

**Prokineticin-2: a novel anti-apoptotic and anti-inflammatory signaling protein in  
Parkinson's disease**

by

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A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY

Major: Genetics

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2016

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## GENERAL ABSTRACT

The mechanisms behind the development and progression of Parkinson's disease (PD) are still not fully understood. Several pathophysiological mechanisms have been implicated in the progression of the disease including oxidative stress, mitochondrial dysfunction, excitotoxicity, protein aggregation, and neuroinflammation. Since current therapeutic options for PD treat only the symptoms without influencing disease progression, recent translational studies have focused on identifying factors that protect against putative pathophysiological mechanisms of PD. We show herein, that the chemokine-like signaling protein, Prokineticin-2 (PK2), is expressed and secreted at higher levels in dopaminergic neurons during the acute phase of inflammatory or neurotoxic stress. Furthermore, we are the first to demonstrate that this protein can protect dopaminergic neurons from MPP<sup>+</sup>-induced cell death through the activation of the MAPK and Akt pathways. PK2 treatment also protected dopaminergic neurons from mitochondrial fragmentation, while increasing the mitochondrial biogenesis mediators Pgc1 $\alpha$  and TFAM. Importantly, overexpression of PK2 by injecting PK2 AAV 2/5 into the mouse striatum significantly protected against MPTP-induced behavioral deficits and dopaminergic neuronal loss, whereas blocking PK2 signaling with the receptor antagonist PKRA7 exacerbates both of these MPTP effects.

Activation of microglia and astrocytes are both hallmarks of PD in the brain, and blocking microglia-produced inflammation has been found to protect dopaminergic neurons in animal models of PD. However, the mechanisms behind the activation of these cells are not fully understood, especially in astrocytes. Both microglia and astrocytes constantly survey their environment and respond to specific stimuli. Microglia can shift their phenotype by expressing

either protective signaling molecules or inflammation-related factors, depending on the type of stimuli. Recent studies indicate that astrocytes undergo similar phenotypic changes that were previously known only for microglia.

During our investigations of PK2, we made novel discoveries about this secreted chemokine-like protein's role in microglial and astrocytic activation, and its contribution to neuroinflammation. PK2 treatment in microglia lead to a shift in the microglial activation state towards a more anti-inflammatory phenotype by reducing basal levels of damaging reactive oxygen species (ROS) and inflammatory cytokines and increasing protective factors such as Arginase1 and Nuclear factor E2-related factor 2 (Nrf2). Similarly, recombinant PK2 (rPK2) attenuated the LPS-induced production of ROS, nitric oxide, pro-inflammatory cytokines and mitochondrial bioenergetic dysfunction. Overexpression of PK2 *in vivo* by injecting PK2 AAV into the striatum led to morphological changes in microglia, such as increased number and length of branch processes. Interestingly, PK2 AAV 2/5 protected microglia from MPTP-induced morphological changes, whereas the PK2 receptor antagonist PKRA7 exacerbated these effects. Our studies in astrocytes produced similar results, wherein PK2 treatment could induce the expression of protective factors such as Arginase1 and Nrf2 while decreasing inflammatory factors such as ROS and reactive nitrogen species (RNS). PK2 treatment in both mouse and human astrocytes increased the production and secretion of the neuroprotective glial cell-line derived neurotrophic factor (GDNF). Importantly, the GDNF produced and secreted by PK2 overexpression significantly protected a human dopaminergic neuronal culture from MPP<sup>+</sup>-induced cell death. Overall, we found that PK2 signaling has a direct neuroprotective effect, and that it can promote microglia and astrocytes to protect neurons through the reduction of inflammation and the production of neuroprotective factors.

## CHAPTER I

## GENERAL INTRODUCTION

## Thesis Layout &amp; Organization

The alternative format was chosen for this thesis and consists of manuscripts that have been published, or are being prepared for submission. The dissertation contains a general introduction, three research papers and a conclusions/future directions section that briefly discusses the overall findings from all chapters, and charts out where the research story will be taken next. The references for each manuscript chapter are listed at the end of that specific section. References pertaining to the background and literature review as well as those used in general conclusion section are listed at the end of the dissertation. The introduction section under Chapter 1 provides a background and overview of Parkinson's disease (PD). The Background and Literature Review-I section covers current evidence implicating the roles of activated microglia and activated astrocytes mediating progressive dopaminergic neuron loss in Parkinson's disease. It also introduces the neurotrophic factor GDNF and studies that have examined the role of this protective factor, therefore an overview of the research objectives pertaining to Chapter V. The Background and Literature Review-II provides an introduction to prokineticin signaling, Prokineticin-2 (PK2) in particular, and the role it plays in the CNS and throughout the body. The manuscript from Chapter 2 was recently accepted for publication in the *Nature Communications*. It explores the induction of PK2 and the role of this signaling protein in mediating dopaminergic neuroprotection in *in-vitro* and *in-vivo* models of PD. Chapter 3 analyzes how the Prokineticin-2 protein, that is a secreted signaling peptide, can induce alternative activation of microglia through the Jmjd3-Irf4 pathway. Chapter 4 examines how Prokineticin-2 can promote astrocyte activation towards a neuroprotective phenotype, through the upregulation of anti-oxidant factors and the glutamate

transporter GLAST. Chapter 5 explores the induction of the neurotrophic factor GDNF by prokineticin signaling in astrocytes. Chapters 3, 4, and 5 are in the process of being submitted for publication.

All of the research described by the author in this thesis was performed during the course of his doctoral studies at Iowa State University under the guidance of his major professors and principal investigators – Dr. Anumantha G. Kanthasamy and Dr. Arthi Kanthasamy.

## Introduction

### **PD overview**

Parkinson's disease (PD) was originally described by James Parkinson in 1817 as the shaking palsy, and is currently the second most common neurodegenerative disease affecting over 4 million people worldwide (de Lau and Breteler 2006, von Bohlen Und Halbach et al. 2004). The median age of onset for idiopathic PD is around 60 years and its risk increases with age. The disease is a chronic, progressive neurodegenerative disorder that is characterized by motor deficits that include akinesia (absence of movement), bradykinesia (slowness of movement), rigidity and postural instability. These symptoms are thought to arise from the pathological hallmark of PD, which is significant loss of the melanized dopaminergic neurons in the substantia nigra pars compacta (SNpc) and subsequent reduction of dopamine in the caudate putamen. By the time the motor symptoms are serious enough to be diagnosed, up to 70% of the dopaminergic neuronal cell bodies are lost in the substantia nigra along with 60-80% depletion of dopamine levels in the striatum (Cheng et al. 2010, Dauer and Przedborski 2003). Interestingly, the death of neurons in other regions, such as the ventral tegmental area (VTA), and locus coeruleus has also been documented (Alberico, Cassell and Narayanan 2015, Gesi et al. 2000). Non-motor symptoms also

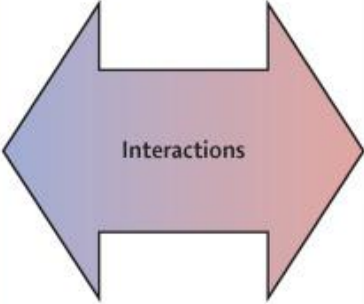


develop in some but not all PD patients, such as sleep disorders, constipation, olfactory deficits and cardiovascular dysfunction. In most cases, these non-motor symptoms can precede the onset of motor symptoms by many years. PD affects 1% of the population over the age of 60, however that percentage jumps to 5% of the population over the age of 85 (de Lau et al. 2006, Wood-Kaczmar, Gandhi and Wood 2006). Dopaminergic neuronal loss is also seen in normal aging, with an estimated loss of 4.7 – 9.8% of neurons per decade of life (Rudow et al. 2008, Fearnley and Lees 1991). However, natural aging does not lead to a PD-like pattern of dopaminergic neuronal loss in the ventral midbrain region, indicating that there are other factors involved in the development of PD (Reeve, Simcox and Turnbull 2014). Although aging appears to be the greatest risk factor for developing PD, the mechanistic basis for the selective vulnerability and progressive loss of nigral dopaminergic neurons has not been uncovered. The emerging hypothesis suggests that PD is a complex heterogeneous disease with multiple genetic mutations and environmental causative factors. Clinical and epidemiological studies have found several potential environmental risk factors for PD. These include heavy metal exposure, pesticide exposure, excess body weight, repeated head trauma and rural living ( Dick et al 2007, Priyadarshi et al 2001).

Most of the cases of PD are sporadic and are not directly linked to a genetic mutation, however, approximately 5-10% of all PD cases show an autosomal dominant or recessive mode of inheritance (Hardy 2010). There are currently 18 chromosomal regions, named PARK genes, that are directly associated with PD. Recently, six of these PARK genes have been implicated in early-onset of PD, such as *α-synuclein* (PARK1/PARK4), *parkin* (PARK2), *PINK1* (PARK6), *DJ-1* (PARK7), *ATP13A2* (PARK9) and *SLC30A10*. Similarly, the genes *LRRK2* (PARK8) and *VPS35* (PARK17) have been linked to late-onset PD (Pankratz and Foroud 2007). The *α-synuclein* and *LRRK2* gene regions are responsible for autosomal dominant PD (AD-PD), whereas the *parkin*,

*PINK1*, *DJ-1* and *ATP13A2* are linked to autosomal recessive PD (AR-PD) (Klein and Westenberger 2012). A mutation in one of these genes is sufficient to cause PD. Several of the genes associated with PD have multifactorial roles that may contribute to PD pathology, including neuroinflammation-related functions. For instance, *LRRK2*, *PINK1*, and *DJ-1* are all implicated in normal autophagy in the cell, and have all been found to promote microglial pro-inflammatory responses (Moehle et al. 2012, Kim et al. 2013). Using accumulated genome-wide association studies (GWAS) over large population cohorts, several loci have been identified as PD risk loci that could contribute to the development of PD (Lill et al. 2012). However, approximately 40% or more of the population-attributable risk remains unexplained by the currently identified PD loci (Nalls et al. 2015).

The sporadic form of PD accounts for more than 90% of PD occurrences where onsets were likely caused by a complex interplay of genetic and environmental factors. Figure 1 details the known factors that either increase or decrease overall risk (OR) of developing PD. The major hallmark of sporadic PD is the cytoplasmic inclusion of aggregated proteins that contain the

Environmental risk factors		Genetic risk factors
<b>Increased risk (OR &gt;1)</b> Pesticide exposure Prior head injury Rural living Beta-blocker use Agricultural occupation Well water drinking	 <b>Interactions</b>	<b>Increased risk (OR &gt;1)</b> GBA (OR >5) <i>VPS13C</i> <i>INPP5F</i> <i>DDRKG1</i> <i>STK39</i> <i>GPNMB</i> <i>LRRK2</i> <i>CCDC62</i> <i>SIPA1L2</i> <i>MIR4697</i> <i>BST1</i> <i>BCKDK-STX1B</i> <i>RAB7L1-NUCKS1</i>
<b>Decreased risk (OR &lt;1)</b> Tobacco smoking Coffee drinking NSAID use Calcium channel blocker use Alcohol consumption		<b>Decreased risk (OR &lt;1)</b> <i>SNCA</i> <i>GCH1</i> <i>MAPT</i> <i>RIT2</i> <i>TMEM175-GAK-DGKQ</i> <i>FAM47E-SCARB2</i> <i>HLA-DQB1</i> <i>FGF20</i> <i>MCCC1</i> <i>SREBF1-RAI1</i> <i>ACMSD-TMEM163</i>

Kalia and Lang, Lancet 2015

**Figure 1. Genetic and environmental risk factors that either increase or decrease the overall risk (OR) of PD**

protein  $\alpha$ -synuclein, these inclusions are known as Lewy bodies and Lewy neurites. However, the presence of Lewy bodies and Lewy neurites is not sufficient in explaining the disease. Other factors such as mitochondrial dysfunction, oxidative stress, dysfunction in the ubiquitin proteasomal system (UPS), and excitotoxicity have been implicated in the development and progression of PD (Savitt, Dawson and Dawson 2006, Gandhi and Wood 2005, McNaught et al. 2003). Another major contributor to PD pathogenesis is the increased presence of activated microglia and pro-inflammatory factors in the substantia nigra, which has been found in clinical subjects and all animal models of the disease (Mosley et al. 2006). It was previously thought that the increase number of activated microglial was just an indication of neuronal injury, however, mounting evidence indicates that reactive microglia and the subsequent inflammatory response plays an active role in the pathogenesis and progression of PD. One hypothesis has been proposed in which different causative factors and multiple neurotoxic insults accumulate throughout the years or possibly converge on the nigrostriatal system, thereby resulting in an obvious idiopathic onset of PD. This is known as the ‘multiple-hit’ hypothesis (Carvey, Punati and Newman 2006). Currently, most drugs and therapies prescribed for PD patients, such as L-DOPA and deep brain stimulation (DBS), only alleviate PD symptoms and do not slow the progression of the disease. Therefore, new therapeutic options are needed that can either protect dopaminergic neurons from cell death or regenerate already lost dopaminergic neurons. Understanding the mechanisms behind dopaminergic neuronal cell death, and the communication signals between neurons and glial cells, such as microglia and astrocytes, would be helpful in finding novel signaling pathways for reducing neuroinflammation and increasing neuroprotection.

## Background and Literature review I

### **Neuroinflammation in PD**

Inflammation is a necessary response that must protect the body against pathogens by performing critical functions such as clearing away dead or dying cell debris, which could otherwise lead to deleterious effects such as infection. However, inflammation also produces factors that can be toxic to cells, potentially leading to significant pathology if the inflammation is not tightly regulated (Glass et al. 2010). Several neurodegenerative diseases, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and Parkinson's disease (PD) have been associated with an exaggerated neuroinflammatory component (Block, Zecca and Hong 2007, Minghetti 2005). The main source of this neuroinflammation is the microglia and astrocytes at the site of neurodegeneration. These two distinct cell populations undergo persistent hyperactivation and exacerbate the disease progression. It was previously thought that reactive gliosis would protect the neurons from cell death by maintaining environmental homeostasis and by increasing phagocytic clearance of dead and dying neurons in the CNS. Both microglia and astrocytes have important roles within the developing brain, and in keeping the brain in a healthy state. Microglia play a crucial role in establishing synapses while the brain is developing, and maintain neuronal health by producing multiple trophic factors (Aarum et al. 2003, Walton et al. 2006). Most neuroinflammatory responses are only transient actions that subside after clearance of the activating stimulus, however the neuroinflammation seen in neurodegenerative diseases such as Parkinson's disease (PD) is chronic and progressive. This progressive inflammatory response has been demonstrated in human PD patients and in most

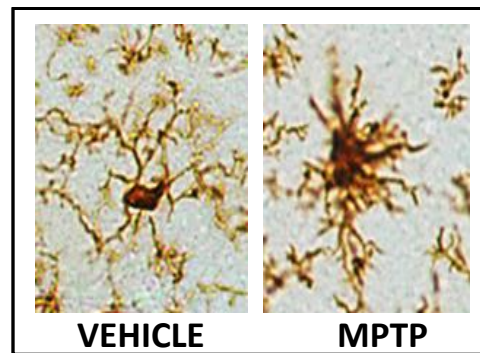
animal models of PD, such as MPTP, 6-OHDA, and LPS models (Imamura et al. 2003, Panicker et al. 2015). Higher amounts of inflammatory cytokines, including TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-2 and IL4, were found in the SN of PD patients compared to age-matched controls (Mogi et al. 1994, Mogi et al. 1996). Several animal studies have demonstrated that inhibiting pro-inflammatory cytokine signaling can reduce dopaminergic neuronal degeneration in mice. Nigral administration of anti-IL-1 $\beta$  neutralizing antibody (Arai et al. 2004, Arai et al. 2006), and the recombinant dominant negative TNF inhibitor XENP345 (McCoy et al. 2006) were both able to significantly protect against dopaminergic neurodegeneration. Neuroinflammation is a key mechanism in the progression of Parkinson's disease and could be a target for therapeutic intervention to prevent dopaminergic neurodegeneration.

### **Microglial role in PD**

Microglia are considered the resident macrophagic cells of the CNS since they were originally found to perform phagocytic functions similar to macrophages, microglia express high levels of MHC class II molecules, making them antigen-presenting cells (Hickey and Kimura 1988). Interestingly, microglia arise from primitive myeloid progenitors that develop from the yolk sac (Ginhoux et al. 2013), which demonstrates that microglia do not arise from the bone-marrow hematopoietic stem cells as originally thought. While microglia comprise 10-15% of the total cells in the brain, their density varies in different areas of the brain, with the highest concentrations found in the hippocampus and ventral midbrain. Microglia in the adult brain typically exist in a 'resting' state, identified by a ramified morphology and small cell soma, in which they can perform

surveillance of the environment. Under normal circumstances, microglia produce and secrete anti-inflammatory and neurotrophic factors that help to promote neuronal health and plasticity (Carson 2002).

Microglia have been found to be highly dynamic and mobile, with the ability to react to any signs neuronal injury. Studies have determined that microglial activation is the first response in the CNS to both acute and chronic neuronal injury (Nimmerjahn, Kirchhoff and Helmchen 2005). Introduction of any potential pathogen or neuroinflammatory agent causes these cells to undergo a rapid transformation into an ‘activated’ state, which is characterized by an amoeboid morphology with de-ramified microglial branches (Figure 2). This ‘activated’ state is also identified by a rapid transcriptional activation of response genes and various cell surface proteins, such as cytokines and chemokine receptors. The secreted or cell surface proteins produced by activated



**Figure 2. DAB immunostaining images of Iba1-positive cells in the mouse striatum of vehicle or MPTP treated mice**

microglia can alter the site of neuronal injury or cell death. For instance, activation of microglia under normal conditions is a transient and highly regulated process, that promotes neuron survival and CNS homeostasis (Glezer, Simard and Rivest 2007, Napoli and Neumann 2010, Simard and Rivest 2007). Microglia have demonstrated the ability to protect neurons through the production of several factors, including BDNF, GDNF, IL-10 and TGF $\beta$ 1 (Patel et al. 2013). Similarly, the increase in the glutamate transporter GLT-1 in microglia protected neurons from excitotoxicity when astrocytic glutamate uptake was impaired (Persson et al. 2005, Shaked et al. 2005). Synapse removal and clearance of dead cells by phagocytosis are two other ways that microglia promote a

healthy environment. Microglia activation and trophic factor secretion around the site of injury promotes a return to homeostasis after infection (Kreutzberg 1996).

Neurodegenerative diseases result in a sustained microglial activation, which is then considered to evolve into hyperactivation at the site of neuronal loss. This sustained hyperactivation of microglia differs from the healthy, transient activation both by the secreted factors produced and by cell morphology (Lull and Block 2010). Along with becoming amoeboid, the activated microglia release reactive oxygen species (ROS), nitrite and pro-inflammatory cytokines that are toxic to neurons. Sustained neuroinflammation has been found to negatively affect neuron health in multiple cell culture models, animal models and post-mortem analyses of human brains (Aschner et al. 1999, Glass et al. 2010). Animal models of Parkinson's disease display this activated microglial response along with an increase in the inflammatory factors that are mainly produced by microglia. MPTP is a selective dopaminergic neuronal toxicant and its acute injection paradigm (4 injections of 18mg/kg every 2 hours) is well characterized to induce de-ramification of microglia and pro-inflammatory factors (Hirsch and Hunot 2009, Sriram et al. 2006, Wu et al. 2002, Wu et al. 2003). Inhibition of the microglial NOS2 gene and the NF- $\kappa$ B pathway can prevent this hyperactivation, thereby attenuating of MPTP-induced neurodegeneration (Du et al. 2001, Ghosh et al. 2007). Similar activation of microglia occurs in an MPTP non-human primate model and in the 6-OHDA mouse model (Stott and Barker 2014, Virgone-Carlotta et al. 2013, Vazquez-Claverie et al. 2009). Evidence is also pointing to an increase in neuroinflammation and activated microglia after genetic overexpression of  $\alpha$ -synuclein and in a relatively new animal model of PD with selective knockout of the transcription factor TFAM specifically in dopaminergic neurons (Chung et al. 2009, Theodore et al. 2008)(Langley et al., unpublished). The strongest evidence comes from animals treated with Lipopolysaccharide

(LPS), which is the most widely used model for testing the role of neuroinflammation in PD. LPS is a component of the gram-negative bacterial cell wall and is a powerful inflammogen that evokes an innate inflammatory response from immune cells by acting as a pathogen associated molecular pattern (PAMP). LPS treatment selectively activates microglia, and the subsequent inflammatory response leads to dopaminergic neuronal death. This effect from LPS treatment has been demonstrated both by direct intranigral injection and by systemic, intraperitoneal administration (Castano et al. 1998, Herrera et al. 2000, Hsieh et al. 2002, Qin et al. 2007). Interestingly, administering LPS together with other neurotoxicants such as pesticides and metals has demonstrated that increased neuroinflammation can amplify the effects of PD-associated environmental insults, thereby resulting in even greater neuron loss (Liu, Gao and Hong 2003, Zhang et al. 2010). Similarly, combining LPS-induced microglial priming and the overall progressive inflammatory status of the aging brain can also increase the chances of developing a neurodegenerative disease (Perry and Holmes 2014).

Along with the studies that found microglial activation to be important in animal models of PD, numerous other studies have examined microglial activation and inflammation in human patients. For instance, post-mortem PD brains contain amoeboid microglia around the regions of dopaminergic neuron loss (Langston et al. 1999, Whitton 2007, McGeer et al. 1988). These reactive microglia displayed characteristics of phagocytic cells that are typically seen with aging (Jyothi et al. 2015). Besides activated microglia, PD patients have an increased level of pro-inflammatory cytokines, such as TNF $\alpha$ , IL-1 $\beta$ , and IFN $\gamma$ , in the cerebrospinal fluid and in the brain itself (Hirsch, Hunot and Hartmann 2005, McGeer et al. 1988, Hunot et al. 1997, Mogi et al. 2000). There is also an inverse correlation of CD68 immunoreactivity with PD progression, therefore finding elevated phagocytosis associated with ongoing tissue degeneration and decreasing in the



final stages (Croisier et al. 2005). Several single nucleotide polymorphisms (SNPs) that have been linked to PD development were identified by genetic linkage studies in PD patient and age-matched control groups. Some of the SNPs are found within the promoter regions of pro-inflammatory genes (Hakansson et al. 2005, Hamza et al. 2010, Nishimura et al. 2000, Nishimura et al. 2001). Microglial activation has been confirmed in living PD patients using positron emission tomography (PET) imaging. Radioligands are used that bind to translocator proteins (TPSO) which is greatly enhanced in the presence of neuroinflammation, and therefore can give an indication of activated microglia (Banati et al. 2014, Papadopoulos and Lecanu 2009). This technique has allowed the visualization of increased inflammation seen in the pons, basal ganglia and frontal and temporal cortices of PD patients compared to control patients (Gerhard 2016). Importantly, it has recently been found and corroborated that regular use of NSAIDs, especially Ibuprofen, reduce the risk for developing PD (Gagne and Power 2010, Gao et al. 2011). Taken together, these results indicate that neuroinflammation plays an important role in PD, and that understanding that role could help to discover therapies to prevent the neuronal loss seen in PD.

### **Microglial activation phenotypes**

The above section on microglial activation describes what is known as ‘classical’ activation of microglia, recognized by its de-ramified, amoeboid morphology along with the increased production of inflammatory factors. However, recent studies are indicating that microglia exist in the brain as a heterogeneous population of activation phenotypes. These different phenotypes perform certain functions depending on the nature, intensity and duration of stimuli presented to the cell (Tansey and Goldberg 2010, Olah et al. 2011). Therefore, microglia seem to respond similar to other tissue macrophages. Their production of different cytokines, inflammatory

mediators, growth factors and cell surface molecules have all been used to classify microglia as either pro-inflammatory or anti-inflammatory. However, as previously stated, microglia exist in a heterogeneous population and activation of these cells cannot be simply classified as pro-inflammatory or anti-inflammatory. Microglia activation was originally perceived to resemble that of T-cell polarization towards pro-inflammatory Th1, or anti-inflammatory Th2, and therefore were given the nomenclature of M1, for pro-inflammatory, or M2, for anti-inflammatory. However, unlike T-cells, microglia do not polarize to a particular set of functions, but can change their activation state depending on environmental situations and subsequent downstream signaling (Martinez and Gordon 2014). Therefore, microglial activation is currently designated as an “M1 phenotype” that is considered cytotoxic and increases inflammatory factors, whereas an “M2 phenotype” promotes tissue repair and the reduction of inflammation (Mills et al. 2000, Mackaness 1962). Many studies have examined the signaling mechanisms driving microglial activation and the different states that microglia exist in under various neurodegenerative diseases, such as PD (Joers et al. 2016).

The M1 microglial phenotype, or ‘classical’ activation, is known to occur with introduction of the stimuli LPS or IFN $\gamma$ . IFN $\gamma$  is the main cytokine associated with this classical activation, and activates the downstream signaling pathways JAK2, STAT1 and IRF1 (Waddell et al. 2010). The classically activated cells produce high levels of inflammatory cytokines, such as TNF $\alpha$ , IL-1 $\beta$ , IL-6, and IL-12, along with cell surface markers such as major histocompatibility complex-II (MHC-II) and CD86, and the toxic nitric oxide molecule (Martinez et al. 2006, Nau and Bruck 2002). These inflammatory factors produced by microglia are toxic to both astrocytes and neurons, and therefore lead to increased neuronal death (Boche, Perry and Nicoll 2013, Fan et al. 2016). A common signaling pathway for any of the M1 stimuli is the activation of the NF $\kappa$ B pathway. The

classical activation of microglia is a necessary function for the protection against any infections of the CNS from various pathogens, including viruses, bacteria, fungi, parasites, and prions (Rock et al. 2004).

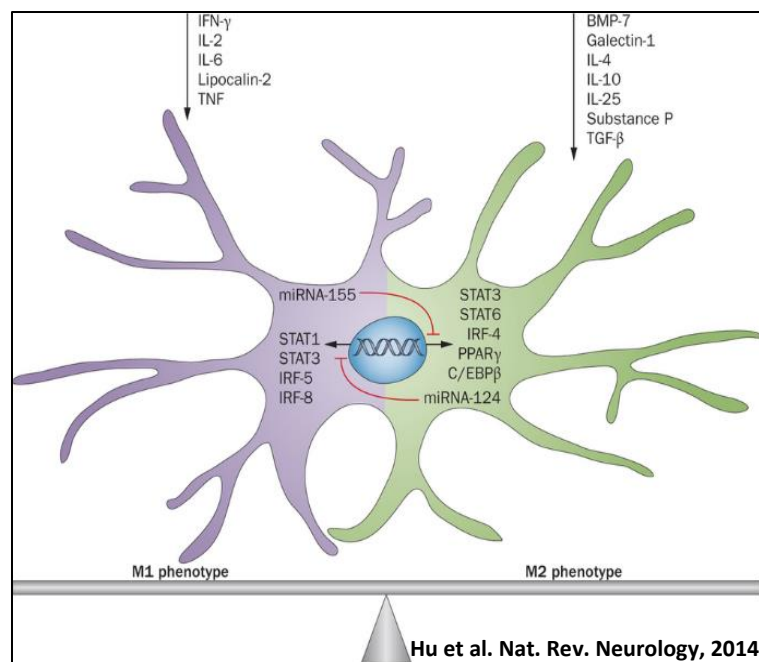
On the other hand, the M2 phenotype, or 'alternative' activation, is associated with wound healing and repair. The alternative activation phenotype has been subdivided into M2a, M2b, and M2c to differentiate between the stimulus that induced the activation and the resulting gene expression changes. The M2a phenotype is induced by the anti-inflammatory cytokines IL-4 and IL-13, and produces protective factors such as IL-10 and Arginase1. The M2b phenotype is a phenotype brought about by IL-1, LPS and certain immune complexes, and is characterized by an increase in IL-10, IL-1, TNF $\alpha$  and IL-6. This phenotype is considered to be immunity regulating. Lastly, the M2c phenotype is induced with IL-10 and TGF $\beta$  treatment, and produces high levels of these two factors. M2c is considered to be the deactivated microglial phenotype associated with tissue repair (Martinez and Gordon 2014, Cherry, Olschowka and O'Banion 2014). Other microglial phenotypes have been discovered after introducing specific stimuli, such as the Mox phenotype seen with oxidative tissue damage (Kadl et al. 2010). The response pattern common to all M2 phenotypes is high expression of the anti-inflammatory cytokine IL-10 and low expression of the pro-inflammatory cytokine IL-12. M2 phenotypes, or 'alternative' activation states, reduce inflammation and promote neuronal survival.

The redox state of microglia has been implicated to be important in determining the phenotype of the cell. Reactive oxygen species, ROS, are damaging molecules that lead to differential cell signaling in microglia. Normally, low levels of ROS are produced by microglia, but with classical activation, ROS levels increase due to a greater amount of the NADPH oxidase and nitric oxide synthase enzymes (Qin and Crews 2012). This increase in ROS acts as a second

messenger to activate downstream signaling, such as the ROS-sensitive NF $\kappa$ B pathway (Rojo et al. 2014). As stated above, most of the classical activation phenotypes of microglia are produced by activating of the NF $\kappa$ B pathway. Introduction of ROS also activates anti-oxidant pathways to counteract this toxic stimulus, and the main pathway that becomes activated is the Nrf2 transcription factor and the Nrf2 target genes. Activation of the Nrf2 transcription factor and subsequent translocation to the nucleus increases the gene expression of anti-oxidant enzymes, such as the peroxiredoxins (Bae et al. 2009, Matte et al. 2015). Interestingly, the M2 markers decrease in Nrf2 knockout mice, thus indicating that Nrf2 is an important mediator of the alternative activation of microglia (Rojo et al. 2010). Mitochondrial dysfunction has also been implicated in classical activation of microglia, with LPS treatment reduction oxidative respiration (Orihuela, McPherson and Harry 2016). This information indicates that the redox state of the cell can determine the phenotype of the microglia, with increased ROS leading to a classically activated phenotype and an increased anti-oxidant response inducing an alternative activation phenotype. Similarly, attenuating mitochondrial dysfunction could protect against the production of these inflammatory mediators.

Reactive microglia have become a hallmark of PD, and have been found to be increased in the vicinity of the few remaining nigral dopaminergic neurons (Jyothi et al. 2015). However, knowing that there are multiple activation states, these reactive microglia could be either helpful or harmful in PD. Since microglia exist as a heterogeneous population of different activation phenotypes there is a complex interplay of stimuli that contribute to the overall status of microglia in the brain. There is a need for homeostasis of the microglial phenotypes, with the availability of both classical activation and alternative activation phenotypes. Interestingly, with aging there is an increase in inflammatory activation of microglia which skews the homeostasis towards a more

damaging phenotype (Raj et al. 2014, Holtman et al. 2015). This age-induced increase in inflammatory microglial activation could explain the increased prevalence of PD with increased age. Similarly, misfolded proteins that are seen in neurodegenerative diseases such as PD can illicit an inflammatory response in microglia. Aggregated, nitrated and oxidized forms of  $\alpha$ -synuclein have been found to increase microglial inflammation (Reynolds et al. 2008, Zhang et al. 2005). Several environmental toxicants such as, the pesticides rotenone and paraquat, have been found to induce neurodegeneration through microglial ROS generation (Bonneh-Barkay et al. 2005, Gao et al. 2002). Microglia respond to various stimuli in a rapid fashion and can switch from classical activation phenotype to an alternative activation phenotype and vice versa. Since microglia can constantly switch phenotypes depending on their environment, it is thought that there is a mixture of different phenotypes throughout the brain at any one time. This would point to the hypothesis



**Figure 3. Input signals and signaling mechanisms that contribute to the shift in microglial phenotypes towards a M1 or M2 status**

that there is a continuum of microglial phenotypes instead of the basic M1/M2, or pro- and anti-inflammatory labels. This means that certain factors and cell signaling pathways that can induce microglia to shift towards a particular side of the continuum (pro- or anti-inflammatory) but that distinct polarizations probably do not occur. See figure 3 for an overview of the input signals and

signaling mechanisms that go into the shift of microglia activation down both sides of the continuum of activation. Understanding the molecular mechanisms behind microglial activation and factors that can induce specific activation phenotypes would be beneficial in finding a therapy for PD.

### **Astrocyte role in PD**

Astrocytes, or astroglia, are found throughout the brain and are the largest population of glial cells in the CNS. The exact ratio of neurons to glial cells throughout the brain is not known, however some reports have estimated that there could be up to 10-17 times as many glial cells than there are neurons in certain areas of the brain (Pakkenberg and Gundersen 1988). However, more recent evidence indicates that the neuron to glia ratio is closer to being 1:1 in the human brain (Herculano-Houzel 2009). Since microglia are only considered to be around 10-15% of the total cells in the brain, depending on the region, the majority of the glial cells in the brain would most likely be astrocytes (Carson 2002). This would indicate that astrocytes play an important role in the CNS and could be a powerful therapeutic option for neurodegenerative disease such as Parkinson's disease.

There are three known types of mature astrocytes, with diverse phenotypes and tissue localization. Two of the three forms are the protoplasmic astrocytes that are found in the grey matter, and the fibrous astrocytes found in the white matter of the brain. These cells are characterized by the spherical bushy morphology in the grey matter, and the process-bearing less bushy morphology in the white matter. The third type of astrocytes is the Bergmann glia of the cerebellum and the Muller cells of the retina, characterized by their elongated morphology (Kimelberg and Nedergaard 2010). There are several astrocyte-specific markers that identify these cells from other cell types in the brain. The most commonly used astrocyte markers currently are

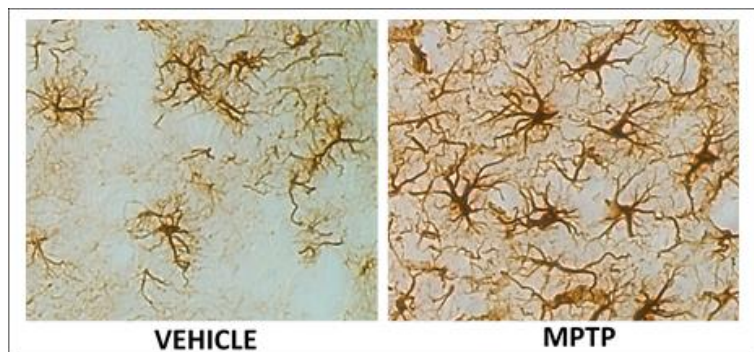
glial fibrillary acidic protein, GFAP, and the glutamate transporters GLT-1/EAAT2 and GLAST/EAAT1. Some of the newer markers include Aquaporin 4 and aldehyde dehydrogenase 1 family member 1 (Aldh1L1) (Kimelberg 2004, Lovatt et al. 2007). All cells that robustly produce the GFAP protein are astrocytes, however, an astrocyte can be GFAP-negative and are defined as astrocytes by the expression of other proteins and physiological properties (Kimelberg 2004). The exact function of the GFAP protein in astrocytes is poorly understood, however, it is thought to promote mechanical strength and help maintain the shape of the cell (Cullen, Simon and LaPlaca 2007). GFAP knockout studies have been immensely helpful in determining the functions of this protein in astrocytes. Liedtke et al. demonstrated that GFAP knockout led to abnormal myelination, deterioration in white matter structure, and impairment of the function and structure of the blood brain barrier (Liedtke et al. 1996). Along the same lines, the average process length of astrocytes lacking GFAP expression were found to be significantly smaller when cultured with neurons, compared to astrocytes normally expressing GFAP (Weinstein, Shelanski and Liem 1991). Therefore, GFAP helps produce the astrocyte processes that could be used to contact neurons. Interestingly, there is increased GFAP expression after any degenerative injury or insult including neurodegenerative diseases such as Alzheimer's and Parkinson's disease. The reason for this increased GFAP is still not fully understood, however the second messenger nitric oxide (NO) has been implicated in increasing GFAP expression in astrocytes (Brahmachari, Fung and Pahan 2006).

Glia, which is from Greek for glue, no longer seems to be apt name for these cells. Astrocytes work very closely with neurons with intricate neuron-glia crosstalk. Astrocytes support neurons through multiple processes, including antioxidant protection, glutamate protection, maintenance of the blood brain barrier, and the production of neurotrophic factors and cytokines

(Volterra and Meldolesi 2005, Hamby and Sofroniew 2010, Barreto et al. 2011). In the healthy CNS, astrocytes are arranged in non-overlapping domains that potentially contact around 160,000 synapses. This allows the astrocytes to therefore control both neural activity and cerebral blood flow by enwrapping the neuronal synapses along with contacting the brain vasculature, and (Maragakis and Rothstein 2006, Zonta et al. 2003). The equal spacing of astrocytes in the healthy CNS is thought to allow these cells to perform their important support functions of potassium buffering (Leis, Bekar and Walz 2005), controlling extracellular pH (Deitmer and Rose 2010), glutamate and GABA uptake (Sattler and Rothstein 2006), and antioxidant functions (Aschner 2000) to protect the neurons throughout the brain. Cui et al. found that inducible ablation of astrocytes in adult mice led to significant neurodegeneration, demonstrating the importance of these cells on neuronal survival (Cui et al. 2001).

### **Astrocyte activation**

Reactive astrogliosis is the mechanism by which astrocytes respond to injury or toxic stimuli, such as infection,  $\alpha$ -synuclein accumulation, ischemia, and neurodegenerative disease such as Parkinson's disease. Astrocyte activation is a constitutive, graded, multi-stage, and evolutionary conserved reaction of astrocytes. This activation of astrocytes is characterized by both molecular and morphological changes, and is a hallmark of Parkinson's disease along with microgliosis, or activated microglia. The morphological changes mainly include hypertrophy of the cell body



**Figure 4. DAB immunostaining images of GFAP-positive cells in the mouse striatum of either vehicle or MPTP treated mice**



and major process branches, and reduction in the number of primary branches (Figure 4). The expression of several proteins increase with astrocyte activation, including GFAP, vimentin and nestin. The increase in GFAP expression leads to the assumption that there is an increase in astrocyte proliferation, however that is not always the case since most astrocytes only express GFAP at low levels under basal conditions. Astrocyte activation is much more complex than originally thought, and researchers are now realizing that astrocyte activation is a gradient process that is not as uniform as previously described. Understanding the mechanism behind astrocyte activation and the effects of this activation could be a key piece to the puzzle of solving neurodegenerative diseases such as Parkinson's disease.

Previously, it was thought that microglia were the only immune cells in the CNS. However, it has recently been determined that astrocytes are also immunocompetent cells, and express the MHCII protein which is crucial for antigen presentation. Similar to microglia, astrocytes activated by an inflammatory stimulus can also produce higher levels of pro-inflammatory cytokines, such as IL-1 $\beta$  and TNF- $\alpha$ , along with increased production of reactive oxygen species (ROS) (Hirsch et al. 2003). Animal models of PD have demonstrated that astrocyte activation is increased, along with an increase in inflammatory cytokines. MPTP, a selective dopaminergic neuronal toxicant, increased astrocyte activation most likely through neuronal damage (Yasuda et al. 2008). Similarly, lipopolysaccharide (LPS) is a bacterial endotoxin that has been found to be a potent activator of microglia and induces the production of inflammatory factors. LPS treatment both in cell culture and in animal models demonstrated the ability to activate astrocytes and cause them to produce inflammatory factors such as nitric oxide (Sola et al. 2002). However, evidence indicates that the increased inflammation from astrocyte cultures due to LPS treatment could be a secondary effect from contaminating microglia (Saura 2007, Sola et al. 2002). Along the same lines,

Tarassishin et al. demonstrated that human astrocytes do not respond to LPS, but strongly respond to the pro-inflammatory cytokine IL-1 $\beta$  (Tarassishin, Suh and Lee 2014). As it relates to Parkinson's disease, excessive uptake of aggregated  $\alpha$ -synuclein by astrocytes can induce the upregulation of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and TNF $\alpha$  (Rappold and Tieu 2010). The cell signaling responsible for the activation of astrocytes in neurodegenerative disease has yet to be uncovered, however the JAK-STAT pathway has been implicated. Interestingly, MPTP-induced activation of the JAK2-STAT3 in mice preceded the increase of GFAP in astrocytes. Similarly, LPS injection induced a similar activation of STAT3 in a mouse model. Both neuronal damage and inflammatory treatment induced this STAT3 activation, indicating that the JAK2-STAT3 pathway is important for astrocyte activation in neurodegenerative diseases (O'Callaghan et al. 2014). Therefore, blocking astrocyte activation under certain conditions could be beneficial by reducing the astrocyte-produced inflammation.

On the other hand, several studies demonstrated that activated astrocytes increase neuroprotection by the increased uptake of glutamate, production of reduced glutathione (GSH), production of superoxide dismutases (SODs), release of adenosine, and formation of glial scars. The production of GSH is important in the protection of neurons because it can prevent the nitric oxide (NO)-induced inhibition of the mitochondrial complex I and therefore reduce the production of reactive oxygen species (ROS) (Dias, Junn and Mouradian 2013a). Increasing the glutathione peroxidase-containing cells could reduce the severity of neurodegeneration in cell culture from PD patients, indicating that production of glutathione could be a crucial protective factor against ROS produced in PD patients (Zeevalk, Razmpour and Bernard 2008). The transcription factor Nrf2 has been demonstrated to increase the production of GSH in astrocytes (Steele et al. 2013). Nrf2 activation leads to the expression of several cytoprotective genes that reduce ROS levels and

directly protect neurons, including the peroxiredoxins (Dowell and Johnson 2013). Interestingly, astrocyte-specific Nrf2 overexpression was able to protect against  $\alpha$ -synuclein-induced neurodegeneration through the increase in autophagy-lysosome degradation of the synuclein protein (Gan et al. 2012). Other protective factors, such as several astrocyte-produced chaperones have been implicated in neuroprotection. DJ-1 is an anti-apoptotic protein that serves as a redox-sensitive chaperone, and is increased in reactive astrocytes (Mullett et al. 2013). Along with another chaperone Hsp70, DJ-1 can lead to increased degradation of  $\alpha$ -synuclein aggregates (Batelli et al. 2008). Several astrocyte-derived neurotrophic factors have been found to protect dopaminergic neurons from cell death. For instance, glial-cell derived neurotrophic factor (GDNF) is secreted by astrocytes and is essential in dopaminergic neuronal survival (Arenas 1996). Implantation of GDNF overexpressing astrocytes into the Substantia Nigra of rats was able to significantly protect against 6-OHDA induced neurodegeneration (Ericson, Georgievska and Lundberg 2005). Similarly, overexpression of brain-derived neurotrophic factor (BDNF) under the control of the GFAP promoter led to significant neuroprotection in a Huntington's disease animal model (Giralt et al. 2010). Finding signaling pathways that can increase neuroprotective mechanisms in astrocytes is an important therapeutic approach for neurodegenerative diseases. Calcium is another important signaling mechanism for astrocytes, and it is proposed that calcium signaling regulates many of the astrocytic functions. Calcium signaling through the Inositol 1,4,5-triphosphate (IP<sub>3</sub>) was found to be required in astrocyte activation and neuroprotection after brain injury (Kanemaru et al. 2013). Astrocytes have demonstrated the ability to be neuroprotective, so directing that neuroprotection could be a powerful approach to protecting against neurodegenerative diseases such as Parkinson's disease.

Astrocyte activation is still poorly understood, and can be both protective and harmful to neurons. Interestingly, only protoplasmic astrocytes, and not fibrous astrocytes, have been found to contain an increase in aggregated  $\alpha$ -synuclein (Halliday and Stevens 2011, Braak et al. 2006). This would indicate that the heterogeneous population of astrocytes can respond differently to certain signals. Similarly, a comparison in astrocyte gene expression in either an ischemic stroke model or endotoxin inflammatory model demonstrated that at least 50% of the injury-altered gene expression was disease specific (Zamanian et al. 2012). Therefore, it stands to reason that astrocyte activation is not a uniform mechanism, but dependent on factors such as area of the brain and activation stimuli (Rappold and Tieu 2010). This was confirmed by Jang et al. by treating primary astrocyte cultures with either the bacterial endotoxin lipopolysaccharide (LPS) along with the inflammatory cytokine IFN $\gamma$ , or the anti-inflammatory cytokine IL-4. The LPS co-treated with IFN $\gamma$  in these primary astrocytes led to an increase in the gene expression of pro-inflammatory factors such as iNOS, IL-1 $\beta$ , and TNF $\alpha$ . On the other hand, the IL-4 treatment induced the gene expression of protective factors such as IL-10 and Arginase1. Activation of STAT6 by the IL-4 treatment is implicated in the shift of astrocyte phenotype towards a more anti-inflammatory and protective function, similar to that which is seen in microglial alternative activation (M2) (Jang et al. 2013b). The differential activation of astrocytes could be a key therapeutic option in neurodegenerative diseases such as AD and PD, where there is an increase in inflammation and the need for more neuroprotective factors.

### **Neurotrophic factors in PD**

One therapeutic avenue for research against PD is to find a protein or chemical that can protect the neurons from cell death. A family of biomolecules known to support the growth,

survival, and differentiation of developing and mature neurons are called neurotrophic factors (NTFs). These biomolecules are mainly proteins, with the one exception being lactacystin which is produced from D-glucose (Corey and Li 1999). The implications of finding a neurotrophic compound would be immensely helpful in any neurodegenerative diseases and could have many other uses in development and maintenance of neurons throughout the body. Interestingly, lactacystin was originally found by screening thousands of culture samples to find something that could induce neuroblastoma differentiation in N2a cells (Omura et al. 1991). There are over 30 proteins that are considered neurotrophic factors, and there are still new NTF proteins that are being discovered. These proteins are currently divided into 6 subfamilies depending on the receptors that they signal through or the overall structure of the protein. The major families of neurotrophic factors that have been studied in neurodegenerative diseases are the neurotrophins and glial cell-line derived neurotrophic factor (GDNF) family, along with the recently discovered cerebral dopaminergic neurotrophic factor (CDNF)/mesencephalic astrocyte-derived neurotrophic factor (MANF) family.

The GDNF family of proteins all have seven conserved cysteine residues that are in the same spacing as member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily of proteins. Although the sequence homology between the GDNF family members and the other TGF- $\beta$  family members is only about 20%, they all share a conformational similarity and are therefore grouped into the cysteine-knot family (Eigenbrot and Gerber 1997). However, the conformational similarity does not extend to functional similarity because the GDNF family signals through a receptor tyrosine kinase (Ret), whereas the other members of the TGF- $\beta$  superfamily signal through receptor serine/threonine kinases (Trupp et al. 1996). There are currently four members of the GDNF family of neurotrophic factors, Glial-cell line derived neurotrophic factor (GDNF),

Artemin, Neurturin and Persephin. These four proteins share between 40-50% homology. The GDNF family of NTFs was first demonstrated to be a potent dopaminergic neuroprotective factor in 1993 when GDNF was able to protect cultured ventral midbrain dopaminergic neurons (Lin et al. 1993). GDNF was also able to promote the survival and cholinergic differentiation of cultured motor neuron, and could therefore be a potential treatment for Amyotrophic sclerosis (ALS) (Henderson et al. 1994, Zurn et al. 1994). GDNF mRNA has been demonstrated to be expressed in multiple regions in the developing and mature brain, including in the striatum and substantia nigra of the mature rat brain (Pochon et al. 1997, Koo and Choi 2001). Interestingly, GDNF is depleted in the neurons and neuropil of the substantia nigra in PD patients compared to age-matched controls, whereas other neurotrophic factors such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) were not decreased nearly as much as GDNF nor as consistently (Chauhan, Siegel and Lee 2001). This would indicate that GDNF is an important factor in the protection of dopaminergic neurons and the reduction in availability of this protein could lead to the progression of PD. Multiple reports have found that nervous tissue up-regulates protective mechanisms to repair damage from various injury (Benn and Woolf 2004). Where normally it is the neurons that produce GDNF for the adult brain, during injury the neurons have reduced GDNF production and glial cells become the predominant source of GDNF (Pochon et al. 1997, Blum and Weickert 1995, Bresjanac and Antauer 2000). Interestingly, Saavedra et al found that mild damage to dopaminergic neurons in neuron-glia co-cultures induced up-regulation of GDNF production in astrocytes, whereas extensive damage did not (Saavedra et al. 2006). The authors went on to propose that GDNF induction in astrocytes requires signaling from injured dopaminergic neurons. This would imply that there is a neuronal factor that is produced with mild damage that can induce astrocytes to produce more GDNF, but these factors are currently

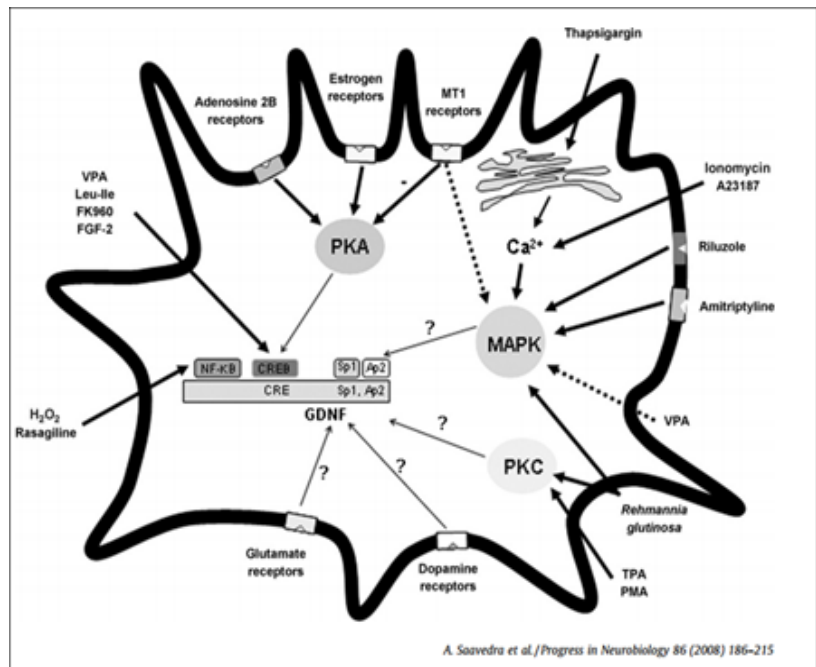
unknown. Finding a signaling molecule that is upregulated in neuronal injury that can signal to astrocytes and increase GDNF would be a promising therapeutic option.

One of the disadvantages of using GDNF as a therapeutic treatment for PD is that it does not cross the blood brain barrier and therefore has to be introduced directly into the brain. Multiple groups have demonstrated that infusion of GDNF protein directly into the brain is neuroprotective and could lead to function improvement in rodent and non-human primate models of PD (Grondin et al. 2002, Tomac et al. 1995, Gash et al. 1996, Bjorklund et al. 2000, Kordower et al. 2000). Two scientific trials showed positive effects in PD patients by direct infusion of GDNF protein into the brain, however, a placebo-controlled study did not show any improvements (Gill et al. 2003, Slevin et al. 2005, Lang et al. 2006b). However, rapid metabolism of this protein would lead to a PD patient needing constant delivery of the GDNF protein, which is not ideal when it has to be delivered directly to the brain. Also, it was found that the patients were developing antibodies directed to the foreign GDNF protein. Development of gene therapy techniques allowed researchers to get around this obstacle by being able to induce overexpression of GDNF with a one-time injection. These methods used viral delivery, with either recombinant Lentiviruses (LVs) or adeno associated viruses (AAVs), to incorporate the GDNF overexpression genes in the nigro-striatal pathway for long-term production of the GDNF protein. Initial studies using both LV-GDNF and AAV-GDNF injections into either the striatum or substantia nigra demonstrated there was effective GDNF delivery for over 3-6 months. These same studies found that both viral vector injections could promote regeneration and functional recovery in both 6-OHDA treated rats and MPTP treated monkeys (Bjorklund et al. 2000). Multiple clinical trials for both direct GDNF protein infusion and AAV-GDNF viral vectors with convection enhanced delivery (CED) are currently underway, with a phase II clinical trial in the UK started in the 2015 (Sullivan and

O’Keeffe 2016). These results demonstrate that prolonged delivery of GDNF can protect dopaminergic neurons in animal models of PD and some human trials have shown improvements as well.

Finding a molecule that increases endogenous GDNF production has several benefits and is a plausible way to get increased GDNF levels to affected areas of the brain. Multiple studies have looked into increasing the endogenous GDNF levels to varying degrees. The pathways

involved in activation of the GDNF gene expression are becoming more clear, however, finding specific transcription factors to drive GDNF production is still underway. Activation of the protein kinase A (PKA), protein kinase C (PKC), and mitogen activated protein kinase (MAPK) pathways have all



**Figure 5. Cell signaling pathways and transcription factors involved in the gene expression of GDNF**

been found to increase GDNF expression in particular cell types (Saavedra, Baltazar and Duarte 2008). Similarly, the transcription factors NFκB, Sp1, Ap2, and cAMP response element-binding protein (CREB) can all bind to the GDNF promoter and have been implicated in the gene expression of GDNF. Activation of multiple receptors, such as the glutamate, dopamine, adenosine 2B, estrogen, and MT1 receptors have also been identified to modulate GDNF gene expression (Ho et al. 1995, D’Astous et al. 2004). Figure 5 diagrams the cellular signaling pathways and



transcription factors that have been associated with GDNF gene expression. Several factors that have been found to increase GDNF expression, such as riluzole (a sodium channel blocker), amitriptyline (an anti-depressant drug), and melatonin, activate the MAPK pathway and then increase downstream transcription factors Sp1 and AP-2 (Caumont, Octave and Hermans 2006, Hisaoka et al. 2001, Armstrong and Niles 2002). Interestingly, activation of the transcription factor NF $\kappa$ B, which is normally seen to increase inflammatory factors, by the reactive oxygen species hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) also binds to the GDNF promoter and increased gene expression (Saavedra et al. 2006). The cell signaling involved in the expression of GDNF is still being investigated, however, several well-known signaling pathways lead to increased gene expression and therefore could be used as therapeutic targets to increase endogenous levels of GDNF. Finding a factor that can activate specific pathways or transcription factors to increase endogenous GDNF levels could be an immensely powerful tool as a therapeutic option in neurodegenerative diseases such as Parkinson's disease.

Although direct GDNF infusion and GDNF overexpression has demonstrated positive improvements in both animal models and human PD patients, there are still concerns with GDNF treatments. The GDNF-induced cell signaling through the Ret receptor that leads to neuroprotection still remains unclear. It has been shown that GDNF can lead to activation of the MAPK and PIP<sub>3</sub>/Akt pathway, but nothing definitive has been found in animal models of PD (Li et al. 2013, Nicole et al. 2001). The use of NTF therapies has an advantage over current PD treatments, since these proteins can protect and possibly regenerate the dopaminergic neurons in the SN compared to the current drugs only treating the symptoms of the disease. However, results indicate that neurotrophic factors alone are not able to fully protect the dopaminergic neurons in

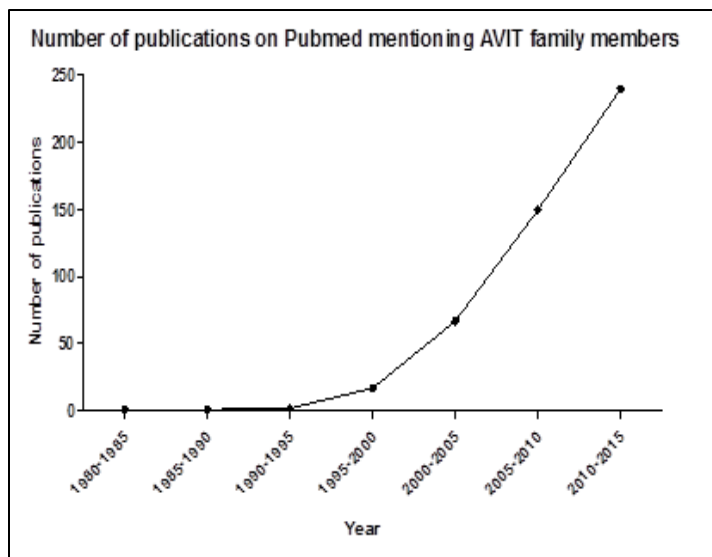
some animal models of PD, such as  $\alpha$ -synuclein models (Decressac et al. 2011), and PD patients. Therefore, it would be beneficial to find a factor that can work with GDNF and improve neuroprotection.

## Background and Literature Review II

### **AVIT proteins**

A non-toxic protein in Mamba snake venom was isolated in 1990 and was titled protein A (Schweitz, Bidard and Lazdunski 1990). This ambiguous title was later changed to Mamba Intestinal Toxin-1 (MIT-1) due to the ability of this protein to potently contract gastrointestinal muscles (Schweitz et al. 1999). Subsequently, proteins homologous to MIT-1 were found in other species, such as frogs and mammals. The frog orthologues were termed Bv8 and Bm8 due to the species it was found in, *Bombina variegata* and *Bombina maxima* respectively (Ferrara et al. 2004). The human orthologues were originally isolated in 2001 by Zhou and colleagues and were termed Prokineticin-1 (PK1) and Prokineticin-2 (PK2) for their ability to contract gastrointestinal muscles (Li et al. 2001). PK1 was then isolated by a different method and also termed Endocrine Gland-Vascular Endothelial Growth Factor (EG-VEGF) (LeCouter et al. 2001). These homologous proteins all share the same amino terminal amino acid signaling sequence of AVITGA. Therefore, this family of proteins became known as the AVIT family (Kaser et al. 2003). Substitution of any one of these amino acids will block any receptor signaling from these proteins (Bullock, Li and Zhou 2004). Along with the AVIT signaling sequence, the members of the AVIT family all contain about 80-90 amino acids and 10 cysteine residues with identical spacing. These 10 cysteine residues form 5 disulfide bonds, and disruption of any of the disulfide bonds prevents any

downstream signaling. Research into the AVIT family has steadily increased over the years (Figure 6), and the diverse biological functions of these proteins continues to expand. For instance, researchers have found a crustacean Prokineticin-like protein, called Astakine, that regulates



**Figure 6. Number of publications that mention AVIT family members on Pubmed**

hematopoietic stem cell proliferation and differentiation, and circadian rhythms in the invertebrate crustaceans. Interestingly, Astakine does not contain the AVIT signal sequence and has demonstrated the ability to bind to different receptors. These results implicate an evolutionary conserved function for the prokineticin domain containing proteins (Wathanasurorot et al. 2011). There is a total of 478 manuscripts on PubMed that mention AVIT family of proteins up to the year 2015. As expected, there is a significant increase in publications starting around 2000, when the human homologues Prokineticin-1 and Prokineticin-2 were isolated. However, the number of publications have continued to increase over every five-year span. This indicates that the AVIT family of proteins is becoming more important as more diverse biological functions are found every year.

## Prokineticins

Prokineticins are human proteins that obtained their name because they were first discovered to contract smooth muscle in the gut (Li et al. 2001). The two prokineticin proteins, Prokineticin-1 (PK1) and Prokineticin-2 (PK2), are members of the AVIT protein family due to

the presence of the particular amino acid signaling sequence AVITGA on the N-terminus and contain 86 and 81 amino acids, respectively. Both PK1 and PK2 share around 45% identity in their amino acid sequences and are expressed on different chromosomes. In humans, the PK1 gene is located on chromosome 1p21 while PK2 maps to 3p21.1. PK2 can also be alternatively spliced with an isoform containing 21 additional amino acids known as Prokineticin-2 long form, or PK2L, and is cleaved to create an active form, PK2 $\beta$  (Chen et al. 2005). PK2 has many physiological functions throughout the human body. For instance, prokineticin signaling modulates the physiological functions the reproductive organs including roles in the ovary, uterus, placenta, and testis. Dysfunction in prokineticin signaling can lead to reproductive pathological conditions such as recurrent pregnancy loss, preeclampsia, and male infertility (Traboulsi et al. 2015).

The prokineticins regulate physiological functions in various tissues including gut motility, circadian rhythms, angiogenesis, pain perception, hematopoiesis, energy expenditure and olfactory bulb biogenesis. Studies also suggests that prokineticins are linked to pathologies myocardial infraction, immune regulation and tissue-specific cancer initiation and propagation. Recently, the prokineticins and their receptors were found increased in the lungs of cystic fibrosis patients compared to control (Chauvet et al. 2015). Prokineticin signaling has been implicated in the induction of cancer cell proliferation and survival through STAT3 signaling (Xin et al. 2013). Interestingly, PKR2 expression could be a used as a prognostic tool for colorectal cancer, with human patients expressing PKR2 having a significantly lower 5-year survival rate (Goi et al. 2015). Prokineticin signaling has been implicated to be an important factor in the development of several organs in the body, such as the reproductive organs, pituitary gland, placenta, and gastrointestinal tract. It is a signaling protein that has several characteristics of a pleiotropic cytokine or a chemokine. PK2 can promote cell survival and growth (LeCouter et al. 2004a), and

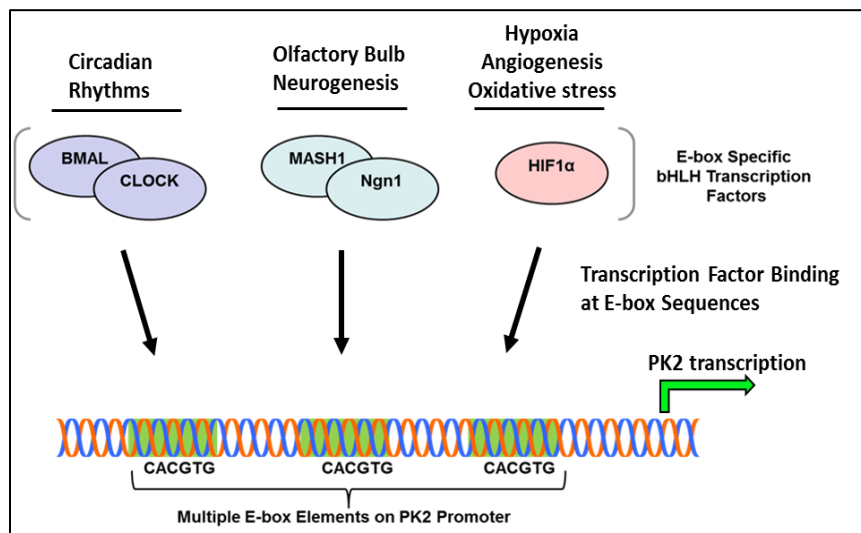
in other tissues PK2 can cause an increase in inflammation and nociception (Negri et al. 2006b). Genetic studies looking into nociception showed that knockout of PK2 or PKR1 caused significant reduction of nociception (Negri et al. 2006a). Since these proteins potently contracted gut muscles they named them prokineticins (Li et al. 2001), and studies looked into function ex vivo (Schweitz et al. 1999) and in vivo (Wade et al. 2010). However, there are conflicting results that showed adding PK2 protein to gut muscles does not induce contraction, but could cause contraction through nerves from the brain (Bassil et al. 2005). Interestingly, Kallmann syndrome, in humans, has been linked to mutations in PK2 or PKR2 (Pitteloud et al. 2007a). Also, some studies have looked into a genetic knockout of one Prokineticin receptor (PKR1) and found heart and kidney defects, possibly due to mitochondrial defects (Boulberdaa, Urayama and Nebigil 2011b). Many research studies have demonstrated important roles for the prokineticins in the course of pregnancy and placental development, with modulation of disorders of both the female and male reproductive system (Alfaidy et al. 2014, Su et al. 2014, Karaer et al. 2014, Traboulsi et al. 2015). Along the same lines, PK1/EG-VEGF was shown to be an important factor produced in Leydig cells and can regulate proliferation of vascular endothelial cells in the testis (LeCouter et al. 2003a, Beltrame, Cerri and Sasso-Cerri 2015). Recently, prokineticins were found to increase insulin sensitivity and reduce adipose tissue expansion, which go along with previous reports that prokineticin signaling to the Hypothalamus acts as an anorectic peptide (Von Hunolstein and Nebigil 2015, Beale et al. 2013). Prokineticin signaling is important for many different functions throughout the body (Scheme 1) and elucidating the signaling mechanism in different areas of the body, and disease conditions, is important in understanding the significance of PK2 expression in different diseases.

