MECHANISMS OF DOPAMINERGIC NEUROTOXIN-INDUCED BLOOD-BRAIN BARRIER DISRUPTION

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MECHANISMS OF DOPAMINERGIC NEUROTOXIN-INDUCED BLOOD-BRAIN

BARRIER DISRUPTION

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Blood-brain barrier disruption in Parkinson's disease and Parkinson's disease models that involve dopaminergic neurodegeneration has been minimally evaluated despite mounting evidence for its involvement. Oxidative stress and neuroinflammation are both involved in Parkinson's disease pathology and also both contribute to blood-brain barrier dysfunction, creating the likelihood that blood-brain barrier disruption is also a pathological feature of the disease. Disruption of the blood-brain barrier can lead to an increased susceptibility to neuronal injury and potentially neurodegeneration due to the invasion of peripheral factors such as immunoglobulins and environmental toxins into the brain. An understanding of mechanisms by which blood-brain barrier disruption occurs may lead to the development of new approaches for the treatment of neurologic diseases such as Parkinson's disease.

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1.0   GENERAL INTRODUCTION

The main goal of the following experiments is to determine a mechanism by which dopaminergic neurotoxins lead to disruption of the blood-brain barrier (BBB). Dopaminergic neurotoxins are often used for the modeling of pathological features of Parkinson's disease (PD) to determine causes, contributing factors, and potential therapeutics. Oxidative stress and neuroinflammation are two of several contributing factors implicated in PD pathogenesis, and are also implicated in causing damage to and disruption of the BBB. Recent evidence suggests that BBB permeability may also be associated with PD (Lee et al., 2004; Kortekaas et al., 2005; Desai et al., 2006; Desai et al., 2007) , as it is in several other disorders involving neuronal injury, and may contribute to the pathogenesis of such diseases by allowing peripheral blood factors that otherwise would not have access to the central nervous system (CNS) to infiltrate the brain.

The examination of BBB integrity following the administration of neurotoxins for the modeling of neurodegenerative disease is of importance for several reasons including:

1.)   Disrupting the BBB via the administration of neurotoxins during modeling presents a confound when examining the outcome of potential therapeutics administered systemically that would otherwise not have brain access,

2.)   Permeability of the BBB may have detrimental effects on the CNS due to the invasion of foreign substances and thus may increase vulnerability to neurodegeneration,

3.)   A dysfunctional BBB does not allow a.) Adequate importation of essential nutrients and b.) exportation of waste and debris, xenobiotics, or environmental toxins that enter the brain, and

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4.)   Disruption of the BBB may be taken advantage of for potential therapeutic implications for neurodegenerative disorders such as PD.

1.1   THE BLOOD-BRAIN BARRIER

The BBB is a highly specialized system of capillary endothelial cells that prevents materials in the blood from entering the brain via both physical tight junctions and metabolic barriers. Large (>400 Daltons) and highly electrically charged molecules cannot cross the BBB easily while lipid-soluble molecules can pass readily. There are highly specific transporters that allow the influx of essential nutrients, amino acids, and peptides into the CNS and as well as transporters that selectively remove metabolites from the brain. Thus, malfunctions of the BBB can have deleterious effects on the CNS due to impaired transport of essential nutrients, the impaired efflux of waste and foreign materials, and the invasion of harmful substances and pathogens that otherwise would not have brain access. Additionally, dysfunction of the BBB has been linked to neurotoxicity and neurological diseases (Schinkel et al., 1994; Banks, 1999; Zlokovic, 2005; Dinapoli et al., 2007; Matsumoto et al., 2007).

1.1.1   Endothelial cells

Endothelial cells that line brain capillaries to comprise the BBB are highly specialized and packed tightly together to provide a physical barrier between the blood stream and the CNS (Ribatti et al., 2006). Cerebrovascular endothelial cells of the BBB restrict the movement of small polar molecules and macromolecules between the blood and the brain interstitial fluid

(Ueno, 2007). Additionally, endothelial cells function to provide the brain with nutrition and rid the brain of waste products through highly specific transporters at luminal and abluminal surfaces (Haseloff et al., 2005). Endothelial cells that comprise the BBB also have notably higher concentrations of mitochondria than endothelial cells in the periphery to handle the demands to high metabolic activity and energy-requiring functions (Ribatti et al., 2006; Ueno, 2007).

1.1.2   Tight junction proteins

Tight junction proteins form a continuous strand of complexes to create a "seal" between adjacent endothelial cells (Haseloff et al., 2005; Ueno, 2007). These complexes are comprised of membrane proteins (claudin, occludin, and junction adhesion molecule) and cytoplasmic accessory proteins (zona occuldins 1-3 and cingulin). The cytoplasmic proteins physically link the membrane proteins to actin to maintain structure and function of the brain endothelium (Depino et al., 2003). Expression and proper formation of cytoplasmic and membrane tight junction proteins is required for maintenance of BBB integrity and function. In patients with multiple sclerosis (MS), increases in serum protein leakage across the BBB is associated with decreases in tight junction protein expression (Leech et al., 2007). Additionally, patients with human immunodeficiency virus (HIV) encephalitis plus concomitant BBB disruption had a significant loss of tight junction protein expression compared to patients without disruption of the BBB (Dallasta et al., 1999). These examples provide evidence for the importance of normal tight junction protein expression in maintaining impermeability of the BBB.

1.1.3 Transporters

Receptors and transporters selectively mediate the uptake of essential nutrients from the blood stream into the brain. There are two types of influx transport mechanisms for molecules moving from the blood stream into the brain. Carrier-mediated transport is utilized to supply the brain with such nutrients as amino acids and glucose. Receptor-mediated endocytosis/transcytosis regulates the influx of transferrin, insulin, and lipoproteins into the brain. These transport mechanisms are highly selective for specific substrates, however pharmacological agents can be developed and targeted for transport into the CNS via these transport mechanisms (de Boer and Gaillard, 2006).

Passive transcellular diffusion is very low because efflux pumps hinder this process (de Boer and Gaillard, 2006). One such efflux transporter is P-glycoprotein (P-gp), located at the plasma membrane, and responsible for the export of xenobiotics from the brain (Bauer et al., 2005). Substrates for P-gp include: pesticides, immunosuppressive agents, opiod analgesics, antibiotics, HIV protease inhibitors, and phospholipids (Ueno, 2007). Tetracycline antidepressants can inhibit P-gp function which can be utilized to increase brain absorption of therapeutic agents that are P-gp substrates (Zhong and Lee, 2007). While inhibition of P-gp can enhance the delivery of therapeutic drugs, there is evidence that decreased P-gp function can lead to undesirable effects. For example, amyloid beta (Aβ) proteins are substrates for P-gp which normally functions to transfer Aβ protein from the brain into the blood stream (Lam et al., 2001). Deficiency of P-gp is associated with increases in Aβ accumulation, one of the main constituents of amyloid plaques in Alzheimer's disease (AD) (Vogelgesang et al., 2002), suggesting that dysfunction of the P-gp transported system could result in the accumulation of toxins in the CNS and this may contribute to neurotoxicity.

1.1.4 Astrocytes

While astrocytes do not structurally comprise the BBB, end feet of astrocytes juxtapose brain capillary endothelial cells and provide structural and nutritional support to the BBB (for review, see Ueno, 2007). Astrocytes can induce tightening of the tight junction complexes (Hurst and Fritz, 1996), influence the upregulation of tight junction proteins (Dehouck et al., 1990), and influence P-gp expression (Schinkel, 1999), thus affecting the efficacy of the BBB at preventing microorganism and environmental toxin invasion from the blood stream into the brain. Such influence on expression of tight junctions and P-gp is likely attributable to secretion of the growth factor, transforming growth factor-beta, by astrocytes (Abbott et al., 2006). Additionally, systemic administration of 3-nitroprpionic acid in rat led to striatal astrocyte cell death which was followed by increasing striatal IgG infiltration from the periphery (Nishino et al., 1997), lending further support for a role for astrocytes in the maintenance of BBB integrity. Finally, astrocytes provide a link in the communication between endothelial cells of the BBB and neuronal cells (Kim et al., 2006), suggesting that astrocytes influence not only maintenance of the BBB but also that of neurons.

1.1.5 Disruption

Disruption of the BBB refers to altered function, expression, or activation of any of the functional components that make up the BBB. Molecular factors known to disrupt function of the BBB include excitatory amino acids, reactive oxygen species (ROS), proinflammatory cytokines, and the activation of matrix metalloproteinases (de Boer and Gaillard, 2006). There is evidence that disruption of BBB function could lead to increased neurotoxicity. In multi drug

resistance (mdr)-1 knockout mice, which lack the P-gp efflux transporter, exposure to the pesticide, ivermectin, a substrate for the P-gp, increased neurotoxicity over wild-type control mice that received the same neurotoxin exposure (Schinkel et al., 1994).

There are many diseases and neurological disorders with neurodegenerative components that are accompanied by changes in the functionality and integrity of the BBB (Lynch et al., 2004; Bazan et al., 2005). Additionally, there is evidence suggesting that compromised cerebral vasculature contributes to the pathogenesis of diseases involving neuronal injury such as AD, MS, acquired immunodeficiency syndrome (AIDS) encephalitis, stroke, and outcome following traumatic brain injury. In neurodegenerative diseases, including MS and AD, evidence suggests that BBB dysfunction precedes neurological deficit as it does in animal models (Huber et al., 2001; Langford and Masliah, 2001; D'Andrea, 2003; Ujiie et al., 2003; Algotsson and Winblad, 2007). There is some evidence for a dysfunctional BBB in PD, however it is not known whether this may contribute to cause, risk of development, or PD pathogenesis.

1.2   PARKINSON'S DISEASE

Parkinson's disease is a progressive neurodegenerative disorder affecting the motor, cognitive, autonomic, and enteric systems, characterized by a loss of dopaminergic neurons in the substantia nigra (SN) and the degeneration of the nigrostriatal projection, as well as the presence of proteinaceous inclusions known as Lewy bodies. The second most common neurodegenerative disease behind Alzheimer's disease, PD affects around one million people over the age of 50 in the United States alone, regardless of sex, race or ethnicity (Kandel, 2000; Dawson and Dawson, 2003).

