 2003; Santonico et al., 2010). The RING domain of RNF11 is important for interactions with proteins involving ubiquitination and degradation (Seki et al., 1999). Although the substrate for RNF11's putative E3 ubiquitin ligase activity has yet to be reported, researchers in previous studies have identified mutations that interfere with the ubiquitination activity of the RING domain in analogous E3 ubiquitin ligases (Joazeiro and Weissman, 2000). Mutations of cysteines or histidines within the RING domain cause structural changes that prevent E2 binding and ubiquitination, whereas an isoleucine mutation within the RING domain prevents interactions with E2 enzymes but keeps the RING domain structure intact (Brzovic et al., 2003). We generated RNF11 mutants for each of these domains to examine their importance with respect to RNF11's influence over neuronal NF-κB signaling (Fig. 4-5A). A myristoylation mutant was made by substituting alanine for glycine 2 (RNF11-G2A) (Santonico et al., 2010), and a PPxY motif mutant was constructed by substituting alanine for tyrosine 40 to abolish binding with Nedd4-like proteins (RNF11-Y40A) (Subramaniam et al., 2003; Santonico et al., 2010). RING domain mutants were generated by substituting alanine for isoleucine 101 to prevent E2 binding (RNF11-I101A) (Brzovic et al., 2003), or by substituting alanine for histidine

119 and histidine 122 to abrogate RING domain structure (RNF11-H2) (Joazeiro and Weissman, 2000), or by substituting alanine for cysteine 99 to abolish proper RING domain folding (RNF11-C99A) (Joazeiro and Weissman, 2000). Importantly, additional site-directed mutagenesis was performed to generate shRNA-resistant forms of these RNF11 mutants. This approach allowed for the expression of RNF11 mutants under reduced expression of endogenous RNF11 in NF-κB-dependent luciferase assays and co-IPs (Fig. 4-5B). Silent mutations were introduced at glutamine 72 and arginine 73 (Shembade et al., 2009) in wild-type RNF11, the myristoylation mutant, the PPxY motif mutant and the three RING domain mutants to generate the following shRNA-resistant RNF11 constructs: V5-WT^{R}, V5-G2A^{R}, V5-Y40A^{R}, V5-I101A^{R}, V5-H2^{R}, and V5-C99A^{R}, respectively (Fig. 4-5A).

To determine the impact of RNF11's functional amino acid motifs with respect to NF-κB signaling, we conducted the luciferase assays described in Fig. 4-2B. SH-SY5Y shRNARNF11 cells were transiently cotransfected with empty vector, V5-WT^{R}, V5-G2A^{R}, V5- Y40A^{R}, V5-I101A^{R}, V5-H2^{R}, or V5-C99A^{R} constructs, as well as plasmids containing an NF- κB response element driving expression of firefly luciferase or the T7 promoter driving expression of *Renilla* luciferase. Cells were exposed to 0 or 10 ng/ml of TNF-α, and luciferase readouts were measured after 6 hours of TNF-α stimulation. Expression of V5-WT^{R}, V5-H2^{R}, or V5-I101A^{R} in shRNA-RNF11 cells decreased NF-κB activity by approximately 75% compared to vector-transfected cells ($P < 0.001$) (Fig. 4-5C). Transfection of V5-G2A^{R} or V5-C99A^{R} did not significantly alter NF-κB activity in comparison to vector transfected cells, but was significantly different from cells

transfected with V5-WT^R ($P < 0.001$). Last, expression of V5-Y40A^R reduced NF-κB activity only 24%, a significant difference from both vector and V5-WT^R ($P < 0.05$ for vector and V5-Y40A^R; $P < 0.001$ for V5-WT^R and V5-Y40A^R). Western blot analyses indicated similar expression levels of RNF11 mutants (Fig. 4-5D). These results suggest that the myristoylation domain is required and the PPxY motif is necessary for the full effect of RNF11's influence over NF-κB activation as measured by luciferase assay. Moreover, cysteine 99 is required, but isoleucine 101 or histidines 199 and 122 are dispensable, for RNF11-mediated suppression of NF-κB activity under these experimental conditions.

To determine whether mutations in the myristoylation domain, PPxY motif or RING domain alter RNF11's association with the A20 ubiquitin-editing protein complex, SH-SY5Y shRNA-RNF11 cells were transfected with vector, V5-WT^R, V5-G2A^R, V5-Y40A^R, V5- I101A^R, V5-H2^R or V5-C99A^R, and co-IPs for endogenous Itch were performed. V5-WT^R, V5-I101A^R, V5-H2^R, and V5-C99A^R immunoprecipitates were enriched with Itch immunoreactivity compared to vector control (Fig. 4-6A, 4-6B), whereas V5-G2A^R immunoprecipitates were absent for Itch (Fig. 4-6A). V5-Y40A^R immunoprecipitates displayed endogenous Itch immunoreactivity, although it was reduced compared to V5-WT^R immunoprecipitates (Fig. 4-6A). Additionally, SH-SY5Y shRNA-RNF11 cells were transfected with vector, V5-WT^R, V5-G2A^R, V5-Y40A^R, V5-I101A^R, V5-H2^R, or V5-C99A^R, as well as FLAG-A20, and co-IPs for A20 were performed. The immunoprecipitate of each RNF11 construct tested was enriched with A20 immunoreactivity compared to vector control (Fig. 4-6C, 4-6D). These studies

revealed that the myristoylation domain is necessary for RNF11 association with Itch and that the PPxY motif is also important. On the basis of these results and the luciferase assays described above, we hypothesize that disrupted RNF11-Itch complex formation contributes to the impaired regulation of NF-κB activation exhibited by the RNF11 myristoylation domain and PPxY motif mutants.

4.5 Altered inflammatory responses with manipulation of RNF11

Inflammation is tightly associated with NF-κB signaling as well as with neurological disease; therefore, we investigated whether RNF11 is linked to inflammatory response induction. To test this hypothesis, SH-SY5Y shRNA-RNF11 and shRNA-Scramble cells were exposed to 0 or 10 ng/ml TNF-α for 4 hours, and RNA was extracted to examine by qRT-PCR the induction of inflammatory cascades. Specifically, mRNA levels of the proinflammatory cytokine TNF-α and the cytokine-inducible protein A20 were measured (Collart et al., 1990; Shakhov et al., 1990a; Shakhov et al., 1990b; Krikos et al., 1992). TNF-α is transcriptionally regulated by NF-κB as well as by other inflammation-related transcription factors; therefore, increased TNF-α mRNA levels are generally indicative of an immune response involving multiple signaling pathways (Feuerstein et al., 1994). Changes in A20 mRNA levels are linked more specifically to activity of NF-κB signaling (McCoy and Tansey, 2008; Lee et al., 2010). TNF-α stimulation increased A20 mRNA levels in shRNA-Scramble cells; however, a more substantial increase in A20 mRNA levels was observed in shRNA-RNF11 cells (shRNA-Scramble: 3.81-fold change, shRNA-RNF11: 38.70-fold change; $P < 0.001$) (Fig. 4-7A). Similarly, TNF-α stimulation increased TNF-α mRNA levels in shRNA-Scramble cells, but to a greater degree in

shRNARNF11 cells (shRNA-Scramble: 3.08-fold change, shRNA-RNF11: 9.70-fold change; $P < 0.001$) (Fig. 4-7B). Importantly, steady-state levels of A20 and TNF-α mRNA were not significantly different between shRNA-RNF11 cells and shRNA-Scramble cells. To further support these findings, we performed a TNF-α ELISA to measure secreted TNF-α protein levels. After serum starvation for 1 hour, cells were stimulated with 0 or 10 ng/ml TNF-α for 4 hours. To minimize detection of the human recombinant TNF-α used to stimulate the cells, cells were rinsed with serum-free media and incubated with a reduced volume of serum-free media for 20 hours. Basal levels of TNF-α were similar in shRNA-Scramble (10.49 pg/ml) and shRNA-RNF11 cells (below the limits of detection) (Fig. 4-7C). Recombinant TNF-α stimulation of the NF-κB pathway increased production of TNF-α protein in shRNAScramble cells and, to a larger extent, in shRNA-RNF11 cells (shRNA-Scramble: 744.51, shRNA-RNF11: 1,285.03 pg/ml; $P < 0.001$).

To expand these studies to primary cells, murine cortical neurons were transduced with lentivirus expressing shRNA-Scramble or shRNA-RNF11 and monocyte chemotactic protein 1 (MCP-1) mRNA levels were measured. MCP-1 is a potent chemokine that attracts monocytes, macrophages and microglia to sites of injury to evoke further release of inflammatory molecules, and it is involved in inflammatory neuropathologies (Fuentes et al., 1995; Chen et al., 2003; Thompson and Van Eldik, 2009; Lee et al., 2010). Importantly, like TNF-α and A20, MCP-1 is transcriptionally regulated by NF-κB (Ueda et al., 1994; Ueda et al., 1997). In our primary neuron-enriched cultures, we specifically examined effects of reduction of the high RNF11 mRNA expression in neurons (in

comparison to glial cells, as shown in Fig. 4-1) due to the cytosine arabinoside treatment of our cultures. Stimulation with 10 ng/ml TNF-α revealed a time-dependent increase in MCP-1 mRNA levels at 4 and 24 hours post-stimulation in shRNA-Scramble neurons, whereas a greater increase was observed at each time point in neurons transduced with shRNA-RNF11 (shRNA-Scramble 0 hours: 1.02-fold change, 4 hours: 6.38-fold change, 24 hours: 14.91-fold change; shRNA-RNF11: 0 hours: 1.81-fold change, 4 hours: 25.73-fold change, 24 hours: 29.04-fold change; $P < 0.05$ for both time and transduction differences) (Fig. 4-7D). This result was confirmed by analyzing MCP-1 protein levels from conditioned media of stimulated cells. Transduced shRNA-Scramble or shRNA-RNF11 primary cortical neurons were stimulated with 0 or 10 ng/ml TNF-α in a reduced volume of serum-free media for 24 hours. Basal levels of MCP-1 were similar in media collected from shRNA-Scramble (9.41 pg/ml) and shRNA-RNF11 cells (9.53 pg/ml). TNF-α stimulation increased MCP-1 protein levels in media collected from shRNA-Scramble cells and, to a greater degree, from shRNA-RNF11 cells (shRNA-Scramble: 1,976.60, shRNA-RNF11: 3,654.02 pg/ml; $P < 0.001$) (Fig. 4-7E). These results suggest that changes in RNF11 expression influence mRNA and protein levels of gene products that are downstream targets of NF-κB signaling. Therefore, we propose that RNF11 likely contributes to the mitigation of inflammatory responses exerted by neurons.

4.6 Discussion

NF-κB activity is essential for peripheral immune cell survival, and proper regulation of NF- κB signaling is critical for mounting a normal immune response (Li and Verma, 2002). Persistent activation of the NF-κB pathway is known to promote inflammation and

inflammatory diseases (Li and Verma, 2002; Karin and Greten, 2005), including progressive neurodegenerative diseases (Mattson and Camandola, 2001; Hunot and Hirsch, 2003; Zhang et al., 2005b; Ghosh et al., 2007; Liang et al., 2007a; Tran et al., 2008; Kaltschmidt and Kaltschmidt, 2009; Glass et al., 2010; Perry, 2010; Bonini et al., 2011). Notably, inhibition of NF-κB activity has been demonstrated to attenuate neuroinflammation and cell death in animal models of PD, AD and ischemia (Ghosh et al., 2007; Liang et al., 2007a; Tran et al., 2008; Cao et al., 2010; van der Kooij et al., 2010). In this study, we investigated the role of neuronal RNF11 as a component of the A20 ubiquitin-editing complex in regulating TNF-α-induced canonical NF-κB activity by targeted knockdown of endogenous RNF11 in human neuroblastoma cells and primary neuronal cultures. Our analyses revealed that (1) neuronal RNF11 acts as a negative regulator of the canonical NF- κB signaling pathway, (2) neuronal RNF11 associates with the A20 ubiquitin-editing protein complex, (3) the myristoylation and PPxY domains of RNF11 are required and necessary, respectively, for RNF11's effects on NF-κB signaling and association with Itch, a member of the A20-ubiquitin editing protein complex and (4) reduced expression of RNF11 in neurons can result in aberrant regulation of inflammatory signaling. Together these findings suggest that RNF11 has a critical role in the regulation of canonical NF-κB signaling in the central nervous system.

NF-κB responds to a diverse series of inflammatory activators, including ultraviolet light, double-stranded RNA, cytokines, vasoactive peptides and viral oncogenes (Pahl, 1999). Stimulation of the NF-κB signaling pathway causes transcription of various inflammatory markers, including inducible chemokines, cell adhesion molecules and vasoactive and

antiapoptotic proteins important in the cellular stress response to effective control of inflammation (Brasier, 2006). The signaling pathway also has several levels of autoregulation, one of which includes the activation and association of the RIP1 and IκB kinases by the A20 ubiquitin-editing protein complex (Brasier, 2006). Through the degradation of RIP1 and the disruption of the association between RIP1 and IKK, the A20 ubiquitin-editing protein complex efficiently halts canonical NF-κB signaling (Brasier, 2006). Outside the nervous system this complex requires the association of A20, Itch, TAX1BP1 and RNF11 to enhance K48-linked ubiquitination of RIP1 and promote RIP1 targeting to the proteasome (Shembade et al., 2009; Parvatiyar and Harhaj, 2011). Recently, a human genomewide siRNA screen identified RNF11 as an important modulator of canonical NF-κB activity in HEK-293 cells (Gewurz et al., 2012). In this report, we have demonstrated that reduction of RNF11 in primary cortical neurons sustained canonical NF-κB signaling, most likely by RNF11's lack of association with the A20 ubiquitin-editing protein complex. A yeast two-hybrid screen revealed that Itch, TAX1BP1, NEMO, and A20 are potential binding partners of RNF11 (Azmi and Seth, 2005). Other researchers have reported associations of RNF11 with glutathione S-transferase-tagged Itch in breast cancer cell lines (Kitching et al., 2003), tagged RNF11 with tagged TAX1BP1 and A20 in HEK cells (Shembade et al., 2009) and RNF11 with A20, TAX1BP1 and RIP1 in mouse embryonic fibroblasts and blood-derived macrophages (Shembade et al., 2009). Our present work reveals that RNF11 associates with Itch and A20 in neuronal systems and that there is an increased association of A20 and Itch with RNF11 after TNF-α stimulation (Fig. 4-4). This phenomenon may be due to enhanced stability of the A20 ubiquitin-editing protein complex through protein changes

such as post-translational modifications. Importantly, our data suggest that neurons, similarly to monocytic cells (Shembade et al., 2009), form an A20 ubiquitin-editing protein complex to garnish transient reactions for regulating the canonical NF-κB signaling pathway.

Our studies involving RNF11 mutants indicate that the myristoylation domain is imperative for RNF11's effects on NF-κB activity as well as for association with the A20 ubiquitin editing protein complex (Fig. 4-5, 4-6). Mutagenesis of glycine 2 disrupts the protein's ability to incorporate myristoylic acid, causing a shift in localization from cytosolic vesiclelike structures to a more diffuse pattern throughout the cell (Santonico et al., 2010). Furthermore, mutation of RNF11's glycine 2 prevents association and ubiquitination by Itch and Nedd4 (Santonico et al., 2010). V5-G2A^{R} most likely does not localize to intracellular compartments, where it interacts with other members of the A20 ubiquitin-editing protein complex and therefore fails to reduce NF-κB-induced luciferase activity and co-IP with Itch (Fig. 4-5C, 4-6A, 4-6C).

RNF11 also harbors a PPxY motif that previously has been shown to be important for associations with Itch and other Nedd4-like proteins (Kitching et al., 2003; Chen et al., 2008; Shembade et al., 2009; Santonico et al., 2010). In contrast, we have shown in this study that V5-Y40AR associated with endogenous Itch in neuroblastoma cells, although Itch immunoreactivity was reduced compared to wild-type RNF11. Santonico *et al*. proposed that RNF11 may have an alternative binding site (VPxY) for Itch and structurally similar proteins (Santonico et al., 2010), which may explain why we

observed some degree of Itch and RNF11 interaction. Santonico *et al*. believe that this nearby site explains why some degree of RNF11 ubiquitination by Itch occurs when the consensus PPxY site is mutated. It would be interesting to explore whether this site is important for interactions with Itch or how a double PPxY and VPxY mutation would affect RNF11 association with the A20 ubiquitin-editing protein complex. Additionally, we observed some reconstitution of negative regulation of NF-κB signaling following expression of V5-Y40AR, whereas Shembade *et al*. did not (Shembade et al., 2009). This contrast may be attributed to experimental differences in measuring NF-κB signaling. Shembade *et al*. used Western blot analysis to examine transient phosphorylation of IκBα and JNK, whereas we employed quantitative luciferase assays.

The three RNF11 constructs we generated harboring RING domain mutations all maintained interactions with Itch and A20 (Fig. 4-6B, 4-6D). Previously, Shembade *et al*. showed that RNF11 with a mutation of cysteine 99 to alanine was unable to associate with A20, TAX1BP1 or RIP1 in TNF-α-stimulated THP-1 cells (Shembade et al., 2009). Similarly to our studies, Santonico *et al*. found that an RNF11 RING domain mutant with serines substituted at cysteines 99 and 102 retained binding to Itch in HEK cells (Santonico et al., 2010). This discrepancy may underlie a putative difference between innate immune cells and other human cell lines in the stability and transient nature of the associations among A20-ubiquitin protein complex members.

The RING domain mutants we examined altered NF-κB-dependent luciferase activity differently, despite maintaining associations with Itch and A20. Although the mutation of

isoleucine 101 and the mutations of histidines 119 and 122 recapitulated effects of wild-type RNF11, the mutation of cysteine 99 did not mimic wild-type RNF11 in this assay. Similarly, Shembade *et al*. showed that mutation of cysteine 99 to alanine was unable to restore negative regulation of NF-κB signaling in siRNA rescue experiments (Shembade et al., 2009). The results of our study support these data and add quantitative assessment of luciferase-dependent NF-κB activity of this mutant (Fig. 4-5C). However, the incongruity between the three RING domain mutants in the NF-κB-dependent luciferase assay highlights an essential remaining question: Is RNF11's E3 ubiquitin ligase activity critical for negative regulation of NF-κB signaling by the A20 ubiquitin-editing protein complex?

NF-κB family proteins and the A20 ubiquitin-editing protein complex, though differentially distributed in various cells, are highly expressed in peripheral immune cells, including lymphocytes and macrophages [38,54,62-64]. RNF11 mRNA and protein levels are higher in neurons than in glial cells, including microglia (Fig. 4-1). The reason for this differential expression in neurons is not clear, but could be a protective strategy in post-mitotic cells which have a limited lifespan and limited tolerance to chronic NF-κB activation and neuroinflammation (Shembade et al., 2009). Our studies show that reduced levels of RNF11 in neurons increase production of the inflammatory cytokine MCP-1 following TNF-α activation (Fig. 4-7D, 4-7E). Other agents targeting NF-κB signaling (that is, Nurr1, alpha-synuclein, amyloid-β) have been shown to increase production of inflammatory cytokines (Lotz et al., 2005; White et al., 2005; Zhang et al., 2005b; Austin et al., 2006; Saijo et al., 2009). In neurodegenerative disease and ischemic attack, it is

hypothesized that signals sent from damaged neurons and glial cells cause activation of NF-κB signaling (Vallabhapurapu and Karin, 2009). Chronic stimulation of the NF-κB signaling cascade brings about increased production of inflammatory molecules such as TNF- α, nitric oxide and IL-1, resulting in oxidative stress and neuronal degeneration (Liu et al., 2002a; McGeer et al., 2005; Sawada et al., 2006). This can be observed in the brains of patients with stroke, AD, PD, ALS and other neurodegenerative diseases (Yamada et al., 1992; Mogi et al., 1994; Kreutzberg, 1996; Hunot and Hirsch, 2003; Liu et al., 2003; Tansey et al., 2007), and it has been hypothesized that the pathogenesis and progression of neurological diseases is in part due to dysregulation or chronic activation of the NF-κB pathway (Nguyen et al., 2002; Wyss-Coray and Mucke, 2002; Glass et al., 2010). Notably, *RNF11* is a candidate gene for PD (Hicks et al., 2002) and RNF11 co-localizes with α-synuclein-positive Lewy bodies and neurites in PD patients (Anderson et al., 2007). Our future studies will focus on investigating the role of RNF11 as a regulator of neuroinflammation in animal models of neurodegenerative diseases.

Given the mounting evidence that neuroinflammation is heightened in neurodegenerative diseases (Ghosh et al., 2007; Liang et al., 2007a; Tran et al., 2008; Frank-Cannon et al., 2009; Cao et al., 2010), we conclude that functionally compromised or reduced expression of RNF11 could result in persistent NF-κB activation and could promote chronic neuroinflammation. Further studies will explore these hypotheses in relevant animal models to ascertain how RNF11 expression levels affect NF-κB signaling *in vivo*, as well as be confirmed in primary neuronal cultures from ventral mesencephalon. Additionally, the impact of post-translational modifications on RNF11 activity will be

explored to gain further understanding of RNF11's role within the A20 ubiquitin-editing protein complex in neurons.

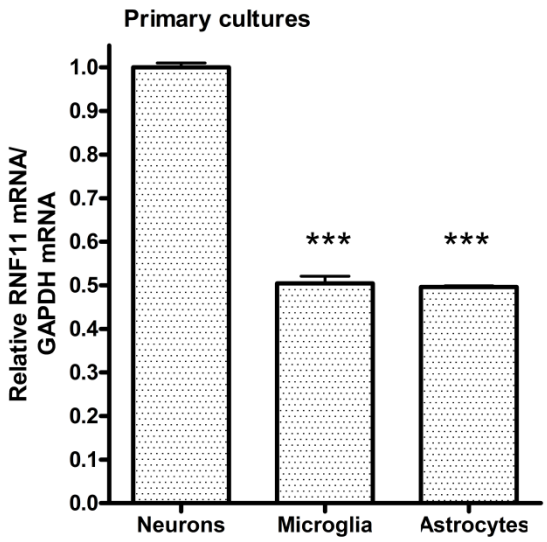

Figure 4-1. RNF11 has robust expression in neurons

Murine primary cultures were harvested, and qRT-PCR was used to measure relative RNF11 mRNA levels. qRT-PCR values are expressed as mean comparative cycle threshold ± SEM with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels as an internal control for three independent experiments. Expression levels were analyzed by one-way ANOVA with Bonferroni post-tests. ***, $P < 0.001$.