## Prokineticin structure

The prokineticin gene and promoter structures are highly conserved in mammals such as humans and mice, indicative of their functional roles in important physiological processes. Mammalian prokineticin genes contain four exons with the third exon being a target for alternative splicing in Prokineticin-2. The prokineticin protein contains a 19-residue signal peptide that targets the proteins for constitutive secretion within the first exon. Six of the structurally important cysteine residues are coded in Exon-2 and Exon-4 encodes for the other four of the ten conserved cysteines. An alternative splice form of prokineticin-2 has been identified, which contains a 63 bp insertion between exon 2 and exon 4 resulting in a longer isoform of PK2 (PK2L) that has an additional 21 amino-acid residues (Kaser et al. 2003, Chen et al. 2005). In the mature PK2L isoform, the 21 lysine and arginine rich amino acid insertion occurs between Lys47 and Val48. Exon-3 insertion contains two putative furin protease cleavage sites. *In vitro* results from co-expression of furin with PK2L demonstrated the production of a shorter cleaved form of the protein (PK2 $\beta$ ) that could still functionally activate prokineticin receptors (Chen et al. 2005). Interestingly, the shorter PK2 $\beta$  fragment showed greater selectivity for PKR1 than PKR2 whereas both PK1 and PK2 show no significant selectivity for either prokineticin receptor. Although some evidence indicates that PK2L is differentially expressed from PK2 in certain tissues, such as the lung, the specific function of this protein is still relatively unknown (Chen et al. 2005). Currently there have not been any alternative splice forms of PK1 detected. The pI of PK2 was determined to be around 8.85 while that of PK2L was 10.68 due to the lysine and arginine rich exon-3 insertion indicating that these proteins are highly basic proteins. PK1 (EG-VEGF) has demonstrated the ability to bind to heparin-sepharose with high affinity and it has been suggested that their bioavailability and

activity may be further regulated by interactions with components of the extracellular matrix due to the highly basic nature of these secreted proteins (LeCouter et al. 2003a).

The transcriptional regulatory elements for PK2 are highly conserved between the mouse and human promoters and have been characterized in detail (LeCouter et al. 2003a, Zhang et al. 2007b). Several transcription factors have demonstrated the ability to regulate the expression of PK2, including CLOCK, BMAL1, Ngn1, MASH1 and HIF1 $\alpha$ . These transcription factors are all in the basic Helix-Loop-Helix (bHLH) family and bind to the specific E-box sequence (CACGTG). The PK2 promoter sequence contains multiple E-box elements in both the mouse and human promoters (Cheng et al. 2002, Zhang et al. 2007b). The CLOCK and BMAL transcription factors have been found to be responsible for the expression of PK2 in the suprachiasmatic nucleus



**Figure 7. Transcriptional regulation of PK2 by basic Helix Loop Helix (bHLH) transcription factors**

(Cheng et al. 2005b, Cheng et al. 2002, Watanabe et al. 2007). Whereas, PK2 expression is regulated by Neurogenin 1 (Ngn1) and MASH1 during olfactory bulb biogenesis. Ngn1 and MASH1 mutant mice also showed significantly reduced PK2 expression (Zhang et al. 2007b). PK2 was shown to be highly induced by hypoxia both in vivo and in cell cultures (LeCouter et al. 2003a, Shojaei et al. 2007). The PK2 promoter contains two putative HIF1 $\alpha$  binding sites. These HIF1 $\alpha$  binding sites are less than 300 nucleotides upstream of the transcriptional start site at -115 and -248. Therefore, PK2 gene expression is

regulated through intricate mechanisms involving several bHLH transcription factors in distinct biological processes. Figure 7 details the functions and bHLH transcription factors involved in PK2 transcription. However, STAT3 was demonstrated to be required for PK2 expression in mouse bone marrow cells, so there are other transcription factors that can control PK2 expression systemically and possibly in the CNS (Qu et al. 2012). Interestingly, PK2 and STAT3 form a feed-forward loop where STAT3 can increase PK2 expression, and PK2 can activate the STAT3 pathway and lead to increased tumor cell proliferation and survival (Xin et al. 2013). PPAR $\gamma$  is another transcription factor recently found to increase PK1 protein expression. Garnier et al found that PK1 expression in placental development was at least partly controlled by PPAR $\gamma$ , and that PPAR $\gamma$  knockout mice produced significantly less PK1 (Garnier et al. 2015).

The protein structure of the prokineticins is a result of the highly conserved cysteine residues that form five disulfide bridges. The N-terminal and C-terminal ends are exposed at the surface of the molecule and are believed to participate in receptor binding and activation. The folded protein is believed to have an approximately ellipsoid shape with a net positive charge on one side and a hydrophobic face on the other. The core of the protein contains many charged amino acids, whereas some of the hydrophobic residues are exposed on the surface of the protein. A potential signaling mechanism emerged from the detailed NMR structure of the amphibian prokineticin homolog where they identified two  $\beta$ -hairpins at the C and N terminus of the protein and a single turn of  $3_{10}$  helix at the N terminus (Kaser et al. 2003, Morales et al. 2010).

Receptor binding and downstream signaling is dependent on the first six N-terminal amino acids (AVITGA) of both prokineticins and are completely conserved in all of their invertebrate homologs. Addition or substitution mutations in any of the six amino acids results in an inactive protein. However, synthetic peptides of the first 6 or 12 amino acid residues alone do not have any



significant functional activity even at high doses, indicating that the N-terminal AVITG segment alone appears to be insufficient for activity (Bullock et al. 2004). Deletion of the AVITG region did not induce a structural change in the Bv8 homologue but resulted in a complete loss of activity. Further, the addition of synthetic AVITG-OH or AVITG-NH<sub>2</sub> peptides in solution could not compensate for the loss of activity in the N-terminal truncated AVIT-mutant. Peptides lacking the AVITG motif did not antagonize any functional activity of the native protein and demonstrated that this sequence is most likely required for both receptor binding and activation (Morales et al. 2010). Studies have also looked into mutating the cysteine residues and C-terminal amino acids. These studies demonstrated that substituting any of the cysteine residues that it would abolish receptor activation, whereas domain swapping of PK1 and PK2 found that the sequences in the C-terminus other than the cysteine residues could be variable and still lead to functional signaling (Bullock et al. 2004). Bullock et al. went on to perform intricate studies using prokineticins with N-terminal AVITG substitution mutants and found that specific mutations at the N-terminus can result in mutant proteins with antagonist activity. The activation of PKR1 and PKR2 by both PK1 and PK2 was antagonized by mutant proteins with a substitution of the N-terminal alanine with methionine (A1MPK1) or the addition of a methionine at the N-terminus (MetPK1). Both *in vitro* cell culture assays and *in vivo* experiments demonstrated that the peptide antagonists could be functional antagonists (Cheng et al. 2002, Cottrell, Zhou and Ferguson 2004, Ng et al. 2005). More research into the protein structure of the Prokineticins could lead to better understanding of the receptor signaling and possibly lead to more directed therapeutic approaches.

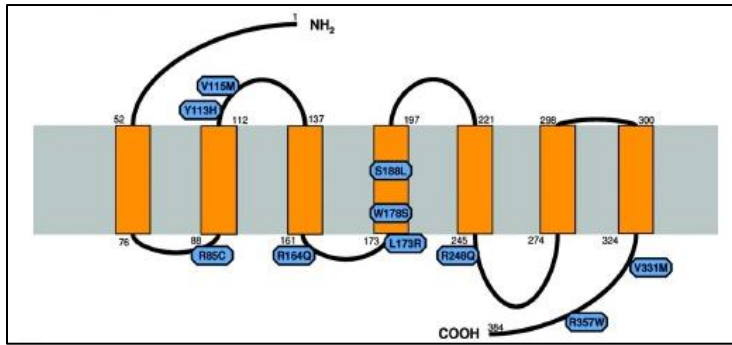
### **Prokineticin receptors**

It is thought that PK2 has such a diverse range of functions due to the fact that it has two known receptors that it can signal through. However, prokineticin signaling is much more complex

than just the existence of two receptors. The prokineticin receptors, PKR1 and PKR2, are G-protein coupled receptors that can both bind PK2 and have the potential to signal through different cascades (Zhou 2006). The two prokineticin receptors belong to the Neuropeptide Y family and have an unusually high degree of similarity (~85% amino acid identity) with most of the variation around the N-terminal region. Human PKR1 is located on chromosome 2q14 and PKR2 on 20p13. Studies have shown mRNA expression of both receptors have been found in several areas of the body, including gastrointestinal organs (Lin et al. 2002b), ovaries, testes, adrenal glands, uterus. In the bone marrow and the blood, both PKR1 and PKR2 have been detected in mature blood cells and hematopoietic stem cells (Lin et al. 2002b, Soga et al. 2002a, LeCouter et al. 2003a, Battersby et al. 2004, Hoffmann, Feige and Alfaidy 2006, Hoffmann, Feige and Alfaidy 2007). However, the expression of PKR1 has been found more in peripheral areas such as the testis, and skin, whereas PKR2 is expressed higher in the CNS. Interestingly, Lin et al. shows that PKR1 is expressed much more than PKR2 in the gut (Lin et al. 2002b). Also, evidence points to the fact that PK2 activates the receptors at slightly different concentrations. PK2 can activate PKR1 at lower concentrations than PKR2 (Soga et al. 2002a). PK2 $\beta$  also has a unique activation concentration, with it binding to PKR1 about the same as PK1 but needs ten times the concentration of PK2 or PK1 to activate PKR2 (Chen et al. 2005). Also, some researchers (Levit et al. 2011) have theorized that the receptors can be phosphorylated on certain intracellular domains and that could lead to differential binding or other signaling factors. To complicate matters further, PKR2 has the ability to dimerize, as either a homodimer or heterodimer, and undergo domain swapping (Marsango et al. 2011). The same group went on to demonstrate that the PKR2 receptor is dimerized in neutrophils, and therefore could exist *in vivo* as a dimer (Marsango et al. 2011). Truncated versions of the PKR2 receptor were used to determine that the

transmembrane domain 5 (TM5) is important for the dimerization of the receptor and could modulate the function of the receptor (Sposini et al. 2015). Dimerized receptors have shown differential binding affinities and could activate different signaling pathways (Munoz et al.).

Multiple proteins have been identified to interact with the prokineticin receptors and modulate their activation and downstream signaling. Recently, Song et al. found that the Snapin protein can interact with the C-terminus of the PKR2 receptor. Snapin deficiency did not affect the calcium mobilization induced by the PKR2 receptor, but led to faster degradation of PKR2 after agonist stimulation. Therefore, Snapin could regulate receptor recycling or cellular trafficking of PKR2 (Song et al. 2016). PKR2 has also been found to associate with the extracellular matrix glycoprotein Anosmin 1, another protein associated with Kallmann's syndrome. Anosmin 1 directly binds to and increases Fibroblast Growth Factor receptor 1 (FGFR1) activation and leads to downstream functions such as neurite outgrowth and cytoskeletal rearrangements (Hu et al. 2004, Murcia-Belmonte, Astillero-Lopez and Esteban 2016). Melanocortin Receptor Accessory Protein 2 (MRAP2) can inhibit PKR1 activation and therefore lead to increased food intake (Chaly et al. 2016). This interaction could be an important therapeutic avenue for patients with eating disorders, with MRAP2 leading to increased food intake whereas PKR1 leads to reduced food intake. Research into other proteins that associate with the prokineticin receptors could lead to new cell signaling pathways that the prokineticins can regulate.



**Figure 8. Known mutations in the PKR2 gene that cause functional impairment and their location in the protein** Zhang et al 2009

The prokineticin receptors, especially PKR2, have been found to be mutated at several different sites (Figure 8), and that some of these mutations could lead to differential downstream signaling (Libri et al. 2014). Levit et al. suggested that Thr178 in human PKR1 and Thr169

in human PKR2 in the second intracellular loop along with Tyr365 in human PKR1 and Gln356 in human PKR2 may represent subtype-specific phosphorylation-related sites in the cytoplasmic side of the receptors by using prediction tools for phosphorylation sites (Levit et al. 2011). The intracellular loop 3 of PKR2 has also been found to impact receptor trafficking and G-protein coupling (Zhou et al. 2013). Interestingly, four different mutations in PKR2 (R85C, R85H, R164Q, V331M) were found to have defective Gq signaling, however the receptor could still recruit  $\beta$ -arrestins with PK2 treatment, which could lead to differential signaling from wild type PKR2 receptor. In particular, the R268C mutation could only signal to Gs and Gq proteins, but not the Gi protein, and could have a protective effect (Sbai et al. 2014). Over 20 mutations PKR2 have been shown to result in either human Kallmann syndrome or hypogonadotropic hypogonadism (HH), with six such mutations affecting cell trafficking of the receptor. The amino acid Arg80 on the intracellular loop of PKR2 is important for the expression of this protein, and an R80C mutation in this intracellular loop 1 of PKR2 leads to a dominant negative effect on wild-type PKR2 (Abreu et al. 2012). Interestingly, the L173R mutation in the PKR2 protein is associated with the gonadotropin releasing hormone (GnRH) deficiency in humans and is estimated to have an age of

9000 years (Avbelj Stefanija et al. 2012). However, Su et al. found that there are two genetic variants of the prokineticin receptors, I379V in PKR1 and V331M in PKR2, could reduce the possibility of recurrent miscarriage (Su et al. 2013). Similarly, the V67I variant of PKR1 could potentially downregulate PK1 expression and regulate human early pregnancy (Su et al. 2016). The differential expression of the prokineticin ligands and receptors throughout tissues in the body leads to differential cell signaling for distinct biological processes, and mutations in the prokineticin receptors have demonstrated the ability to change the signaling pathways. Prokineticin receptor mutations in humans could have diverse biological consequences in both health and disease, and require further study to investigate the functions of these mutations.

The role of each prokineticin receptor is still not fully known in most physiological processes, however overexpression and knockout studies have provided insight into the potential roles for each prokineticin receptor in certain physiological processes. Chen et al. demonstrated that a PKR2 antagonist and glycerol could induce cellular trafficking of the PKR2 receptor to the cell surface and potentially treat patients with Kallmann's syndrome with this trafficking issue (Chen et al. 2014). Along the same lines, PKR2 knockouts or null mutations could recapitulate Kallmann's syndrome and HH in mouse models (Pitteloud et al. 2007a, Abreu et al. 2008). Similarly, PKR2 knockout mice demonstrate a dysfunction in olfactory bulb development to go along with hypoplasia and severe atrophy of the reproductive organs. PK2-PKR1 signaling has been studied using PKR1 knockout mice, and found impaired pain sensitization and nociceptive responses, similar to that which is found with PK2 knockout mice (Hu et al. 2006a, Negri et al. 2006a, Giannini et al. 2009). The two Prokineticin receptors have drastically different roles in the cardiovascular system, where overexpression of PKR2 in the mouse heart induces hypertrophy and capillary vessel leakage without angiogenesis. However, the targeted overexpression of PKR1

induced neovascularization of the heart (Urayama et al. 2008). Similarly, Urayama et al. went on to demonstrate that signaling through PKR1 was able to protect cardiomyocytes from cell death, whereas signaling through PKR2 induced hypertrophy and impaired endothelial integrity (Urayama et al. 2007a, Urayama et al. 2009). An antibody directed to PK1/EG-VEGF found that reduction of PK1 signaling and demonstrated that PK1 induced angiogenesis in embryonic tissue (Feflea et al. 2012). Mitochondrial defects and increased apoptosis in both heart and kidney tissues were a result of PKR1 knockout (Boulberdaa et al. 2011a). These knockout and overexpression studies verify that differential expression and availability of the two prokineticin receptors regulate a broad range of physiological processes.

The development of chemical agonists and antagonists to both of the prokineticin receptors would elucidate many of the unknown roles prokineticins play in health and disease, and allow the characterization of signaling pathways that are controlled by either of these receptors. Chemical antagonists of the prokineticin receptors were first developed in 2008 based on triazine and pyrimidindione derivatives (Balboni et al. 2008, Giannini et al. 2009, Ralbovsky et al. 2009). The triazine-based compounds were found to be highly selective for PKR1 in functional assays with almost 70-fold less potency as a PKR2 antagonist. PC-1, the first generation of the triazine compounds, was used to inhibit PK2 signaling in the chronic constriction model, and found that PK2 increases peripheral nerve neuropathic pain (Lattanzi et al. 2015). Similarly, the next generation of triazine antagonists were tested in models of inflammatory pain and found to be highly effective as anti-hyperalgesic drugs (Giannini et al. 2009). The newest generation of triazine compounds (PC-27) actually has an IC<sub>50</sub> that is 100 times more potent than the first generation of triazine compounds (PC-1) (Congiu et al. 2014). Another antagonist was produced as a brain permeant 3-pyrrolidine carboxamide, with an IC<sub>50</sub> of 5nM and 8nM for PKR1 and PKR2,

respectively (Curtis et al. 2013). Gasser et al. then formulated a chemical non-peptide agonist to the PKR1 receptor, and found that this agonist was able to protect cardiomyocytes from cell death through the MAPK and Akt pathways (Gasser et al. 2015). Currently there are no specific PKR2 agonists, and all of the chemical antagonists inhibit both of the prokineticin receptors. Genetic knockdown of the two receptors using siRNA were useful in demonstrated that the signaling is more dominantly through PKR1 (Guilini et al. 2010). Therefore, more research into separating the functions of the two prokineticin receptors could elucidate how to control prokineticin signaling in different tissues and disease models.

### **Prokineticin signaling pathways**

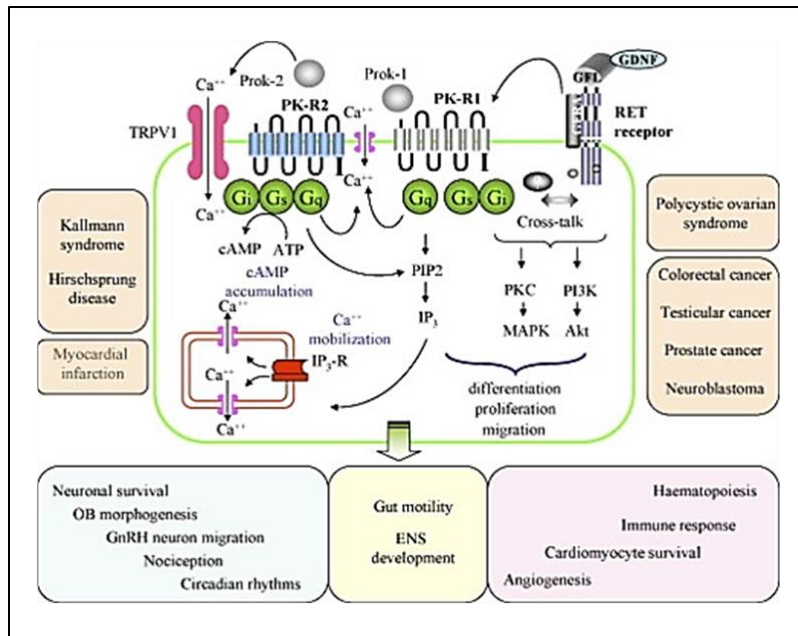
The two prokineticin receptors belong to the neuropeptide Y (NPY) receptor class of GPCRs which can promote intracellular calcium mobilization, cAMP accumulation and phosphoinositol turnover (Lin et al. 2002b, Masuda et al. 2002a). GTP $\gamma$ S displacement experiments were the first findings to demonstrate that prokineticins signaled through GPCRs (Li et al. 2001). Since the receptors are GPCRs, signaling also depends on the G-protein that is activated by the receptor. The prokineticin receptors signal through the Gi, Gq, and Gs proteins (Chen et al. 2005). The signaling pathways downstream of the receptors have begun to be elucidated in recent years and have been shown to depend on the G-protein involved. The majority of the known cell signaling pathways and functions of prokineticin signaling are shown in Figure 9. Prokineticins activate the MAP kinase pathway most likely through Gi signaling, calcium mobilization through Gq signaling, and increased Akt and cAMP levels through Gs signaling (Chen et al. 2005). Different tissues can therefore express the 3 ligands, 2 receptors, or 3 G-proteins in distinct manners along with receptor phosphorylation and therefore lead to tissue

specific functions. Specific downstream pathways activated however remain to be elucidated, particularly the transcription factors activated by prokineticin signaling to mediate their functional effects have not been defined. Both ligands activate the two receptors almost equally, therefore indicating that signaling studies can be related to both proteins. Li et al. performed the first study to characterize the prokineticins and found that recombinant PK1 and PK2, even in the nanomolar ranges, induced calcium influx into the cell. They went on to find that this calcium influx was required for prokineticin-induced contraction of the gastrointestinal smooth muscle (Li et al. 2001). Several common signaling pathways are activated by prokineticin signaling in different cell types and areas of the body.

Several studies have characterized a similar downstream pathway leading to cell survival and proliferation. Lin et al. used a treatment with Prokineticin-1 in adrenal cortex-derived endothelial (ACE) cells which led to a dose and time-dependent increase in cell proliferation and migration through the activation of p44/42 MAPK pathway. The same group went on to demonstrate that PK1 treatment also activated the Akt pathway, which is a known survival signal for these cells. The Akt activation then led to increased endothelial nitric-oxide synthase (eNOS) (Lin et al. 2002b). The Akt pathway is also activated by PK1 treatment and similarly induces proliferation and survival of neuroblastoma cells. The cell survival induced in these neuroblastoma cells by PK1 treatment was mediated by PKR2, whereas the cell proliferation needed the expression of both prokineticin receptors. Similarly, the Mitogen-activated protein kinase (MAPK) and Akt pathways were activated by PK1 treatment in enteric neural crest cells, which also led to an increase in cell proliferation and differentiation (Ngan et al. 2007). Phosphorylation of the MAPK, Akt pathways was also demonstrated with PK1 treatment in multiple myeloma cells. Li et al. found that multiple survival signaling pathways could be activated downstream of the



prokineticin receptors. PK1 signaling also caused the activation of STAT3 and upregulated Sphingosine kinase (SphK) along with the potent anti-apoptotic Bcl-2 family member Mcl-1 in myeloma cells (Li et al. 2010). Similarly, prokineticins act as autocrine mitogenic factor in



**Figure 9. Cell signaling pathways involved for prokineticin signaling through PKR1 and PKR2, and the functions/disease this signaling modulates**

endocrine endothelial cells, which was demonstrated with PK1 treatment resulting in phosphorylation and activation of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Keramidas et al. 2008). PK1 also acts as a survival factor by activating the Akt and MAPK pathways in placental cell extracts and umbilical vein-derived macrovascular endothelial cells (HUVEC) (Brouillet et al. 2010). Interestingly, PK2-PKR1 signaling activated Akt in cardiomyocytes to protect against oxidative stress and promote angiogenesis (Urayama et al. 2007a). These results were confirmed with *in vivo* studies using a selective PKR1 agonist, that demonstrated cardiomyocyte protection from a myocardial infarction through the MAPK and Akt pathways (Gasser et al. 2015).

Prokineticin treatment has been found to activate signaling pathways other than MAPK and Akt, leading to an expanded physiological role throughout the body. In endometrial epithelial Ishikawa cells, PK1 signaling went through the calcineurin/NFAT pathway to induce the expression and secretion of IL-8 and IL-11 cytokines (Maldonado-Perez et al. 2009, Cook et al.

2010). Waddell et al. found that PK1 treatment in these same cells increased CTGF, or connective tissue growth factor, via Gq, phospholipase C (PLC), cSrc, growth factor (EGFR), and the MAPK signaling pathways (Waddell et al. 2011). Nitric oxide release was increased with PK1 treatment in longitudinal muscle-myenteric plexus cultures, which then inhibits spontaneous giant contractions in the circular muscle of the mouse proximal colon (Hoogerwerf 2006). Prokineticin-1 signaling is important for normal angiogenesis of the placenta and could modulate placental pathologies (Alfaidy et al. 2014). Interestingly, PK1 is highly expressed and was defined as a novel mediator of the inflammatory response in the third-trimester placenta, based on the induction in the expression of IL-8 and COX-2 (Denison et al. 2008). Seidmann et al. demonstrate that a balance between PK1 expression and FGF is necessary to prevent fetal hypoxia, and that PK1 modulates functional capillarization in late pregnancy (Seidmann et al. 2013). Another function of prokineticin signaling is the down regulation of B7-1, CD-14, CC chemokine receptor 5 and CXC chemokine receptor 4 in human peripheral blood monocytes induced by PK1 treatment and induces morphological changes in these cells (Dorsch et al. 2005).

Prokineticin signaling has also been studied some in the CNS, with the presumed signaling through the more dominantly expressed PK2 receptor. For instance, PK2 is able to induce a dose-dependent depolarization of subfornical organ neurons and can regulate voltage-activated potassium channels through the MAPK signaling pathway and sodium channels (Cottrell et al. 2004, Fry, Cottrell and Ferguson 2008). PK2 is also able to increase the activity of acid-sensing ion channels, which is dependent on the PKC signaling pathway in rat primary sensory neurons. This indicates that PK2 increases pain sensation from acidosis through acid sensing ion channels (Qiu et al. 2012). Similarly, through a PKC-epsilon dependent signaling pathway, Bv8 was able to increase in the inward currents caused by heat and the transient receptor potential vanilloid 1

(TRPV1) capsaicin receptor (Vellani et al. 2006). Another PKC-dependent role for prokineticin signaling was demonstrated in rat trigeminal ganglion neurons, where PK2 treatment reduced inward currents produced upon GABA(A) receptor activation involved in pain perception. This effect was blocked with a pan PKC inhibitor but the PKA inhibitor H89 was not able to inhibit the inward current reduction.

The current information about prokineticin signaling is not very detailed. The majority of the cell signaling studies for downstream pathways of the prokineticin receptor involve the commonly activated MAPK and Akt pathways. However, detailed signaling mechanisms have yet to be elucidated for many cell types and tissues. Collectively, the current information related to prokineticin signaling demonstrate that there is a potential for diverse signaling pathways activation downstream of the prokineticin receptors. Cell survival, differentiation, migration, and cell excitability are the main cellular functions controlled by prokineticin signaling, depending on the cell type and physiological function of the tissue. Several cell types, including cardiomyocytes, endothelial cells, neural crest cells and myeloid cells have increased cell survival from prokineticin-induced activation of Akt and ERK1/2. More research into the signaling pathways for the prokineticin receptors could elucidate many of the physiological roles that these signaling peptides play throughout the body.

### **Prokineticin-2 in inflammation**

Prokineticin-2 expression has been found to be increased with tissue inflammation in different areas throughout the body. Recently, the prokineticins have been found to be increased with tissue inflammation in lung (Chauvet et al. 2015), gut (Watson et al. 2012), bladder (Chen et al. 2015a), testis (Chen et al. 2016), spinal cord (Abou-Hamdan et al. 2015), and brain (Cheng et al. 2012). The role of prokineticin signaling in the propagation of inflammation has been studied

by several groups that demonstrate blockage of PK2 signaling can reduce the level of inflammation in the tissue. Martucci et al. demonstrated that sub-nanomolar ranges of PK2 could induce chemotaxis of macrophages and potentiate LPS-induced IL-1 and IL-12 proinflammatory cytokines, while reducing the anti-inflammatory cytokine IL-10 (Martucci et al. 2006). The Gq proteins seem to be responsible for the effects of PK2 on macrophages, due to the fact that the phospholipase inhibitor U73122 blocked the signaling. In primary mouse splenocytes, PK2-PKR1 signaling was demonstrated *in vitro* and *in vivo* using receptor knockout mice. Amphibious Bv8 treatment induced the production of IL-1beta and treatment at levels between picomolar and low nanomolar decreased IL-4 and IL-10 levels in these cultured mouse splenocytes. A PKR1 knockout mouse was unable to elicit this PK2-induced effect, therefore pointing to the PKR1 receptor as being responsible for skewing the cell towards the production of proinflammatory factors (Franchi et al. 2008). Interestingly, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) increased the expression of PK2 in isolated neutrophils. PK2 was also demonstrated to be a potent chemotactic factor for neutrophils most likely via PKR2 signal transduction since PKR1 mRNA was not detected in these cells. PK2 was highly expressed in tumor-infiltrating neutrophils and isolated PK2 from neutrophils was found to be fully active *in vitro*. The involvement of G<sub>i</sub> proteins in prokineticin GPCR signal transduction in haematopoietic cells was further demonstrated by the discovery that PK2-induced hematopoiesis and mobilization of hematopoietic cells were found to be pertussis toxin sensitive (LeCouter et al. 2004a, Zhong et al. 2009). Dorsch et al. found that monocytes treated with PK1 had increased levels of IL-12 and TNF $\alpha$  and decreased IL-10 production in response to LPS

stimulation. Whereas other groups demonstrated an increase in CCL4, CXCL1 and CXCL8 with PK1 treatment in monocytes but not macrophages or dendritic cells (Dorsch et al. 2005, Monnier et al. 2008).

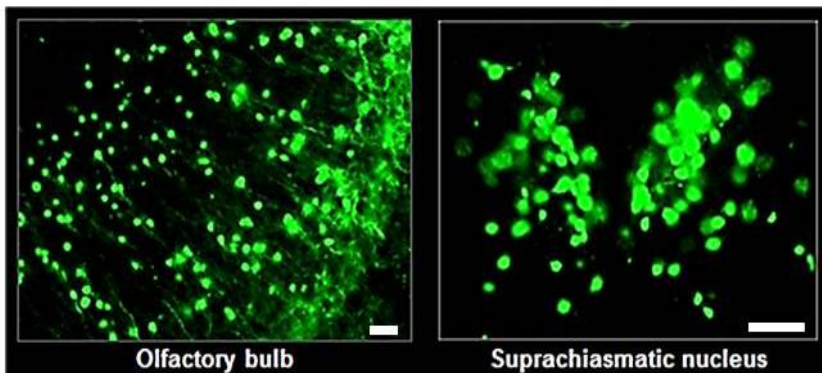
There have been many studies on the effects of prokineticin signaling in the different cell types of the blood. However, there have not been any extensive studies to detail signaling pathways downstream of the prokineticin receptor that would lead to an increase in inflammation. Additionally, the studies that have implicated prokineticin signaling in the induction of inflammation use the recombinant proteins mainly in the picomolar range or with amphibious Bv8 in mammalian cells. Due to the fact that there are two receptors and that the signaling of this molecule is highly regulated at the receptor level, with heterodimerization and phosphorylation leading to differential signaling, studies into higher doses and more intricate cell signaling would be appropriate.

### **Prokineticin-2 in the CNS**

Prokineticin 2 signaling has been studied in several areas of the brain, and the prokineticin receptors are expressed in many areas of the central nervous system. Interestingly, PK1 has only been found expressed in low levels throughout the CNS except in the nucleus tractus solitarius and lateral reticular nucleus of the hindbrain, and therefore PK2 is the dominant prokineticin signaling molecule in the CNS. In situ hybridization experiments performed by Cheng et al. detailed the regional expression of PK2 and its two prokineticin receptors across the entire mouse brain (Cheng et al. 2002). PK2 expression was also determined using the GENSAT EGFP-PK2 transgenic mouse in which the expression of the EGFP reporter protein is driven by the PK2 promoter (Zhang, Truong and Zhou 2009). Images from the olfactory bulb and superchiasmatic nucleus from eGFP-

PK2 transgenic mice is shown in Figure 10. PKR2 is expressed higher in the CNS than PKR1, however, studies have indicated that PKR1 is expressed at a higher rate in immune cells such as microglia (Zhou 2006). Olfactory bulb neurogenesis is an important function that has been attributed to PK2 signaling in the CNS. Ng et al. demonstrated that if you remove PK2 using knockout mice, that there was a reduction in the size and architecture of the olfactory lobe (Ng et al. 2005). The transcription of PK2 in this region of the brain was found to be under the regulation of the proneural transcription factors Neurogenin 1 and MASH1 which bind at conserved E-box sites on the PK2 promoter to activate transcription (Zhang et al. 2007b). Interestingly, migrating neuroblasts and subventricular zone transient-amplifying progenitors in the adult mouse brain were found to express PKR2 (Puverel et al. 2009). PK2 expression was also identified to be associated with areas of active neuronal migration and proliferation in the adult zebrafish brain during normal development and during post-injury telencephalon regeneration (Ayari et al. 2010). This research indicates that PK2 acts as a chemoattractant that causes neural progenitor cells to migrate through the rostral migratory stream towards the olfactory bulb and possibly induce neuronal differentiation. Recent studies have indicated that PK2 is an important factor in the superchiasmatic nucleus regulating circadian rhythms (Zhou and Cheng 2005, Li, Hu and Zhou 2009, Li et al. 2006b, Burton et al. 2016). In response to light there is an induction of PK2 within the superchiasmatic nucleus possibly in AVP-positive cells, and circadian rhythms become dysfunctional when either PK2 or PKR2 is knocked out (Mieda et al. 2015). PK2 also has the ability to induce its own transcription in the SCN through PKR2 signaling in a positive feedback loop. The PKR2 receptor is expressed in various SCN output areas including the paraventricular hypothalamic nucleus (PVN), dorsal medial hypothalamic nucleus (DMH), paraventricular thalamic nuclei (PVT). Therefore, PK2 is increased in the SCN and could potentiate signaling in

output areas through PKR2 signaling. Along the same lines, PK2 signaling has also been demonstrated to modulate the excitability of certain subsets of subfornical neurons, which would suggest a potential role for regulating autonomic function (Yuill et al. 2007). Collectively these studies indicate that PK2 is an important mediator for circadian rhythms and the autonomic functions associated with them. Zhou et al. also used PK2 knockout mice to show that PK2 is involved in thermo-regulation and energy expenditure (Zhou et al. 2012). This study demonstrated that PK2 expression was increased in the paraventricular nucleus (PVN) after fasting, and then returned to normal after the addition of food. This research indicates that PK2 is at least partly responsible for energy uptake and expenditure and that PK2 increase in the PVN could help animals survive better when there is little food uptake. Interestingly, PK2 expression in the hypothalamus was reduced when an animal was fasting, and acute or chronic administration of



**Figure 10. Immunofluorescent images of GFP-positive cells in the mouse olfactory bulb and suprachiasmatic nucleus of untreated PK2-eGFP mice**

PK2 caused the animals to eat less food (Gardiner et al. 2010). Along the same lines, exogenously added PK2 signals through the PKR1 receptor in the brainstem and leads to reduced food intake and

body weight in mice (Beale et al. 2013). Therefore, PK2 can signal in several areas of the brain that can lead to reduced food intake and weight loss, indicating that it is an anorectic peptide. PK2 signaling has been widely studied in the different areas of the brain and they have demonstrated a wide range of important functions throughout the CNS.

There have been mixed results with PK2 in diseases of the CNS, with neuroprotection seen in some models and neurodegeneration seen in others. The first results of PK2 signaling in neurodegeneration were reported in 2001 by Melchiorri et al., demonstrating that mouse Bv8 could support neuronal survival against NMDA excitotoxicity in the mouse cortex and cerebellum through the MAPK/PI3K pathway (Melchiorri et al. 2001). The neuroprotection conferred by mouse Bv8 was seen in a mouse co-culture of cortical neurons and astrocytes. However, it took another decade before more research went into prokineticin signaling in neurodegeneration. Interestingly, Cheng et al. found that Prokineticin-2 is increased in a model of stroke. While picomolar concentrations of PK2 increased the infarct volume, a prokineticin receptor antagonist was able to reduce the infarct volume (Cheng et al. 2012). However, in 2016 Landucci et al. demonstrated that prokineticin signaling could be neuroprotective in a cell culture model of ischemia through the ERK1/2 and Akt pathways (Landucci et al. 2016). These results are more in line with the cell signaling that has been seen with prokineticin receptor activation. The largest difference in the two studies is the dose of Prokineticin-2 or Bv8 used, with Cheng et al. looking at a picomolar dose and Landucci et al. with a nanomolar concentration. There has only been one study on prokineticin signaling in Alzheimer's disease (AD) that came out in 2015. They found that PK2 expression was increased in animal models of AD and that using a prokineticin receptor antagonist there was a reduction in Amyloid $\beta$ -induced cell death of cortical neurons (Severini et al. 2015). Recently, a study found that PK2 expression is increased in CNS autoimmunity animal models and that blocking this increased PK2 in the spinal cord of animals led to a reduction in inflammation and demyelination. However, the study also points out that using the prokineticin



receptor antagonist reduced production of IL-6, which is normally pro-inflammatory (Abou-Hamdan et al. 2015). Prokineticin signaling is a very complicated mechanism and has shown differing disease outcomes depending on the situation and dose of PK2. Severini et al. went on to discuss that their results could be due to the use of low (picomolar) ranges of PK2. Currently, there has been no research into overexpressing PK2 in the healthy or diseased CNS (Severini et al. 2015).

## CHAPTER II

PROKINETICIN-2 UPREGULATION DURING NEURONAL INJURY MEDIATES A  
COMPENSATORY PROTECTIVE RESPONSE AGAINST DOPAMINERGIC NEURONAL  
DEGENERATION

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### Abstract

Prokineticin-2 (PK2), a recently discovered secreted protein, regulates important physiological functions including olfactory biogenesis and circadian rhythms in the CNS. Interestingly, although PK2 expression is low in the nigral system, its receptors are constitutively expressed on nigrostriatal neurons. Herein, we demonstrate that PK2 expression is highly induced in nigral dopaminergic neurons during early stages of degeneration in multiple models of Parkinson's disease (PD), including PK2 reporter mice and MitoPark mice. Functional studies demonstrate that PK2 promotes mitochondrial biogenesis and activates ERK and Akt survival signaling pathways, thereby driving neuroprotection. Importantly, PK2 overexpression is protective whereas PK2 receptor antagonism exacerbates dopaminergic degeneration in experimental PD. Furthermore, PK2 expression increased in surviving nigral dopaminergic neurons from PD brains, indicating that PK2 upregulation is clinically relevant to human PD. Collectively, our results identify a paradigm of compensatory neuroprotective PK2 signaling in nigral dopaminergic neurons that could have important therapeutic implications for PD.

## Introduction

Prokineticin-2 (PK2), of the AVIT protein family, regulates diverse physiological processes including hematopoiesis, angiogenesis, reproductive functions, and pain perception (Cheng et al. 2002, Hu et al. 2006b, Li et al. 2006a, Lin et al. 2002a, Ng et al. 2005, Pitteloud et al. 2007b). In the brain, PK2 regulates circadian rhythms by functioning as an output molecule from the suprachiasmatic nucleus (SCN) and neurogenesis by directing the migration of progenitor cells from the subventricular zone during olfactory bulb biogenesis (Ng et al. 2005, Cheng et al. 2002, Cheng et al. 2005b). Recent evidence also links PK2 signaling to thermoregulation and energy expenditure via the hypothalamus (Zhou et al. 2012). Constitutive expression of PK2 is largely restricted to these aforementioned regions, however, its two highly similar G-protein-coupled receptors (GPCR), PKR1 and PKR2, are ubiquitously and differentially expressed across the central nervous system (CNS), reflecting PK2's distinct functions throughout the brain (Cheng, Leslie and Zhou 2006a).

While investigating changes in gene expression during dopaminergic cell death via PCR-array, we surprisingly found that PK2 mRNA was highly induced during dopaminergic (DAergic) cell death triggered by tumor necrosis factor alpha (TNF $\alpha$ ) (Gordon et al. 2012). We therefore hypothesized that PK2 is a novel signaling mediator secreted during dopaminergic degeneration. Supporting this notion, our mouse models of PD revealed that PK2 is induced in nigral dopaminergic neurons early during degeneration, prior to the onset of motor deficits. We also found that PK2 expression is elevated in the substantia nigra (SN) of PD patients, corroborating PK2's clinical relevance in human PD. PK2 activates PKR1 and PKR2, both of which are differentially expressed in the CNS (Lin et al. 2002a, Soga et al. 2002b, LeCouter et al. 2003b, Shojaei et al. 2007). We found that PKR2 is constitutively expressed in dopaminergic neurons of

the nigrostriatal system in mice and human PD cases, suggesting that PK2 signaling via PKR2 could be relevant during dopaminergic degeneration. Indeed, our functional studies using PK2 overexpression and recombinant PK2 (rPK2) demonstrated that PK2 signaling protects against oxidative stress, mitochondrial dysfunction and dopaminergic degeneration induced by the Parkinsonian neurotoxicant MPP<sup>+</sup>. Blocking PK2 signaling using a PK2 receptor antagonist augmented dopaminergic degeneration in a PD mouse model, while overexpression of PK2 by AAV gene delivery was neuroprotective. Given that prokineticin GPCRs are druggable targets and that PK2 signaling is neuroprotective against dopaminergic degeneration, our results have potential therapeutic implications for mitigating dopaminergic neuron loss in PD using prokineticin receptor agonists.

## Results

### **PK2 is induced during MPP<sup>+</sup>- and TNF $\alpha$ -induced N27 cell death**

Using qPCR-array, we serendipitously discovered that PK2 mRNA expression is highly induced (~7-fold over control) early during dopaminergic cell death (Supplementary Table. 1). Thus, we hypothesized that PK2 is likely a compensatory bioactive signaling mediator secreted early during dopaminergic degeneration. To validate this hypothesis, we determined both TNF $\alpha$  (30 ng/ml) and the Parkinsonian toxicant MPP<sup>+</sup> (200  $\mu$ M) increased PK2 protein expression in N27 cells (Fig. 1A). PK2 supernatant levels also increased, demonstrating that PK2 is both upregulated and secreted by dopaminergic neuronal cells following neurotoxic stress. A rabbit polyclonal antibody that recognizes an epitope within the internal region of PK2 was used for immunofluorescence detection of PK2 expression and localization in N27 dopaminergic cells (Fig.

1B). Typical of a secreted protein, PK2 accumulated prominently around the perinuclear zone (arrow heads). Indeed, PK2 contains a signal peptide at its N-terminus targeting it for constitutive secretion (LeCouter et al. 2003b, Li et al. 2001). Our results identify PK2 as an inducible secreted mediator during early stages of neurotoxic stress.

### **PK2 is induced in nigral DAergic neurons in experimental PD**

Previous studies characterizing PK2 expression in the adult mouse brain reported it either absent or sparse in the ventral midbrain, including the SN (Cheng et al. 2006a, Zhang et al. 2009). Since PK2 receptors were constitutively expressed mostly on neurons, it's possible that PK2 ligand expression could mediate specific signaling functions via its cognate receptors. Initially, using the standard acute (4 x 18 mg/kg, 2-h intervals) MPTP model of PD, we verified, in concordance with our *in vitro* results, that MPTP significantly increased PK2 levels in nigral tissue lysates. Western blot (Fig. 2A) and densitometric analysis (Fig. 2B) revealed that upregulation occurred as early as 3 h after MPTP treatment and peaked at 24 h in MPTP-treated mice. Reduced nigral levels of tyrosine hydroxylase (TH), a marker of dopaminergic neurons, was not observed until 3 days post-treatment, suggesting MPTP-induced PK2 upregulation precedes dopaminergic degeneration (Fig. 2A). No significant difference in PKR2 expression existed between MPTP-treated and saline-injected control mice (Fig. 2A). We confirmed that PK2 staining co-localized largely with TH-positive (TH<sup>+</sup>) dopaminergic neurons (Fig. 2C). PK2 upregulation was not observed in the striatum or cortex (Supplementary Fig. 1). In saline-treated mice, we observed little to no PK2 expression, and no distinct cellular staining patterns, which supports Cheng et al. (Cheng, Leslie and Zhou 2006b), who characterized PK2 expression throughout the entire adult mouse brain by *in situ* hybridization without finding significant expression in the SN pars compacta (SNpc). We also

confirmed PK2 mRNA upregulation in the nigra (Fig. 2D). Two GPCRs for PK2, prokineticin receptors 1 and 2 (PKR1/2), have been identified and cloned by independent groups (Lin et al. 2002a, Masuda et al. 2002b, Soga et al. 2002b). We determined their expression in the SN of both saline- and MPTP-treated mice. Cellular PKR2 was highly expressed on dopaminergic neurons and colocalized with TH staining revealing a punctate pattern of PKR2 staining evident at higher magnification (Fig. 2E). Unlike the PK2 ligand, MPTP did not alter the constitutive expression of PKR2 in the SN (Fig. 2E).

To further confirm PK2 upregulation in dopaminergic nigral neurons, we used GENSAT BAC PK2 eGFP transgenic mice [Tg (Prok2-eGFP) FH64Gsat]. This transgenic mouse contains an eGFP reporter transgene and a polyadenylation sequence introduced between the PK2 promoter and the first coding exon of the PK2 gene (Heintz 2004, Gong et al. 2003). Transcription of the eGFP reporter is driven by PK2 promoter activation, and is terminated at the polyadenylation site, so that reporter expression occurs without any changes in native PK2 expression. Consistent with reported constitutive PK2 expression in the SCN and olfactory bulb (Cheng et al. 2002, Cheng et al. 2005b), we observed high eGFP fluorescence in these regions in the adult PK2-eGFP mouse brain (Supplementary Fig. 2A), confirming the utility of this model for our studies. PK2-eGFP mice were treated with MPTP (4 x 20 mg/kg, 2-h intervals). In nigral sections from saline-treated mice, eGFP fluorescence was undetectable (Fig. 2F). However, in MPTP-treated mice at 24 h, strong eGFP fluorescence was evident along the nigral tract with most of the eGFP-positive cells being TH-positive dopaminergic neurons. Some TH-negative cells in the nigral tract were also labelled with eGFP (Fig. 2F), representing less than 5% of the PK2-positive cells in the SN; however, the identity of these PK2-positive cells is unclear. We also confirmed our IHC findings

by Western blotting PK2 and GFP (Supplementary Fig. 2B). Together, these results demonstrate that PK2 is highly expressed in nigral dopaminergic neurons of PK2-eGFP mice following MPTP-induced degeneration.

The acute MPTP model exhibits rapid dopaminergic degeneration. To verify if PK2 is upregulated in a more gradual and progressive model of dopaminergic degeneration, we utilized the MitoPark mouse, which is an L-DOPA-responsive transgenic model of PD generated by targeted inactivation of the mitochondrial transcription factor A (Tfam) gene in dopaminergic neurons. Thus, mitochondrial dysfunction drives progressive dopaminergic degeneration in MitoPark mice over a period of 32 weeks and is accompanied by behavioral motor deficits (Ekstrand and Galter 2009, Ekstrand et al. 2007). We investigated whether PK2 upregulation occurred at time points preceding the onset of motor deficits in MitoPark mice, which have been reported to begin at around age 12 weeks (Ekstrand and Galter 2009, Ekstrand et al. 2007). First, we confirmed the time course of MitoPark motor deficits and found no significant motor impairments at 8 weeks compared to age-matched wild-type C57 controls (Fig. 2G-H, and Supplementary Fig. 3). PK2 expression increased significantly at 8 and 12 wk (Fig. 2I). Consistent with our observation that PK2 upregulation precedes dopaminergic neurodegeneration, no significant loss of nigral TH protein was evident in MitoPark mice at 8 weeks (Fig. 2I). We also confirmed that PK2 was localized to TH<sup>+</sup> nigral dopaminergic neurons in 12-wk-old MitoPark mice (Fig. 2J). Overall, these results demonstrate that PK2 is upregulated specifically in nigral dopaminergic neurons following both acute and progressive dopaminergic degeneration. Constitutive PK2 receptor expression on adjacent nigral dopaminergic neurons suggests that PK2 has autocrine/paracrine signaling roles as a compensatory response following dopaminergic neuron injury and degeneration.



### **PK2 is elevated in the substantia nigra of PD brains**

Nigral tissue lysates from the postmortem brains of PD patients (Supplementary Table 2) and age-matched controls were probed for PK2 protein expression by Western blotting. Increased PK2 expression was evident in PD brains with significantly lower expression in age-matched control subjects (Fig. 3A and Supplementary Fig. 4A). Densitometric analysis of PK2 band intensities from 10 controls and 10 PD samples indicated that PK2 levels in PD brain lysates were about 2-fold higher (Fig. 3B). For reasons not clear at present, in contrast to mouse brains, we observed a small amount of PK2 in control human brains. Perhaps a higher basal level of PK2 expression occurs in human brains compared to mice, or the effect is age-related. We also confirmed increased neuronal PK2 expression in DAB-stained postmortem nigral tissue sections obtained from PD patients relative to age-matched controls (Fig. 3C). PK2 and TH co-labeling (Fig. 3D) revealed that PK2 was largely absent on TH<sup>+</sup> dopaminergic neurons in control brain sections. However, in PD brain sections, intense and specific PK2 staining was localized to surviving TH<sup>+</sup> dopaminergic neurons. Together, these results demonstrate for the first time that PK2 expression is elevated in PD patients, providing clinical relevance to our findings from experimental PD models.

We also confirmed that the PKR2 receptor is expressed on dopaminergic neurons in the human brain (Fig. 3E and Supplementary Fig. 4B) using nigral samples from control and PD cases. Again, constitutive PKR2 expression showed no significant difference between control and diseased patient samples. Although PKR2 is constitutively expressed on neurons across different brain regions (Li et al. 2001, Zhang et al. 2009), a significant decrease in PKR2 expression is not evident in nigral lysates of PD brains perhaps because it would still be expressed in other neuronal

populations at substantial levels. Supplementary Fig. 5 shows that PKR2 is expressed in many MAP2-positive neurons in human SN samples. These results confirm that secreted PK2 can act directly on dopaminergic neurons via the constitutively expressed PKR2 receptor.

### **PK2 reduces MPP<sup>+</sup>-induced dopaminergic neuronal cell death**

We next identified the functional role of secreted PK2 during dopaminergic cell death. We found that PKR2 was highly expressed on N27 dopaminergic cells, and consistent with our *in vivo* findings, its expression did not change with MPP<sup>+</sup>-induced degeneration (Fig. 4A). We also confirmed that rPK2 treatment mobilized calcium in these cells (Fig. 4B), indicating that functional PK2 receptors are present on dopaminergic neuronal cells. When we treated N27 dopaminergic cells with 200  $\mu$ M MPP<sup>+</sup> in the presence or absence of nanomolar doses of rPK2, the rPK2 (25 nM) co-treatment significantly reduced MPP<sup>+</sup>-induced activation of caspase-3 and DNA fragmentation measured at 8-h and 16-h time points, respectively (Fig. 4C-D), suggesting that PK2 is protective in dopaminergic cells during neuronal cell death. Furthermore, co-treatment with a specific PK2 receptor antagonist, PKRA7 (Curtis et al. 2013) effectively blocked the neuroprotective action of rPK2 (Fig. 4E-F). We then showed that co-treatment with 5-50 nM rPK2 mitigated MPP<sup>+</sup>-induced intracellular ROS levels in a dose-dependent manner (Fig. 5A). Next, we visualized mitochondrial integrity by using MitoTracker Red fluorescent dye in MPP<sup>+</sup>-treated N27 cells in the presence and absence of rPK2. MPP<sup>+</sup> caused considerable fragmentation of mitochondria compared to untreated controls, whereas in rPK2 co-treated cells, mitochondrial fragmentation was significantly reduced (Fig. 5B, top panel). These changes were further evidenced by quantifying key morphometric parameters including mitochondrial circularity and solidity (Fig. 5B, bottom panels). To understand the mechanism by which PK2 signaling reduces

mitochondrial dysfunction in dopaminergic cells, we examined the relevant protective signaling pathways. Both rPK2 co-treatment with MPP<sup>+</sup> and rPK2-alone significantly increased mRNA and protein levels of the mitochondrial anti-apoptotic protein Bcl-2 in N27 cells (Fig. 5C-E). To further investigate PK2's neuroprotection in greater mechanistic detail, we evaluated the contribution of key downstream effectors of PK2 signaling in other systems, including ERKs (p44/42) and Akt (Guilini et al. 2010, Lin et al. 2002b, Ngan and Tam 2008, Martin et al. 2011). In N27 dopaminergic cells, phosphorylation of Akt and ERKs was observed 3 h after rPK2 stimulation (Fig. 6A-B), confirming that these pathways are activated downstream of PK2 signaling. The PK2-stimulated Akt and ERK activations were both completely blocked by the PK2 receptor antagonist PKRA7 (Curtis et al. 2013), indicating the specificity of PK2 signaling in activating these kinases. The ERK-specific inhibitor PD98059 (Crews, Alessandrini and Erikson 1992) significantly attenuated the protection from MPP<sup>+</sup>-induced cell death afforded by rPK2 treatment (Fig. 6C), whereas PD98059-alone had no effect on cell viability. Similar results were obtained with the Akt inhibitor API-1 (Kim et al. 2010) (Fig. 6C). These results indicate that the neuroprotective effect of rPK2 was mediated, at least in part, by activating Akt and ERK/MAPK signaling pathways. Finally, we tested whether a genetic knockdown of endogenous PK2 sensitizes N27 cells to MPP<sup>+</sup>-induced toxicity. Transduction of N27 cells with the CRISPR/Cas9-based PK2 KO lentivirus almost completely abolished PK2 protein and mRNA expression in N27 cells (Fig. 6D-E). Our cytotoxicity results demonstrated that PK2 deficiency via CRISPR/Cas9 indeed sensitized the cells to MPP<sup>+</sup> toxicity, and that the addition of 25 nM rPK2 reversed this sensitivity (Fig. 6F). Knockdown of PK2 significantly reduced Akt activity in both untreated and MPP<sup>+</sup>-treated N27 cells (Fig. 6G). Together, these results demonstrate for the first time that PK2 signaling protects dopaminergic cells against MPP<sup>+</sup>-mediated oxidative stress, mitochondrial dysfunction and cell

death by activating ERK and Akt signaling pathways, implying that PK2 secreted from dopaminergic neurons is a compensatory protective factor acting locally against neuronal death.

### **PK2 overexpression promotes mitochondrial biogenesis**

Having confirmed that exogenous rPK2 is neuroprotective, we next tested endogenous PK2 overexpression. We engineered dopaminergic MN9D cells to express human wild-type PK2 by stably transfecting them with pCMV6-PK2-myc expression plasmid or control vector. Stable PK2 overexpression was validated by Western blotting using the PK2 antibody (Supplementary Fig. 6A) and immunostaining for PK2-myc using a myc-tag antibody (Supplementary Fig. 6B). Basal levels of its PKR2 receptor in these cells were unaffected (Supplementary Fig. 6A). After vector and PK2-overexpressing cells were treated with MPP<sup>+</sup> (300  $\mu$ M), we confirmed that, similar to rPK2 treatment, PK2-expressing cells were significantly protected against MPP<sup>+</sup> neurotoxicity compared to vector control cells (Supplementary Fig. 6C-E). This protective effect of PK2 was attenuated by co-treating with 2  $\mu$ M PKRA7. To investigate whether stable PK2 expression protects against MPP<sup>+</sup>-induced mitochondrial dysfunction, we first performed MitoTracker Red imaging in an N27 cell line stably expressing human PK2, established by a lentivirus-based gene delivery method. MPP<sup>+</sup> caused mitochondrial fragmentation in N27 vector control cells, as evidenced by donut-shaped morphology (Fig. 7A). However, in MPP<sup>+</sup>-treated PK2-expressing cells, longer strand-like mitochondria were preserved. Quantification of mitochondrial circularity, solidity and length confirmed these findings (Fig. 7B-D). The fluorescent signals from MitoTracker Red (Fig. 7E) revealed a larger mitochondria area in PK2-expressing cells relative to vector cells, suggesting a role for PK2 in regulating mitochondrial biogenesis. We also examined whether PK2 protected against MPP<sup>+</sup>-induced cellular ATP depletion, which could result from

mitochondrial dysfunction. PK2-expressing and vector control cells were treated with or without MPP<sup>+</sup> for 16 h. PK2 overexpression increased basal levels of ATP production; however no significant differences were observed between MPP<sup>+</sup>-treated PK2-expressing and vector control cells (Fig. 7F). Consistent with our rPK2 treatment results, stable PK2 overexpression also increased basal levels of Bcl-2 mRNA, and it mitigated the loss of Bcl-2 caused by MPP<sup>+</sup> treatment relative to vector control cells (Fig. 7G). Furthermore, the transcription of PGC1 $\alpha$  (Fig. 7H) and TFAM (Fig. 7I), key genes controlling mitochondrial biogenesis, were both markedly upregulated in PK2-expressing cells compared to vector cells. In line with these results, stably expressing PK2 increased protein levels of TFAM as well as a mitochondria marker, mitochondrial import receptor subunit TOM20 (Fig. 7J and Supplementary Fig. 7). Finally, PK2 signaling increased the mitochondrial DNA copy number in both control and MPP<sup>+</sup>-treated N27 cells (Fig. 7K). Together, these data demonstrate for the first time that PK2 protects dopaminergic cells by activating mitochondrial biogenesis pathways. These findings also establish an exciting new link between PK2 signaling and the PGC1 $\alpha$  pathway as the mechanism by which PK2 mediates protection against mitochondrial dysfunction and death in dopaminergic cells.

### **PK2 reduces primary DAergic neurons from MPP<sup>+</sup>-induced loss**

Prior to testing if PK2 protects primary dopaminergic neurons from MPP<sup>+</sup>-induced neurodegeneration, we verified that primary dopaminergic neurons expressed both PKR1 and PKR2 (Fig. 8A), and rPK2-induced calcium mobilization confirmed that these receptors were functionally active (Fig. 8B). Given the constitutive expression of PKR2 in various neuronal subpopulations, the observed calcium changes are not specific for dopaminergic cells, which compose about 5% of the total cells in this population. We then treated primary mesencephalic neuronal

cultures for 48 h with 5  $\mu$ M MPP<sup>+</sup>-alone or co-treated with 25 nM rPK2, which was added again 24 h later. MPP<sup>+</sup> reduced the number of TH<sup>+</sup> dopaminergic neurons to almost 30% of the vehicle-treated group, indicating extensive dopaminergic degeneration. PK2 treatment significantly (#,  $p < 0.05$  vs MPP<sup>+</sup>-alone) mitigated MPP<sup>+</sup>-induced dopaminergic degeneration (Fig. 8C-D). MPP<sup>+</sup> induced a dramatic decrease (~85%) in neurite length of TH-immunoreactive neurons, which was significantly improved by rPK2 treatment (Fig. 8C and 8E). The functional activity of surviving primary dopaminergic neurons was confirmed by dopamine uptake assays. PK2 significantly ameliorated the MPP<sup>+</sup>-induced loss of dopamine uptake activity (Fig. 8F). Together, these results further corroborate a novel neuroprotective role for PK2 in dopaminergic neurons *in vitro*.

### **PKRA7 increases susceptibility to MPTP toxicity in mice**

We utilized the small-molecule PK2 receptor antagonist PKRA7 (Curtis et al. 2013) to block PK2 signaling in the MPTP model of PD because knockout mice for PK2 and its receptors show significant defects in brain development and architecture, potentially involving dopamine pathways which would confound our studies (Cheng et al. 2002, Li et al. 2006a, Ng et al. 2005, Pitteloud et al. 2007b). Since the acute MPTP treatment paradigm causes severe loss of dopaminergic neurons, it is not amenable to testing the effect of PK2 receptor antagonists, which we anticipated would exacerbate neuronal loss. Therefore, we adopted a subacute MPTP treatment paradigm that results in partial nigral dopaminergic degeneration (Ghosh et al. 2012, Ghosh et al. 2013, Thomas et al. 2011). Furthermore, the time course of neurodegeneration in the sub-acute model is linear and well characterized, therefore the dose and treatment regimen can be tailored to achieve the partial dopaminergic degeneration required to study the effect of blocking PK2 signaling. Once-daily injections of MPTP (18 mg/kg/day, i.p.) were administered for three

consecutive days to induce moderate neurochemical and behavioral deficits. One day before the first MPTP treatment, we initiated once-daily i.p. injections of PKRA7 (20 mg/kg/day) for 10 days. TH immunostaining of coronal sections from the SN and striatum showed that while PKRA7-alone had no effect on the nigrostriatal dopaminergic system, it augmented MPTP-induced neurodegeneration in both the striatum and SN (Fig. 9A). These findings were confirmed by stereological quantification of TH<sup>+</sup> neurons and Nissl-positive cells in the SN as well as densitometry of the striatal TH innervation (Fig. 9B-D). We found that the number of Nissl-positive total neurons did not significantly differ from that of TH<sup>+</sup> neurons, which is consistent with previous findings (De Miranda et al. 2015, St-Amour et al. 2012). Western blotting of nigral tissue lysates showed moderately reduced TH expression in the SN of MPTP-treated mice when compared to control mice, whereas PKRA7 significantly enhanced nigral TH loss in MPTP-treated mice (Fig. 9E). In addition, PKRA7 did not alter PK2 and PKR2 levels in the SN of either PKRA7-alone- or PKRA7/MPTP-treated mice compared to saline and MPTP-treated mice (Fig. 9F). Nigral PK2 levels did not differ significantly between vehicle- or MPTP-injected mice after 7 days (Fig. 9F). This result is not surprising since we have observed that the MPTP-induced PK2 expression started to decrease by the third day post-MPTP injection (Fig. 2A). As expected, three doses of MPTP produced a ~51% loss of striatal dopamine compared to vehicle-treated control mice as measured by HPLC at day 7 after last administration of saline or MPTP, a more sensitive and precise measure of MPTP-induced dopaminergic degeneration. However, PKRA7 treatment significantly exacerbated MPTP-induced dopamine depletion by ~75%, whereas PKRA7-alone had no effect on striatal dopamine levels (Fig. 9G). In line with the histological, biochemical and neurochemical evidence, both the representative locomotor activity maps of individual open-field movements (Fig. 9J) and the group averages of horizontal activity and total number of movements

(Fig. 9H-I) indicated that PKRA7 worsens the behavioral deficits of MPTP-treated mice. Together, our results from a preclinical model of PD further support that blocking endogenous PK2 signaling exacerbates dopaminergic degeneration, resulting in increased neuropathology and behavioral deficits.