1.2.1 Symptoms, pathology, and incidence

Symptoms for those with PD include muscle rigidity, resting tremor, lack of balance and coordination of movement, bradykinesia, and a loss of facial expression. Autonomic dysfunction, sensory disturbances, and cognitive symptoms, including depression and dementia, are also prevalent in PD (Samii et al., 2004). Pathologically, a characteristic feature of PD is the loss of pigmented dopaminergic neurons from the SN however, dopaminergic neuron loss in the SN of greater than 50% and 70-80% of striatal dopamine (DA) is required for mild, classic PD motor symptoms to appear (Zigmond et al., 2002; Przuntek et al., 2004). This loss is progressive and therefore the loss of neurons required to elicit classic motor symptoms normally does not occur until a later stage of the disease. Prior to the substantial degeneration, dopaminergic neurons compensate for the decrease in neuronal number by producing more DA (Zigmond et al., 2002) which contributes to lack of motor symptoms associated with dopaminergic deficits. There are, however symptoms and pathology that precede this extensive SN neuronal loss, including decreased motility of the gastrointestinal system, olfactory dysfunction, sleep disturbances, and psychological impairments (Przuntek et al., 2004). Therefore, these non-motor symptoms develop and persist before the onset of motor symptoms associated with the loss of neurons from the midbrain.

Another pathological characteristic of PD is the formation of proteinaceous inclusions called Lewy bodies, of which a primary structural component is α-synuclein (Spillantini et al., 1997; Trojanowski and Lee, 1998). The function of α-synuclein is unclear, though α-synuclein has been shown to bind tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis, and inhibits TH activity (Perez et al., 2002). These factors, in addition to genetic forms of PD which are associated with alterations in α-synuclein (Polymeropoulos et al., 1997; Gasser et al.,

1998), make α-synuclein a protein of interest when investigating causes of PD. The pathological stages of the progression of PD have been described and are largely based on the presence of Lewy bodies and Lewy neurites in the cells of a specific affected region (Braak et al., 2002; Braak et al., 2003; Braak et al., 2004). The role and consequence of Lewy bodies and Lewy neurites, however, remains unclear (Caughey and Lansbury, 2003).

There are currently no known causes of sporadic PD. Some genetic factors have been implicated but a large number of cases, approximately 90% of all PD cases, are considered to be sporadic (Hunot and Hirsch, 2003). Biological contributors to the formation and progression of the neurodegenerative component of the disease have been identified and include mitochondrial dysfunction, dysfunction of the ubiquitin-proteasome system, neuroinflammation, and oxidative stress. Despite that the etiology of PD remains largely unknown, some risk factors for PD have been identified and offer insight into potential causes. It is currently accepted that environmental and genetic factors as well as aging are likely involved in a susceptibility for PD without being mutually exclusive of one another (Dauer and Przedborski, 2003; Eells, 2003; Greenamyre and Hastings, 2004).

1.2.2   The BBB and PD

Permeability of the BBB in PD has been minimally examined, despite that it is a pathological feature in other neurodegenerative diseases. Samples of cerebrospinal fluid (CSF) taken from PD patients and analyzed for serum albumin content revealed no difference between PD patients and controls, indicating normal function of the blood-cerebrospinal fluid barrier (BCB; Haussermann et al, 2001). Recently, attention has shifted to a possible dysfunction of the BBB efflux transporter P-gp, indicating a genetic susceptibility for PD due to the increased

accumulation of environmental neurotoxins into the CNS. Functional polymorphisms of the *MDR1* gene which produces P-gp were explored in three separate studies. One study involved Italian PD and control patients and found no significant differences between the groups (Furuno et al., 2002). However, a second study involving Chinese patients found three single nucleotide polymorphisms of the *MDR1* gene associated significantly with PD patients compared to control patients (Lee et al., 2004). Another study involving PD patients found reduced P-gp-inhibitor uptake via positron emission tomography (PET) when compared to age-matched control subjects (Kortekaas et al., 2005). This supports the hypothesis that BBB disruption is a clinical feature of the disease.

1.3 OXIDATIVE STRESS

Oxidative stress has been implicated as harmful to components of the BBB and also as a molecular contributor to PD pathogenesis. Oxidative stress results from an imbalance between ROS and antioxidant production. In normal, healthy brain there is an abundance of oxygen which contributes to the production of ROS via the normal functioning of several different sources. To counteract this, healthy brain cells also produce enzymatic and small molecule antioxidants (Schulz et al., 2000). When there is 1) excessive production of ROS, 2) a decrease in antioxidant production or activity, or 3) both, then oxidative stress occurs. This is detrimental to the cell leading to protein, lipid, and DNA oxidation and ultimately to cell death.

1.3.1   Mechanisms of Oxidative Stress in Parkinson's Disease

There is substantial evidence suggesting that oxidative stress plays a key role in the degeneration of the nigrostriatal pathway that occurs in PD. The SN is known to have inherently less of the antioxidant glutathione (GSH) than other brain regions (Loeffer DA, 1994) and is therefore more vulnerable to oxidative stress. Additionally, markers for protein, lipid, and DNA oxidation are found to be abundant in the affected brain areas in PD. Since oxidative stress is known to be harmful to cells, it is likely that the observed increases in markers of oxidation indicate oxidative damage to cells, contributing to PD.

1.3.1.1 The contribution of dopamine to oxidative stress

Dopamine-dependent oxidative stress may contribute to the vulnerability of dopaminergic neurons in PD. Cytoplasmic and extracellular DA is able to oxidize to form ROS and the DA quinone (DAQ) which leads to oxidative stress and cellular damage (Graham et al., 1978; Hastings TG, 1996). Sequestration of DA in vesicles prevents DA-induced oxidative stress due to the vesicular environment having a low pH and monoamine oxidase (MAO) not being present for DA metabolism (Lotharius and Brundin, 2002). However upon release into the extracellular space and following DA reuptake into the cytoplasm, DA oxidation can occur. Production of ROS results from DA metabolism by MAO into dihydroxyphenylacetic acid (DOPAC; Fig. 1). A consequence of this reaction is the production of hydrogen peroxide (H_2O_2), an ROS which by itself is not terribly damaging to cells, but can lead to the formation of highly reactive hydroxyl radicals ($\cdot OH$) through interaction with iron via the Fenton reaction (Halliwell, 1992; Fig. 2).
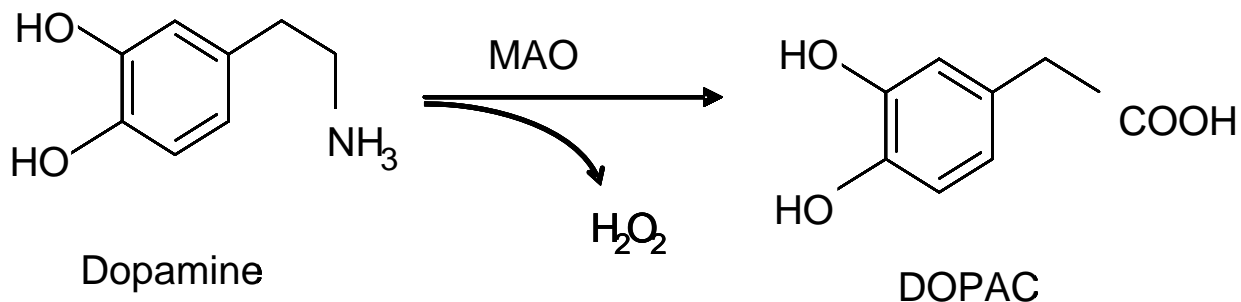
Figure 1: Dopamine metabolism. Metabolism of DA to DOPAC via MAO leads to production of H_2O_2 which itself is an ROS and can therefore contribute to cell damage via oxidative stress**.**

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^-$$
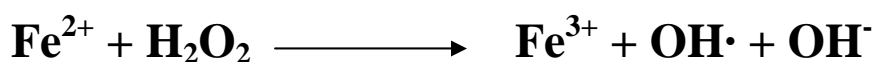
Figure 2 The Fenton reaction. Following DA metabolism, H_2O_2 interacts with free ferrous iron (Fe^{2+}) via the Fenton reaction to form the highly reactive hydroxyl radical ($OH\cdot$).

Oxidation of DA to form the DAQ occurs spontaneously and is catalyzed by the presence of transition metals (Graham et al., 1978; Hastings et al., 1996). The DAQ is electron-deficient and therefore is susceptible to nucleophilic attack. Strong nucleophiles include thiol groups (sulfhydryl group: R—SH) found on GSH, free cysteine, and cysteinyl residues on proteins. The reaction of the DAQ and cysteine leads to the formation of 5-cysteinyl-DA (Fig. 3). Cysteine residues are often found at the active sites of proteins and therefore, modification of cysteines by DAQ could lead to protein inactivation/impairment and subsequent cellular damage. Auto-oxidation of DA can also lead to the formation of neuromelanin, the substance responsible for the pigmentation of dopaminergic neurons in the SN (Fahn and Cohen, 1992; Hastings, 1995; Zecca et al., 2001; Gotz et al., 2004; Zucca et al., 2006).
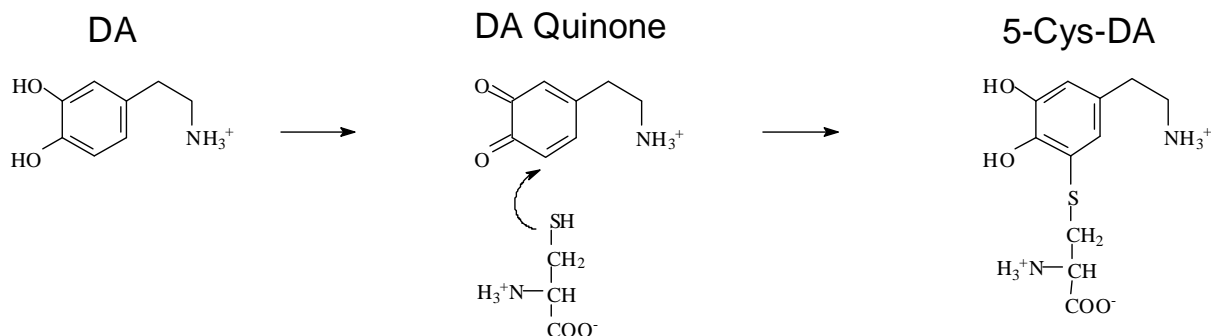
Figure 3: Formation of the DAQ from DA can occur spontaneously and the reaction is catalyzed by the presence of transition metals. The electron-deficient DAQ is susceptible to attack by strong nucleophiles such as free or protein-bound thiols (-SH groups), leading to 5-cysteinyl-DA (5-Cys-DA) conjugation and covalent modification of proteins by DA.