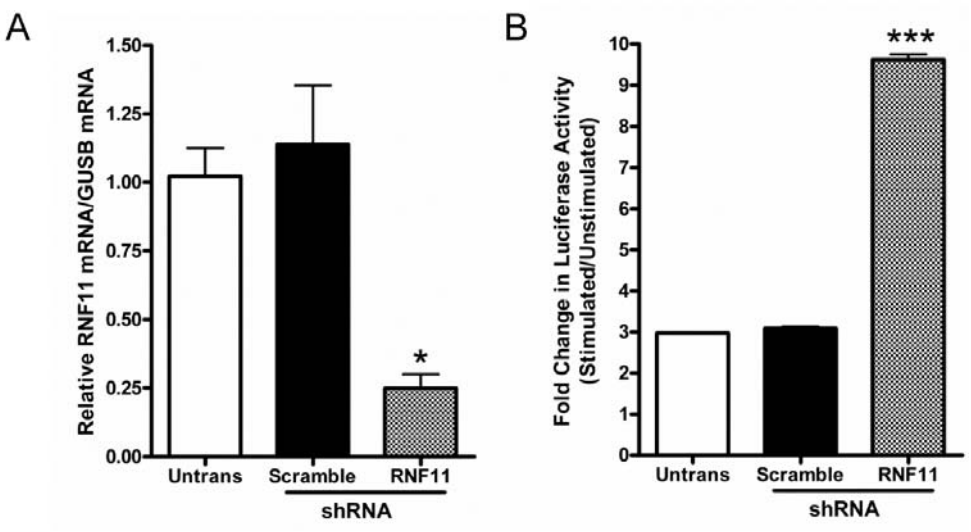

Figure 4-2. Increased NF-κB luciferase activity in neuronal cells with knockdown of RNF11

A. Stable SH-SY5Y cell lines were generated using lentiviruses containing a control scramble small hairpin RNA (shRNA) sequence (shRNA-Scramble cells) or shRNA targeted against RNF11 (shRNA-RNF11 cells). Quantitative RT-PCR was used to measure relative RNF11 mRNA levels with glucuronidase β as an internal control. Values are expressed as mean comparative cycle threshold ± SEM of triplicate experiments. B. Stable SH-SY5Y cell lines, as well as untransduced cells, were transiently co-transfected with luciferase and *Renilla* plasmids and stimulated with 10 ng/ml TNF-α or PBS for 6 hours. Values represent the fold change in NF-κB-dependent activity as measured by the ratio of luciferase to *Renilla* luminescence in stimulated over unstimulated samples ± SEM in triplicate experiments. Results were analyzed by one-way ANOVA with Bonferroni post-tests. *$P < 0.05$; ***$P < 0.001$.

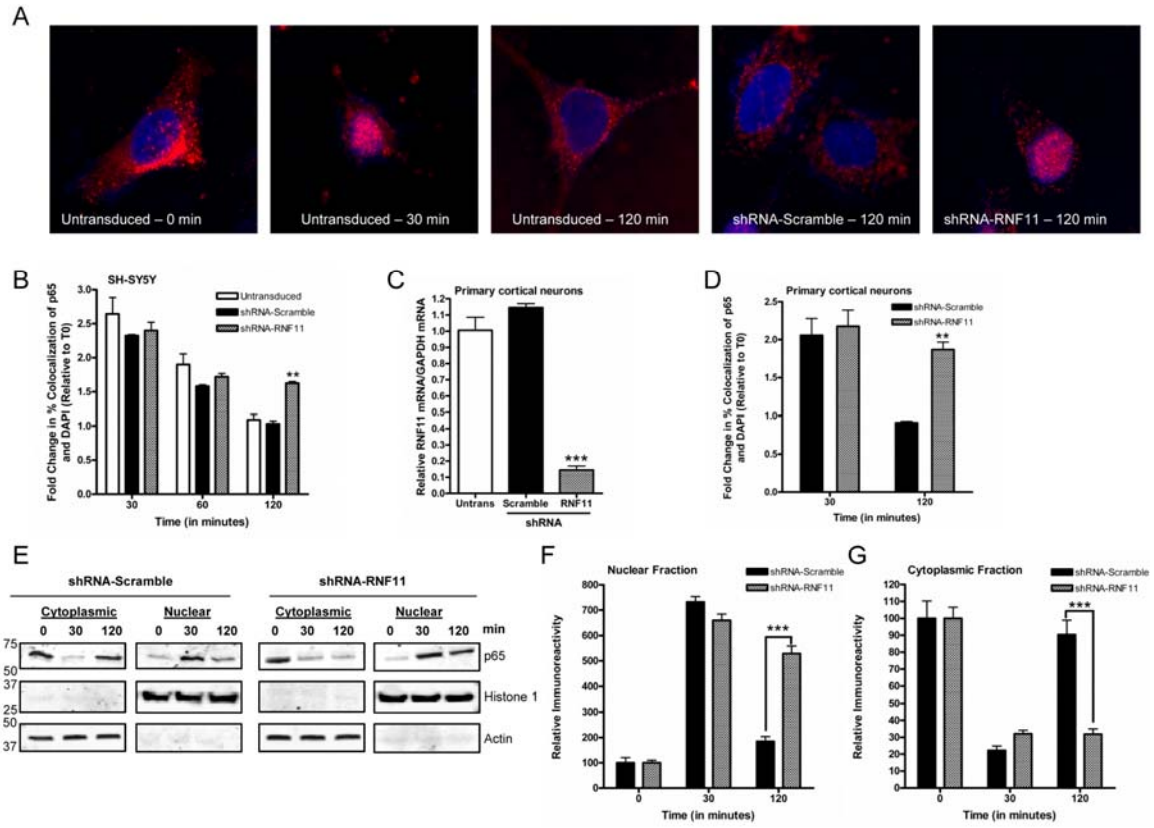

Figure 4-3. Prolonged nuclear p65 translocation after TNF-α stimulation observed in neuronal cells with knockdown of RNF11

A. SH-SY5Y cell lines (untransduced, small hairpin RNA (shRNA)-RNF11, shRNA-Scramble) were stimulated with TNF-α for 0, 30, 60 or 120 minutes. Cells were immunostained for p65 (red) and nuclear DNA (Hoechst 333258, blue). Representative images obtained at 0, 30 and 120 minutes after stimulation are shown. B. Cells were analyzed for overlap of p65-positive and Hoechst 333258-positive pixels. The fold change in percentage overlap of p65- and Hoechst 333258-positive pixels was calculated relative to unstimulated conditions. Results were analyzed by two-way repeated measures ANOVA with Bonferroni post-tests. C. Murine primary cortical neurons were treated with cytosine arabinoside and transduced with lentiviruses containing shRNA targeted against RNF11 (shRNA-RNF11 neurons) or scramble shRNA sequence (shRNA-

Scramble neurons). Quantitative RT-PCR (qRT-PCR) was used to measure relative RNF11 mRNA levels. Results were analyzed by one-way ANOVA with Bonferroni post-tests. D. shRNA-RNF11 or shRNA-Scramble neurons were stimulated for 0, 30 or 120min before being immunostained for p65 and Hoechst 333258. Transduced cells were analyzed for overlap of p65- and Hoechst 333258-positive cells. The fold change in percentage overlap of p65- and Hoechst 333258-positive pixels was calculated relative to unstimulated conditions. Results were analyzed by two-way repeated measures ANOVA with Bonferroni post-tests. E. shRNA-Scramble and shRNA-RNF11 cells were stimulated for 0, 30 or 120 minutes. Cytoplasmic and nuclear fractions were resolved by SDS-PAGE. F. ImageJ software was used to quantify the ratio of the densitometry of the p65 bands in the nuclear fractions to the density of histone 1 immunoreactivity. The ratio for each time point was compared relative to the steady-state ratio, which was set at 100%. Results were analyzed by two-way repeated measures ANOVA with Bonferroni post-tests. G. ImageJ software was used to quantify the ratio of the densitometry of the p65 bands in the cytoplasmic fractions in a manner similar to that described in F. All p65 co-localization values are means ± SEM of triplicate experiments. qRT-PCR values are expressed as mean comparative cycle threshold ± SEM of triplicate experiments. Western blots of cytoplasmic and nuclear fractions were run from triplicate experiments. Results were analyzed by two-way repeated measures ANOVA with Bonferroni post-tests. $^{**}P < 0.01$; $^{***}P < 0.001$.

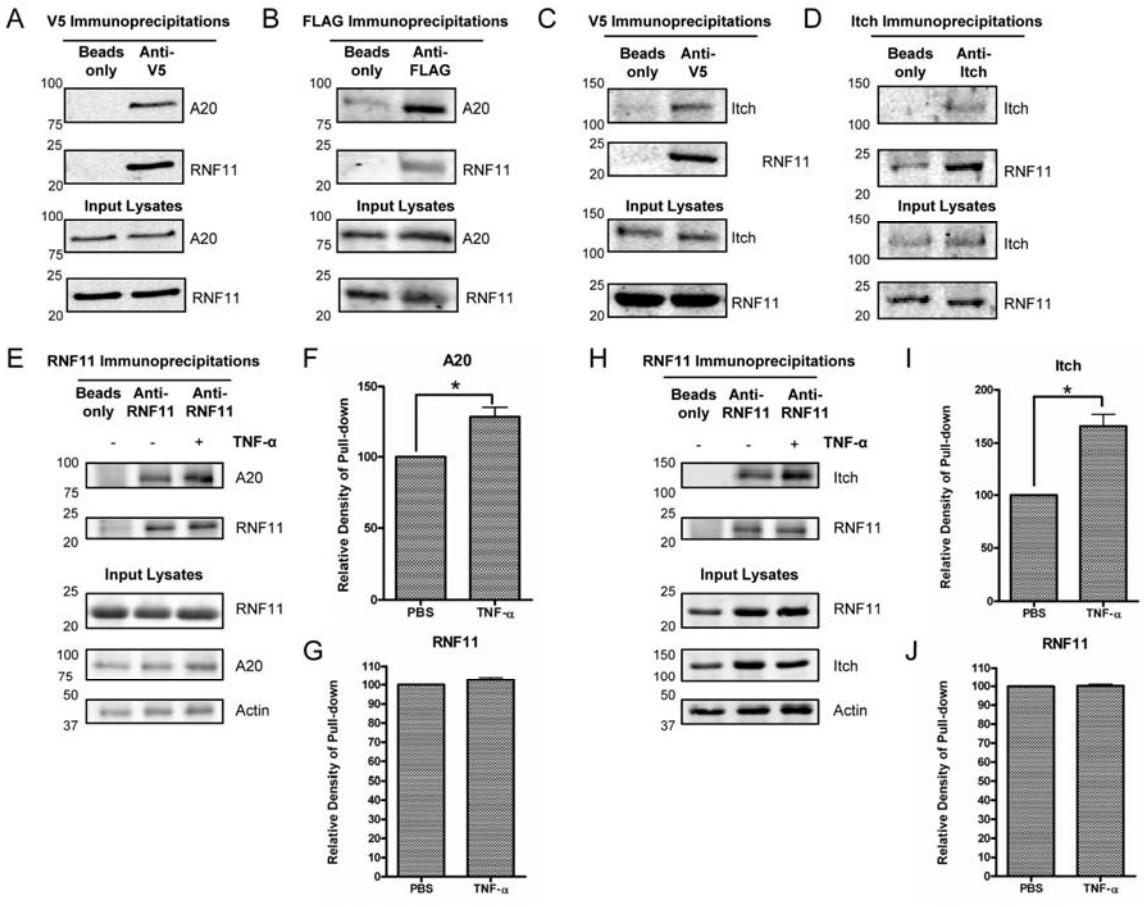

Figure 4-4. Dynamic associations of RNF11 with both A20 and Itch in primary neurons

N2A cells transduced with V5-RNF11 lentivirus (N2A V5-RNF11) and transfected with FLAG-A20 were harvested for immunoprecipitation (IP) with V5 antibody (A) or harvested for IP with FLAG antibody (B). Proteins were resolved by SDS-PAGE and immunoblotted with anti-A20 and RNF11. In parallel, pull-down assays with V5 antibody (C) or with Itch antibody (D) from N2A V5-RNF11 cell lysates were resolved by SDS-PAGE. Immunoprecipitates and lysates were immunoblotted with anti-Itch and RNF11. (E) and (H) Murine primary cortical neurons were stimulated with 10 ng/ml TNF-α for 0 or 30 minutes and harvested for IP with RNF11 antibody. Control IP experiments were performed with antibody omitted. Proteins were resolved by SDS-

PAGE and immunoblotted with anti-A20, Itch, RNF11 and actin. (F), (G), (I) and (J) ImageJ software was used to quantify the densitometry of the immunoprecipitated bands relative to the 0-minutes time point. Each input sample's immunoreactivity was used as a loading control. All IPs are representative of at least three independent experiments. Results were analyzed by paired *T*-tests. $*P < 0.05$.

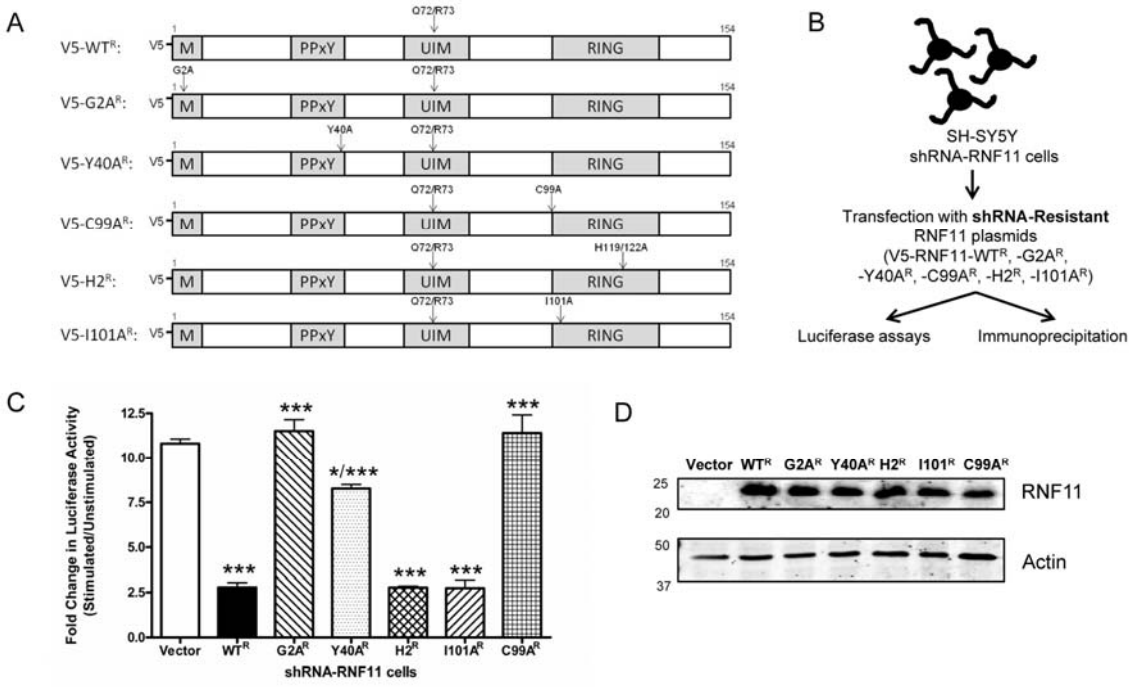

Figure 4-5. Myristoylation mutant of RNF11 is unable to alter NF-κB signaling

A. Site-directed mutagenesis of V5-tagged wild-type RNF11 was used to create the myristoylation mutant (G2A), the PPxY motif mutant (Y40A) and RING domain mutants (C99A, H2, I101A). Small hairpin (shRNA)-resistant mutants were created using Q72/R73 mutagenesis. The domains were as follows: M, myristoylation domain; PPxY, PPxY motif; UIM, ubiquitin-interacting motif; and RING, RING-H2 domain. B. Model of resistance experiments in SH-SY5Y cells. Stably transduced shRNA-RNF11 cells were transfected with shRNA-resistant RNF11 plasmids to determine which mutants behaved similarly to the endogenous protein in luciferase and coimmunoprecipitation experiments. C. SH-SY5Y shRNA-RNF11 cells were transfected with shRNA-resistant RNF11 mutants and luciferase and *Renilla* constructs. Cells were exposed to 10 ng/ml TNF-α for 6 hours and subjected to a luciferase assay. Values represent the fold changes in the ratios ± SEM of luciferase to *Renilla* luminescence of stimulated over unstimulated samples of triplicate samples in three experiments. Results were analyzed by one-way

ANOVA with Tukey's post-tests. D. Samples from C were resolved by SDS-PAGE and immunoblotted with RNF11 antibody. Blots are representative of three independent experiments. *$P < 0.05$; ***$P < 0.001$.

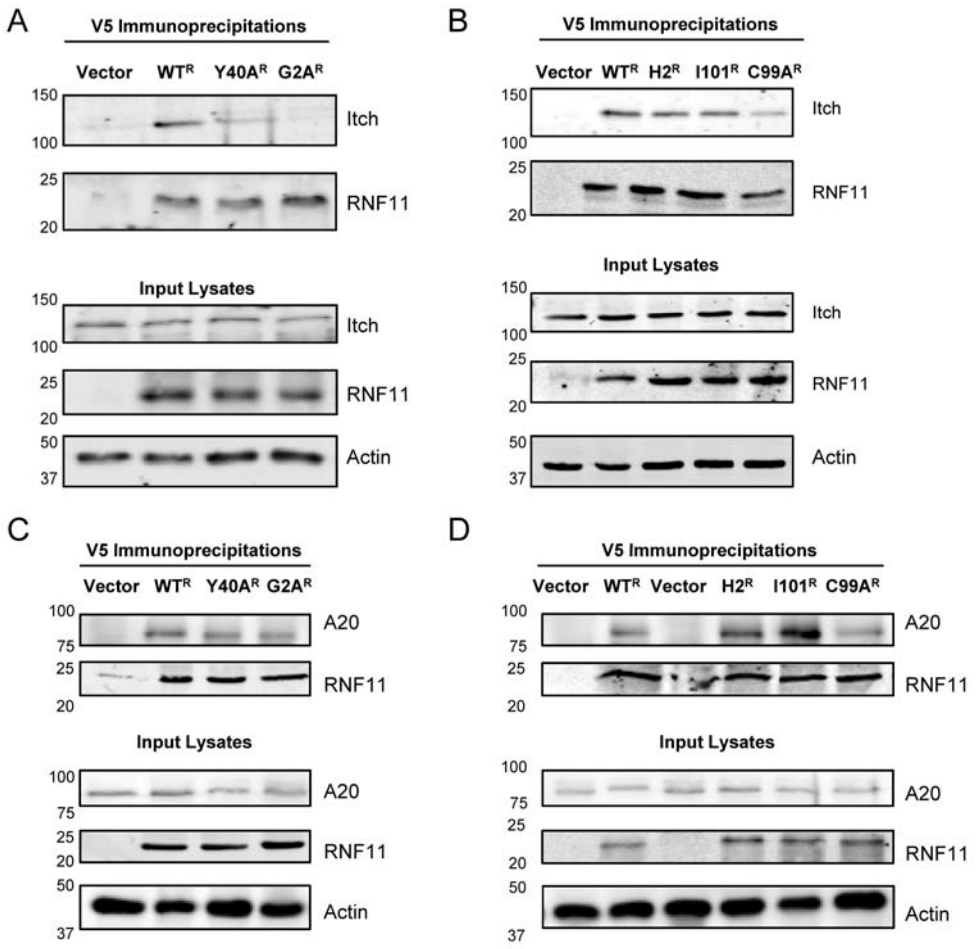

Figure 4-6. Myristoylation mutant of RNF11 is unable to associate with Itch

SH-SY5Y shRNA-RNF11 cells were transfected with shRNA-resistant RNF11 constructs or vector. Coimmunoprecipitation experiments using V5 antibody were performed 24 hours after transfection. Immunoprecipitates and lysates were resolved by SDS-PAGE and immunoblotted with anti-A20, Itch, RNF11 or actin. Blots are representative of three independent experiments.