### **rAAV-mediated PK2 overexpression is neuroprotective in mice**

To further demonstrate the neuroprotective action of PK2 *in vivo*, we investigated whether recombinant adeno-associated virus (rAAV) 2/5-mediated PK2 overexpression confers MPTP resistance in mice. Recombinant AAVs encoding GFP-tagged human PK2 or GFP-alone were stereotaxically injected into the striatum of C57BL/6 mice. Striatal delivery of neuroprotective growth factors with rAAV gene delivery system has been more effective than nigral delivery (Kirik et al. 2000, Klein et al. 1999). Four weeks after the virus injection, we performed GFP/PK2 double IHC, confirming that rAAV-mediated delivery of GFP-PK2 or GFP resulted in widespread and efficient transgene expression in the striatum (Fig. 10A). Consistent with the initial observation of low basal PK2 expression in the striatum, only marginal endogenous PK2 immunoreactivity was observed in the striatum of AAV-GFP-transduced mice. We confirmed GFP-PK2 or GFP transgene expression in AAV-injected mice by Western blotting striatal lysates with a hamster monoclonal anti-PK2 (top panel) or anti-GFP (middle panel) antibody (Fig. 10B). GFP-PK2-AAV expression was lower than that of GFP-AAV (Fig. 10B), possibly because PK2 is a secreted protein, and therefore, GFP-tagged PK2 can be released from GFP-PK2-AAV-infected cells. To confirm the retrograde transport of PK2 from the striatum to the SN, we determined PK2 expression in the SN from animals that received viral gene delivery in the striatum. IHC revealed GFP-PK2 co-labeled in TH<sup>+</sup> neurons of the SN, indicating retrograde transport of the GFP-PK2



transgene from the striatum to the SN (Supplementary Fig. 8). Four weeks after virus injection, mice were exposed to a sub-acute MPTP paradigm, treating mice with 20 mg/kg MPTP daily for four days to produce significant dopaminergic neurodegeneration in the SN and striatum; the subacute paradigm is routinely used to assess the efficacy of putative neuroprotective agents (Chen et al. 2015b, Carta et al. 2009). Saline- and MPTP-alone groups were also included. Spontaneous open-field movements lasting 10 min were recorded 24 h after the last MPTP dose and individual track plots were generated for each mouse (e.g., Fig. 10C). The locomotor activity of AAV-PK2-transduced mice treated with MPTP was much improved relative to MPTP-alone and AAV-GFP/MPTP groups (Fig. 10D-E). Similarly, AAV-PK2 transduction significantly restored the 20-rpm rotarod performance of MPTP-treated mice (Fig. 10F). Based on these results, we conclude that AAV-mediated PK2 overexpression reverses MPTP-induced motor deficits. Furthermore, PK2 overexpression protected against MPTP-induced dopaminergic terminal loss in the striatum (Fig. 10G-H). TH<sup>+</sup> DAB staining (Fig. 10I) and stereological counts of TH<sup>+</sup> and Nissl<sup>+</sup> neurons (Fig. 10J-K) revealed a severe loss of nigral dopaminergic neurons (72% in MPTP-alone group and 74% in AAV-GFP/MPTP group compared with saline controls;  $p < 0.001$  for both groups) and total neurons (56% in MPTP-alone group and 55% in AAV-GFP/MPTP group compared with saline controls;  $p < 0.001$  for both groups) in both MPTP-alone and AAV-GFP/MPTP groups. In contrast, AAV-mediated delivery of PK2 significantly prevented the loss of nigral dopamine neurons and total neurons after MPTP treatment (Fig. 10J-K), with only 36% of dopamine neurons and 23% of total neurons degenerated compared to saline controls, further supporting the neuroprotective efficacy of PK2 overexpression ( $p < 0.001$  compared with MPTP-alone group;  $p < 0.001$  compared with AAV-GFP/MPTP group). The difference between TH<sup>+</sup> and total neuronal loss is consistent with other reports from Przedborski and colleagues (Jackson-Lewis et al. 1995)

using the same dose (80 mg/kg of total MPTP) that we have used in an acute treatment paradigm, and could be due to the fact that TH protein levels can be transiently decreased after MPTP toxicity without directly causing neuronal death (Meredith and Rademacher 2011a). Combined with the abovementioned observation that blocking PK2 signaling *in vivo* potentiates MPTP toxicity, these results convincingly demonstrate a protective role for PK2 signaling in the nigrostriatal dopaminergic system and suggest that modulating PK2 levels may be a novel therapeutic strategy for PD.

## Discussion

PD is a progressive neurodegenerative disorder with a complex multifactorial etiology that makes therapeutic intervention a major challenge. Therapeutic approaches that can slow or halt disease progression are urgently needed. This study uncovers a novel neuroprotective role for the AVIT protein family member PK2 in nigral dopaminergic neurons with clinical relevance to PD pathophysiology. We demonstrate for the first time that PK2 is rapidly induced following neuronal injury in cell culture and preclinical mouse models of PD. We also confirmed elevated PK2 levels in nigral tissues of postmortem PD patients, thereby establishing the clinical importance of these findings. Since the PKR2 receptor for PK2 is constitutively expressed on nigral dopaminergic neurons, pharmacological targeting of PK2 signaling could be explored for therapeutic development in PD. Our mechanistic studies demonstrate that both endogenous PK2 overexpression and soluble rPK2 protect dopaminergic neurons against mitochondrial dysfunction and cell death by promoting mitochondrial biogenesis through PGC1 $\alpha$ - and TFAM-dependent mechanisms and by activating ERK and Akt signaling pathways. AAV-mediated PK2 gene delivery significantly protects mice against MPTP-induced behavioral deficits and dopaminergic

degeneration in the nigrostriatal system, indicating that modulating PK2 activity may benefit the treatment of PD. We show that blocking the endogenous protective function of PK2 with the PK2 receptor antagonist PKRA7 worsens MPTP-induced histological, neurochemical and behavioral deficits, further demonstrating the neuroprotective function of PK2 in the dopaminergic system.

Our *in vivo* data from preclinical mouse models suggest that PK2 upregulation occurs during the early phase of dopaminergic degeneration prior to the onset of motor deficits. In the MPTP model, PK2 protein levels in nigral dopaminergic neurons increased 3 h post-MPTP administration and remained elevated for up to 3 days when nigral TH protein began declining (Fig. 2A). In the MitoPark transgenic mouse model of PD, PK2 increased by 8 weeks (Fig. 2I), prior to the onset of motor symptoms and remained elevated until 12 weeks when significant motor deficits are evident (Fig. 2G-H). Basal PK2 levels were either low or undetectable in the nigral brain region of healthy control mice. This is in agreement with previous reports showing little or no PK2 expression in regions of the midbrain (Zhang et al. 2009, Cheng et al. 2006a). Our finding that PK2 expression is upregulated early during the disease course prior to the onset of motor symptoms, together with our clinical data demonstrating that PK2 is elevated in PD patients, suggests that soluble circulating PK2 could serve as a potential marker of disease onset or progression. Understanding the transcriptional mechanisms underlying PK2 upregulation in dopaminergic neurons is crucial for targeting this pathway to achieve neuroprotection. Previous studies have shown that constitutive PK2 expression in the CNS is controlled by the circadian regulatory genes CLOCK/BMAL1 in the SCN and by Ngn1/MASH1 during olfactory bulb biogenesis (Cheng et al. 2002, Cheng et al. 2005a, Zhang et al. 2007a). The PK2 promoter is replete with multiple E-box sequences (CACGTG) that bind basic helix-loop-helix (bHLH) transcription factors such as HIF1 $\alpha$ , which regulates PK2 expression during angiogenesis (LeCouter et al.

2003b). Others have reported HIF1 $\alpha$  activation following MPTP-induced oxidative stress in nigral dopaminergic neurons and also in cell culture models of PD, making it a likely candidate for transcriptional upregulation of PK2 as a protective response (Lee et al. 2009, Correia and Moreira 2010, Sharp and Bernaudin 2004). Our laboratory is currently studying the transcriptional mechanisms regulating PK2 upregulation in dopaminergic neurons.

PK2 signaling supports the survival and proliferation of specific cell types outside the CNS (LeCouter et al. 2003b, LeCouter et al. 2004b, Shojaei et al. 2007). For example, PK2 is required for angiogenesis and hematopoiesis and regulates the survival of cardiomyocytes and myeloid cells via the AKT and STAT3 signaling pathways, respectively (Urayama et al. 2007b, Xin et al. 2013). Also, MAPK/ERK activation is involved in PK2-mediated angiogenesis (Guilini et al. 2010, Lin et al. 2002b). Biphasic PK2 expression has been demonstrated in the ischemic striatum (Cheng et al. 2012). In agreement with these findings, we provide the first evidence that PK2 protects dopaminergic neuronal cells by activating ERK and Akt signaling pathways (Fig. 6). Additionally, our results demonstrate for the first time that PK2 signaling via PKR2 on dopaminergic neurons upregulates neuroprotective antioxidant response pathways, particularly PGC-1 $\alpha$  and TFAM (Fig. 7). Recent large-scale genome-wide association studies (GWAS) have shown that PGC-1 $\alpha$ -responsive genes, which regulate cellular bioenergetics, are specifically down-regulated in early PD patients (Zheng et al. 2010, Mudo et al. 2012). Also, PGC-1 $\alpha$  overexpression protects against dopaminergic degeneration in PD models (Mudo et al. 2012, O'Donnell et al. 2014). The loss of functional TFAM signaling also results in dopaminergic degeneration and underlies the recently developed MitoPark genetic mouse model of PD. Our results suggest that PK2 is an upstream positive regulator of PGC-1 $\alpha$ , and thus PK2 receptor agonists could have therapeutic potential in restoring mitochondrial dysfunction and mitigating progressive dopaminergic neuron loss in PD.

Overall, our results have important implications for understanding dopaminergic degeneration in PD and PK2 biology. To our knowledge, this is the first report showing that PK2 is induced early during dopaminergic neuronal injury in the adult brain and that recovery of PK2 levels translates into dopaminergic neuronal protection through activation of cell survival signaling events and restoration of mitochondrial function in preclinical models of PD. Thus, developing agonists for PK2 GPCRs could be a novel therapeutic strategy for slowing or arresting the progression of dopaminergic neuronal degeneration in PD.

## Methods

### Chemicals and Reagents

DMEM/F-12, RPMI, neurobasal medium, B27 supplement, fetal bovine serum (FBS), L-glutamine, Lipofectamine 2000, G418, IR-dye tagged secondary antibodies, Hoechst nuclear stain, penicillin, streptomycin, MitoTracker Red dye and other cell culture reagents were purchased from Life Technologies (Gaithersburg, MD). Recombinant TNF $\alpha$  was purchased from Peprotech. Antibodies for rabbit (Cat# sc-67176) and goat (Cat# sc-48069) PK2, BCL-2 (Cat# sc-7382), TFAM (Cat# sc-23588), TOM20 (Cat# sc-11021) and c-Myc (Cat# sc-40X) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Specificity of the PK2 rabbit antibody was confirmed by pre-absorption of antibody with rPK2 peptide (Supplementary Fig. 9). Mouse TH (Cat# MAB318) and Tubulin (Cat# MAB1637) antibodies were purchased from Millipore (Temecula, CA). Prokineticin receptor R2 antibody (Cat# CVL-PAB0638-0) was obtained from Covalab (Villeurbanne, France). Antibodies for p44/42 (Cat# 9102), phospho-p44/42 (Thr202/Tyr204) (Cat# 4370s), Akt (Cat# 4691s) and phospho-Akt (Ser473) (Cat# 4060s) were

purchased from Cell Signaling (Danvers, MA), and goat polyclonal GFP antibody (Cat# ab6673) was obtained from Abcam (Cambridge, MA). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). The Cell Titer Glo Luminescent Cell Viability assay kit was purchased from Promega. Caspase-3 assay substrate was purchased from MP Biomedicals (Solon, OH). The DNA fragmentation assay kit was purchased from Roche Applied Science. The PK2 antagonist PKRA7 (Curtis et al. 2013) and hamster monoclonal PK2 antibody were kindly provided by Dr. Qun-Yong Zhou (University of California, Irvine). The ROS generation determinant DCF-DA was purchased from Calbiochem (San Diego, CA). Mouse  $\beta$ -actin antibody (Cat# A5441) and MPTP were obtained from Sigma (St. Louis, MO).

### **Animal studies**

All animal procedures were approved by Iowa State University's Institutional Animal Care and Use Committee (IACUC). All mice were housed under a 12-h light cycle in a climate-controlled mouse facility ( $22 \pm 1^\circ\text{C}$ ) with food and water available *ad libitum*. Based upon the outcomes of preliminary studies, we estimated error variances of 15% for biochemical and neurochemical endpoints and 20% for histological measures. Setting  $\alpha=0.05$ , we calculated sample sizes (n) of 8-10 animals per group, which has worked well for us in previous animal studies conducted in our laboratory (Ghosh et al. 2012, Ghosh et al. 2013). We used all male animals for all studies. All mice were pre-screened for normal baseline performance during behavioral assessments conducted prior to randomly assigning animals to experimental groups. Investigators involved with data collection and analysis were not blinded to group allocation. MPTP studies were performed as previously described (Zhang et al. 2007c), using C57BL/6NCrl mice (8-10 weeks of age) to determine levels of PK2 in the mouse brain. Over an 8 h period, one

i.p. injection of 18 mg/kg MPTP or equal volumes of saline (vehicle) was administered every 2 h. Mice were euthanized at the indicated time points and samples processed for either IHC, qRT-PCR or Western blotting. For PKRA7 experiments, C57BL/6 mice were injected i.p. with 18 mg/kg MPTP, or equal volumes of saline (vehicle), once daily for three consecutive days. PKRA7 (20 mg/kg/day) was given i.p. 24 h before MPTP treatment and continued once daily for 10 consecutive days until sacrifice. For the PK2 rAAV neuroprotection experiments, C57BL/6NCrl mice were injected i.p. with 20 mg/kg MPTP, or equal volumes of saline (vehicle), once daily for four consecutive days and sacrificed 24 h after the last MPTP injection.

### **PK2-eGFP transgenic mice**

The PK2-eGFP transgenic FVB/N-Swiss mice were originally generated at Rockefeller University by the GENSAT project using bacterial artificial chromosome (BAC) clone RP23-12A18, as described previously (Gong et al. 2003). The eGFP reporter cassette included a polyadenylation site that prevents PK2 overexpression. The PK2-eGFP male mice (8-10 weeks of age) were obtained from NIH-supported Mutant Mouse Regional Resource Center, UC Davis, CA, and bred at Iowa State University's animal facility. Mice were housed under a 12-h light cycle in a climate-controlled mouse facility ( $22 \pm 1^\circ\text{C}$ ) with food and water available *ad libitum*. Intraperitoneal injections of either 20 mg/kg MPTP or saline (vehicle) were administered every 2 h with a total of four injections.

### **MitoPark transgenic mice**

MitoPark transgenic mice were kindly provided by Dr. Nils-Göran Larsson of Karolinska Institute, Stockholm, and was originally generated in his laboratory at the Max Planck Institute for Biology of Ageing by conditionally knocking out the mitochondrial transcription factor TFAM through control of the dopamine transporter (DAT) promoter as previously described (Ekstrand and Galter 2009, Ekstrand et al. 2007). All mice used for this study were from the MitoPark breeding colony at Iowa State University. Housing was under a 12-h light cycle in a climate-controlled facility with food and water provided *ad libitum*. After behavioral experiments were performed at the indicated ages, mice were perfused for IHC or euthanized and dissected for Western blot analysis. All animal procedures were approved by Iowa State University's Institutional Animal Care and Use Committee (IACUC).

### **Human post-mortem PD brain samples**

We obtained freshly frozen SN tissue samples and cryostat sections from the brains of confirmed post-mortem human PD patients and age-matched neurologically normal individuals from the brain banks at the Miller School of Medicine, University of Miami, FL, the Australian Brain Bank Network and at the Banner Sun Health Research Institute, AZ. Case details of human post-mortem tissues are shown in Table S1. For Western blot experiments, tissue homogenates from freshly frozen tissue were prepared at a final concentration of 1 mg/ml total protein from which 40 µg of total protein was used. Nigral tissue blocks were fixed with 4% paraformaldehyde (PFA) solution (in 0.1 M phosphate-buffered saline, pH 7.4). Cryostat sections were used for IHC experiments. All human post-mortem samples were procured, stored, and distributed according to applicable regulations and guidelines involving consent, protection of human subjects and donor



anonymity. For samples obtained from the Australian Brain Bank Network, Institutional Human Research Ethics Approval was obtained from the Medical Research Ethics Committee at the University of Queensland, Brisbane, Australia. Since the post-mortem human brain tissues were obtained from approved national brain banks, Institutional Review Board (IRB) approval from Iowa State University was not required.

### **Fluorescent Western blotting**

Cells and micro-dissected brain tissues were collected at the end of treatment and lysed using a modified RIPA buffer as previously described (Kanthasamy et al. 2006, Latchoumycandane et al. 2011). Proteins were separated by Sodium Dodecyl Sulfate (SDS) gel electrophoresis. Normalized protein samples were loaded into each well and first separated in a 4% stacking gel and then through a 12-18% resolving gel. Proteins were then transferred to a nitrocellulose membrane and blocked using fluorescent Western blocking buffer (Rockland Immunochemicals). Membranes were washed several times in wash buffers comprising either PBS containing 0.05% Tween (PBST) or Tris-buffered saline containing 0.05% Tween (TBST). Primary antibodies, in either blocking buffer or 5% bovine serum albumin (BSA) solution, were then added to the membranes and incubated overnight at 4°C. After another 4-5 washes, an infra-red (IR) dye-tagged secondary antibody was added for 1 h. Actin or  $\alpha$ -tubulin was used as a loading control. Membranes were scanned using the Odyssey IR imaging system (LI-COR).

### **SYBR Green qRT-PCR**

Total RNA was isolated from fresh cell pellets or animal tissues using the Absolutely RNA Miniprep kit from Agilent Technologies. First strand cDNA synthesis was performed using an

Affinity Script qPCR cDNA synthesis system (Agilent Technologies). Real-time PCR was performed with the RT<sup>2</sup> SYBR Green master mix (Qiagen) and pre-validated qPCR mouse primer sets purchased from Qiagen. For normalization of each sample, the 18S rRNA gene (mouse and rat) was used as the housekeeping gene. The amount of each template was optimized empirically to maximize efficiency without inhibiting the PCR reaction. According to manufacturer's guidelines, dissociation curves were run to ensure a single amplicon peak was obtained. The results are reported as fold change in gene expression, which was determined via the  $\Delta\Delta C_t$  method using the threshold cycle ( $C_t$ ) value for the housekeeping gene and for the respective gene of interest in each sample.

### **Mitochondrial fragmentation analysis**

For Mitochondrial dysfunction experiments, cells were plated onto glass-bottom dishes coated with 0.1% Poly-D-Lysine in RPMI with 10% FBS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and 2 mM L-glutamine. Cells were treated in RPMI with 2% FBS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and 2 mM L-glutamine for 16 h. After treatment, MitoTracker Red dye was added to each dish at a concentration of 1:2000  $\mu$ l from a stock of 1 mM. The cells were incubated with the dye for 15-20 min at 37°C. After incubation, cells were fixed with 4% PFA for 1 h and then washed several times with PBS. High resolution confocal images were obtained using a Leica SP5 X confocal microscope system at the Confocal Microscopy and Image Analysis Facility at Iowa State University. Quantification of mitochondrial morphology was performed using a custom Image J macro program as described previously (Dagda et al. 2009, Merrill et al. 2011). To determine the mitochondria/cell ratio, mitochondrial area was measured by thresholding the MitoTracker fluorescence in ImageJ and dividing by the whole cell area.

### **Mitochondrial DNA copy number**

Total DNA containing both nuclear and mitochondrial DNA was isolated from N27 cells using the DNeasy Blood & Tissue kit (Qiagene). DNA concentrations were measured using Nanodrop UV absorbance spectrophotometry. Real-time PCR primers for mitochondrially encoded NADH dehydrogenase 1 (ND1) (forward, TGACCAACTAATGCACCTCCTA; reverse, GAAAATTGGCAGGGAAATGT) and nuclear-encoded glyceraldehyde phosphate dehydrogenase (GAPDH) (forward, AAACCCATCACCATCTTCCA; reverse, CCTCGAAGTACCCTGTGCAT) were synthesized as previously described (McInerny, Brown and Smith 2009). For qPCR assay, each reaction contained 200 nM each of forward and reverse primers and 20 ng of sample DNA, plus appropriate amounts of Universal Master Mix (Qiagene). The standard cycling conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, and 60°C for 30 s. The mtDNA levels were expressed as relative ND1 levels (ND1/GAPDH).

### **CRISPR/Cas-based knockdown of PK2 in N27 cells**

The lentivirus-based CRISPR/Cas9 KO plasmid, pLV-U6gRNA-Ef1aPuroCas9GFP-PK2, with the PK2 gRNA target sequence CTGTTACACCCGCCCGCCGGGG, was purchased from Sigma-Aldrich. To make lentivirus, the lenti-CRISPR/Cas9 PK2 KO plasmid and control plasmid were transfected into 293FT cells using the Mission Lentiviral Packaging Mix from Sigma-Aldrich according to manufacturer's instructions. The lentivirus was harvested 48 h post-transfection and titers were measured using the Lenti-X™ p24 Rapid Titer Kit (Clontech). For stable knockdown of PK2 in N27 cells, cells were grown in 6-well plates with a seeding density of  $0.1 \times 10^6$  cells/well

in growth media, and lentivirus was added to the media at an MOI of 100 the following morning. After 24 h, fresh media supplemented with puromycin (50  $\mu\text{g}/\text{mL}$ ) was added to the cells for stable cell selection.

### **Plasmid construction, generation and purification of rAAV**

PK2 mRNA sequence was obtained from NCBI and codon-optimized for expression in the mouse using the GeneArt Gene Synthesis service at Life Technologies. The finished plasmid was sent to the Viral Vector Core Facility at the University of Iowa for cloning. Briefly, Kpn1 and Xho1 restriction enzymes were used to subclone the codon-optimized PK2 sequence into the pFBAAV2/5CMVmcsBgHpA viral vector. To generate rAAV, the pFBAAV2/5CMV-PK2-eGFP-BgHpA or AAV2/5CMVeGFP control viral plasmid was transfected into SF-9 insect cells along with the helper plasmid pAAV-RC containing Rep/Cap sequences for viral packaging. The resulting packaged cis-acting AAV baculovirus carries the AAV expression cassette flanked by the AAV inverted terminal repeats (ITR). P1 baculovirus stock was used to amplify the P2 virus. For production of the virus, SF-9 cells were grown in bioreactors and infected with the P2 virus. Cells were harvested after signs of infection and collected in 50 mL tubes. Cells were pelleted and mechanically lysed with glass beads and a bead blender to release the virus. The lysate was treated with detergent to further lyse cells and dissociate virus from membranes and proteins. Benzonase was added to digest genomic, proviral, and plasmid DNA while leaving packaged viral DNA intact. The lysate was then clarified by centrifugation to obtain viral particles. Viral particles were purified using Iodixanol step gradients of 25%, 40%, and 60%, and full viral particles were then collected at the 40-60% interface. The viral particles were further purified using a Mustang Q

Anion Exchange disk as per manufacturer's instructions. A qPCR assay verified AAV2/5CMV-PK2-eGFP-BgHpa viral genome titer of  $2.98 \times 10^{12}$ .

### **Stereotaxic surgery**

C57BL/6NCrl mice (8-10 weeks of age) were anesthetized using a ketamine/xylazine mixture. The Angle 2 stereotaxic instrument was used with a 10-mL Hamilton syringe to inject the rAAV directly into the striatum at the following stereotaxic coordinates in relation to Bregma (mm): -2 ML, 0.5 AP, -4 DV. After drilling a hole in the skull, 3  $\mu$ l of rAAV viral particles ( $\sim 9 \times 10^{12}$  total viral particles) were injected into the brain. The animal was allowed to recover for four weeks to maximize viral gene expression before behavioral testing, sacrificing or any treatment paradigm.

### **Behavioral measurements**

The automated VersaMax Monitor (model RXYZCM-16, AccuScan, Columbus, OH) was used to measure the spontaneous open-field locomotor activity of mice in an activity chamber made of clear Plexiglas and covered with a ventilated Plexiglas lid, as described previously (Ngwa et al. 2013, Harischandra et al. 2014). Horizontal and vertical activity data were collected and analyzed by a VersaMax Analyzer (model CDA-8, AccuScan). Locomotor activities were monitored during a 10-min test session following a 2-min acclimation period. For rotarod experiments, mice were placed on the rod as it rotated at a constant speed of 20 rpm and latency to fall was recorded during 20-min sessions.

### **HPLC analysis of striatal dopamine levels**

HPLC samples were prepared and processed as described previously (Ghosh et al. 2013). Briefly, mice were sacrificed, striata were collected, and neurotransmitters were extracted in 0.2 M perchloric acid solution containing 0.05% Na<sub>2</sub>EDTA, 0.1% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, and isoproterenol (internal standard). Dopamine and metabolites were separated isocratically by a reversed-phase column with a flow rate of 0.6 ml/min using a Dionex Ultimate 3000 HPLC system (pump ISO-3100SD, Thermo Scientific, Bannockburn, IL) equipped with a refrigerated automatic sampler (model WPS-3000TSL). The electrochemical detection system included a CoulArray model 5600A coupled with an analytical cell (microdialysis cell 5014B) and a guard cell (model 5020). Data acquisition and analysis were performed using Chromeleon 7 and ESA Coullarray 3.10 HPLC Software.

### **Immunocytochemistry and histology**

For immunocytochemistry, cells were plated onto coverslips in 24-well plates coated with 0.1% poly-D-lysine. After treatment, 4% formaldehyde was used to fix the cells for 30 min. The cells were washed 4-5 times with PBS. Blocking buffer containing 2% BSA, 0.1 % Triton X-100, and 0.05% Tween 20 was added to the wells for 1 h to permeabilize the cells. The cells were incubated with primary PK2 (Rabbit polyclonal, 1:500 dilution), Bcl-2 (Mouse polyclonal, 1:500 dilution) or c-Myc (mouse monoclonal, 1:2000) antibodies in 2% BSA at 4°C overnight. After 4-5 washes, an Alexa dye-conjugated secondary antibody in 2% BSA was added and incubated at room temperature on a shaker for 1 h. Cells were then washed 4-5 times before adding Hoechst counterstain for 15-20 min to label nuclei. Coverslips were mounted onto slides with Fluoromount mounting media (Sigma). Cells were imaged under an Eclipse TE2000-U microscope (Nikon,

Tokyo, Japan) with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI), and all photomicrographs were processed in MetaMorph 5.7 (Universal Imaging, Downingtown, PA).

For histology, mice were perfused with saline and 4% PFA after being anesthetized using a ketamine-xylazine mixture. Extracted brains were then post-fixed in PFA for 24 h and cryoprotected in 30% sucrose before being embedded in OCT compound, frozen, and sectioned on a Cryostat (CryoStar NX70, Thermo Scientific) at  $-20^{\circ}\text{C}$ . Antigen retrieval was performed using citrate buffer (10 mM sodium citrate, pH 8.5) at  $90^{\circ}\text{C}$  for 30 min. Sections were then washed with PBS and permeabilized with blocking buffer (2% BSA, 0.1% Triton X-100, and 0.05% Tween 20 in PBS) for 1 h at room temperature. Antibodies directed against PK2 (rabbit polyclonal, 1:500), PKR1 (goat polyclonal, 1:1000), PKR2 (rabbit polyclonal, 1:1000), and TH (mouse monoclonal, 1:2000) were incubated with the sections overnight at  $4^{\circ}\text{C}$ . After several washes with PBS, sections were incubated with Alexa dye-conjugated secondary antibodies for 1 h at room temperature. Hoechst stain was added to the sections for 20 min at room temperature to label nuclei. Sections were then mounted on slides using the ProLong<sup>®</sup> Gold antifade mounting medium (Molecular probes) according to the manufacturer's instructions. A SPOT digital camera attached to an inverted fluorescence microscope (Nikon TE2000-U) was used to capture photomicrographs of sections.

DAB immunostaining was performed on striatal and SN sections, as described previously (Ghosh et al. 2013, Roy et al. 2012). Briefly, 30- $\mu\text{m}$  sections were incubated with either anti-TH antibody (Calbiochem, Billerica, MA, USA; rabbit anti-mouse, 1:1800 dilution) or anti-PK2 followed by incubation with biotinylated anti-rabbit or goat or mouse secondary antibody. Separate sections were stained with cresyl violet to count the total number of Nissl-positive neurons in the same animals. Total numbers of TH<sup>+</sup> and Nissl<sup>+</sup> neurons in every 6<sup>th</sup> section of the

SN were counted stereologically with Stereo Investigator software (MBF Bioscience, Williston, VT, USA) using an optical fractionator as described previously (Ghosh et al. 2010).

### **DAergic cell cultures and generation of stable cell lines**

A rat mesencephalic dopaminergic clonal neural cell model (N27 neuronal cells) was a kind gift from Dr. Kedar N. Prasad (University of Colorado Health Sciences Center, Denver, CO) and grown in RPMI 1640 media supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine in 175-mm<sup>3</sup> flasks, as previously described (Clarkson et al. 1998, Kaul et al. 2003, Zhang et al. 2007c). N27 cells were maintained in an incubator at 37°C with 5% CO<sub>2</sub>. To generate N27 cell lines stably overexpressing human PK2, N27 cells were stably infected with lentiviral particles encoding human PK2-GFP or GFP-alone. For the production of lentiviruses, lentiviral constructs (pLenti-PK2-mGFP and pLenti-C-mGFP, Origene, Rockville, MD) were packaged into viruses via transient transfection of the 293FT packaging cells (Invitrogen) using Lipofectamine 2000 reagent and a Lentiviral packaging kit from Systems Biosciences (SBI), as previously described (Jin et al. 2011b). The viruses were collected by centrifuging 48-72 h post-transfection. Transduction of N27 cells was performed at a multiplicity of infection (MOI) of 1 in the presence of polybrene (6 µg/ml). Two days after transduction, GFP-expressing cells were sorted once per week for three weeks by flow cytometry, each time taking the brightest 1% of GFP-expressing cells to guarantee that 100% of the cells were GFP-positive.

The mouse dopaminergic MN9D cell line was kindly obtained from Dr. Syed Ali (National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR) and cultured as described previously (Jin et al. 2011a). MN9D cells stably expressing human PK2 were created by stable transfection with the pCMV6-PK2-myc plasmid or empty vector control (Origene) using



Lipofectamine 2000 reagent according to manufacturer's recommendations. The stable transfectants were selected in 400 µg/ml G418 and further maintained in 200 µg/ml G418 added to MN9D growth media. We used ciprofloxacin for mycoplasma elimination (GenHunter, Nashville, TN). The widely used N27 and MN9D neuronal cell models both represent a homogeneous population of TH-positive dopaminergic cells and are highly useful for studying degenerative mechanisms in PD (Zhang et al. 2007d, Jin et al. 2011a). Primary neurons were obtained from the ventral mesencephalon of gestational 14-day-old (E14) mouse embryos as described previously (Jin et al. 2011b). Quantification of TH<sup>+</sup> cell counts and neuronal processes in primary neuronal cultures was performed as described previously (Ghosh et al. 2013). TH<sup>+</sup> neurons and their processes were counted from three experiments in which over 8 fields were imaged in each well per group.

### **Caspase-3 activity, DNA fragmentation and ATP production**

Enzymatic assays for caspase-3 activity were performed using acetyl-DEVD-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC, 25 µM) as the fluorometric substrate for the reaction, as described previously (Anantharam et al. 2002, Kaul et al. 2003). A Synergy-2 multi-mode microplate reader (BioTek Instruments, Inc) was used to detect fluorescent signals generated upon cleavage of the AFC peptide substrate by caspase-3 with excitation at 510 nm and emission at 400 nm. Caspase-3 activity was normalized by determining protein concentrations with the Bradford assay. For the DNA fragmentation assay, N27 cells were plated in 6-well plates at  $0.8 \times 10^6$  cells/well and treated the next day. At the end of treatment, cells were gently lysed using the lysis buffer provided with the kit. Lysates were then spun down at 200 x g for 10 min to collect supernatants. The extent of DNA fragmentation was then quantified using the Cell Death Detection

ELISA Plus assay kit (Roche Applied Science) according to previously published methods (Anantharam et al. 2002, Kaul et al. 2003, Zhang et al. 2007c). The plates were read by a Synergy-2 multi-mode microplate reader with absorbance at 405 nm and an ABTS solution (reference wavelength at ~490 nm) as a blank. The amount of protein in each sample lysate was used to normalize the raw absorbance values. For measurement of ATP production, cells were plated onto black-walled clear-bottom plates at  $0.7 \times 10^4$  cells/well and exposed to 300  $\mu\text{M}$  of  $\text{MPP}^+$  for 16 h in 2% FBS medium. Following treatment, the plates were equilibrated for 15 min at room temperature. After equilibration, Cell Titer-Glo Luminescent reagent was added and then incubated at room temperature for 30 min to get a steady luminescence signal. Luminescence readout was recorded using the Synergy-2 multi-mode microplate reader.

### **Intracellular ROS generation**

The fluorescent probe 2',7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA) (Calbiochem) was used to determine the level of intracellular reactive oxygen species (iROS). N27 cells were treated with 200  $\mu\text{M}$   $\text{MPP}^+$  in RPMI media with 2% FBS or co-treated with different doses of recombinant PK2 (rPK2), ranging from 5 to 50 nM, along with the  $\text{MPP}^+$  treatment. After treatment for 8 h, the cells were washed with warm HBSS media, and then incubated with 100  $\mu\text{l}$  of 40  $\mu\text{M}$  DCFH-DA in HBSS for 1 h. The fluorescence intensity of the signal was determined using a Synergy-2 multi-mode microplate reader at an excitation of 485 nm and an emission of 530 nm. After subtracting the control fluorescent signal as background, an increase in fluorescence intensity of treatments was expressed as an increase in iROS, as previously described (Qian et al. 2007, Zhang et al. 2005).

### **Fluo-4 calcium mobilization assays**

A Fluo-4 NW assay kit (Molecular probes) was used to test calcium mobilization in N27 cells and primary neurons resulting from nanomolar concentrations of rPK2. Cells were plated overnight into 96-well plates with their respective growth media. Cells were then washed with HBSS and incubated with the Fluo-4 NW dye. Each plate was then read kinetically using a Synergy-2 multi-mode microplate reader. After obtaining background readings by reading each plate every 50 ms for 20 sec, rPK2 was injected at the indicated nanomolar concentrations. The net change in fluorescent signal ( $D_t$ ), read every 50 ms for 3 min, was obtained after subtracting the background from the maximum signal for each sample.

### **Data analysis and statistics**

All *in vitro* data were determined from at least two biologically independent experiments, each done with a minimum of three biological replicates. Data analysis was performed using Prism 4.0 software (GraphPad Software, San Diego, CA). Data were analyzed using one-way ANOVA and then Bonferroni's post-tests or two-way ANOVA to compare all treatment groups. Because our simple one-way and two-way ANOVAs were performed on completely balanced data sets, we ignored group variance inequalities which were all generated by large treatment effects. Where the normality assumption was violated we conducted nonparametric tests, however, in no case did the nonparametric results change the overall interpretation of our parametric results. Differences with  $p < 0.05$  were considered statistically significant. Student's t-test (two-tailed) was used when two groups were being compared.

**Data availability**

All data are available from the authors on request.

**Author contributions**

R.G. conceived, designed and performed experiments, analyzed data and wrote the manuscript. M.L.N. designed, performed experiments and analyzed data. J.L., M.R.L., D.S.H., N.P. and A.C. performed experiments. H.J. and V.A. provided intellectual input on experimental design, data analysis, interpretation, and manuscript preparation. T.M.W. and Q.Y.Z. provided key reagents and assistance with experimental design and interpretation. A.G.K. and A.K. led the investigation, conceived the project and wrote the manuscript. All authors reviewed and edited the manuscript.

**Acknowledgements**

This study was supported by NIH grant NS 78247. The W. Eugene and Linda Lloyd Endowed Chair to AGK and the Dean Endowed Professorship to AK are also acknowledged. We also thank Mr. Gary Zenitsky for assistance in preparing this manuscript.

**Conflicts of Interest**

A.G.K. and V.A. are shareholders of PK Biosciences Corporation (Ames, IA), which is interested in translating mechanistic studies into therapies targeting PK2 signaling. The other authors have no conflicts of interest.

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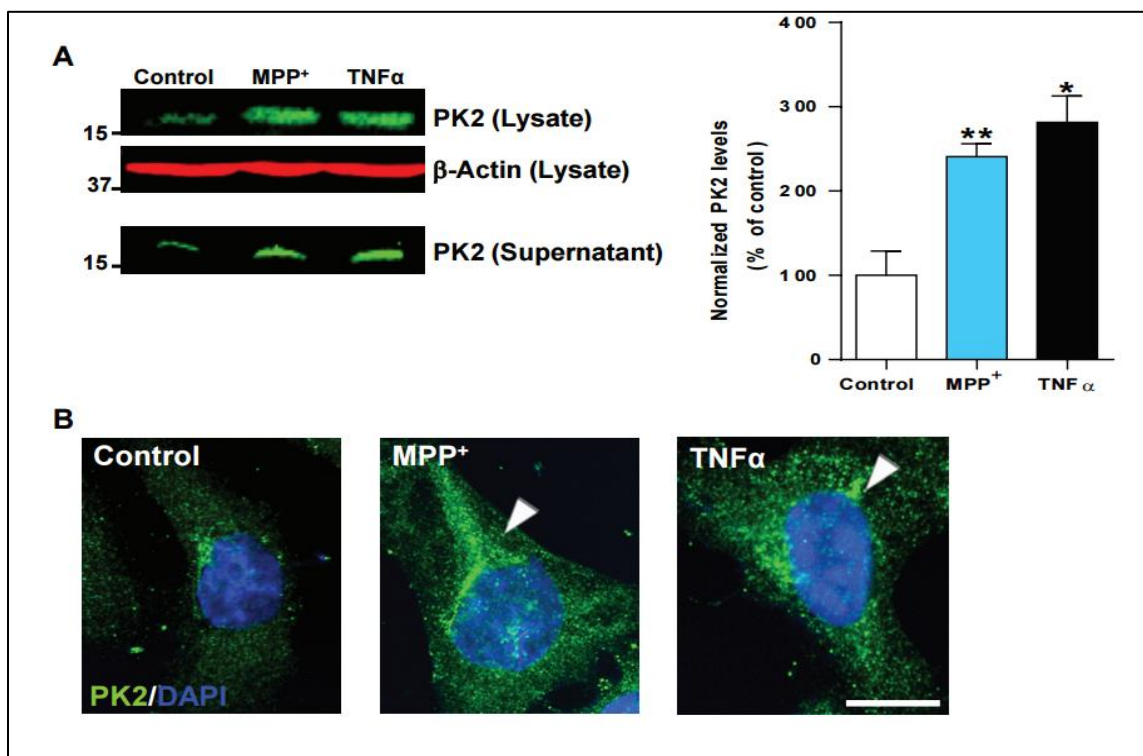
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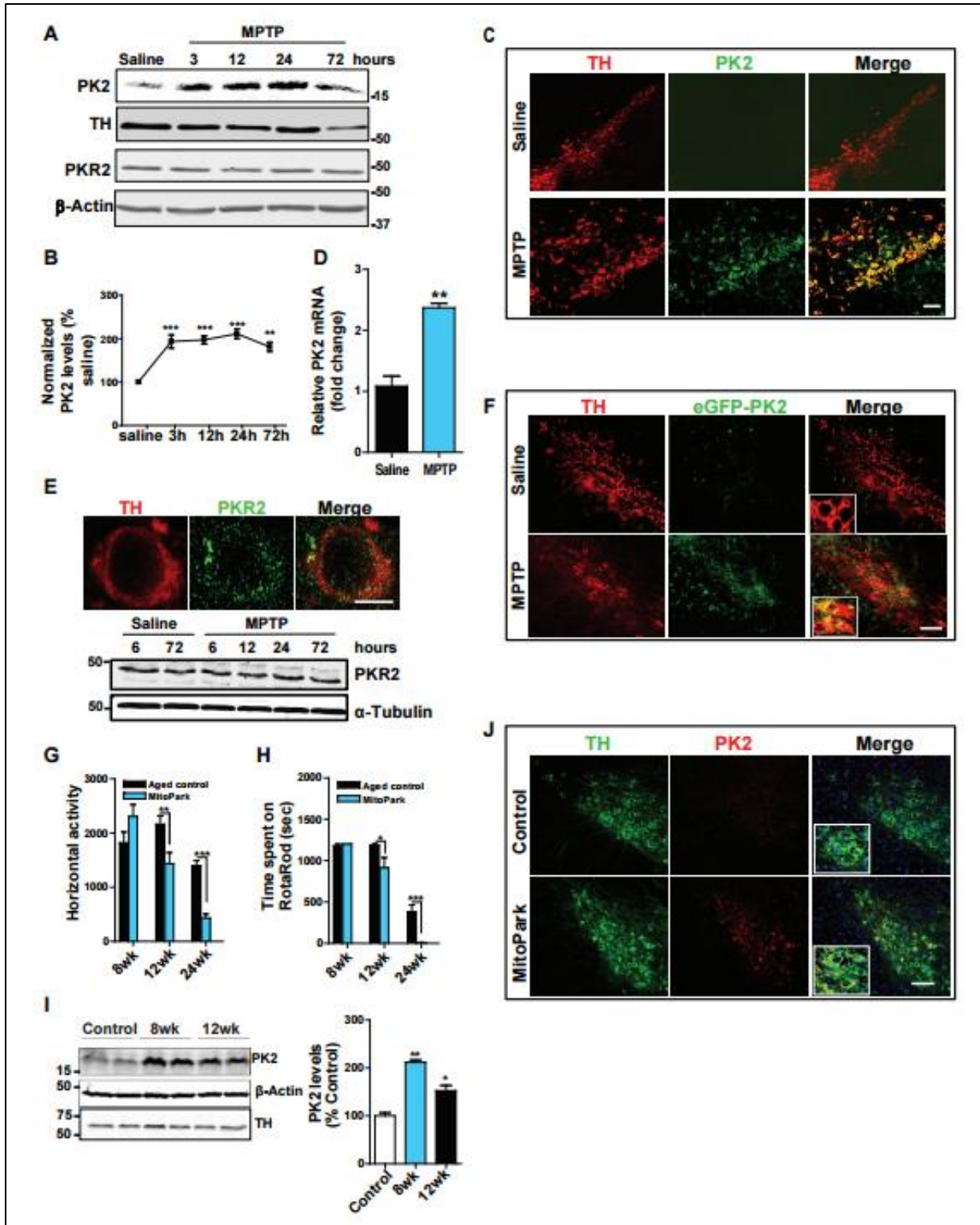
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## Figures



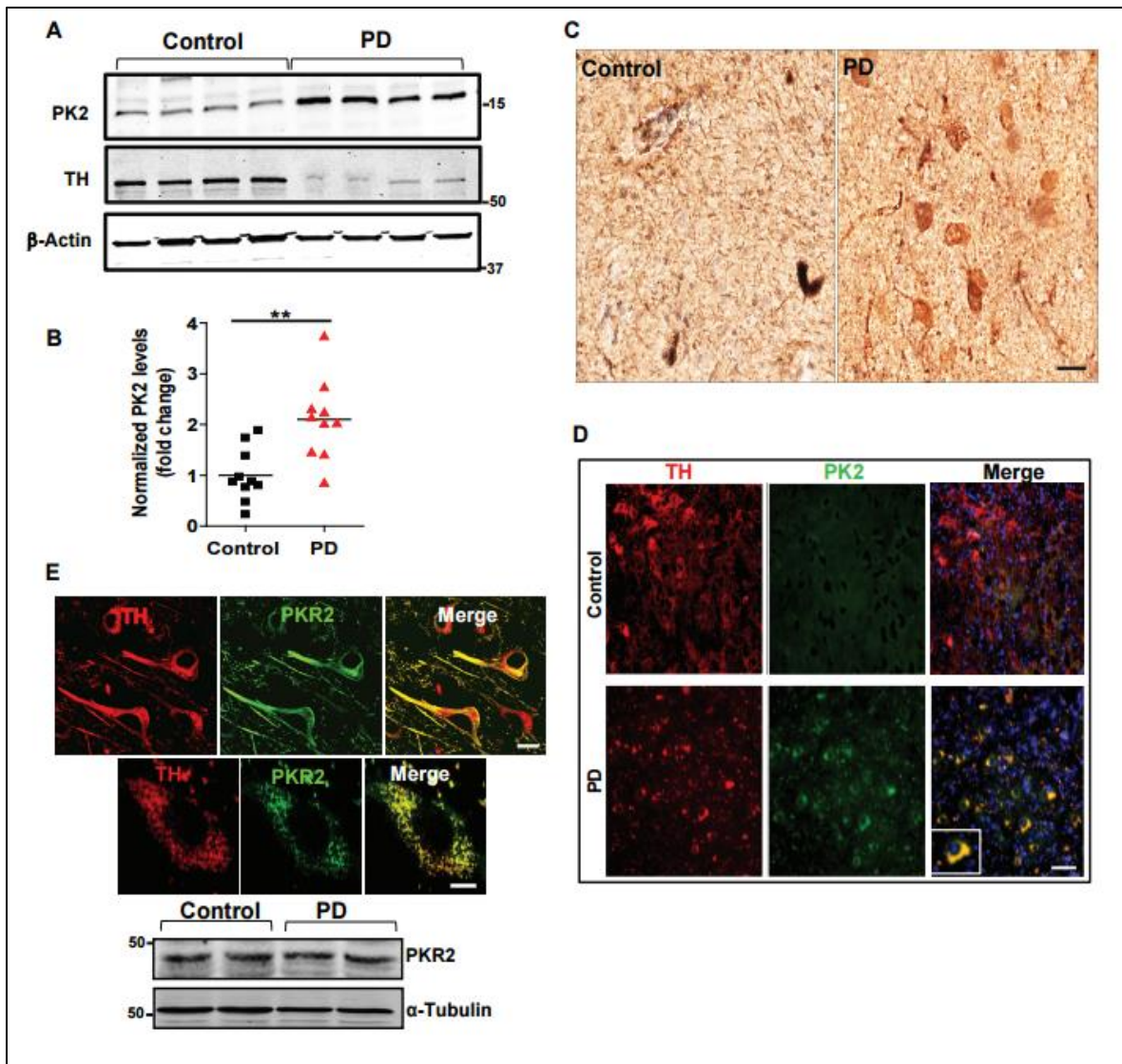
**Figure 1.** *Prokineticin-2 is upregulated and secreted following neurotoxic stress in N27 dopaminergic neuronal cells.* (A) Upregulation and release of PK2 in dopaminergic N27 cells. Representative (left) Western blot analysis of intracellular and secreted PK2 protein following treatment of N27 cells with TNF $\alpha$  (30 ng/ml) and MPP $^{+}$  (300  $\mu$ M) for 12 h. At the end of treatment, supernatants were collected and concentrated using a Vivaspin centrifugal concentrator with a 3-kDa molecular weight cutoff. Experiments were repeated twice with a total n = 3. Data represented as mean  $\pm$  SEM and asterisks denote a significant (\* p<0.05 and \*\* p<0.01) difference between control and TNF $\alpha$ - or MPP $^{+}$ -treated groups using one-way ANOVA. (B) Localization of PK2 (green) in N27 cells was determined via IHC after exposure to TNF $\alpha$  and MPP $^{+}$  for 12 h. Hoechst dye stained the nuclei (blue). Cytosolic expression of PK2 was localized to the peri-nuclear region (white arrows). Scale bar, 10  $\mu$ m.



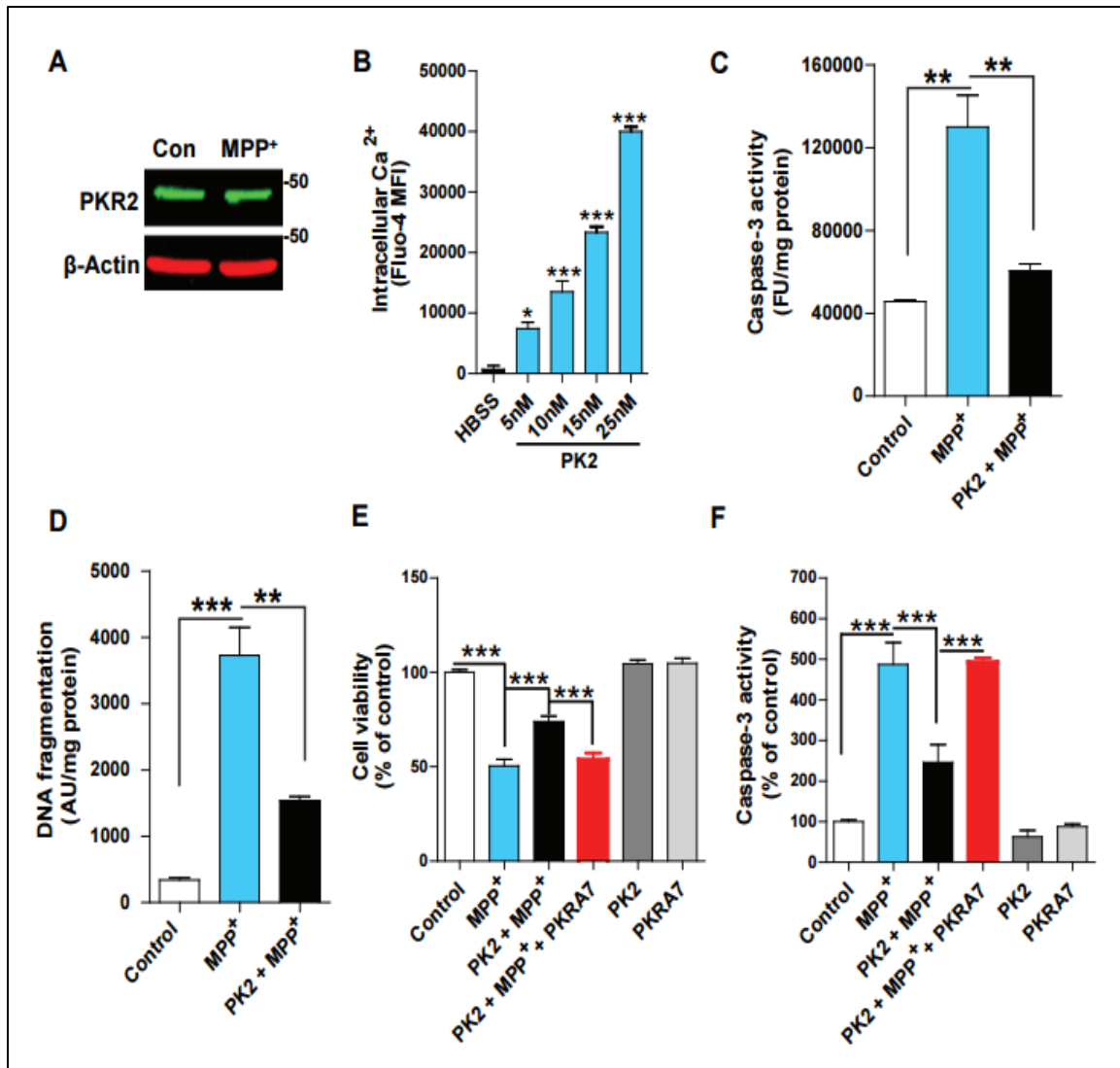


**Figure 2.** *Prokineticin-2 is highly induced in nigral dopaminergic neurons in animal models of Parkinson's disease.* (A-D) Mice were injected with 4 MPTP doses (18 mg/kg, i.p.) at 2-h intervals. (A-B) Representative Western blots (A) of PK2, PKR2 and TH expression in nigral lysates. Mice were sacrificed at 3, 12, 24 and 72 h post-MPTP. Experiments were repeated three times with three mice/group and quantified by densitometric analysis after normalization to  $\beta$ -actin (B). (C) Double-labeling IHC for PK2 and TH in mouse nigral sections stained for TH (red) and PK2 (green). Strong PK2 expression occurred in TH<sup>+</sup> neurons along the nigral tract. Scale bar, 40  $\mu$ m. (D) Real-time PCR for PK2 mRNA expression in the SN, 24 h post-MPTP treatment, with 18s as the internal standard for normalization. Data represented by group mean  $\pm$  SEM from experiments repeated twice with a total n = 4. Asterisks denote a significant difference between control and MPTP treatment (\*\*\*)  $p < 0.001$ ) using Student's t-test. (E) Top: Double-labeling IHC shows expression of PKR2 (green) on TH<sup>+</sup> neurons. Image magnification, 60X. Scale bar, 10  $\mu$ m. Bottom: Western blot for PKR2 in mouse nigral lysates. Mice receiving 4 doses of MPTP (18 mg/kg, i.p.) were sacrificed at the indicated time points. Tubulin was used as the loading control. Representative blots indicate that PKR2 is constitutively expressed. (F) Visualization of PK2 using GENSAT Prok2-eGFP reporter transgenic mice. Coronal sections from saline- or MPTP-treated transgenic mice were stained for TH (red) with eGFP directly imaged at 488nm excitation. Image magnification, 20X. Scale bar, 40  $\mu$ m. Representative images are shown and experiments were repeated at least three times. (G-J) PK2 induced in nigral dopaminergic neurons in transgenic MitoPark mice. Horizontal activity (G) and rotarod performance (H) showing no significant differences in motor function at 8 wk and onset of motor deficits at 12 wk (n = 7-10 mice/group). Data represented as mean  $\pm$  SEM (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) using Two-way ANOVA. (I) Western blot of PK2 and TH expression revealed significantly elevated PK2 in 8- and 12-wk

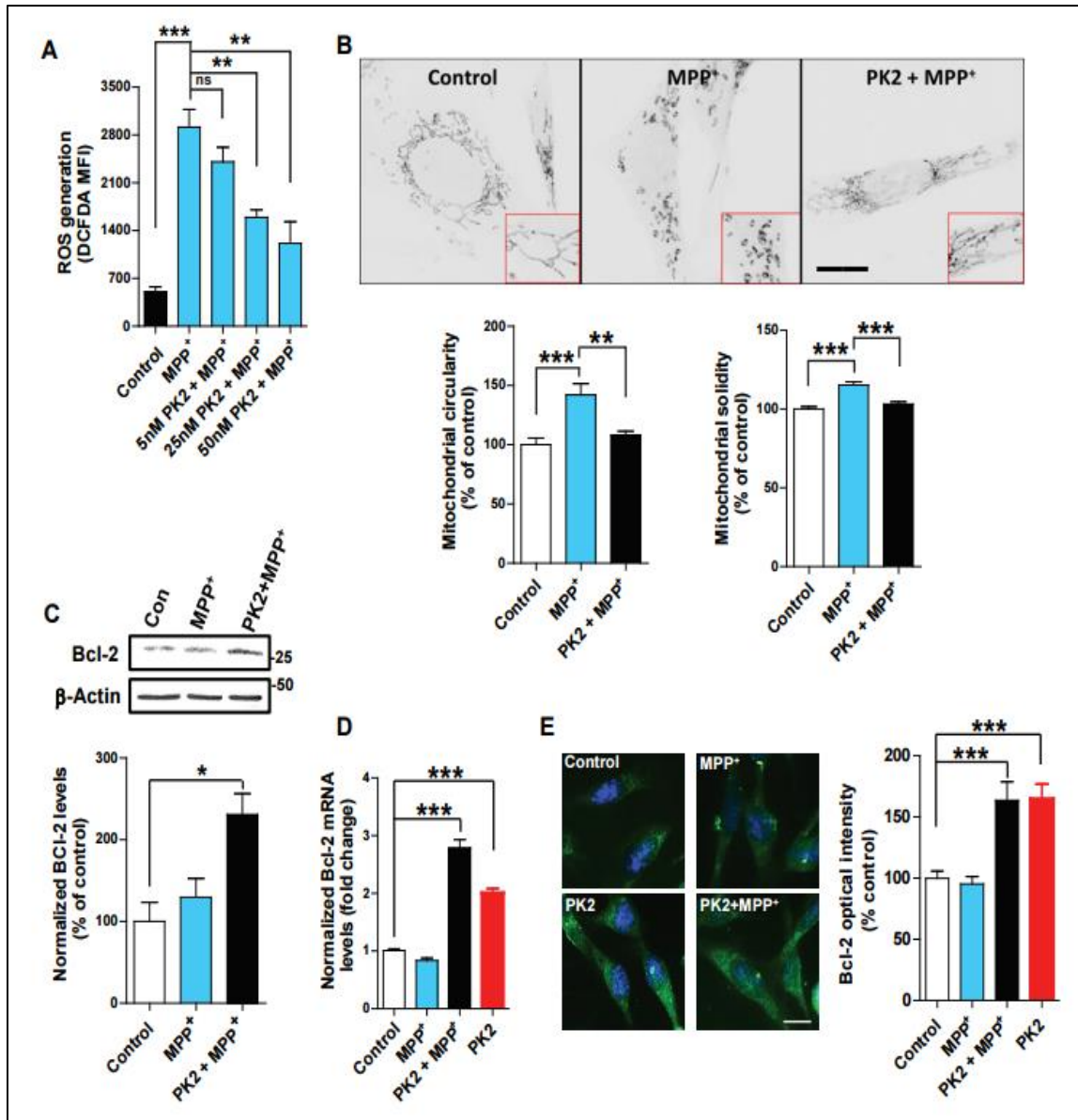
MitoPark mice. Quantification of PK2 band is shown on the right. (J) IHC staining for TH (green) and PK2 (red) showing increased PK2 expression in nigral dopaminergic neurons at 12 wk. Scale bar, 40  $\mu$ m. Data represented as mean  $\pm$  SEM (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001) with an  $n = 2-8$  using one-way ANOVA with Bonferroni post test.



**Figure 3.** *PK2 is elevated in the substantia nigra of human PD patients.* (A) Representative Western blot for PK2 expression in nigral lysates from PD patients and age-matched controls probed using a rabbit polyclonal antibody directed against the human PK2 protein. Increased levels of PK2 were evident in PD patient samples compared to control subjects. A representative TH Western blot is also shown. (B) Densitometric band intensities for PK2, determined from 10 control and 10 PD samples normalized using  $\beta$ -actin as the internal control. Data represented as mean  $\pm$  SEM and expressed as fold change over control with a total n = 10. Asterisks denote a significant (\*\*  $p < 0.01$ ) difference between control and PD samples using Student's t-test. (C) IHC for PK2 in human nigral sections using DAB immunostaining showed increased neuronal PK2 expression in PD brains. Scale bar, 40  $\mu$ m. (D) Double-labeling immunofluorescence for PK2 (Green), TH (Red) and nuclei (blue). Scale bar, 40  $\mu$ m. Representative images from one of three experiments are shown. (E) **Top:** Double-labeling IHC shows expression of PKR2 (green) on human nigral TH<sup>+</sup> (red) neurons. The top panel is 60X magnification to show PKR2 merging with several TH positive neurons (scale bar, 20  $\mu$ m), and the bottom panel is 63X magnification with 2.5X zoom to get a high magnification image of PKR2 in one TH-positive cell (scale bar, 10  $\mu$ m). **Bottom:** Western blots for PKR2 expression in nigral lysates from control and PD brains (3 cases each) revealed that PKR2 was highly expressed in control brains and its expression levels did not change in PD patients. A representative TH Western blot is also shown.



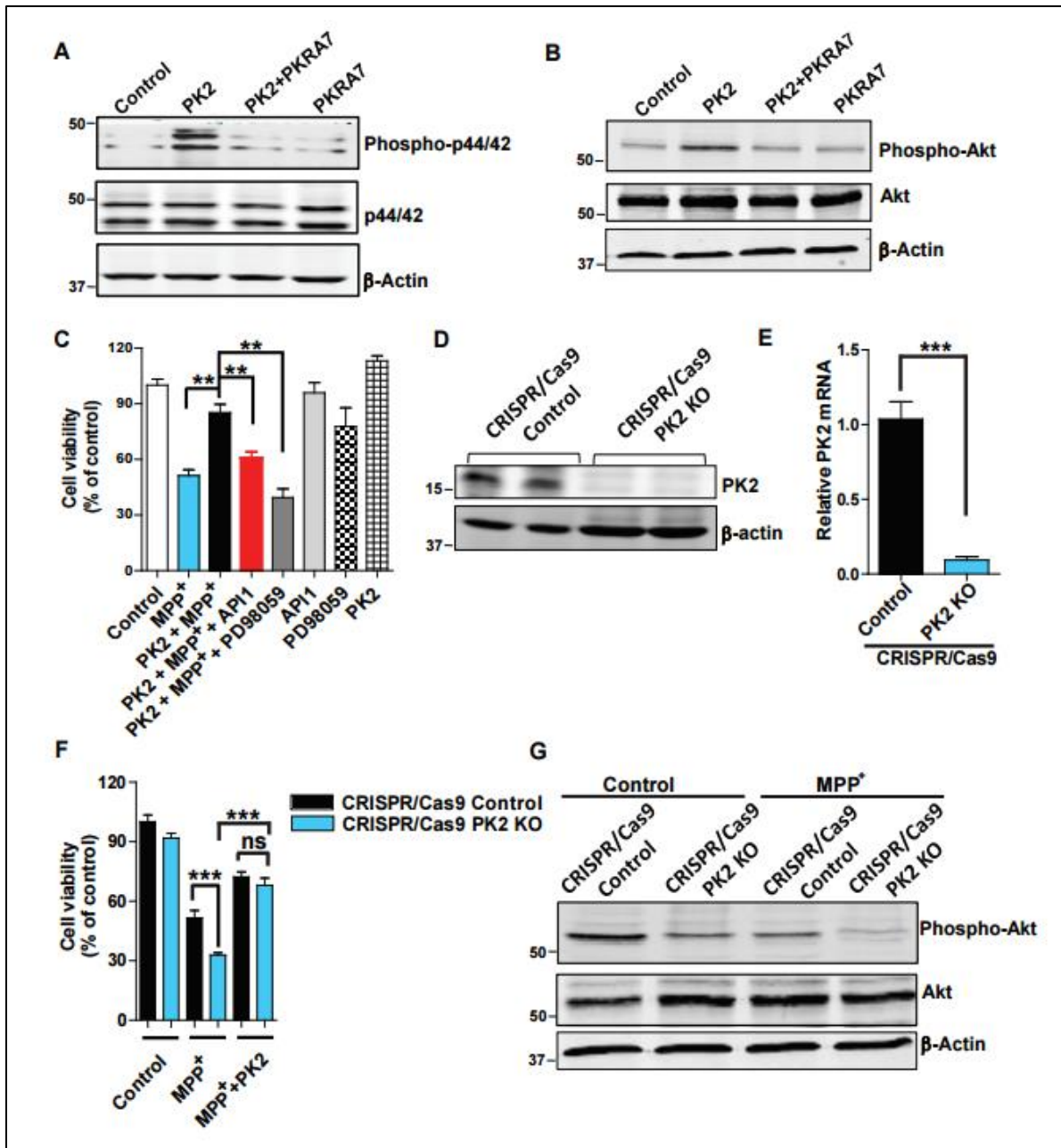
**Figure 4.** *Recombinant PK2 protects dopaminergic N27 cells against MPP<sup>+</sup>-induced dopaminergic neuronal cell death.* (A) Representative Western blot from one of three experiments showing no changes in PKR2 expression during MPP<sup>+</sup>-induced cell death in N27 dopaminergic cells. (B) Calcium mobilization (flux) induced by rPK2 in N27 cells. Calcium-free HBSS was used on the control group (One-way ANOVA with Bonferroni post test and total n = 3). (C-D) Exogenously-stimulated PK2 signaling attenuated caspase-3 activation induced by MPP<sup>+</sup> at 8 h (C) and DNA fragmentation at 16 h after MPP<sup>+</sup> treatment (D). N27 cells were treated with MPP<sup>+</sup> (200  $\mu$ M) or co-treated with PK2 (25 nM) and cells were collected for assays of caspase-3 activity at 8 h and DNA fragmentation at 16 h. The data were expressed as fluorescence units (FU) per mg of protein for caspase-3 activity and absorbance units (AU) per mg protein for DNA fragmentation (One-way ANOVA with Bonferroni post test and total n = 3). (E-F) Co-treatment with the specific PK2 receptor antagonist PKRA7 blocked PK2's neuroprotection in MTS (E) (One-way ANOVA with Bonferroni post test and total n = 4-8) and caspase-3 activity (F) (One-way ANOVA with Bonferroni post test and total n = 3) assays. N27 cells were treated with 200  $\mu$ M MPP<sup>+</sup> for 24 h in the presence or absence of 25 nM PK2 and 2  $\mu$ M PKRA7. Data represented by group mean  $\pm$  SEM (\* p<0.05, \*\* p<0.01 and \*\*\* p<0.001; ## p<0.01 and ### p<0.001 comparing MPP<sup>+</sup> and MPP<sup>+</sup>/PK2 co-treatment groups).