1.3.1.2 Oxidative stress through mitochondrial dysfunction

Oxidative stress can also occur in PD through mitochondrial dysfunction. Mitochondria are major sources of ROS under basal conditions. It is reported that as much as 2% of the oxygen consumed by mitochondria results in superoxide production (Beal, 2003). Superoxide is a free radical that contributes to oxidative stress and cell death by 1) making free iron available to increase hydroxyl radical production, 2) reacting with nitric oxide (NO) to form the highly reactive oxidant, peroxynitrite ($ONOO^-$), and 3) participating in lipid and protein oxidation (Halliwell, 1992, 2001). Within mitochondria are specialized antioxidant defense enzymes to counteract this ongoing ROS production and prevent oxidative stress (Sturtz et al., 2001). When complex I is inhibited, increased production of ROS occurs, leading to oxidative stress (Liu et al., 2002). There is a 30-40% decrease in complex I activity in the SN of PD post-mortem brain tissue and reduced immunoreactivity for complex I subunits (Bindoff et al., 1989; Schapira et al., 1990), suggesting that mitochondrial ROS production via complex I deficiencies is especially high in PD patients. There is also reduced complex I activity in platelets from PD patients which

occurs during early stages of the disease (Haas et al., 1995), revealing systemic complex I inhibition and mitochondrial dysfunction in PD.

1.3.2 Evidence of Oxidative Stress in Parkinson's disease

Oxidative stress is a well-documented pathological feature of PD. Whether oxidative stress is a primary cause or consequence of PD is unknown, but it is likely that oxidative stress contributes to both. Direct evidence of oxidative stress leading to damage of lipids, proteins, and DNA comes from post-mortem brain tissue.

1.3.2.1 Lipid oxidation

Oxidative stress can lead to lipid oxidation and damage which has been found to occur in PD. The reaction of ROS with the double-bond of unsaturated fatty acids leads to lipid peroxidation and peroxyradicals which form toxic byproducts that induce cellular toxicity (Dexter et al., 1986; Pall et al., 1986; Dexter et al., 1989b). When the SN of normal versus PD brain was analyzed for oxidized lipids, there was found to be a 10-fold increase in levels of malondialdehyde and lipid hyperoxides in PD brains (Dexter et al., 1989b; Dexter et al., 1989a). A by-product of lipid oxidation, 4-hydroxynonenal, was present in 58% of the dopaminergic neurons in the SN of PD patients compared to only 9% of dopaminergic neurons in the SN of normal, non-diseased brains (Yoritaka et al., 1996). Finally, in a study that examined peripheral blood markers of oxidative stress in PD patients versus controls, plasma concentrations of markers for lipid peroxidation were significantly increased in PD patients (Younes-Mhenni et al., 2007).

1.3.2.2 DNA oxidation

Oxidative stress can also lead to both nuclear and mitochondrial DNA damage which can lead to mutations and strand breakage, ultimately leading to cell damage and death. There is significant evidence for DNA oxidation in other neurodegenerative diseases such as AD, amyotrophic lateral sclerosis, and Huntington's disease (Browne et al., 1997; Ferrante et al., 1997; Gabbita et al., 1998; Lovell et al., 1999). Oxidized DNA has also been reported in PD, specifically oxidative modification to the base guanine. An increase in oxidized DNA and RNA, examined via immunohistochemical detection for 8-hydroxyguanosine, was observed in dopaminergic neurons in the SN in PD patients compared to normal controls (Alam et al., 1997b; Zhang et al., 1999). Additionally, increased levels of oxidized deoxyguanosine has been identified in the caudate/putamen, SN, and cortex in PD patients compared to non-diseased controls (Halliwell, 2001). An increase in oxidation products of mitochondrial DNA has also been observed (Zhang et al., 1999), providing further evidence for oxidative stress playing a role in PD pathology.

1.3.2.3 Protein oxidation

Oxidative damage to proteins has also been observed in PD. Protein carbonyls, which are derived from the oxidation of amino acid side chains, are used to measure protein oxidation and were found to be increased 2-fold in the SN of PD patients over other brain regions (Floor and Wetzel, 1998). Increases in protein carbonyls have also been found in other brain areas in PD such as the basal ganglia, cortex, globus pallidus, and cerebellum (Alam et al., 1997a). Additionally, specific proteins are the target of oxidative modification in PD. For example, post-mortem cortical tissue from sporadic PD cases revealed oxidative modification of DJ-1 (Choi et al., 2006). DJ-1 is a potential antioxidant for which loss of function has been associated with a

genetic form of PD and increased susceptibility to dopaminergic degeneration (Bonifati et al., 2003; Kim et al., 2005). Oxidative modification of DJ-1 and other proteins could lead to inactivation of the protein, leading to cellular damage.

1.3.3   Antioxidant systems

Decreases in antioxidant enzymes and impaired antioxidant systems have been reported in PD, contributing to the increase in oxidative stress-induced damage. Dopaminergic neurons in the SN appear to be particularly vulnerable to the effects of decreased antioxidant function (Dexter et al., 1994), which may be the result of increases for antioxidant demand by dopaminergic neurons because of the potential for DA-induced oxidant production.

Reduced GSH is an endogenous antioxidant found in high (mM) concentrations in the brain (Hjelle et al., 1994) and in the cytoplasm and mitochondria of dopaminergic neurons (Schulz et al., 2000). One way by which GSH acts to protect dopaminergic cells against oxidative damage is by reducing the DAQ back to DA to inhibit DA-modification of proteins or by binding to the DAQ and thereby directly competing with DAQ binding to cysteinyl residues on proteins (Hastings, 1995). Notably, post-mortem examination of PD brain tissue revealed decreased GSH levels in the SN (Perry et al., 1982; Riederer et al., 1989; Loeffer DA, 1994; Schulz et al., 2000), and another study found a decrease in GSH peroxidase and catalase, both of which normally inactivate H_2O_2 (Kish et al., 1985; Kish et al., 1986). Examination of peripheral blood markers in PD patients revealed a significant increase in oxidized glutathione (Younes-Mhenni et al., 2007), suggesting cellular oxidative stress. There were, however, no differences in blood levels of GSH between PD patients and age-matched controls in this study (Younes-Mhenni et al., 2007). Additionally, increases in the antioxidant superoxide dismutase (SOD) in

nigral tissue (Saggu et al., 1989) and increased activity of blood SOD have been observed in PD patients compared to normal, age-matched controls (Younes-Mhenni et al., 2007). This increase in SOD activity in PD patients may be a compensatory protective response to keep up with the demands of the cell upon increases in superoxide production.

1.3.4   Oxidative stress and the BBB

There are several lines of evidence which indicate that increased oxidative stress is associated with a dysfunctional BBB. Acute hypertension following ischemic/hypoxic insult resulted in increased ROS leading to neuronal damage (Ischiropoulos and Beckman, 2003) and this was also associated with increased BBB permeability (Poulet et al., 2006). BBB permeability was demonstrated *in vitro* following brain endothelial cell exposure to xenobiotics that promote ROS generation. This effect was dose-dependent and blocked when endothelial cells were pre-incubated with the antioxidants SOD and catalase (Lagrange et al., 1999), suggesting that ROS contributed to BBB disruption. Additionally, cultured brain endothelial cells exposed to lipopolysaccharide (LPS), which was shown to increase ROS production, led to dysfunction of the endothelial cells. This dysfunction was also prevented with antioxidant treatment (de Boer and Gaillard, 2006).

Oxidative stress has been shown to increase matrix metalloproteinase-9 (MMP-9) activity (Kim et al., 2003), which is involved in degradation of the basal lamina. ROS-induced MMP-9 activity has been linked to BBB disruption (Gasche et al., 1999; Asahi et al., 2001; Kim et al., 2003) and leads to increased leukocyte infiltration into the brain, increased brain endothelial cell dysfunction, and disruption of tight junction assembly in human stroke patients and *in vitro* (Rosell et al., 2005; Rosell et al., 2006; Haorah et al., 2007). Antioxidant administration to

cultured brain endothelial cells prevented increases in MMP-9 activity and was protective against MMP-9-induced tight junction disassembly (Haorah et al., 2007). Another consequence of oxidative stress is the dysfunction of mitochondria, which can contribute to cellular toxicity (For review, see Beal, 2003). Thus, one of the factors that may render the endothelial cells of the BBB more sensitive to oxidative stress is that they have substantially increased mitochondrial content as compared to endothelial cells that make up the peripheral vasculature (Hawkins & Davis, 2005). Additionally, DA has been shown to result in oxidative stress-induced mitochondrial dysfunction (Berman and Hastings, 1999), which may contribute to BBB permeability.

1.4   NEUROINFLAMMATION

Neuroinflammation can result in altered function of components that comprise the BBB, and is suggested to play a role in the pathogenesis of many neurodegenerative diseases, including PD. Neuroinflammation refers to the inflammatory response by the innate immune cells of the brain which can be both detrimental and beneficial to cells. The main immune effectors of the CNS are glial cells, which function to support neuronal and endothelial cells and contribute homeostasis of the brain environment through active surveillance of the brain parenchyma (Nimmerjahn et al., 2005) and maintenance of communication with the periphery (Streit et al., 1999; Kim et al., 2006; Lok et al., 2007).

1.4.1   Microglia

Under normal conditions, microglia are considered to be the resident immune cells of the brain and are among the first cells to respond to damage of the CNS (Liberto et al., 2004). Upon damage or the invasion of potentially deleterious microorganisms into the brain parenchyma, microglia become activated, upon which their primary function is the phagocytosis of cellular debris (Streit et al., 1999). It should be noted, however, that microglia are active surveyors of the brain environment and phagocytose even during "rest" (Nimmerjahn et al., 2005). The multi-faceted functions of microglia allow the cells to play a role in the maintenance, destruction, and repair of neurons.

1.4.1.1 Activated microglia

Microglia are able to participate in destruction and repair functions upon neuronal injury through morphological and functional transitions or different activation states. While it is unclear exactly how functional states of activated microglia correspond with morphology, there are some general guidelines to the activation states of microglia based on morphological criteria. It is clear that amoeboid-shaped microglia are present at development and likely participate in neuronal death and development during embryonic stages (Streit et al., 1999; Streit et al., 2004). In the adult brain, Streit et al. (1999) has characterized four distinct phases of microglial activation based on morphology. 1) Resting microglia have small cell bodies with thin, ramified processes. Upon an activation stimulus, microglia can become 2) reactive, with an enlarged cell body and a thickening of the ramified processes. However, microglia can also transform morphologically into 3) an intermediate phase, whereby they become hyper-ramified while cell-body size remains similar to that of resting microglia. Following the reactive stage, microglia can become 4)

phagocytic, which is characterized by a retraction of the processes and swelling of the cell body. In this stage, microglia take on the appearance of other brain and body macrophages (See Appendix A).

There are a number of events that can lead to the activation of microglia *in vivo*. As part of the inflammatory response, microglia can be activated by immunological challenges, acute or chronic CNS trauma, and hemorrhagic or ischemic stroke (Imamura et al., 2003). Degenerating neurons, themselves, may also signal and trigger reactive gliosis at the site of degeneration (Wilhelmsson et al., 2004). The DAQ can lead to changes in expression levels of several genes, many of which are involved in the inflammatory cascade, and can lead to direct activation of microglia *in vitro* (Kuhn et al., 2006). Finally, alterations in the integrity of the BBB can lead to an immediate microglial response including migration to the affected area, cell proliferation, and morphological activation (Nimmerjahn et al., 2005).