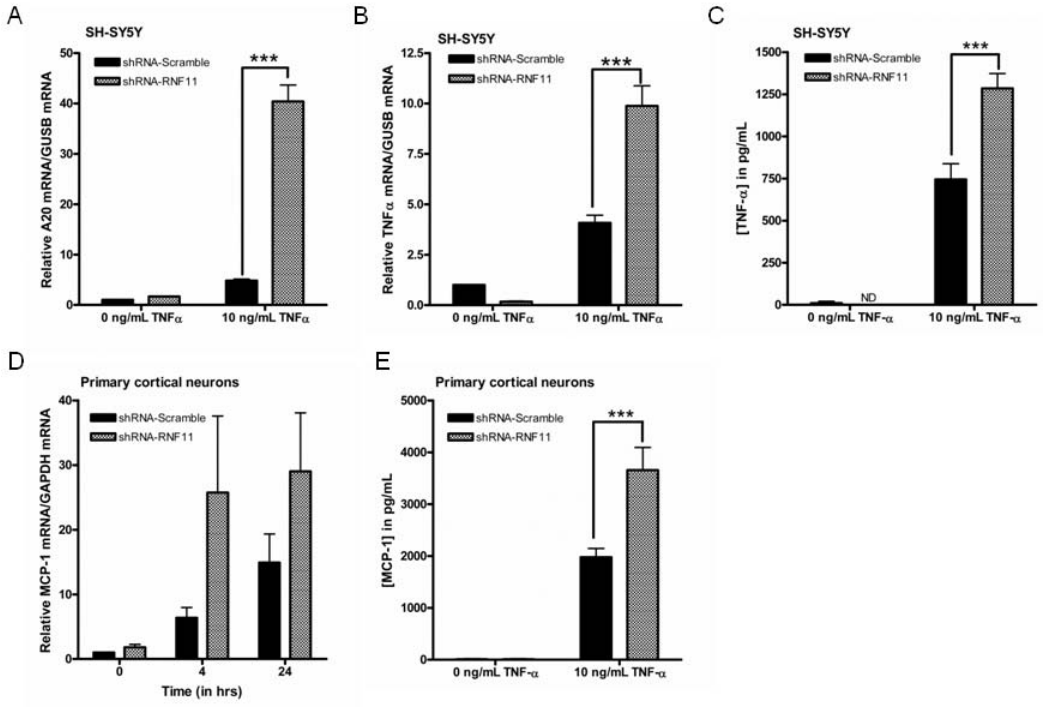

Figure 4-7. Knockdown of RNF11 exaggerates inflammatory responses following TNF-α treatment

A, B. Stably transduced SH-SY5Y short hairpin RNA (shRNA)-RNF11 and shRNA-Scramble cells were exposed to 0 or 10 ng/ml TNF-α for 24 hours. The relative amount of mRNA expression in stimulated and unstimulated cells was calculated using quantitative RT-PCR (qRT-PCR) and glucuronidase β (GUSB) as an internal control. C. Stably transduced SH-SY5Y shRNA-RNF11 and shRNA-Scramble cells were exposed to 0 or 10 ng/ml TNF-α for 4 hours before cells were rinsed and fresh media were replaced in the plate. Media were collected after 20 hours and analyzed for TNF-α protein levels using a human TNF-α ELISA. ND, not detectable. D. Murine primary cortical neurons were treated with cytosine arabinoside and transduced with shRNA-Scramble or shRNA-RNF11 lentivirus. Cells were exposed to 0 or 10 ng/ml TNF-α for 4 or 24 hours, and mRNA levels were examined by qRT-PCR. E. Murine primary cortical neurons were

transduced with shRNA-Scramble or shRNA-RNF11 lentivirus. Cells were exposed to 0 or 10 ng/ml TNF-α for 4 or 24 hours, and protein levels of monocyte chemoattractant protein 1 (MCP-1) were measured using a mouse MCP-1 ELISA. qRT-PCR values are expressed as mean comparative cycle threshold ± SEM with GUSB (for SH-SY5Y cells) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (for primary cultures) levels as an internal control for three independent experiments. ELISA values are expressed as averages of protein levels extrapolated from standard curve analysis from three independent experiments. Results were analyzed by two-way ANOVA with Bonferroni post-tests. ***$P < 0.001$.

Chapter 5

CG32850, the *Drosophila melanogaster* homologue of RNF11, is a negative regulator of NF-κB signaling

5.1 Introduction

Drosophila melanogaster has served as a valuable model system for the molecular genetics of innate immunity since the 1990s (Lemaitre and Hoffmann, 2007; Hetru and Hoffmann, 2009). Innate immunity can be divided into two main systems, the cellular response and the humoral response (Lemaitre and Hoffmann, 2007). The *Drosophila* cellular response is mediated by the lymph gland and the hemocytes. This system is responsible for phagocytosis of microbes, encapsulation of macroparasites, and generation of melanin and free radicals for coagulation, wound healing, and pathogen killing (Meister and Lagueux, 2003). The cellular response is useful for understanding the

immune systems of other insects, such as crop pollinators and agricultural pests (Schneider and Shahabuddin, 2000; Evans et al., 2006).

The *Drosophila* humoral response has been intensely studied for its role in combating bacterial and fungal infections. The humoral response is initiated by recognition of conserved molecules that lead to the activation of signaling cascades and translocation of transcription factors which induce the transcription of immune-responsive peptides and proteins (De Gregorio et al., 2002; Hultmark, 2003). The fat body, similar to the mammalian liver, controls the production and release of extracellular proteins related to immunity such as antimicrobial peptides (AMPs) and complement-like proteins (Lemaitre and Hoffmann, 2007; Hetru and Hoffmann, 2009). These proteins are secreted into the hemolymph, which baths the entire innards of the fly, to create a systemic immune response (Hetru and Hoffmann, 2009).

There are two major humoral response pathways operating in *Drosophila*: the NF-κB pathways Toll and immune deficiency (Imd) (Boutros et al., 2002). These pathways operate in manners analogous to the innate immune pathways of mammals, with similar signaling cascades and regulatory mechanisms (Silverman and Maniatis, 2001; Evans et al., 2003). Evidence for specificity for induction of the Toll pathway by gram-positive bacteria and fungi (Ochiai and Ashida, 2000; Michel et al., 2001; Leulier et al., 2003; Gottar et al., 2006), as well as specificity for induction of the Imd pathway by gram-negative bacteria, exists but this is not an absolute and there still exists cross talk between these two pathways (Lemaitre and Hoffmann, 2007). These pathways might also be

involved in defense against viruses (Zambon et al., 2005). Although the humoral response is largely dependent on the Toll and Imd pathways, the janus kinase-signal transducer and activator of transcription (JAK-STAT) and JNK pathways also play auxiliary roles (Boutros et al., 2002; Agaisse and Perrimon, 2004).

In the Toll pathway (Fig. 5-1), microbial inducers interact with peptidoglycan recognition proteins (PGRPs) (Kang et al., 1998; Werner et al., 2000; Steiner, 2004) or glucan-binding proteins (Lee et al., 1996; Gobert et al., 2003; Gottar et al., 2006; Wang et al., 2006) to activate proteolytic cascades that terminate in the cleavage of the cytokine Spaetzle. Binding of Spaetzle to the Toll extracellular domain activates a downstream signaling cascade (Hashimoto et al., 1988; Morisato and Anderson, 1994; Hoffmann, 2003; Brennan and Anderson, 2004; Tanji and Ip, 2005; Leulier and Lemaitre, 2008). Toll, encoded by the *Tl* gene, is a transmembrane protein with an intracellular signaling domain that is similar to the mammalian IL-1 receptor and all mammalian TLRs (Hashimoto et al., 1988; Hoffmann, 2003; Brennan and Anderson, 2004; Tanji and Ip, 2005; Leulier and Lemaitre, 2008). The Toll signaling cascade terminates in the translocation of two NF-κB-like transcription factors, Dorsal and Dif (for dorsal-related immunity factor) to the nucleus, and subsequent regulation of their target genes, similar to the immunity and inflammation pathway in mammals (Ip et al., 1993; Wu and Anderson, 1998; Manfruelli et al., 1999; Meng et al., 1999; Rutschmann et al., 2000; Hoffmann, 2003; Brennan and Anderson, 2004; Tanji and Ip, 2005; Leulier and Lemaitre, 2008). These transcription factors are retained in the cytoplasm due to binding with Cactus, similar to the mammalian IκBα (Schüpbach and Wieschaus, 1989; Geisler et al.,

1992; Huguet et al., 1997). Activation of the signaling pathway induces phosphorylation of Cactus and subsequent K48-linked ubiquitination, which targets it for degradation by the proteasome (Hashimoto et al., 1988; Belvin et al., 1995; Fernandez et al., 2001; Hoffmann, 2003; Brennan and Anderson, 2004; Tanji and Ip, 2005; Leulier and Lemaitre, 2008), releasing Dorsal and Dif to translocate to the nucleus.

In the Imd pathway (Fig. 5-2), activation is dependent upon direct interaction of gram-negative bacteria with the transmembrane receptor PGRP-LC (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002; Choe et al., 2005). PGRP-LC contains an extracellular conserved peptidoglycan recognition domain and an intracellular signaling domain, but has distinct splice isoforms that can give rise to slightly different exodomains (Werner et al., 2000). Activation of the intracellular signaling cascade begins with the recruitment of Imd, similar to mammalian RIP1 (Lemaitre et al., 1995; Georgel et al., 2001). Through mechanisms hypothesized to involve K63 ubiquitination, Imd and a complex of proteins activate transforming growth factor β–activated kinase 1 (TAK1), a MAP3 kinase (Zhou et al., 2005). Activation of TAK1 leads to JNK pathway activation, as well as activation of the NF-κB-like transcription factor Relish (Vidal et al., 2001; Zhuang et al., 2006). Relish, similar to mammalian p100 and p105, requires proteolytic cleavage for its activation (Hultmark, 2003). Phosphorylation of Relish releases an amino-terminal transcriptional regulatory domain which binds to Relish response elements in the nucleus and induces gene expression (Stöven et al., 2000; De Gregorio et al., 2002).

Investigation into the negative regulation of the Toll response has been limited; necrotic, a serpin responsible for inhibition of the cleavage of Spaetzle, has been reported to potentially contribute to the down-regulation of the pathway (Irving et al., 2001). In the Imd pathway, negative regulation has been investigated at the level of the PGRP-LC receptor binding (Mellroth et al., 2003; Mellroth and Steiner, 2006; Zaidman-Rémy et al., 2006), poor Imd response upon knock-in (Pirk) associations with Imd (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008), and Relish cleavage (Kim et al., 2006).

The extensive similarities between the humoral immunity pathways in *Drosophila melanogaster* and mammals have prompted us to investigate if the functional role of RNF11, as a regulator of NF-κB pathway and immune response, is conserved throughout different species. If RNF11 is conserved in *Drosophila*, there are a number of valuable genetic tools that could be utilized in future research to learn more about RNF11's basic biology, as well as role in disease models.

5.2 CG32850 is homologous to mammalian RNF11

Our analysis of RNF11 cDNA has revealed a homologue of the full-length mammalian protein in *Drosophila melanogaster* at the unidentified gene *CG32850* (Fig. 5-3). Like Kitching and colleagues reported (Kitching et al., 2003), this gene lacks the PPxY domain found in mammalian *RNF11*. Despite a rearrangement of exon structure, *CG32850* maintains high sequence identity with human *RNF11* throughout the sequence, unlike other potential homologues in other species (Kitching et al., 2003).

To confirm the similarity of CG32850 to human RNF11, we used immunohistochemistry to stain *Drosophila* tissue with the polyclonal RNF11 antibody. In the fat body, we see abundant staining of fat body cells for RNF11 in control (OregonR x C833-gal4) flies (Fig. 5-4A). However, in CG32850-RNAi (*CG32850*-RNAi x C833-gal4) flies, we observe a significant decrease in RNF11-immunoreactivity suggesting that the RNF11 antibody was specifically staining CG32850. Furthermore, to confirm the specificity of the knockdown in CG32850-RNAi flies, we performed qRT-PCR on tissue from control and CG32850-RNAi flies and found a 73% decrease in *CG32850* expression (Fig. 5-4B) ($P < 0.001$).

5.3 CG32850 is a negative regulator of NF-κB signaling

To examine CG32850's effect on NF-κB signaling, we measured expression levels of previously reported *Drosophila* immune genes induced following septic injury. *Drosomycin* and *Diptericin* are AMPs commonly used to assess activation of the two immunity signaling pathways Toll and Imd, respectively (Lemaitre and Hoffmann, 2007). The response to septic injury begins to show specificity at time points 6 to 12 hours post-infection (Lemaitre et al., 1997; Bettencourt et al., 2004; Keebaugh and Schlenke, 2012), with maximum activation of the Toll pathway at 24 to 48 hours (Lemaitre et al., 1997). Thus, we examined expression at 6 and 24 hours. Additionally, activation of the Toll pathway following microbial infection specifically induces the expression of the fly IkB homologue *Cactus*, responsible for retaining NF-κB-like transcription factors in the cytoplasm (Boutros et al., 2002; De Gregorio et al., 2002). Expression of *Relish*, the NF-κB-like transcription factor similar to mammalian p100 and p105, is induced following

activation of the Imd pathway. Knockdown of *CG32850* in the fat body decreased the endogenous expression of *Drosomycin* by 33% (Fig. 5-5A, $P < 0.01$) and *Diptericin B* by 27% (Fig. 5-5C, $P < 0.001$) compared to control flies, and increased the endogenous expression of *Cactus* by 43% (Fig. 5-5B, $P < 0.01$). Endogenous expression of *Relish* did not change with *CG32850* knockdown in the fat body (Fig. 5-5D).

Young control flies and flies with knockdown of *CG32850* in the fat body were pricked with sterile pins dipped in luria broth without any bacteria (mock), *E. faecalis* (gram-positive), or *S. mercesens* (gram-negative) to induce an immune response and were collected at 6 and 24 hours post-injury for analysis of mRNA levels by qRT-PCR. In all conditions, the expression of RNF11 did not change in the CG32850-RNAi and control flies (Fig. 5-6A, 5-7A).

For induction of the Toll pathway, we examined the mRNA expression of *Drosomycin* and *Cactus* following septic injury. *Drosomycin* expression was increased with gram-positive and gram-negative infections after 24 hours in control flies (gram-positive: 14.64 fold, gram-negative: 5.57-fold change) ($P < 0.001$), as well as in CG32850-RNAi flies (gram-positive: 34.53-fold change, gram-negative: 9.05-fold change) ($P < 0.001$) (Fig. 5-6B, 5-7B). Additionally, a 5.38-fold increase in *Drosomycin* expression was observed in CG32850-RNAi flies 6 hours after gram-positive infection and a 2.75-fold increase 6 hours after gram-negative infection ($P < 0.05$ for both). A significant increase in expression of *Cactus* was observed in both fly lines at 6 hours (control: 1.33-fold change, CG32850-RNAi: 1.53-fold change) and at 24 hours (control: 1.60-fold change,

CG32850-RNAi: 2.96-fold change) after gram-positive infection ($P < 0.001$ for both fly lines at both times) (Fig. 5-6C). Also, there was a significant increase in *Cactus* expression after gram-negative infection; a 1.78-fold increase in *Cactus* expression was observed in CG32850-RNAi flies 6 hours after infection ($P < 0.001$) and a 1.57-fold increase observed in control flies 24 hours after infection ($P < 0.01$) (Fig. 5-7C). While there are differences in the degree of activation between the two types of septic injuries related to specificity, in each case there is a larger induced response in *Drosomycin* and *Cactus* expression in the flies with *CG32850* knockdown indicating an enhanced activation of the Toll pathway with gram-positive infection. The induced responses by gram-positive bacteria can be characterized as both heightened and longer-lasting in the CG32850-RNAi flies compared to control flies, suggesting that CG32850 is critical for regulation of the Toll pathway.

For activation of the Imd pathway, we examined the mRNA expression of *Diptericin B* and *Relish* following septic injury. A significant increase in expression of *Diptericin B* was observed in control flies only at 6 hours following a gram-positive infection (11.55-fold change, $P < 0.001$) (Fig. 5-6D). In CG32850-RNAi flies, significant increases in expression of *Diptericin B* were observed at 6 and 24 hours following gram-positive infections (6 hours: 12.92-fold change, 24 hours: 10.37-fold change, $P < 0.001$) (Fig. 5-6D) and at 6 hours following gram-negative infection (7.62-fold change, $P < 0.001$) (Fig. 5-7D). A significant increase in *Relish* expression was only observed in CG32850-RNAi flies 6 hours after infection (gram-positive: 1.91-fold change, gram-negative: 2.57-fold change, $P < 0.001$) (Fig. 5-6E, 5-7E). The persistent heightened activation of NF-κB

signaling in CG32850-RNAi flies following gram-negative bacterial infection suggests that CG32850 is imperative for negative regulation of the Imd pathway. In contrast to the response induced by gram-positive bacteria, the induced expression of AMPs and immunity-related proteins is much more transient with gram-negative infection. Thus, from our documented data, it appears that CG32850 is a crucial negative regulator of both the Toll and Imd pathways.

Next, the survival of septic-infected flies was followed for 35 days to determine if the induction of a systemic immune response was beneficial or detrimental to the animal. Animals that died within the first four hours were not used in survival estimates. As expected, flies that were injured with a sterile pin dipped in luria broth (mock) did not have significant differences in survival from flies that were anesthetized and moved to food vials, regardless of knockdown of *CG32850* in the fat body (Fig. 5-8A). Additionally, bacterial injury significantly shortened survival. With gram- positive infection, knockdown of *CG32850* significantly reduced the median survival compared to control flies (control: 20.95 days, CG32850-RNAi: 3.30 days, $P < 0.0001$) (Fig. 5-8B). Conversely, with gram-negative infection, knockdown of *CG32850* significantly increased the median survival compared to control flies (control: 11.45 days, CG32850-RNAi: 20.95 days, $P < 0.0001$) (Fig. 5-8C). In this experiment, we have CG32850-RNAi–mediated activation of NF-κB signaling through two different signaling pathways, with significantly distinct outcomes. This suggests that NF-κB can be beneficial or detrimental to the organism in different contexts and prompts further investigation for systems where NF-κB signaling is crucially involved.

5.4 Discussion

In this study, we have investigated the signaling pathways activated during innate immunity in adult *Drosophila* and the role that CG32850 plays in their regulation. Our results show that CG32850 has a homologous function to RNF11 as a negative regulator of NF-κB signaling induced following septic injury such that heightened activation of induced inflammatory responses are observed with knockdown of *CG32850*, analogous to the increased expression of cytokines and chemokines with RNF11 knockdown in neurons (Chapter 4). Additionally, we find that sustained and heightened activation of the Toll pathway in CG32850-RNAi flies following infection has deleterious effects on survival. Conversely, we find that protection against the infection toxicity is conferred in CG32850-RNAi flies with sustained and heightened activation of the Imd pathway. Altogether, these results suggest that RNF11 serves a conserved role in the regulation of immune responses and provides insights into the connection of toxicity and activation of distinct signaling pathways.

NF-κB signaling plays a critical role in the innate and adaptive immune response in mammals. Sustained activation of the pathway is observed in a number of diseases; thus, regulation of NF-κB signaling is tightly controlled at many distinct levels. The A20 ubiquitin-editing protein complex, comprised of RNF11 and other proteins, is one of the crucial components for negative regulation of canonical NF-κB signaling. In *Drosophila*, the A20 ubiquitin-editing protein complex has not yet been described. Suppressor of deltex (Su(dx)) and Traf2 have been reported as homologues for Itch and Traf6, respectively (Khush and Lemaitre, 2000; Chastagner et al., 2008). RIP1, whose

degradation is impacted by the A20 ubiquitin-editing protein complex, has a similar death domain as Imd (Georgel et al., 2001). Lastly, there are no reported homologues for A20 or TAX1BP1. Given the high homology that *CG32850* shares with *RNF11*, we hypothesize that CG32850 either works by itself to confer negative regulation, or works in concert with Su(dx) and/or Traf2 to create a *Drosophila* version of the A20 ubiquitin-editing protein complex. Future studies should examine genetic mutants of Su(dx) and Traf2 to identify their role in immune regulation and interactions with CG32850.

In *Drosophila*, two distinct NF-κB pathways, Toll and Imd, have been shown to mediate immune responses to gram-positive and gram-negative bacteria, respectively. We show that CG32850 plays an important role in the regulation of both the Toll and Imd pathways. Indeed, potential protein interactors for CG32850 have been identified in the Toll pathway (dorsal, cactus) as well as the Ras pathway (sty, Shc, Rap21, Rab7, dos), a pathway important for the negative regulation of the Imd pathway (Guruharsha et al., 2011). However, we found important differences in the immune response to bacteria with modulation of CG32850 expression. With gram-positive infections, we show that knockdown of *CG32850* induces heighted and more sustained immune responses in both the Toll and Imd pathways (Fig. 5-6). Further, gram-positive infection combined with knockdown of *CG32850* is matched with a decreased survival rate (Fig. 5-8). In contrast, with gram-negative infections, we show that knockdown of *CG32850* induces heightened immune responses in both pathways that have a more transient nature (Fig. 5-7). Unlike gram-positive infections, gram-negative infection combined with knockdown of *CG32850* confers protection against the bacterial infection (Fig. 5-8). These results

suggest that transient up-regulation of the Imd pathway is protective within *Drosophila*, whereas sustained up-regulation of the Toll pathway is detrimental for the organism's health. Similarly, NF-κB activation in mammalian systems is linked to both cell survival and cell toxicity (Mattson and Meffert, 2006; Costanzo et al., 2011; Galardi et al., 2011; Gordon et al., 2011; Li et al., 2012), indicating that NF-κB activation may produce different effects under different circumstances. Indeed, NF-κB activation can induce transcription of both pro- and anti-apoptotic factors (Blum et al., 2001; McCoy and Tansey, 2008). To confirm the regulatory role of CG32850 in the *Drosophila* immune pathways, it would be important to combine known mutants of the Toll and Imd pathway with knockdown of *CG32850* to examine changes in induced immune responses and survival following septic injury. Additionally, such experiments would allow us to identify at what level of the signaling pathway CG32850 was working at.

Analysis of the contribution of CG32850 to each of these signaling pathways in *Drosophila* would be a useful undertaking to dissect the complex immune processes in mammals. With knowledge that CG32850 is structurally and functionally homologous to RNF11, this analysis could lead to deeper understanding of the exact molecular mechanism conferred by RNF11 and the A20 ubiquitin-editing protein complex in mammalian canonical NF-κB signaling. Additionally, the breadth of genetic PD models available in *Drosophila* would make it easy to identify genetic interactions between known PD-associated genes and *CG32850/RNF11*. This could lead to a greater understanding of the pathogenic mechanisms of PD and to novel therapeutic targets.