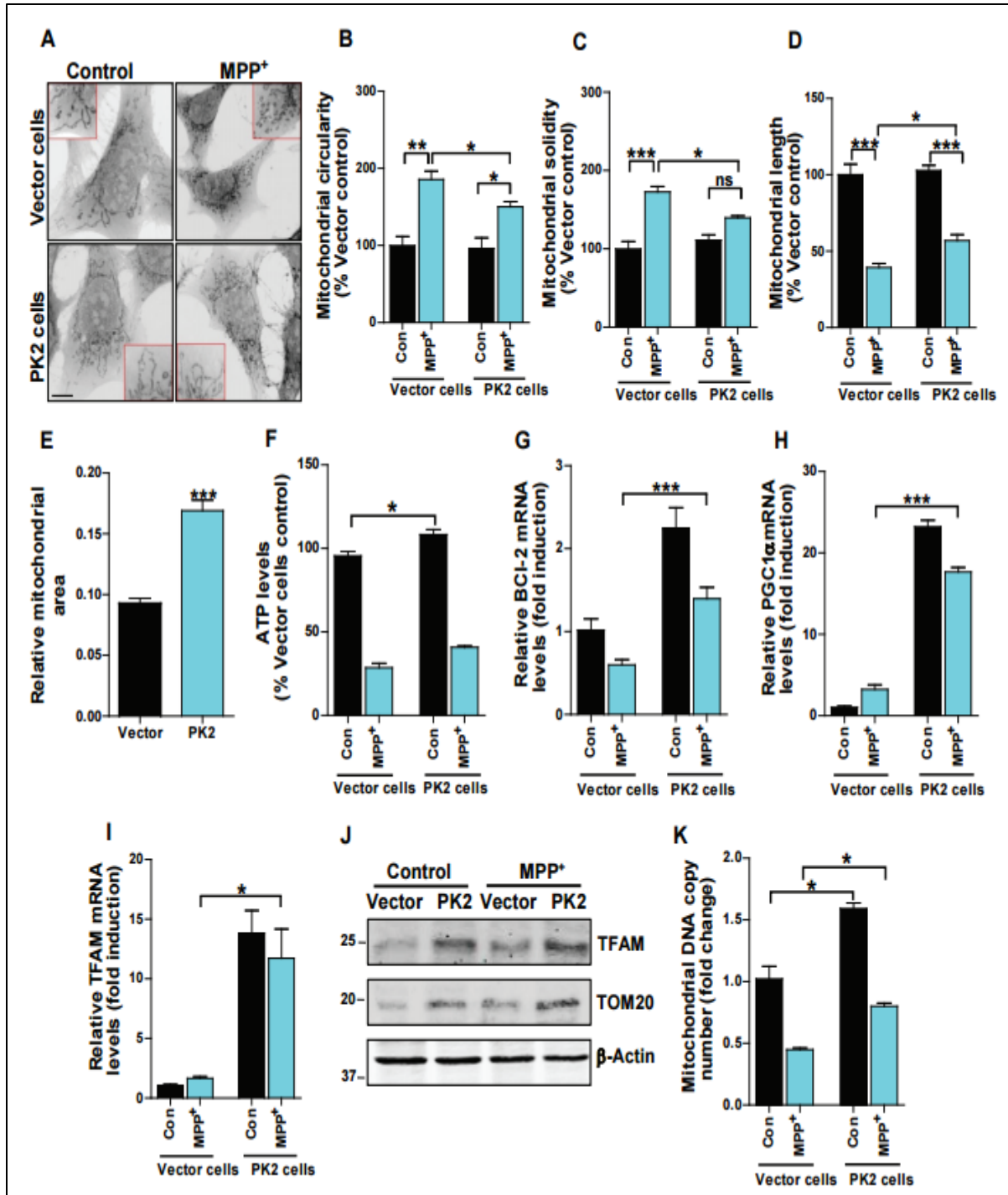


**Figure 5.** *Recombinant PK2 protects dopaminergic N27 cells against MPP<sup>+</sup>-mediated oxidative stress and mitochondrial dysfunction.* **(A)** Co-treatment with nanomolar doses of rPK2 attenuated MPP<sup>+</sup>-induced ROS generation in a dose-dependent manner. N27 cells were treated with 300  $\mu$ M MPP<sup>+</sup> for 8 h and total iROS was determined by quantifying the DCF-DA dye fluorescence using a plate reader with a total n = 3. “ns” = non-significant with an exact p-value = 0.4939. **(B)** PK2 signaling blocked MPP<sup>+</sup>-induced mitochondrial fragmentation. **Top:** N27 cells were treated with MPP<sup>+</sup> (300  $\mu$ M) for 16 h and incubated with MitoTracker Red dye to visualize mitochondria. Scale bar, 20 $\mu$ m. **Bottom:** Morphometric analysis (ImageJ plug-in (Dagda et al. 2009)) of mitochondrial structures showed that PK2 co-treatment significantly reduced MPP<sup>+</sup>-induced mitochondrial circularity (left) and solidity (right) with a total n = 8-10. **(C-E)** PK2 upregulates anti-apoptotic protein Bcl2. Bcl-2 protein (C) (n = 2-3) and mRNA expression (D) (n = 3-15) was determined by Western blotting and qPCR, respectively. PK2 treatment upregulated the basal level of Bcl-2 expression, which remained elevated during treatment with MPP<sup>+</sup>. Bcl-2 expression was also visualized by immunofluorescence (E) with a total n = 3. Scale bar, 10  $\mu$ m. Data represented by group mean  $\pm$  SEM (\* p<0.05 and \*\*\* p<0.001; ## p<0.01 and ### p<0.001 comparing MPP<sup>+</sup> and MPP<sup>+</sup>/PK2 co-treatment groups). All statistics for this figure were determined using one-way ANOVA with Bonferroni post test.



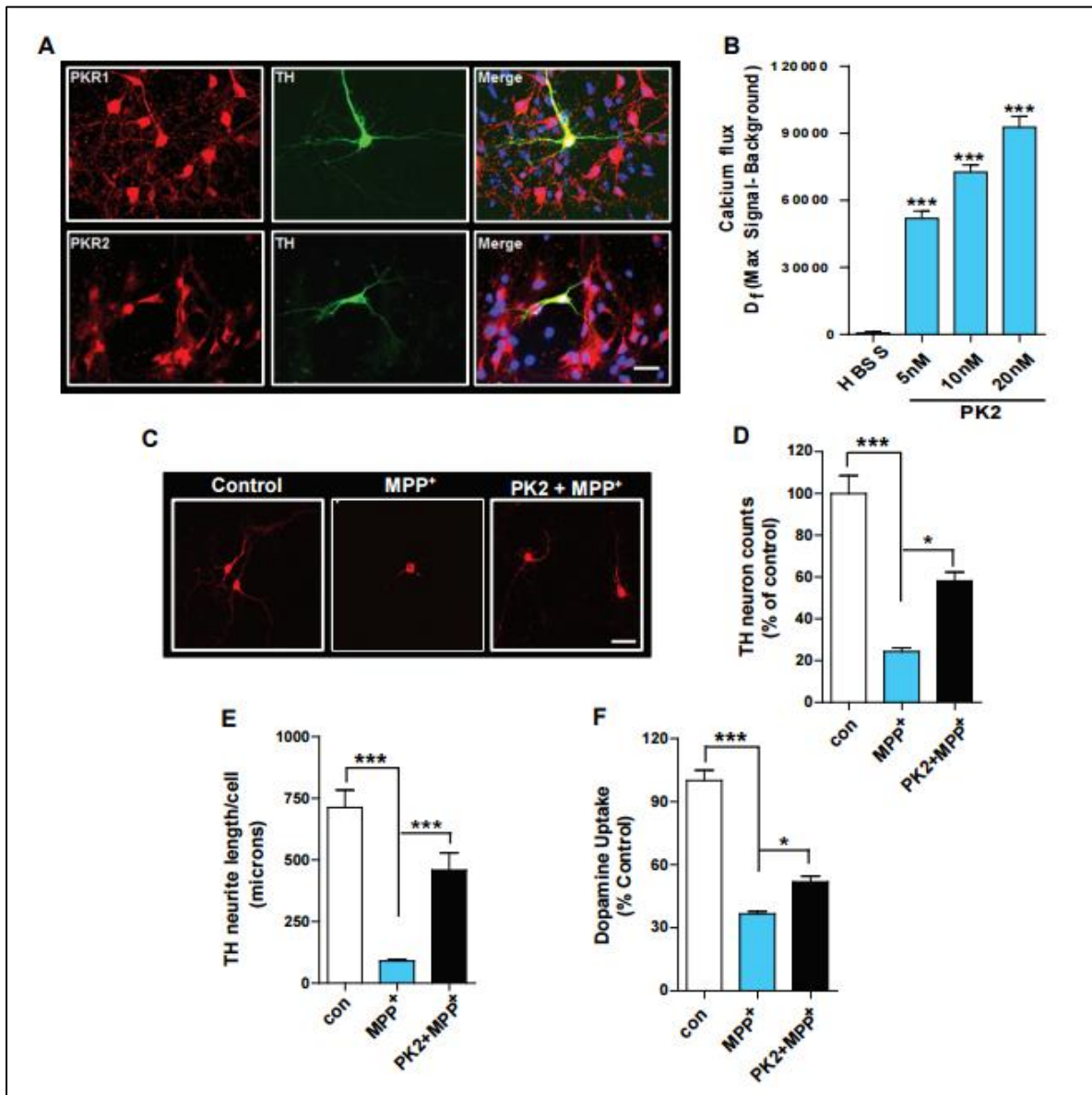


**Figure 6.** Recombinant PK2 (rPK2) activates Akt and ERK (p44/42) signaling pathways to protect N27 cells from MPP<sup>+</sup> toxicity. **(A-B)** N27 cells were untreated or treated with 25 nM rPK2 in the absence or presence of 2  $\mu$ M PKRA7 for 3 h. Cells were collected and subjected to Western blot analysis of ERK (A) and Akt (B) activation. **(C)** N27 cells were untreated or treated with 300  $\mu$ M MPP<sup>+</sup> in the absence or presence of 25 nM rPK2, 100 nM Akt inhibitor API1 or 50  $\mu$ M ERK inhibitor PD98059 for 24 h. Cell viability was then measured by MTS assay. Data are expressed as the mean  $\pm$  SEM of three independent experiments using one-way ANOVA with Bonferroni post test with a total n = 7-22. **(D-E)** CRISPR/Cas9-based knockdown of PK2 in N27 cells infected with CRISPR/Cas9 PK2 KO lentivirus or CRISPR/Cas9 control scrambled lentivirus. PK2 expression was analyzed by Western blot (D) and real time RT-PCR (E) (Student's t-test with total n = 6). **(F-G)** CRISPR/Cas9 PK2 KO or CRISPR/Cas9 control lentivirus-infected N27 cells were treated with or without 300  $\mu$ M MPP<sup>+</sup> in the presence or absence of 25 nM rPK2. Cell viability was analyzed by MTS assay (F) (one-way ANOVA with Bonferroni post test and total n = 8, ns stands for non-significant with an exact p-value = 0.9065) and Akt activation was measured by Western blot (G) 24 h after MPP<sup>+</sup> treatment. Asterisks denote statistical significance (\*, p < 0.05, \*\*, p < 0.01 and \*\*\*, p < 0.001. ns, non-significant).



**Figure 7.** *Stable expression of PK2 protects against MPP<sup>+</sup>-mediated mitochondrial dysfunction and promotes mitochondrial biogenesis.* (A-D) Reduced mitochondrial fragmentation in PK2-overexpressing cells. Mitochondrial fragmentation was visualized using MitoTracker Red in vector and PK2-overexpressing cells treated with MPP<sup>+</sup> (300  $\mu$ M) for 16 h. Scale bar, 10 $\mu$ m. Reduced mitochondrial circularity (B), solidity (C) and increased mitochondrial length (D) were evident in cells overexpressing PK2 (Student's t-test with a total n = 3, ns stands for non-significant with an exact p-value = 0.0767). (E) Increased mitochondrial area in PK2 overexpressing cells. Mitochondrial area was measured by thresholding the MitoTracker Red images to compare to the total area of the cell. PK2 overexpressing cells had significantly more mitochondrial area/total cell area compared to vector cells (Student's t-test with total n = 6-7). (F) Overexpression of PK2 increased ATP levels. PK2-overexpressing and vector cells were exposed to 300  $\mu$ M MPP<sup>+</sup> for 16 h, and the amount of cellular ATP produced was measured using the Luminescent Cell Viability assay kit (One-way ANOVA with Bonferroni post test and total n = 4). (G-K) Overexpression of PK2 upregulated mitochondrial biogenesis pathways. Real-time qPCR analysis of Bcl-2 (G) (Student's t-test with total n = 3), PGC1 $\alpha$  (H) (One-way ANOVA with Bonferroni post test and total n = 3) and TFAM (I) (One-way ANOVA with Bonferroni post test and total n = 3-6) shows increased mRNA expression of Bcl-2, PGC1 $\alpha$  and TFAM in PK2 cells compared to vector cells 24 h post-MPP<sup>+</sup> (300  $\mu$ M). Increased TFAM and TOM20 protein levels were found in PK2 cells compared to vector cells 24 h post-MPP<sup>+</sup> (300  $\mu$ M) (J). Analysis of mitochondrial ND1 as a marker of mitochondrial DNA content shows increased mitochondrial DNA copy number (K) in PK2 cells

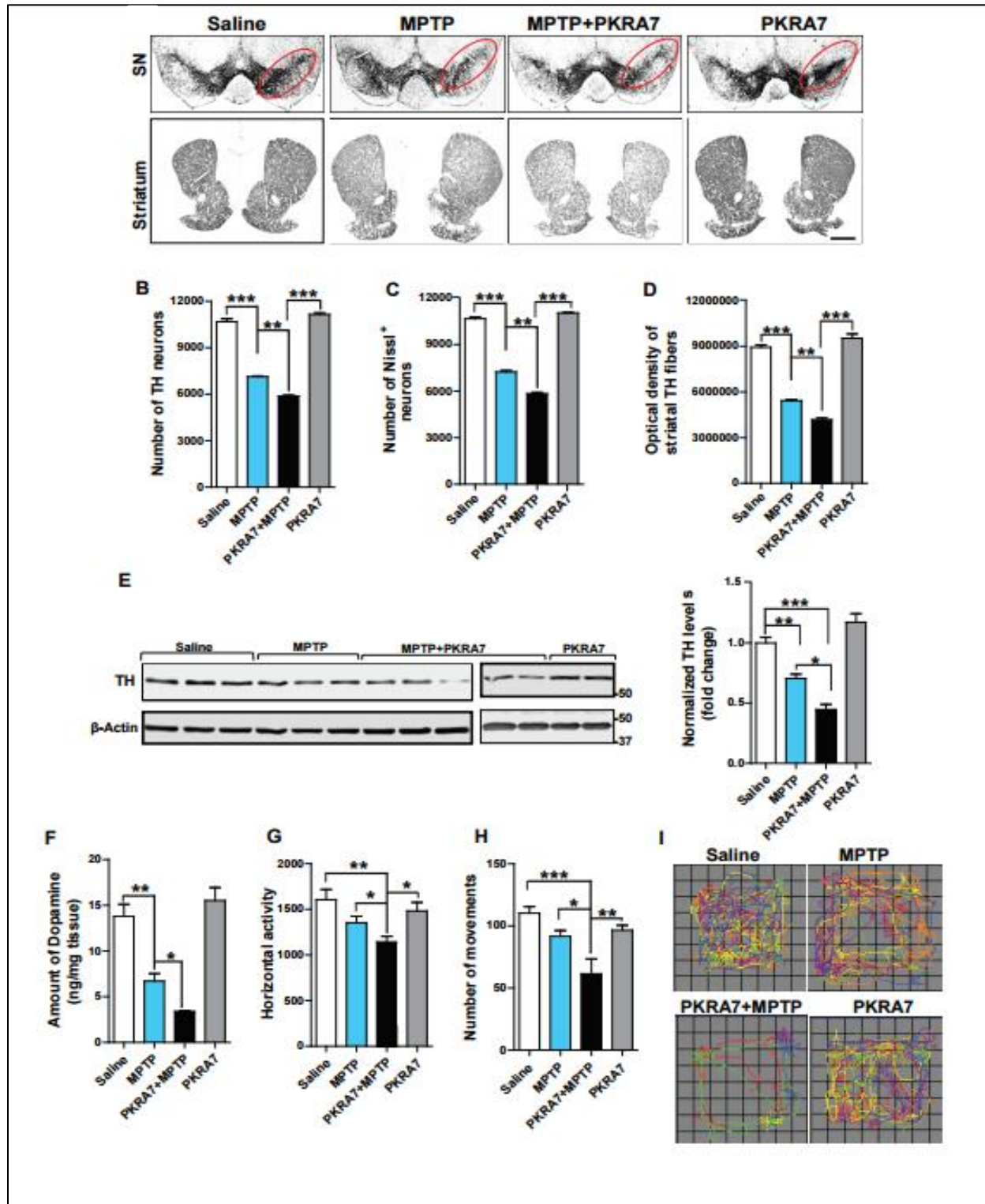
compared to vector cells 24 h after 300- $\mu$ M MPP<sup>+</sup> (One-way ANOVA with Bonferroni post test and total n = 6). Data represented by group mean  $\pm$  SEM (\* p<0.05, \*\* p<0.01 and \*\*\* p<0.001, relative to relevant control or MPP<sup>+</sup>-treatment groups).



**Figure 8.** *Recombinant PK2 protects primary dopaminergic neurons from MPP<sup>+</sup> neurotoxicity.*

(A) Expression of prokineticin receptors on primary dopaminergic neurons. Primary mesencephalic neuron cultures were stained for PKR1 and PKR2 (red) and TH (green). Both PKR1 and PKR2 were expressed on TH<sup>+</sup> dopaminergic neurons. Scale bar, 10  $\mu$ m. (B) Calcium mobilization by rPK2 in primary mesencephalic neuron cultures. Primary neuron cultures were treated with nanomolar doses of rPK2 and the calcium flux was quantified via Fluo-4 NW calcium assay. Control cells were treated with calcium-free HBSS **containing** PK2. Calcium-flux was expressed as the net change in fluorescence signal ( $D_f$ ) represented as the maximum signal after baseline subtraction for each dose of PK2. Calcium was mobilized by PK2 in a dose-dependent manner. Data represented by group mean  $\pm$  SEM. Asterisks denote significant (\*\*\*)  $p < 0.001$ ) differences between HBSS-treated controls and PK2-treated groups. (C) Immunocytochemistry for TH<sup>+</sup> dopaminergic neurons in EVM cultures treated with MPP<sup>+</sup> and PK2. The PK2-treated cultures significantly improved dopaminergic neuron survival and function compared to MPP<sup>+</sup>-alone, showing less degeneration of dopaminergic cell bodies and neurites. Representative images of dopaminergic neurons in primary EVM cultures were acquired at 20X using a Nikon TE2000-U fluorescence microscope. MPP<sup>+</sup> caused extensive degeneration of dopaminergic neurons, which was attenuated by rPK2. Scale bar, 10  $\mu$ m. (D-F) TH<sup>+</sup> neuron counts (D) (One-way ANOVA with Bonferroni post test and total n = 4), TH<sup>+</sup> neurite length measurements (E) (One-way ANOVA with Bonferroni post test and total n = 3) and dopamine uptake functional assay (F) (Student's t-test with total n = 3) in primary EVM neuronal cultures. Primary EVM cultures were treated with MPP<sup>+</sup> (5  $\mu$ M) for 48 h or co-treated with rPK2 (25 nM), which was added initially and again 24 h later. Data represented by group mean  $\pm$  SEM. Asterisks denote significant (\*\*\*)  $p < 0.001$ )

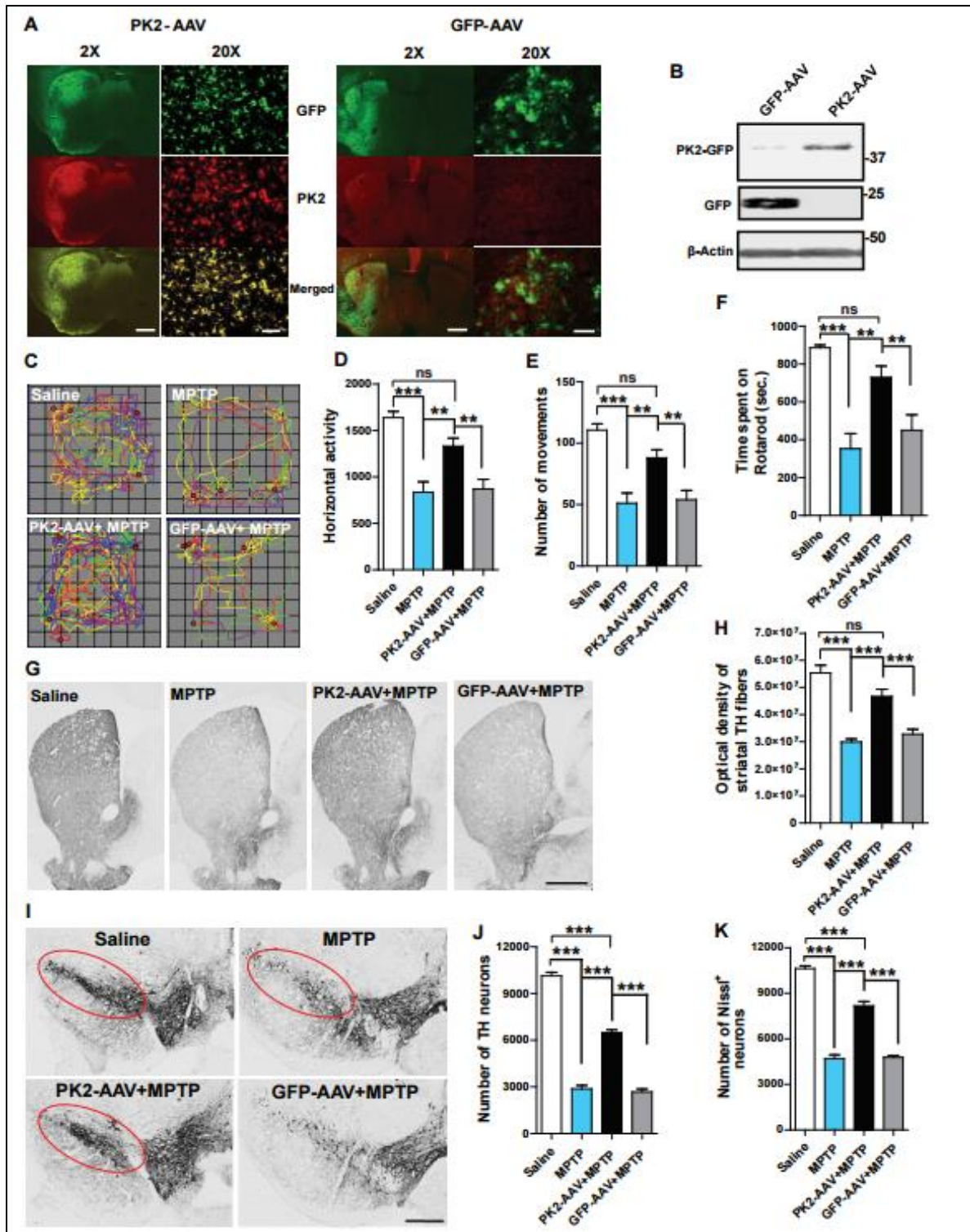
differences between the control and MPP<sup>+</sup>-treated groups. # and ### denotes  $p < 0.05$  and  $p < 0.001$  between the MPP<sup>+</sup> and MPP<sup>+</sup>/PK2 co-treatment groups.



**Figure 9** *Inhibition of endogenous PK2 signaling by a receptor blocker exacerbates MPTP-induced toxicity in mice.* Mice were intraperitoneally injected with 18 mg/kg MPTP or equal volumes of saline (vehicle), once daily for three consecutive days. PKRA7 (20 mg/kg/day) was given i.p. 24 h before MPTP treatment and continued once daily for 10 consecutive days until sacrifice. **(A)** TH-DAB immunostaining in the substantia nigra (SN) and striatum (2X magnification). Scale bar, 200  $\mu$ m. **(B-C)** Stereological counts of TH-positive (B) and Nissl-positive (C) neurons in the SN of ventral midbrain. **(D)** Integrated density quantification of DAB immunostaining in the striatum. Data represented by group mean  $\pm$  SEM of 3 mice per group using one-way ANOVA with Bonferroni post test and total n = 3 (\*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ , relative to relevant control or MPTP-treatment groups). **(E)** Western blot analysis of SN samples probed for TH and its quantification (right panel) show reduced TH levels when PKRA7 is administered along with MPTP. Data represented by group mean  $\pm$  SEM using one-way ANOVA with Bonferroni post test and 5-7 mice per group (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ , relative to control or MPTP-treated groups). **(F)** Western blot analysis of SN samples demonstrates that PKRA7 treatment does not change PK2 or PKR2 levels. Data represented by group mean  $\pm$  SEM using one-way ANOVA with Bonferroni post test and 4 mice per group. **(G)** Compared to MPTP-alone, PKRA7 treatment significantly reduced striatal dopamine levels, as measured using high-performance liquid chromatography (HPLC). Data represented by group mean  $\pm$  SEM using Student's t-test and a total n = 2-3 mice per group (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ , relative to relevant control or MPTP-treatment groups). **(H-J)** Mice were tested for motor activities 7 days



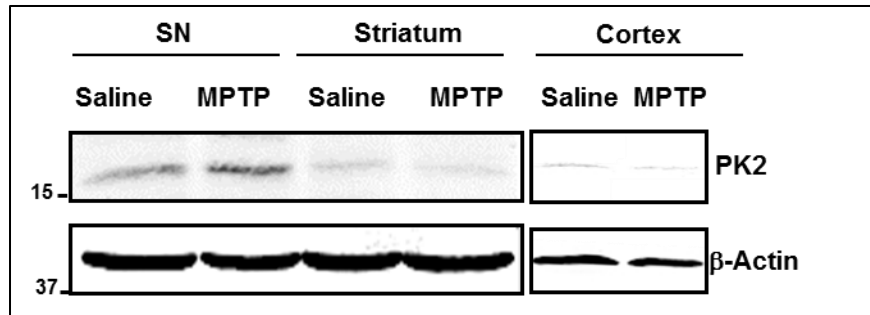
after the last dose of MPTP. (J) Representative movement tracks of mice are shown. Behavior analysis showing horizontal activity (H) (Student's t-test with total  $n = 7$ ) and number of movements (I). Data represented as the mean  $\pm$  SEM using one-way ANOVA with Bonferroni post test and total  $n = 7-8$  mice per group (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).



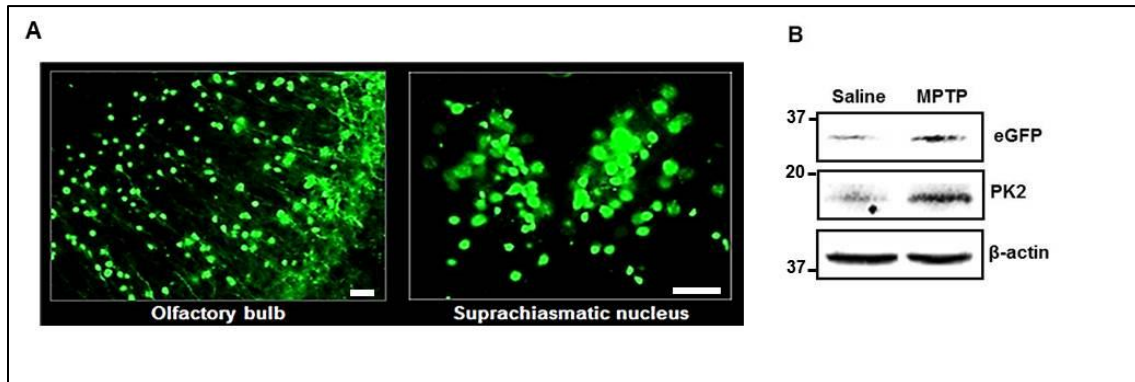
**Figure 10.** *PK2 overexpression protects against MPTP-induced neurotoxicity in mice.* Mice were stereotaxically injected in the striatum with either GFP-AAV or GFP tagged PK2-AAV and allowed to reach maximal viral expression for four weeks. The mice were then intraperitoneally

injected with 20 mg/kg MPTP or equal volumes of saline (vehicle), once daily for four consecutive days and sacrificed 24 h after the last injection. **(A)** Expression of PK2 virus in the striatum by immunofluorescence microscopy. Brain sections were probed with anti-GFP (Green) and anti-PK2 (Red) antibodies and imaged to show that the two proteins co-localize together (2X and 20X images). Scale bar, 200  $\mu\text{m}$  (2 X images); 40  $\mu\text{m}$  (20 X images). **(B)** PK2 overexpression levels by Western blotting. Striatal protein lysates probed for either GFP or PK2 show that the PK2 virus was able to overexpress PK2 in the striatum. Since GFP was directly fused to PK2, we observed only one band corresponding to the tagged PK2-GFP fusion protein and did not detect a separate 25-kDa GFP band in the GFP tagged PK2-AAV samples. **(C-F)** Mice were tested for motor activities 1 day after the last dose of MPTP. **(C)** Representative VersaPlot movement tracks of mice are shown. Behavioral data showing horizontal activity **(D)**, number of movements **(E)** and rotarod activity **(F)**. Data represented by group mean  $\pm$  SEM using one-way ANOVA with Bonferroni post test and a total  $n = 12-24$  mice per group (\*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ , relative to relevant control or MPTP-treatment groups). “ns” stands for non-significant with an exact  $p$ -value = 0.0912 **(D)**, 0.1048 **(E)**, 0.55 **(F)**, and 0.054 **(H)**. **(G-J)** PK2-AAV protects against MPTP-induced loss of dopaminergic neurons. **(G)** TH-DAB immunostaining in the striatum (2X magnification). Scale bar, 200 $\mu\text{m}$ . **(H)** Integrated density quantification of DAB immunostaining in the striatum (One-way ANOVA with Bonferroni post test and a total  $n = 12-16$ ). **(I)** TH-DAB immunostaining (2X magnification) in the substantia nigra (SN). Scale bar, 100 $\mu\text{m}$ . **(J-K)** Stereological counts of TH-positive **(J)** and Nissl-positive **(K)** neurons in the SN of the ventral midbrain. Data represented by group mean  $\pm$  SEM using one-way ANOVA with Bonferroni post test and a total  $n = 3$  mice per group (\*\*\*  $p < 0.001$ , relative to relevant control or MPTP-treatment groups).

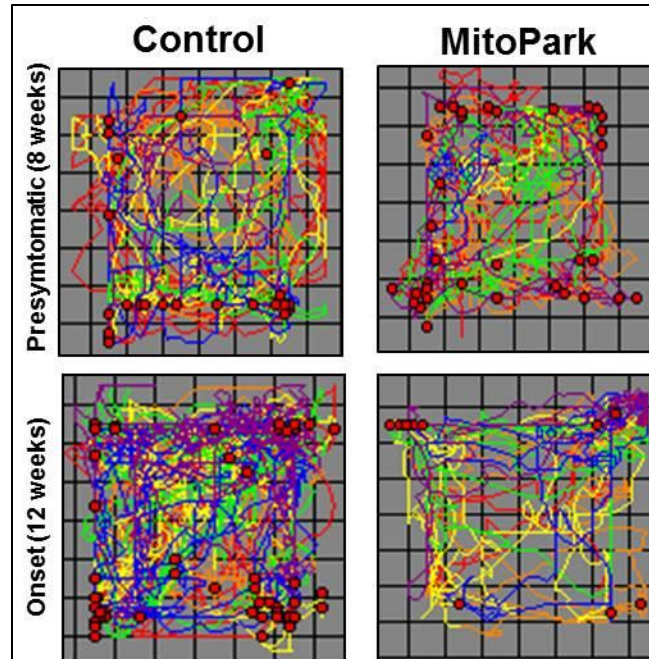
## Supplemental Figures



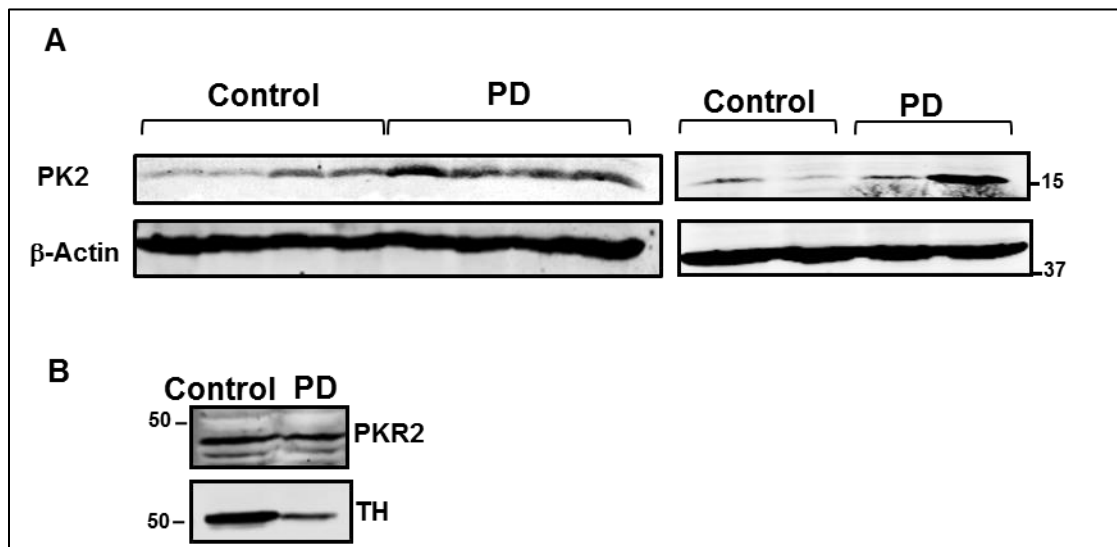
**Supplementary Figure 1.** Prokineticin-2 is induced in the substantia nigra, but not in the striatum, in MPTP-treated mice. C57BL/6 mice were injected with 4 doses of MPTP (18 mg/kg, i.p.) each separated by 2-h intervals. Representative Western blots of PK2 expression in nigral, striatal and cortical lysates.



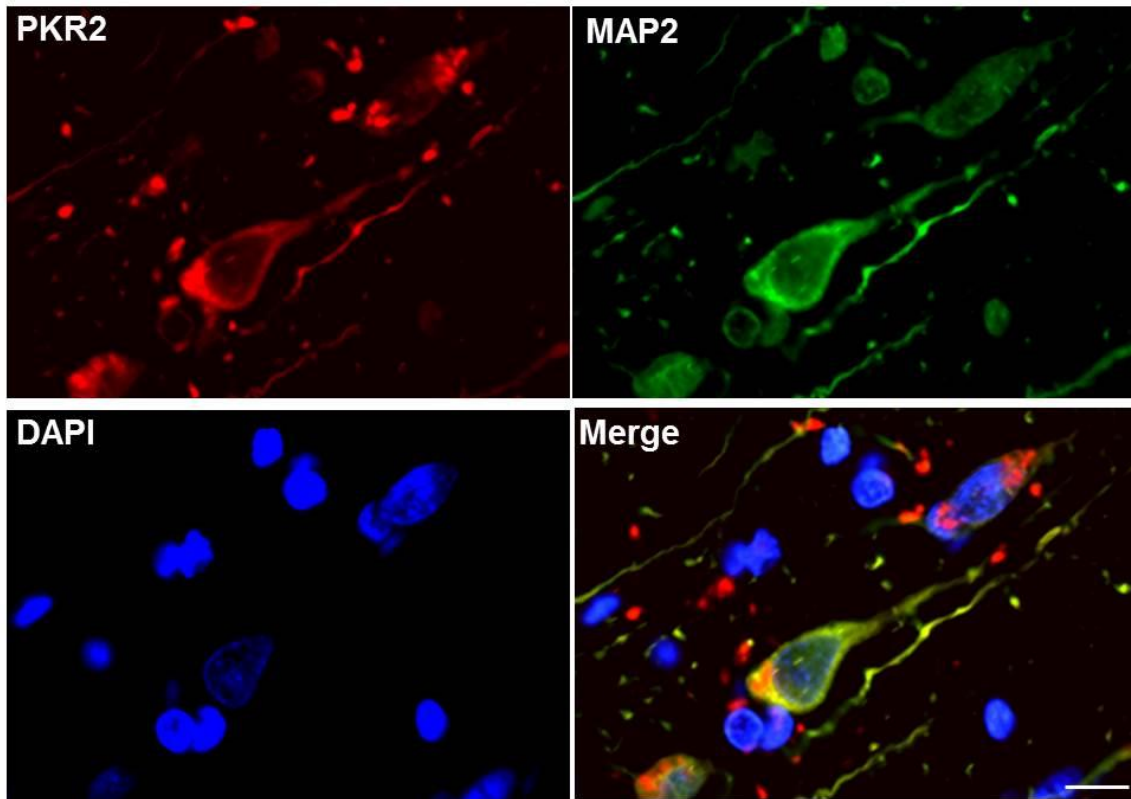
**Supplementary Figure 2.** (A) Direct visualization of eGFP-PK2 in the olfactory bulb and suprachiasmatic nucleus where PK2 is known to be constitutively expressed at high levels in the adult PK2-eGFP reporter transgenic mouse brain. Representative images are shown. Scale bars, 40  $\mu$ m. (B) Saline- or MPTP-treated transgenic mice were sacrificed 24 h after the last injection and subjected to immunoblotting analysis of eGFP and PK2 expression in substantia nigra;  $\beta$ -Actin was used as a loading control.



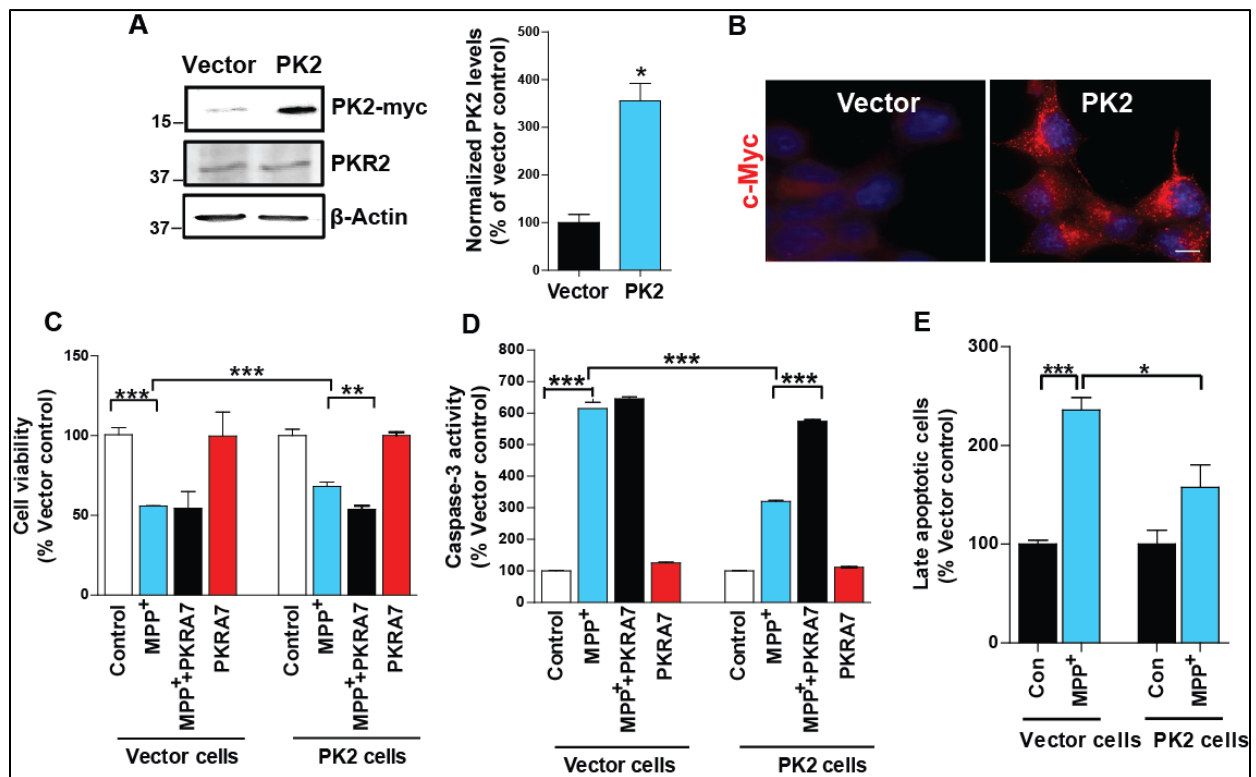
**Supplementary Figure 3.** Spontaneous locomotor activity plots of MitoPark mice and age-matched controls at 8 weeks (presymptomatic) and 12 weeks (onset) showing decreased activity in MitoPark mice with disease onset at 12 weeks.



**Supplementary Figure 4.** Additional Western blots of PK2 (**A**) and PKR2 (**B**) expression in nigral lysates PD patients and age-matched controls.



**Supplementary Figure 5.** PKR2 is expressed ubiquitously in MAP2-positive neurons in the substantia nigra of human brains. Double-labeling immunofluorescence for PKR2 (Red), MAP2 (Green) and nuclei (blue) in substantia nigra of human brains. Scale bar, 10  $\mu$ m. Representative images are shown.

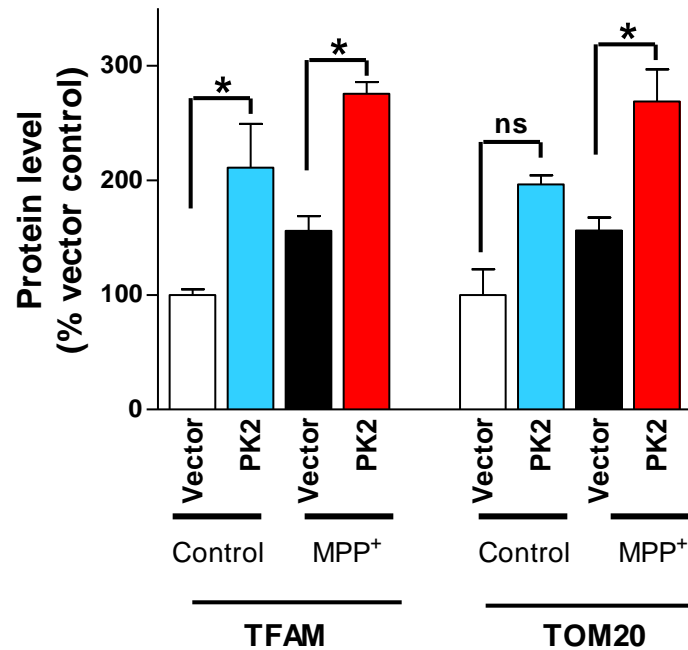


**Supplementary Figure 6. Stable expression of PK2 protects against dopaminergic cell death.**

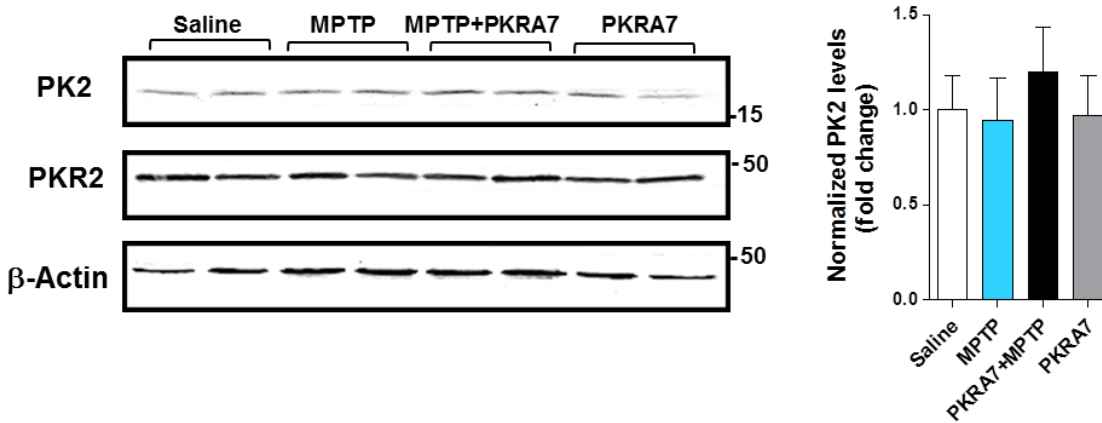
**(A)** Stable expression of PK2 in dopaminergic MN9D cells was confirmed by Western blot. Overexpression of PK2 did not affect PK2 receptor expression. Densitometric analysis of PK2 band intensity is shown in right panel with a total  $n = 2$  and analyzed using student's t-test. **(B)** Immunofluorescence staining for PK2-myc expression in vector and PK2 stable cells using an myc-antibody. Scale bar, 10 μm. **(C-D)** MN9D cells expressing PK2 were partially protected from MPP<sup>+</sup>-induced neurotoxic insult as assessed by MTS (C) (student's t-test;  $n = 3-5$ ) and caspase-3 activity assays (D) (one-way ANOVA with Bonferroni post-test;  $n = 3$ ). The protective effect of PK2 overexpression was abolished using the specific PK2 receptor antagonist PKRA7 (2 μM). **(E)** The number of late-apoptotic cells following MPP<sup>+</sup> treatment was abolished in PK2-



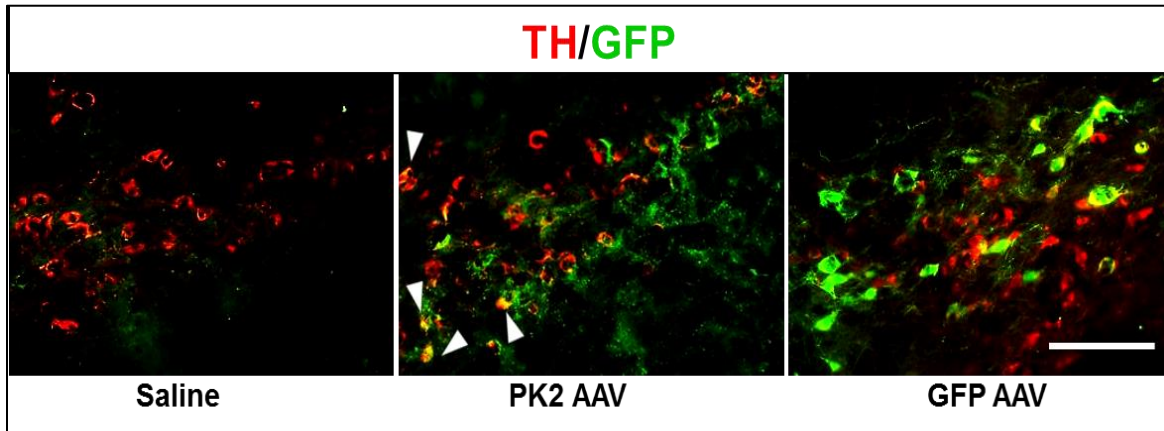
overexpressing cells at 24 h, as quantified by flow cytometric analysis of Annexin V staining (one-way ANOVA with Bonferroni post-test;  $n = 3$ ). Data represented by group mean  $\pm$  SEM and asterisks denote statistical significance (\*  $p < 0.05$  and \*\*\*  $p < 0.001$ , relative to relevant control or MPP<sup>+</sup>-treatment groups).



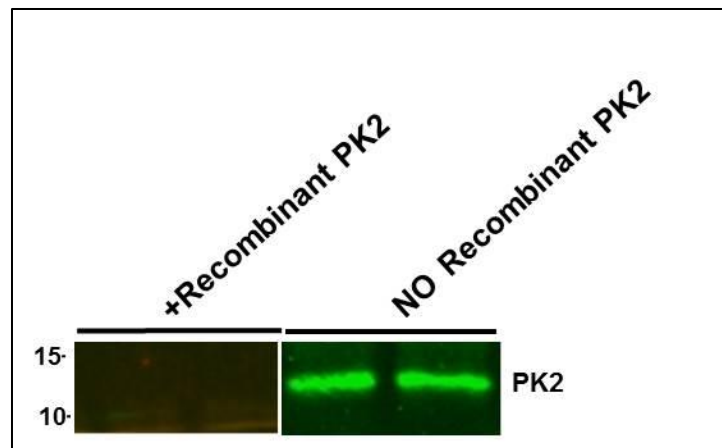
**Supplementary Figure 7.** Densitometric analysis of the Western blots in Figure 7J. Data are representative of at least three independent experiments (Means  $\pm$  SEMs of three samples) (\*  $p < 0.05$ , relative to Vector control or MPP<sup>+</sup>-treatment groups, ns, non-significant  $p = 0.1618$ ).



**Supplementary Figure 8.** Mice were intraperitoneally injected with 18 mg/kg MPTP or equal volumes of saline (vehicle), once daily for three consecutive days. PKRA7 (20 mg/kg/day) was given i.p. 24 h before MPTP treatment and continued once daily for 10 consecutive days until sacrifice. Western blot analysis of SN samples demonstrates that PKRA7 treatment does not change PK2 or PKR2 levels. Data represented by group mean  $\pm$  SEM using one-way ANOVA with Bonferroni post-test and 4 mice per group.



**Supplementary Figure 9.** Expression of GFP-PK2 in the substantia nigra following PK2-AAV injection into the striatum at 4 weeks post injection (white arrows). Double labeling immunofluorescence for GFP (Green) and TH (Red) in substantia nigra region of mouse brains stereotaxically injected with Saline, PK2-AAV, or GFP-AAV in the striatum. Scale bar, 40  $\mu$ m.



**Supplementary Figure 10.** Rabbit PK2 polyclonal antibody was pre-absorbed by incubating 5  $\mu$ g of antibody together with 25  $\mu$ g of recombinant PK2 protein overnight and Western blot was run with N27 cell lysates and incubated overnight with either pre-absorbed antibody (left) or normal antibody (right).

**Supplementary Table 1.** TNF $\alpha$ -induced PK2 gene upregulation in N27 dopaminergic cells in qPCR array

<b>Gene</b>	<b>Fold change (TNF<math>\alpha</math>/Control)</b>
<i><b>Prok2</b></i>	6.81
<i>Ripk2</i>	3.80
<i>Tnfrsf5</i>	2.86

PK2 was discovered as a candidate in a Qiagen mouse apoptosis qPCR array study of 84 apoptosis-related genes. The three most upregulated genes are shown with PK2 mRNA expression induced by over 6-fold.

**Supplementary Table 2.** Human post-mortem tissues used for immunoblot and IHC in Fig. 3 and supplementary Fig. 4. (N/A= not available)

<b>Sample Code</b>	<b>Gender</b>	<b>Age (yrs)</b>	<b>Post-mortem intervals</b>	<b>Braak Score</b>	<b>Neuropathological Diagnosis</b>
<b>454</b>	M	69	40	I-II	Parkinson's disease
<b>580</b>	M	65	27	III-IV	Parkinson's disease
<b>588</b>	M	72	12	III-IV	Parkinson's disease
<b>582</b>	F	76	36	N/A	Parkinson's disease
<b>526</b>	F	54	23		Normal control
<b>540</b>	M	64	28		Normal control
<b>562</b>	M	42	13		Normal control
<b>560</b>	F	56	5.42		Normal control

<b>Code</b>	<b>Gender</b>	<b>Age (yrs)</b>	<b>Race</b>	<b>Post-mortem intervals</b>	<b>Braak Score</b>	<b>Neuropathological Diagnosis</b>
<b>AK-2013-1</b>	M	83	C	10.5	N/A	Parkinson's disease
<b>AK-2013-2</b>	F	88	C	3.8	N/A	Parkinson's disease
<b>AK-2013-3</b>	M	81	C	6	N/A	Parkinson's disease
<b>AK-12-03</b>	F	87	C	2.08	IV	Parkinson's disease
<b>AK-11-54</b>	F	76	C	3.5	III	Parkinson's disease
<b>AK-11-90</b>	M	78	C	2.75	III	Parkinson's disease
<b>AK-2013-4</b>	F	87	C	12		Normal control
<b>AK-2013-5</b>	F	72	C	7.5		Normal control
<b>AK-2013-6</b>	F	87	C	8		Normal control
<b>AK-00-14</b>	M	90	C	4		Normal control
<b>AK-05-12</b>	M	88	C	2		Normal control
<b>AK-11-11</b>	M	93	C	2.15		Normal control

## CHAPTER III

PROKINETICIN-2 PROMOTES ALTERNATIVE ACTIVATION OF MICROGLIA  
THROUGH THE JMJD3-IRF4 PATHWAY

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## Abstract

Microglial activation has been implicated in the progression of Parkinson's disease (PD), and anti-inflammatories have demonstrated the ability to protect against dopaminergic degeneration in animal models of PD. Alternative activation of microglia towards an anti-inflammatory phenotype can resolve the increased inflammation and lead to neuronal protection. Prokineticin-2 (PK2) is a small, secreted chemokine-like signaling protein with wide-ranging biological functions throughout the body. Microglia express the prokineticin receptors and recombinant PK2 [rPK2] can activate downstream pathways in microglia. In the present study, we demonstrate that rPK2, PK2 overexpression and a PKR1 agonist induces basal levels of alternative activation markers such as Jmjd3 and Arginase-1, while decreasing classical activation markers such as reactive oxygen species (ROS), nitric oxide and pro-inflammatory cytokines. Recombinant

PK2 can also attenuate LPS-induced production of reactive oxygen, nitric oxide, pro-inflammatory cytokines and mitochondrial bioenergetics dysfunction. Overexpression studies with PK2-AAV 2/5 in the mouse striatum induces alternative activation in microglia, with increased ramification of branches. PK2-AAV 2/5 injection rescues MPTP-induced microglial deramification, whereas the prokineticin receptor antagonist PKRA7 exacerbates this effect. Collectively, our results establish a novel paradigm of neuron-microglia signaling leading to alternative activation of microglia that could be an important therapeutic avenue for PD.

### Introduction

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disease that is characterized by the loss of dopamine producing neurons in the substantia nigra region of the brain. The exact cause of this neuronal death is not known, however, several factors have been implicated in the progression of the disease. These factors include mitochondrial dysfunction, oxidative stress, and neuroinflammation. Several studies have demonstrated that neuroinflammation is major part of PD progression (Hirsch and Hunot 2009), but the initial cause of the inflammation is not well understood. The hallmark of neuroinflammation includes increased number of activated microglia in the nigrostriatal pathway and the production of proinflammatory cytokines such as TNF $\alpha$  and IL-1 $\beta$ . Interestingly, several animal models of PD, including the neurotoxin MPTP that can cause Parkinsonian symptoms, has also been shown to increase inflammation and proinflammatory cytokines (Lofrumento et al.). Recently, our group discovered that a chemokine-like protein, Prokineticin-2 or PK-2, is upregulated in dopaminergic neurons on stimulation with inflammatory factors or neurotoxicants.

Prokineticins are proteins that obtained their name because they were first discovered to contract smooth muscle in the gut (Li et al. 2001). The two prokineticin proteins, Prokineticin-1 and Prokineticin-2, are members of the AVIT protein family due to the presence of the particular amino acid signaling sequence AVITGA on the N-terminus. PK2 mediates diverse physiological functions throughout the human body, from the testis, ovaries, heart, to the brain. It is a signaling protein that has several characteristics of a pleiotropic cytokine or a chemokine. This is because PK2 has been demonstrated to promote cell survival and growth (LeCouter et al. 2004a), however in other systemic tissues PK2 can cause an increase in inflammation (Martucci et al. 2006) and nociception (Negri et al. 2006a). Prokineticins activate the ERK p44/42 kinase in the MAPK pathway most likely through Gi signaling, calcium mobilization through Gq signaling, and increased Akt and cAMP levels through Gs signaling (Lin et al. 2002b). In the central nervous system (CNS), PK2 plays a role in regulating circadian rhythms (Zhou and Cheng 2005), olfactory bulb biogenesis (Ng et al. 2005), thermoregulation, and energy expenditure (Zhou et al. 2012). Most importantly to Parkinson's disease though, Prokineticin-2 has demonstrated the ability to protect dopaminergic neurons from neurotoxic insult *in vitro* (OUR PAPER).

Tissue inflammation can induce increased PK2 levels in different tissues, including the brain (Gordon and Neal et al., unpublished) (Cheng et al.). However, very little is known of the role that PK2 plays in neuroinflammation of the CNS. Microglial cells are the resident immune cells in the central nervous system and have the ability to fight off infections. These microglial cells are constantly surveying their surroundings to check for infection, but can be polarized into an activated state to produce inflammation in order to suppress the infection. This activated inflammatory state has been labeled M1, or classical activation, and is characterized by an increase in inflammatory cytokines such as TNF $\alpha$  and IL-1 $\beta$  along with damaging reactive oxygen and



reactive nitrogen species (ROS and RNS, respectively) (Lull and Block, 2010). This M1 phenotype can be brought about by the bacterial endotoxin lipopolysaccharide (LPS) or Th1 cytokines such as interferon,  $IFN\gamma$ . Interestingly, activated microglial cells have been detected in the substantia nigra region of the brain in PD patients (Langston et al. 1999, Whitton 2007, McGeer et al. 1988). Evidence indicates that the activated microglia are polarized to a classical activation state due to the increase in inflammatory cytokines  $TNF\alpha$  and  $IL-1\beta$ . Recently, it was discovered that microglia can be alternatively activated, labeled M2, by the Th2 cytokines IL-4, IL-13 and IL-10 (Olah et al. 2011, Mills et al. 2000). IL-4 and IL-13 can activate signal transducer and activators of transcription 6 (STAT6), which can then regulate the transcription of M2 related genes (Li et al. 2014, Wang, Liang and Zen 2014). This M2-like alternative activation leads to the expression of anti-inflammatory factors such as  $PPAR\gamma$ , Arginase1 and IL-10. These genes are involved in tissue repair and regeneration, such as the reduction of reactive nitrogen species by Arginase1. Recent results from Tang et al. demonstrated that reduced microglial alternative activation, by knocking out the demethylase Jmjd3, led to more toxicant-induced neuronal death. It has been determined that microglia, unlike T helper cells, do not polarize, and continually shift their phenotype depending on their environment. Similarly, there are not a distinct populations of microglia that are either classically or alternatively activated, instead these cells are in a mixed state with no discernible phenotype. However, specific stimuli can elicit a phenotypic response in microglia and could shift the entire populations phenotype towards more of a pro- or anti-inflammatory status. Therefore, it is important to elucidate mechanisms that could lead to the promotion of microglia towards an alternative activation phenotype (Tang et al. 2014). Switching microglia phenotypes

from a pro-inflammatory activation towards more of an anti-inflammatory activation could be a potential treatment option for the therapy of neuroinflammatory diseases, such as Alzheimer's and Parkinson's disease.

Most importantly in association to PD, PK2 has demonstrated the ability to protect dopaminergic neurons from neurotoxic insult *in vitro*. Our lab previously found that PK2 expression is induced in Parkinson's disease patients when compared to age-matched controls, and this increase is localized to the remaining dopaminergic neurons. We went on to demonstrate that dopaminergic neurons *in vitro* secreted increased levels of PK2 with neurotoxic insult (Gordon and Neal et al., unpublished). Both astrocytes and microglia express both prokineticin receptors, PKR1 and PKR2, and PK2 is able to signal through these receptors in both of these cell types (Koyama et al. 2006). However, there is currently no research published regarding microglial prokineticin signaling in neurodegenerative disease models. With the knowledge that PK2 can protect neurons, the current study went on to investigate the role of PK2 in microglial cells and its role in alternative activation.

## Results

### **Prokineticin receptors R1 and R2 are expressed in microglial cells**

PK2 is a small chemokine-like protein that signals through two G protein-coupled receptors, PKR1 and PKR2. PKR1 and R2 can both bind PK2 and have the potential to signal through different cascades (Zhou 2006). Studies have shown mRNA expression of both receptors in several areas of the body, including the brain (Lin et al. 2002b). However, the expression of PKR1 has been found more in peripheral areas such as the testis and skin, whereas PKR2 is

expressed higher in the CNS. Also, evidence points to the fact that PK2 activates the receptors at slightly different concentrations. PK2 can activate PKR1 at lower concentrations than PKR2 (Soga et al. 2002a). Although PKR2 has been seen at a higher level than PKR1 in the CNS, Koyama et al. demonstrated that PKR1 is expressed higher in the glial cells of the brain, including astrocytes and microglia (Koyama et al. 2006). Since PK2 had previously been demonstrated to modulate systemic inflammation and is increased in PD, we therefore wanted to determine the effect of PK2 on the resident immune cells of the CNS, microglial cells. To begin with, we wanted to confirm that our cultured primary mouse microglia expressed the prokineticin receptors and that recombinant PK2 can signal to these cells. We found that both receptors were expressed in the primary mouse microglia by ICC (Figure 1A) and Western blotting (Figure 1B). The microscopy showed a differential cellular localization of R1 and R2, with R1 localized more around the periphery and R2 more throughout the cytoplasm. Consistent with previous results, the Western blots showed more PKR1 expression compared to PKR2. To verify these results we ran qPCR to determine the gene expression levels of both receptors in both mouse astrocytes and microglia (Figure 1C). These results indicate that the prokineticin receptors are expressed on our cultured mouse microglial cells. PK2 signaling in mouse astrocytes has been demonstrated to result in an increase in intracellular calcium, cell proliferation, and MAPK pathway activation (Koyama et al. 2006). Since we confirmed that our cultured glial cells express the prokineticin receptors, the next step was to confirm that the human recombinant PK2 (Peprotech) could elicit the functional signaling in primary mouse microglial cells. Previous groups measured the increase in intracellular calcium to determine that PK2 activated one of the receptors in a particular cell type (Ngan and Tam 2008). Therefore, we used the Fluo4 fluorescent calcium dye to determine intracellular calcium levels as an experiment to test PK2 signaling in the microglial culture. We found that there

was a dose-dependent increase in intracellular calcium levels with PK-2 treatment in a primary mouse microglial cell culture (Figure 1D). Together, these results demonstrate that human recombinant PK2 can signal in our primary mouse microglial cells and cause functional changes such as increased intracellular calcium mobilization.