1.4.1.2 Microglial activation and oxidative stress

Oxidative stress, implicated in PD pathogenesis and disruption of the BBB, leads to cell damage and can also incite an inflammatory response (Raivich et al., 1999) via activation of microglia. Methamphetamine-induced DAQ formation induces a pro-inflammatory gene profile and directly induces microglial activation (LaVoie et al., 2004; Thomas et al., 2004; Kuhn et al., 2006), providing evidence for a mechanism by which ROS may lead to microglial activation.

In turn, activated microglia can also release free radicals, leading to increased ROS production and thus perpetuating or increasing oxidative stress. A main pathway through which microglia contribute to oxidative stress is via the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The microglial NADPH oxidase is comprised of cytosolic subunits (p47^{phox}, p67^{phox}, p40^{phox}, and the G-protein Rac) and a membrane protein complex known as

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cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$). Upon microglial activation from pro-inflammatory stimuli, signaling events occur, which promote phosphorylation of $p47^{phox}$ and $p67^{phox}$ and subsequent binding of the cytosolic components. The cytosolic complex then translocates to the membrane where $p47^{phox}$ binds to $gp91^{phox}$, initiating an electron flow and catalyzing the generation of superoxide to be released from the cell (See Appendix B). Stimulation of microglial cells in mice via LPS administration revealed that NADPH oxidase activation is a main mechanism by which microglial activation leads to neurotoxicity (Qin et al., 2004). Further, inhibition of NADPH oxidase activation by apocynin, a methoxy-substituted catechol that inhibits NADPH oxidase activation, protects neurons from a variety of ROS-generating insults (Gao et al., 2003a; Gao et al., 2003b; Choi et al., 2005; Wang et al., 2006; Anantharam et al., 2007; Lo et al., 2007; Rodriguez-Pallares et al., 2007), providing additional evidence that NADPH oxidase-induced free radical production is a significant pathway by which activated microglia contribute to oxidative stress and cellular toxicity.

1.4.2 Neuroinflammation and PD

Neuroinflammatory processes may either play a role in or be a consequence of the pathogenesis of PD. Glial factors have been observed in the brains of PD patients both *in vivo* through PET imaging and post-mortem in brain tissue. There are also immunomodulatory factors that have been identified in PD patients and post-mortem in brain tissue. Together, these factors provide evidence for neuroinflammation during PD.

Microgliosis is found consistently in the brains of PD patients while astrogliosis is observed in PD brains sporadically (Depino et al., 2003). It is unclear whether microglial

activation is a cause or consequence of PD pathogenesis, however microglial activation has been implicated in dopaminergic cell death because the appearance of activated microglia precedes dopaminergic neuronal loss following medial forebrain bundle axotomy in rats (Sugama et al, 2002), indicating that microglia may contribute to the initiation of dopaminergic neurodegeneration. Additionally, activated microglia are found in the immediate proximity to degenerating DA neurons in PD patients (McGeer et al., 1988). Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine secreted by activated microglia. Treatment of cells with TNF-α leads to a decrease in GSH levels (Hayter et al., 2001), and it is therefore noteworthy that GSH levels are decreased while TNF-α levels are increased in post-mortem PD brains.

Even under basal conditions, the SN is particularly enriched with resident microglia (Kim et al., 2000). In the SN of PD patients, there are increased numbers of glial cells that express inducible nitric oxide synthase (iNOS) which mediates the synthesis of NO (Hirsch et al., 2005). High levels of NO can disrupt iron homeostasis by upregulating ferritin expression, which can result in increased iron concentration. Correspondingly, increased iron levels have been observed in postmortem PD patients (Sofic et al., 1988; Dexter et al., 1989a; Gotz et al., 2004). Some of the highest levels of brain iron under normal conditions are bound to ferritin, a protein which stores iron and is expressed predominantly in glia. Increased numbers of ferritin-immunoreactive microglial cells were observed in neurons of the SN of PD patients (Jellinger et al., 1990). The ferritin stores in the SN of PD patients were also found to be denser and contain more iron than those of healthy subjects (Gotz et al., 2004). Increasing iron levels may allow more free iron to be available for participation in the Fenton reaction, thus allowing the generation of the highly toxic hydroxyl radical (Hunot and Hirsch, 2003) and further contribute to the demise of DA neurons due to oxidative damage.

Microglial activation has also been observed in animal models following the administration of dopaminergic neurotoxins such as intrastriatal 6-hydroxydopamine (6-OHDA) (Depino et al., 2003) or LPS (Tomas-Camardiel et al., 2004) in rat, systemic 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) administration in mice (Delgado and Ganea, 2003; Thomas et al., 2004), treatment of mice with methamphetamine (METH) (LaVoie et al., 2004; Thomas et al., 2004), and systemic administration of the organic pesticide rotenone (Gao et al., 2003c; Sherer et al., 2003b). In many of these models microglial activation precedes dopaminergic cell death, providing support for the notion that neuroinflammation contributes to dopaminergic neuronal pathogenesis. Models for PD have also identified activated microglial-induced NADPH oxidase activation to be damaging to dopaminergic neurons both *in vitro* (Gao et al., 2003a; Gao et al., 2003b; Anantharam et al., 2007; Zhang et al., 2007) and *in vivo* (Wu et al., 2002; Wu et al., 2003).

1.4.3   Neuroinflammation and the BBB

Immune privilege of the CNS is severely undermined once inflammation occurs due to breakdown of BBB integrity (Galea et al., 2007). Small breaks in the BBB from irradiation initiate a microglial response almost immediately (Nimmerjahn et al., 2005), indicating that disruption of the BBB itself elicits an inflammatory reaction and an immune response in the brain (Galea et al., 2007). However an inflammatory response from within the CNS can also alter BBB integrity. For example, chronic production of the proinflammatory cytokine interleukin-1, which is increased in the CSF and brain from PD patients, is associated with BBB dysfunction (Ferrari et al., 2004). Additionally, TNF-α has been shown to alter functions of BBB components, rendering the BBB permeable (Huber et al., 2001), and there is a positive

correlation between CSF levels of TNF-α and observable BBB permeability in MS patients (Dopp et al., 1997). Exposure of cultured brain endothelial cells to TNF-α also resulted in decreased P-gp activity (Hartz et al., 2006), which corresponds with the observation that chronic brain inflammation reduced P-gp activity in brain capillary endothelial cells (Bauer et al., 2005; Hartz et al., 2006), thus reducing efflux of potentially harmful toxins from the CNS into the blood stream. Endothelial cell tight junction properties are also altered under pro-inflammatory conditions (Lossinsky and Shivers, 2004), affecting CNS homeostasis which can contribute to cellular toxicity (Huber et al., 2001; Lossinsky and Shivers, 2004; Krizanac-Bengez et al., 2006; Quan, 2006).

Intracranial administration of LPS, a bacterial endotoxin that is known to promote microglial activation and a pro-inflammatory response, leads to disruption of the BBB (Dyatlov et al., 1998; Tomas-Camardiel et al., 2004; Ahishali et al., 2005). LPS-induced BBB disruption in rats was prevented by systemic treatment with the anti-inflammatory agent, minocycline (Tomas-Camardiel et al., 2004), providing additional evidence for the role of neuroinflammation in disruption of the BBB. LPS also leads to oxidative stress through the production of superoxide following activated microglial-induced NADPH oxidase activation (Qin et al., 2004). Treatment with apocynin, an NADPH oxidase inhibitor, is protective against BBB disruption following traumatic brain injury in rat (Lo et al., 2007; Titova et al., 2007) and in animal models for stroke (Wang et al., 2006; Xian et al., 2007), suggesting that NADPH oxidase activation contributes to BBB disruption.

1.5   MODELS OF DOPAMINERGIC NEUROTOXICITY

1.5.1   Dopamine

One of the factors that may make dopaminergic neurons vulnerable to degeneration in PD may be DA itself. The addition of DA and its metabolites is toxic to dopaminergic neurons *in vitro* (Graham et al., 1978; Michel and Hefti, 1990). Additionally, an intrastriatal injection of DA in rats is dose-dependently toxic to dopaminergic terminals, providing further evidence for DA as a dopaminergic neurotoxin (Filloux and Townsend, 1993; Hastings et al., 1996). Several reports from the Hastings lab have characterized the selective loss of dopaminergic neurons following an intrastriatal DA injection into rats, and the lab currently utilizes exogenous DA as a model for dopaminergic neurodegeneration.

1.5.1.1 How DA acts as a neurotoxin

Dopamine acts as a neurotoxin by contributing to cellular oxidative stress via two primary mechanisms. The first mechanism is via the production of ROS during normal metabolism of DA. In the extracellular or cytoplasmic space, DA is metabolized by MAO into DOPAC. A byproduct of this reaction is H_2O_2, (Fig. 1), which on its own is not highly toxic but can lead to the formation of the highly reactive ·OH in the presence of iron (Fig. 2), if H_2O_2 is not reduced adequately by antioxidant systems (Halliwell, 1992).

A second, well-documented means by which DA can contribute to cellular toxicity is via oxidation of the catechol ring to form the reactive DAQ (Graham et al., 1978). This oxidation can occur either spontaneously or in the presence of transition metals, peroxynitrite (LaVoie and Hastings, 1999a), or enzymes such as cyclooxygenase (Hastings, 1995). The DAQ is electron-

deficient and readily reacts with cellular nucleophiles such as reduced sulfhydryl groups found on free cysteine, GSH, or protein cysteinyl residues (Graham et al., 1978; Hastings and Zigmond, 1994). Since cysteines are often found at the active sites of proteins, then covalent modification of such sites by the DAQ can lead to inactivation of the proteins, which can result in cellular toxicity.

1.5.1.2 Evidence for DA as a dopaminergic neurotoxin

There are several lines of evidence that DA is a dopaminergic neurotoxin *in vivo*. When injected intrastriatally, DA leads to a loss of dopaminergic terminals (Filloux and Townsend, 1993; Hastings et al., 1996) and degeneration occurs by 24h following injection (Rabinovic et al., 2000). This loss is dose-dependent and is prevented upon co-injection with the antioxidants GSH and ascorbate (Hastings et al., 1996). Additionally, an intrastriatal DA injection led to decreases in striatal GSH and treatment with L-buthionine sulfoximine, and inhibitor of GSH synthesis, potentiated DA-induced protein cysteinyl-catechol formation (Rabinovic and Hastings, 1998), providing evidence for intrastriatal DA contributing to oxidative stress. The loss of dopaminergic terminals in striatum is also associated with increasing production of free and protein-bound cysteinyl-catechol conjugates (Hastings et al., 1996), suggesting that DA oxidation is responsible for the neurodegenerative effect of an intrastriatal DA injection. Additionally, an intrastriatal DA injection is associated with gliosis at the injection site (Filloux and Townsend, 1993; Hastings et al., 1996), which has been suggested to contribute to neurotoxicity.