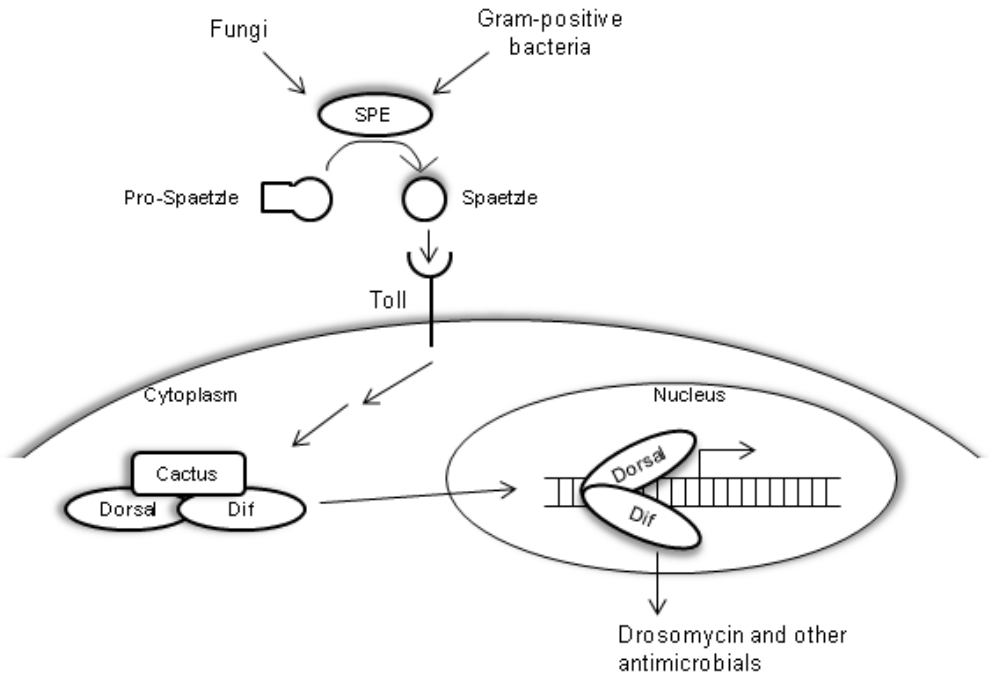

Figure 5-1. The Toll immunity signaling pathway in *Drosophila melanogaster*

In the Toll pathway, microbes interact with PGRPs or glucan-binding proteins to activate proteolytic cascades that terminate in the cleavage of the cytokine Spaetzle. Binding of Spaetzle to Toll activates a downstream signaling cascade that terminates on the Cactus/Dorsal/Dif complex. Dorsal and Dif are two NF-κB-like transcription factors that are retained in the cytoplasm due to binding with Cactus. Activation of the signaling pathway induces phosphorylation of Cactus and subsequent K48-linked ubiquitination, which targets it for degradation by the proteasome, releasing Dorsal and Dif to translocate to the nucleus. Translocation of Dorsal and Dif into the nucleus influences the transcription of *Drosomycin* and other immune-related genes(Ip et al., 1993; Wu and Anderson, 1998; Manfruelli et al., 1999; Meng et al., 1999; Rutschmann et al., 2000; Hoffmann, 2003; Brennan and Anderson, 2004; Tanji and Ip, 2005; Leulier and Lemaitre, 2008).

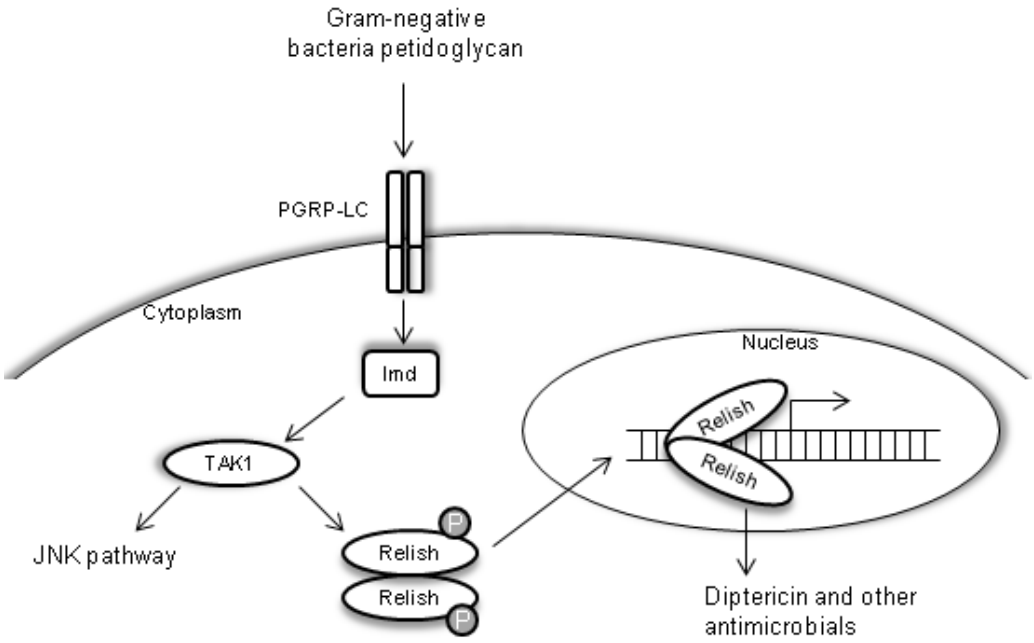

Figure 5-2. The Imd immunity signaling pathway in *Drosophila melanogaster*

In the Imd pathway, activation is dependent upon direct interaction of gram-negative bacteria with the transmembrane receptor PGRP-LC. Activation of the intracellular signaling cascade begins with the recruitment of Imd(Lemaitre et al., 1995; Georgel et al., 2001). Through unconfirmed mechanisms, Imd and a complex of proteins activate TAK1, a MAP3 kinase(Zhou et al., 2005). Activation of TAK1 leads to JNK pathway activation, as well as activation of the NF-κB-like transcription factor Relish. Relish requires proteolytic cleavage for its activation(Hultmark, 2003). Phosphorylation of Relish releases an amino-terminal transcriptional regulatory domain which binds to Relish response elements in the nucleus and influences transcription of *Diptericin* and other immune-related genes

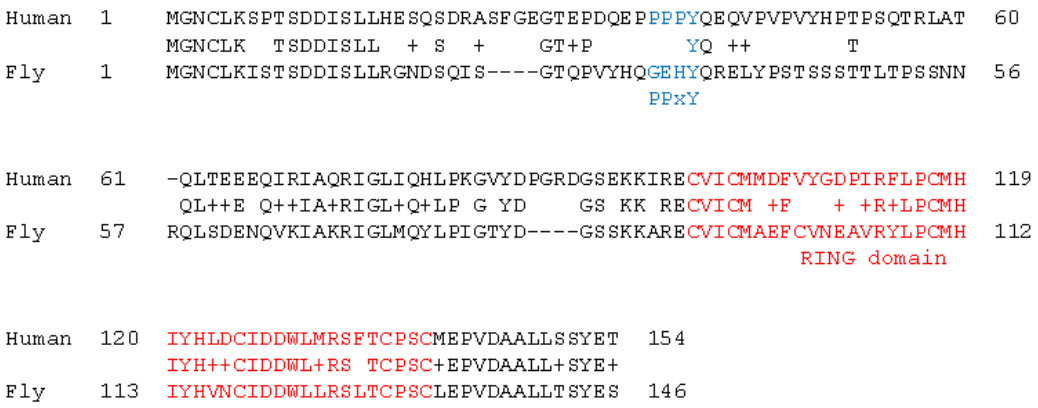

Figure 5-3. CG32850 is homologous to mammalian RNF11

Alignment of the protein sequence of human RNF11 (top) and fly CG32850 (bottom). Highlighted in blue is the human PPxY motif and in red is the human RING-H2 domain. 53% of the identities in CG32850 are the same in RNF11, with 68% of the identities in CG32850's RING domain the same as RNF11's RING domain.

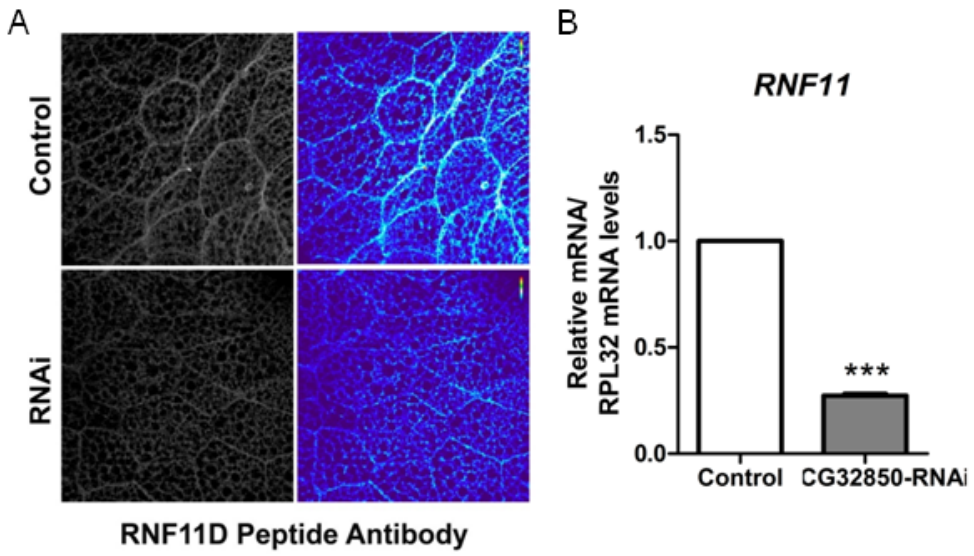

Figure 5-4. Immunohistochemical and mRNA analysis of CG32850 knockdown in the fat body

A. Fat bodies from larval OregonR x C833-gal4 (control) and CG32850-RNAi x C833-gal4 (RNAi) flies were stained for RNF11. Pictured is the heat map of the intensity of the staining. B. Adult (n=10) OregonR x C833-gal4 (control) and CG32850-RNAi x C833-gal4 (CG32850-RNAi) flies were harvested at 3 days post-eclosion for analysis of mRNA levels of *CG32850* by qRT-PCR. Values are expressed as mean comparative cycle threshold ± SEM of triplicate experiments. Results were analyzed by paired *T*-test. *** $P < 0.001$.

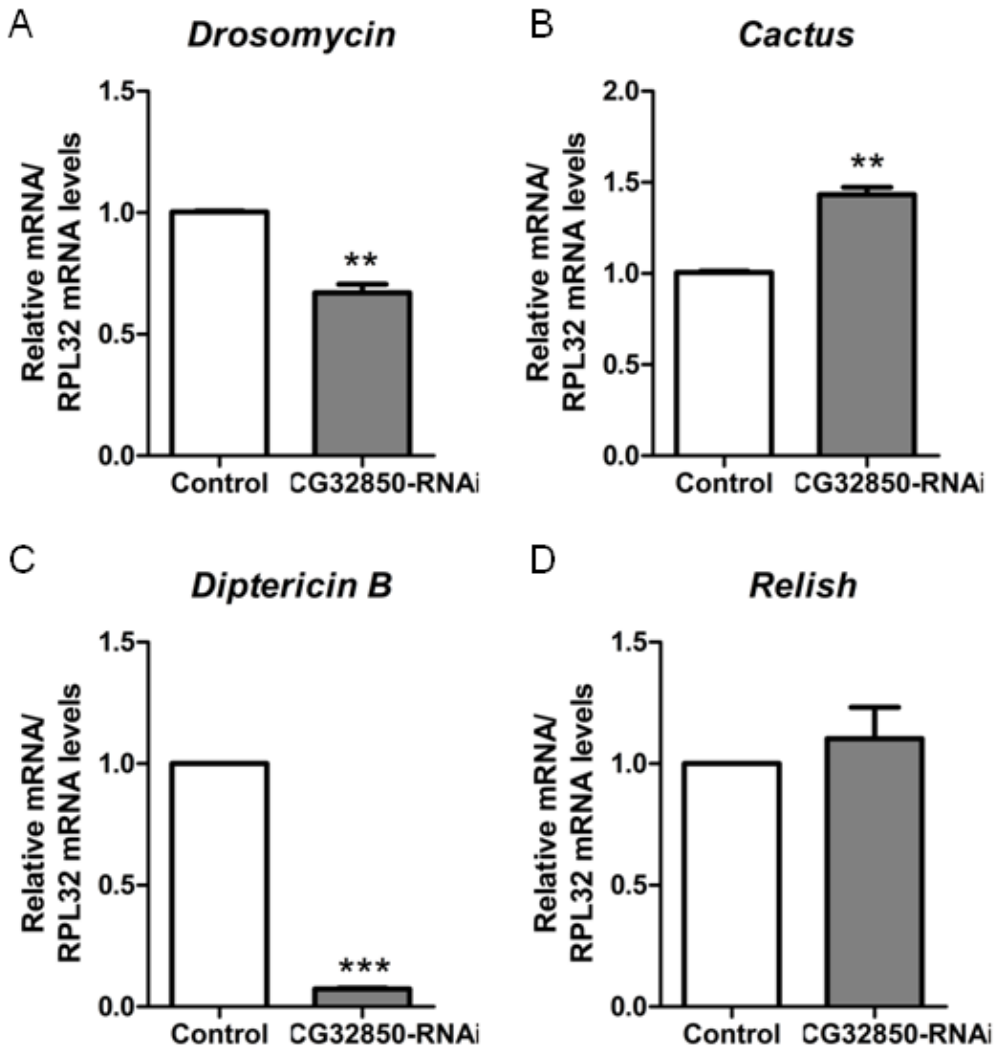

Figure 5-5. Differences in endogenous expression of immune-related genes exist with knockdown of CG32850 in the fat body

Adult (n=10) OregonR x C833-gal4 (control) and CG32850-RNAi x C833-gal4 (CG32850-RNAi) flies were harvested at 3 days post-eclosion for analysis of mRNA levels of *Drosomycin* (A), *Cactus* (B), *Diptericin B* (C), and *Relish* (D) by qRT-PCR. Values are expressed as mean comparative cycle threshold ± SEM of triplicate

experiments. Results were analyzed by paired two-tailed *T*-tests. **, $P < 0.01$; ***, $P < 0.001$.

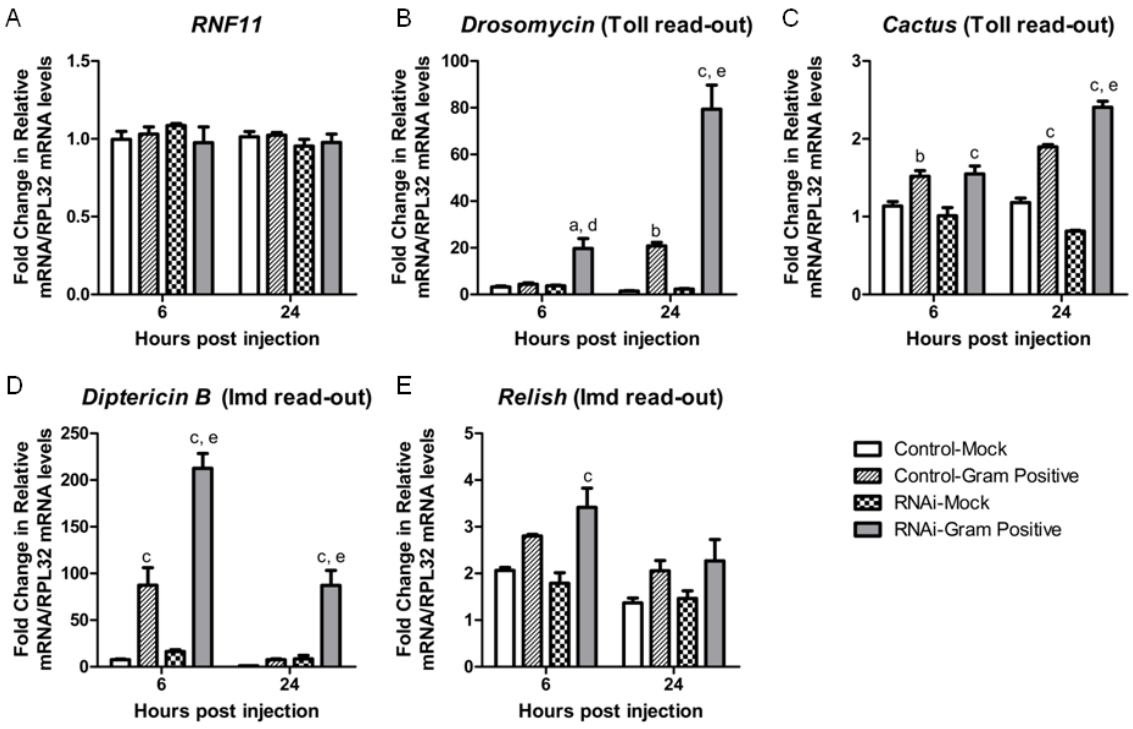

Figure 5-6. **Knockdown of CG32850 in the fat body induces heightened and sustained immune responses in both Toll and Imd pathways following gram-positive septic injury**

Adult (5 days post-eclosion, n=10) OregonR x C833-gal4 (control) and CG32850-RNAi x C833-gal4 (CG32850-RNAi) flies were pricked in the thorax with a needle dipped in LURIA broth (mock) or a culture of *E. faecalis* (gram-positive). Flies were collected 6 or 24 hours post-septic injury and analyzed by qRT-PCR for mRNA levels of *RNF11* (A), *Drosomycin* (B), *Cactus* (C), *Diptericin B* (D), and *Relish* (E). Values represent the fold change in the mean comparative cycle threshold ± SEM at 6 or 24 hours compared to animals collected immediately after injury of triplicate experiments. Results were analyzed by two-way repeated measures ANOVA with Bonferroni post-tests.a, $P < 0.05$ from mock; b, $P < 0.01$ from mock; c, $P < 0.001$ from mock; d, $P < 0.05$ from control

gram-positive; e, $P < 0.001$ from control gram-positive.

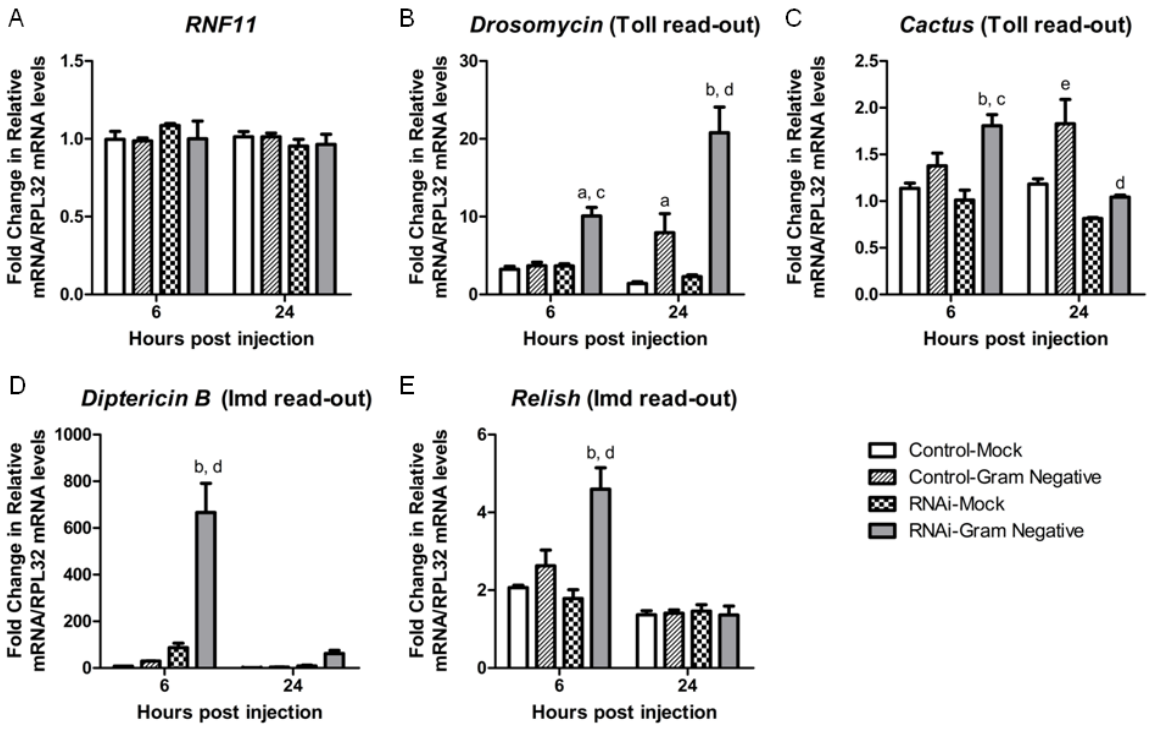

Figure 5-7 Knockdown of CG32850 in the fat body induces heightened immune responses in both Toll and Imd pathways following gram-negative septic injury with sustained responses in the Toll pathway

Adult (5 days post-eclosion, n=10) OregonR x C833-gal4 (control) and CG32850-RNAi x C833-gal4 (CG32850-RNAi) flies were pricked in the thorax with a needle dipped in LURIA broth (mock) or a culture of *S. mercesens* (gram-negative). Flies were collected 6 or 24 hours post-septic injury and analyzed by qRT-PCR for mRNA levels of *RNF11* (A), *Drosomycin* (B), *Cactus* (C), *Diptericin B* (D), and *Relish* (E). Values represent the fold change in the mean comparative cycle threshold ± SEM at 6 or 24 hours compared to animals collected immediately after injury of triplicate experiments. Results were analyzed by two-way repeated measures ANOVA with Bonferroni post-tests. a, $P < 0.05$ from mock; b, $P < 0.001$ from mock; c, $P < 0.05$ from control gram-negative; d, $P <$

0.001 from control gram-negative; e, $P < 0.01$ from mock.