### **Prokineticin-2 promotes alternative activation of mouse microglia**

It was previously thought that activation of microglia would always lead to a pro-inflammatory phenotype, known as classical activation, or M1. It is now known that certain stimuli can induce microglia to become activated but have an anti-inflammatory or protective phenotype, this is called alternative activation of microglia, or M2 (Boche et al. 2013). However, the mechanism underlying microglial polarity is not completely understood and seems to be more of a spectrum of phenotypes, with many different levels between classical activation, ‘resting’ (surveillant) and alternative activation. Certain factors, such as the IL-4 and IL-13 cytokines can polarize microglia towards an M2-like phenotype through STAT6 signaling (Li et al. 2014). Jmjd3 is a histone demethylase that is mediated by STAT6 signaling and has previously been implicated in the induction of genes that are important for alternative activation in microglia. Jmjd3 is able to protect dopaminergic neurons from toxicant-induced cell death (Tang et al. 2014) through polarizing microglia. The demethylase activity of Jmjd3 leads to activation of M2 related genes, such as Arginase1 and mannose receptor, through the induction of the Irf4 transcription factor (Sato et al. 2010). PK2 has demonstrated the ability to mediate inflammatory responses systemically, including macrophages (Martucci et al. 2006, Monnier and Samson 2008). We wanted to characterize the effect of exogenous PK2 on microglial activation states. Interestingly, we found that with exogenously added recombinant human PK2 (rPK2) on isolated primary mouse microglial cells significantly induced Jmjd3 gene expression (Figure 2A). This increase was also

attenuated with co-treatment with PC-7, a PK2 receptor antagonist, therefore showing that the increase of Jmjd3 expression was due to PK2 signaling. Following the discovery that PK2 can increase Jmjd3 expression, we wanted to measure several alternative activation marker genes to confirm that the increased Jmjd3 led to an alternative activation phenotype, such as what has been previously classified as M2. Gene expression studies of Irf4, Arginase-1 and mannose receptor c type-1 (Mrc1) showed a significant increase in the expression of these genes with rPK2 treatment, similar to the effect of IL-4, that was attenuated with co-treatment with PC7 (Figure 2B- 2D, respectively). Following the gene expression results, we wanted to examine the protein level of Jmjd3 and an important M2-associated marker, Arginase-1, using by Western blotting techniques. Consistent with the qPCR results we found that PK2 increases both Jmjd3 and Arginase-1 protein level compared to control, and PC-7 is able to significantly reduce this increase (Figure 2E). Similarly, immunocytochemistry (ICC) of Jmjd3 and Arginase-1 in primary mouse microglia allows the visual representation of the PK2-induced increase in these proteins that can be attenuated with PC-7 (Figure 2F). By quantifying the fluorescent signal, we were able to show consistency with the previous results of PK2 significantly increasing (~2 fold) Jmjd3 (Figure 2G) and Arginase-1 (Figure 2H). Collectively, this data demonstrated that exogenously added rPK2 can induce an alternative activation, or M2-like, phenotype of mouse primary microglial cell cultures through the STAT6-Jmjd3-Irf4 signaling pathway.

### **Prokineticin-2 attenuates toxicant-induced ROS and RNS in microglial cells**

Neuroinflammation seen in PD includes increases in proinflammatory cytokines, such as TNF $\alpha$ , and IL-1 $\beta$ , along with ROS and RNS (Imamura et al. 2003, Panicker et al. 2015). The main sources of these inflammatory factors are the glial cells, and more specifically microglia. Previous

studies have demonstrated that anti-oxidant drugs can actually protect dopaminergic neurons from apoptosis (Jin et al. 2014a). Similar results were seen by reducing nitric oxide (NO) levels (Wong and Lerner 2015). Our previous results with PK2 signaling in neurons demonstrated that recombinant PK2 treatment could protect neurons by increasing Bcl2 and protecting the mitochondria (Gordon and Neal et al., unpublished). In that study we have shown that PK2 can attenuate toxin-induced ROS production in dopaminergic neurons. In this study, we wanted to investigate whether PK2 could have a similar reduction of ROS on microglial cells, and also look into microglial-derived RNS production. First, we used the endotoxin LPS to activate cultured primary microglial cells. After measuring intracellular ROS (iROS) levels using DCFDA fluorescent dye, we found that LPS significantly increased iROS production in these primary microglial cells and that cotreatment with 25nM of PK2 completely attenuated this increase (Figure 3A). These results are consistent with what we found in neurons, in that PK-2 cotreatment can reduce toxin-induced iROS levels. Nrf2 is the transcription factor mainly seen for anti-oxidant response genes, and an increase in Nrf2 activity leads to a reduction in ROS damage. Interestingly, we found that the gene expression for Nrf2 and one of the target genes of Nrf2, Prdx2, is significantly increased with rPK2 treatment (Figure 3B). Similarly, Nrf2 protein levels, measured by Western blot, also increased with rPK2 treatment, and using the receptor antagonist PC7 we were able to attenuate the PK2-induced increase of the Nrf2 protein (Figure 3C). Therefore, rPK2 treatment in cultured primary microglial cells attenuated LPS-induced iROS levels, possibly through the upregulation of Nrf2 and its target gene Prdx2. We previously found that PK2 treatment in dopaminergic neurons led to an increased expression of the anti-apoptotic mitochondrial protein Bcl2. We found similar results with rPK2 treatment in cultured primary microglia, with a significant increase in Bcl2 gene expression that can be attenuated with co-

treatment with the PK2 receptor antagonist PC-7 (Figure 3D). Some reports have found that Bcl2 acts as an anti-inflammatory mediator through inhibition of NF $\kappa$ B (Badrichani et al. 1999). Therefore, PK2 can induce several factors that can reduce or protect against oxidative stress.

Increased Bcl2 has been implicated in protecting the cell against mitochondrial dysfunction (Thomenius and Distelhorst 2003). Therefore, the increase in these anti-oxidant and mitochondrial protective proteins led us to examine the effect of rPK2 treatment on mitochondrial bioenergetics in microglia. Previous studies found that LPS treatment could reduce oxidative respiration in microglial cell cultures using the Seahorse XFe24 instrument (Orihuela et al. 2016). We found similar results with an 8 hour LPS treatment in primary microglia (Figure 3E), where basal respiration (Figure 3F, left panel) and ATP production (Figure 3F, right panel) were both significantly reduced compared to control. Interestingly, rPK2 co-treatment with LPS significantly attenuated the reduction in both basal respiration and ATP production and brought these parameters back to control levels. Recombinant PK2 treatment alone did not change either parameter compared to control. These results are the first to indicate that PK2 signaling can attenuate LPS-induced mitochondrial dysfunction in primary mouse microglia.

We then went on to measure the reactive nitrogen species (RNS), nitric oxide (NO) levels produced in primary microglial cells treated with either LPS alone or co-treated with rPK2. Recombinant PK2 co-treatment was able to significantly attenuate LPS-induced NO production in primary microglial cells (Figure 3E). This reduction was not specific to LPS treatment, because we found that PK-2 also significantly attenuated TNF $\alpha$ -induced NO production in BV2 cells (not shown). The enzyme responsible for the production of nitric oxide in microglial cells is inducible nitric oxide synthase, or iNOS. Using qPCR to determine gene expression, we found that rPK2 treatment was able to significantly reduce basal levels of NOS2 gene expression that was

attenuated with co-treatment with PC-7 (Figure 3F). Oxidative stress has been implicated in the progression of Parkinson's disease, and reduction of these damaging molecules has demonstrated the ability to have a positive impact in fighting the disease progression. Herein, we found that cotreatment with recombinant human PK2 is able to significantly attenuate LPS-induced ROS and NO production, and mitochondrial energetics dysfunction in microglial cells.

### **Prokineticin-2 reduces inflammatory cytokines production in primary microglial cells**

Microglial cells are able to fight off infections using inflammatory molecules such as ROS, RNS and cytokines. It is normal for microglia to become classically activated and secrete inflammatory cytokines for short periods of time, but when this activation persists into chronic activation then it can lead to neurodegenerative diseases such as Parkinson's disease (Castano et al. 1998, Herrera et al. 2000, Qin et al. 2007). It is also known that as ageing occurs, microglial cells are more prone to become classically activated into an inflammatory state (Perry and Holmes 2014). If there were a way to reduce inflammatory cytokine production from these microglial cells, then it could help to slow the progression of a number of neurodegenerative diseases. Since rPK2 was able to increase M2-associated markers in these microglial cultures, we wanted to determine if rPK2 treatment could also reduce M1-associated inflammatory markers. Surprisingly, we found that rPK2 treatment on primary mouse microglial cell cultures lead to a significant decrease in the gene expression of TNF $\alpha$ , IL-6 and IL-12 (Figure 4 A,B,and C). Consistent with this data, after a 12-hour treatment with rPK2 there was a significant reduction in the secreted protein level pro-inflammatory cytokines TNF $\alpha$ , IL-12, and IL-6 (Figure 4D). Age is the major factor that increases likelihood of having Parkinson's disease and microglial cells change to a more pro-inflammatory activation state with an increase in age, which this leads to more pro-inflammatory cytokines.



These are the first reported results indicating that human rPK2 can reduce pro-inflammatory cytokines in primary microglial cells. Therefore, this further indicates that PK2 can have an impact in polarizing microglia towards an alternative or anti-inflammatory phenotype and could have a positive impact on ageing and neurodegenerative disease such as Parkinson's disease. Studies into PK2 reveal that it is produced higher in inflamed systemic tissues compared to healthy tissues (Chauvet et al. 2015, Chen et al. 2015a, Chen et al. 2016, Abou-Hamdan et al. 2015, Watson et al. 2012). With this in mind, we wanted to investigate what role PK2 treatment would play in primary mouse microglia in an inflammatory setting. LPS is a powerful endotoxin that has been demonstrated to cause a significant increase in M1-associated pro-inflammatory cytokines in microglia (Castano et al. 1998, Herrera et al. 2000). Therefore, we treated primary mouse microglia with LPS and co-treated with rPK2, then measured secreted cytokine levels. Interestingly, we found that co-treatment with PK2 significantly reduced the production of several LPS-induced M1-associated pro-inflammatory cytokines, such as  $\text{TNF}\alpha$ , IL-6, and IL-12 (Figure 4E-4G, respectively). This is the first study to show that Prokineticin-2 can significantly reduce toxin-induced pro-inflammatory cytokines in primary mouse microglial cells. Therefore, PK2 could play a role in reversing the neuroinflammation seen in neurodegenerative diseases such as Parkinson's disease by polarizing the microglia away from a pro-inflammatory and towards a more anti-inflammatory phenotype.

### **PK2 overexpression *in vitro* induces an alternative activation microglial phenotype**

Since microglial activation is important in health and disease, understanding the pathways involved in the switch from one phenotype to another would be beneficial to understanding how to protect against many inflammatory diseases. Following our cell culture results that showed rPK2 treatment can induce an alternative activation phenotype and reduce production of pro-

inflammatory factors, we wanted to determine if PK2 overexpression (PK2 OE) would have the same effect both *in vitro* and *in vivo*. We used a lenti-viral delivery system to induce PK2 OE in primary mouse microglial cultures. Microglia were infected with the lenti-virus for 24 hours, cells were washed and kept in growth media for 24 hours and then collected. We confirmed PK2 OE in the microglial cells by using Western blotting techniques to show increased GFP protein level (Figure 5A). Similar to what was seen with rPK2 treatment, PK2 OE significantly increased the protein level of microglial alternative activation phenotype markers Jmjd3, Arginase1 and Nrf2 (Figure 5B, 5C and 5D respectively) compared to control and GFP overexpression (GFP OE).

Classical activation of primary microglial cell culture induces morphological changes towards a more amoeboid phenotype (Kettenmann et al. 2011, Stence, Waite and Dailey 2001). Interestingly, IL-4 anti-inflammatory cytokine treatment leads to a re-ramification of these activated microglia, indicating that morphology can be a determinant of the microglial activation states. Therefore, we wanted to characterize any microglial morphological changes that might be induced by PK2 OE in cell culture. Iba1 immunofluorescent images (Figure 5E top panel) were converted to binary in ImageJ and then skeletonized (Figure 5E middle panel). The skeletonized images were then analyzed using a specific ImageJ plugin called Analyze Skeleton (Arganda-Carreras et al. 2010), according to previous literature that used it to measure microglial morphology (Morrison and Filosa 2013). This plugin analyzes the skeleton image to count the number of branches, junction, endpoints and determine process length (Figure 5E bottom panel). Using these morphological measurements, we were able to find that PK2 overexpression significantly increased the number of microglial branches, junctions and endpoints (Figure 5F, 5G and 5H respectively) compared to control and GFP OE. The overall length of the microglial branches was also significantly increased with PK2 overexpression compared to control and GFP

overexpression by looking at the longest/shortest branch, which gives the diameter of the cell's branch reach. This is a way to calculate the length from the endpoint of one branch to the furthest endpoint on the opposite side of the cell, therefore measuring the entire breadth of the microglial branches. The longest/short path calculation and applications come from a previous source (Fraas, Niehoff and Luthen 2014). These morphological results are another indication of PK2 inducing alternative activation of microglia. This is because the morphology seen with PK2 OE leads to an increase in branches length, unlike what is seen with classical activation of microglia which leads to a more amoeboid morphology with less process branches. Together, this data demonstrates that PK2 OE, similar to recombinant PK2, can lead to an increase in alternative activation of microglia.

### **PK2-AAV 2/5 induced an alternative activation microglial phenotype *in vivo***

We developed an Adeno associated virus (AAV) viral expression system that can overexpress PK2 tagged to GFP. We injected the PK2-AAV into the mouse striatum stereotaxically, and were able to get PK2 overexpression stably for at least 2 months (data not shown). Once we determined that the PK2-AAV injection was successful *in vivo*, we wanted to determine if the PK2-AAV could induce alternative activation of microglia in the mouse striatum. Interestingly, using qPCR gene expression studies we found that the PK2-AAV was able to significantly increase the expression of multiple alternative activation genes, such as *Jmjd3*, *Irf4*, *Arginase1*, *Mrc1* (CD206), *Nrf2*, and *Bcl2* (Figure 6A), when compared to control animals injected with GFP AAV in same site of the striatum. Next, we wanted to determine if PK2-AAV can reduce classical activation markers, such as pro-inflammatory cytokines. We found PK2-AAV significantly reduced basal  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ ,  $\text{IL-6}$ ,  $\text{IL-12}$  and  $\text{NOS2}$  (Figure 6A) when compared to GFP-AAV injected animals. This data indicates that PK2 overexpression can lead to alternative activation of microglia *in vivo* by increasing alternative activation markers while reducing classical

activation markers. Next, we analyzed used Western blotting techniques to measure the protein level of microglial activation markers in the mouse striatum. We found a significant increase in both Jmjd3 (Figure 6B left panel) and Arginase1 (Figure 6B right panel) protein level in the mouse striatum with PK2-AAV when compared to control and GFP-AAV. To show that this increase in Jmjd3 and Arginase1 were coming from microglia, we used triple-label immunofluorescence in striatum sections to visualize the protein levels. As expected, we found that PK2-AAV was able to increase both Jmjd3 (Figure 6C) and Arginase1 (Figure 6D) in Iba1-positive cells in the striatum compared to GFP-AAV injected animals. Therefore, the increases in Jmjd3 and Arginase1 are seen specifically in microglial cells, although these proteins might also be increased in other cell types. Overall, these results demonstrate for the first time that PK2 overexpression is able to induce alternative activation of microglial cells *in vivo*.

Classical activation of microglia leads to well characterized morphological changes *in vivo*. These changes include the de-ramification of the microglial branches and cell soma hypertrophy. Therefore, measuring microglial morphological parameters can give insight into the activation state of microglia in the brain. We wanted to investigate the effects of PK2-AAV injection in the mouse striatum on microglial morphology to give an indication of the microglial activation states. We used Iba1 DAB immunostaining to label the microglia in the striatum, and this staining gave a clear image of the microglia all the way down to the fine processes. The DAB phase contrast images (Figure 6E top inset) were then converted to binary and skeletonized (Figure 6E middle inset). The Analyze skeleton plugin then converted the skeletonized image to count the number of branches, junctions, endpoints and length of branches (Figure 6E bottom inset). Analyzing over 200 microglia across at least three animals for each treatment showed us that PK2-AAV increased the number of branches, junctions and endpoints per cell (Figure 6F, 6G and 6H, respectively)

compared to GFP-AAV injected animals. Similar to the results from microglial cell cultures, we also found an increase in the longest-shortest path (Figure 6I), which is an indication of the total breadth of the microglial branches from the endpoint of one branch to the endpoint of the furthest branch. We went on to measure cell soma descriptors such as cell soma total area and roundness using ImageJ. This is because some reports have described alternative phenotype morphology with a thin cell body, whereas classical activation leads to a larger cell soma (Gonzalez et al. 2014). Interestingly, we found that PK2-AAV was able to significantly reduce the roundness of the cell soma, and therefore more of a thinner morphology (Figure 6J). However, PK2-AAV did not increase cell soma size compared to control, whereas the GFP AAV injection did significantly increase microglial cell soma area (Figure 6K). This increase of the cell soma by GFP-AAV could be due to injury from the stereotaxic injection used to add the virus, whereas PK2-AAV was able to attenuate this injection-induced change. Taken together, these data demonstrate that PK2-AAV is able to induce a morphological change in microglial cells that is similar to what has been described as alternatively activated microglia. These results also demonstrate that the Analyze skeleton ImageJ plugin can be a tool used to determine activation states of microglia.

### **PK2-AAV 2/5 attenuates MPTP-induced microglial morphology changes**

Several groups, including our lab (Ghosh et al. 2012, Ghosh et al. 2016), have shown that MPTP leads to an increase in microglial activation and inflammation (Costa et al. 2013, Kim et al. 2012, Lofrumento et al. 2011). Our lab previously demonstrated that PK2-AAV could protect dopaminergic neurons from MPTP-induced cell death. Therefore, we wanted to determine if PK2-AAV could attenuate the MPTP-induced activation of microglial cells in the mouse striatum. Using Iba1 DAB immunostaining to look at the microglial cells in the striatum (Figure 7A top

panel), we converted the images into binary and then the images were skeletonized (Figure 7A middle panel). Using the Analyze skeleton ImageJ plugin, the skeletonized images were converted to analyze the microglial branches, junctions, endpoints and branch length. As expected, MPTP induced a significant reduction in microglial branches, junctions, endpoints and branch length (Figure 7B, 7C, 7D and 7E, respectively) compared to saline injected animals. Interestingly, PK2-AAV not only significantly attenuated the MPTP-induced morphological changes, but increased all of the metrics significantly more than the saline group. Importantly, GFP-AAV had no effect on MPTP-induced microglial morphology changes, so the injection and the virus did not lead to any protection. We then went on to show that MPTP caused a significant increase in the microglial cell soma size, that PK2-AAV was able to significantly attenuate and bring back to control levels (Figure 7F). These results demonstrate that PK2 overexpression *in vivo* can attenuate the MPTP-induced microglial classical activation morphology.

### **PK2 receptor antagonist PKRA7 exacerbates MPTP-induced microglial morphology changes**

Our lab previously found that MPTP treatment increases PK2 production and secretion in dopaminergic neurons. Since we found that PK2 overexpression can lead to attenuation of MPTP-induced morphological changes, we wanted to use a prokineticin receptor antagonist to determine if blocking prokineticin signaling would exacerbate of the phenotype. We injected PKRA7, a specific prokineticin receptor antagonist, intraperitoneally once a day starting one day before the first MPTP injection and continued giving PKRA7 injections daily until sacrifice 12 days after the first injection. We injected MPTP 18mg/kg once a day for 5 days and then sacrificed the animals 7 days after the last MPTP injection. This is a well characterized MPTP subacute paradigm that

leads to neuronal loss and neuroinflammation (Petroske et al. 2001, Meredith and Rademacher 2011b). Again, we used the Iba1 DAB immunostaining and Analyzed skeleton ImageJ plugin to measure the morphology changes (Figure 8A). We found that this MPTP paradigm also led to a microglial morphology shift towards a classical phenotype, and PKRA7 significantly exacerbated the MPTP-induced loss of microglial branches, junctions, endpoints (Figure 8B, 8C, and 8D, respectively). Interestingly, PKRA7 also exacerbated the de-ramification of the microglia, leading to both significantly reduced branch length and increased cell soma area (Figure 8E and 8F). These results demonstrate that blocking the MPTP-increased PK2 signaling to microglial cells that it exacerbates the MPTP-induced microglial classical activation morphology.

### **PKR1 chemical agonist IS20 induces alternative activation phenotype in microglial cells**

Recently, Gasser et al. created a selective PKR1 agonist, IS20, and demonstrated that this agonist could protect cardiomyocytes from a myocardial infarction (Gasser et al. 2015). Dr. Desaubry was gracious enough to send us some of the compound so that we could test the ability of this agonist to perform a similar function as rPK2 and PK2 OE in a microglial cell culture. Interestingly, we found that IS20 treatment on primary microglia for 8 hours significantly increased the gene expression of alternative activation markers such as *Jmjd3*, *Arginase1*, *Mrc1*, and *IGF-1*. While at the same time significantly decreasing or having no effect on classical activation markers such as *NOS2*, *IL-1 $\beta$* , *IL-6*, and *TNF $\alpha$*  compared to control (Figure 9A). We followed these results with Western blotting (Figure 9B) of *Jmjd3* (Figure 9C) and *Arginase1* (Figure 9D) to demonstrate that these alternative activation proteins are significantly increased with IS20 treatment compared to control. We went on to perform immunofluorescence microscopy for both *Jmjd3* and *Arginase-1* to visualize the protein levels (Figure 9E). Analyzing the

immunofluorescence from these images showed us that IS20 significantly increased both Jmjd3 (Figure 9F) and Arginase-1 (Figure 9G) in these cultured primary microglial cells compared to control. These results were confirmed by using IL-4 as a positive control, and that treatment led to an even greater increase in the immunofluorescence of both Jmjd3 and Arginase-1. These results demonstrate that the PKR1 agonist IS20 can induce alternative activation of cultured microglial cells.

Similar to PK2 overexpression, we wanted to examine the morphology of the cultured primary microglia with IS20 treatment. Iba1 immunofluorescent images were used to skeletonize and then analyzed using the Analyze skeleton ImageJ plugin (Figure 9H). We found that IS20 treatment was able to increase microglial branches, junctions, and endpoints (Figure 9I, 9J, and 9K, respectively). Overall microglial branch length was also significantly increased with IS20 treatment (Figure 9L). A positive control of IL-4 treatment was used to demonstrate that a protein known to induce alternative activation led to a similar phenotype to what was seen with both PK2 overexpression and IS20 treatment. Taken together, these results demonstrate that the PK2-induced alternative activation is most likely through the PKR1 receptor, however some signaling through PKR2 cannot be ruled out. This is the first example known to the authors of a non-peptide chemical that can induce alternative activation in microglial cells.

## Discussion

Inflammation is a normal, beneficial function that can help to keep the CNS in a healthy state. However, in neurodegenerative diseases such as Parkinson's disease, the neuroinflammation becomes excessive and can exacerbate neurodegeneration (Imamura et al. 2003, Panicker et al.



2015). In PD, there is an increase in classically activated microglia in the nigro-striatal region which is accompanied by an increase in inflammatory factors. Several anti-inflammatory drugs have demonstrated the ability to help protect dopaminergic neurons both in cell culture and animal models of PD (Asanuma and Miyazaki 2008). However, it is still unclear if anti-inflammatory drugs, such as NSAIDs, have a significant effect on the progression of the disease. Therefore, there is a need for research into pathways that can reduce this increased inflammatory response and protecting the dopaminergic neurons from cell death. Our lab previously found that Prokineticin-2 production and secretion is increased in dopaminergic neurons both in animal models of PD and in post-mortem PD patients compared to age-matched controls (Gordon and Neal et al., unpublished.). Koyama et al demonstrated that microglia express the prokineticin receptors, therefore the increased PK2 secreted from dopaminergic neurons could signal to the microglia (Koyama et al. 2006). We confirmed that primary mouse microglia produce both of the prokineticin receptors, with significantly more PKR1 expressed compared to PKR2. Human recombinant PK2 addition onto cultured primary mouse microglia led to a dose dependent increase in calcium, an expected outcome with successful PK2 signaling. Therefore, PK2 could play an important role as a neuron-microglia signaling molecule in the progression of Parkinson's disease.

Microglial activation has been seen in many of the neurodegenerative disorders, and this activation is implicated in the disease progression. However, the activation states of microglia have become a hot topic in the neuroscience field, with many reports demonstrating that certain stimuli can lead to a specific phenotype in microglia (Tang and Le 2016). With excessive sustained inflammation found in PD there is a need to find therapies that can shift microglia towards more of an alternative activation phenotype. The *Jmjd3-Irf4* axis has been used to determine alternative activation in both macrophages and microglia (Satoh et al. 2010). *Jmjd3* was found to be essential

in microglial alternative activation, and knockout of *Jmjd3* led to a skewing towards more classical, or pro-inflammatory, phenotypes (Tang et al. 2014). The induction of *Jmjd3* by recombinant human PK2, PK2 overexpression and the PKR1 agonist, along with the increase in Arginase-1 and *Mrc1* in cultured primary mouse microglia indicate that PK2 signaling leads to the shift towards an alternative activation phenotype. This is further evident with the increase in the transcription factor *Irf4*, which has been implicated in shifting microglia to an alternative phenotype. Similarly, the rPK2-induced reduction of classical activation markers basal levels such as intracellular ROS, nitrite and proinflammatory cytokine production in cultured primary microglia demonstrates that PK2 signaling does not increase basal levels of any classical activation markers. This is the first instance known to the authors of a neuronal protein that can modulate microglial phenotype towards an alternative phenotype through the activation of the *Jmjd3-Irf4* signaling pathway.

Inflammatory factors such as reactive oxygen and nitrogen species are increased in neurodegenerative diseases, such as PD. Lipopolysaccharide is a membrane component of gram-negative bacteria that can illicit an inflammatory response in microglia, and this molecule has been used to study neuroinflammation for many years. We found that LPS increased both intracellular reactive oxygen and nitric oxide in primary microglia, and that rPK2 treatment could significantly attenuate the production of these damaging molecules. Mitochondrial dynamics can signify the energy demands of the cell, and particularly in microglia it can indicate whether the cell is activated. Olah et al found that LPS stimulated microglia had a reduced basal oxidative respiration compared to control, whereas IL-4 stimulation was not able to induce a similar effect (Orihuela et al. 2016). We found that recombinant PK2 can attenuate the LPS-induced reduction in mitochondrial basal respiration and ATP production. An increase in the gene expression of both

Nrf2 and Bcl2 with rPK2 treatment indicates that prokineticin signaling can increase protection against mitochondrial dysfunction and therefore reduce the effects from a pro-inflammatory stimulus. Similarly, rPK2 treatment was able to significantly attenuate LPS-induced gene expression and protein level of pro-inflammatory cytokines TNF $\alpha$ , IL-6, and IL-12. This opens up the idea that PK2 can reduce the production of inflammatory mediators through the protection against mitochondrial dysfunction. More studies are required to determine if prokineticin signaling can have a similar protective effect against other toxicants that affect microglial mitochondria, such as aggregated proteins or heavy metals.

Overexpression of PK2 *in vivo* by injecting an AAV 2/5 into the striatum can protect dopaminergic neurons from MPTP-induced cell death (Gordon and Neal et al., unpublished). This same PK2-AAV is able to increase gene expression and protein levels of alternative activation markers in the mouse striatum while significantly reducing the classical activation marker basal gene expression. The specific localization of the PK2-AAV-induced increases in Jmjd3 and Arginase-1 protein levels in Iba1-positive cells demonstrates that PK2 signaling can shift microglia towards an alternative activation phenotype. This data confirms that the cell culture results and demonstrates that PK2 signaling can induce alternative activation of microglia *in vivo*.

Morphological assessment of microglial phenotypes both *in vitro* and *in vivo* can be a powerful tool to investigate how microglia act in health and disease. This method gives reproducible results and can be used across different treatment paradigms and even disease models. There are only a few reports that look into a morphological assessment of microglial phenotypes, with no clear description for alternatively activation. It is known that classical activation leads to a de-ramification of the microglial processes and hypertrophy of the cell soma. However, alternative activation has only been described as having a thin cell body and increased branches

(Nimmerjahn et al. 2005, Ransohoff and Perry 2009). Both in primary mouse microglial cell culture and in the mouse striatum PK2 overexpression led to an increase in the number of microglial branches, junctions and endpoints while not increasing the cell soma size. Therefore, PK2 signaling does not lead to the de-ramification of microglial processes or the hypertrophy of the cell soma seen with toxicant treatment in cell culture and animal models of PD. This was confirmed with similar results using the PKR1 agonist and IL-4, a known inducer of alternative activation in microglia. Importantly, PK2-AAV was able to rescue the MPTP-induced de-ramification and cell soma hypertrophy of microglia in the striatum, similar to what IL-4 has been shown to do in cell culture (Wirjatijasa et al. 2002). Importantly, preventing MPTP-increased PK2 signaling with a receptor antagonist can exacerbate the MPTP-induced morphological effects in microglia. PK2 signaling is able to reduce the MPTP-induced classical activation of microglia in the mouse striatum and in the absence of PK2 signaling there is an increase in classically activated microglia. Therefore, Prokineticin-2 is the only known signaling molecule other than IL-4 that can signal from neurons to microglia and shift the microglia to an alternatively activated phenotype. This study demonstrates that Prokineticin-2 is a protective factor in Parkinson's disease by inducing alternative activation of microglia and reducing inflammation to go along with the previous results of PK2 directly signaling to protective pathways in dopaminergic neurons.

## Materials and Methods

### Reagents

DMEM/F12, fetal bovine serum (FBS), L-glutamine, IR-dye tagged secondary antibodies, Hoechst nuclear stain, penicillin, streptomycin, and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Recombinant PK2 was purchased from Peptidech. The

primary antibody for Iba1 was ordered from Abcam. The Arginase1 antibody was ordered from Thermo. The Jmjd3 antibody was purchased from The Bradford protein assay kit was purchased from Millipore. Bio-Rad Laboratories (Hercules, CA). DCF-DA intracellular ROS fluorescent dye was purchased from Calbiochem.

### **Cell culture and animal studies**

Primary mouse microglia were obtained from whole brain homogenate of 0-3 day mouse pups. Microglia were collected, isolated and maintained according to previous methods (Gordon et al. 2011) using DMEM/F12 media(GIBCO) (10% heat-inactive FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine, 2mM sodium pyruvate, 100µM non-essential amino acids (Invitrogen)) to grow the cells in an incubator at 37°C with 5% CO<sub>2</sub>. The media was changed on the 5<sup>th</sup> day of culture and then the microglia were isolated on the 16<sup>th</sup> day of culture. Isolation of microglia was performed using the CD11b labeling kit from StemCell, according to previous methods (Gordon et al. 2011). The isolated microglia were counted and seeded into cell culture plates for treatment.

All animal procedures were approved by Iowa State University's Institutional Animal Care and Use Committee (IACUC). All mice were housed under a 12-h light cycle in a climate-controlled mouse facility (22 ± 1°C) with food and water available *ad libitum*. Based upon the outcomes of preliminary studies, we estimated error variances of 15% for biochemical and neurochemical endpoints and 20% for histological measures. Setting alpha=0.05, we calculated sample sizes (n) of 8-10 animals per group, which has worked well for us in previous animal studies conducted in our laboratory (Ghosh et al. 2012, Ghosh et al. 2013). We used all male animals for all studies. All mice were pre-screened for normal baseline performance during behavioral assessments conducted prior to randomly assigning animals to experimental groups.

Investigators involved with data collection and analysis were not blinded to group allocation. MPTP studies were performed as previously described (Zhang et al. 2007c), using C57BL/6NCrl mice (8-10 weeks of age). For the PK2 rAAV neuroprotection experiments, C57BL/6NCrl mice were injected i.p. with 20 mg/kg MPTP, or equal volumes of saline (vehicle), once daily for four consecutive days and sacrificed 24 h after the last MPTP injection. For PKRA7 experiments, C57BL/6 mice were injected i.p. with 18 mg/kg MPTP, or equal volumes of saline (vehicle), once daily for three consecutive days. PKRA7 (20 mg/kg/day) was given i.p. 24 h before MPTP treatment and continued once daily for 10 consecutive days until sacrifice. Mice were euthanized at the indicated time points and samples processed for either IHC, qRT-PCR or Western blotting.

### **Stereotaxic surgery**

C57BL/6NCrl mice (8-10 weeks of age) were anesthetized using a ketamine/xylazine mixture. The Angle 2 stereotaxic instrument was used with a 10-mL Hamilton syringe to inject the rAAV directly into the striatum at the following stereotaxic coordinates in relation to Bregma (mm): -2 ML, 0.5 AP, -4 DV. After drilling a hole in the skull, 3  $\mu$ l of rAAV viral particles ( $\sim 9 \times 10^{12}$  total viral particles) were injected into the brain. The animal was allowed to recover for four weeks to maximize viral gene expression before behavioral testing, sacrificing or any treatment paradigm.

### **Quantitative PCR**

Primary mouse microglia were seeded out into 6 well plates at  $2 \times 10^6$  cells per well. Treatments were performed in DMEM/F12 media supplemented with 2% FBS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 2 mM L-glutamine, 2mM sodium pyruvate and 100 $\mu$ M non-essential amino acids. Cells were treated for 8 h with rPK2 (25nM), rPK2 cotreated with PC-7 (1 $\mu$ M), IS20 (10 $\mu$ M), or rIL-4 (20ng/mL). After treatment, cells were collected, pelleted, and

resuspended in lysis buffer with  $\beta$ -mercaptoethanol. RNA was isolated using Absolutely RNA Miniprep, an RNA isolation kit from Stratagene. RT-PCR was performed using an cDNA synthesis system (Applied Biosystems) to convert the RNA into cDNA. Expression levels were determined using real-time PCR with Qiagen RT<sup>2</sup> SYBR Green master mix and prevalidated using the qPCR mouse primers from SuperArray/Qiagen, or from previous literature (SA Biosciences)(Tang et al. 2014). For normalization of each sample, the mouse gene 18SrRNA (Cat. No. PPM57735E) was used as the housekeeping gene. The amount of each template was optimized empirically to maximize efficiency without inhibiting the PCR reaction. According to manufacturer's guidelines, dissociation curves and melting curves were run to ensure that single amplicon peaks were obtained without any non-specific amplicons. The results are reported as fold change in gene expression, which was determined using the  $\Delta\Delta C_t$  method using the threshold cycle ( $C_t$ ) value for the housekeeping gene and for the respective gene of interest in each sample.

### **Western blotting**

Microglia were collected after treatment, lysed using modified RIPA buffer, homogenized, sonicated, and centrifuged as previously described (Kaul et al. 2003, Kitazawa et al. 2002). The supernatants were collected and proteins were normalized, then stored with loading buffer and DTT. Samples were run on a Sodium Dodecyl Sulfate (SDS) gel electrophoresis. Normalized protein samples were loaded into each well and first separated in a 4% stacking gel and then through a 12-18% resolution gel. Proteins were then transferred to a nitrocellulose membrane. After transfer, the membranes were blocked using Western blocking buffer (Rockland Immunochemicals). Primary antibodies directed to the protein of interest in Rockland blocking buffer were then added to the membranes and stored overnight at 4°C. A Secondary antibody

specific to the primary antibody was added to the membrane in blocking buffer for 1 h. Either  $\beta$ -actin or  $\alpha$ -tubulin was used to confirm equal loading into each well of the gels. The membranes were read using the Odyssey infra-red imaging system.

### **Immunocytochemistry and Immunohistochemistry**

For immunocytochemistry, cells were plated onto coverslips in 24-well plates coated with 0.1% poly-D-Lysine. After cells were treated, 4% formaldehyde was used to fix the cells for 30 min. The cells were washed with PBS wash buffer. Then blocking buffer containing 2% BSA, 0.2% Triton X-100, and Tween was added to the wells for 1 hour. The cells were incubated with primary antibodies in 2% BSA at 4°C overnight. Next, an Alexafluor dye-conjugated secondary antibody in 2% BSA was added and incubated at room temperature on a shaker for 1 h. After washing Hoechst counterstain was added to the cells for 5-6 min to label nuclei. Coverslips were washed several times then mounted onto slides with Fluoromount mounting media. Cells were imaged under a Nikon 2000U microscope with a SPOT camera, and all images were processed in MetaMorph 5.7 (Universal Imaging, Downingtown, PA).

For histology, mice were anesthetized using the ketamine-xylazine mixture and cardiac perfusion of 4% PFA was used to fix the brain before being extracted and stored in 4% PFA at 4°C. After 48 h, brains were washed with PBS, and placed into a 30% sucrose solution for at least 24 h. The brains were then embedded in Optimal Cutting Temperature (OCT) compound and frozen into blocks for sectioning. Brains were sectioned on a Cryostat (CryoStar NX70, Thermo Scientific) at -20°C and placed into Cryosolution (30% Sucrose, Ethylene glycol, and PBS). Sections were then washed with PBS and permeabilized with blocking buffer (2% BSA, 0.1% Triton X-100, and Tween) for 1 h at room temperature. Antibodies directed to the protein of interest were then incubated with the sections overnight at 4°C. After washing with PBS, the



sections were incubated with Alexafluor dye-conjugated secondary antibodies for 1 h at room temperature. After washing with PBS, Hoechst dye (1:5000) was added to the sections for 5 min at room temperature to stain nuclei. Sections were then mounted on slides using the Fluoromount mounting medium (Molecular probes) according to the manufacturer's instructions. An inverted fluorescent microscope (Nikon TE-2000U) was used to visualize the sections, and a Spot digital camera (Diagnostic Instruments, Inc) was used to capture images.

### **Microglia morphological assessment**

Iba1 immunofluorescence in primary mouse microglia (species and dilution) and DAB immunostaining of mouse striatum sections (Species and dilution) was used to mark the microglia. 20X and 60X images were taken using an inverted fluorescent microscope (Nikon TE-2000U). Images were imported into ImageJ for morphological analysis. Morrison et al. demonstrated that the microglial processes could be analyzed using an ImageJ plugin named Analyze Skeleton (Morrison and Filosa 2013). Fluorescent and DAB images were converted to binary, and then skeletonized in the ImageJ software. The skeletonized image is then fed into the Analyze Skeleton plug in (Arganda-Carreras et al. 2010) without pruning ends and measuring the long/short branch distance. The longest/short path calculation and applications come from a previous source (Fraas et al. 2014). Detailed information of branch, junction and endpoint number along with branch length metrics is displayed in the results window for the entire image. The results were transferred to an Excel spreadsheet and sorted to only show information with branch numbers between 10 and 300 to remove any non-cellular background. Results were then compared to the number of counted cells in the field to then determine each of the morphology metrics per cell. Multiple fields were used for each coverslip or section, and then 2-3 biological replicates were used for each experiment. At least 200 cells were measured for each of the morphology assessments. Cell soma

size and roundness is measured in the same Iba1 images in ImageJ by freehand selection outlining each cell soma and then select the “area” and “shape descriptors” analytes to measure. A minimum of 30 cells were analyzed for each treatment in analyzing the soma descriptors, with at least 2 biological replicates for each experiment.

### **Mitochondrial dynamics**

To analyze the mitochondrial energy dynamics in primary mouse microglia, the XFe24 Seahorse analyzer was used to measure OCR and ECAR levels. Microglia were plated on Seahorse plates at 100,000 cells in each well and allowed to attach overnight in growth media. Cells were then washed with serum free DMEM/F12 media 3 times and 500ul of treatment was added to the wells in serum free DMEM/F12 for 8 hours. After the 8 hours, the cells were washed 2 times with the Seahorse base media supplemented with sodium pyruvate and glutamine. The MitoStress test was performed according to the manufacturer’s guidelines. The cells were left in the 500ul of the Seahorse base media supplemented with sodium pyruvate and glutamate. After testing correct concentrations of the chemicals, 0.75uM of oligomycin, 2uM of FCCP, and 0.5uM rotenone/antimycin was added to the cells to measure mitochondrial dynamics. Analysis was performed through the MitoStress Results Generator found on the Agilent website.

### **Intracellular ROS generation and Nitric Oxide measurement**

The fluorescent probe 2',7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA) (Calbiochem) was used to determine the level of intracellular reactive oxygen species (iROS). Primary mouse microglia were treated with 200 nM LPS in RPMI media with 2% FBS or co-treated with 25nM recombinant PK2 (rPK2) along with the LPS treatment. After treatment, the cells were washed with warm HBSS media, and then incubated with 100  $\mu$ l of 40  $\mu$ M DCFH-DA

in HBSS for 1 h. The fluorescence intensity of the signal was determined using a Synergy-2 multi-mode microplate reader at an excitation of 485 nm and an emission of 530 nm. After subtracting the control fluorescent signal as background, an increase in fluorescence intensity of treatments was expressed as an increase in iROS, as previously described (Gordon et al. 2012).

Nitric oxide production by primary microglia was measured indirectly by quantification of nitrite in the supernatant using the Griess reagent (Sigma-Aldrich). Microglia were plated in poly-D-lysine-coated 96-well plates at  $1 \times 10^5$  cells/well. Cells were treated with 250ng/ml of LPS for 24 h; and after 100  $\mu$ l of supernatant was collected from each well, an equal volume of the Griess reagent was added. The samples were incubated on a plate shaker at room temperature for 15 min until a stable color was obtained. The absorbance at 540 nm was measured using the Synergy 2 multimode microplate reader, and the nitrite concentration was determined from a sodium nitrite standard curve.

#### **Fluo-4 calcium mobilization assay**

A Fluo-4 NW assay kit (Molecular probes) was used to test calcium mobilization in primary mouse microglia resulting from nanomolar concentrations of rPK2. Cells were plated overnight into 96-well plates in growth media. Cells were then washed with HBSS and incubated with the Fluo-4 NW dye for one hour. Each plate was then read kinetically using a Synergy-2 multi-mode microplate reader. After obtaining background readings by reading each plate every 50 ms for 20 sec, rPK2 was injected at the indicated nanomolar concentrations. The net change in fluorescent signal ( $\Delta t$ ), read every 50 ms for 3 min, was obtained after subtracting the background from the maximum signal for each sample.

## Data analysis

Data analysis was performed using the Prism 4.0 software package (GraphPad Software, San Diego, CA). The data was first analyzed using one-way ANOVA and then Tukey's post-test was performed to compare all treatment groups. Differences of  $p < 0.05$  were considered statistically significant. The Student's t-test was used when two groups were being compared.

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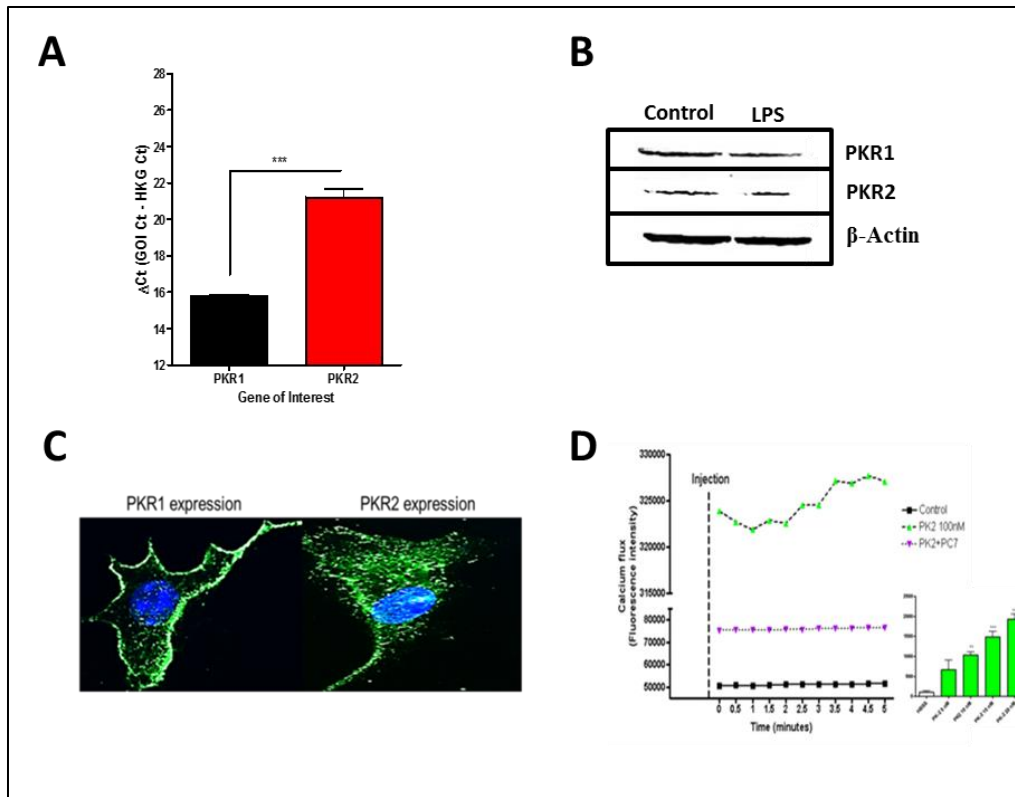
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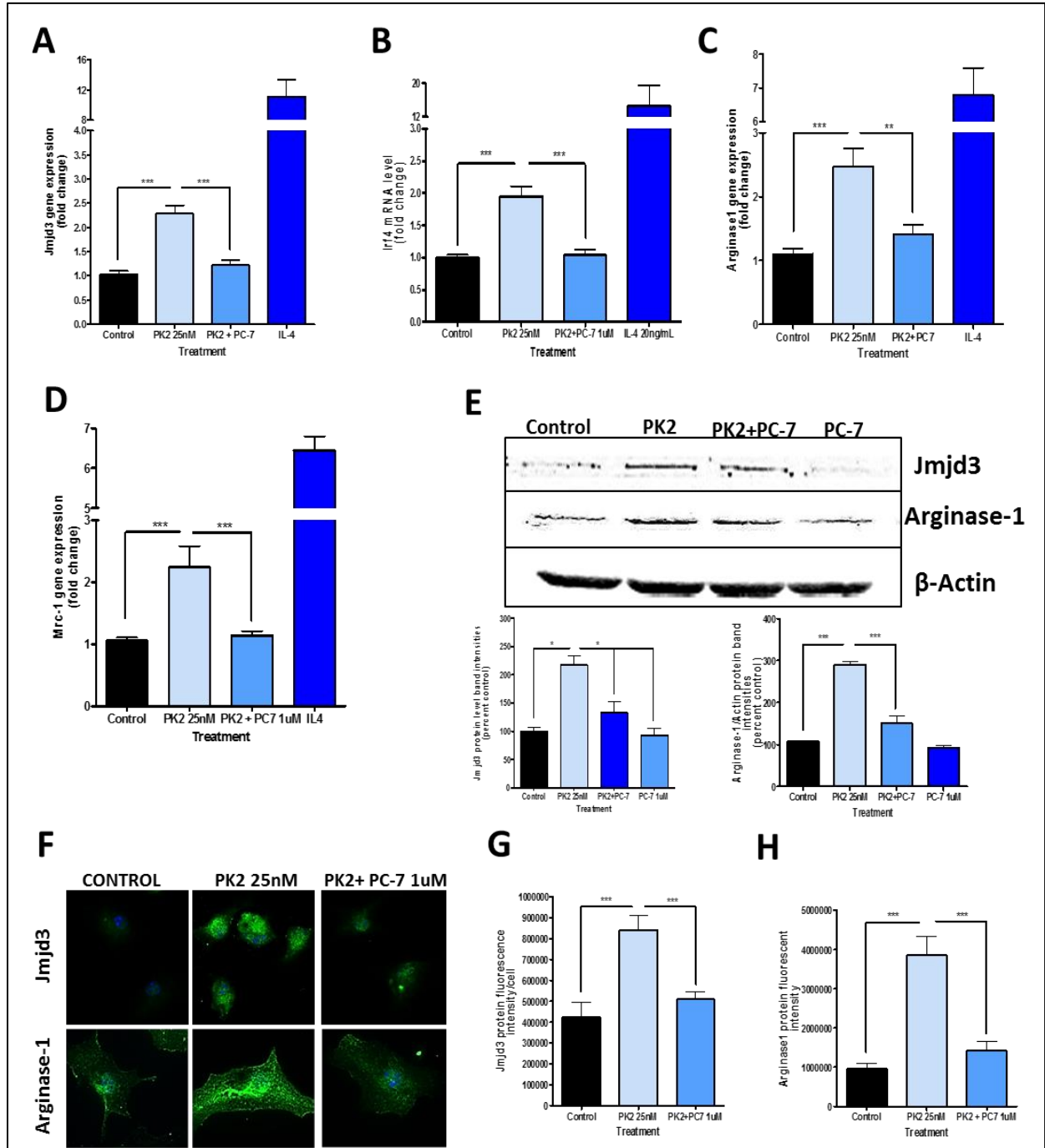
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## Figures

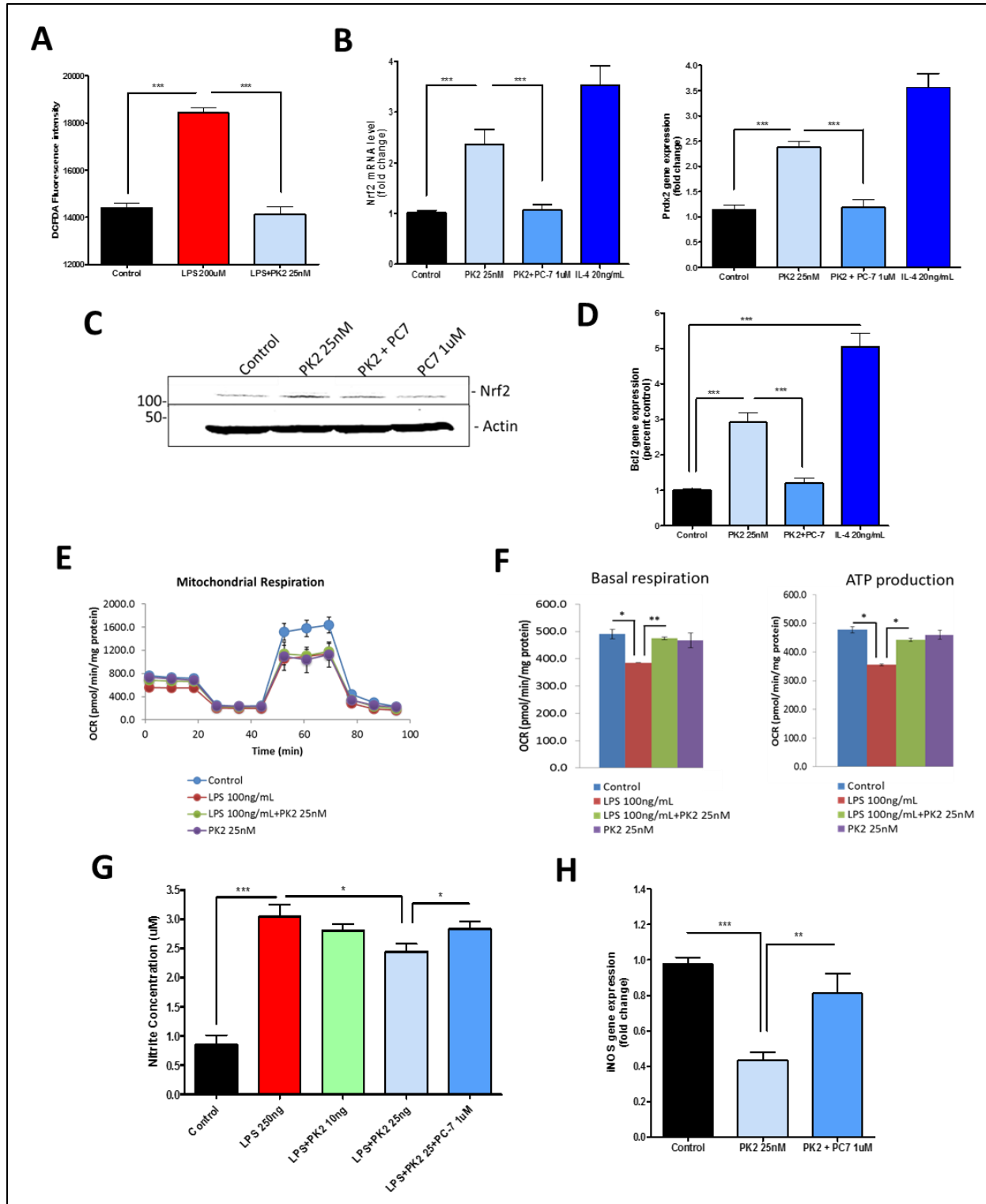


**Figure 1.** *Prokineticin-2 can signal to primary mouse microglia and increases intracellular calcium* (A) Quantitative real time PCR for Prokineticin receptor gene expression, with PKR1 expressed significantly higher than PKR2. (B) Representative Western blot for PKR1 and PKR2 in primary mouse microglia showing higher PKR1 compared to PKR2 and no significant change with LPS treatment. (C) Different cellular localization pattern for PKR2 (Green, right panel) and PKR1 (Green, left panel) with the nucleus stained with Hoechst (Blue). Expression of PKR2 was peri-nuclear whereas PKR1 expression was localized more on the periphery of the cell. (D) Calcium mobilization (flux) dose-dependently induced by rPK2 in primary mouse microglia, with calcium-free HBSS used as a control treatment. Data represented by group mean  $\pm$  SEM and experiments were repeated at least twice. Asterisks denote a significant difference between rPK2 treatment and the control (\*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).



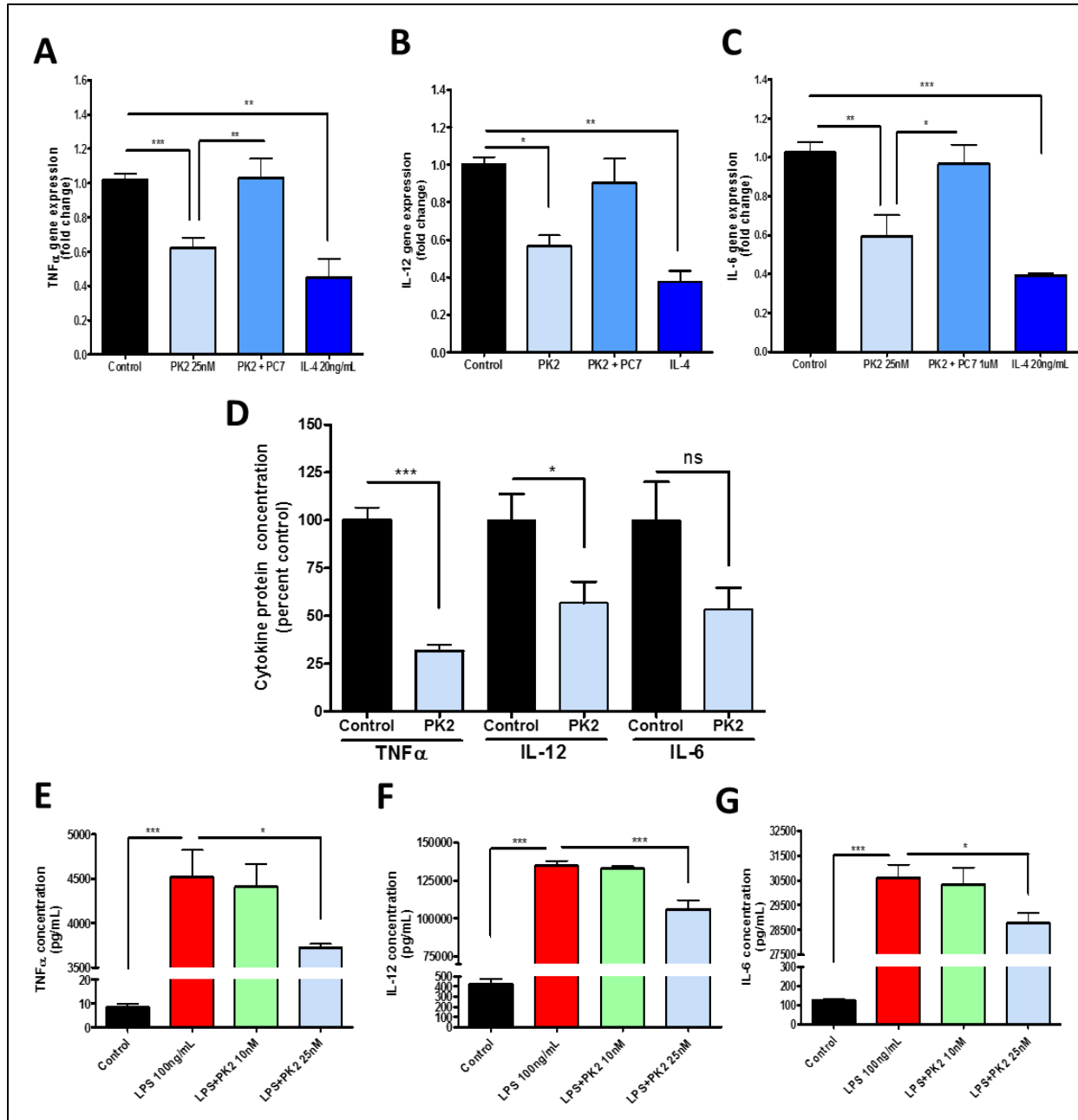
**Figure 2.** *Prokineticin-2 induces alternative activation phenotype in primary mouse microglia.*

(A-D) Quantitative real-time PCR analysis for the gene expression of alternative activation markers Jmjd3 (A), Irf4 (B), Arginase-1 (C) and Mrc1 (D). Data represented by group mean  $\pm$  SEM and experiments were repeated at least three times. Asterisks denote a significant difference between rPK2 treatment and the control or rPK2 co-treated with PC-7 (\*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ). (E) Representative Western blot with immunofluorescence band intensity quantification for Jmjd3 (left panel) and Arginase-1 (right panel). (F) Immunofluorescence microscopy for Jmjd3 (Green, top panel) and Arginase-1 (Green, bottom panel) with the nucleus stained with Hoechst (Blue). (G-H) Integrated density of immunofluorescence quantification for Jmjd3 (G) and Arginase-1 (H), showing that rPK2 increases the protein level of both proteins compared to control and rPK2 co-treated with PC-7. Data represented by group mean  $\pm$  SEM and experiments were repeated at least twice. Asterisks denote a significant difference between rPK2 treatment and the control or rPK2 co-treated with PC-7 with experiments repeated at least twice (\*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).

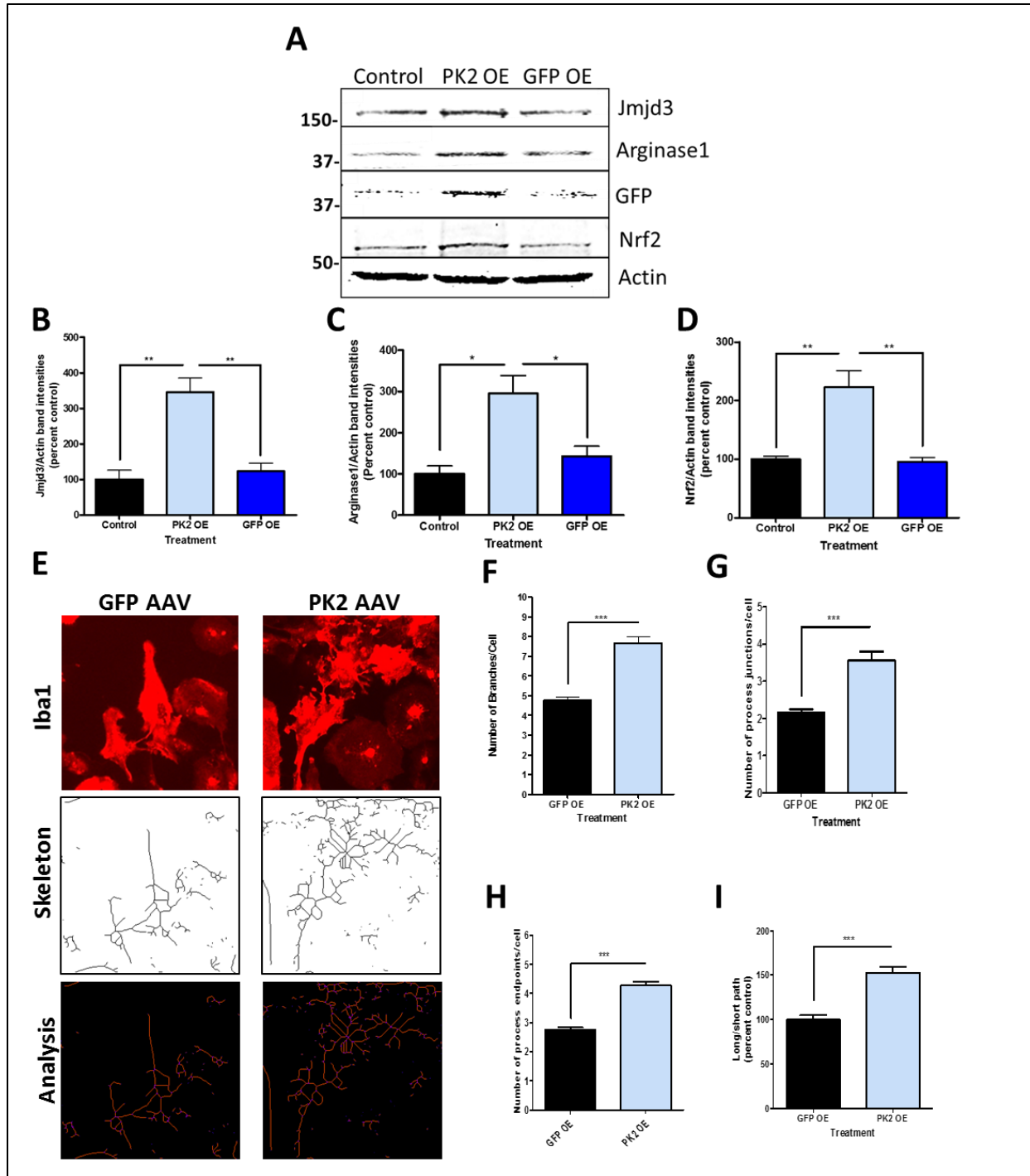




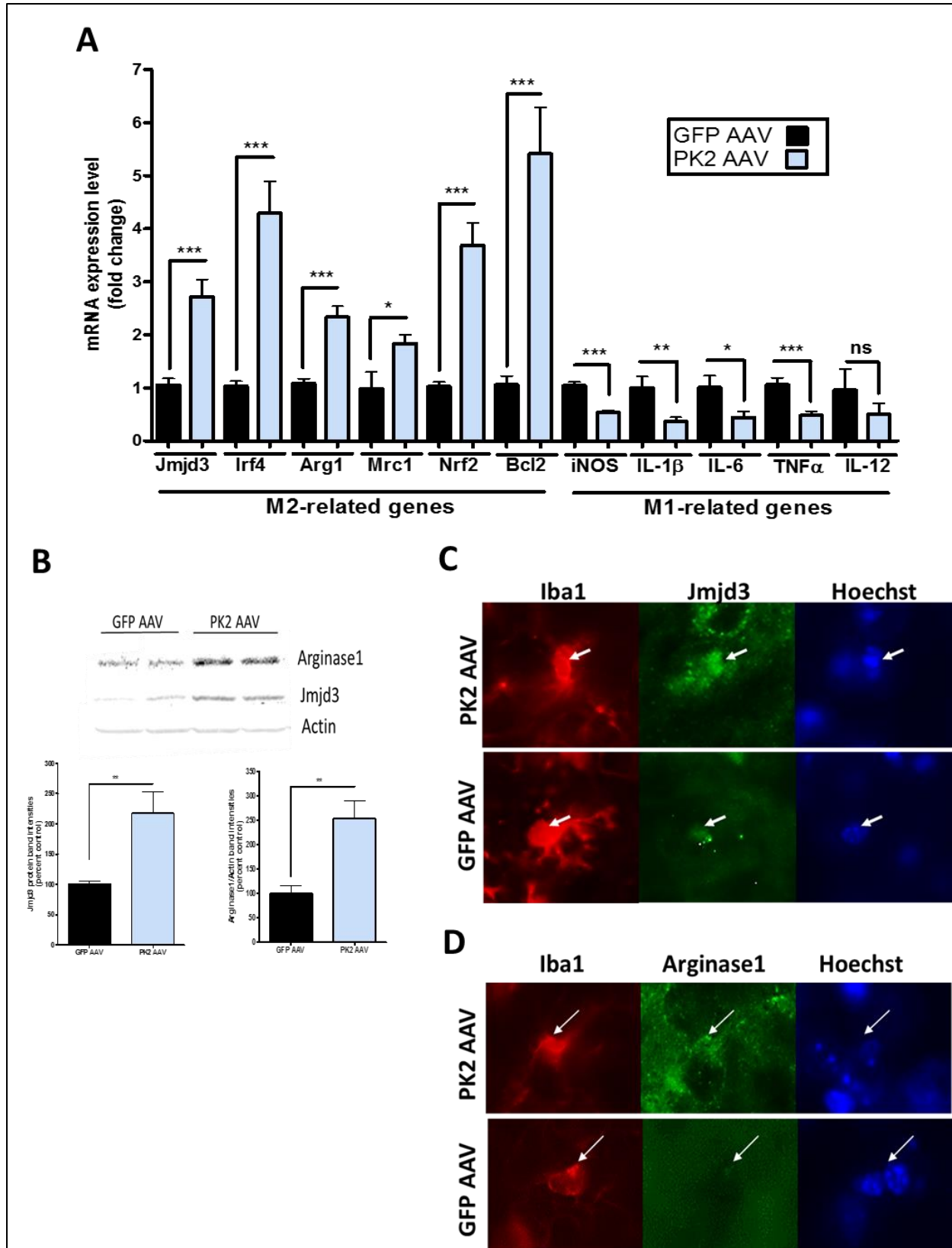
**Figure 3.** *Prokineticin-2 attenuates LPS-induced mitochondrial bioenergetic dysfunction and production of ROS and NO production in primary mouse microglia.* (A) DCF-DA fluorescent dye measurement for intracellular ROS, shows that rPK2 treatment can significantly reduce LPS-induced iROS levels. (B) Quantitative real-time PCR gene expression analysis for anti-oxidant genes, demonstrating that rPK2 treatment can induce Nrf2, and Prdx2 (C) Representative Western blot for Nrf2 protein level. (D) Quantitative real-time PCR gene expression analysis for Bcl2. Data represented by group mean  $\pm$  SEM and experiments were repeated at least three times. Asterisks denote a significant difference between rPK2 treatment and the control or rPK2 co-treated with PC-7 (\*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ). (E-F) Mitochondrial dynamics in primary microglia with LPS and PK2 treatment. Recombinant PK2 treatment attenuates LPS-induced reduction in mitochondrial dysfunction, demonstrated by oxygen consumption rate (OCR) plot (F), quantification of basal respiration (G, left panel) and quantification of ATP production (G, right panel). (G) Griess assay for measurement of nitric oxide in supernatants from treated microglia, showing that rPK2 can significantly attenuate LPS-induced nitric oxide production. (H) Quantitative real-time PCR gene expression analysis for NOS2. Data represented by group mean  $\pm$  SEM and experiments were repeated at least two times. Asterisks denote a significant difference between rPK2 treatment and the control or rPK2 co-treated with PC-7 (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).