Striatal toxicity as a result of an intrastriatal DA injection is selective for dopaminergic terminals. Evidence indicates that a DA injection does not lead to loss of serotinergic neurons from the striatum (Hastings et al., 1996) or a loss microtubule-associated protein 2-positive

neurons in striatum at the sites of specific loss of TH-positive terminals (Rabinovic et al., 2000). Additionally, the lesion produced by an intrastriatal DA injection is not due to a downregulation of TH, but rather to degeneration of the dopaminergic terminals (Rabinovic et al., 2000).

1.5.2   6-OHDA

The neurotoxin 6-OHDA is commonly used to model PD both *in vitro* and *in vivo*. Unilateral, intracranial injections into the striatum, SN, and medial forebrain bundle (MFB) are often used to cause selective toxicity to catecholaminergic neurons. 6-OHDA is a substrate for the DAT and the norepinephrine transporter. Therefore, to increase selectivity for dopaminergic neurons, norepinephrine uptake inhibitors may be used in conjunction with 6-OHDA treatment (Kandel, 2000).

1.5.2.1 How 6-OHDA acts as a neurotoxin

Similar to DA, 6-OHDA acts as a neurotoxin by oxidizing to the *para*-quinone and covalently binding to nucleophiles, such as cysteinyl proteins, leading to protein inactivation and cellular toxicity (Saner and Thoenen, 1971). Oxidation of 6-OHDA is showed in Fig. 4 and is reviewed in Przedborski and Ischiropoulos, 2005 (Przedborski and Ischiropoulos, 2005). Oxidation of 6-OHDA requires oxygen, as 6-OHDA will not oxidize under anaerobic conditions (Gee and Davison, 1984), and trace amounts of transition metals (Gee and Davison, 1989; Gee et al., 1992). Also similarly to DA, oxidation of 6-OHDA leads to the formation of ROS such as H_2O_2 which can lead to increased production of $\cdot OH$, thus increasing neurotoxicity. Correspondingly, co-treatment with 6-OHDA and SOD, a free radical antioxidant, protects against 6-OHDA-induced neurotoxicity (Heikkila and Cohen, 1973; Sullivan and Stern, 1981).
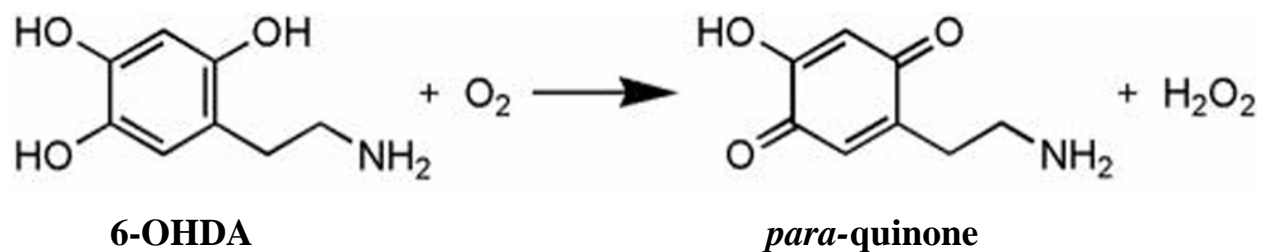
6-OHDA　　　　　　　　　　　　　　*para*-quinone

Figure 4: Oxidation of 6-OHDA. The neurotoxin 6-OHDA oxidizes to form the *para*-quinone and ROS such as H_2O_2, leading to increases in cellular oxidative stress and toxicity.

1.5.2.2 Evidence for 6-OHDA as a dopaminergic neurotoxin

Injection of 6-OHDA into the SN or medial forebrain bundle of rat leads to degeneration of dopaminergic neurons within 24h (Jeon et al., 1995). Intrastriatal injection of 6-OHDA leads to a loss of striatal TH-ir and also retrograde degeneration of the nigrostriatal projection, evidenced by a loss of TH-ir cell bodies in the SN 1-3w following 6-OHDA (Sauer and Oertel, 1994; Przedborski et al., 1995). Injections of 6-OHDA into rat are also associated with gliosis, in particular microglial activation (Stromberg et al., 1986; Rodrigues et al., 2001). Evidence suggests that microglial activation contributes to 6-OHDA-induced neurotoxicity because treatment of mice with minocycline, an inhibitor of microglial activation, was protective against 6-OHDA-induced dopaminergic neurodegeneration (He et al., 2001). Since 6-OHDA is a commonly utilized model that leads to oxidative stress and neuroinflammation, we chose to explore the effects of this dopaminergic neurotoxin on the BBB. A previous study showed that MFB injections of 6-OHDA into rat led to disruption of the BBB in the SN and striatum several days following 6-OHDA treatment (Carvey et al., 2005).

1.5.3   Rotenone

Rotenone is a naturally occurring substance used to rid waters of nuisance fish and as an organic pesticide/insecticide (Fang and Casida, 1999). Rotenone exerts its pesticide function by inhibiting complex I of the mitochondrial electron transport chain, which can lead to cellular toxicity. It is possible that chronic rotenone exposure could increase susceptibility for the development of PD since systemic complex I inhibition has been observed in PD patients (Parker et al., 1989; Haas et al., 1995), and pesticide exposure is considered to be an environmental risk factor for the disease (Fall et al., 1999; Lai et al., 2002; Liu et al., 2003). Moreover, when administered systemically in rats, several characteristic pathological features of PD are present such as behavioral and postural abnormalities, selective dopaminergic degeneration, robust microglial activation, the formation of cytoplasmic inclusion bodies in the SN, and altered proteasomal activity in TH-positive neurons (Betarbet et al., 2000; Sherer et al., 2003b; Sherer et al., 2003c; Sherer et al., 2003a; Betarbet et al., 2006).

1.5.3.1 How rotenone acts as a neurotoxin

As mentioned previously, rotenone inhibits complex I of the mitochondrial electron transport chain. Since the compound is lipophilic and can readily cross membranes, it is likely that rotenone inhibits complex I in all cells. Inhibition of complex I leads to the increased production of ROS (Pitkanen and Robinson, 1996; Cassarino et al., 1997; Votyakova and Reynolds, 2001; Liu et al., 2002), which can contribute to mitochondrial dysfunction and increase cellular oxidative stress. As evidence for rotenone-induced oxidative stress, elevated protein carbonyl levels were observed following rotenone treatment in rat in the affected brain regions (Sherer et al., 2003c). Additionally, depletion of cellular GSH increased the cytotoxic

effects of rotenone *in vitro* (Sherer et al., 2003c). Moreover, treatment with the antioxidant α-tocopherol attenuated the neurotoxic effects of rotenone *in vitro* (Sherer et al., 2003c). Finally, it was observed that rotenone oxidatively modified DJ-1 and resulted in the upregulation and accumulation of α-synuclein (Betarbet et al., 2006), two proteins that are found to be altered in some genetic forms of PD. However, antioxidant treatment prevented alterations in DJ-1 modification and alterations in α-synuclein expression (Betarbet et al., 2006), providing further evidence that rotenone-induced oxidative stress leads to neurotoxicity.

1.5.3.2 Evidence for rotenone as a dopaminergic neurotoxin

There are several complex I inhibitors that are toxic to dopaminergic neurons, however rotenone leads to selective dopaminergic neurodegeneration despite inhibiting complex I in all cells of the body (Betarbet et al., 2000; Giasson and Lee, 2000). When administered systemically (i.v. or s.c.) in Lewis rats, rotenone leads to a progressive, focal loss of TH-positive terminals in the striatum and neurons in the SN. Degeneration of the nigrostriatal projection may occur in a retrograde fashion since it is not until severe depletion of TH-positive terminals that a loss of TH-positive neurons in the SN pars compacta is observed (Betarbet et al., 2000). The neurodegeneration that occurs is highly selective for dopaminergic regions that degenerate in PD, as dopaminergic neurons from the ventral tegmental area, as well as glutamic acid decarboxylase-positive and acetylcholine esterase-positive neurons of the striatum are spared (Betarbet et al., 2000).

Since rotenone is administered systemically and affects all cells, a reason for the selective degenerative effect on dopaminergic neurons is unknown. It is speculated that dopaminergic neurons are more susceptible to oxidative stress-induced damage because of the excessive ROS production and oxidative modification that results from normal dopamine metabolism and

oxidation (Graham et al., 1978; Hastings et al., 1996). Indeed, rotenone increases protein cysteinyl catechol levels in a dopaminergic *in vitro* cell system (Dukes et al., 2005), suggesting that rotenone may potentiate cellular oxidative stress, making dopaminergic neurons especially vulnerable to degeneration.

1.6   AIMS OF DISSERTATION

The BBB is an interface between the blood stream and the CNS that is highly specialized to allow influx of essential nutrients and molecules while disallowing the invasion of circulating microorganisms and environmental toxins that may pose a threat to homeostasis of the CNS. There is evidence in PD and several other neurodegenerative diseases for a dysfunctional BBB, which may contribute to or be a result of disease pathogenesis. Additionally, there is evidence for BBB disruption in animal models that involve dopaminergic degeneration. Examination of the BBB is important because a dysfunctional BBB may contribute to the pathogenesis of neurological diseases by allowing peripheral blood factors that otherwise would not have brain access to infiltrate the CNS. Additionally, characterization of BBB integrity in PD and animal models may have implications for therapeutics. There is substantial evidence that oxidative stress and neuroinflammation, two factors thought to contribute to PD pathogenesis, are harmful to components that comprise the BBB and therefore may lead to a dysfunctional BBB. The main goal of the following experiments is to determine mechanisms by which dopaminergic neurotoxins lead to the disruption of the BBB. The roles of both oxidative stress and neuroinflammation in BBB disruption will be examined in models of dopaminergic neurotoxicity.