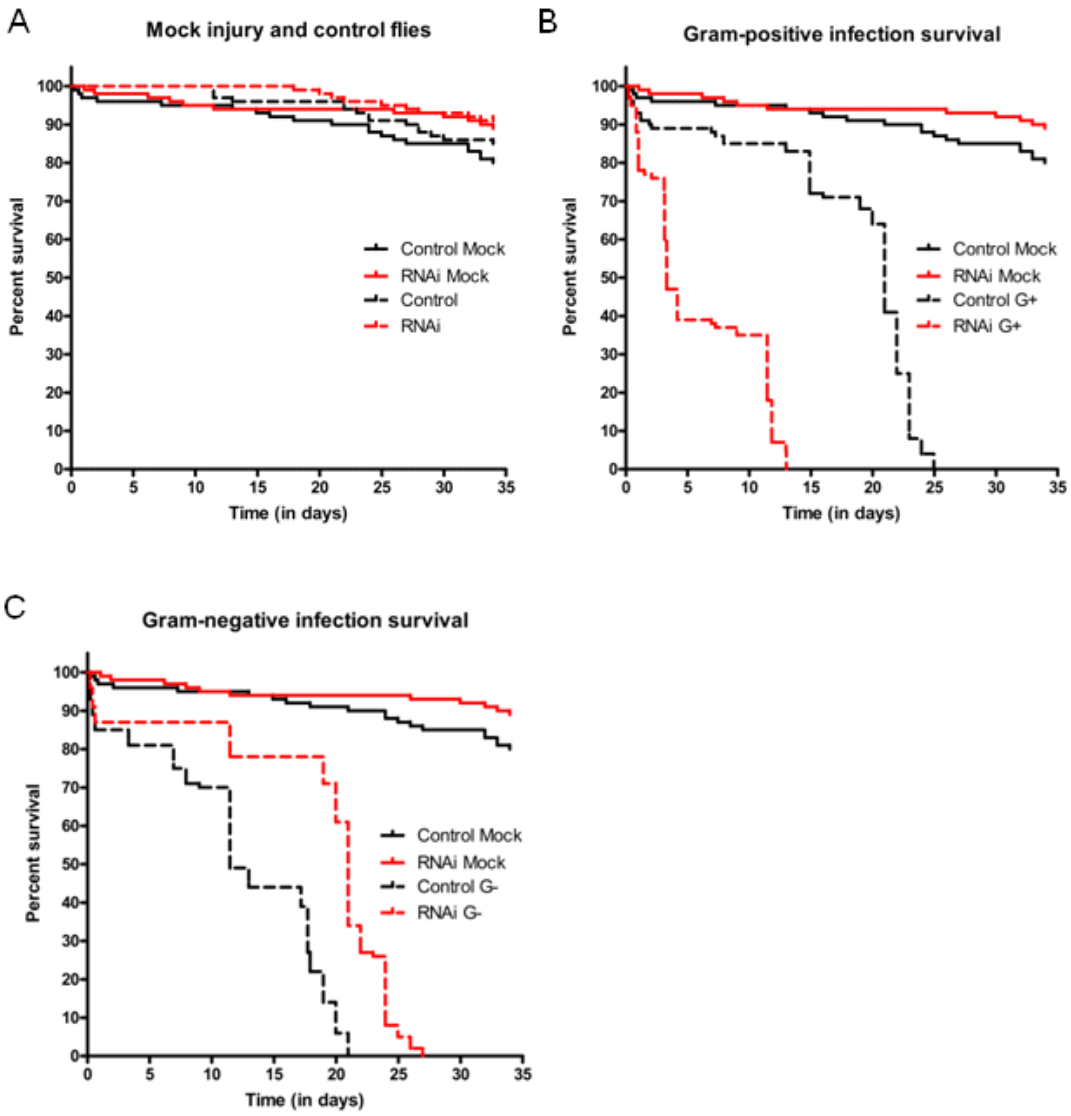

Figure 5-8. Knockdown of CG32850 in the fat body alters survival following septic injury

Adult (5 days post-eclosion, n=100) OregonR x C833-gal4 (control, black) and CG32850-RNAi x C833-gal4 (CG32850-RNAi, red) flies were pricked in the thorax with a needle dipped in LURIA broth (mock, solid), a culture of *E. faecalis* (gram-positive), or a culture of *S. mercesens* (gram-negative). Flies were counted every 3 hours for the first 72 hours and every day thereafter for 35 days. The survival of mock injured animals did not differ from control flies that were anesthetized and moved to new food vials (A). The

survival curves of the gram-positive (B) and gram-negative (C) animals were significantly different by the Log-rank (Mantel-Cox) test, $P < 0.0001$.

Chapter 6

RING finger protein 11 (RNF11) modulates susceptibility to 6-OHDA-induced nigral degeneration and behavioral deficits through NF-κB signaling in dopaminergic cells

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6.1 Introduction

PD is a chronic, progressive neurological disorder with motor abnormalities as the

cardinal clinical symptom (Fahn, 2003; Coelho and Ferreira, 2012). A pathological hallmark of PD is degeneration of dopaminergic neurons in the SN. This selective dopaminergic cell loss results in reduction of striatal dopamine and motor dysfunction (Crossman, 1989), however the underlying disease-causing mechanisms remain to be elucidated. Recently, human clinical imaging, epidemiological studies and genetic associations have highlighted and confirmed a role for neuroinflammation in PD (Chen et al., 2005; Hald and Lotharius, 2005; Hirsch et al., 2005; Gerhard et al., 2006; Ton et al., 2006; Frank-Cannon et al., 2009; Hamza et al., 2010) as well as raise the possibility that chronic inflammatory responses may promote progressive degeneration of dopaminergic neurons.

Inflammatory response in the central nervous system is primarily mediated by microglia through activation of NF-κB, and this mechanism elevates levels of inflammatory cytokines (ie., TNF-α), apoptotic factors and oxidative stress. NF-κB, a transcription factor, is expressed in neurons and glia (O'Neill and Kaltschmidt, 1997) and is activated in response to various toxic stimulations. Moreover, the NF-κB pathway has divergent roles as both promoter and inhibitor of neurodegeneration (Tansey and Goldberg, 2010). To maintain cellular homeostasis, activation of NF-κB pathway is tightly controlled with several layers of regulation (Ruland, 2011). One such regulator is A20, an NF-κB transcribed gene (Krikos et al., 1992).

A20, through its associations with TAX1BP1, Itch, and RNF11 forms the A20 ubiquitin editing complex (Shembade et al., 2007; Shembade et al., 2008; Shembade et al., 2009),

which negatively modulates NF-κB-mediated inflammatory responses. However the regulatory role of A20 ubiquitin-editing protein complex in NF-κB-mediated inflammatory response and cell degeneration has not been reported in the central nervous system. Our recent studies show that the A20 ubiquitin-editing protein complex components are expressed throughout the brain (Chapter 3) and that RNF11 was the only complex component with significantly altered expression in PD. RNF11 is predominantly expressed in neurons (Anderson et al., 2007) and is essential for regulation of neuronal NF-κB signaling (Chapter 4). We therefore hypothesized that RNF11 in dopaminergic neurons may modulate NF-κB signaling, mediate inflammatory responses and possibly affect cell survival. This hypothesis was tested by genetic manipulation of RNF11 expression in both cellular and rat 6-OHDA models of PD. Our results suggest that RNF11-mediated activation of NF-κB signaling in dopaminergic cells is protective against 6-OHDA toxicity through inflammatory responses involving the transcription of TNF-α, antioxidants and anti-apoptotic factors. This report highlights the importance of neuronal RNF11, as a negative modulator of NF-κB responses with major implications for targeted therapeutics in PD.

6.2 Validation of AAV-mediated targeted knockdown and over-expression of RNF11 *in vitro* and *in vivo*

Subsequent to the association of reduced RNF11 expression that we observed in PD cases (Chapter 3), we investigated the effects of RNF11 on dopaminergic cell loss, a phenotype essential to PD pathology and movement dysfunction. These experiments enabled us to confirm RNF11's association with PD and determine RNF11's functional role in PD-

related degeneration in an *in vivo* system. The design for the experimental paradigm (Fig. 6-1) was to alter RNF11 expression in nigral dopaminergic neurons, followed by intrastriatal administration of neurotoxin 6-OHDA two weeks later. Animals were examined after four weeks through immunohistochemistry or qRT-PCR.

To manipulate RNF11 expression, we generated and tested the ability of AAV2 to specifically alter neuronal expression within the SNpc as previously described (Bartus et al., 2011). The three viruses used were AAV2-shScramble-GFP, AAV2-shRNF11-GFP, and AAV2-V5-RNF11. The AAV2-V5-RNF11 was developed without GFP due to the size constraints associated with AAV. First, we confirmed the ability of AAV2 to manipulate RNF11 expression in primary neuronal cultures. In primary neurons, transduction of AAV2 expressing shRNA targeted against RNF11 (AAV2-shRNF11-GFP) decreased RNF11 mRNA levels by approximately 60% by qRT-PCR when compared to untransduced as well as following transduction of AAV2 expressing a scramble shRNA sequence (AAV2-shScramble-GFP), while transduction of AAV2 over-expressing V5-RNF11 (AAV2-V5-RNF11) increased RNF11 mRNA levels by approximately 50% ($P < 0.001$ for both) (Fig. 6-2A). Additionally, we found that transduction of AAV2-shScramble-GFP did not significantly alter RNF11 mRNA levels when compared to untransduced cells. Next, we verified that manipulation of RNF11 levels by AAV2 produced the expected changes in NF-κB signaling as previously reported (Chapter 4). To measure changes in NF-κB signaling, we examined the translocation of the NF-κB transcription factor, p65, into the nucleus following TNF-α stimulation by immunocytochemistry for p65 and the nuclear stain, Hoechst 333258. As

described before, we observed increased co-localization of p65 with Hoechst 333258 at 120 minutes after TNF-α stimulation in AAV2-shRNF11-GFP transduced cells in comparison to AAV2-shScramble-GFP transduced cells (AAV2-shScramble-GFP: 18.62%, AAV2-shRNF11-GFP: 118.30%) (Fig. 6-2B). Also, a significant decrease in p65 co-localization with the nucleus was seen at 120 minutes after TNF-α stimulation in the AAV2-V5-RNF11transduced cells in comparison to AAV2-shScramble-GFP transduced cells (AAV2-V5-RNF11: 2.11%) ($P < 0.001$). These experiments confirm that AAV2 was altering RNF11 mRNA levels which resulted in negative modulation of the NF-κB signaling pathway.

6.3 *In vivo* targeted knockdown of nigral dopaminergic RNF11 imparts neuroprotection against 6-OHDA-induced toxicity and attenuates rotational behavior

Validation of AAV2 *in vivo*

For targeted manipulation of RNF11 expression in dopaminergic neurons, a unilateral intranigral injection of AAV2 was performed. As described previously, the three AAV2 used were AAV2-shScramble-GFP, AAV2-shRNF11-GFP, and AAV2-V5-RNF11. Thus, the three groups of rats used for this set of experiments were (1) shScramble, (2) shRNF11 and (3) V5-RNF11. The AAV2 serotype was used since it has been shown previously to primarily transduce neurons (as opposed to glia) within the SNpc (Gasmi et al., 2007; Bartus et al., 2011). Using AAV2 GFP virus we observed extensive infection of dopaminergic neurons, as evident from GFP and TH co-localization throughout the SNpc on the ipsilateral hemisphere (Fig. 6-2C). Additionally, we observed GFP-positive

immunoreactivity in the ipsilateral striatum suggesting that our construct was not only being expressed throughout the cell body but also extended to the processes and terminals of the nigral dopaminergic neurons (Fig. 6-2D). The transduced cells in the nigra were nearly all MAP2-positive neurons (Fig. 6-2E), with little to no detectable infection of Iba1-positive microglia (Fig. 6-2F) or GFAP-positive astrocytes (data not shown). Furthermore, we did not observe an up-regulation of microglia in response to the AAV2 injections (Roca et al., 2011). Most importantly, AAV2 was able to manipulate RNF11 expression *in vivo* as expected. We qualitatively observed appreciable differences in RNF11 immunoreactivity in cells expressing the different viruses (Fig. 6-2G), which mirrored the mRNA expression results. Using qRT-PCR to measure relative RNF11 expression in ventral midbrain (VM) brain samples, we saw an approximately 50% decrease in the shRNF11 animals and an approximately 20% increase in the V5-RNF11 animals in comparison to shScramble animals ($P < 0.001$) (Fig. 6-2H). These results show that AAV2 is able to manipulate RNF11 mRNA levels *in vitro* and *in vivo* as expected, and that AAV2 targeted TH-positive neurons, allowing us to confidently use these reagents to manipulate expression levels of RNF11 in 6-OHDA rat model of PD.

Behavioral analysis

6-OHDA is a hydroxylated analog of dopamine and is commonly used as a neurotoxin to induce dopaminergic degeneration (Blum et al., 2001) primarily through reactive oxygen species-dependent apoptosis (Lotharius et al., 1999; Holtz et al., 2006). In these experiments, a unilateral intrastriatal preterminal injection of 6-OHDA was performed ipsilateral to the AAV2 injection while the contralateral hemisphere served as an internal

control. As a physiological measure of 6-OHDA-induced lesion of the nigrostriatal pathway, we tested amphetamine-induced rotation behavior three weeks after striatal 6-OHDA injections. We found 6-OHDA induced amphetamine (5 mg/kg, i.p.) rotations in the shScramble animals were attenuated in the shRNF11 animals, but drastically exaggerated in the V5-RNF11 animals (shScramble: 8.4, shRNF11: 5.3, V5-RNF11: 11.1 rotations/minute) (Fig. 6-3A), suggesting that 6-OHDA induced lesion (ie., loss of striatal dopamine) was reduced in the shRNF11 group versus the shScramble group while the lesion was greater in the RNF11 over-expressing group. These behavioral phenotypes implied that knockdown of RNF11 may be protective while RNF11 over-expression could potentiate 6-OHDA toxicity.

Histological and mRNA analysis

The RNF11-induced differences in 6-OHDA-induced lesions in the three animal groups, indicating differential loss of nigral dopaminergic neurons, was confirmed by both qualitative and quantitative analysis of nigral dopaminergic cell loss and TH mRNA levels in the three groups of rats. Similar to previous reports (Kirik et al., 1998), striatal injections of 6-OHDA resulted in retrograde loss of nigral dopaminergic cells in all three groups of rats. To quantify and differentiate the loss of dopaminergic cells, the number of TH-positive soma was estimated within the SNpc using unbiased stereology. We found that AAV2-shRNF11-GFP injection in the SN rescued 15% of the nigral dopaminergic neurons from 6-OHDA-induced cell death, while AAV2-V5-RNF11 injection in the SN enhanced 6-OHDA-induced death of nigral dopaminergic neurons by 12%, (Fig. 6-3B, 6-3D) as compared to control group with AAV2-shScramble-GFP injection ($P < 0.001$).

These statistically significant differences in the three groups were further emphasized following analysis of relative TH mRNA levels in the VM by qRT-PCR. In comparison to the unlesioned side, a 12% increase in TH mRNA in the shRNF11 group ($P < 0.01$) and a 20% decrease in TH mRNA in the V5-RNF11 group ($P < 0.001$) was observed compared to the shScramble group (Fig. 6-3C). The slight discrepancy in the TH mRNA data versus the stereological cell counts could possibly be due to the inclusion of ventral tegmental area (VTA) in the VM samples; VTA neurons were more compromised in the V5-RNF11 group (Fig. 6-3D).

Lastly, qualitative analysis of fluorescence immunohistochemistry and double labeling for GFP or V5 and TH demonstrated the presence of more GFP-TH positive cells in the shRNF11 group in comparison to the shScramble group (Fig. 6-3E). Additionally, very few V5/TH-positive cells and processes were present in the SN in the V5-RNF11 group (Fig. 6-3E), further confirming the differences in 6-OHDA toxicity in the three groups. Moreover, these double-labeling experiments demonstrated that AAV2-V5-RNF11 resulted in loss of dopaminergic cells and not just loss of TH expression as evident from absence of both V5 and TH-positive immunoreactivity in the V5-RNF11 group.

Thus, the protection of dopaminergic neurons in the SNpc achieved with nigral delivery of targeted knockdown of neuronal RNF11 correlated with attenuated ipsiversive circling behavior induced by amphetamine (Fig. 6-3). In reverse, the enhanced dopaminergic degeneration in the SNpc with nigral delivery of RNF11 over-expression correlated with

increased ipsiversive circling behavior induced by amphetamine. This correlation of the behavioral data with TH mRNA levels, quantitative analysis of TH cells and double labeling experiments in the SN confirmed the differences in 6-OHDA-induced lesions in the three groups, suggesting that targeted manipulation of RNF11 expression in nigral dopaminergic neurons can impact 6-OHDA-induced toxicity.

6.4 *In vivo* targeted knockdown of nigral dopaminergic RNF11 increases NF-κB signaling

Since RNF11 is a negative modulator of NF-κB pathway (Chapters 4, 5), we hypothesized that the differences in protection against 6-OHDA toxicity conferred by RNF11 was due to differential NF-κB activation in the three experimental groups. Therefore we examined the expression level of NF-κB and its target genes in VM tissue from rat brain. This also enabled us to determine the NF-κB-mediated responses that could be accountable for the differences in 6-OHDA-induced dopaminergic degeneration. We examined the mRNA expression of a NF-κB subunit (p50), cytokine (TNF-α), brain derived neurotrophic factor (BDNF), oxidative antioxidants (GSS, SOD1), and anti-apoptosis or pro-survival (BCL2) factor using qRT-PCR. The mRNA level for the p50 subunit of NF-κB and TNF-α, a cytokine produced following activation of NF-κB signaling, was elevated in the 6-OHDA lesioned hemisphere in all groups, with the largest increase observed in the shRNF11 group ($P < 0.001$) and minimum increase in the V5-RNF11 group ($P < 0.05$) (shScramble: 118.9, shRNF11: 137.9%, V5-RNF11: 111.2% expression of control side) (Fig. 6-4A, 6-4B). mRNA expression for BDNF, a molecule important for neurogenesis and survival of nigral dopaminergic neurons

(Nagahara and Tuszynski, 2011; Stahl et al., 2011), as well as glutathione synthase (GSS), the enzyme responsible for catalyzing the reaction producing glutathione, was decreased on the lesioned side of the shScramble group (BDNF: 77.0%, GSS:79.6% expression of control side) (Fig. 6-4C, 6-4D). A more substantial decrease in BDNF and GSS mRNA expression was seen in the lesioned V5-RNF11 group, while a significant increase in BDNF mRNA expression was observed in lesioned shRNF11 group (BDNF shRNF11: 131.0%, V5-RNF11: 59.9% expression of control side, $P < 0.001$ for both; GSS shRNF11: 302.1%, $P < 0.001$, V5-RNF11: 66.1%, $P < 0.05$). mRNA level for superoxide dismutase 1 (SOD1), an enzyme that catalyzes the reaction to prevent oxidative damage by $\cdot O_2^-$, was reduced on the lesioned side in both the shScramble and V5-RNF11 groups (shScramble: 60.4%, V5-RNF11: 49.6% expression of control side) (Fig. 6-4E). However, there was a marked increase in SOD1 mRNA expression in the lesioned hemisphere of the shRNF11 group in comparison to shScramble group (shRNF11: 201.7% expression of control side, $P < 0.001$). Lastly, we measured the mRNA levels of BCL2, a protective anti-apoptotic factor. While increased mRNA expression was seen in all the lesioned animals, a significant increase was observed in the shRNF11group and a significant decrease was observed in the V5-RNF11group in comparison to the shScramble group (shScramble: 247.5%, shRNF11: 379.8%, V5-RNF11: 142.9% expression of control side, $P < 0.001$ for both) (Fig. 6-4F). From these results, we concluded that the degree of vulnerability to 6-OHDA conferred by the targeted manipulation of RNF11 in dopaminergic neurons correlated negatively with NF-κB mRNA expression levels and expression of its target genes including TNF-α, antioxidants, neurotrophic factors, and anti-apoptotic factors. Thus, knockdown of

RNF11 related to increased NF-κB activity and dopaminergic cell survival, while RNF11 overexpression inhibited NF-κB activity and increased dopaminergic cell loss.

6.5 *In vitro* knockdown of RNF11 in dopaminergic cells imparts neuroprotection against 6-OHDA-induced toxicity

To confirm the differential *in vivo* susceptibility to 6-OHDA toxicity was mainly due to RNF11 expression levels in dopaminergic neurons and consequent NF-κB activation, we examined the effects of manipulation of RNF11 expression in a cell culture model of PD. PC12 cells have a dopaminergic phenotype and are responsive to 6-OHDA (Elkon et al., 2004; Zhu et al., 2012). We transfected PC12 cells with the plasmids used to generate AAV2 used *in vivo* (generating shScramble-transfected, shRNF11-transfected, and V5-RNF11-transfected cells) and monitored cell death after 18 hours of 6-OHDA exposure as described previously (Zhu et al., 2012). In response to 6-OHDA exposure, we observed a dose-dependent response in each of the cell lines (Fig. 6-5A). Similar to the *in vivo* results, we observe increased susceptibility to cell death in V5-RNF11-transfected cells and decreased susceptibility in shRNF11-transfected cells when compared to untransfected or shScramble-transfected cells, again implicating RNF11 as a modulator of 6-OHDA toxicity.

Parallel samples were analyzed for mRNA expression by qRT-PCR. RNF11 mRNA levels were as expected in the different cell lines, with a 53% knockdown in the shRNF11-transfected cells and an approximately 150% increase with V5-RNF11 transfection (Fig. 6-5B). Moreover, exposure to 6-OHDA did not alter RNF11 mRNA

expression by qRT-PCR. Similar to the *in vivo* system, exposure to 6-OHDA induced NF-κB activation as measured by mRNA levels of NF-κB (p50 subunit) and TNF-α in shScramble-transfected cells (NF-κB: 10.1-fold change at 100 μM, $P < 0.001$, TNF-α: 8.3-fold change at 100 μM, $P < 0.001$) (Fig. 6-5C, 6-5D). This activation was enhanced in shRNF11-transfected cells and was not observed in V5-RNF11-transfected cells (NF-κB: shRNF11: 40.8-fold change at 100 μM, $P < 0.001$, V5-RNF11: 0.9-fold change at 100 μM; TNF-α: shRNF11: 21.9-fold change at 100 μM, $P < 0.001$, V5-RNF11: 0.7-fold change at 100 μM), confirming the role of RNF11 as a negative modulator of NF-κB pathway. We also observed decreased mRNA expression of BDNF (shScramble: 0.3-fold change, $P < 0.05$; V5-RNF11: 0.4-fold change), GSS (shScramble: 0.3-fold change, $P < 0.001$; V5-RNF11: 0.3-fold change, $P < 0.05$), SOD1 (shScramble: 0.4-fold change, $P < 0.05$; V5-RNF11: 0.4-fold change), and BCL2 (shScramble: 0.3-fold change, $P < 0.001$; V5-RNF11: 0.4-fold change, $P < 0.001$) in all the cell lines except for shRNF11-transfected cells following 6-OHDA exposure (Fig. 6-5E, 6-5F, 6-5G, 6-5H). shRNF11-transfected cells expressed significantly increased levels of BDNF (9.8-fold change, $P < 0.001$), GSS (7.2-fold change, $P < 0.001$), SOD1 (7.7-fold change, $P < 0.001$), and BCL2 (4.1-fold change, $P < 0.001$) following 6-OHDA exposure suggesting the important role of these NF-κB transcribed factors (Pahl, 1999) in the observed differences in 6-OHDA-induced cell death. These *in vitro* studies further validated our *in vivo* demonstration that RNF11-mediated NF-κB activation in dopaminergic cells can impact vulnerability to 6-OHDA toxicity through NF-κB transcribed genes.