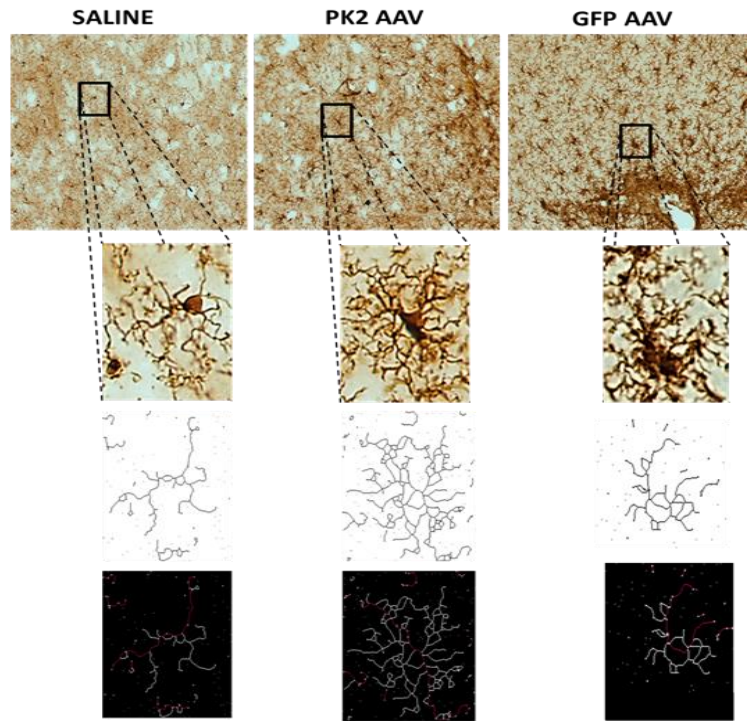
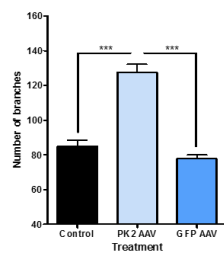
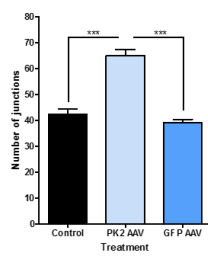
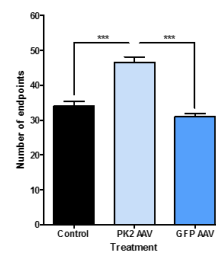
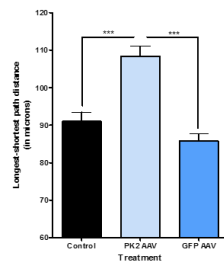
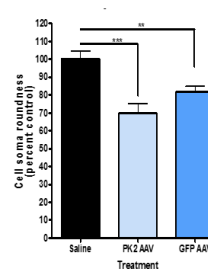
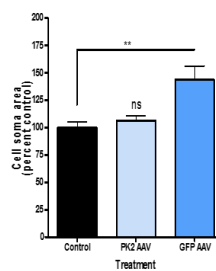


**Figure 4.** *Reduction in pro-inflammatory cytokine production with Prokineticin treatment in primary mouse microglia.* (A-C) Quantitative real-time PCR gene expression analysis for proinflammatory cytokines, which showed that rPK2 treatment reduced basal TNF (A), IL-12 (B), and IL-6 (C) gene expression compared to control. (D) Pro-inflammatory cytokine protein levels measured in the primary mouse microglia conditioned media treated with rPK2 using the Bioplex 200 multi-plex system for TNF $\alpha$ , IL-12 and IL-6. (E-G) Pro-inflammatory cytokine protein levels measured in the primary mouse microglia conditioned media treated with LPS and LPS co-treated with PK2 using the Bioplex 200 multi-plex system for TNF $\alpha$  (E), IL-12 (F) and IL-6 (G). Data represented by group mean  $\pm$  SEM and experiments were repeated at least two times. Asterisks denote a significant difference between rPK2 treatment and the control or rPK2 co-treated with PC-7 (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).



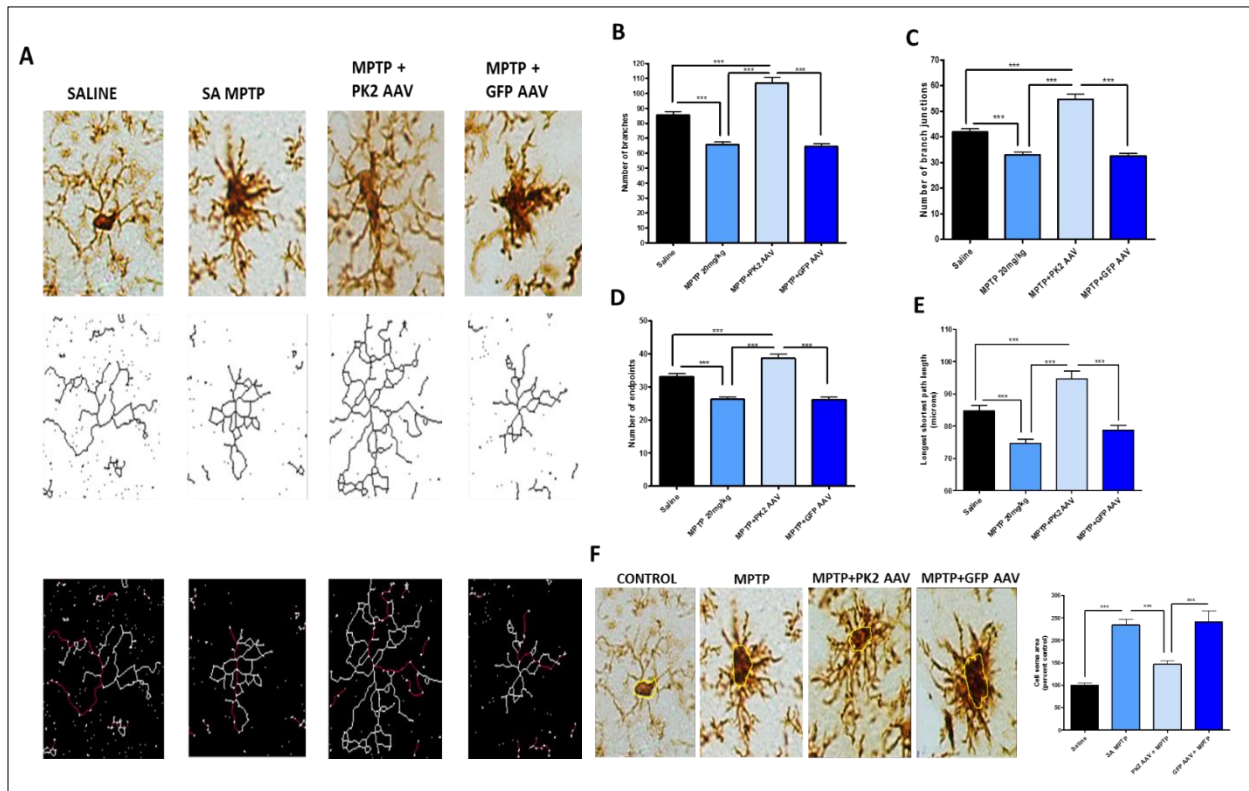
**Figure 5.** *Prokineticin-2 overexpression in vitro induces alternative activation of primary mouse microglia.* (A) Representative Western blots for Jmjd3, Arginase-1, and GFP show that Lenti-viral PK2 overexpression was successful with increased GFP levels. (B-C) Protein band densitometric quantification for Jmjd3 (B), Arginase-1 (C) and Nrf2 (D) demonstrating that PK2 overexpression increases these alternative activation marker protein levels. (E) ImageJ skeletonization after converting the Iba1 immunofluorescent image (top panel) into a binary image. ImageJ Analyze Skeleton plug-in image (bottom panel) from the skeletonized image (middle panel) with process branches (Orange), junctions (Purple), and endpoints (Blue). (F-I) Measurements of the microglia processes. Analysis for the average number of process branches (F), process junctions (G), process endpoints (H) and longest shortest branch path (I) per Iba1-positive cell shows that PK2 overexpression significantly increased average cell process metrics compared to Control, and GFP overexpression. Data represented by group mean  $\pm$  SEM and experiments were repeated at least twice. Asterisks denote a significant difference between PK2 OE and the control and GFP OE (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).



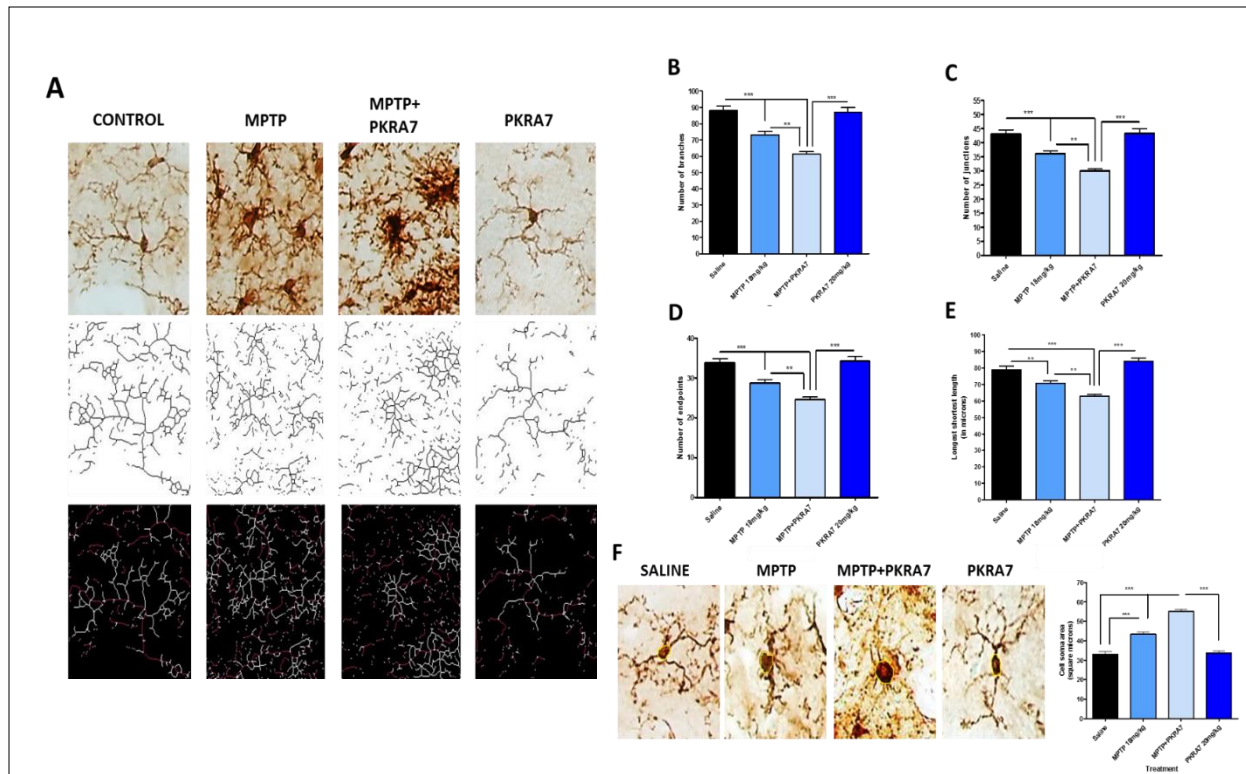
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**Figure 6.** *PK2-AAV 2/5 overexpression induces alternative activation of microglia in the mouse striatum.* C57/Bl6 mice were stereotaxically injected in the striatum with either PK2-AAV 2/5 or GFP-AAV 2/5 and allowed to reach maximal viral gene expression for four weeks. **(A)** Quantitative real-time PCR gene expression analysis for classical or alternative activation markers. PK2-AAV significantly increased alternative activation markers such as *Jmjd3*, *Irf4*, *Arginase-1* and *Mrc1*, while significantly decreasing classical activation markers such as *iNOS*, *TNF $\alpha$* , *IL-1 $\beta$* , *IL-6* and *IL-12*. **(B)** Representative Western blot for *Jmjd3* and *Arginase-1* with densitometric quantification of the bands showing that PK2-AAV significantly increases *Jmjd3* (left panel) and *Arginase-1* (right panel). Data represented by group mean  $\pm$  SEM and experiments were performed with at least 4 animals run with technical replicates. Asterisks denote a significant difference between PK2 OE and the control and GFP OE (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ). **(C-D)** Immunofluorescent images of *Jmjd3* (C) and *Arginase-1* (D)(Green) in *Iba1*-positive cells (Red) in the mouse striatum with the nucleus stained with Hoechst (Blue). **(E)** *Iba1* DAB immunostain image (top panel) was converted into a binary image and skeletonized (middle panel). ImageJ Analyze Skeleton plug-in image (bottom panel) with process branches (Orange), junctions (Purple), and endpoints (Blue). **(F-I)** Measurements of the microglia processes. Analysis for the average number of process branches (F), process junctions (G), process endpoints (H) and longest shortest branch path (I) shows that PK2-AAV significantly increased average cell process metrics compared to Control, and GFP-AAV. **(J-K)** Microglial cell soma shape descriptors. (J) ImageJ quantification of cell soma roundness in *Iba1*-positive cells. (K) ImageJ quantification of cell soma area in *Iba1*-positive cells. Data represented by group mean  $\pm$  SEM and experiments were performed with at least 3 animals with multiple visual fields per animal section. Asterisks denote a significant difference between PK2 OE and the control and GFP OE (\*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).

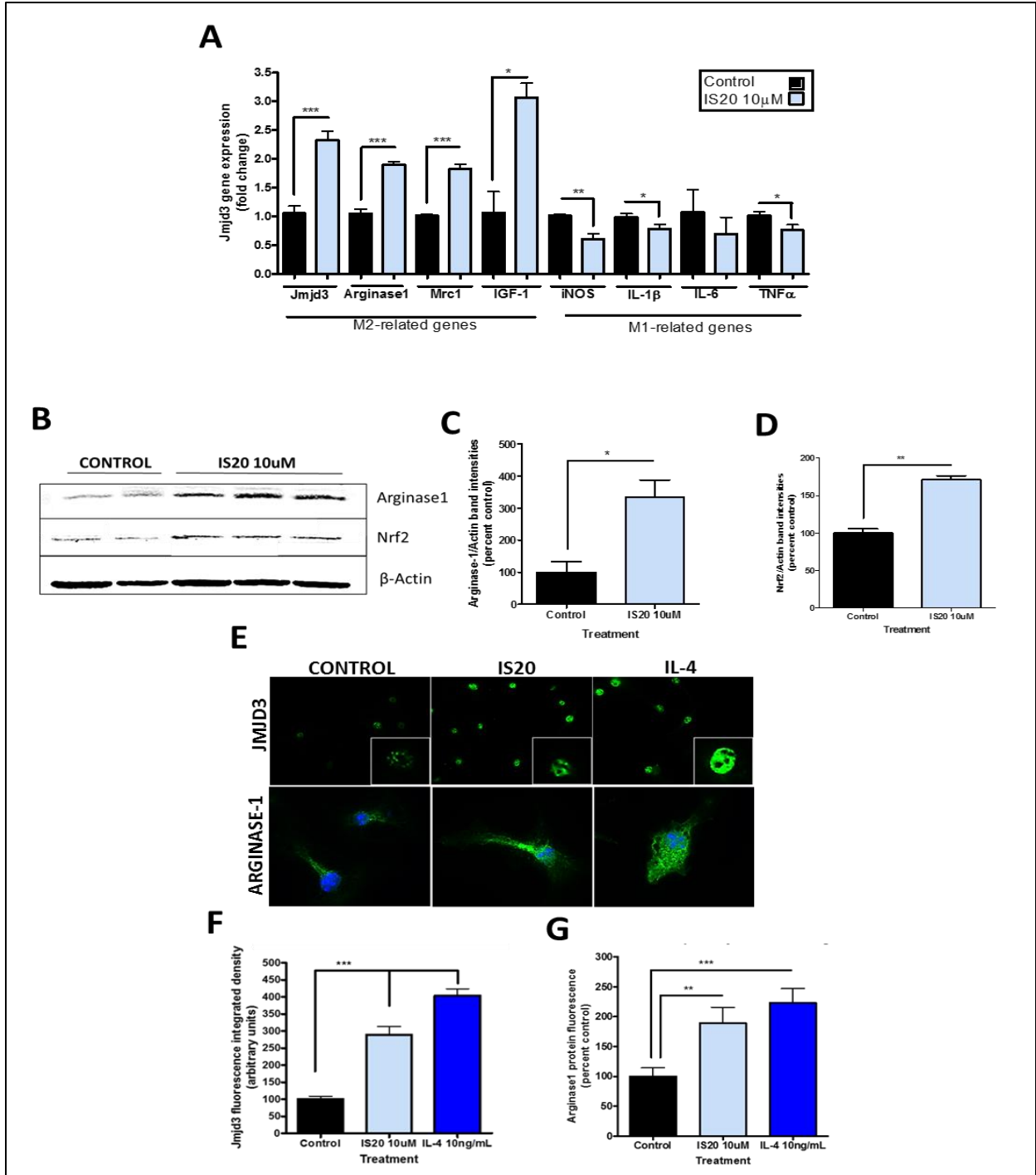


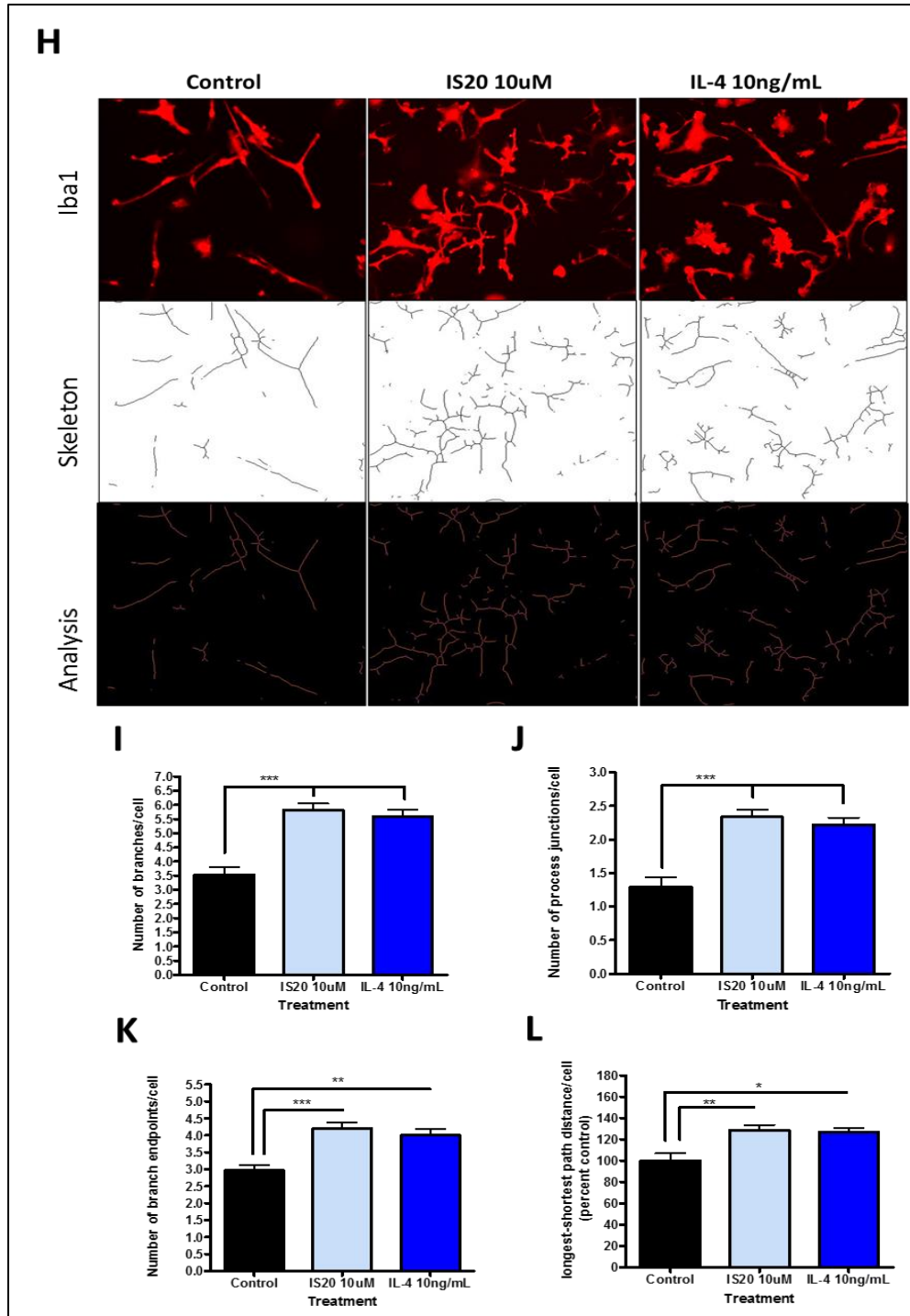


**Figure 7.** PK2-AAV 2/5 attenuates MPTP-induced classical activation of microglia in the mouse striatum. (A) Iba1 DAB immunostain image (top panel) was converted into a binary image and skeletonized (middle panel). ImageJ Analyze Skeleton plug-in image (bottom panel) with process branches (Orange), junctions (Purple), and endpoints (Blue). (B-E) Measurements of the microglia processes. Analysis for the average number of process branches (B), process junctions (C), process endpoints (D) and longest shortest branch path (E) shows that PK2-AAV significantly attenuated the MPTP-induced loss of cell process metrics. (F) Iba1 immunostaining microscopy images in ImageJ with the cell soma outlined and the quantification of the cell soma area (right panel). Data represented by group mean  $\pm$  SEM and experiments were performed with at least 3 animals with multiple visual fields per animal section. Asterisks denote a significant difference between PK2 OE and the control and GFP OE (\*\*\*)  $p < 0.001$ .



**Figure 8.** Prokineticin receptor antagonist exacerbates MPTP-induced classical activation morphology in microglia of the mouse striatum. (A) Iba1 DAB immunostain image (top panel) was converted into a binary image and skeletonized (middle panel). ImageJ Analyze Skeleton plug-in image (bottom panel) with process branches (Orange), junctions (Purple), and endpoints (Blue). (B-E) Measurements of the microglia processes. Analysis for the average number of process branches (B), process junctions (C), process endpoints (D) and longest shortest branch path (E) shows that PKRA7 significantly increased the MPTP-induced loss of cell process metrics. (F) Iba1 immunostaining microscopy images in ImageJ with the cell soma outlined and the quantification of the cell soma area (right panel). Data represented by group mean  $\pm$  SEM and experiments were performed with at least 3 animals with multiple visual fields per animal section. Asterisks denote a significant difference between MPTP+PKRA7 and the control and PKRA7 alone groups (\*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).





**Figure 9.** *PKR1* agonist, IS20, promotes alternative activation of primary mouse microglia. **(A)** Quantitative real-time PCR gene expression analysis for classical or alternative activation markers. IS20 significantly increased alternative activation markers such as *Jmjd3*, Arginase-1 and *Mrc1*, while significantly decreasing classical activation markers such as iNOS, TNF $\alpha$ , IL-1 $\beta$ , and IL-6. **(B-D)** Representative Western blot **(B)** for Arginase-1 and Nrf2 with densitometric quantification of the bands showing that IS20 significantly increases Arginase-1 **(C)** and Nrf2 **(D)**. Data represented by group mean  $\pm$  SEM and experiments were performed with at least two times. Asterisks denote a significant difference between IS20 and the control (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ). **(E-G)** Immunofluorescence microscopy for *Jmjd3* (Green, top panel) and Arginase-1 (Green, bottom panel) with the nucleus stained with Hoechst (Blue) **(E)**. Integrated density of immunofluorescence quantification for *Jmjd3* **(F)** and Arginase-1 **(G)**, showing that IS20 increases the protein level of both proteins compared to control. **(H)** ImageJ skeletonization after converting the Iba1 immunofluorescent image (top panel) into a binary image. ImageJ Analyze Skeleton plug-in image (bottom panel) from the skeletonized image (middle panel) with process branches (Orange), junctions (Purple), and endpoints (Blue). **(I-L)** Measurements of the microglia processes. Analysis for the average number of process branches **(I)**, process junctions **(J)**, process endpoints **(K)** and longest shortest branch path **(L)** per Iba1-positive cell shows that IS20 treatment significantly increased average cell process metrics compared to control. Data represented by group mean  $\pm$  SEM and experiments were repeated at least twice. Asterisks denote a significant difference between IS20 and the control (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).

## CHAPTER IV

PROKINETICIN-2 INDUCES CHEMOTAXIS AND ALTERNATIVE ACTIVATION OF  
ASTROCYTES WITH INCREASED ANTI-OXIDANT RESPONSE AND GLUTAMATE  
UPTAKE

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## Abstract

The mechanisms behind astrocyte activation in Parkinson's disease (PD) are poorly understood. With significant crosstalk between neurons and astrocytes, neuronal-secreted factors could play an important role as modulators of astrocyte activation. Previously, our lab found that dopaminergic neurons produce and secrete higher levels of the chemokine-like signaling protein Prokineticin-2 (PK2) in cell culture, animal models of PD, and in post-mortem PD patients compared to age-matched controls. Astrocytes express the prokineticin receptors, and exogenously added PK2 can activate signaling pathways in cultured astrocytes. Herein, we demonstrate that PK2 can induce astrocyte activation, both *in vitro* and *in vivo*. Importantly, functional studies in primary mouse astrocytes reveal that PK2 signaling in astrocytes reduces pro-inflammatory factors, while increasing anti-oxidant proteins such as Arginase1 and Nrf2. Furthermore, we found that a non-peptide prokineticin receptor 1 chemical agonist is able to similarly induce this

alternative activation of astrocytes. Importantly, PK2 treatment was also able to increase extracellular glutamate uptake by increasing gene expression of the glutamate transporter GLAST. Collectively, our results reveal a novel neuron-astrocyte signaling pathway important for alternative astrocyte activation, and a new potential therapeutic avenue for Parkinson's disease and other neurodegenerative diseases.

### Introduction

Astrocytes are important for brain homeostasis and neuronal health, including the production of neurotrophic factors and uptake of extracellular glutamate. Also, during central nervous system (CNS) injury, astrocytes become activated in a process commonly referred to as astrogliosis, and some studies use astrocyte activation as a marker for damaged neurons (Ferraguti et al. 2001, Hu et al. 2016). Activated astrocytes have also been identified in Parkinson's disease (PD) patients (Forno et al. 1992, Wakabayashi et al. 1999), and multiple animal models of PD have demonstrated an increase in astrogliosis (Yasuda et al. 2008, Teismann et al. 2003, Saura et al. 2003). However, the mechanisms behind astrocyte activation are not completely understood. Currently, researchers are debating whether activated astrocytes are beneficial or harmful in CNS injury, including PD. Interestingly, some results indicate that astrocytes respond to different stimuli by shifting to different activation phenotypes, similar to the classical and alternative phenotype activation states seen in macrophages and microglia (Jang et al. 2013a). Therefore, ligands that activate astrocytes could lead to either a classical, pro-inflammatory activation or alternative, anti-inflammatory activation state, and should be investigated in the search for potential therapeutics for neurodegenerative diseases.

Prokineticin-2 (PK2) is a small chemokine-like signaling protein that was originally found to potently contract gastrointestinal muscles (Li et al. 2001). It is a homologue to MIT1, a non-venomous protein in mamba snake venom, and a frog skin protein, Bv8 (Schweitz et al. 1999, Ferrara et al. 2004). Subsequently, two G-protein coupled receptors were identified as prokineticin receptors, PKR1 and PKR2 (Lin et al. 2002b). Signaling through these receptors mediates various physiological functions throughout the body, including hematopoiesis, angiogenesis, reproductive functions, innate immunity (Monnier and Samson 2008) and cardiomyocyte protection (Urayama et al. 2007a). In the healthy CNS, PK2 also plays a role in modulating circadian rhythms, olfactory bulb biogenesis (Ng et al. 2005, Zhou and Cheng 2005), energy expenditure and homeostasis (Zhou et al. 2012), and neuroprotection (Melchiorri et al. 2001, Landucci et al. 2016). PK2 expression is generally induced in areas of increased inflammation, including the gut (Watson et al. 2012), and the brain (Cheng et al. 2012). Our lab previously established that dopaminergic neurons secrete soluble PK2 when treated with TNF $\alpha$  *in vitro*, and in multiple *in vivo* mouse models of PD, and we detected it in human postmortem PD patients (Gordon and Neal et al., unpublished). In that study, we found that PK2 inhibited MPTP neurotoxicant-mediated dopaminergic neuronal death both *in vitro* and *in vivo*. We concluded that dopaminergic neurons produced PK2 as a potential compensatory reaction to stressful conditions, such as mitochondrial dysfunction and neuroinflammation. We were also able to determine that PKR2 is expressed in dopaminergic neurons *in vitro* and in the mouse substantia nigra. Previous studies found that the PKR2 gene is expressed at a higher level in the brain compared to PKR1 (Lin et al. 2002b). However, the PKR1 gene is expressed at a higher level in astrocytes and microglia in the brain compared to PKR2 (Zhou 2006). Interestingly, PK2 treatment induces cell proliferation and



intracellular calcium mobilization in cultured astrocytes. Based on the observations of soluble PK2 being secreted from damaged dopaminergic neurons and that PK2 signals to astrocytes, we investigated the role of PK2 signaling in astrocytes of the brain.

## Results

### **Receptors for secreted Prokineticin-2 are expressed on primary mouse and human astrocytes**

To determine what role the chemokine-like protein PK2 plays in astrocytes, we first tested our primary mouse astrocytes to characterize prokineticin receptor expression. Using qRT-PCR with validated primers (Qiagen) we found that both the PKR1 and PKR2 genes are expressed in primary mouse astrocyte cells and primary human astrocytes (Figure 1A). However, PKR1 was expressed over 12-fold higher than the expression of PKR2 in both mouse and human astrocytes, which is consistent with previous results in mouse astrocytes (Koyama et al. 2006). Additionally, we characterized the protein level of the prokineticin receptors using both Western blot (Figure 1B) and fluorescence microscopy (Figure 1C). The Western blot clearly shows higher PKR1 protein when compared to PKR2 protein levels in the same primary mouse astrocyte samples. The fluorescence microscopy images demonstrate that PKR1 is localized to the periphery of the cell, whereas PKR2 has higher expression closer to the nucleus. Prokineticin receptors are GPCRs that can be linked to Gi, Gq or Gs (Zhou 2006). Signaling through Gq induces intracellular calcium mobilization, Gs increases cAMP levels, whereas Gi inhibits the other two pathways. Therefore, we tested whether adding recombinant human PK2 (rPK2) to isolated primary mouse astrocytes could cause functional changes, such as increased intracellular calcium, and cell proliferation. The Fluo4-NW intracellular calcium dye revealed that intracellular calcium levels spiked significantly

in an rPK2 dose-dependent manner when compared to adding calcium-free HBSS alone (Figure 1D). The spike in calcium was blocked using a prokineticin receptor inhibitor, PC-7, thus indicating its specificity to the PK2 signaling. These results verify that rPK2 is capable of eliciting a signaling response in astrocytes through one of the prokineticin receptors. Recombinant PK2 treatment was also able to significantly increase primary mouse astrocyte cell proliferation when compared to control, as measured by the CyQuant dye assay of astrocyte cell numbers (Figure 1E). Taken together, these results verify previous research on PK2 in astrocytes, elucidate cellular localization of the two prokineticin receptors in astrocytes and demonstrate that our model will work for prokineticin signaling in astrocytes.

### **Prokineticin-2 overexpression increases GFAP level, astrocyte numbers and soma size**

Normal resting astrocytes have thin cell bodies, thin processes, and express a low level of glial fibrillary acidic protein (GFAP). When astrocytes become activated, the soma and primary processes undergo hypertrophy, and the astrocytes express higher levels of GFAP (Pekny and Pekna 2014). Previous results demonstrated that PK2 could activate immune cells and modulate systemic inflammation (Monnier and Samson 2008), therefore we investigated if PK2 could similarly affect astrocytes in the mouse striatum. We developed a way to overexpress PK2 tagged to GFP *in vivo* using an AAV 2/5 viral delivery system under the power of the CMV promoter. We injected PK2-AAV into the mouse striatum and waited 4 weeks for maximal expression. The PK2 tagged to GFP was found throughout most of the striatum as seen by fluorescence microscopy. Interestingly, GFAP increased significantly in the striatum of PK2-AAV injected animals (Figure 2A). Similarly, Western blotting of the striatum demonstrated that GFAP protein levels had

increased in the striatum of PK2-AAV injected animals (Figure 2B). These results indicate that overexpression of PK2 in the striatum increases protein levels of GFAP, and therefore astrocyte activation.

To further characterize the astrocyte activation elicited by PK2 overexpression, we performed DAB immunostaining for GFAP on sections from either GFP-AAV or PK2-AAV injected animals. The control striatum contained cells with characteristics resembling resting astrocytes with thin cell bodies, thin processes, low GFAP expression and fewer cells that were GFAP-positive. Consistent with Western blotting results, the PK2-AAV injection increased the number of GFAP-positive cells in the striatum, which also exhibited cell soma hypertrophy, obvious hypertrophy of primary processes, and an increased amount of GFAP protein immunostaining (Figure 2C). In contrast, GFP-AAV failed to significantly increase any of these characteristics when compared to control. Taken together, these results demonstrate that PK2 overexpression induces significant morphological changes in GFAP-positive cells that are indicative of astrocyte activation.

### **Prokineticin-2 overexpression reduces the number of astrocyte processes and endpoints**

Another indication of astrocyte activation is the retraction of cytoplasmic processes (Kang et al. 2014). Since images of GFAP DAB immunostained mouse striatal sections seemed to reveal fewer astrocytic processes in PK2-AAV injected animals, we analyzed astrocyte processes using an ImageJ plugin developed to measure branching (Morrison and Filosa 2013). The GFAP DAB immunostain images were uploaded into ImageJ (Figure 3A), converted into binary images, and finally reduced to a skeleton image (Figure 3B). This skeleton image was then analyzed to show

every branch (orange), junction (purple) and endpoint (blue) (Figure 3C), allowing us to determine the number of branches and junctions, length of processes and number of endpoints. PK2-AAV significantly reduced the number of astrocyte processes when compared to control, whereas GFP-AAV did not (Figure 3D). Also, the number of astrocytic process endpoints per cell was significantly reduced by the PK2-AAV injection (Figure 3E). Taken together, these results add another quantitative measure demonstrating that PK2 overexpression can activate astrocytes *in vivo*.

### **Recombinant soluble PK2 induced wound healing in primary mouse and human astrocytes**

Since a reduction in the number of processes and endpoints in astrocytes *in vivo* could be because those cells are currently migrating or preparing for migration, we tested whether rPK2 stimulates astrocyte migration. To measure cell migration, we utilized a scratch-wound technique, wherein a pipet tip was used to create a “wound” on a confluent layer of astrocytes. Comparing the width of the “wound” at 0 hours and 24 hours after rPK2 treatment will give the distance that the cells migrated (Figure 4A). The results demonstrate that recombinant PK2 treatment in both the U373 human astrocytoma cell line and primary human astrocytes significantly increased wound closure (healing) over control treatment (Figure 4B and C). This is the first report demonstrating that prokineticin signaling can lead to increased wound healing in astrocytes.

### **Recombinant human PK2 induces chemotaxis in the U373 human astrocyte cell-line**

In the injured or diseased CNS, astrocytes become activated and migrate to the site of injury. Several factors, including TGF $\beta$  and bFGF, have demonstrated the ability to cause astrocyte

migration (Huang et al. 2012, Holland and Varmus 1998). Prokineticin-2 is a chemokine-like protein that has been demonstrated to modulate the activation and migration of several systemic cell types, such as macrophages and monocytes (Martucci et al. 2006, Ferrara et al. 2004). The ability of PK2 to induce the migration of other cell types and increase astrocyte wound healing, prompted us to examine whether PK2 could be a chemotactic protein for astrocytes. We tested chemotaxis with PK2 treatment in the U373 human astrocytoma cell line using the 2D chemotaxis slide (Ibidi, Martinsried, Germany), on which the cells are seeded into a strip in between two media reservoirs (Figure 5A). For the experiment, one of these reservoirs holds only media, while the other holds the putative chemotactic factor in media. The design principle behind the Ibidi chemotaxis slide is for the slide to allow the chemotactic protein to reach the cells at a concentration curve that is stable for 48 hours. Therefore, the cells in the middle strip will migrate more towards the chemotactic protein-containing reservoir than towards the media-only reservoir if the protein is able to cause chemotaxis in astrocytes. The U373 human astrocytes were seeded onto the slide about 10 hours before the treatment was started. Images were taken of the cells after seeding, and before and after adding the treatment media, to ensure cells were present and appeared healthy. Two migration experimental platforms were used. One platform created a chemical gradient between PK2 in one media reservoir and control in the opposing reservoir, while the other platform lacked a chemical gradient with identical controls in both of its media reservoirs as a negative control. Images were taken at 10x magnification once every hour for 18 hours after the treatment was added to the reservoirs. The images were compiled and stitched together for manual tracking of cells. Images taken at 0 hr and 18 hr for the PK2 migration platform are shown, with two red arrows (0 hr and 18 hr) indicating migration of one cell and two blue arrows (0 hr and 18 hr) indicating migration of a different cell (Figure 5B). The Ibidi manual cell tracking plugin for

ImageJ was used to quantify migration parameters. One individual cell was manually selected at its 0 hr time point and then manually selected at each subsequent time point to form a migration track for that cell. Migration tracks were performed for over 50 cells on each experimental platform and then compiled to get migration metrics (Figure 5C). The tracks were analyzed with respect to both of the slide's two dimensional axes, with the treatment reservoirs representing the y-axis and horizontal movement on the x-axis. The forward migration index on the y-axis (yFMI) indicates the distance traveled by a single cell towards a treatment reservoir, with a larger index indicating a stronger chemotactic effect. The PK2 experimental platform generated a significant increase in the yFMI towards the reservoir containing rPK2, and as expected, the control platform showed no significant yFMI towards either reservoir (Figure 5D). The x-axis forward migration index (xFMI) showed that neither platform induced an increase in xFMI, or lateral movement in the cell reservoir perpendicular to the chemical gradient being tested. Therefore, rPK2 induced a directed migration towards one reservoir because directed migration comes from an increased yFMI and little to no change in the xFMI, which was what we observed. PK2 also increased the migration velocity, or distance travelled each hour, of the analyzed cells (Figure 5E), indicating that the cell migration towards the PK2 reservoir was faster than was the cell migration in the control platform. Similarly, rPK2 treatment increased the total accumulated distance of the astrocytes as well as the Euclidean distance, or the straight line distance travelled from the origin. Comparing the accumulated distance and the Euclidean distance informs us about the directionality of cell migration, with a directionality score close to 1 indicating a straight line cell migration. Recombinant PK2 treatment significantly increased the directionality score over control treatment. Collectively, these results demonstrate for the first time that PK2 is a chemotactic factor increasing astrocyte cell migration, similar to what was seen in systemic immune cells.

### **Prokineticin-2 shifts mitochondrial metabolism towards an energetic phenotype**

Prokineticin-2 can induce mitochondrial biogenesis and help protect the mitochondria from toxicant-induced stress in neurons (Gordon and Neal et al, unpublished), therefore we wanted to examine the possible role of PK2 acting on the astrocyte mitochondria. Powerful and accurate descriptor for shifts in astrocyte cellular responses include mitochondrial metabolism, oxidative respiration, and glycolysis. Studies reported that astrocytes primarily rely on mitochondrial oxidative metabolism for energy production and that mitochondrial dynamics shift when exposed to pro-inflammatory stimuli or in response to neuronal activity (Motori et al. 2013, Hertz, Peng and Dienel 2007), indicating that mitochondrial dynamics can be a strong marker of astrocyte activation. Therefore, we wanted to determine whether PK2-induced activation of astrocytes can change the cellular mitochondrial dynamics. We used the Seahorse XFe24 instrument looking at both primary mouse astrocytes (Fig. 6A) and the U373 human astrocytoma cell line (Fig. 6E). In the primary mouse astrocytes, treatment with recombinant PK2 (rPK2) for 8 hours leads to an increase in the basal oxygen consumption rate, or OCR, (Fig. 6B) and ATP production (Fig. 6C). We also looked into the cell energy phenotype by comparing the OCR, and the extracellular acidification rate, or ECAR, to determine the response to treatment. Higher OCR would indicate a shift in mitochondrial dynamics towards a more aerobic phenotype, whereas a higher ECAR would indicate a shift towards more a more glycolytic phenotype. Interestingly, rPK2 treatment induced the astrocyte cells to increase both OCR and ECAR levels compared to the control. This increase by rPK2 leads to a cell energy phenotype that would be more energetic, because it increased both energy production pathways in the primary mouse astrocytes (Fig. 6D). When we performed similar treatment of rPK2 for 8 hours in the U373 human astrocytoma cell line we found similar

results. Basal OCR (Fig. 6F) and ATP production (Fig. 6G) were significantly increased with rPK2 treatment compared to the control. The rPK2 treatment was also able to increase the ECAR levels in the U373 cell line, which along with the increased OCR leads to a shift towards a more energetic cell energy phenotype (Fig. 6H). Taken together, these data demonstrate that recombinant PK2 is able to induce a mitochondrial dynamic shift towards a more energetic phenotype indicating astrocyte activation.

### **Recombinant human PK2 and PK2 overexpression reduces production of pro-inflammatory cytokines and increases astrocyte anti-oxidant response elements**

Studies have demonstrated that activated astrocytes can either exacerbate inflammation, by producing pro-inflammatory cytokines (Hirsch et al. 2003, Rappold and Tieu 2010), or help protect against inflammation, through anti-oxidant response elements such as Nrf2 and reduced glutathione (Vargas et al. 2008, Dias, Junn and Mouradian 2013b). Therefore, astrocyte activation could be detrimental or therapeutic, depending upon the signals that the astrocytes produce. Some studies postulate that it could be a situation similar to microglia, where depending upon the signal the cell will activate specific pathways that leads either to M1, classic activation and pro-inflammatory, or an M2, alternative activation and anti-inflammatory, phenotype (Jang et al. 2013a). Knowing that PK2 can activate astrocytes, we then wanted to investigate whether PK2-induced astrocyte activation would lead to the production of inflammatory or anti-oxidant elements. First, we wanted to determine whether recombinant PK2 would lead to an increase in the production and release of pro-inflammatory cytokines. Recombinant PK2 was added to primary mouse astrocytes for 8 hours for qPCR analysis for pro-inflammatory cytokines or 12 hours to check for cytokine secretion in the media. Gene expression analysis with rPK2 treatment showed



that there was no change in inflammatory cytokines IL-1 $\beta$ , IL-6, and TNF $\alpha$  (Figure 7A). The conditioned media from the 12-hour treatment was then run on a Bioplex 200 for pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and TNF $\alpha$ . Interestingly, we surprisingly found that rPK2 conditioned media significantly reduced the basal levels of all three of these pro-inflammatory cytokines (Figure 7B). These results indicate that PK2 can lead to activation of astrocytes, but there is no increase in the production or release of pro-inflammatory cytokines. Next, we wanted to determine whether rPK2 could induce an anti-oxidant or anti-inflammatory response in primary mouse astrocytes.

Arginase-1 and iNOS, or nitric oxide synthase, are enzymes that compete for l-arginine. This allows arginase to modulate the amount of nitric oxide, or NO, that is produced by iNOS. Therefore, the ratio of Arginase-1 to iNOS gene expression and protein level is an important indicator of whether a cell is inducing inflammation or protecting against it. Previous studies indicate that astrocytes can produce more arginase1 during stressful conditions, such as a stroke, in the brain. Using quantitative PCR for primary mouse astrocyte samples treated with rPK2 for 8hrs, we tested the gene expression for Arginase1 and iNOS. Interestingly, PK2 treatment significantly increased Arginase1 gene expression, while also significantly decreased iNOS gene expression (Figure 7C). Protein levels of iNOS were also found to be decreased by immunofluorescence microscopy (Figure 7D). Overexpression of Nrf2 in astrocytes has demonstrated the ability to protect dopaminergic neurons from neurotoxicity (Gan et al. 2012). Therefore, we wanted to check the expression of Nrf2 and an anti-oxidant gene downstream of Nrf2 in peroxiredoxin-2, or Prdx2. We found that rPK2 treatment significantly increased the gene expression of both Nrf2 and Prdx2 (Figure 7E) and the protein level of Nrf2 by immunofluorescence microscopy and Western blotting techniques (Figure 7F and 7G). These

results indicate that exogenously added recombinant PK2 can induce astrocytes to produce anti-oxidant and anti-inflammatory factors such as arginase1 and Prdx2.

Next, we used a lenti-viral delivery system to either overexpress PK2 tagged to GFP or control GFP overexpression in primary mouse astrocytes (Figure 8A). Gene expression analysis shows that PK2 overexpression increased both Arginase1 and Nrf2, while there was no change in the iNOS gene expression level (Figure 8B). We then wanted to examine the secretion of pro-inflammatory cytokines IL-1 $\beta$ , IL-6, TNF $\alpha$  in conditioned media of primary mouse astrocytes overexpressing PK2. Interestingly, there was no change in the protein levels in the conditioned media from PK2 overexpressing cells compared to GFP infected cells (Figure 8C). Similarly, we found Nrf2 protein level increased with PK2 overexpression compared to control cells and GFP overexpression cells (Figure 8D bottom left panel). STAT6 phosphorylation has been implicated in alternative activation of microglia and macrophages, therefore we wanted to measure STAT6 phosphorylation in PK2-induced astrocyte activation. Interestingly, we found that PK2 overexpression significantly increased phosphorylated STAT6 protein level compared to GFP overexpression, whereas total STAT6 protein level did not change (Figure 8D bottom middle and right panel). Taken together, these results demonstrate that PK2 overexpression shift astrocytes to produce more anti-oxidant proteins such as Arginase-1 and Nrf2 with no change in pro-inflammatory cytokine secretion, similarly to our results with recombinant PK2.

### **PKR1 agonist promotes alternative activation of astrocytes in cell culture**

The prokineticin receptors are GPCRs, or G-protein coupled receptors. This receptor class has been exploited for pharmaceutical therapies in different disease models (Ghanemi 2015). Our

previous results of PK2 activating astrocytes led us to use a non-peptide compound to activate the prokineticin signaling pathways in astrocytes. Gasser et al. originally tested PKR1 agonist compounds to use for protection of cardiomyocytes in the heart. They found that IS20 was able to activate the prokineticin signaling pathways, such as Akt and ERK, and that it was able to protect cardiomyocytes from a myocardial infarction (Gasser et al. 2015). Therefore, we wanted to determine if IS20 was capable of activating astrocytes in a similar manner to recombinant and overexpression of PK2 since astrocytes mainly produce the PKR1 receptor compared to PKR2. First, we found that IS20 could significantly increase mitochondrial respiration and ATP production similar to that seen with rPK2 treatment (Figure 9A). Also, we found a similar shift in the cellular energy phenotype towards a more energetic role compared to control treatment. These results indicate that IS20 can activate astrocytes in a similar manner to recombinant PK2 protein treatment. Next, we used quantitative real-time PCR to examine the gene expression of genes previously seen in astrocyte activation. We found that anti-oxidant genes such as Arginase-1 and Nrf2 were significantly increased, whereas iNOS gene expression decreased compared to the control (Figure 9B). We validated the gene expression results by examining the protein level of these key activation proteins using Western blotting techniques. In concordance with our qPCR results, we found Arginase-1 and Nrf2 significantly increased compared to control (Figure 9C). Taken together, these results demonstrate that IS20, a non-peptide PKR1 agonist, is able to activate astrocytes and increase anti-oxidant protein expression of Arginase-1 and Nrf2.

## **Prokineticin signaling increases glutamate uptake through upregulation of GLAST in cultured astrocytes**

Astrocytes are known to help protect neurons through multiple ways, with one of those protective mechanisms being the uptake of glutamate to prevent excitotoxicity (Sattler and Rothstein 2006). Pro-inflammatory cytokines, such as TNF $\alpha$ , have been found to decrease the expression of glutamate transporters, especially GLAST (Dumont et al. 2014). Therefore, we wanted to determine whether rPK2 treatment could lead to a change in the ability of primary mouse astrocytes to take up extracellular glutamate. Using radio-labeled glutamate, we found that treating primary mouse astrocytes for 8 hours with PK2 or the PKR1 chemical agonist IS20, along with IL-4 as a positive control, could increase overall glutamate uptake in these cells compared to control cells (Figure 10A). Astrocytes that are cultured without the presence of neurons will express very low levels of GLT-1, however GLAST is expressed at a normal level (Swanson et al. 1997). Therefore, we measured the gene expression of GLAST in primary mouse astrocytes, and found that recombinant PK2 treatment increased the gene expression of GLAST, in a similar manner of IL-4 treatment (Figure 10B). Taken together, these data indicate that PK2 signaling can lead to functional changes in astrocytes that would lead to a reduction of extracellular glutamate through upregulation of the glutamate transporter GLAST, and could potentially protect neurons from excitotoxicity.

## Discussion

Astrocytes play important roles in maintaining a healthy CNS and become activated during the progression of Parkinson's disease, and other neurodegenerative diseases. Astrocyte activation

in not very well understood, and only recently has it been discovered that astrocytes can respond differently depending on the stimuli. Proinflammatory cytokines such as  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  can stimulate astrocytes to propagate inflammation through the production of more inflammatory cytokines, whereas stimulation with the anti-inflammatory cytokine  $\text{IL-4}$  led to a reduction in astrocyte-produced pro-inflammatory cytokines (Jang et al. 2013a). Neuron-astrocyte signaling therefore could play a key role in the progression of PD, and finding factors from neurons that can influence astrocyte activation is an important research avenue for neurodegenerative diseases. Previously, our lab found that dopaminergic neurons produce and secrete the chemokine-like signaling protein Prokineticin-2 both in Parkinson's disease patients and in animal models of PD. Interestingly, astrocytes express the mRNA for the two prokineticin receptors, mainly PKR1, and that PK2 can increase astrocyte proliferation (Koyama et al. 2006). In the present study we found that the prokineticin receptors protein level is similar to what was seen at the mRNA level, and recombinant human PK2 was able to dose-dependently increase both intracellular calcium levels, and cell proliferation in primary mouse astrocytes and a human astrocyte cell line. Also, this is the first study to show the protein levels and cellular localization of the prokineticin receptors in astrocytes. Interestingly, by analyzing the branch patterns of astrocytes with PK2 we found reduced number of astrocyte branches and endpoints compared to GFP overexpression. This could be due to the cell migrating and will remove some of the processes that are connected to other cells. Prokineticin signaling has been implicated in the migration of several cell types (Lin et al. 2002b, Ayari et al. 2010, Monnier and Samson 2008), so it was not surprising to find that PK2 was able to increase both wound healing and migration of human astrocytes. Collectively, these results

along with previous knowledge demonstrate an important finding, that PK2 secreted from damaged neurons can signal to astrocytes, causing them to migrate to the site of injury and proliferate.

Astrocyte activation has been used as a marker for neurodegeneration and neuroinflammation, but it still not understood what role this activation plays in the normal or diseased states of the CNS. Herein, we demonstrated that PK2 can induce astrocyte activation both *in vitro*, by analyzing mitochondrial dynamics, and *in vivo* in the striatum of mice evident by the PK2-induced increase in the number of GFAP-positive cells, the amount of GFAP immunostaining per cell, and the increased soma size of astrocytes. We previously found that PK2 can increase mitochondrial biogenesis in neurons, and the increase in basal respiration of astrocytes could indicate a similar mechanism. However, it is more likely that activated astrocytes require more energy for proliferation and migration similar to previous reports for mitochondrial dynamics in microglia (Jiang and Cadenas 2014, Orihuela et al. 2016). This would explain the increase in migration and proliferation of astrocytes with PK2 treatment, because astrocyte activation increases both of these functions. These results implicate Prokineticin-2 as a factor behind the increased number of activated astrocytes seen in the nigro-striatal pathway in animal models of PD and in PD patients. In PD there is an increase in PK2 production and secretion from dopaminergic neurons and PK2 then can signal to cause astrocytes to activate and migrate to the damaged neurons.

Astrocytes are traditionally viewed as support cells for the neurons of the CNS, however astrocytes can contribute to inflammatory damage of the CNS with certain stimuli. Interestingly, studies have shown that overexpression of Nrf2 in astrocytes is able to protect dopaminergic neurons from cell death (Gan et al. 2012). Therefore, it could be an important Parkinson's disease

therapeutic avenue to look for stimuli that increase antioxidant proteins, such as Nrf2, in astrocytes. Importantly, Prokineticin-2 signaling in astrocytes does not increase inflammatory factors such as pro-inflammatory cytokines, or iNOS. On the other hand, PK2 is able to increase protective factors in such as Arginase-1, but most importantly an increase in Nrf2 both at a gene level and protein level in astrocytes. Interestingly, the non-peptide PKR1 agonist was also able to activate cultured astrocytes and increased both Arginase-1 and Nrf2 protein level. Therefore, this increase in antioxidant proteins paired with the lack of increase in pro-inflammatory cytokines means that PK2-activated astrocytes could lead to a more neuroprotective phenotype. Further, prokineticin signaling increases glutamate uptake by increasing the gene expression of GLAST in cultured astrocytes and therefore leading protection of neuroprotection. This is the first study to show that Prokineticin-2 signaling can alternatively activate astrocytes, induce migration and produce neuroprotective factors such as Nrf2, along with increased glutamate uptake. This opens the opportunity for using prokineticin signaling as a possible therapeutic opportunity to protect dopaminergic neurons in Parkinson's disease through reducing astrocyte-produced inflammation. Further studies into neuron-glia crosstalk could be an important avenue for therapeutic approaches of neurodegenerative diseases.

## Materials and Methods

### **Reagents**

DMEM, MEM media, fetal bovine serum (FBS), L-glutamine, IR-dye tagged secondary antibodies, Hoechst nuclear stain, penicillin, streptomycin, and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Recombinant PK2 was purchased from Peprotech. The primary antibody for PKR1, PKR2, Nrf2 was ordered from Santa Cruz Biotechnology Inc.

(Santa Cruz, CA). GFAP was obtained from Millipore. We purchased the phosphorylated STAT6 and Native STAT6 antibodies from Cell Signaling. The Arginase1 antibody was ordered from Thermo. The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Fluo4 calcium assay and CyQuant kit was ordered through Invitrogen.

### **Cell culture and animal studies**

Human U373 astrocytoma cells (ATCC), were grown in MEM media supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), according to ATCC instructions. U373 cells were maintained in an incubator at 37°C with 5% CO<sub>2</sub>. Primary mouse astrocytes were obtained from whole brain homogenate of 0-3 day mouse pups. Astrocytes were collected, isolated and maintained according to previous methods (Gordon *et al.*, 2011) using DMEM media (10% heat-inactive FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, glutamate 2mM) to grow the cells. Primary human astrocytes were obtained from Lonza.

### **Quantitative PCR**

Mouse astrocyte cells or U373 cells were seeded out into 75-mm<sup>3</sup> flasks at 2.5 x 10<sup>6</sup> cells per flask. Treatments were performed in either MEM or DMEM media supplemented with 2% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine. Cells were treated for 8 h with rPK2 (25nM) or rPK2 cotreated with PC-7 (1µM). After treatment, cells were collected, pelleted, and resuspended in lysis buffer with β-mercaptoethanol. RNA was isolated using Absolutely RNA Miniprep, an RNA isolation kit from Stratagene. RT-PCR was performed using an cDNA synthesis system (Applied Biosystems) to convert the RNA into cDNA. Expression levels were determined using real-time PCR with Qiagen RT<sup>2</sup> SYBR Green master mix and prevalidated using the qPCR mouse primers from SuperArray/Qiagen (Qiagen). For normalization of each sample, the mouse gene 18SrRNA (Cat. No. PPM57735E) was used as the housekeeping



gene. The amount of each template was optimized empirically to maximize efficiency without inhibiting the PCR reaction. According to manufacturer's guidelines, dissociation curves and melting curves were run to ensure that single amplicon peaks were obtained without any non-specific amplicons. The results are reported as fold change in gene expression, which was determined using the  $\Delta\Delta C_t$  method using the threshold cycle ( $C_t$ ) value for the housekeeping gene and for the respective gene of interest in each sample.

### **Western blotting**

Astrocytes were collected after treatment, lysed using modified RIPA buffer, homogenized, sonicated, and centrifuged as previously described (Kaul *et al.*, 2003; Kitazawa *et al.*, 2002). Supernatants were collected and proteins were normalized, then stored with loading buffer and DTT. Samples were run on a Sodium Dodecyl Sulfate (SDS) gel electrophoresis. Normalized protein samples were loaded into each well and first separated in a 4% stacking gel and then through a 12-18% resolution gel. Proteins were then transferred to a nitrocellulose membrane. After transfer, the membranes were blocked using Western blocking buffer (Rockland Immunochemicals). Primary antibodies, in blocking buffer were then added to the membranes and stored overnight at 4°C. A Secondary antibody specific to the primary antibody was added (in either blocking buffer or in 5% low-fat dry milk solution) to the membrane for 1 h. Either  $\beta$ -Actin or  $\alpha$ -Tubulin was used to confirm equal loading into each well of the gels. The membranes were read using the Odyssey infra-red imaging system.

### **Immunocytochemistry**

For immunocytochemistry, cells were plated onto coverslips in 24-well plates coated with 0.1% poly-D-Lysine. After cells were treated, 4% formaldehyde was used to fix the cells for 30 min. The cells were washed with PBS wash buffer. Then blocking buffer containing 2% BSA, 0.2%

Triton X-100, and Tween was added to the wells for 1 hour. The cells were incubated with primary antibodies in 2% BSA at 4°C overnight. Next, an Alexafluor dye-conjugated secondary antibody in 2% BSA was added and incubated at room temperature on a shaker for 1 h. After washing Hoechst counterstain was added to the cells for 5-6 min to label nuclei. Coverslips were washed several times then mounted onto slides with Fluoromount mounting media. Cells were imaged under a Nikon 2000U microscope with a SPOT camera, and all images were processed in MetaMorph 5.7 (Universal Imaging, Downingtown, PA).

For histology, mice were anesthetized using the ketamine-xylazine mixture and cardiac perfusion of 4% PFA was used to fix the brain before being extracted and stored in 4% PFA at 4°C. After 48 h, brains were washed with PBS, and placed into a 30% sucrose solution for at least 24 h. The brains were then embedded in Optimal Cutting Temperature (OCT) compound and frozen into blocks for sectioning. Brains were sectioned on a Cryostat (CryoStar NX70, Thermo Scientific) at -20°C and placed into Cryosolution (30% Sucrose, Ethylene glycol, and PBS). Sections were then washed with PBS and permeabilized with blocking buffer (2% BSA, 0.1% Triton X-100, and Tween) for 1 h at room temperature. Antibodies directed to the protein of interest were then incubated with the sections overnight at 4°C. After washing with PBS, the sections were incubated with Alexafluor dye-conjugated secondary antibodies for 1 h at room temperature. After washing with PBS, Hoechst dye (1:5000) was added to the sections for 5 min at room temperature to stain nuclei. Sections were then mounted on slides using the Fluoromount mounting medium (Molecular probes) according to the manufacturer's instructions. An inverted fluorescent microscope (Nikon TE-2000U) was used to visualize the sections, and a Spot digital camera (Diagnostic Instruments, Inc) was used to capture images.