Hypothesis: *1) The dopaminergic neurotoxins DA, 6-OHDA, and rotenone result in BBB disruption, 2.) a mechanism by which dopaminergic neurotoxins contribute to BBB disruption is via oxidative stress, 3) a mechanism by which dopaminergic neurotoxins contribute to BBB disruption is via neuroinflammation.*

1.6.1 To examine the integrity of the blood-brain barrier in response to dopaminergic neurotoxins.

Previous data from our lab suggested that an intrastriatal DA injection may lead to disruption of the BBB. Additionally, previous studies showed that an intrastriatal injection of DA resulted in a specific loss of DA terminals as well as increases in oxidation products such as ROS and the DAQ which contribute to a state of oxidative stress leading to cellular toxicity. It has also been well established that 6-OHDA contributes to oxidative stress and inflammation, both of which can be harmful to components that comprise the BBB. Carvey, et al. reported in 2005 that striatal and MFB injections of 6-OHDA result in disruption of the BBB. Finally, when given systemically, rotenone results in systemic complex I inhibition, neuroinflammation, and oxidative stress-mediated dopaminergic neuronal toxicity. Endothelial cells that comprise the BBB are susceptible to oxidative stress-and neuroinflammation-induced damage, therefore, the effects of DA, 6-OHDA, and rotenone, known dopaminergic neurotoxins, on integrity of the BBB were examined.

1.6.2   To examine the role of oxidative stress in dopaminergic neurotoxin-induced blood-brain barrier disruption.

A primary mechanism by which the dopaminergic neurotoxins are thought to elicit their neurotoxicity is via oxidative stress. There is also substantial evidence that components of the BBB are vulnerable to oxidative stress-induced damage. Therefore, the effects of dopaminergic neurotoxin-induced oxidative stress on BBB integrity were examined. The effects of an intrastriatal injection of DOPAC, an inducer of extracellular oxidative stress that does not lead to dopaminergic neuronal toxicity, on the BBB were examined. This was to elucidate whether dopaminergic neurotoxin-induced BBB disruption was due to dopaminergic degeneration or oxidative stress. The effects of an antioxidant, N-acetylcysteine, on DA-induced BBB disruption were also examined to determine the role of oxidative stress.

1.6.3   To examine the role of neuroinflammation in dopaminergic neurotoxin-induced blood-brain barrier disruption.

Another mechanism by which dopaminergic neurotoxins have been proposed to lead to toxicity is via neuroinflammation. There is also substantial evidence for neuroinflammation leading to a dysfunctional BBB. Therefore, the effects of DA-induced microglial activation on integrity of the BBB were evaluated. To examine the role of microglial activation in DA-induced BBB disruption, animals were treated with an anti-inflammatory agent and known inhibitor of microglial activation, minocycline, following an intrastriatal DA injection, to determine if DA-induced microglial activation contributed to disruption of the BBB. A role for DA-induced

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microglial activation was further elucidated by inhibiting NADPH oxidase activation by treating intrastriatal DA-injected animals with apocynin, a known NADPH oxidase inhibitor. This was to determine a mechanism by which DA-induced microglial activation contributed to BBB disruption.

2.0   DOPAMINERGIC NEUROTOXIN-INDUCED BLOOD-BRAIN BARRIER

DISRUPTION

2.1   INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized in part by a loss of dopaminergic neurons in the substantia nigra (SN) and degeneration of the nigrostriatal projection. Most cases of PD are sporadic, with no known etiology. There is, however, substantial evidence suggesting that oxidative stress plays a key role in degeneration of the nigrostriatal pathway in PD (Schulz et al., 2000; Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Greenamyre and Hastings, 2004). The SN is known to have inherently less of the antioxidant glutathione (GSH) and is therefore more vulnerable to oxidative stress (Loeffler et al., 1994). Other indicators of oxidative stress, including increased iron levels (Gotz et al., 2004) and lipid peroxidation products (Olanow, 1990; Fahn and Cohen, 1992), have been reported in the SN of PD patients and may contribute to dopaminergic degeneration.

Dopaminergic neurons may become vulnerable to oxidative stress because they contain the neurotransmitter dopamine (DA), and DA-dependent oxidative stress may contribute to the degeneration of dopaminergic neurons in PD. DA is able to oxidize to form reactive oxygen species (ROS) and the DA quinone (DAQ) which leads to oxidative stress and cellular damage (Graham et al., 1978; Hastings et al., 1996). Production of ROS occurs during DA metabolism

by monoamine oxidase (MAO) into dihydroxyphenylacetic acid (DOPAC). A consequence of this reaction is the formation of hydrogen peroxide (H_2O_2), an ROS which is less reactive on its own, but can lead to the formation of highly damaging hydroxyl radicals through interaction with iron via the Fenton reaction (Halliwell, 1992). Oxidation of the DA catechol ring to DAQ can occur spontaneously and is catalyzed by the presence of transition metals (Graham et al., 1978; Przedborski and Ischiropoulos, 2005), peroxynitrite (LaVoie and Hastings, 1999a), and enzymes such as cyclooxygenase (Hastings, 1995). The DAQ is electron-deficient and is therefore susceptible to nucleophilic attack. Strong nucleophiles include thiol groups found on GSH, free cysteine, and cysteinyl residues on proteins. Cysteines are often found at the active sites of proteins and therefore, modification of proteins by DAQ could lead to protein inactivation/impairment and subsequent cellular damage. An intrastriatal injection of DA into rat results in the loss of tyrosine hydroxylase (TH)-positive dopaminergic terminals of the striatum (Hastings et al., 1996; Rabinovic et al., 2000). The DA-induced lesion is dose-dependent and correlates with increases in free and protein cysteinyl catechols (Hastings et al., 1996; Rabinovic et al., 2000). Further, loss of dopaminergic terminals is prevented by co-injection with antioxidants (Hastings et al., 1996), providing evidence that oxidative stress is a mechanism by which DA acts as a neurotoxin.

In addition to DA, activated microglia also contribute to oxidative stress in the brain through the production of cytotoxic ROS. The SN is particularly enriched with resident resting microglia under normal conditions (Lawson et al., 1990; Kim et al., 2000), and in the SN of PD patients, activated microglia are found in immediate proximity to degenerating dopaminergic neurons (McGeer et al., 1988). Activated microglia are part of the neuroinflammatory response which, in addition to the production of ROS, can release cytotoxic compounds such as nitric

oxide (NO), prostaglandins, and pro-inflammatory cytokines (Streit et al., 1999). In particular, the presence of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) has been identified in the brain and cerebrospinal fluid of PD patients (Mogi et al., 1994; Mogi et al., 2000), suggestive of a role for neuroinflammation in PD pathogenesis.

Another dopaminergic neurotoxin, rotenone, an organic pesticide that causes mitochondrial complex I inhibition, also leads to selective degeneration of nigrostriatal dopaminergic neurons (Betarbet et al., 2000) and recapitulates several other features of PD when systemically administered to rats (Betarbet et al., 2000; Greenamyre et al., 2003; Sherer et al., 2003b; Sherer et al., 2003a; Testa et al., 2005). Similar to intrastriatal DA, a likely contributor to rotenone-induced neurodegeneration is ROS, which are formed during mitochondrial respiration (Sherer et al., 2003c). Formation of ROS is enhanced upon inhibition of complex I activity (Cassarino et al., 1997). Chronic rotenone exposure leads to increases in oxidized proteins both *in vivo* and *in vitro* and to neuronal toxicity. This is potentiated *in vitro* upon GSH depletion, and attenuated by inclusion of the antioxidant α-tocopherol (Sherer et al., 2003c), supporting a role for oxidative stress in rotenone-induced neurotoxicity. Additionally, systemic administration of rotenone in rats leads to microglial activation (Sherer et al., 2003b), which may also contribute to rotenone-induced oxidative stress.

While oxidative stress and neuroinflammation are two factors that contribute to neurodegeneration, they are also likely to be harmful to components of the blood-brain barrier (BBB), the physical and chemical interface restricting influx of harmful toxins and microorganisms from the blood stream into the brain (Ribatti et al., 2006; Bechmann et al., 2007). Tight junction proteins and endothelial cells that help maintain the integrity of the BBB are functionally and physically altered under conditions of oxidative stress (Lagrange et al.,

1999; Krizbai et al., 2005), which is prevented by antioxidant treatment (Agarwal and Shukla, 1999; Lagrange et al., 1999; Schulz et al., 2000; Dong et al., 2007). In addition, components of the BBB are susceptible to neuroinflammation-induced damage via ROS, NO, and proinflammatory cytokine production (Banks, 1999; Hawkins and Davis, 2005; de Boer and Gaillard, 2006), supporting a role for both oxidative stress and neuroinflammation in the breakdown of the BBB. Therefore, features of the DA-injection and rotenone models, such as increases in oxidative stress and neuroinflammation, suggest that these models may affect BBB integrity. This has been shown recently in other models involving dopaminergic neurodegeneration and may also be a feature of these toxin models (Carvey et al., 2005; Westin et al., 2006; Zhao et al., 2007).

The present study sought to examine whether dysfunction of the BBB occurred in animal models for dopaminergic degeneration. Integrity of the BBB was examined following an intrastriatal DA injection and in this paradigm we observed that DA leads to disruption of the BBB. The observed disruption of the BBB is likely due to neurotoxic properties of DA and is associated with microglial activation both spatially and temporally. BBB integrity was also examined following systemic rotenone treatment and extravasation of serum markers into the brain was observed, indicating a compromise of the BBB in this model as well. These findings may have implications for further elucidating factors involved in disease pathogenesis and for examining current and developing new therapeutics for PD.

2.2   METHODS AND MATERIALS

Animals.

Male Sprague-Dawley or Lewis rats (300g; Hilltop Laboratories, Allison Park, PA) were used throughout the current sets of experiments. The rats were kept in standard caging in a temperature- and light-controlled room that was on a 12h light/dark cycle. Food and water were available *ad libitum*. All experimental protocols were in accordance with the guidelines developed by the NIH Guide to the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

DA and 6-OHDA intrastriatal injections and tissue collection.

Prior to all surgical procedures, male Sprague-Dawley rats were anesthetized with Equithesin (3.0ml/kg i.p.). A 26.5 gauge injection cannula was placed stereotaxically into the right striatum (Coordinates from Bregma: -2.7 mm medial/lateral and +0.8 mm anterior/posterior. Coordinates from dura: -5.0 mm dorsal ventral). Dopamine (0.4 μmol/2μl sterilized water), 6-hydroxydopamine (6-OHDA; 10μg/2μl 0.02% ascorbate), or saline (2μl) were unilaterally injected into the right striatum over 20 min via a microfusion injection pump (model 341B; Sage Instruments, Boston, MA). All injected solutions were prepared immediately prior to surgery and kept on ice with minimal light exposure.