6.6 Protection against 6-OHDA-induced cell death in PC12 cells is attenuated with inhibition of NF-κB activation

For additional corroboration that RNF11-mediated NF-κB activation was indeed responsible for the observed differences in 6-OHDA-induced cell death, we pretreated cells with an NF-κB activator or inhibitor prior to exposure with 6-OHDA and measured cell death and alterations in mRNA expression of NF-κB and its target genes. To inhibit NF-κB signaling, we used Bay 11-7085, a molecule that blocks the phosphorylation of IκB and the subsequent dissolvement of the complex rendering NF-κB transcription factors inactive (Pierce et al., 1997). With Bay 11-7085 pretreatment alone, we observed no changes in cell viability (Fig. 6-6A). However, pretreatment of Bay 11-7085 followed by exposure to 6-OHDA increased susceptibility to cell death in the control, shScramble-transfected, and shRNF11-transfected cells (control: 24.89%, shScramble: 22.27%, shRNF11: 21.36% cell viability) to that observed in the V5-RNF11-transfected cells with 6-OHDA treatment alone (26.16% cell viability). While Bay 11-7085 did not alter RNF11 mRNA levels (Fig. 6-6B), the induced expression of the p50 subunit of NF-κB and TNF-α mRNA following 6-OHDA exposure was completely abolished (Fig. 6-6C, 6-6D). Also, the increased mRNA levels of GSS, SOD1, and BCL2 observed in shRNF11-transfected cells were eliminated with Bay 11-7085 pretreatment, with all cell types being identical to V5-RNF11-transfected cells (Fig. 6-6F, 6-6H). Bay 11-7085 pretreatment, however, did not alter mRNA expression of BDNF in shRNF11-transfected cells (Fig. 6-6E). The results of this experiment suggest that the protection granted with knockdown of RNF11 is related to the NF-κB dependent up-regulation of antioxidants and anti-apoptotic factors.

6.7 Protection against 6-OHDA-induced cell death in PC12 cells is enhanced with TNF-α pretreatment

To confirm and extend these results, we activated NF-κB signaling prior to exposure with 6-OHDA in order to bestow additional protection through up-regulation of antioxidants and anti-apoptotic factors. To activate NF-κB signaling, we pretreated PC12 cells with recombinant TNF-α. With TNF-α pretreatment, we observed no changes in cell viability (Fig. 6-7A). However, pretreatment of TNF-α followed by exposure to 6-OHDA was found to increase protection to 6-OHDA-induced cell death in all cell lines; the viability of control and shScramble-transfected cells was similar to shRNF11-transfected cells (control: 94.97%, shScramble: 95.47%, shRNF11: 100.9%, V5-RNF11: 42.90% cell viability). While TNF-α pretreatment did not alter RNF11 mRNA expression (Fig. 6-7B), only modest increases in the expression of the p50 subunit of NF-κB (shScramble: 11.3-fold change, shRNF11: 45.9-fold change, V5-RNF11: 0.9-fold change) and TNF-α (shScramble: 9.2-fold change, shRNF11: 32.0-fold change, V5-RNF11: 0.7-fold change) mRNA were observed in all cell lines, with only a significant increase in the shRNF11 cells ($P < 0.001$ for both) (Fig. 6-7C, 6-7D). Also, we observe significant increases in expression of GSS (shScramble: 1.2-fold change, $P < 0.01$; shRNF11: 12.6-fold change, $P < 0.001$; V5-RNF11: 1.4-fold change, $P < 0.05$), SOD1 (shScramble: 1.2-fold change, $P < 0.05$; shRNF11: 11.1-fold change, $P < 0.001$; V5-RNF11: 1.4-fold change, $P < 0.05$), and BCL2 (shScramble: 1.7-fold change, $P < 0.001$; shRNF11: 3.9-fold change; V5-RNF11: 2.0-fold change, $P < 0.001$) mRNA in all cell lines with TNF-α pretreatment (Fig. 6-7F, 6-7G, 6-7H), while expression of BDNF mRNA remained similar following 6-OHDA exposure regardless of pretreatment conditions (Fig. 6-7E). Consistent with our

results in the Bay 11-7085 pretreatment experiments, this experiment suggests that RNF11-mediated NF-κB activation in dopaminergic cells and subsequent upregulation of antioxidants and anti-apoptotic factors contribute to the protection we observe against 6-OHDA-induced cell death.

Together our *in vivo* and *in vitro* data provide strong evidence that RNF11 modulated NF-κB activation in dopaminergic cells is imperative for survival mechanisms against 6-OHDA toxicity and that the down-regulation of RNF11 expression we observed in PD brain samples may represent a protective mechanism against a toxic environment.

6.8 Discussion

Regulation of NF-κB signaling, through mechanisms such as the A20 ubiquitin-editing protein complex, is necessary to maintain normal homeostasis (Ruland, 2011) in the central nervous system. Regulation of NF-κB becomes highly relevant in neurodegenerative disease, such as PD, where persistent NF-κB activation is associated with increased inflammatory responses and degeneration of dopaminergic neurons (Mattson and Meffert, 2006; Hirsch and Hunot, 2009; Glass et al., 2010; Tansey and Goldberg, 2010). In this study, we investigated the role of RNF11, an essential component of the A20 ubiquitin-editing protein complex, in PD-associated neurodegeneration since RNF11 was the only A20 ubiquitin-editing protein complex component to have significantly altered expression in PD. The functional role of RNF11 was determined by manipulation of RNF11 expression in *in vivo* and *in vitro* models of PD, demonstrating that in dopaminergic cells, (1) RNF11 is a negative regulator of NF-

κB and (2) RNF11 can modulate susceptibility to 6-OHDA toxicity through NF-κB mediated responses, including induction of TNF-α, antioxidants and anti-apoptotic factors (Fig. 6-8). Thus, RNF11 over-expression enhanced dopaminergic cell death through decreased NF-κB responses while RNF11 knock-down substantially protected against 6-OHDA toxicity via enhanced NF-κB activation. Together, our *in vivo* and *in vitro* studies provide compelling evidence that RNF11, a component of A20 ubiquitin-editing protein complex, can modulate NF-κB activation in dopaminergic neurons with critical influences on PD associated neurodegeneration.

The A20 ubiquitin-editing protein complex is crucial for termination of NF-κB signaling and is dependent upon interactions between A20, Itch, RNF11, and TAX1BP1 (Shembade et al., 2007; Shembade et al., 2008; Shembade et al., 2009; Verstrepen et al., 2010). We have previously shown that each of these complex components has predominantly neuronal expression throughout the human brain (Chapter 3). However, the expression of A20, along with the proteins it associates with, had not been examined in neurodegenerative diseases. Our analysis of diseased human brain tissue showed a specific but significant reduction in RNF11 expression in PD brains while protein levels of TAX1BP1 and Itch and mRNA levels of A20 remained unchanged. *RNF11*, a candidate gene for late-onset PD at the PARK10 locus (Hicks et al., 2002) also co-localizes to alpha-synuclein-positive Lewy bodies (Anderson et al., 2007) further implicating RNF11's role in PD pathogenesis.

RNF11, as a negative regulator of the canonical NF-κB signaling pathway (Shembade et al., 2009) (Chapters 3, 4), is expected to have a crucial role in most NF-κB-mediated cellular function including inflammatory responses, similar to A20 (Lee et al., 2000). In this study we chose to examine the influence of RNF11 on vulnerability of dopaminergic cells to 6-OHDA, a PD toxin. 6-OHDA has been extensively used in cell culture systems (Cova et al., 2012; Grau and Greene, 2012; Lu et al., 2012) and in animals to model PD (Betarbet et al., 2002; Bezard et al., 2012). Moreover, 6-OHDA has been used in various cell culture systems to demonstrate that activation of NF-κB signaling pathway (Liang et al., 2007b; Zhang et al., 2011) and TNF-α (McCoy et al., 2006; McCoy et al., 2008; Lee et al., 2009) are toxic to nigral dopaminergic neurons. The unique aspect of our experimental paradigm, however, was that we manipulated NF-κB activation through expression of RNF11 in the dopaminergic neurons specifically targeted by 6-OHDA. Interestingly, our *in vivo* and *in vitro* experiments demonstrated that RNF11-mediated inhibition of NF-κB exacerbated 6-OHDA toxicity while reduced expression of RNF11 and consequent increased NF-κB activation protected against 6-OHDA toxicity.

This outcome was surprising to us since it is generally believed that in PD, NF-κB activation and increased expression of TNF-α are toxic for dopaminergic neurons and promote neurodegeneration (Saijo et al., 2009; Cao et al., 2010; Zhang et al., 2011). Moreover, inhibitors of the NF-κB pathway have been shown to confer neuroprotection in PD models (Ghosh et al., 2007; Tran et al., 2008; Flood et al., 2011) by diminishing inflammatory responses. An inflammatory response is commonly defined as a response mounted by immune cells, including microglial cells of the central nervous system, that

includes NF-κB activation and subsequent transcription of numerous cytokines (ie., TNF-α) and chemokines that perpetuate this response (Glass et al., 2010; Flood et al., 2011). Indeed, the importance of microglial NF-κB activation and TNF-α in PD is well documented (Kaltschmidt et al., 2005; Minghetti et al., 2005; Wilms et al., 2007; Aoki et al., 2009). Modulation of microglial NF-κB through various factors including regulator of G-protein signaling 10 (RGS10), nuclear receptor related 1 protein (NURR1), Fcγ receptors, and cluster of differentiation 200 (CD200)-CD200 receptor (CD200R) (Wang et al., 2007; Saijo et al., 2009; Cao et al., 2010; Lee et al., 2011) have specifically shown that it is microglial NF-κB activation and the subsequent increase in TNF-α that are harmful to dopaminergic neurons. In addition, others have demonstrated that increased microglial activation and TNF-α levels are detrimental to dopaminergic cell survival in various PD models (ie., alpha-synuclein, and toxin models) (Ferger et al., 2004; McCoy et al., 2006; Aoki et al., 2009). Thus neurons appear to be passive targets of immune offensives following microglial NF-κB activation.

Our study, however, suggests that NF-κB modulators such as RNF11 can influence neurons, including dopaminergic neurons, to mount a 'protective response' characterized by NF-κB activation and TNF-α when exposed to an insult. Though we have demonstrated this phenomenon for the first time in a PD model, there are numerous reports that show activation of neuronal NF-κB and increased TNF-α expression confers protection against neuronal toxicity (Kaltschmidt et al., 2005; Mattson and Meffert, 2006). For instance, pretreatment with TNF-α conferred protection in hippocampal neurons exposed to metabolic and excitotoxic insults (Cheng et al., 1994; Mattson et al.,

1997; Tamatani et al., 1999). This protection was dependent upon NF-κB activation which increased production of anti-apoptotic proteins such as BCL2, consistent with what we found. Similar protective effects of NF-κB activation are observed in cortical and sensory neurons (Marchetti et al., 2004; Fernyhough et al., 2005). Likewise, similar protection with NF-κB activation is found in disease models. With AD, there is increased neuronal vulnerability to amyloid β with inhibition of NF-κB that can be reversed with TNF-α-mediated NF-κB activation (Barger et al., 1995). It is hypothesized that NF-κB activation is protective when observed in AD patients, as NF-κB activation around plaques diminishes with disease progression (Kaltschmidt et al., 1999). Similarly, inhibitors of NF-κB enhanced apoptosis in response to neurotoxins in dopaminergic cells (Taglialatela et al., 1997), hippocampal neurons (Albensi and Mattson, 2000), and sympathetic neurons (Maggirwar et al., 1998). Further, TNF-α null mice show increased neuronal death after ischemia (Bruce et al., 1996) and TNFR knockout mice have exacerbated cortical neuron death in a traumatic brain injury model (Sullivan et al., 1999).

This protective role of neuronal NF-κB activation is not entirely surprising given the diverse roles of NF-κB. Persistent NF-κB activation and subsequent increase in TNF-α have been linked to both cell survival and cell toxicity (Mattson and Meffert, 2006; Costanzo et al., 2011; Galardi et al., 2011; Gordon et al., 2011; Li et al., 2012) against multitude of cellular insults in different disease model systems, indicating that NF-κB signaling may induce different outcomes in a context-specific manner. Furthermore, NF-κB is expressed in both neurons and glia (O'Neill and Kaltschmidt, 1997). Additionally,

NF-κB activation can regulate transcription of both pro- and anti-survival factors (Blum et al., 2001; McCoy and Tansey, 2008).

In fact it is the NF-κB transcribed pro-survival factors that seem to be the underlying cause for neuroprotection observed in our studies. Dopaminergic cells are particularly susceptible to oxidative stress (Graham et al., 1978), while neurotrophic and anti-apoptotic factors can confer protection against environmental and genetic risk factors associated with PD pathogenesis (Mangano et al., 2011; Nagahara and Tuszynski, 2011; Stahl et al., 2011; Rieker et al., 2012; Yin et al., 2012). We showed that in cases of increased protection against 6-OHDA-induced death, such as following neuronal RNF11 knockdown, there is an associated increase in expression of (1) antioxidants GSS and SOD1, (2) neurotrophic factor BDNF and (3) anti- apoptosis factor BCL2. Additionally, we showed that this increase is dependent on NF-κB activation, suggesting that NF-κB transcribed expression of antioxidants and anti-apoptotic factors may contribute to dopaminergic cell survival. BDNF, though still conferring protection, appears to be transcribed by an IκB independent pathway. Thus our *in vitro* and *in vivo* data lead us to conclude that RNF11-mediated NF-κB responses in dopaminergic cells can alter cell survival in PD models of degeneration.

Normal expression of RNF11 is important for normal homeostasis in non-dividing neurons to maintain optimum NF-κB function since this pathway is important for synaptic plasticity and neuronal stability (Mattson and Camandola, 2001; Boersma et al., 2011; Imielski et al., 2012). However, in neurodegenerative disease such as PD, RNF11,

as a member of the A20 ubiquitin-editing protein complex, could activate beneficial processes to alleviate stress and toxicity (Fig. 6-8). Our finding that PD patients have reduced mRNA and protein expression of RNF11 may thus represent a protective and compensatory response by neurons to PD associated stressors. Further investigation to better understand the modulation of NF-κB signaling in contexts of diverse central nervous system cell types (including glial cells) and specific insults is critical for our understanding of the contribution of NF-κB signaling to PD pathogenesis. Additionally, it will be important to explore factors that regulate expression of RNF11, such as miR-19 RNA (Gantier et al., 2012) to create a more targeted therapeutic intervention for PD. For now, our work has made a significant contribution in characterizing RNF11 as a negative regulator of NF-κB signaling in PD-related neurodegeneration and provides a foundation for future investigations into PD therapeutics.

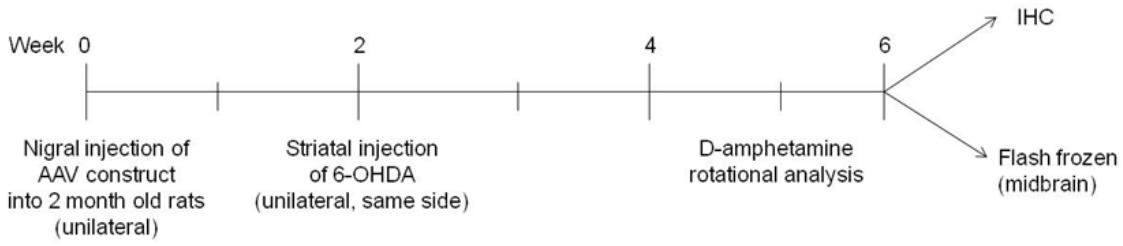


Figure 6-1. Schematic for in vivo experiments

AAV2 constructs (GFP-shScramble, GFP-shRNF11, V5-RNF11) were unilaterally injected into the substantia nigra pars compacta of two month old male rats. Two weeks later, a unilateral striatal lesion was induced by injecting 6-OHDA (10 μg) into the striatum of rats. Three weeks after lesion, rotational behavior induced by an intraperotoneal injection of 5 mg/kg D-amphetamine was measured in all animals as a physiological measure of striatal dopamine depletion. Four weeks after lesion, animals were anesthetized and brains were either fixed for immunohistochemical analysis or microdissected and flash frozen for qRT-PCR analysis.

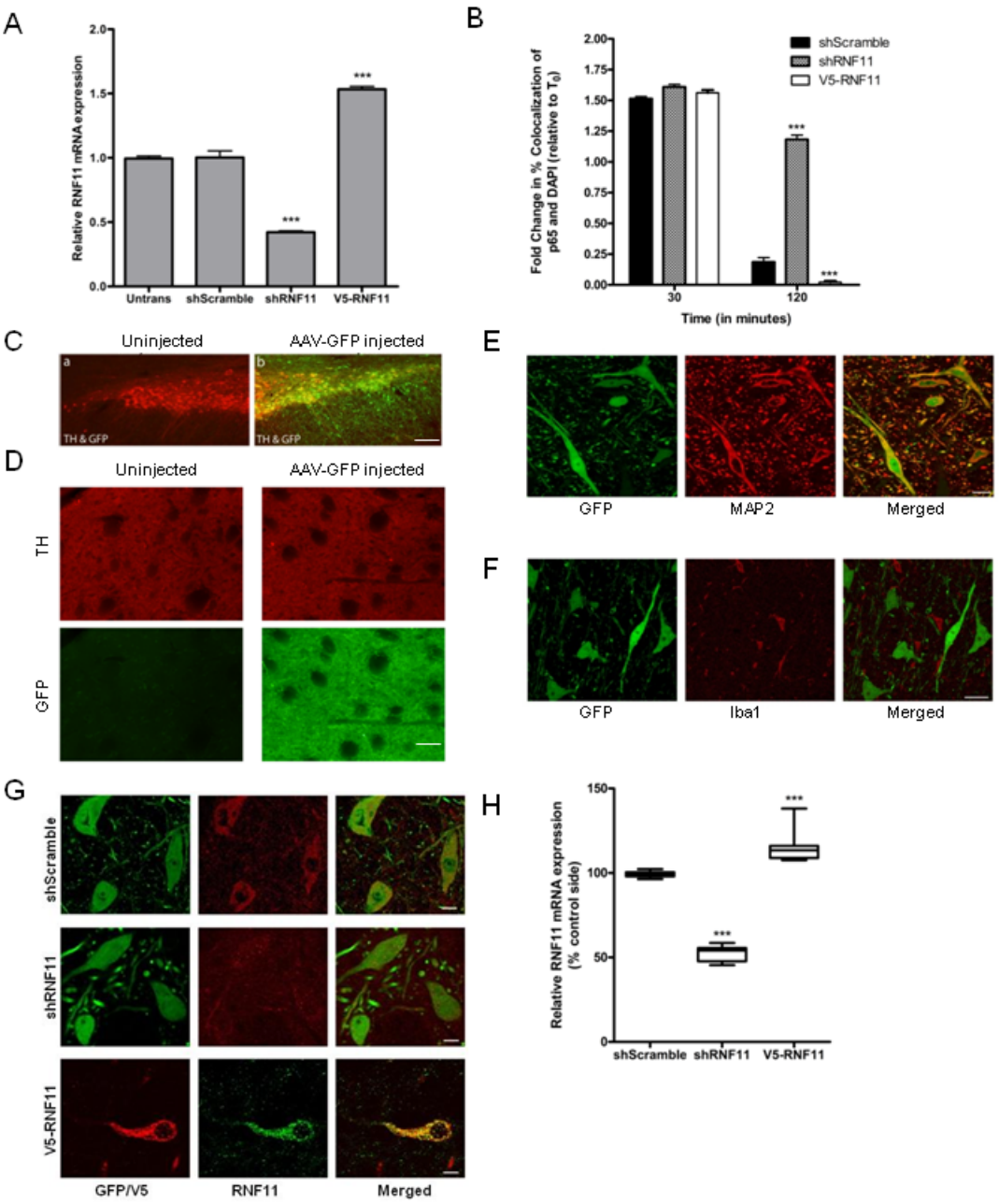


Figure 6-2. Validation of AAV-mediated targeted knockdown and over-expression of RNF11 *in vitro* and in vivo

A. Total RNA was extracted from murine primary neurons that were untransduced or transduced with shScramble, shRNF11, or V5-RNF11 AAV2 constructs, and examined by qRT-PCR for expression of RNF11 mRNA. Values expressed are mean comparative

cycle threshold ± SEM with GAPDH levels as an internal control. Results were analyzed by one-way ANOVA with Dunnett's multiple comparisons post-tests.***, $P < 0.001$. B. Murine primary neurons were transduced with shScramble, shRNF11, or V5-RNF11 AAV2 constructs and stimulated for 0, 30, or 120 min with TNF-α before being immunostained for p65 and Hoechst 333258. Transduced cells were analyzed for overlap of p65- and Hoechst 333258-positive pixels. The fold change in percentage overlap of p65- and Hoechst 333258-positive pixels was calculated relative to unstimulated conditions. Values represent means ± SEM of at least 50 cells in triplicate experiments. Results were analyzed by two-way ANOVA with Bonferroni selected multiple comparisons post-tests.***, $P < 0.001$. C, D. Animals were stereotaxically injected with AAV2 (tagged with GFP or V5) constructs in the nigra. Representative images of TH (red) and GFP (green) staining at the level of the substantia nigra (C) and striatum (D) in the uninjected and AAV-injected hemispheres showing spread of AAV infection throughout the nigrostriatal pathway. Scale bar for C: 200 μM. Scale bar for D: 50 μM. E. Representative images of GFP- (green) and MAP2-positive (red) cells at the level of the substantia nigra in AAV2-injected hemisphere. Note the extensive co-localization of GFP with MAP2. Scale bar: 20 μM. F, Representative images of GFP- (green) and Iba1-positive (red) cells at the level of the substantia nigra in AAV2-injected hemisphere. Note the absence of co-localization. Scale bar: 20 μM. G. Representative images of tag expression (GFP or V5) and RNF11 at the level of the substantia nigra in AAV2-injected hemisphere demonstrating knock down (middle panel) and over-expression (bottom panel) of RNF11 in nigral neurons. Scale bar: 10 μM. H. Total RNA was extracted from flash frozen ventral midbrain samples and examined by qRT-PCR for expression of

RNF11 mRNA. Values expressed are mean comparative cycle threshold ± SEM with GAPDH levels as an internal control. Results were analyzed by one-way ANOVA with Dunnett's post-tests. ***, $P < 0.001$.