### **Scratch-wound migration assay**

To assess wound healing, astrocytes were allowed to grow to a confluent monolayer in 6 well plates. Once the cells are fully confluent, a 1000ul pipet tip was used to make the scratch through the cells. A scratch was made from top to bottom and from left to right, to form a plus sign of a scratch to identify similar imaging areas. Cells were washed 3 times with serum free media, treatments were added and wells were imaged for 0 hour timepoint. Endpoint images were taken at 24 hours after treatment using an inverted fluorescent microscope (Nikon TE-2000U). Measurement of the scratch width was randomly assessed in each group and expressed in microns. This experiment was repeated with a three total wells for each treatment.

### **2D Chemotaxis assay**

The chemotaxis assay was performed using Ibidi 2D chemotaxis chambers that were purchased from Ibidi. The assay was performed according to the manufacturer's guidelines. The U373 astrocytoma cell line was seeded into the middle line of each chamber and allowed to adhere to the plate overnight. The 10% serum containing DMEM growth media was washed twice with serum free DMEM media, then either serum free media or recombinant PK2 was introduced through one of the experimental reservoirs. The cells were imaged at 0 hours to ensure that the cells were still attached and then imaged every hour for 18 hours at 10X magnification. Images were taken using an inverted fluorescent microscope (Nikon TE-2000U) and collated using ImageJ software. Cells were tracked to measure migration parameters with the Ibidi Manual Tracking software. A minimum of 50 cells were followed for each treatment to determine overall migration parameters.

### **Mitochondrial dynamics**

To analyze the mitochondrial energy dynamics in both primary mouse astrocytes and the U373 human astrocyte cell line, the XFe24 Seahorse analyzer was used to measure OCR and ECAR levels. Astrocytes were plated on Seahorse plates at 80,000 cells in each well and allowed to attach overnight in growth media. Cells were then washed with serum free DMEM media 3 times and 500ul of treatment was added to the wells in serum free DMEM for 8 hours. After the 8 hours, the cells were washed 2 times with the Seahorse base media supplemented with sodium pyruvate and glutamine. The MitoStress test was performed according to the manufacturer's guidelines. The cells were left in the 500ul of the Seahorse base media supplemented with sodium pyruvate and glutamate. After testing correct concentrations of the chemicals, 0.75uM of oligomycin, 1uM of FCCP, and 0.5uM rotenone/antimycin was added to the cells to measure mitochondrial dynamics. Analysis was performed through the MitoStress Results Generator found on the Agilent website.

### **Fluo-4 calcium mobilization and CyQuant proliferation assay**

A Fluo-4 NW assay kit (Molecular probes) was used to test calcium mobilization in primary mouse astrocytes resulting from nanomolar concentrations of rPK2. Cells were plated overnight into 96-well plates in growth media. Cells were then washed with HBSS and incubated with the Fluo-4 NW dye for one hour. Each plate was then read kinetically using a Synergy-2 multi-mode microplate reader. After obtaining background readings by reading each plate every 50 ms for 20 sec, rPK2 was injected at the indicated nanomolar concentrations. The net change in fluorescent signal ( $\Delta t$ ), read every 50 ms for 3 min, was obtained after subtracting the background from the maximum signal for each sample.

Astrocyte proliferation was measured using the CyQuant dye according to manufacturer's guidelines. A standard curve of cell numbers was generated by seeding out the astrocytes into a 24-well plate. The same number of astrocytes were seeded into the experimental wells. After 48 hour of incubation with treatment of control or different doses of rPK2 (5, 10, 25 or 50nM), 50  $\mu$ l of CyQUANT NF Cell Proliferation Assay reagent was added to each well after aspiration of medium. After incubation for 30 minutes at 37°C, fluorescence was measured (excitation 485 nm, emission 538 nm) using the Synergy-2 multi-mode microplate reader. Fluorescent values were compared to the standard curve of cell numbers to generate the treatment induced cell number.

### **Glutamate uptake assay**

Primary mouse astrocytes were plated into a 24-well plate with 120,000 cells per well. The astrocytes were treated with rPK2, IS20 or neither in serum free DMEM media for 8 hours. After the 8 hour treatment, three washes with Krebs-Ringer buffer was used to wash away the treatment media. The cells were then incubated with 2 $\mu$ Ci radioactive Glutamate for 15 minutes along with 100 $\mu$ M L-Glutamine in warm Krebs-Ringer buffer. Remove media and washed three times ice cold Krebs-Ringer buffer to stop uptake. Lyse the cells in 1N NaOH and collect the sample. The radioactivity was measured with a liquid scintillation counter (Tri-Crab 4000, Packard Instrument Co.) after the addition of a 5-ml scintillation mixture to each vial. The specific dopamine uptake was expressed as mean values of counts.

## Data analysis

Data analysis was performed using the Prism 4.0 software package (GraphPad Software, San Diego, CA). The data was first analyzed using one-way ANOVA and then Tukey's post-test was performed to compare all treatment groups. Differences of  $p < 0.05$  were considered statistically significant. The Student's t-test was used when two groups were being compared.

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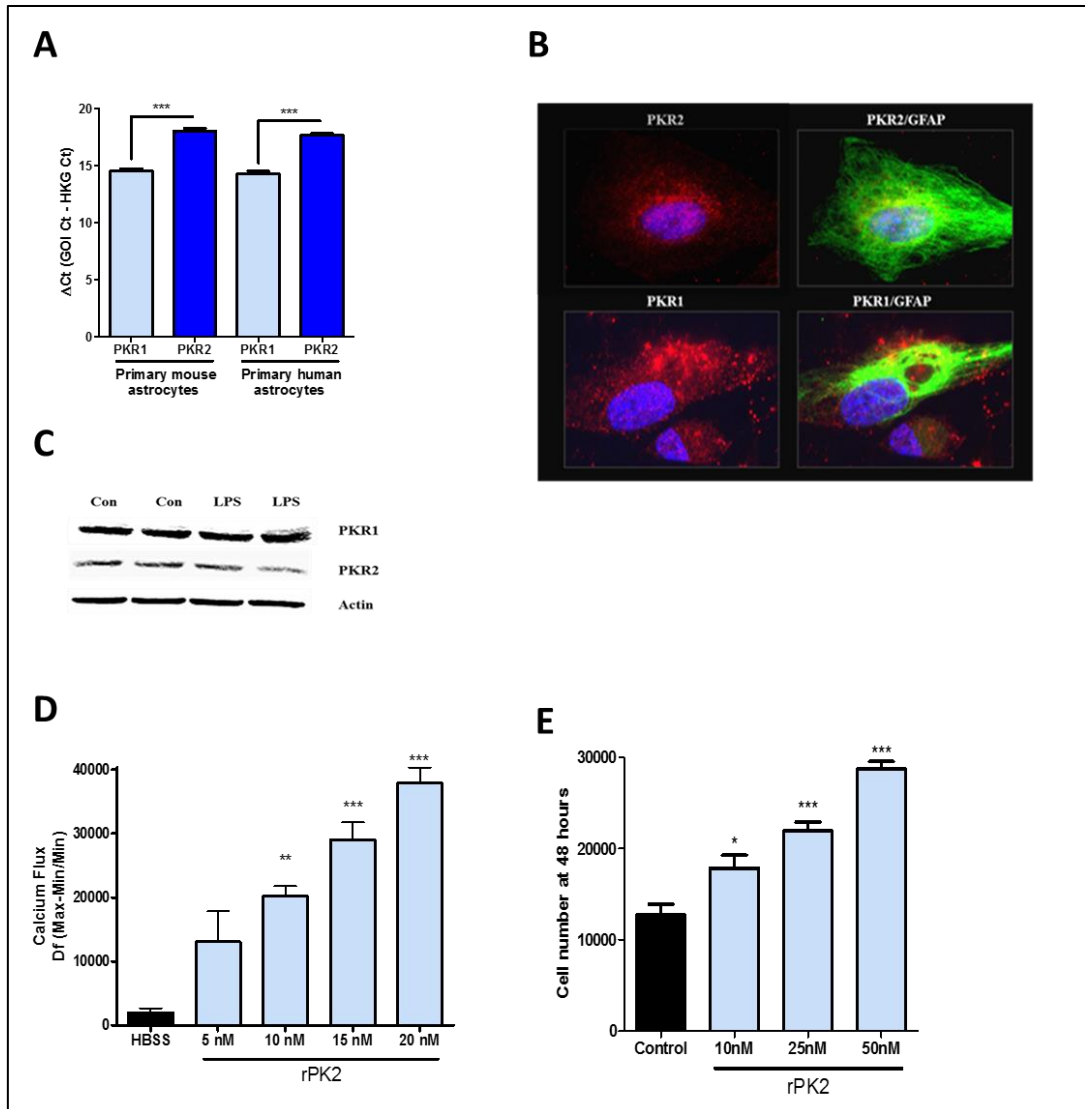
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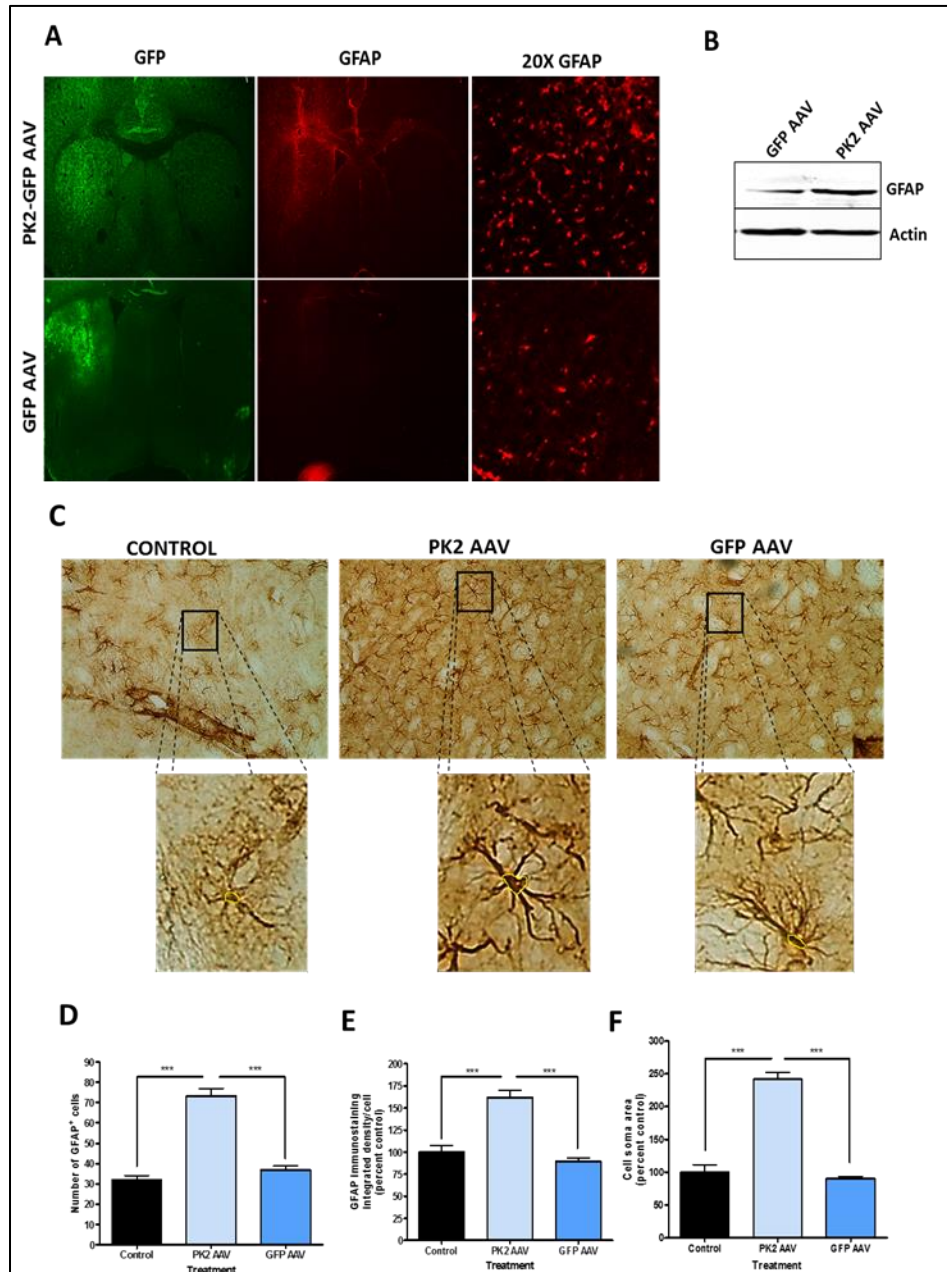
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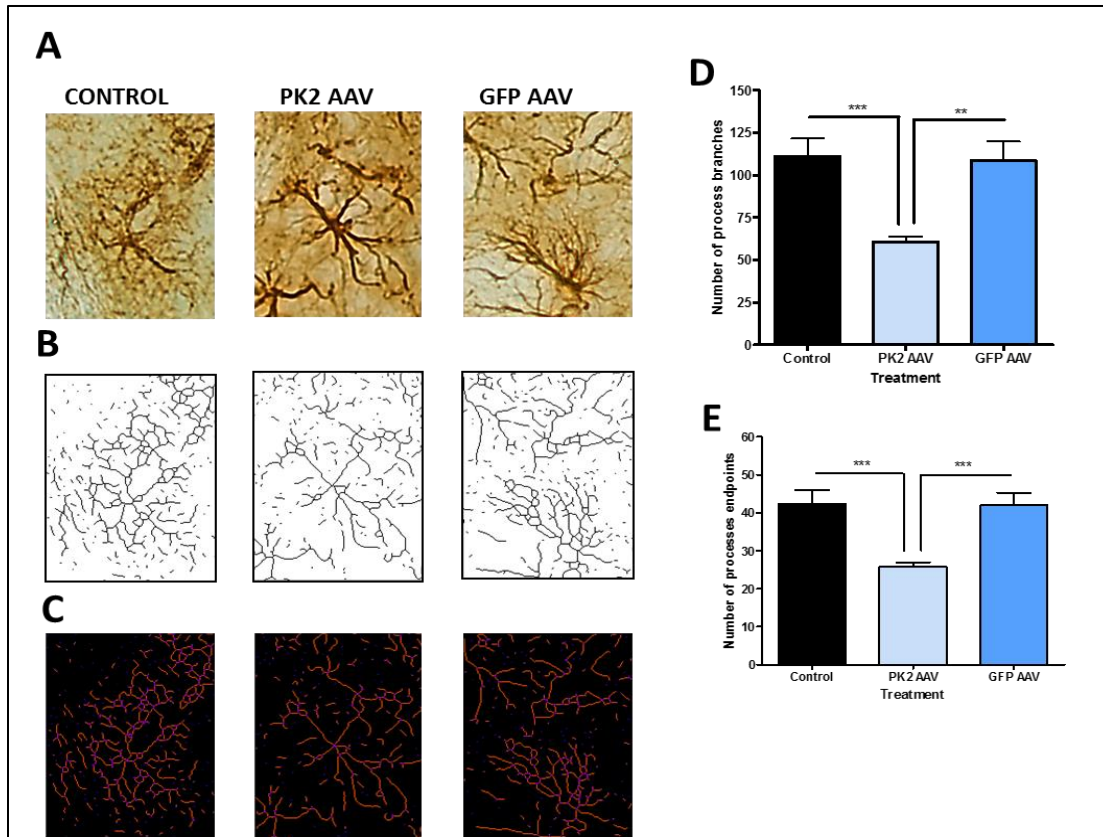
## Figures



**Figure 1.** *Prokineticin-2 can signal to primary mouse astrocytes through increased intracellular calcium and cell proliferation.* (A) Quantitative real time PCR for prokineticin receptor gene expression, with PKR1 expressed over 2000 fold higher than PKR2. (B) Different cellular localization pattern for PKR2 (Red, top panel) and PKR1 (Red, bottom panel) in GFAP (Green) positive astrocytes and the nucleus stained with Hoechst (Blue). Expression of PKR2 was perinuclear whereas PKR1 expression was localized more on the periphery of the cell. (C) Representative Western blot for PKR1 and PKR2 in primary mouse astrocytes showing higher PKR1 compared to PKR2 and no significant change with LPS treatment. (D) Calcium mobilization (flux) dose-dependently induced by rPK2 in primary mouse astrocytes, with calcium-free HBSS used as a control treatment. (E) CyQuant cell proliferation assay used to show dose-dependent increase in primary mouse astrocyte cell proliferation. Asterisks denote a significant difference between rPK2 treatment and the control (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).

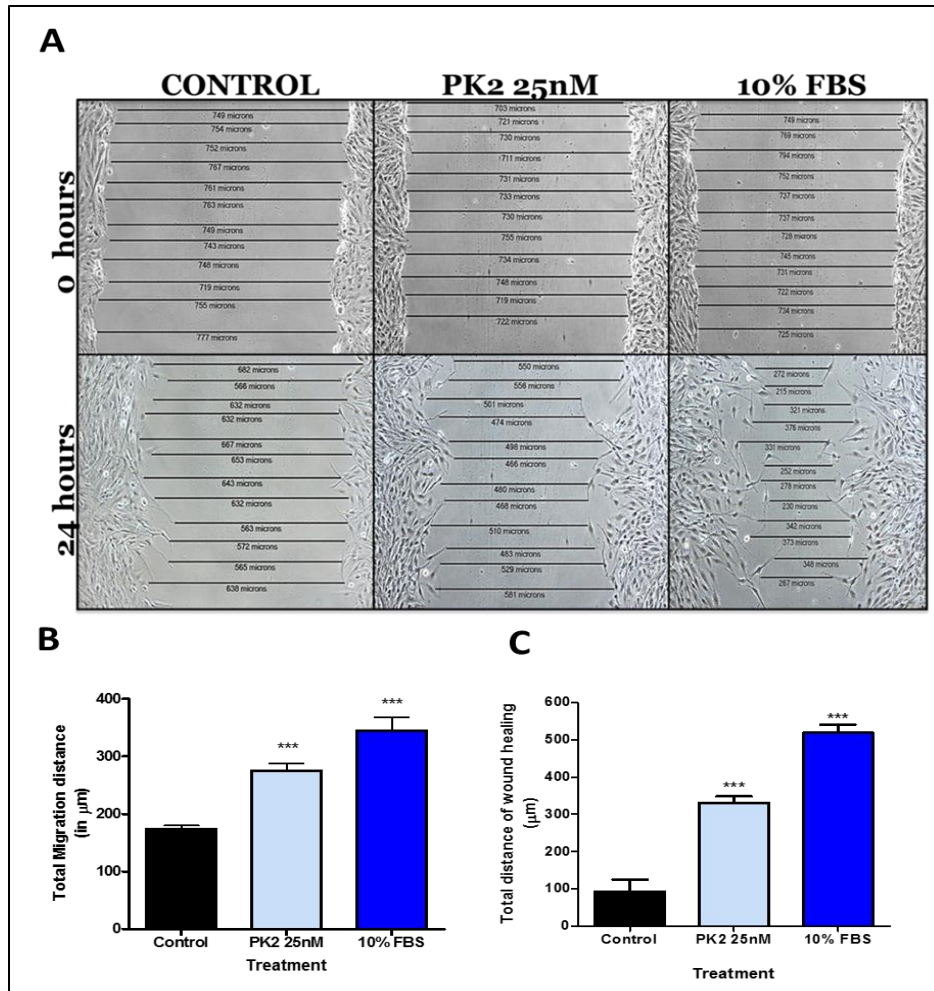


**Figure 2.** *Prokineticin-2 overexpression induces astrocyte activation in vivo.* C57/B16 mice were stereotaxically injected in the striatum with either PK2-AAV 2/5 or GFP AAV 2/5 and allowed to reach maximal viral gene expression for four weeks. **(A)** Expression of the PK2 tagged to GFP and GFAP protein levels by fluorescence microscopy. Brain sections were probed with anti-GFP (Green) and anti-GFAP (Red) antibodies and imaged to show an increase in GFAP around the areas of PK2-tagged GFP expression (2X left panel and 20X right panel). **(B)** Representative Western blot for GFAP in the mouse striatum showing that there is an increase in GFAP with the PK2-AAV. **(C)** GFAP-DAB immunostaining of the striatum (20X magnification top panel) with insets zoomed into one cell. **(D-F)** Cell counting of GFAP-positive cells (D), immunostaining quantification of GFAP for each cell (E) and cell soma size measurement of GFAP-positive cells (F) in the mouse striatum showing that PK2-AAV increases both the number of GFAP-positive cells and the amount of GFAP, and increases cell soma size in these cells compared to GFP-AAV injected and control striatum.

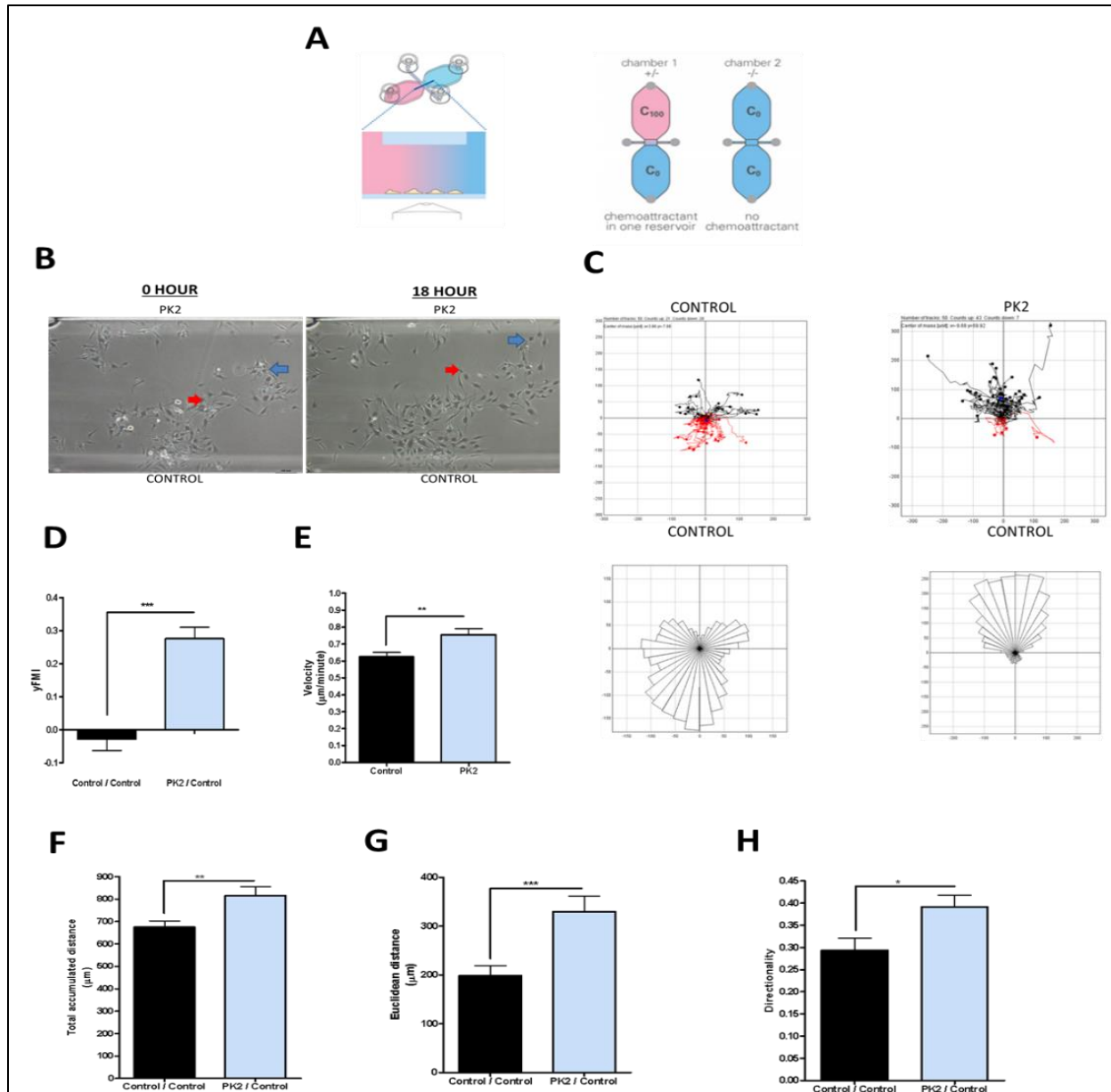


**Figure 3.** *Prokineticin-2 overexpression reduces number of astrocyte processes.* (A-C) Converting GFAP-DAB images for measurement of astrocyte processes with PK2-AAV or GFP-AAV injection in the striatum. (A) GFAP-DAB immunostaining for control, PK2-AAV and GFP-AAV injected striatum (zoomed in images from 20X magnification). (B) ImageJ skeletonization after converting the GFAP-DAB image into a binary image. (C) ImageJ analyze skeleton plug-in image from the skeletonized image with process branches (Orange), junctions (Purple), and endpoints (Blue). (D-E) Measurement of the astrocyte processes. Analysis for the average number of process branches (D) and process endpoints (E) per GFAP-positive astrocyte shows that PK2-AAV significantly reduced average cell process branches compared to Control, and GFP-AAV.

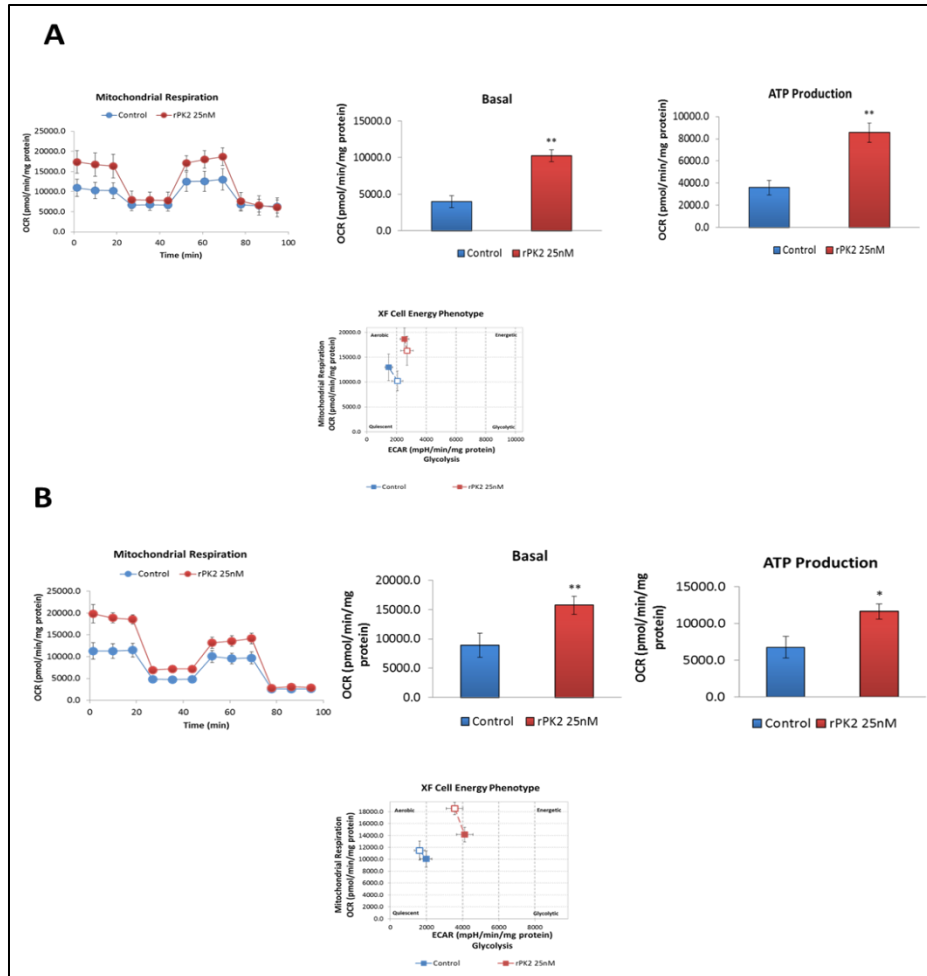




**Figure 4.** Recombinant PK2 treatment induces astrocyte wound healing. (A) Scratch-wound assay images for control, rPK2 and 10% FBS as a positive control from 0 hours (top panel) and 24 hours post scratch (bottom panel) with scale bars randomly placed down the scratch to measure the wound width. (B-C) Analysis of total migration distance travelled from 0 hour to 24 hours showing that rPK2 treatment increases wound healing in the U373 human astrocyte cell line (B) and in primary human astrocytes (C). Experiment was repeated twice with three biological replicates in each treatment. Asterisks denote statistical significance between treatments and the control (\*\*\*)  $p < 0.001$ ).

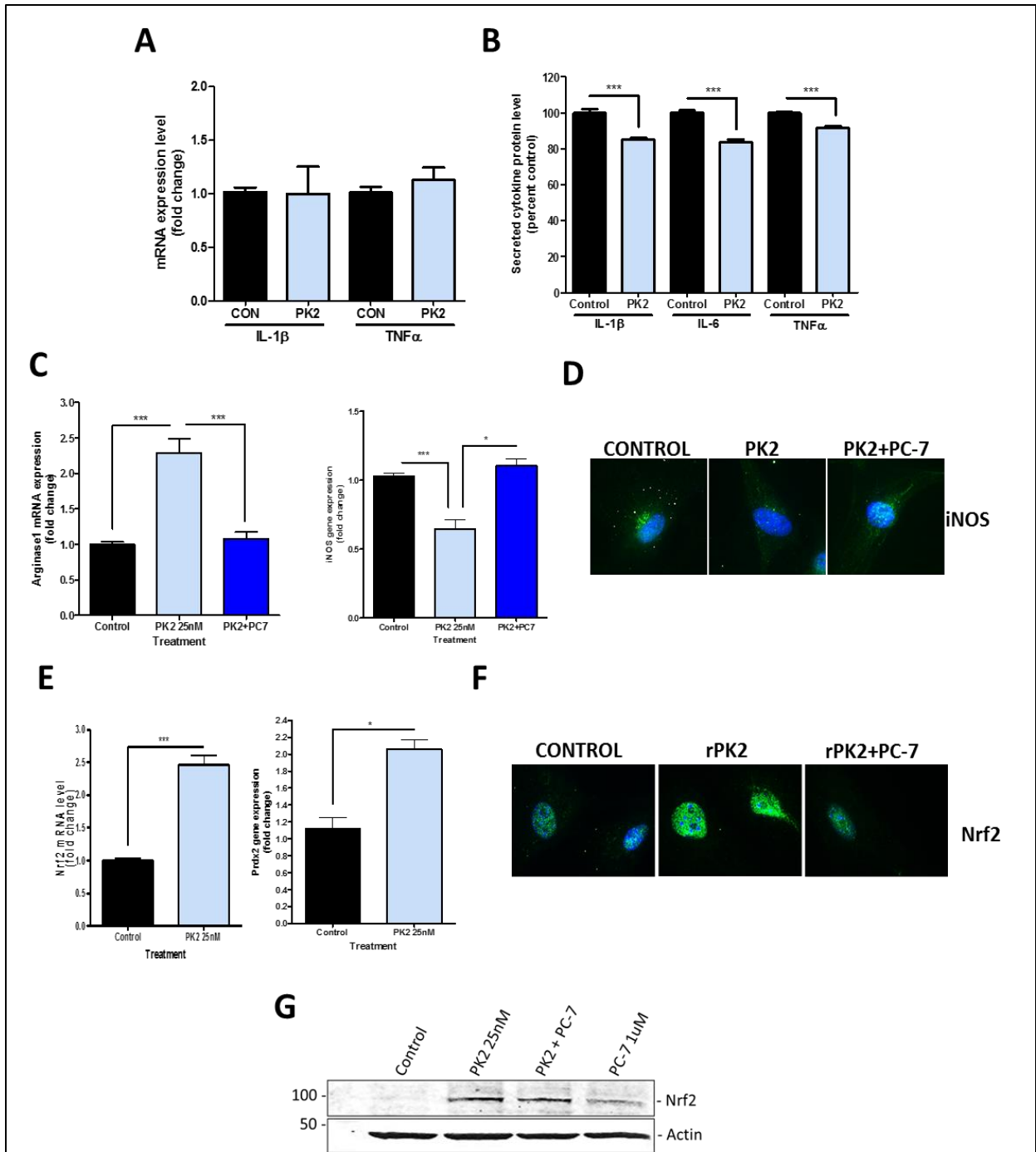


**Figure 5.** *Prokineticin-2 increases U373 astrocyte migration.* (A) Ibidi 2D migration chamber setup to demonstrate experimental design of testing a chemoattractant, such as PK2, from one chamber to measure directional migration. (B) Phase contrast images from 0 hour (left panel) and 18 hours (right panel) after addition of treatment to the reservoirs for the slide with PK2 treatment coming from the top of the image. Two separate individual cell are marked both in the 0 hour and the 18 hour (Red and Blue arrows). (C) Migration tracking plots of individual cell migration tracks with either control (left panel) or PK2 treated (right panel) platforms, along with a rose plot for the migration tracks (bottom panels). (D-H) Analysis of the 2D migration chambers comparing either control or PK2 treated platforms. Measurement for the single cell y-axis forward migration index (D), migration velocity (E), total accumulated migration distance (F), Euclidean migration distance (G), and migration directionality all shows that PK2 significantly increases the directional migration of U373 astrocytes. Measurements were performed with at least 50 cells in each treatment group, and asterisks denote statistical significance between the PK2 and control treatments (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).

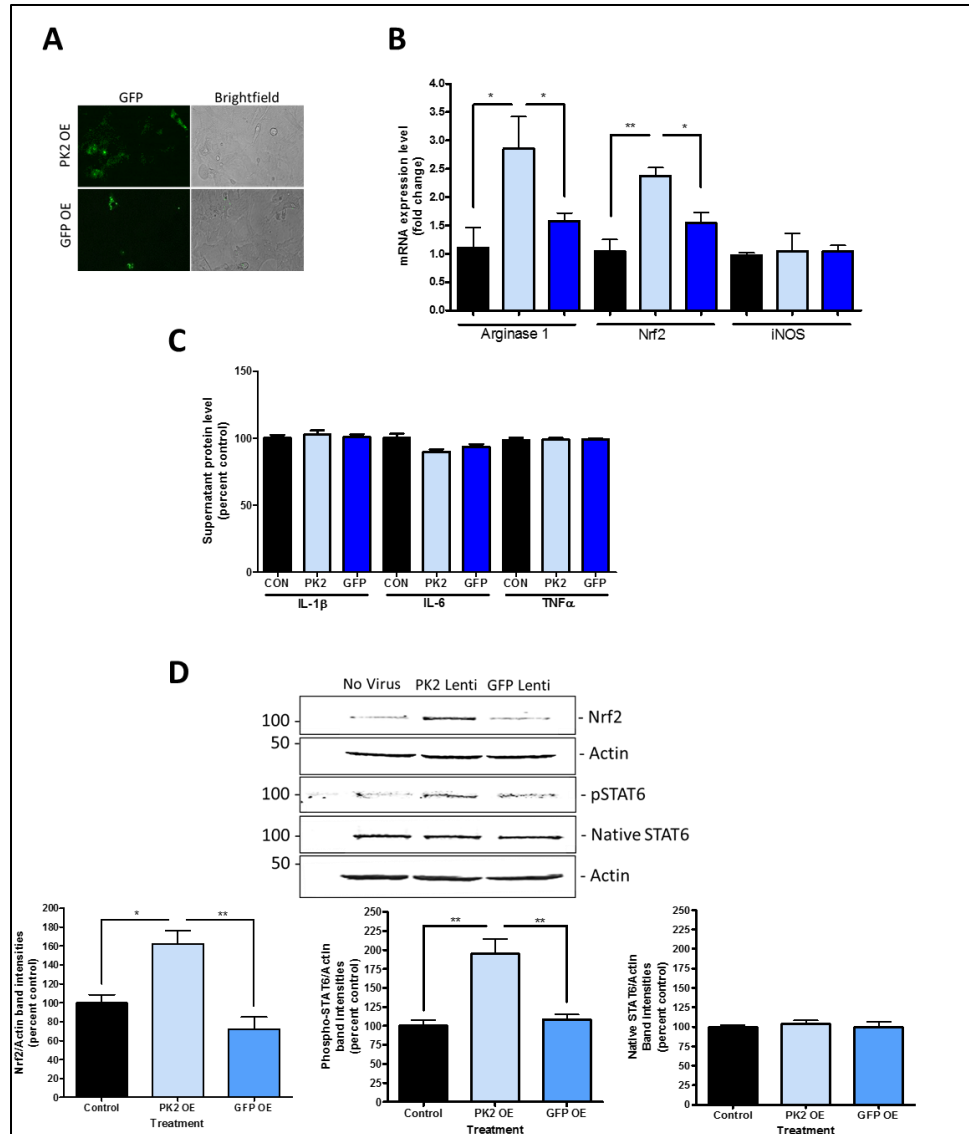


**Figure 6.** PK2 increases induces shift in astrocyte mitochondrial dynamics and energy production.

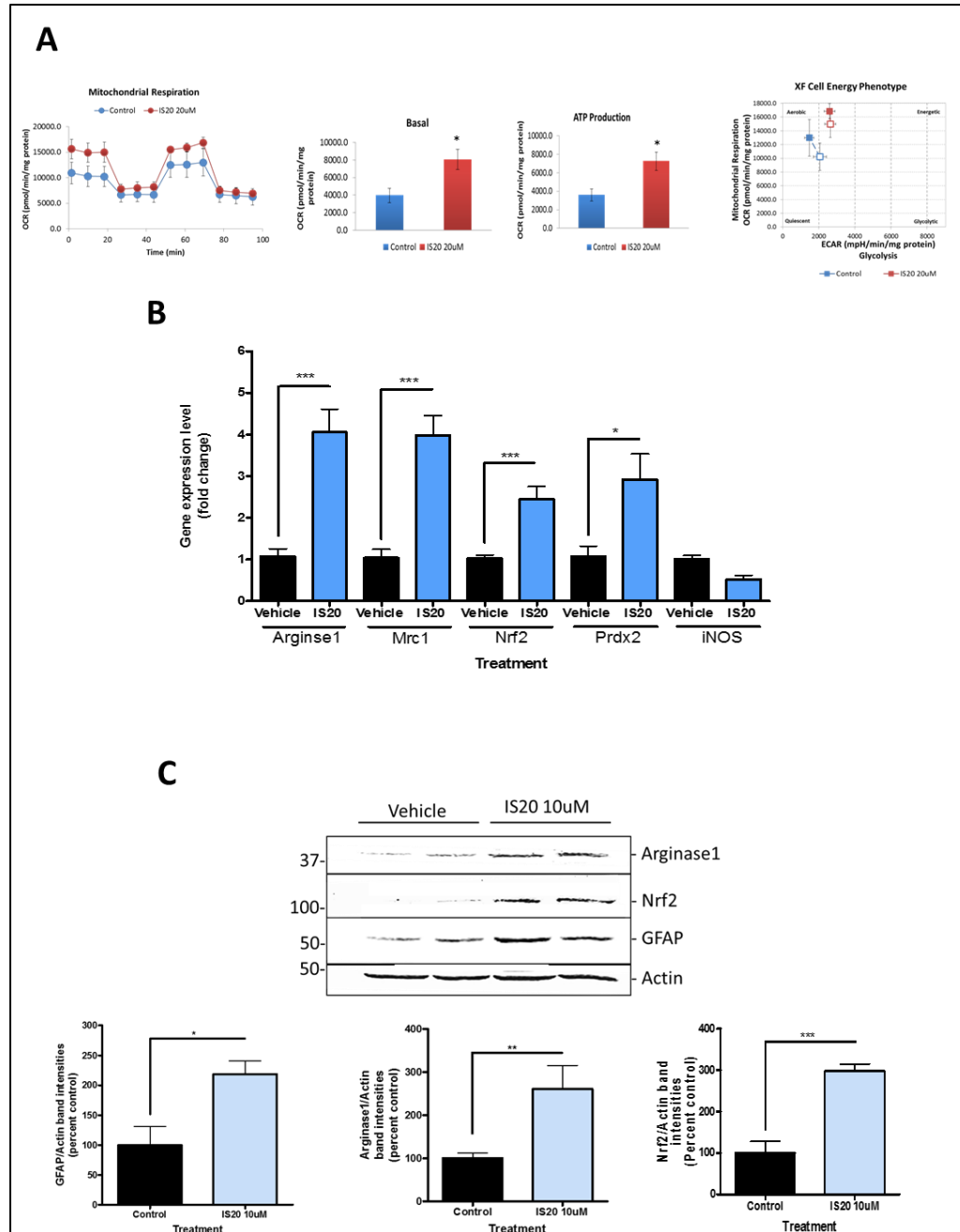
(A-B) XFe24 Seahorse Mitochondrial stress test on primary mouse astrocytes(A) and the U373 human astrocyte cell line (B). Analysis of the mitochondrial respiration rate with the respiration plot (A and B, left panel) along with basal respiration measurement (A and B, middle panel) and ATP production measurement (A and B, right panel) shows that PK2 treatment causes a significant induction of oxidative respiration in astrocytes. Cellular phenotype plot comparing OCR on the y-axis and ECAR on the x-axis (A and B, bottom panel) shows that PK2 treatment increases overall energy production in primary mouse astrocytes and the U373 human astrocyte cell line. At least four biological replicates for each treatment with asterisks denote statistical significance of SEM between PK2 and control treatments (\*  $p < 0.05$ , and \*\* $p < 0.01$ ).



**Figure 7.** *PK2 induces alternative activation of primary mouse astrocytes.* (A) Quantitative real time PCR analysis for the pro-inflammatory cytokines IL-1 $\beta$  and TNF $\alpha$ . (B) Pro-inflammatory cytokine protein levels measured in the primary mouse astrocyte conditioned media using the Bioplex 200 multi-plex system for IL-1 $\beta$  (left panel), IL-6 (middle panel), and TNF $\alpha$  (right panel). (C-D) Recombinant PK2 treatment increases anti-oxidant factors in primary mouse astrocytes. (C) Quantitative real-time PCR analysis for Arginase-1 (Left panel) and iNOS (Right panel). (D) Fluorescence microscopy images of iNOS protein level. (E-F) Recombinant PK2 treatment increases anti-oxidant factors in primary mouse astrocytes. (E) Quantitative real-time PCR gene expression analysis for Nrf2 (Left panel) and Prdx2 (Right panel). (F) Fluorescence microscopy images detecting iNOS protein levels. (G) Western blot analysis for Nrf2 shows that PK2 treatment can increase Nrf2 protein level where PC7 can attenuate this increase, with  $\beta$ -Actin as an internal loading control.



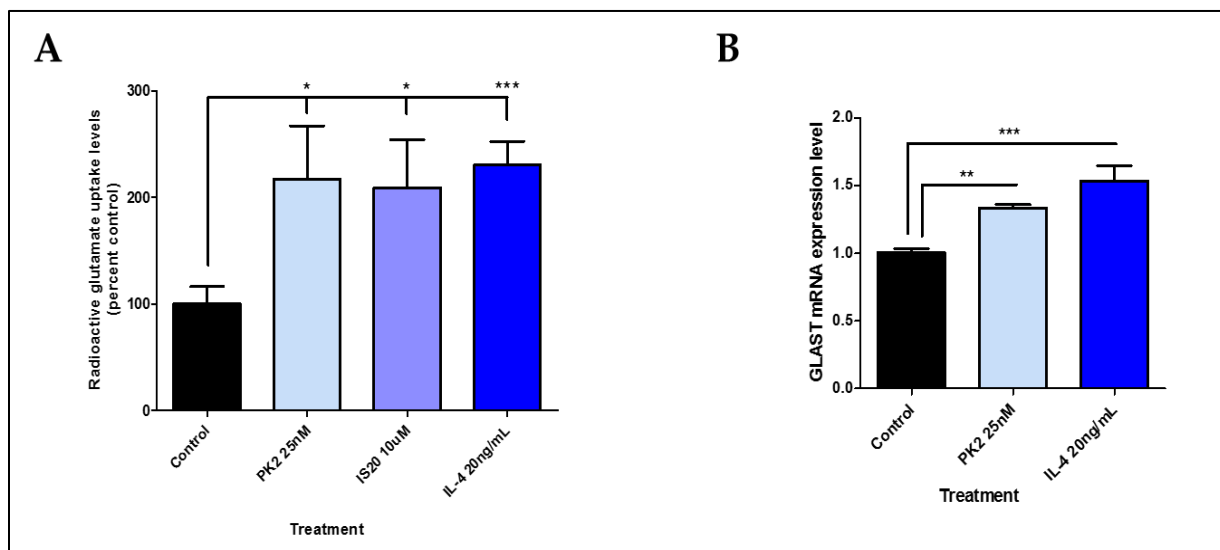
**Figure 8.** PK2 overexpression promotes alternative activation of primary mouse astrocytes. (A) Fluorescence microscopy detects GFP to confirm Lenti-viral overexpression of PK2. (B) Quantitative real-time PCR gene expression analysis for Arginase1, Nrf2 and iNOS with PK2 overexpression. (C) Western blot analysis of PK2 overexpression for Nrf2, phosphorylated STAT6 and native STAT6, with  $\beta$ -Actin as an internal loading control. Asterisks denote statistical significance between PK2 treatment and either control or PC7 (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).



**Figure 9.** *PKR1* agonist promotes alternative activation of primary mouse astrocytes. (A) Mitochondrial dynamics measured with XFe24 Seahorse instrument and mitochondrial stress test (Left panel) to compare IS20 to control for basal respiration and ATP production (Middle panels). Cell energy phenotype plot (right panel) comparing OCR (y-axis) and ECAR (x-axis) shows that



IS20 shifts astrocyte mitochondria to a more energetic phenotype. **(B)** Quantitative real-time PCR gene expression analysis for Arginase1, Mrc1, Nrf2, Prdx2 and iNOS with IS20 treatment compared to control. **(C)** Western blot for Arginase1 and Nrf2 comparing IS20 treatment and control, with  $\beta$ -Actin as an internal loading control. Asterisks denote statistical significance between IS20 treatment and control (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).



**Figure 10.** Prokineticin signaling increases glutamate uptake in cultured astrocytes through induction of GLAST gene expression. **(A)** Exogenously added radioactive glutamate levels in primary mouse astrocytes. **(B)** Quantitative real-time PCR gene expression analysis for GLAST with PK2 and IL-4 treatment compared to control. Asterisks denote statistical significance between PK2, IS20 or IL-4 treatment and control (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).

## CHAPTER V

THE CHEMOKINE-LIKE SIGNALING PROTEIN PROKINETICIN-2 INDUCES GDNF  
EXPRESSION AND SECRETION IN ASTROCYTES

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## Abstract

Parkinson's disease (PD) is characterized by the selective loss of dopaminergic neurons in the substantia nigra region of the brain. A major scientific focus has been developing ways to protecting these neurons from cell death. One such avenue of research has been investigating the use of proteins that are known to activate cell survival signals in neurons, such as neurotrophic factors. The most studied neurotrophic factor in PD is glial cell-line derived neurotrophic factor, or GDNF. GDNF has been found to protect dopaminergic neurons from cell death in both cell culture and animal models of PD. However, clinical trials for GDNF therapies in PD patients have demonstrated mixed results. Therefore, the search continues for factors that increase endogenous GDNF and could protect neurons through other pathways. Our lab previously found that dopaminergic neurons produce higher levels of the chemokine-like protein Prokineticin-2 (PK2) in animal models of PD and in PD patients compared to age-matched controls. PK2 is a secreted signaling protein that can signal to both microglia and astrocytes in the brain. Herein, we found

that prokineticin signaling can lead to increased gene expression and protein level of GDNF in mouse and human astrocytes. Interestingly, the PK2-induced GDNF increase was able to protect human dopaminergic neuronal cells from MPP<sup>+</sup>-induced cell death. Collectively, Prokineticin-2 is a novel protein that we found which could modulate GDNF expression in astrocytes, and therefore induce astrocytes to protect dopaminergic neurons through the increased GDNF protein levels.

### Introduction

Parkinson's disease is characterized by the selective loss of dopamine producing neurons in the Substantia Nigra (SN) region of the brain. It is currently unknown what causes these neurons to undergo apoptosis, and there is no treatment available to prevent this cell loss. Research in the Parkinson's disease field has focused on finding a treatment that can either protect or restore these dopaminergic neurons. Glial-derived neurotrophic factor, GDNF, was first identified as a protein in the TGF $\beta$  family that is secreted from astrocytes in the central nervous system. Neurotoxic studies *in vitro* demonstrated that exogenously added GDNF could be neuroprotective against dopaminergic neuronal apoptosis (Lin et al. 1993). Interestingly, Parkinson's disease patients were found to have significantly reduced levels of GDNF in the SN region in comparison to age-matched controls (Chauhan et al. 2001). Therefore, GDNF has become an interesting factor for current study for a possible treatment of Parkinson's disease due to the ability to protect and restore dopaminergic neurons. Several studies in the last decade have demonstrated that GDNF overexpression can increase neuroprotection both in cell culture and animal models of Parkinson's disease (Andereggen et al. 2009, Elsworth et al. 2008, Sandhu et al. 2009).

Similar to the *in vitro* studies that showed GDNF could induce neuronal survival and neurite outgrowth (Lin et al. 1993, Kaddis et al. 1996), many studies have demonstrated that GDNF can protect against MPTP (Tomac et al. 1995, Gash et al. 1996) or 6-OHDA (Lu and Hagg 1997) induced behavioral deficits and neuronal loss with GDNF injections either intraventricular, intrastriatal and intranigral injections. However, clinical trials in humans have offered mixed results to GDNF therapy of Parkinson's disease. Firstly, GDNF is not able to cross the blood brain barrier (BBB) and therefore intracranial infusion through injections or pumps is the only feasible option for a GDNF protein therapy. Unfortunately, GDNF intracranial infusion in human patients was not able to significantly improve the unified Parkinson's disease rating scale (UPDRS), and actually lead to adverse effects such as anorexia and weight loss (Lang et al. 2006a, Hovland et al. 2007). Researchers have since tried to deliver GDNF by different routes, such as intranasal (Migliore et al. 2014), transplantation of GDNF expressing cells (Liu et al. 2013), and gene therapy using viral vectors (Wang et al. 2002, Tereshchenko et al. 2014). Results from these studies are encouraging, however, it seems as though increased GDNF alone is not enough to significantly affect the progression of Parkinson's disease.

Prokineticin-2 is a small chemokine-like protein first identified to potently contract gastrointestinal muscles (Li et al. 2001). PK-2 is an 86 amino acid signaling protein that is the mammalian homologue of MIT1, a non-toxic protein in mamba snake venom, and Bv8, found in frog skin. Prokineticin-2 has many physiological functions throughout the human body, from the testis, ovaries, heart, to the brain. Among other functions, PK2 can promote cell survival and growth, and in other tissues it can cause an increase in inflammation and nociception. Prokineticin-2 signaling has been studied in several areas of the brain, and the prokineticin receptors, PKR1 and PKR2, are expressed throughout most of the central nervous system. PK2 plays a role in

everything from olfactory bulb neurogenesis (Ng et al. 2005), regulating circadian rhythms and autonomic functions (Zhou and Cheng 2005, Li et al. 2006b, Li et al. 2009, Yuill et al. 2007), thermo regulation and energy expenditure (Zhou et al. 2012), and leads to reduced food intake and weight loss (Beale et al. 2013, Gardiner et al. 2010). One of the most interesting functions of PK2 is the ability to be neuroprotective against several different toxicants. Melchiorri et al. first demonstrated that PK2 could protect against NMDA excitotoxicity in the cerebellum (Melchiorri et al. 2001). Then, our group was the first to identify that PK2 can protect dopaminergic neurons from MPP<sup>+</sup>-induced cell death (unpublished). PK2 is a secreted protein, and both microglia and astrocytes produce the receptors to bind PK2 (Koyama et al. 2006). Therefore, we wanted to investigate the role PK2 could play in astrocytes, the support cells of the CNS.

## Results

### **Prokineticin-2 intracellular signaling leads to an induction of GDNF gene expression**

Prokineticin-2 signals through two cognate G-protein coupled receptors (GPCRs), PKR1 and PKR2. These two receptors are highly, and Zhou et al demonstrated that both receptors can signal through either G<sub>i</sub>, G<sub>q</sub>, or G<sub>s</sub>, depending on cell type or location of the cell (Zhou 2006). Prokineticin-2 signaling is therefore known to cause an increase in intracellular calcium, cell proliferation and migration, and activation of the MAPK pathway that can be blocked by a prokineticin receptor antagonist or inhibitors of the specific pathways (Chen et al. 2005). Since we confirmed that astrocytes express both prokineticin receptors (Neal et al., unpublished)(Koyama et al. 2006), we wanted to characterize the cell signaling pathways that could be activated by this signaling protein in primary mouse astrocytes. Consistent with previous reports that PK2 can

activate the MAPK pathway, we found an increase in the protein levels of both phosphorylated MEK and phosphorylated ERK with recombinant PK2 (rPK2) treatment (Figure 1A). Activation of the MAPK pathways can have many different downstream effects, one of which is phosphorylation and activation of the CRE binding protein, or CREB (Cargnello and Roux 2011). Similarly, PK2 has been implicated in increasing levels of cyclic AMP, which has been found to increase activation of the CREB transcription factor. Measuring the activation of the CREB protein, by phosphorylation, using Western blotting techniques we found that rPK2 treatment in primary mouse astrocytes increased the levels of phosphorylated CREB. The CREB transcription factor has been implicated in the induction of several neurotrophic factors, such as GDNF and BDNF (Brain derived neurotrophic factor)(Saavedra et al. 2008). Therefore, we wanted to determine whether rPK2 treatment on primary human astrocytes could lead to changes in neurotrophic factor gene expression by using a quantitative PCR array that can measure 80 different genes in a single sample. Interestingly, we found that rPK2 treatment on primary human astrocytes led to changes in several genes, including an increase in GDNF gene expression (Fig 1A).

Astrocytes can protect neurons by several different mechanisms, including glutamate synthesis and neurotrophic factor release. Glial cell-line derived neurotrophic factor, GDNF, is a protein that is can be secreted from astrocytes and has demonstrated the ability to protect dopaminergic neurons from apoptosis (Lin et al. 1993). GDNF gene expression studies have indicated that increased intracellular calcium levels and activation of the MAPK pathway could induce GDNF expression levels (Saavedra et al. 2008). After finding that rPK2 could activate these specific cell signaling pathways, we wanted to investigate whether rPK2 could induce GDNF gene expression in primary mouse astrocytes. Several different transcription factors have been

found to regulate the GDNF promoter, such as CREB and SP1. To confirm our previous findings that rPK2 treatment can induce GDNF gene expression, we performed promoter studies to determine if rPK2 treatment can activate the GDNF promoter. Our lab was able to obtain a 3.6kb fragment of the GDNF promoter already cloned into a luciferase plasmid that has already been proven to be an effective measurement of GDNF promoter activity (Brodbeck, Besenbeck and Englert 2004). We transfected the U373 astrocytoma cell-line with the GDNF promoter plasmid and then treated the cells with or without rPK2 for 8 hours. Interestingly, we found that rPK-2 treatment significantly increased activity of the GDNF promoter compared to GFP vector control (Fig 1D). This data indicates that recombinant human PK2 treatment in a human astrocyte cells can induce GDNF promoter activation. Taken together, this is the first evidence that Prokineticin-2 can activate cell signaling pathways and transcription factors that can lead to activation of the GDNF promoter in astrocytes.

Several studies have looked into the effect of adding either exogenous GDNF or using gene therapy techniques to increase the level of GDNF to protect dopaminergic neurons in Parkinson's disease models and clinical trials on patients (Grondin et al. 2002, Tomac et al. 1995, Bjorklund et al. 2000, Kordower et al. 2000). However, the clinical trials for GDNF replacement therapy have had mixed results depending on the location and method of delivery (Gill et al. 2003, Slevin et al. 2005, Lang et al. 2006a). With the knowledge that Prokineticin-2 can signal to astrocytes through PKR1/2 and increase intracellular calcium levels and activate the MAPK pathway, our lab investigated whether PK2 can induce astrocytes to produce more GDNF. To begin, we used qPCR with validated primers for mouse GDNF, to analyze primary mouse astrocyte cell culture after adding exogenous rPK2. We found that there was a significant induction of GDNF gene expression with 25nM rPK2 treatment (Figure 1E). This induction was partially blocked when cells were co-

treated with the prokineticin receptor antagonist PC-7. We then used Western blotting to confirm that exogenous PK2 can also increase the GDNF protein. After treating primary mouse astrocytes with rPK2 for 10hrs we discovered that there was a significant increase in GDNF protein levels with rPK2 treatment using Western blotting when compared to control (Figure 1F). Since GDNF needs to be secreted to have a neuroprotective effect, we used an ELISA to determine whether rPK2 treatment on primary mouse astrocytes can increase the amount of secreted GDNF. We used a chemical, Golgi-block, which inhibits the secretory pathway, and therefore would leave any GDNF protein that is produced to remain inside the cell. Recombinant PK2 treatment caused a significant increase in GDNF protein level of astrocyte cell lysates treated with the secretion inhibitor when compared to control treatment (Figure 1G). GDNF protein levels also increased slightly with rPK2 even without the Golgi-block, however it was not significant (data not shown). Overall these results indicate that exogenously added PK2 can increase levels of GDNF in primary mouse astrocytes, and that the PK2-induced increase in GDNF production is also secreted from the cells.

### **Soluble exogenous PK2 increases production and secretion of GDNF in U373 human astrocytoma cell line**

Previous literature has indicated that the expression of GDNF is different between mouse and human cells (Hunot et al. 1996). Following the results from the mouse astrocytes, we wanted to determine whether PK2 could induce an increase in GDNF production in human astrocytes. To test this, our lab added exogenous rPK2 to the U373 human astrocytoma cell-line and checked GDNF levels by qPCR for gene expression, along with Western blotting, ELISA and ICC for protein levels. The gene expression levels were tested with validated qPCR primers for the human



GDNF and 18s ribosomal genes. We found that an 8 hour rPK2 treatment on the U373 cells induced a 3-fold increase in GDNF gene expression (Figure 2A). Following the gene expression results, our lab wanted to investigate whether rPK2 could induce a change in GDNF protein level in this human astrocytoma cell-line. Using immunocytochemistry techniques our lab was able to find that rPK2 treatment on the U373 cells induces a visible difference in GDNF protein levels. We quantified the fluorescence from the GDNF ICC and found that the rPK2 treatment lead to about a 50% increase in GDNF protein levels inside the cell (Figure 2B). Similarly, we used Western blotting and ELISA to confirm the protein changes that we found with the ICC. Western blotting techniques demonstrate that rPK2 treatment on U373 cells induces over a 2.5-fold increase in GDNF protein levels that is significantly attenuated when co-treated with a PK2-receptor antagonist, PC-7 (Figure 2C). Consistent with the GDNF Western blot, when we performed an ELISA for GDNF on rPK2 treated U373 conditioned media, we discovered that there was a significant increase in secreted GDNF protein levels in the rPK2 treated samples compared to the control, and PC-7 was able to significantly attenuate this increase (Figure 2D). Together, these results indicate that recombinant PK2 treatment can induce GDNF production and secretion in human astrocytes

### **PK2 overexpression in the human astrocyte cell line increases GDNF-induced neuroprotection of human dopaminergic neurons**

Since we determined that exogenously added recombinant human PK2 protein could induce GDNF production in both mouse and human astrocytes, we next wanted to investigate whether genetic overexpression of GDNF could elicit a similar response in the U373 human astrocyte cell line. We infected U373 human astrocytes with PK2 tagged to GFP or GFP alone for 24 hours and then changed the media. The cells were collected 24 hours later to ensure maximal

expression of the protein. First, we confirmed that the U373 cells were successfully overexpressing the proteins by GFP immunofluorescence (Figure 3A) and by Western blotting for the GFP protein (Figure 3B). Once we confirmed that the cells were overexpressing PK2, we used Western blotting techniques to measure the levels of the GDNF protein and activation of the CREB transcription factor (Figure 3C). Interestingly, we found that PK2 overexpression was able to increase the levels of phosphorylated CREB (Figure 3D, left panel), similar to that which was seen with rPK2 treatment, whereas total CREB protein levels did not change (Figure 3D, right panel). GDNF protein levels were also significantly increase in the U373 cell lysates with PK2 overexpression compared to both control and GFP overexpressing cells (Figure 3E). To determine if the amount of PK2-induced GDNF protein could be neuroprotective, we overexpressed PK2 in U373 cells and conditioned the media for 24 hours. We added the conditioned media with or without MPP<sup>+</sup> on differentiated human LUHMES cells. PK2 conditioned media was able to significantly protect against cell death when compared to MPP<sup>+</sup> alone by using an MTS assay (Figure 3F). This protection was attenuated with pre-treatment of LUHMES cells with RPI-1, a Ret inhibitor. Therefore, the amount of GDNF produced and secreted from PK2 overexpressing astrocytes could protect neurons from cell death, and importantly the Ret inhibitor was able to attenuate this protection. Collectively, this data demonstrates that PK2 signaling can not only induce GDNF in mouse astrocytes, but can do the same action in a human astrocytoma cell line and produce enough GDNF to be neuroprotective against MPP treatment. These results have important implications in translating this work from mouse cell culture into finding the potential role PK2 may play in human PD patients.

### **Soluble exogenous PK2 induces an increase in gene expression and protein level of GDNF in primary human astrocytes**

Cultured cell lines have historically been found to differ in expression of certain proteins when compared to primary cells. Therefore, we wanted to test our hypothesis in primary human astrocytes to get as close to that of the human CNS. Similar to U373 cells the primary human astrocytes treated with rPK2 for 8 hours showed significantly increased GDNF gene expression compared to control (Figure 4A). Western blotting also demonstrated that human recombinant PK2 can significantly increase GDNF protein level in human primary astrocyte cultures (Figure 5B). We then used confocal microscopy images to visually demonstrate that rPK2 treatment induced GDNF protein level in these primary human astrocytes, and fluorescent intensity quantification shows that it is significantly more GDNF compared to the control (Figure 5C, bottom panel). Interestingly, recombinant PK2 treatment also increased GDNF protein levels in the media after 12 hours of treatment and was attenuated with co-treatment of PC-7 (Figure 5D). These results indicate that PK2 signaling in primary human astrocytes can also lead to increased production of GDNF.