At the indicated time points following surgery, animals were deeply anesthetized and brain tissue was collected for immunohistochemical analysis. Rats were transcardially perfused

with ice cold 1% paraformaldehyde followed by 4% paraformaldehyde, in phosphate buffer, at a steady flow rate. Whole brains were rapidly removed and placed in 4% paraformaldehyde for 24h and then in 30% sucrose for 48h at 4°C. Fixed rat brains were sliced coronally (40µm) on a cryostat at -20°C. Tissue sections were placed serially into a 24-well plate with anti-freezing solution and stored at -20°C until further use. Animals sacrificed for biochemical analyses were perfused with saline at 4°C. Brains were rapidly removed and striatal tissue surrounding the injection site as well as tissue from the contralateral, non-injected striatum were dissected using a 3mm biopsy punch determined by an area 2mm rostral to and 1mm caudal from the optic chiasm (10-15 mg tissue). Tissue was then weighed, snap-frozen on dry ice, and stored at -80°C until analysis.

Rotenone administration and tissue collection.

Male Lewis rats were fully anesthetized with isoflurane and an osmotic minipump (Alzet Corporation, Palo Alto, CA; model 2ML4) was inserted subcutaneously below the scapulae. Rats (n=32) received 3mg/kg/day rotenone in vehicle, DMSO/PEG (1:1), for up to 4 weeks. Control rats (n=8) received vehicle for 28d. Some rotenone-treated rats (25%) displayed significant weight loss, stopped grooming and feeding and were subsequently sacrificed for tissue collection prior to 28d.

Following rotenone treatment rats were anesthetized and perfused with ice-cold saline. Because the rotenone-induced lesion is symmetrical, brains were bisected midsagitally into two hemispheres. The right hemisphere was collected for biochemical analysis, snap frozen on dry ice, and stored at -80°C until future use. The left hemisphere was post-fixed in 4% paraformaldehyde for 7d and then placed in 30% sucrose for 48h at 4°C. Fixed brains were

sectioned coronally (30µm) using a microtome. Tissue sections were collected serially and stored at -20°C in a cryoprotectant solution until use.

Evans blue injections.

At the indicated time points following an intrastriatal injection of DA or saline, and 4 h prior to sacrifice, animals were injected, i.v., with a 2% solution of Evans blue dye (2ml/kg) in saline. At 4h following Evans blue injection, animals were anesthetized and transcardially perfused with 1% paraformaldehyde, followed by 4% paraformaldehyde, and the brains were removed, processed, and sectioned as described. Tissue sections used to visualize Evans blue extravasation were mounted directly onto slides following slicing, and cover-slipped with Fluoromount-G (Southern Biotechnology Associates, Inc. Birmingham, AL), and protected from light at 4°C until examined under a fluorescence microscope (Olympus BX-51).

Immunohistochemistry.

Mouse anti-CD11b (OX-42; 1:1000; Serotec, Raleigh, NC) specific for rat microglia, rabbit anti-rat IgG (1:1000; Vector Laboratories, Burlingame, CA) specific for rat IgG, mouse anti-rat tyrosine hydroxylase (TH; 1:2000; Chemicon International, Temecula, CA) with specificity for rat TH, and mouse anti-rat endothelial brain antigen (EBA; SMI71; 1:1000; Sternberger Monoclonals, Berkeley, CA) specific for endothelial cells of the BBB, were used as primary antibodies for immunohistochemical detection. Immunohistochemical processing was standardized by processing tissue sections from each group of animals at the same time to reduce variability of staining within each group of animals.

Free-floating striatal sections were immersed into 1% H_2O_2 for 15min to remove endogenous peroxidase, followed by incubation with normal serum to reduce non-specific binding. Tissue sections were then incubated in primary antibody for 24h or 48h at 4°C, followed by incubation with the appropriate biotinylated secondary antibody and processing by the avidin-biotin (ABC) method using the Vectastain kit (Vector Laboratories, Burlingame, CA). Processed sections were visualized with diaminobenzidine, mounted onto slides, and coverslipped. Striatal tissue sections were examined using a bright field microscope (BX-51, or stereoscope (SX-61, Olympus America, Inc. Center Valley, PA).

Optical density measurements.

Optical density measurements of tissue sections processed for immunohistochemistry were obtained by outlining the entire striatum using a computer analysis system (MCID M5 5.1, Imaging Research, Inc.) to quantify immunoreactivity of TH, IgG, or OX-42 from a tissue section within each animal (n). To quantify the extent of dopaminergic terminal loss following an intrastriatal injection of DA, 6-OHDA, or saline TH-ir was quantified by measuring the adjusted optical density at the site of injection. Adjusted optical density was calculated using the following equation:

Adjusted Optical Density = log [density of background / density of striatum]

The section containing the injection site was viewed on a microscope and displayed on a monitor linked to a computer equipped with an MCID M5 image analysis system (Imaging Research Inc., Ontario, Canada). The background was obtained by measuring the optical density

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in corpus callosum, an area devoid of TH labeling. The density of TH-ir in striatum was obtained

by outlining the entire striatum and measuring the optical density of the delineated area. Optical

density measurements of IgG-ir and OX-42-ir were obtained in a similar manner. All density

measurements were calibrated to an arbitrary scale of 0-255 with 0 being black and 255 being

white. Measurements were then expressed as a percent of control based on comparison of optical

density readings from the injected striatum to the contralateral, non-injected striatum within the

same tissue section and animal. A Student's *t*-test was used to determine significance at p<0.05.

Western blot analysis.

Two days following intrastriatal DA, animals were deeply anesthetized and perfused

transcardially with ice-cold saline, brains were immediately removed, and 3mm striatal punches

were removed from both the injected and non-injected striata, as previously described

(Rabinovic et al., 2000). Striatal samples were homogenized in lysis buffer (9M urea, 2% w/v

CHAPS, 10mM Tris-base, and protease inhibitor cocktail [2.5µl/mg protein], pH 8.5), kept on

ice for 20 min, centrifuged, and protein amounts in the supernatant were assayed (Bradford,

1976). Samples (50µg protein) were loaded onto a 12% SDS-polyacrylamide gel and

electrophoresed, followed by transfer onto a nitrocellulose membrane. The blot was then

incubated with rabbit anti-rat IgG antibody (1:2000; Vector Laboratories, Burlingame, CA)

overnight at 4°C, and incubated for 1 h at room temperature anti-rabbit IgG secondary antibody

(BioRad, Hercules, CA). Protein bands were visualized by chemiluminescent detection and

GAPDH (1:10,000; Novus Biologicals, Littleton, CO) was used as a loading control. Protein

band density was measured using UN-SCAN-IT version 5.1 software (Silk Scientific, Inc. Orem,

UT, USA). The density of the IgG light chain band was expressed as a ratio of the GAPDH band.

Values from the injected striatum were compared to the contralateral, non-injected striatum and expressed as percent of control. Significance was set at $p < 0.05$ using a Student's *t*-test.

2.3 RESULTS

2.3.1 Effect of DA on terminal degeneration and BBB disruption.

Intrastriatal injection of DA (0.4µmol) has been previously shown to result in the loss of TH-ir and selective degeneration of the dopaminergic terminals in striatum (Hastings et al., 1996; Rabinovic et al., 2000). Preliminary data from our lab suggested that alterations of the BBB may exist, thus this model was utilized to directly examine the integrity of the BBB following intrastriatal DA. To confirm the DA-induced toxicity model, we examined striatal TH-ir at several time points. No loss of TH-ir was observed at 8h following intrastriatal DA; however by 2d a significant loss (-12%) of TH-ir was observed compared to the contralateral, non-injected striatum (Figs. 5A, C). This loss occurred in a halo-type pattern surrounding the actual injection site, as shown previously (Rabinovic et al., 2000). By 1w following DA, there was an even greater loss (-30%) of TH-ir, observed as a clearly defined, circular lesion surrounding the striatal injection site (Figs. 5A, C). No loss of TH-ir following an intrastriatal saline injection was detected (Figs. 5B, 4C).
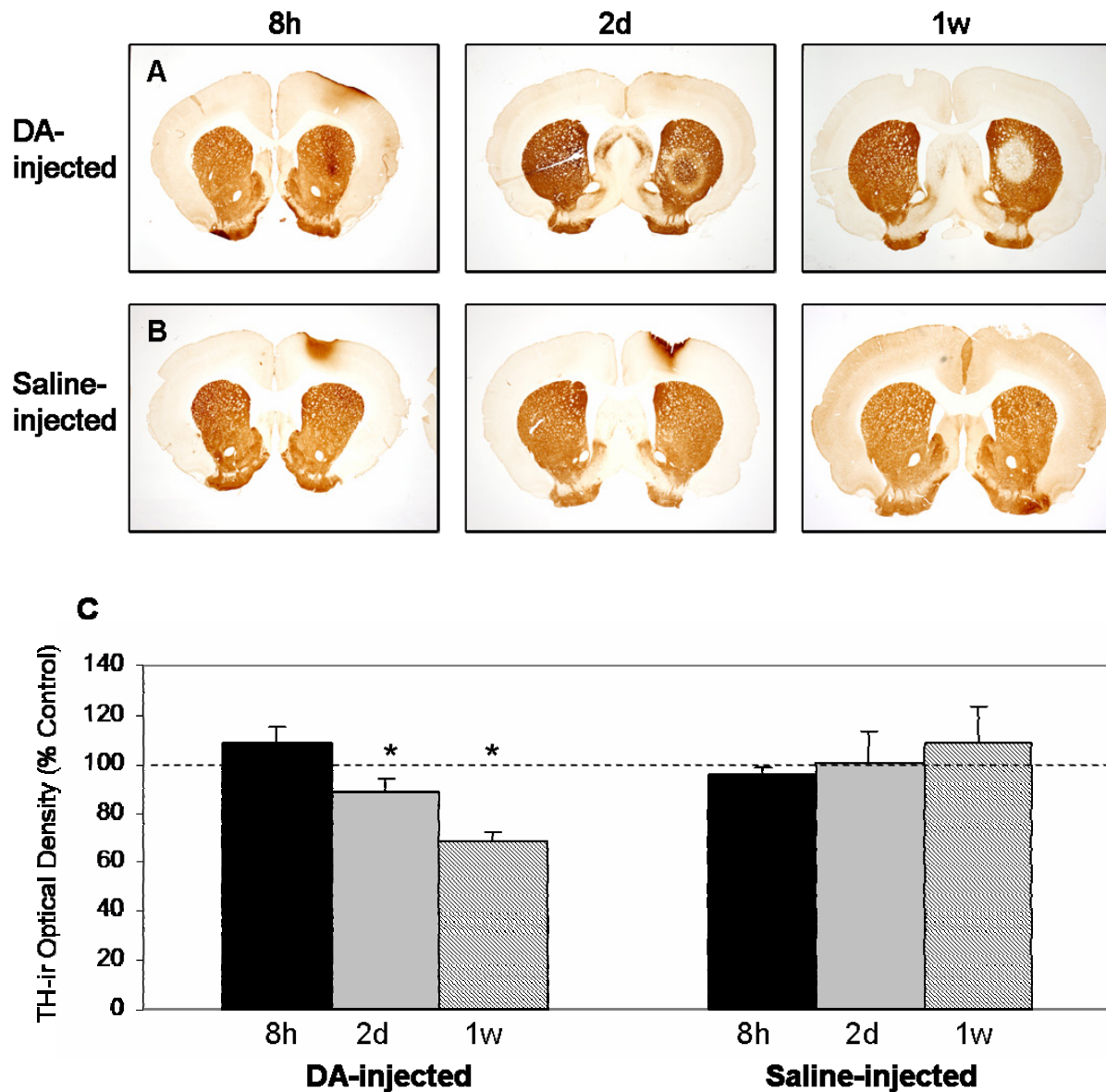
Figure 5: Effect of DA on the loss of striatal TH-immunoreactivity. Striatal sections were examined for TH-ir at 8h, 2d, and 1w following an intrastriatal injection of either *A*. DA (0.4μmol/2μl) or *B*. saline (2μl;). *C*. Optical density of the DA- or saline-injected striatum was measured and compared to the contralateral, non-injected striatum (control) to determine differences in TH-ir following intrastriatal DA or saline. Data are expressed as percent of control (Mean ± SEM; n=3-5), *p<0.05, significantly different from control.