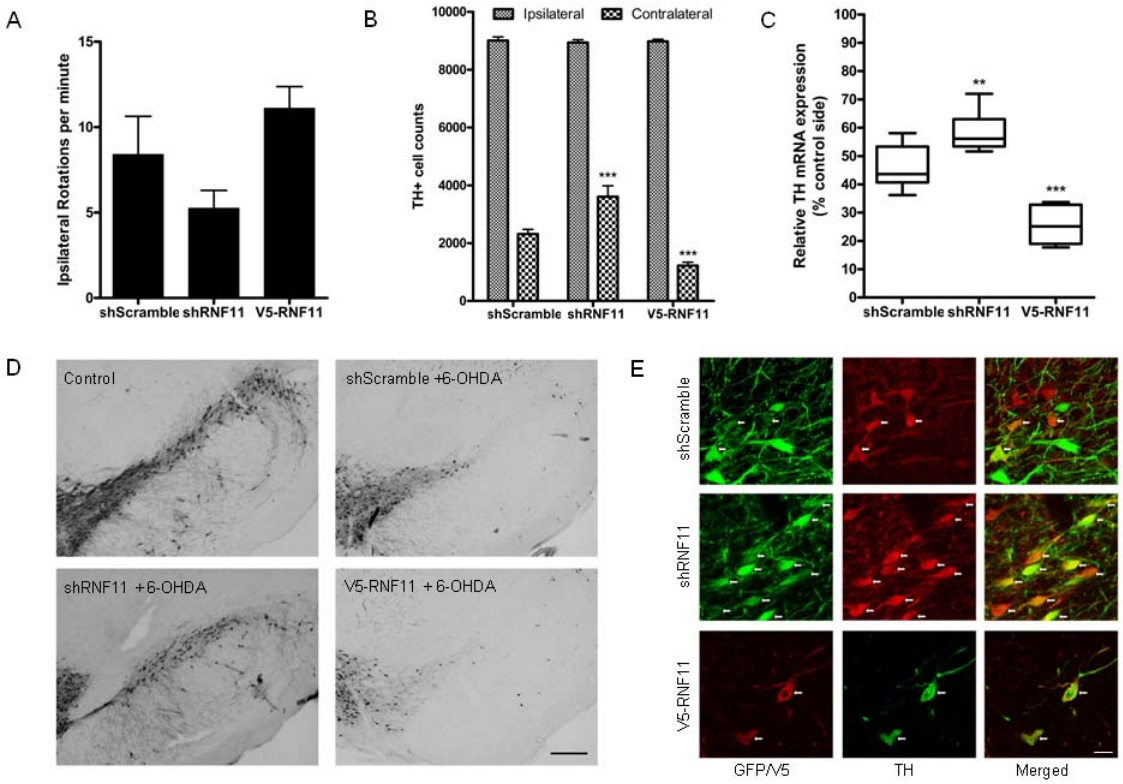


Figure 6-3. *In vivo* targeted knockdown of nigral dopaminergic RNF11 imparts neuroprotection against 6-OHDA-induced toxicity and attenuates rotational behavior

A. Three weeks after lesion, rotational behavior induced by an intraperitoneal injection of 5 mg/kg D-amphetamine was measured in all animals as a physiological measure of striatal dopamine depletion and expressed as the number of ipsilateral turns per minute. While a one-way ANOVA between groups was significant ($P = 0.01$), no significant differences between groups were found using Dunnett's multiple comparison test. Values expressed are group mean ± SEM. B. Stereological estimates of nigral dopaminergic neuron number (TH-immunoreactive neurons) after 6-OHDA injection on the unlesioned (contralateral) and lesioned (ipsilateral) sides. Left versus right differences from the same animals were analyzed using two-tailed paired Student's *T*-test. Values were expressed as

the group mean ± SEM; ***, $P < 0.001$, significantly different from unlesioned side. C. Total RNA was extracted from flash frozen ventral midbrain samples and examined by qRT-PCR for expression of TH mRNA. Values expressed are mean comparative cycle threshold ± SEM with GAPDH levels as an internal control. Results were analyzed by one-way ANOVA with Dunnett's post-tests.***, $P < 0.001$. D. Representative sections of TH-immunoreactivity from an uninjected hemisphere (control) or hemispheres receiving injections of shScramble-GFP, shRNF11-GFP, or V5-RNF11 AAV and 6-OHDA. Scale bar: 200 μM. E. Representative images of co-localization (indicated by white arrows) of expression of GFP or V5 and TH at the level of the substantia nigra in AAV2-injected hemisphere. Note the numerous GFP/TH-positive cells in shRNF11 group. Scale bar: 20 μM.

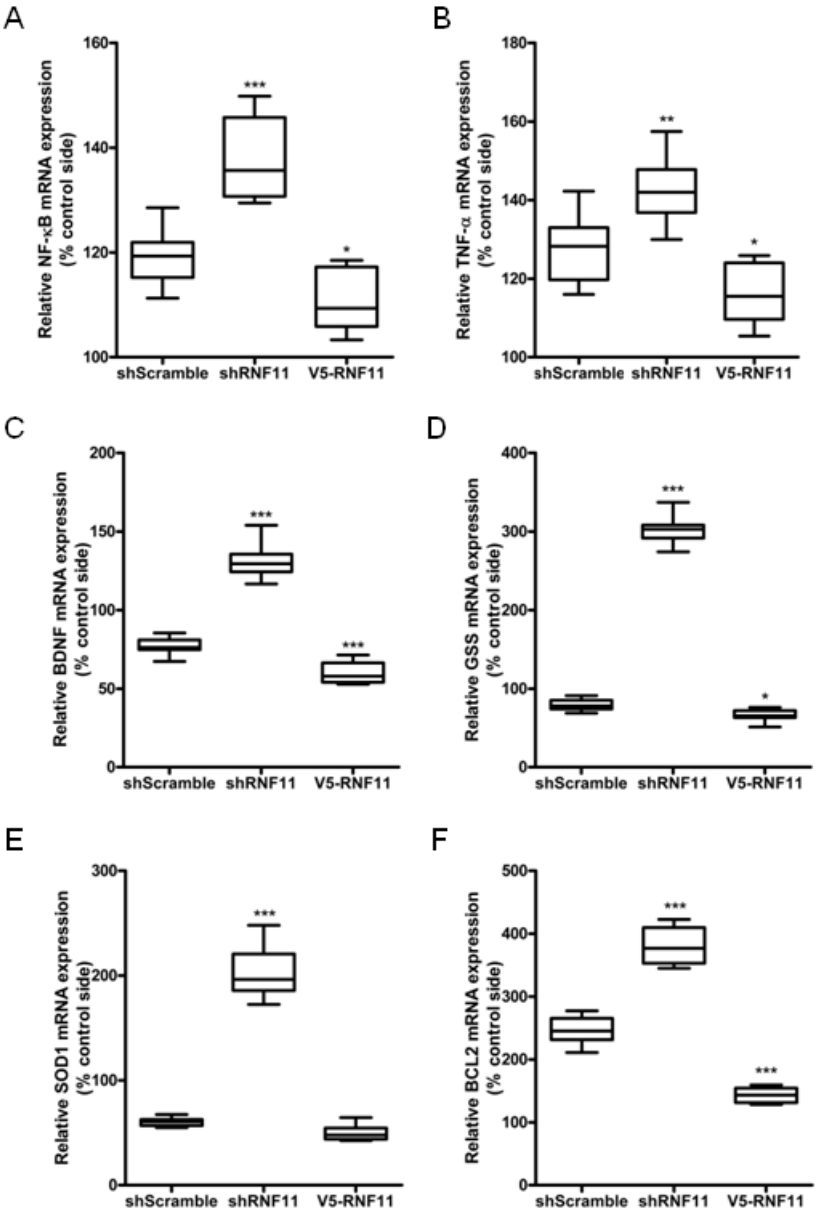
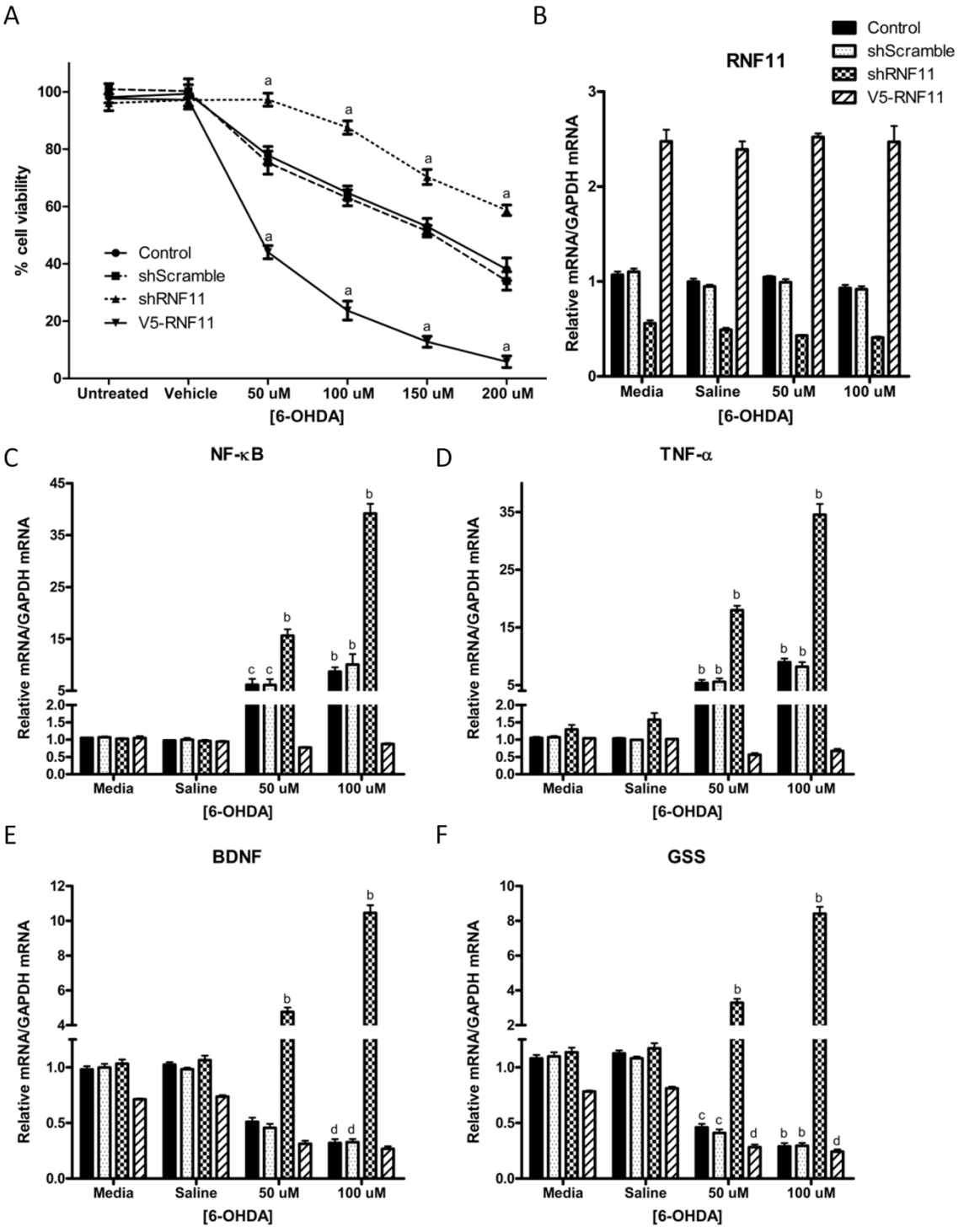


Figure 6-4. *In vivo* targeted knockdown of nigral dopaminergic RNF11 increases NF-κB signaling

Total RNA was extracted from flash frozen ventral midbrain samples and examined by qRT-PCR for expression of the p50 subunit of NF-κB (A), TNF-α (B), BDNF (C), GSS (D), SOD1 (E), and BCL2 (F) mRNA. Values expressed are mean comparative cycle

threshold ± SEM with GAPDH levels as an internal control. Results were analyzed by one-way ANOVA with Dunnett's post-tests.*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.


A
% cell viability
Control
shScramble
shRNF11
V5-RNF11
Untreated
Vehicle
50 uM
100 uM
150 uM
200 uM
[6-OHDA]
B
RNF11
Relative mRNA/GAPDH mRNA
Media
Saline
50 uM
100 uM
[6-OHDA]
C
NF-κB
D
TNF-α
E
BDNF
F
GSS

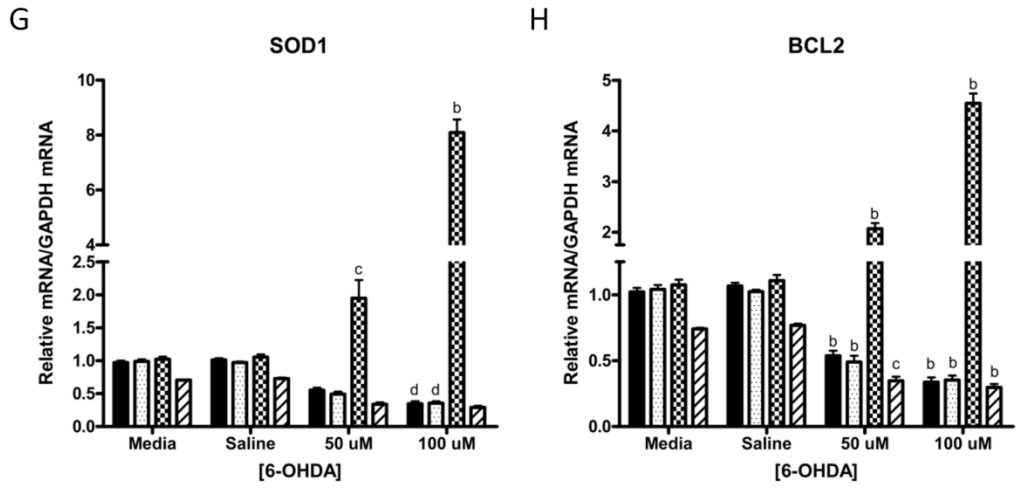
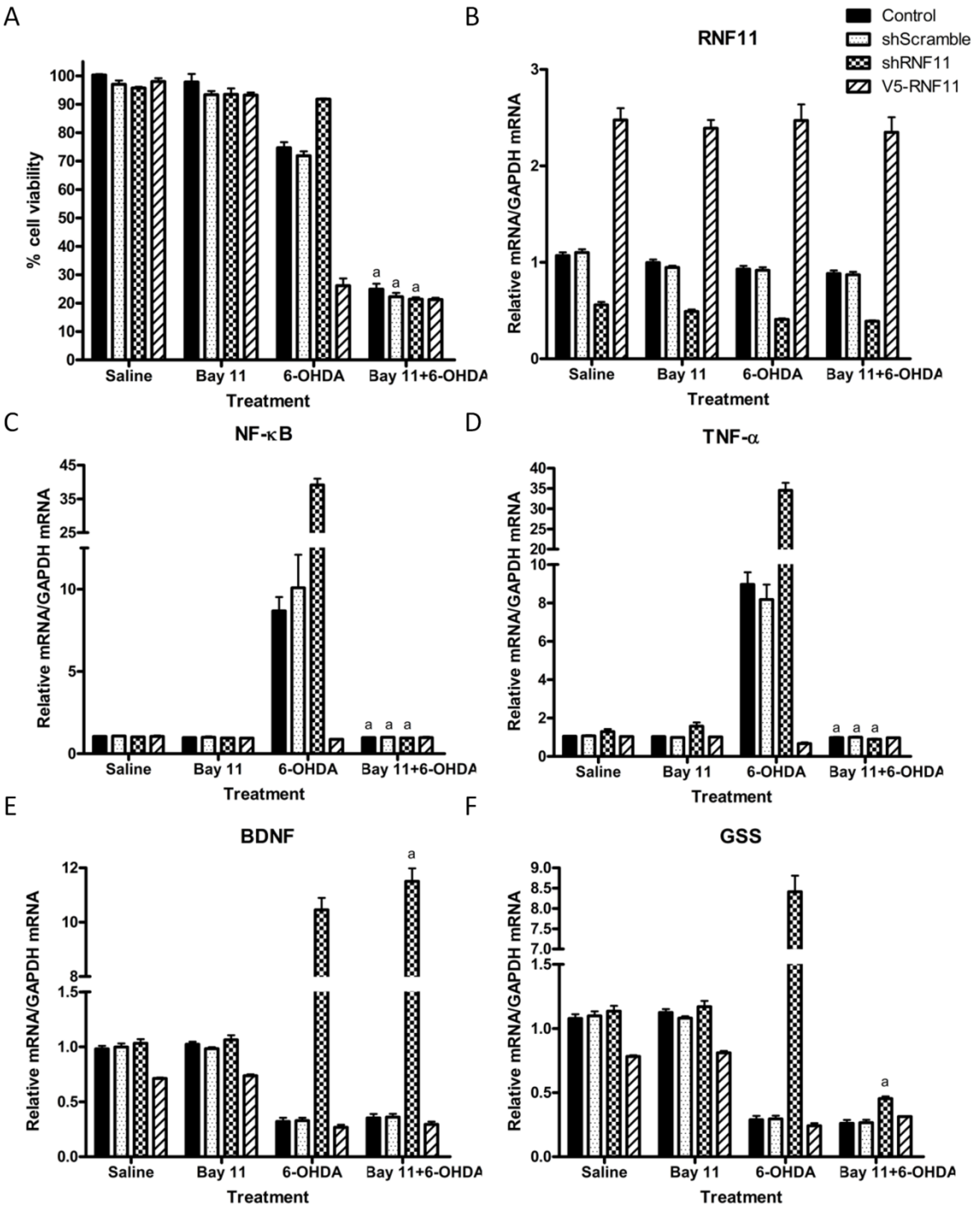


Figure 6-5. *In vitro* knockdown of RNF11 in dopaminergic cells imparts neuroprotection against 6-OHDA-induced toxicity

A. PC12 cells were transfected with empty vector (control), shScramble, shRNF11, or V5-RNF11 AAV constructs and exposed to media (untreated), saline (vehicle), or 50-200 μM 6-OHDA for 18 hours before measuring cell viability. Values expressed are mean percentage of viable cells using untreated control cells as the standard. Results were analyzed by two-way ANOVA with Bonferroni post-tests. a, $P < 0.001$ compared to control cells. Total RNA was extracted from parallel PC12 cell samples and examined by qRT-PCR for expression of RNF11 (B), the p50 subunit of NF-κB (C), TNF-α (D), BDNF (E), GSS (F), SOD1 (G), and BCL2 (H) mRNA. Values expressed are mean comparative cycle threshold ± SEM with GAPDH levels as an internal control. Results were analyzed by two-way ANOVA with Bonferroni post-tests. b, $P < 0.001$, c, $P < 0.01$, d, $P < 0.05$ compared to vehicle treated cells.