### **PK2 overexpression AAV infection induces an increase in GDNF levels in the mouse Striatum compared to GFP AAV infected tissues**

Several groups have used an AAV delivery system to increase neuroprotection by overexpressing GDNF in animal models of PD (Bjorklund et al. 2000, Kordower 2003). We wanted to use a similar approach to overexpress PK2 in the striatum and confer neuroprotection. Our lab previously demonstrated that this PK2-AAV 2/5 injection strategy could protect

dopaminergic neurons against MPTP-induced cell death (unpublished). We stereotaxically injected the PK2-AAV 2/5 or GFP-AAV 2/5 into the mouse striatum and let the gene reach maximal expression at 4 weeks, and then we sacrificed the mice. Interestingly, using quantitative real-time PCR we found that PK2-AAV was able to significantly increase GDNF gene expression compared to GFP AAV injected animals (Figure 5A). This was confirmed by using Western blotting techniques for the GDNF protein, showing that PK2-AAV significantly increases GDNF protein level in the mouse striatum compared to GFP-AAV (Figure 5B). We then used immunofluorescent microscopy 2X magnification images to demonstrate that the striatum with PK2-AAV injection had increased GDNF protein levels compared to the control striatum and the GFP-AAV injected striatum (Figure 5C). Similarly, 10X images of the same striatum indicate that the cells producing the PK2 are not the cells that have increased GDNF levels (Figure 5D). Taken together, these data confirm the cell culture results and demonstrate that PK2 overexpression *in vivo* can induced GDNF production in the mouse striatum.

### **The PKR1 agonist, IS20, increases GDNF production in primary mouse astrocytes**

Gasser et al. recently developed a non-peptide chemical agonist for the PK2 receptor PKR1 (Gasser et al. 2015). They used this chemical, IS20, to protect cardiomyocytes during myocardial infarctions in mice, mainly through activation of the ERK and Akt pathway. We used this chemical agonist in primary mouse astrocytes to determine whether activation of PKR1 would lead to the increased GDNF that we have found using recombinant and overexpression PK2. First, we treated primary mouse astrocytes with IS20 10 $\mu$ M for 8 hours and found that GDNF gene expression significantly higher compared to control treatment (Figure 6A). Similarly, Western blot of for the GDNF protein showed that IS20 also increased the GDNF protein level in the primary mouse

astrocyte protein lysates (Figure 6B). Next, we used immunofluorescence microscopy to confirm that the GDNF protein is higher in the cell cytosol with rPK2 treatment, and fluorescence intensity quantification shows that rPK2 treatment significantly increased the GDNF protein level compared to control (Figure 6C). Taken together, these data implicate the PKR1 receptor as being at least partially responsible for relaying the signal of PK2 treatment into the production of GDNF in astrocytes. However, it cannot be ruled out that PKR2 can also have a role, since there is no selective PKR2 receptor agonist available to test. Astrocytes normally express PKR1 at a much higher level than PKR2 under basal conditions, and therefore would indicate that PKR1 is the major signaling receptor of the exogenous PK2 protein to astrocytes.

## Discussion

GDNF is a major potential therapeutic option for Parkinson's disease by providing neurotrophic support for dopaminergic neurons of the substantia nigra. GDNF has demonstrated the ability to protect neurons both *in vitro* and *in vivo*, and reduce behavioral deficits in animal models of PD (Lin et al. 1993, Kramer and Liss 2015). However, clinical trials of GDNF therapies have had mixed results but have shown some promise. Therefore, any factor that can induce GDNF expression and release in the CNS can be relevant for PD research. This report has uncovered a novel role for Prokineticin-2 signaling in the brain and particularly astrocytes. First, consistent with previous findings (Koyama et al. 2006) in mouse astrocytes, we found that recombinant human PK2 (rPK2) increased activation of the MAPK pathway. Similarly, rPK2 treatment was able to induce activation of the transcription factor CREB. GDNF transcription is known to be regulated by several transcription factors in the CNS, including CREB, NF $\kappa$ B and SP1. Several signaling pathways, including MAPK, PKC and PKA, have also been implicated in the induction

of GDNF gene expression (Saavedra et al. 2008). We confirmed that PK2-induced activation of these signaling pathways induced GDNF gene expression, by performing a promoter analysis to show that PK2 signaling can increase the GDNF promoter activity. This is the first evidence that the chemokine-like protein Prokineticin-2, or prokineticin signaling in general, can lead to the activation of the GDNF promoter.

We then demonstrated for the first time that recombinant human Prokineticin-2 is able to induce GDNF production and secretion in primary mouse astrocytes and a human astrocyte cell line *in vitro*. Following these results, we went on to show that rPK2 can also increase GDNF gene expression and protein levels in primary human astrocytes. Importantly, we confirmed that the GDNF produced by PK2 overexpression in the human astrocyte cell line is able to significantly attenuate MPP<sup>+</sup> neurotoxicity of differentiated human dopaminergic neuronal LUHMES cells that was attenuated with RPI-1 blocking the GDNF protection. These results indicate that PK2 can induce astrocytes to produce and secrete sufficient amounts of GDNF to protect dopaminergic neurons from MPP<sup>+</sup> induced neurotoxicity *in vitro*. Therefore, we are the first to demonstrate that a protein increased in dopaminergic neurons during inflammatory or toxic conditions can induce neuronal protection through the increase of the neurotrophic factor GDNF from astrocytes.

Prokineticin signaling has been implicated in the survival of several different systemic cell types (LeCouter et al. 2004a, Lin et al. 2002b, Urayama et al. 2007a), neurogenesis (Ng et al. 2005) and neuroprotection (Melchiorri et al. 2001, Landucci et al. 2016). The basis for these studies was our discovery that PK2 is produced and secreted by dopaminergic (DA) neurons after neurotoxic insult *in vitro* and that postmortem Parkinson's disease patients had an elevated level of PK2 when compared to age-matched controls. Interestingly, we went on to find that exogenously added recombinant PK2 could protect DA neurons from MPTP-induced toxicity *in vitro* (unpublished).

Our previous results demonstrating that PK2 is neuroprotective, taken together with the current findings indicate that Prokineticin-2 is a potential therapeutic target for Parkinson's disease. To our knowledge, this is the first report showing that PK2 can signal in human astrocytes and increase the GDNF promoter activity, gene expression and protein level. More research is needed to determine which of the two receptors the PK2 uses to signal through in astrocytes, but our results indicate that the prokineticin-induced cell signaling in astrocytes is most likely through the PKR1 receptor. This study suggests that the use of the prokineticin receptor 1 agonist, IS20, could have wide-ranging implications for Parkinson's disease and possibly other neurodegenerative diseases. However, knowledge of prokineticin signaling in diseases of the CNS is limited and specific cell types involved in this signaling could lead to other therapeutic options.

## Materials and Methods

### **Reagents**

DMEM, MEM media, fetal bovine serum (FBS), L-glutamine, IR-dye tagged secondary antibodies, Hoechst nuclear stain, penicillin, streptomycin, and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Recombinant human PK2 was purchased from Peptidech. The primary antibody for GDNF was ordered from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The GFP antibody was purchased from Abcam. The Phosphorylated CREB and the Native CREB were ordered from Cell Signaling. The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). The GDNF E<sub>MAX</sub> ELISA kit was obtained from Promega.

### **Cell culture and animal studies**

Human U373 astrocytoma cells (ATCC), were grown in MEM media supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), according to ATCC instructions. U373 cells were maintained in an incubator at 37°C with 5% CO<sub>2</sub>. Primary astrocytes were obtained from whole brain homogenate of 0-3 day mouse pups. Astrocytes were collected, isolated and maintained according to previous methods (Gordon *et al.*, 2011) using DMEM media (10% heat-inactive FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, glutamate 2mM) to grow the cells. Primary human astrocytes were obtained from Lonza.

### **Quantitative PCR**

Mouse astrocyte cells or U373 cells were seeded out into 75-mm<sup>3</sup> flasks at 2.5 x 10<sup>6</sup> cells per flask. Treatments were performed in either MEM or DMEM media supplemented with 2% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine. Cells were treated for 8 h with rPK2 (25nM) or rPK2 cotreated with PC-7 (1µM). After treatment, cells were collected, pelleted, and resuspended in lysis buffer with β-mercaptoethanol. RNA was isolated using Absolutely RNA Miniprep, an RNA isolation kit from Stratagene. RT-PCR was performed using an cDNA synthesis system (Applied Biosystems) to convert the RNA into cDNA. Expression levels were determined using real-time PCR with Qiagen RT<sup>2</sup> SYBR Green master mix and prevalidated using the qPCR mouse primers from SuperArray/Qiagen. For normalization of each sample, the mouse gene 18SrRNA (Cat. No. PPM57735E) was used as the housekeeping gene. The amount of each template was optimized empirically to maximize efficiency without inhibiting the PCR reaction. According to manufacturer's guidelines, dissociation curves and melting curves were run to ensure that single amplicon peaks were obtained without any non-specific amplicons. The results are reported as fold change in gene expression, which was determined using the  $\Delta\Delta C_t$



method using the threshold cycle ( $C_t$ ) value for the housekeeping gene and for the respective gene of interest in each sample.

### **Western blotting**

Astrocytes were collected after treatment, lysed using modified RIPA buffer, homogenized, sonicated, and centrifuged as previously described (Kaul *et al.*, 2003; Kitazawa *et al.*, 2002). Supernatants were collected and proteins were normalized, then stored with loading buffer and DTT. Samples were run on a Sodium Dodecyl Sulfate (SDS) gel electrophoresis. Normalized protein samples were loaded into each well and first separated in a 4% stacking gel and then through a 12-18% resolution gel. Proteins were then transferred to a nitrocellulose membrane and allowed to transfer overnight in a 4°C refrigerator at 23-30 volts. After transfer, the membranes were blocked using either Western blocking buffer (Rockland Immunochemicals) or with 5% nonfat dry milk powder. Membranes were washed several times in wash buffers comprising either PBS containing 0.05% Tween (PBST) or Tris buffer saline containing 0.05% Tween (TBST). Primary antibodies, in either blocking buffer or in a 5% bovine serum albumin (BSA) solution, were then added to the membranes and stored overnight at 4°C. After another 4-5 washes, a secondary antibody specific to the primary antibody is added (in either blocking buffer or in 5% low-fat dry milk solution) to the membrane for 1 h. Either  $\beta$ -Actin or  $\alpha$ -Tubulin was used to confirm equal loading into each well of the gels. The membranes were washed another 4-5 times with wash buffer, and then the membranes were read using the Odyssey infra-red imaging system.

### **GDNF ELISA**

Astrocyte cells were seeded into either 96-well plate or 75mm<sup>3</sup> flask. Treated cells in DMEM or MEM media supplemented with 2% FBS for 12 hours, then collected the media and the cells separately. The astrocyte conditioned media (ACM) was then centrifuged at max speed for 5

minutes to remove any cell debris. The GDNF ELISA was performed according to the manufacturer's instructions. The signal was measured using the Synergy-2 multi-mode microplate reader.

### **Immunocytochemistry**

For immunocytochemistry, cells were plated onto coverslips in 24-well plates coated with 0.1% poly-D-Lysine. After cells were treated, 4% formaldehyde was used to fix the cells for 30 min. The cells were washed 4-5 times with PBS wash buffer. Then blocking buffer containing 2% BSA, 0.2% Triton X-100, and Tween was added to the wells for 1 h to permeabilize the cells. The cells were incubated with primary GDNF (Mouse polyclonal, 1:500 dilution) antibodies in 2% BSA at 4°C overnight. After 4-5 more washes, an Alexafluor dye-conjugated secondary antibody in 2% BSA was added and incubated at room temperature on a shaker for 1 h. Cells were then washed 4-5 times before adding Hoechst counterstain for 5-6 min to label nuclei. After 4-5 more washes, coverslips were mounted onto slides with Fluoromount mounting media. Cells were imaged under a Nikon 2000U microscope with a SPOT camera, and all images were processed in MetaMorph 5.7 (Universal Imaging, Downingtown, PA).

For histology, mice were anesthetized using the ketamine-xylazine mixture and cardiac perfusion of 4% PFA was used to fix the brain before being extracted and stored in 4% PFA at 4°C. After 48 h, brains were washed 5 times with PBS, and placed into a 30% sucrose solution for at least 24 h. The brains were then embedded in Optimal Cutting Temperature (OCT) compound and frozen into blocks for sectioning. Brains were sectioned on a Cryostat (CryoStar NX70, Thermo Scientific) at -20°C and placed into Cryosolution (30% Sucrose, Ethylene glycol, and PBS). Sections were then washed with PBS and permeabilized with blocking buffer (2% BSA, 0.1%

Triton X-100, and Tween) for 1 h at room temperature. Antibodies directed to GDNF (Mouse polyclonal, 1:500) were then incubated with the sections overnight at 4°C. After 4 washes with PBS, the sections were incubated with Alexafluor dye-conjugated secondary antibodies for 1 h at room temperature. After several more washes with PBS, Hoechst dye (1:5000) was added to the sections for 5 min at room temperature to stain nuclei. Sections were then mounted on slides using the Fluoromount mounting medium (Molecular probes) according to the manufacturer's instructions. An inverted fluorescent microscope (Nikon TE-2000U) was used to visualize the sections, and a Spot digital camera (Diagnostic Instruments, Inc) was used to capture images.

### **GDNF Promoter studies**

The GDNF promoter luciferase plasmid was generously provided by Dr. Englert's lab from their manuscript (Brodbeck et al. 2004). First, the plasmid was transfected into U373 cells by home-made Amaxa solutions and electroporation and allowed the cells to recover for 24 hours. Each transfection was performed with 2 µg of reporter constructs along with 0.2 µg of pcDNA3-LacZ to normalize transfection efficiencies. Then the cells were treated with PK2 for 8 hours. Cells were harvested and lysed in 150 µl of Reporter Lysis Buffer (Promega), and assayed for luciferase activity using the Synergy-2 multi-mode microplate reader.

### **MTS cell survival assay**

MTS cell viability assay was performed, as described previously (Jin et al. 2014b). Briefly, differentiated LUHMES cells were seeded onto 96-well plates and treated at differentiation day 5. After treatment, 20 µl of MTS solution was added to each well, and the plates were incubated at 37°C and 5% CO<sub>2</sub> for 2 h. Measurements were made at 490 nm using a fluorescence microplate reader (SpectraMax Gemini XS, Molecular Devices).

## Data analysis

Data analysis was performed using the Prism 4.0 software package (GraphPad Software, San Diego, CA). The data was first analyzed using one-way ANOVA and then Tukey's post-test was performed to compare all treatment groups. Differences of  $p < 0.05$  were considered statistically significant. The Student's t-test was used when two groups were being compared.

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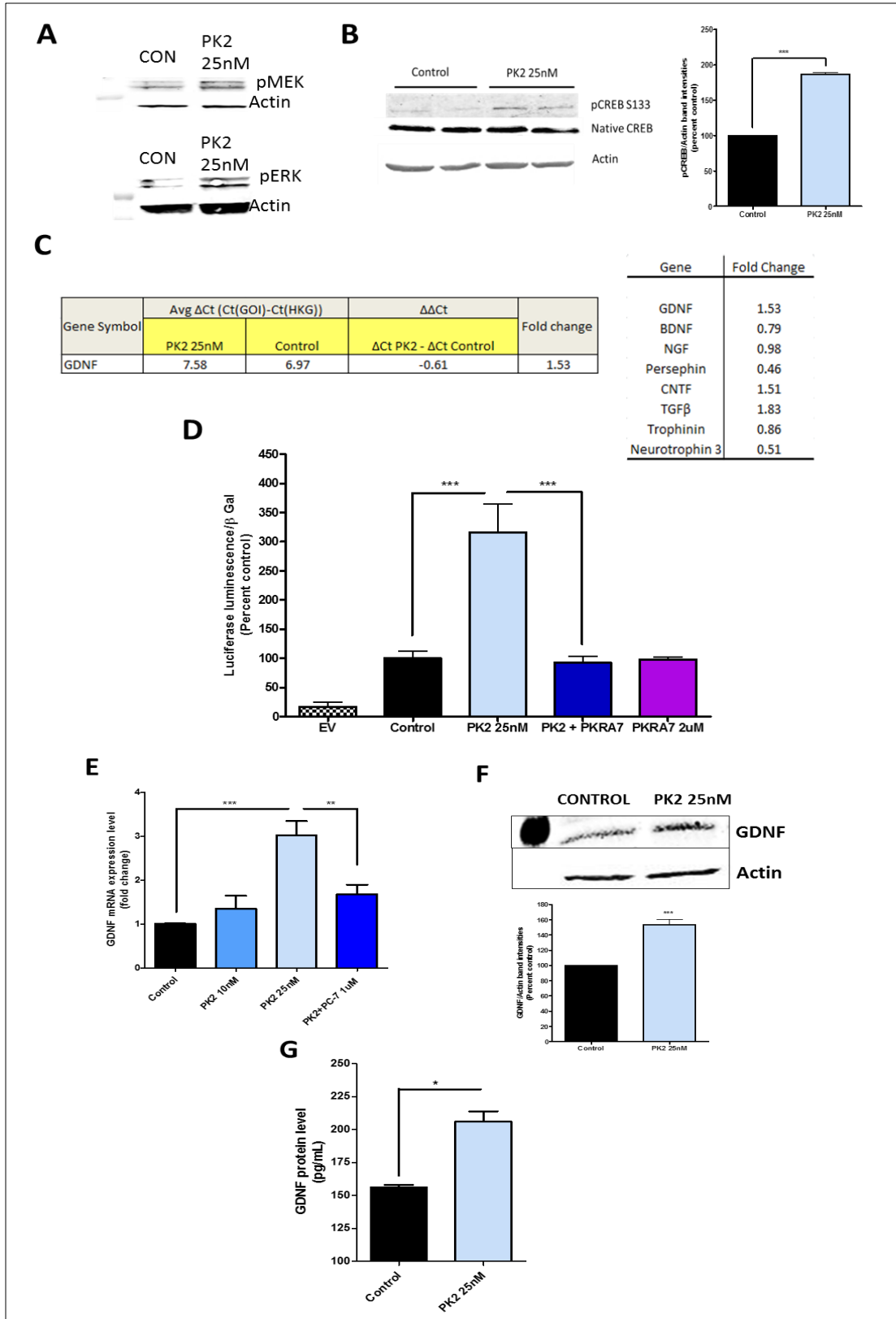
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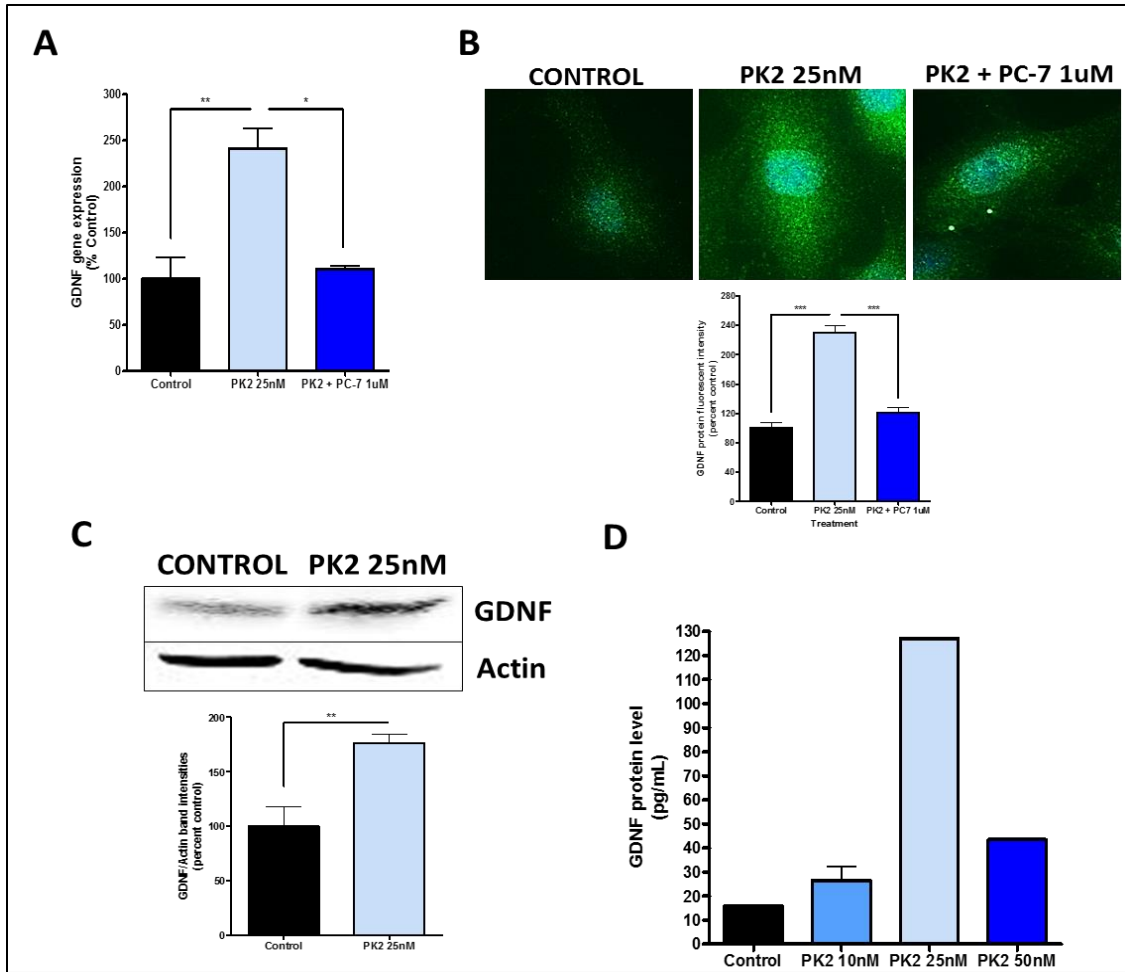
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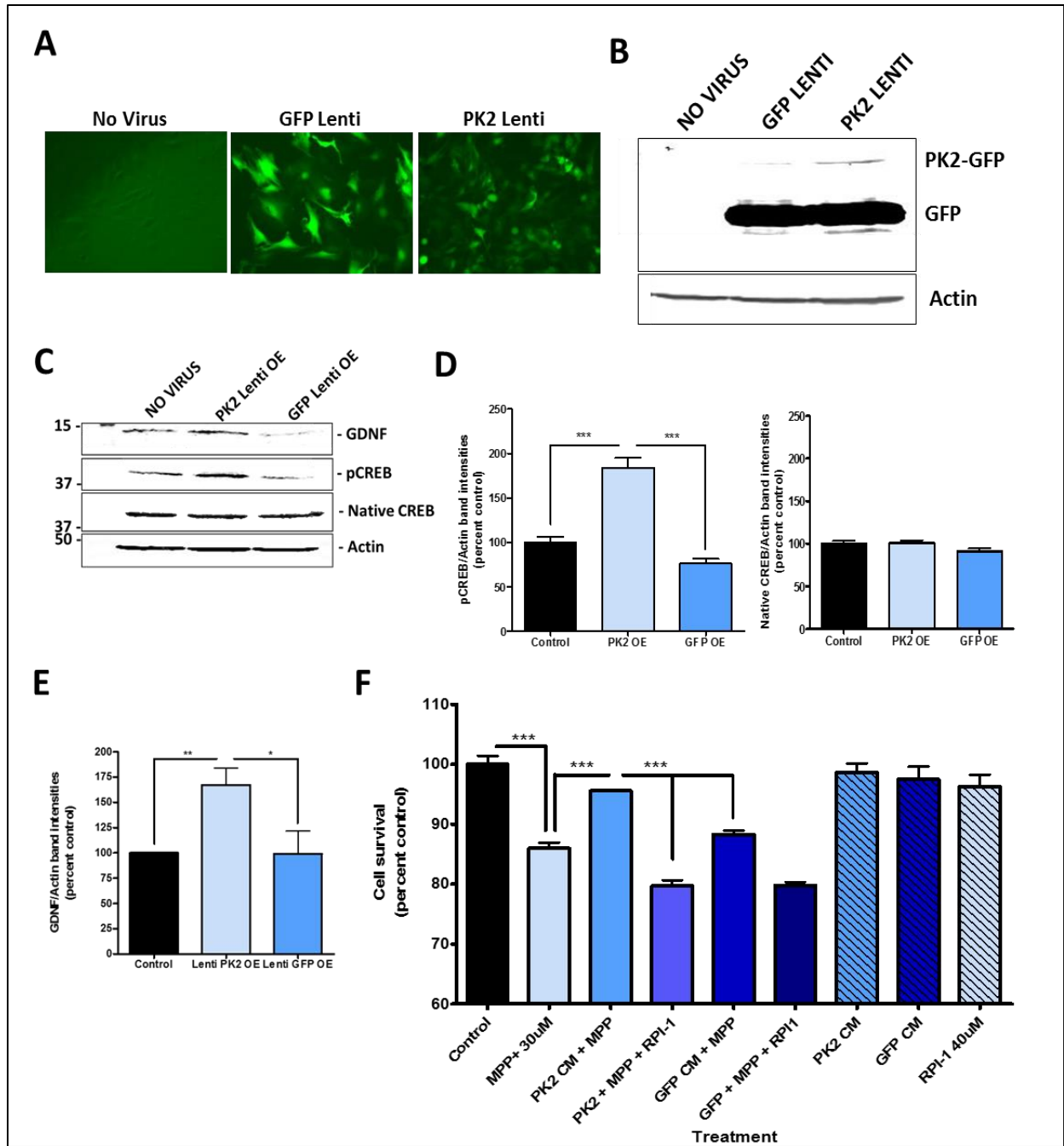
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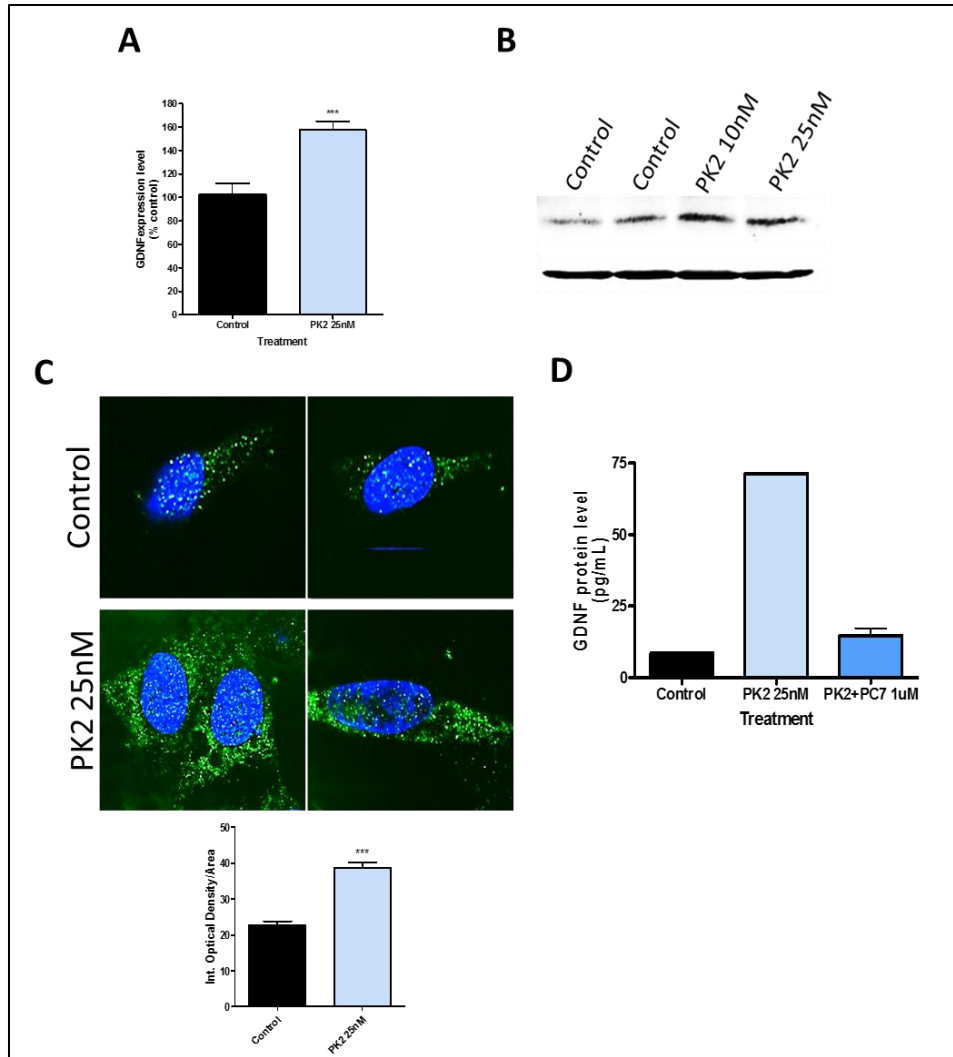
**Figure 1.** *Prokineticin-2 can signal to astrocytes and induces GDNF production.* (A) Representative Western blot for phosphorylated MEK and phosphorylated ERK with control or rPK2 treatment. (B) Representative Western blot for phosphorylated CREB. (C) Quantitative real time PCR array results in primary human astrocytes using the  $\Delta\Delta C_t$  method, with a table of common neurotrophic factors and their gene expression fold change. (D) Analysis of the GDNF promoter upstream of the luciferase gene, values were normalized using  $\beta$ -galactosidase values. (E) Quantitative real time PCR results for gene expression of GDNF in primary mouse astrocytes. (F) Representative Western blot for GDNF protein levels with control of PK2 treatment in primary mouse astrocytes, with  $\beta$ -Actin used as a loading control. (G) GDNF ELISA results for primary mouse astrocytes conditioned media to detect secreted GDNF protein levels. Asterisks denote a significant difference between rPK2 treatment and the control (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).



**Figure 2.** Recombinant Prokineticin-2 induces GDNF expression in the U373 human astrocytoma cell line. (A) Quantitative real time PCR results for gene expression of GDNF in U373 cells expressed as fold change compared to control. (B) Fluorescence microscopy images for GDNF protein level, with control, PK2 or PK2 co-treated with PC-7. (C) Representative Western blot for GDNF in the U373 astrocytes showing that there is an increase in GDNF with the rPK2 treatment, with  $\beta$ -Actin used as a loading control. Densitometric band quantification in the bottom panel. (D) GDNF ELISA results for the U373 astrocytes conditioned media to detect secreted GDNF protein levels. Asterisks denote a significant difference between rPK2 treatment and the control (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).



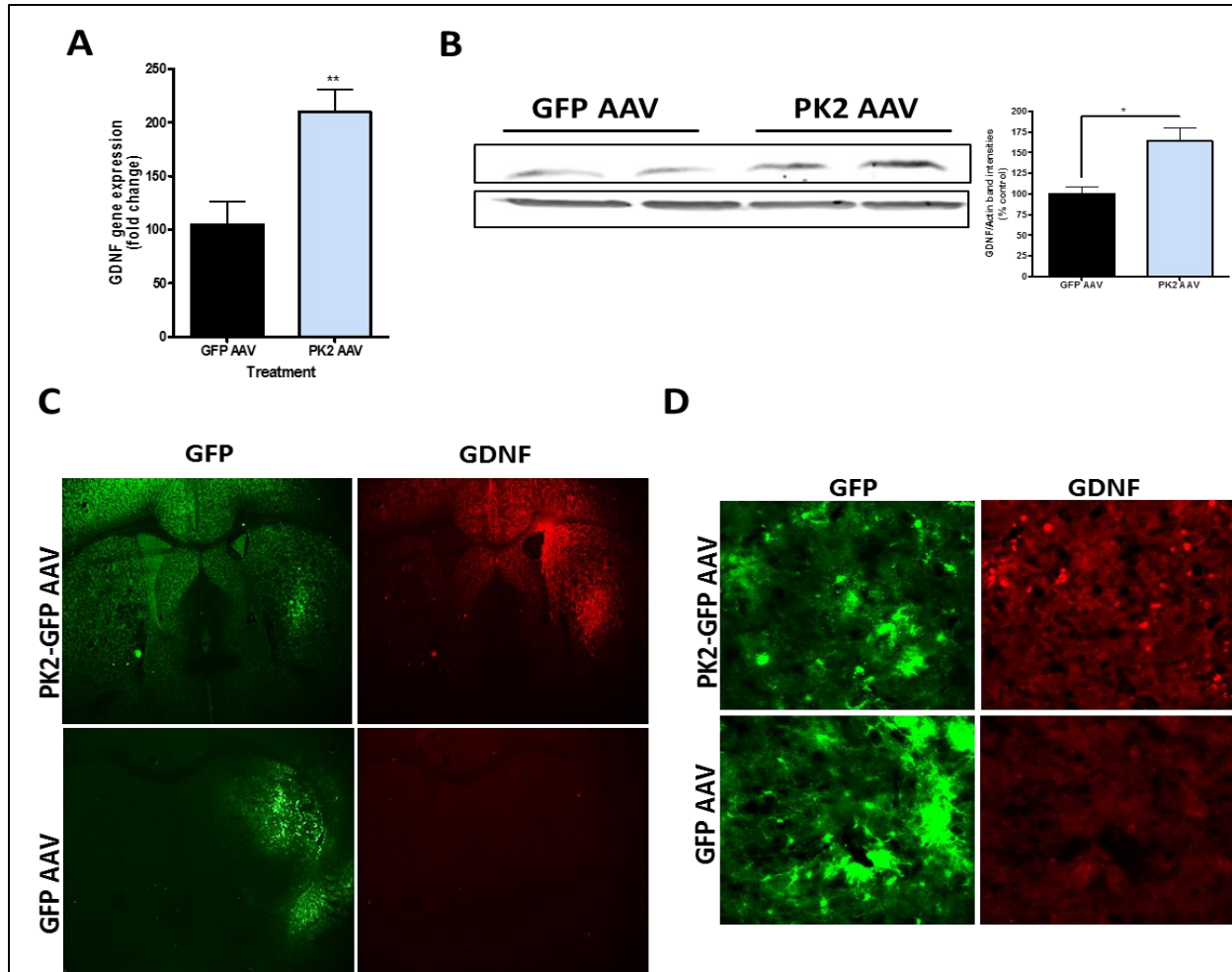
**Figure 3.** *Prokineticin-2 overexpression increases GDNF expression in the U373 human astrocytoma cells.* (A) Fluorescent microscopy images to detect GFP protein levels ensuring either GFP or PK2-GFP overexpression. (B) Representative Western blot for GFP in PK2 or GFP overexpressing astrocytes, with  $\beta$ -Actin used as a loading control. (C) Representative Western blot for GDNF, phosphorylated CREB at S133, and Native CREB, with  $\beta$ -Actin used as a loading control. (D-E) Densitometric quantification of Western blot protein bands in PK2 or GFP overexpressing astrocytes. Quantification of Western blot proteins for phosphohorylated CREB (D, left panel) and native CREB (D, right panel) indicate that PK2 overexpression increases phosphorylated CREB levels. Similarly, quantification of the GDNF protein band (E) found an increase with PK2 overexpression compared to control and GFP overexpression. (F) MTS assay results quantifying cell survival of human LUHMES cells treated with astrocyte conditioned media, from either PK2 or GFP overexpressing cells, along with the Ret inhibitor, RPI-1. Asterisks denote a significant difference between rPK2 treatment and the control (\*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ ).



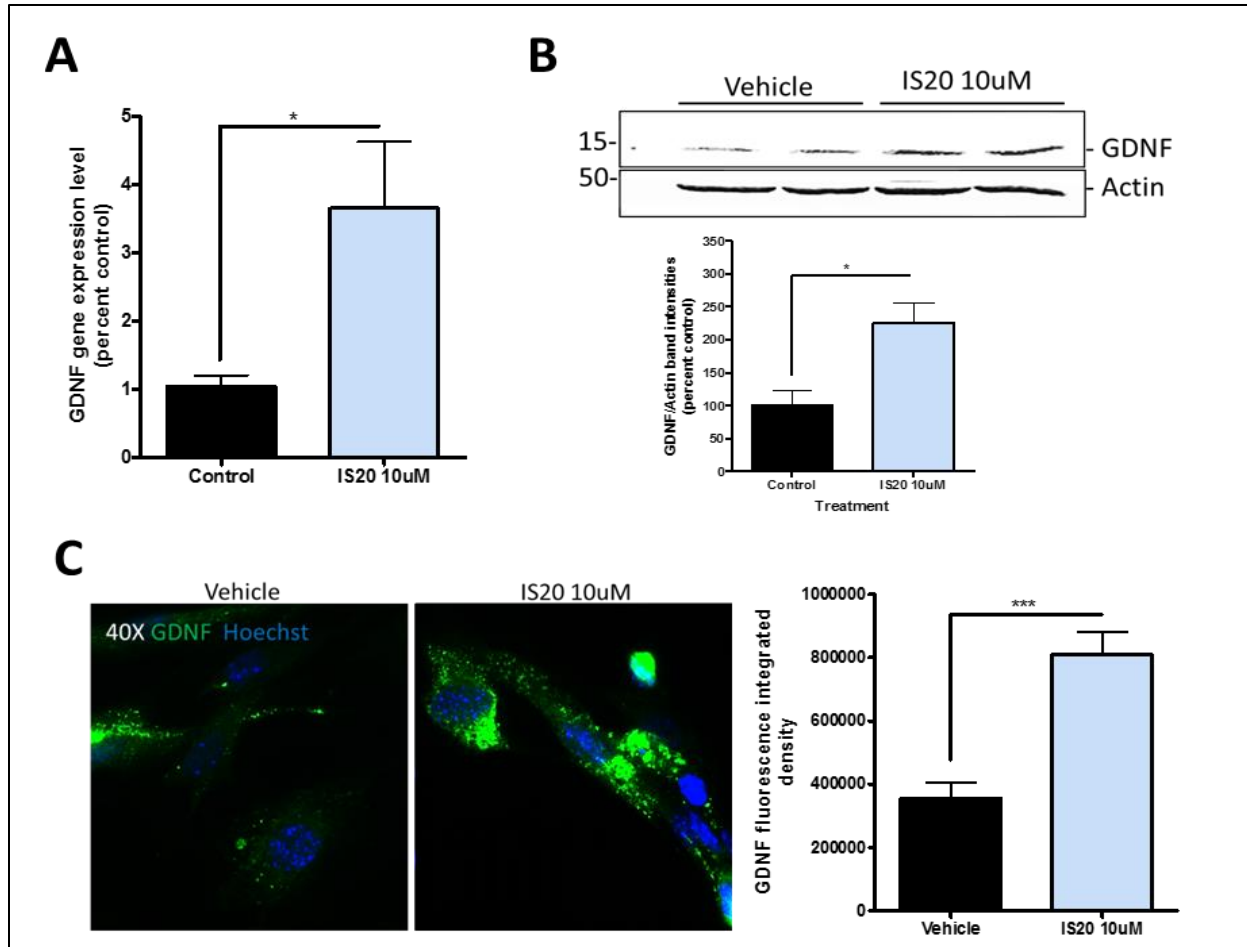
**Figure 4.** Recombinant Prokineticin-2 induces GDNF expression in the primary human astrocytes.

(A) Quantitative real time PCR results for gene expression of GDNF in primary human astrocytes expressed as fold change compared to control. (B) Representative Western blot for GDNF in the U373 astrocytes showing that there is an increase in GDNF with the rPK2 treatment. (C) Fluorescence microscopy images for GDNF protein level, treated with control or rPK2. Fluorescence intensity quantification in the bottom panel. (D) GDNF ELISA results for the U373 astrocytes conditioned media to detect secreted GDNF protein levels. Asterisks denote a significant difference between rPK2 treatment and the control (\*\*\*)  $p < 0.001$ .





**Figure 5.** PK2-AAV 2/5 induces GDNF expression in the mouse striatum. (A) Quantitative real time PCR results for gene expression of GDNF in striatum tissue of mice injected with either GFP or PK2-AAV. (B) Representative Western blot for GDNF in the U373 astrocytes showing that there is an increase in GDNF with the rPK2 treatment. (C) Fluorescence microscopy 2X magnification images for GFP and GDNF protein level in the mouse striatum. Demonstrating an increase in the GDNF protein level in the striatum injected with PK2-AAV and not with GFP-AAV injection. (D) Fluorescence microscopy 10X magnification images for GFP and GDNF protein level in the mouse striatum. Asterisks denote a significant difference between rPK2 treatment and the control (\*  $p < 0.05$  and \*  $p < 0.01$ ).

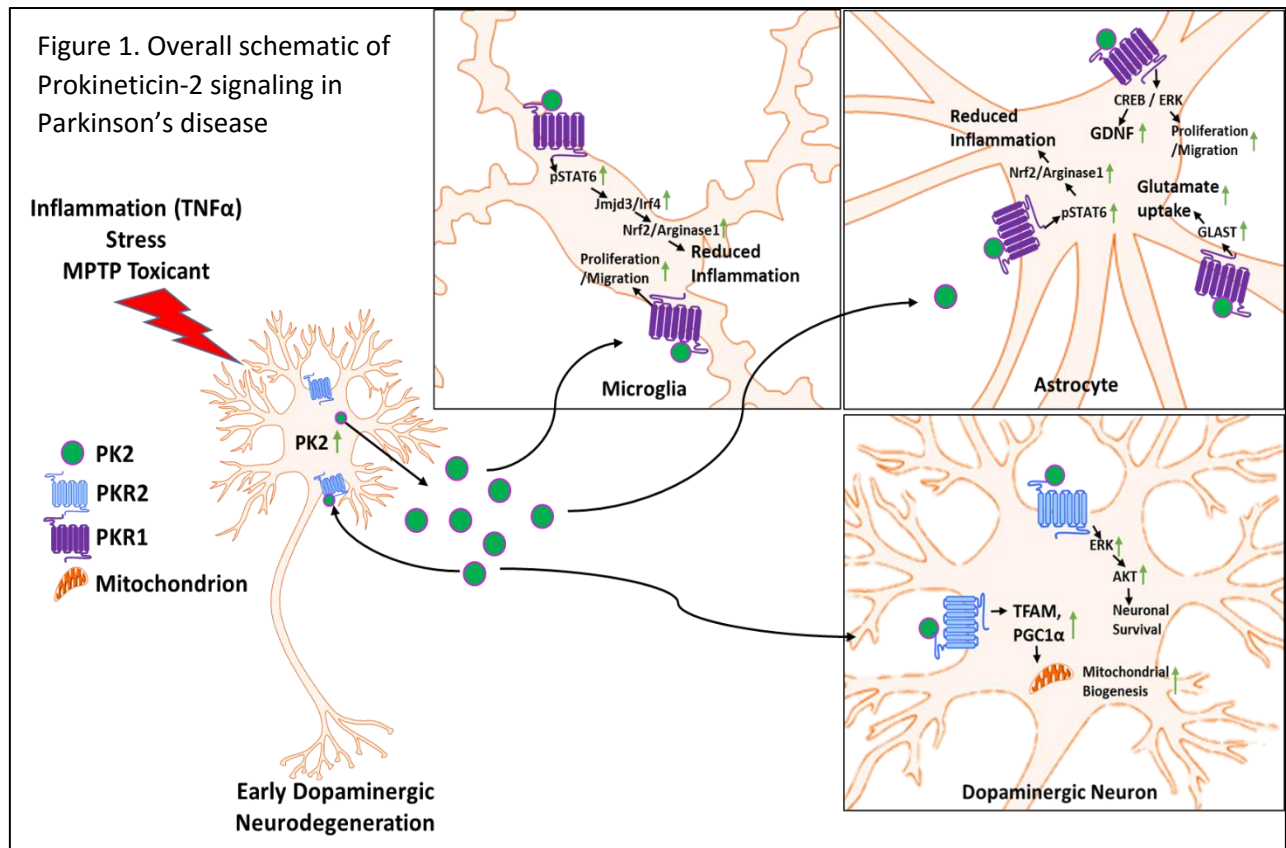


**Figure 6.** *PKR1* agonist, IS20, induces GDNF expression in the primary mouse astrocytes. (A) Quantitative real time PCR results for gene expression of GDNF in primary mouse astrocytes expressed as fold change compared to control. Mouse 18srRNA used as a loading control. (B) Representative Western blot for GFDF in the primary mouse astrocytes showing that there is an increase in GDNF with the IS20 treatment. (C) Fluorescence microscopy images for GDNF protein level, treated with control or IS20. Fluorescence intensity quantification in the panel to the right. Asterisks denote a significant difference between rPK2 treatment and the control (\*  $p < 0.05$  and \*\*\*  $p < 0.001$ ).

## CHAPTER VI

## GENERAL CONCLUSION AND FUTURE DIRECTIONS

This section presents a general overview of the results described in the thesis, with special emphasis on future directions and overall implications of these findings for the pathogenesis and progression of PD. The major findings pertaining to each research manuscript and their specific implications are covered in the ‘Results’ and ‘Discussion’ sections of each chapter.



### **Prokineticin-2 neuroprotective signaling is mediated through the ERK and Akt pathways**

The findings reported in Chapter II demonstrate that PK2 is highly induced following dopaminergic neuron injury in cell culture and animal models of PD and is also elevated in postmortem human PD brains. This is the first evidence for the induction of PK2 expression in dopaminergic neurons following injury, thus indicating an intriguing new function for PK2 in the pathophysiology of PD. Our results suggest that PK2 expression is most likely induced at early stages of PD by physiological insults including oxidative stress, mitochondrial dysfunction and neuroinflammation. Evidence for this was manifested by increased PK2 levels in MitoPark mice, which have a genetic deletion of the TFAM protein specifically in dopaminergic neurons. Mitochondrial dysfunction occurs in the MitoPark mouse model because the loss of TFAM cripples mitochondrial DNA replication and transcription. Additional evidence came from TNF $\alpha$  stimulation, which rapidly enhanced PK2 expression in dopaminergic neurons. Further studies are being performed to determine whether environmental stressors induce PK2 expression during early stages to protect dopaminergic neurons from neurotoxic insults. Identifying the transcription factors involved in this mitochondrial dysfunction-induced PK2 activation would facilitate the development of novel ways to induce PK2 and broaden our understanding its role in PD. Studies are currently being conducted to identify the molecular underpinnings of PK2 upregulation in dopaminergic neurons.

Studies using models of cerebellar excitotoxicity and ischemia suggest that PK2 could be a neuroprotective protein (Melchiorri et al. 2001, Landucci et al. 2016). The PKR2 receptor is expressed in dopaminergic neurons in cell culture, and *in vivo* in both mouse and human dopaminergic neurons. We found that either the recombinant protein or overexpression of PK2 protects dopaminergic neurons from MPP<sup>+</sup>/MPTP-induced cell death. PK2 treatment increased the

activation of both ERK and Akt signaling pathways in the N27 dopaminergic neuronal cell line. We found that the neuroprotection induced by PK2 treatment was abolished by PD98059 and API-1, which are pharmacological inhibitors of ERK and Akt, respectively. Several different neurotrophic factors exert their protective effects through the Akt pathway (Duronio 2008, Downward 2004, Orike et al. 2001, Brunet, Datta and Greenberg 2001). Activation of the Akt pathway was reported to protect neurons in disease models of ischemia, PD and Alzheimer's disease (Zhang et al. 2016, Ries et al. 2009, Wang et al. 2015). Therefore, PK2 could potentially act through similar cell signaling pathways used by neurotrophic factors to protect neurons. Our MPTP and PKRA7 experiments provided further evidence for PK2 acting like a neurotrophic factor. Using PKRA7 to block MPTP-induced endogenous PK2 signaling significantly increased dopaminergic neuronal cell death. This indicates that PK2 expression by dopaminergic neurons during toxic insults could protect against some neurodegeneration as depicted in Figure 1. Therefore, PK2 signaling could be harnessed to slow the progression of PD by increasing PK2 above endogenously produced levels. PK2-AAV overexpression in the mouse nigrostriatal system demonstrated that higher levels of PK2 protected against MPTP-induced behavioral deficits and TH<sup>+</sup> neuronal cell loss. Further studies are still needed to examine the effectiveness of PK2 in other genetic models of PD including LRRK mutant mice.

### **Importance of PK2-induced mitochondrial biogenesis in neurons**

Mitochondrial dysfunction has been implicated in the progression of PD (Schon and Przedborski 2011). Mitochondrial complex I activity is lower in the substantia nigra of PD patients compared to age-matched controls, and several PD toxicants such as rotenone, MPTP, and 6-OHDA are potent mitochondrial complex I inhibitors. Interestingly, genetic knockout of the master regulator of mitochondrial biogenesis, TFAM, in mice leads to the development of

neurodegenerative diseases such as PD (Ekstrand et al. 2007). Similarly, a genetic mutation in TFAM is also a moderate risk factor for AD (Belin et al. 2007). Mitochondrial biogenesis can become compromised with aging, thereby increasing the risk of developing age related neurodegenerative diseases such as PD. Recombinant PK2 treatment and overexpression of PK2 in dopaminergic neuronal cell lines led to protection against MPP<sup>+</sup>-induced mitochondrial dysfunction and increased the expression of mitochondrial protective proteins such as Bcl2. Interestingly, overexpression of PK2 also led to a significant increase in the gene expression of the mitochondrial biogenesis regulators PGC-1 $\alpha$  and TFAM, and increased the total number of mitochondria in cells. The action of PK2 signaling to increase mitochondrial biogenesis and mitochondrial protective proteins could help neurons survive against any toxicants or destructive factors that target the mitochondria. Similarly, this indicates that PK2 signaling could play a major part in preventing age-related problems in the CNS.

### **PK2 promotes glial cell-induced neuroprotection by reducing of inflammatory factors**

In Chapters III and IV, we investigated the roles that PK2 plays in both microglia and astrocytes, with Figure 1 summarizing the PK2 signaling seen in these two cell types. Due to previous reports that PK2 could increase inflammation in systemic immune cells and that blocking PK2 signaling could reduce some systemic inflammation (Martucci et al. 2006, Giannini et al. 2009), we hypothesized that PK2 signaling in glial cells would lead to an increased inflammatory response. Surprisingly, we found the complete opposite result. Both recombinant PK2 treatment and PK2 overexpression reduced basal levels of inflammatory factors such as nitric oxide and intracellular ROS in microglia, while increasing anti-inflammatory factors such as Arginase1 and Nrf2. Similarly increased Nrf2 and Arginase1 levels were found in astrocytes. Moreover, in both cell types, basal levels of the pro-inflammatory cytokines TNF $\alpha$ , IL-6 and IL-12 were either

decreased or unchanged compared to controls. We reproduced the same effects seen in cell culture in an *in vivo* mouse model via PK2-AAV overexpression in the nigrostriatal system, which decreased the gene expression of pro-inflammatory factors such as nitric oxide synthase (NOS2) and pro-inflammatory cytokines relative to GFP-AAV control injections. Interestingly, rPK2 treatment significantly attenuated LPS-induced production of ROS, RNS, pro-inflammatory cytokines and mitochondrial dysfunction in primary mouse microglia. Taken together, these data indicate that PK2 signaling increases anti-oxidant and anti-inflammatory factors, while reducing pro-inflammatory mediators increased by an inflammatory stimulus. This is the first report of PK2 attenuating inflammation and promoting anti-inflammatory factors, and therefore points to prokineticin signaling as a potential therapeutic anti-inflammatory molecule for treatment of neurodegenerative diseases.

These interesting results conflict with previous results found in peripheral tissues due to several factors. First, and perhaps most importantly, is that microglia found in the brain respond differently than microglia found in the brain stem and especially macrophages found more peripherally (Olson 2010, Greter, Lelios and Croxford 2015). Therefore, the cell type-specific differences could lead to differential responses to factors such as PK2. Secondly, prokineticin signaling involves complex interactions between differential receptor expression, phosphorylation and heterodimerization. Along with this, prokineticin receptors can signal through different G-proteins and could therefore lead to distinct downstream signaling pathways in different cells and conditions. Currently, no studies have investigated cell signaling in peripheral immune cells such as macrophages that could be compared with what we found in brain-resident microglia. Lastly, any detrimental effects reported for PK2/Bv8 signaling originated from the use of picomolar ranges of the recombinant protein, or blocking prokineticin signaling using a receptor antagonist.

However, all the beneficial effects of PK2 signaling have been found in the nanomolar ranges of recombinant PK2 or overexpression. This indicates dual roles for PK2, wherein it either increases peripheral inflammation at lower (picomolar) concentrations or reduces in CNS inflammation at higher (nanomolar or above) concentrations.

### **PK2 promotes astrocyte-induced neuroprotection by producing the neurotrophic factor GDNF**

Neurotrophic factors are important in the development and maintenance of neurons. Several neurotrophic factors have been found to potently protect dopaminergic neurons from cell death, including GDNF, Neurturin, BDNF, and CDNF. Prokineticin signaling activates particular cell signaling pathways, including the cyclic AMP and MAPK pathways (Ngan and Tam 2008). Increased cAMP can lead to activation of the CREB transcription factor. Both pathways, CREB and MAPK, have been implicated in activating the gene expression of several neurotrophic factors, including GDNF. We found that recombinant and overexpressed PK2 increased GDNF mRNA and protein expression in cultured mouse and human astrocytes. Astrocyte-conditioned media treated with PK2 could protect N27 dopaminergic neurons from MPP<sup>+</sup>-induced cell death that could be attenuated by blocking GDNF signaling through the Ret receptor using the chemical antagonist RPI-1. This demonstrates that PK2-induced GDNF is produced at high enough levels to protect dopaminergic neurons from toxicant-induced cell death. Therefore, PK2 signaling could offer druggable targets for increasing GDNF levels in the CNS.

Our preliminary data suggests that PK2 also increases GDNF in other cell types such as cultured microglia, neurons, and bone marrow derived stem cells (BMSCs). Importantly, if PK2 signaling could increase GDNF from multiple cell types, then the therapeutic potential drastically increases to where the increase in GDNF could reach across the entire CNS if necessary. The



increased GDNF production in the BMSCs also opens up the option for PK2-enhancing stem cell replacement therapies, since previous studies have demonstrated that increased GDNF and other neurotrophic factors can enhance stem cell incorporation and survival (Ren et al. 2013). Studies are currently underway in our lab to investigate the potential for PK2 to increase GDNF in other cell types such as neurons, microglia, and BMSCs.

The cell signaling required to produce other neurotrophic factors is similar to that of GDNF, mainly in the form of CREB and MAPK signaling. Therefore, it is possible that PK2 could increase neurotrophic factors other than GDNF. For instance, BDNF is protective in animal models of PD and its expression can be increased with activation of the CREB transcription factor (Spina et al. 1992, Tao et al. 1998). Similarly, cerebral dopamine neurotrophic factor (CDNF) is a potent neurotrophic factor for dopaminergic neurons (Lindholm and Saarma 2010). Preliminary data in our lab shows that PK2 can increase CDFN in astrocyte cultures, and possibly BDNF as well. We are currently characterizing the PK2-induced CDFN increase in astrocytes. More studies are needed to find other cell type- specific neurotrophic factors that PK2 could potentially increase.

### **PK2 in non-motor symptoms of PD**

The motor symptoms of PD are well-known and aid diagnosis of the disease. However, non-motor symptoms associated with PD include anosmia (loss of the sense of smell), sleep deficits, sexual dysfunction, weight loss and gastrointestinal problems. These non-motor symptoms typically emerge several years before the motor symptoms of PD. Interestingly, several of the known functions of PK2 signaling overlap with these non-motor symptoms. For instance, in peripheral tissues, PK2 functions in the formation and proper health of reproductive organs, such as the testes, and gastrointestinal motility. Also, the known functions of PK2 in the CNS include the formation and maintenance of the olfactory bulbs, regulation of circadian rhythms, and

energy homeostasis in the hypothalamus (Cheng et al. 2002, Ng et al. 2005, Beale et al. 2013, Matsumoto et al. 2006, Hu et al. 2007). The evidence for PK2 affecting these non-motor symptoms is currently only circumstantial. However, the finding of differential PK2 signaling in PD models and in PD patients demonstrates that this protein might play a role in other areas of the disease. This includes other areas of the CNS, such as the olfactory bulbs or superchiasmatic nucleus, and peripherally, such as the gastrointestinal tract and reproductive organs. Further studies would be required to investigate differential PK2 expression in these areas in animal models and post-mortem PD patients. Currently, our lab is investigating whether PK2 is a novel mediator in the non-motor symptoms of PD to better determine its potential for improving the quality of life for PD patients.

### **Potential roles for PK2 in other neurodegenerative diseases**

Oxidative stress and neuroinflammation are common disease mechanisms for most neurodegenerative diseases. Besides PD, other diseases like AD, amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), ischemia and glaucoma have elevated levels of oxidative damage and neuroinflammation (Heneka et al. 2015, Lewis et al. 2012, Crotti and Glass 2015, Uttara et al. 2009, Soto and Howell 2014). Therefore, since PK2 can reduce neuroinflammation and oxidative stress in cell culture and animal models of PD, it is plausible that it could also have a similar effect in these other neurodegenerative diseases. We found that PK2 could directly reduce mitochondrial damage in neurons and reduce ROS levels in neuronal cells. Similarly, PK2 signaling induced both astrocytes and microglia to produce factors that reduce inflammation and oxidative damage in neurons, such as Nrf2 and Arginase1. Figure 1 summarizes the PK2 signaling pathways that we have found in neurons, microglia and astrocytes. Interestingly, a recent AD study implicated PK2 in exacerbating amyloid- $\beta$ -induced neurodegeneration (Severini et al. 2015).

However, the authors stated that the effects seen in their studies could be due to the use of picomolar amounts of PK2, and that higher doses did not show any adverse effects and could possibly be protective. The advent of PK2-AAV allows for the overexpression of PK2 in animal models of different diseases as well as the directed expression of the protein to specific regions-of-interest without overexpressing PK2 throughout the body. Therefore, more studies are required for directed PK2 overexpression in different neurodegenerative diseases and models of ageing to determine if PK2 could induce positive effects on these different diseases. Similarly, the tissue-specific and stimulus-specific activation of PK2 cell signaling in the brain would be worth investigating for a detailed understanding of the diverse biological functions of PK2 in the CNS. Overall, PK2 is emerging as a key endogenous protein that could regulate physiological function of neurons as well as glial cells, impacting the course of disease onset and progression in the brain. Further studies are warranted to better understand the role of PK2 in PD and other neurodegenerative diseases.

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## Abbreviations

- 6-OHDA – 6-hydroxydopamine, or 2,4,5-trihydroxyphenethylamine
- AAV – Adeno-associated virus
- AD – Alzheimer’s disease
- AD-PD – Autosomal dominant Parkinson’s disease
- ALS – Amyotrophic Lateral Sclerosis
- AR-PD – Autosomal recessive Parkinson’s disease
- BBB – Blood brain barrier
- Bcl2 – B-cell lymphoma 2
- bHLH – basic Helix Loop Helix
- BDNF – Brain derived neurotrophic factor
- bFGF – basic Fibroblast growth factor
- Bv8 – Bombina Variegata 8
- CDNF – Cerebral dopamine neurotrophic factor
- CNS – Central Nervous System
- CREB – cyclic AMP response element binding protein
- DAB – 3,3’-Diaminobenzidine
- DAT – Dopamine transporter
- DBS – Deep Brain Stimulation
- DCFH-DA – 2’,7’-Dichlorodi-hydrofluorescein diacetate
- ECAR – Extracellular acidification rate
- EG-VEGF – Endocrine gland-vascular endothelial growth factor, also known as Prokineticin-1
- ELISA – Enzyme-linked immunosorbent assay
- ERK – Extracellular signal-regulated kinase
- GDNF – Glial cell-line derived neurotrophic factor
- GFP – Green fluorescent protein
- GFAP – Glial fibrillary acidic protein
- GnRh – Gonadotropin releasing hormone

GPCR – G-protein coupled receptor  
GSH – Reduced glutathione  
HD – Huntington’s disease  
HH – Hypogonadotropin hypogonadism  
HPLC – High performance liquid chromatography  
Iba1 – Ionized calcium-binding adaptor molecule 1  
IL – Interleukin  
ICC – Immunocytochemistry  
IHC – Immunohistochemistry  
Irf4 – Interferon regulatory factor 4  
IS20 – Chemical Prokineticin receptor 1 agonist  
Jmjd3 – Jumonji-domain containing protein 3  
LPS – Lipopolysaccharide  
LUHMES – Lund human mesencephalic cells  
LV - Lentivirus  
MANF – Mesencephalic astrocyte-derived neurotrophic factor  
MAPK – Mitogen-activated protein kinase  
MIT-1 – Mamba intestinal toxin 1  
MPP<sup>+</sup> - 1-methyl-4-phenylpyridinium  
MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine  
Mrc1 – Mannose receptor c-type 1, also known as CD206  
MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium  
NGF – Nerve growth factor  
NO – Nitric Oxide  
NOS – Nitric oxide synthase  
Nrf2 – Nuclear factor E2-related factor 2  
NSAID – Non-steroidal anti-inflammatory drug  
NTF – Neurotrophic factor

OCR – Oxygen consumption rate

OE - Overexpression

OR – Overall risk

PAMP – Pathogen associated molecular pattern

PC-7 – Prokineticin receptor antagonist, N-{2-[5-(4-fluoro-benzyl)-1-(4-methoxy-benzyl)-4,6-dioxo-1,4,5,6-tetrahydro-[1,3,5]triazin-2-ylamino]-ethyl}-guanidine

PD – Parkinson's disease

PET – Positron emission tomography

Pgc1 $\alpha$  – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PK1 – Prokineticin-1

PK2 – Prokineticin-2

PK2 $\beta$  – Active, cleaved form of PK2L

PK2L – Prokineticin-2 long isoform

PKR1 – Prokineticin receptor 1

PKR2 – Prokineticin receptor 2

PKRA7 – chemical prokineticin receptor antagonist, C<sub>27</sub>H<sub>34</sub>CLFN<sub>2</sub>O<sub>4</sub>

ROS – Reactive oxygen species

RNS – Reactive nitrogen species

rPK2 – Recombinant human Prokineticin-2

SNpc – Substantia Nigra pars compacta

SOD – Superoxide dismutase

STAT – Signal transducer and activator of transcription

STR – Striatum

TFAM – Transcription factor A, Mitochondrial

TH – Tyrosine hydroxylase

TNF $\alpha$  – Tumor necrosis factor alpha

UPDRS – Unified Parkinson's disease rating scale

UPS – Ubiquitin proteasomal system

VTA – Ventral Tegmental Area



### Acknowledgements

I would like to thank Dr. Anumantha Kanthasamy for allowing me to be a part of this fantastic lab and exposing me to this exciting field of research. He has always encouraged me to become the best possible scientist and been very understanding when things have not always gone according to plan. I will always appreciate the advice and direction that he has given me, especially in the fact that he allowed me to pursue the research directions that interested me. He pushed me to grow into someone that can find a new project, plan the experiments, analyze and interpret the results, and write up the manuscript. This lab has been a wonderful place to work, and the best place for me to grow and learn. I definitely need to thank my co-major professor Dr. Arthi Kanthasamy and Dr. Vellareddy Anantharam for their great ideas, guidance, and research advice that have been an immense help. I would like to thank the members of my committee for their great advice and directions, as well as their important inputs during my POS meeting and prelims. The member of the DRKLAB have been an indispensable resource during my time in the lab. I will always remember the great times that we have shared and all of the help that I have received from these amazing scientists. Special recognition for Richard, Dilshan, Nikhil, Souvarish, Huajun, Monica, Dan, and Gary because they have elevated my research to heights that I could not achieve without all of their help. A very special and important thanks has to go to my beautiful and amazing wife, Megan Smith. She has been there for me through the ups and downs of grad school, and supported me through it all. Words cannot describe how thankful I am to have her by my side through this process. I have to send a thank you to my son, Layne, for always putting a

smile on my face, even on the worst days. Lastly, I would like to thank my family, especially my supportive parents, for always being there for me and pushing me to be the best at everything that I do. There is absolutely no possible way that I could have reached this point without so many caring and supportive individuals that have made me a better person by knowing them.