A
% cell viability
Saline
Bay 11
6-OHDA
Bay 11+6-OHDA
Treatment
B
RNF11
Control
shScramble
shRNF11
V5-RNF11
Relative mRNA/GAPDH mRNA
C
NF-κB
D
TNF-α
E
BDNF
F
GSS

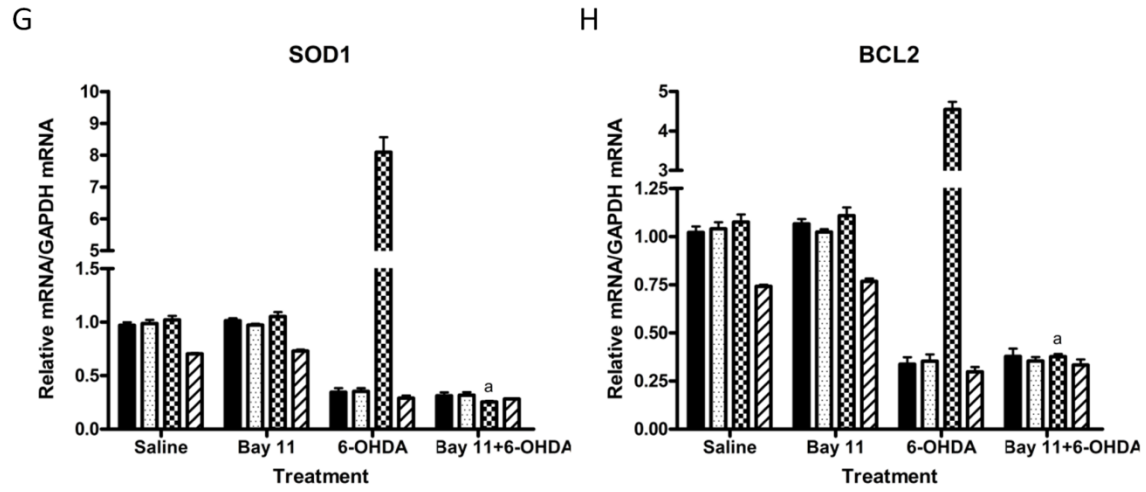
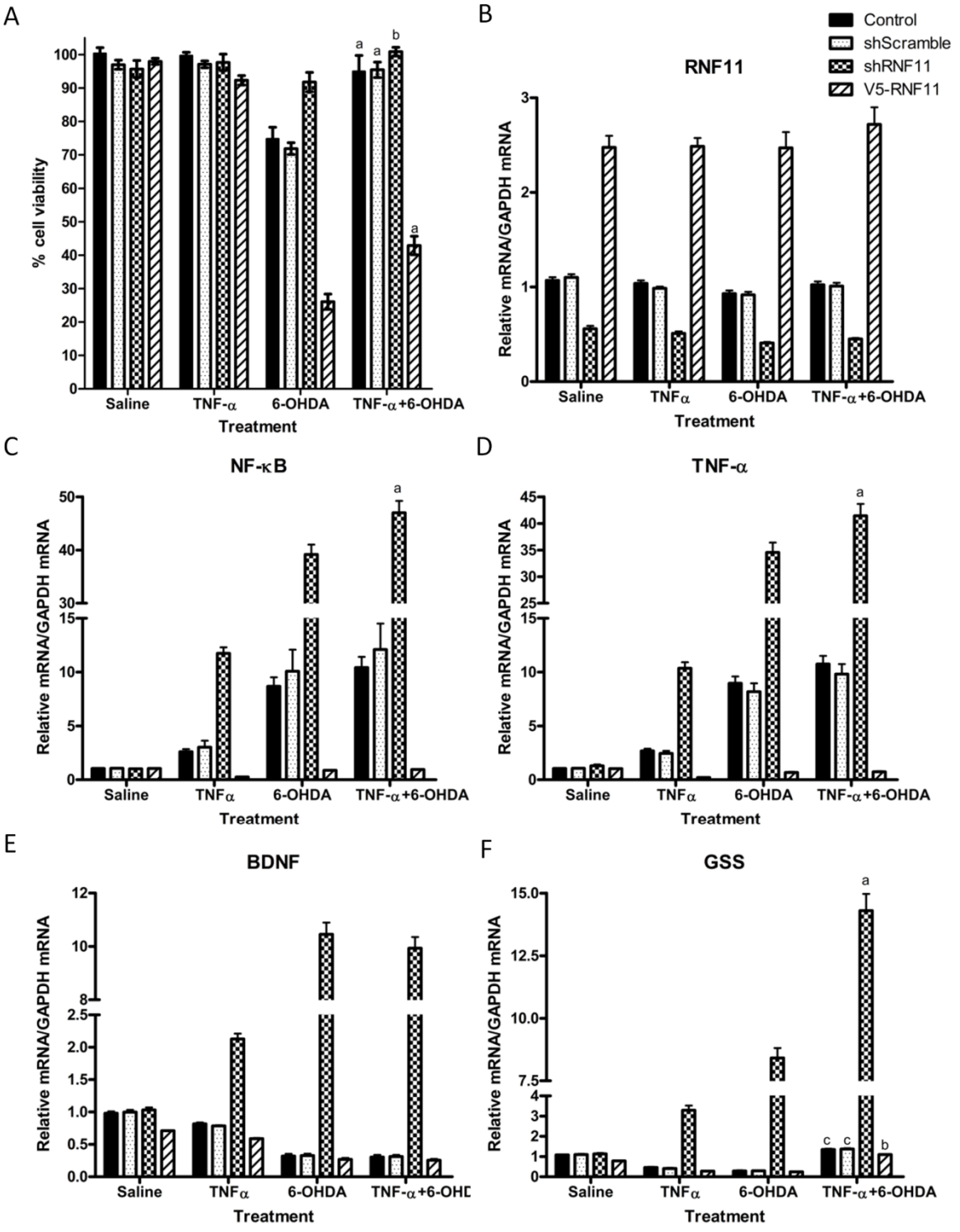


Figure 6-6. Protection against 6-OHDA-induced cell death in PC12 cells is attenuated with Bay 11-7085, an inhibitor of NF-κB activation

A. PC12 cells were transfected with empty vector (control), shScramble, shRNF11, or V5-RNF11 AAV constructs and exposed to 20 μM Bay11-7085 for 1 hour followed by saline or 100 μM 6-OHDA for 18 hours before measuring cell viability. Values expressed are mean percentage of viable cells using untreated control cells as the standard. Total RNA was extracted from parallel PC12 cell samples and examined by qRT-PCR for expression of RNF11 (B), the p50 subunit of NF-κB (C), TNF-α (D), BDNF (E), GSS (F), SOD1 (G), and BCL2 (H) mRNA. Values expressed are mean comparative cycle threshold ± SEM with GAPDH levels as an internal control. Results were analyzed by two-way ANOVA with Bonferroni post-tests. a, $P < 0.001$ compared to 6-OHDA treated cells.


A
% cell viability
Saline
TNF-α
6-OHDA
TNF-α+6-OHDA
Treatment
B
RNF11
Control
shScramble
shRNF11
V5-RNF11
Relative mRNA/GAPDH mRNA
C
NF-κB
D
TNF-α
E
BDNF
F
GSS

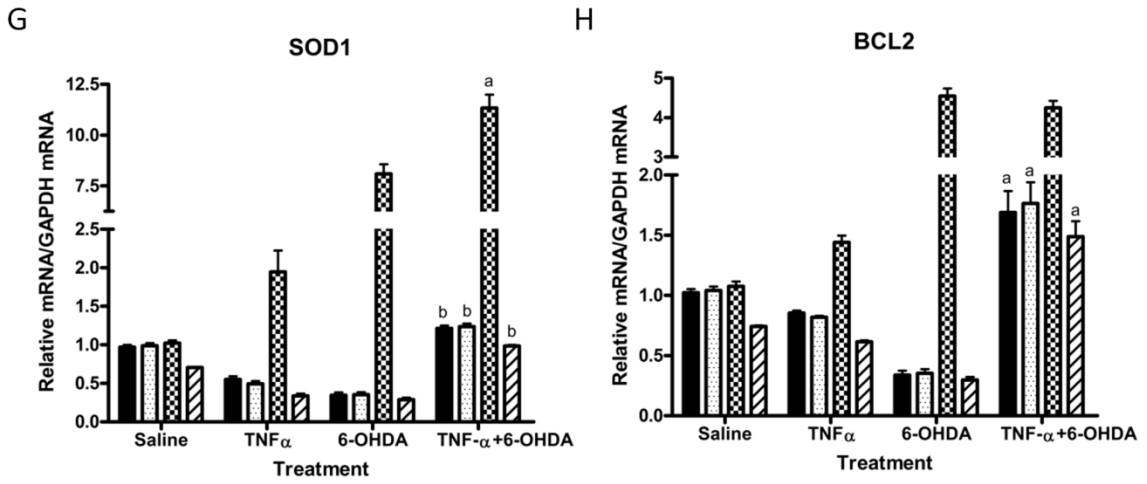


Figure 6-7. Protection against 6-OHDA-induced cell death in PC12 cells is enhanced with TNF-α pretreatment

A. PC12 cells were transfected with empty vector (control), shScramble, shRNF11, or V5-RNF11 AAV constructs and exposed to 10 ng/ml TNF-α for 1 hour followed by saline or 100 μM 6-OHDA for 18 hours before measuring cell viability. Values expressed are mean percentage of viable cells using untreated control cells as the standard. Total RNA was extracted from parallel PC12 cell samples and examined by qRT- PCR for expression of RNF11 (B), the p50 subunit of NF-κB (C), TNF-α (D), BDNF (E), GSS (F), SOD1 (G), and BCL2 (H) mRNA. Values expressed are mean comparative cycle threshold ± SEM with GAPDH levels as an internal control. Results were analyzed by two-way ANOVA with Bonferroni post-tests. a, $P < 0.001$, b, $P < 0.05$, c, $P < 0.01$ compared to 6-OHDA treated cells.

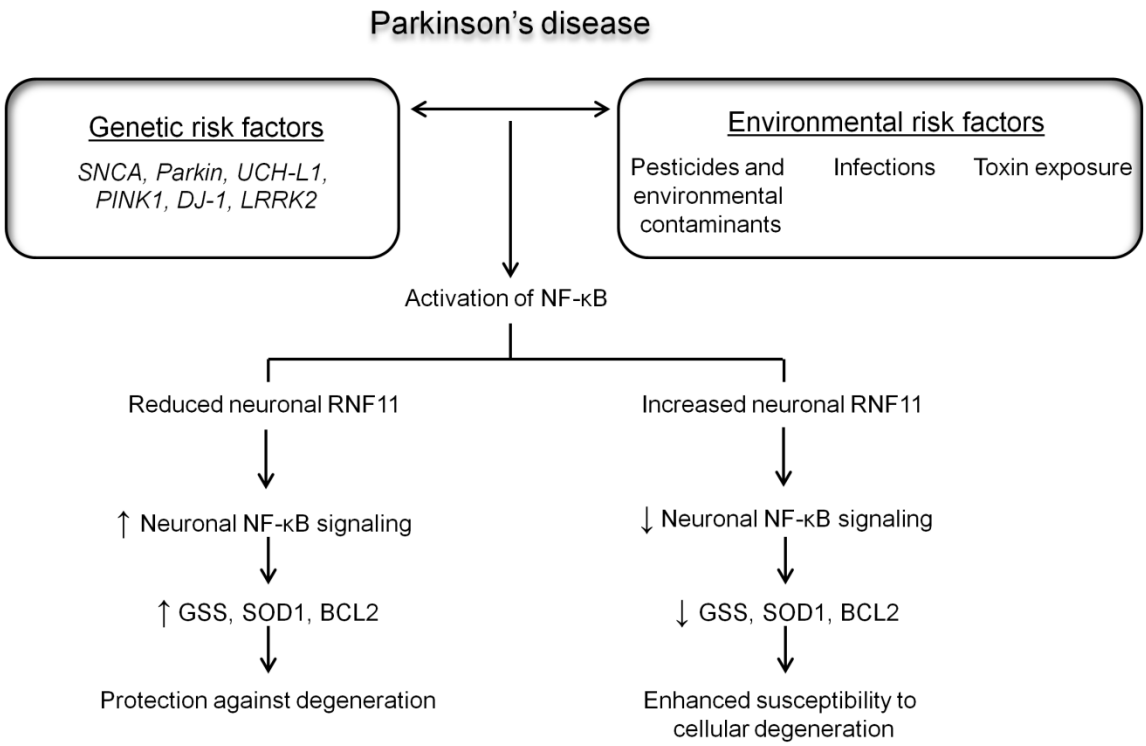


Figure 6-8. Model for the perceived functional role of RNF11 in neurodegeneration

In PD, the known genetic risk factors as well as environmental risk factors both contribute to the pathology and pathogenesis of the disease. In patients, activation of p65 is observed within the brain. We prospose that RNF11, as a negative regulator of NF-κB signaling in the brain, can mediate two distinct outcomes as a result of NF-κB activation. When neuronal RNF11 is reduced, there is an increase in NF-κB signaling due to the decreased inhibition of the pathway. This activation causes increased expression of protective factors such as GSS, SOD1, and BCL2. Expression of these factors operates to protect against degeneration. In contrast, when there is increased neuronal RNF11, there is a decrease in neuronal NF-κB signaling due to the inhibition conferred by RNF11. This decrease in NF-κB signaling leads to the decreased expression of protective anti-apoptotic factors, which promotes cellular degeneration. Thus, targeted inhibition of

neuronal RNF11 could be an interesting therapeutic avenue for slowing degeneration of dopaminergic neurons.

Chapter 7

Summary and future directions

7.1 Summary of results

This dissertation provides a foundation for the role of RNF11 in PD pathogenesis. First, in Chapter 3, we show that the A20 ubiquitin-editing protein complex is expressed in neurons and that RNF11, as a member of the A20 ubiquitin-editing protein complex, was the only reported complex member to show significant alterations in diseased human brain tissue. In particular, decreased expression of RNF11 appears to be specific to parkinsonian diseases. Second, in Chapter 4, we show that neuronal RNF11 negatively regulates canonical NF-κB signaling. Furthermore, neuronal RNF11 associates with A20 and Itch suggesting the existence of an A20 ubiquitin-editing protein complex within neurons. The localization of RNF11, conferred by its myristoylation domain, is

imperative to its regulatory function within the NF-κB pathway. Third, in Chapter 6, we show that knockdown of RNF11 and subsequent upregulation of NF-κB signaling bestows protection against 6-OHDA-induced toxicity in dopaminergic cells both *in vivo* and *in vitro*. This is the first reported manipulation of neuronal NF-κB signaling in an animal model of PD. This work also highlights differences in NF-κB activation in neuronal and glial cells. These important observations suggest that loss of RNF11 in PD may potentially influence the progression of the disease.

7.2 Expression of the A20 complex in the brain

In this project, the expression of the A20 ubiquitin-editing protein complex components in the brain was examined (Chapter 3) first to determine which component should be targeted for the remaining experiments. Expression of the components was examined throughout the brain (cortex, hippocampus, striatum, pons, and medulla) and found to be high in neuronal populations (Fig. 3-2, 3-3, 3-6). The only notable exception was in the white matter underlying the cortical tissue, where expression was also observed in microglia cells. Next, I examined expression in diseased tissue. With RNF11 as the only complex component with specifically altered expression in PD (Fig. 3-7), I focused on the effect of manipulation of RNF11 expression in neurons on NF-κB signaling and cytotoxicity.

Interestingly, RNF11 has been previously reported to be sequestered into cytoplasmic Lewy body inclusions, the pathological hallmark of PD (Anderson et al., 2007). RNF11 does not localize to all Lewy body pathology, suggesting that it does not play a role in the

formation of the Lewy body but is a part of the aggregated species. The localization of RNF11 to the Lewy body could thus represent a mechanism to alter susceptibility of SN dopaminergic neurons to PD insults. Our *in vivo* experiments (summarized later) begin to answer this underlying question about RNF11's role in disease pathogenesis and cell survival.

7.3 Neuronal RNF11 as a negative regulator of canonical NF-κB signaling

While RNF11 and the A20 ubiquitin-editing protein complex have been described as negative regulators of NF-κB signaling in peripheral immune cells (Shembade et al., 2009), we next confirmed that RNF11's function was conserved in neurons, cells that are both post-mitotic and non-immune cells (Chapter 4). With TNF-α stimulation, knockdown of RNF11 increased luciferase expression with a NF-κB-dependent luciferase assay (Fig. 4-2). Additionally, knockdown of RNF11 was associated with more extended heightened levels of p65 within the nucleus by immunocytochemistry and Western blotting (Fig. 4-3). These orthogonal approaches confirm RNF11's role as a negative regulator of NF-κB signaling in neurons. Furthermore, we showed that neuronal RNF11 associates with A20 and Itch and, interestingly, that this association is enhanced with TNF-α stimulation (Fig. 4-4). Lastly, knockdown of RNF11 in primary neurons lead to increased mRNA expression of NF-κB target genes (Fig. 4-7).

Recent work by Shembade and colleagues (Shembade et al., 2009) show that RNF11 is required for A20 to interact with and degrade RIP1, however, the exact molecular

contribution of RNF11 within the A20 ubiquitin-editing protein complex has not been yet been described. The complex components serve non-redundant roles in the inactivation of canonical NF-κB signaling (Shembade et al., 2009). It is hypothesized that RNF11 may be involved in the K48-linked polyubiquitination of RIP1 or may serve as a regulator of Itch function within the complex; however, further work is needed to identify the specific role of both Itch and RNF11 within the A20 ubiquitin-editing protein complex. Through examination of the role of RNF11 within the A20 ubiquitin-editing protein complex, we may gain additional insight into mechanisms and potential therapeutics to regulate the activity of RNF11 as well as the A20 ubiquitin-editing protein complex and activation of NF-κB signaling that may play a role in PD pathogenesis.

We also examined the function of *CG32850*, a gene in *Drosophila melanogaster* that is homologous to mammalian RNF11 (Kitching et al., 2003), in the immune pathways of the fly (Chapter 5). With knockdown of *CG32850* (Fig. 5-4), we observed significant up-regulation of expression of immunity molecules suggesting functional homology between *RNF11* and *CG32850* (Fig. 5-6, 5-7). Depending upon the type of bacteria used to induce an immune reaction, knockdown of *CG32850* could either be detrimental or protective to the health of the fly, with gram-positive infections inducing death quicker than controls and gram-negative infections protecting flies against death (Fig. 5-8). With these experiments, we see that induction of NF-κB signaling with different stimuli can lead to diverse outcomes that could either be beneficial or detrimental to the animal. In future work, it would be interesting to look at the effect of altering CG32850 expression within

the brain, specifically the dopaminergic neurons, to identify contexts related to PD that augment or diminish neuronal survival.

7.4 Activation of neuronal NF-κB signaling is cytoprotective in dopaminergic neurons

In this thesis, we show that neurodegeneration in hemiparkinsonian rats induced by 6-OHDA can be attenuated with knockdown of RNF11 in neurons by AAV2 (Chapter 6). This attenuation is supported by stereological estimates of TH-positive neurons, mRNA expression of TH in VM samples, and decreased ipsilateral turning in response to d-amphetamine (Fig. 6-3). Conversely, increased neurodegeneration is observed with over-expression of RNF11 by AAV2. These results suggest that neuronal NF-κB activation is actually protective against PD insults. Previously, inhibitors of NF-κB have shown neuroprotection in PD models (Ghosh et al., 2007; Flood et al., 2011); however, the mechanism behind this protection was the inactivation of an immune response mounted by glial cells. This represents an important contrast to the work presented in this thesis: activation of NF-κB signaling in neurons does not activate a "toxic" immune response, but rather activates a protective response that we hypothesize is important for neuronal health. Specifically, we show that NF-κB activation of antioxidants and anti-apoptotic factors may confer the beneficial effects observed against 6-OHDA-induced toxicity (Fig. 6-4).

While 6-OHDA is an established animal model for PD (Blum et al., 2001), it does not recapitulate all of the symptoms and phenotypes that accompany PD. There are many

different types of disease modifiers, both genetic and environmental factors that cause PD and may induce cell death through different mechanisms. Therefore, a logical extension of our work would be to look at other animal models of PD to determine if RNF11-mediated inactivation of NF-κB signaling is also detrimental in these models. Moreover, to confirm the cell-type differences in the response mediated by activation of NF-κB signaling, it would be important for experiments utilizing manipulation of RNF11 levels in glial cells to be performed. If activation of glial NF-κB signaling opposes neuronal NF-κB activation, one would find that knockdown of glial RNF11 would increase transcription of NF-κB target genes, and this increase in expression would have unfavorable effects on the survival of neurons.

The *in vivo* work describing RNF11 as a protective factor against neurodegeneration in the 6-OHDA model was extended into cell culture studies to determine the contribution of RNF11 and subsequent NF-κB signaling to dopaminergic cell survival. We show that manipulation of RNF11 expression in dopaminergic cells can recapitulate the neuroprotective and neurodegenerative phenotypes observed *in vivo* (Fig. 6-5). Importantly, we show that activation of specific anti-apoptotic factors (that are protein products of NF-κB target genes) is induced following stimulation of NF-κB signaling to promote protection of dopaminergic cells. Finally, we show that increasing or decreasing NF-κB signaling can promote or diminish expression of these anti-apoptotic factors and accompanying cytotoxicity, respectively (Fig. 6-6, 6-7).

Interestingly, *RNF11* is a candidate gene at the PARK10 locus (Hicks et al., 2002). While another study has confirmed the importance of the PARK10 region in sporadic PD in a separate population (Li et al., 2002), a specific gene underlying the PARK10 linkage has not been identified. Genes reported in the linkage cannot be confirmed in other populations (Farrer et al., 2004; Maraganore et al., 2005; Oliveira et al., 2005; Clarimon et al., 2006) and there are sound criticisms for the methodologies used in some studies (Goris et al., 2006; Myers, 2006). A more recent study addressed these concerns and was able to confirm RNF11 as a possible gene underlying the PARK10 linkage (Haugarvoll et al., 2008). While *RNF11* may or may not be the candidate gene at the PARK10 locus, it is crucial to further characterize factors that may alter the risk of PD pathogenesis regardless of whether genetic support can be garnished.

A natural extension from the work within this thesis would be to utilize therapeutics to promote NF-κB activation to delay the progression of PD pathogenesis. However, this line of thinking should incite caution and further research before being pursued. NF-κB signaling is important for many different cellular functions within the central nervous system, ranging from neuronal development, synaptic signaling that underlies learning and memory, and coordination of immune responses to toxic stimuli (Ea et al., 2004; Imielski et al., 2012). Without specific targeting to neurons, uncontrolled and prolonged NF-κB activation could have deleterious effects in the central nervous system. Additionally, our research shows effects with specific targeting to the dopaminergic neurons within the SN (Fig. 6-2).

7.5 Final Thoughts

While inflammation is generally thought to help an organism return to homeostasis, it is clear that the chronic inflammation in glial cells observed in neurodegenerative disease and acute brain injury plays a critical role in brain damage (Mattson and Meffert, 2006; Hirsch and Hunot, 2009; Tansey and Goldberg, 2010). Indeed, in PD we observe increased nuclear translocation of p65 in the SN is observed in PD patients (Hunot et al., 1997) and enhanced expression of inflammatory mediators from activated microglia (Hirsch et al., 1998). However, it is unclear whether this inflammation is an initiating factor for PD pathogenesis or a resulting factor from the death of neurons (Glass et al., 2010; Tansey and Goldberg, 2010; Flood et al., 2011). Therefore, in order to better understand and design therapeutic interventions for PD, it is important to investigate the role of inflammation as a precipitating factor in this disease. Our work has contributed to the characterization of the A20 ubiquitin-editing protein complex and the role that RNF11, a crucial factor of the complex, plays in neurodegeneration related to PD and has created a foundation for further investigation for a new therapeutic target for PD.

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