The current study examined whether DA-induced toxicity in striatum was also causing disruption of the BBB. When the BBB is intact, the immunoglobulin protein, IgG, is not present in rat striatum (Yoshimi et al., 2002). The appearance of IgG in striatum is likely indicative of peripheral infiltration of blood proteins due to BBB disruption. As early as 8h, a 6.9-fold increase in IgG-ir was observed in the DA-injected striatum as compared to the contralateral, non-injected striatum (Fig. 6A, C). By 2d following intrastriatal DA, there was a substantial increase (21-fold) in striatal IgG-ir as compared to the control side, non-injected striatum (Fig 6A, C). By 1w, an 6.3-fold increase in striatal IgG was still observed in the DA-injected striatum compared to the non-injected, control side (Fig. 6A, C). This indicates that an intrastriatal DA injection leads not only to dopaminergic neurodegeneration, but also to BBB disruption and that disruption of the BBB at 8h precedes any measureable loss of TH-ir. To determine that the striatal IgG-ir observed was not due entirely to physical trauma from insertion of the cannula and injection, we examined IgG infiltration following an intrastriatal saline injection. Small increases in IgG-ir were observed in the saline-injected striatum at all time points (Fig 6B, C), suggesting some disruption of the BBB due to the injection itself. However, quantification by optical density showed that levels of IgG-ir were not significantly different from the non-injected, contralateral striatum (Fig. 6C).
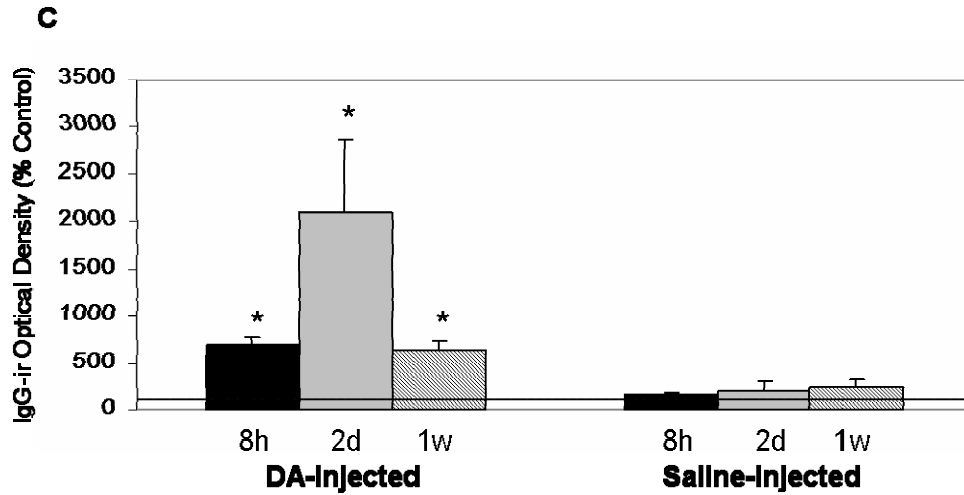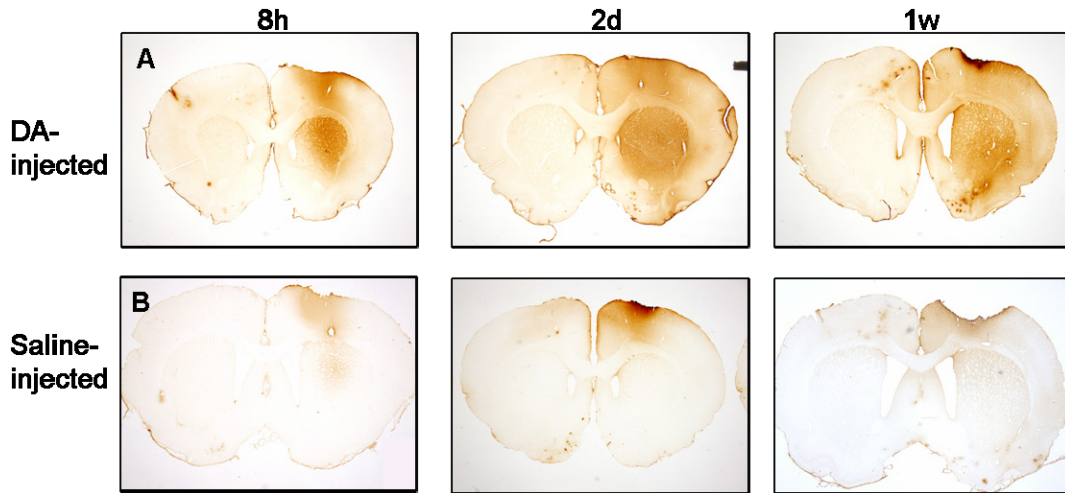
Figure 6: Effect of DA on integrity of the BBB. Striatal sections were examined for IgG-ir at 8h, 2d, and 1w following an intrastriatal *A*. DA (0.4μmol/2μl) or *B*. saline (2μl) injection. *C*. Optical density measurements of striatum were taken to examine differences in density of IgG-ir between the DA- or saline-injected striatum and its contralateral, non-injected side (control). Data are expressed as percent control, (Mean ± SEM; n=4-6), *p<0.05, significantly different from control.

2.3.2 Effect of intrastriatal 6-OHDA on BBB disruption.

To examine a more frequently utilized PD model, the integrity of the BBB was also examined following an intrastriatal injection of 6-OHDA. As previously described (Jeon et al., 1995), an intrastriatal injection of 6-OHDA (10µg) led to a 26.4% loss of striatal TH-ir at 2d following treatment and a 58.4% loss of TH-ir at 1w (Fig. 7A, C). Similar to the DA injection, as early as 8h following an intrastriatal 6-OHDA, IgG-ir increased by 14.2-fold in the injected striatum and compared to the non-injected, control striatum (Fig. 7B, C). By 2d, striatal IgG staining was further increased in the 6-OHDA-injected striatum (by 18.7-fold) compared to the non-injected striatum and an increase in IgG-ir (6.4-fold) over the contralateral, non-injected striatum persisted at 1w (Fig. 7C).
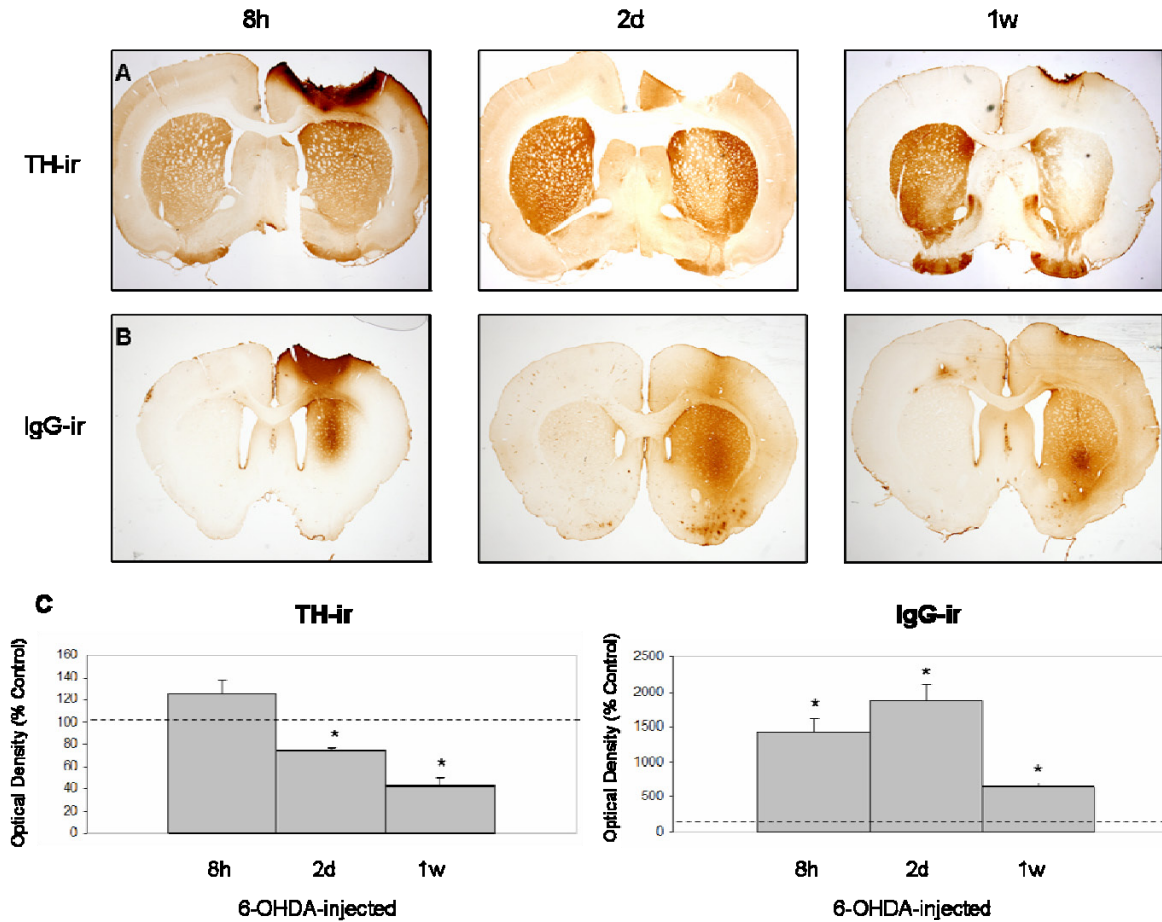
Figure 7: Effect of intrastriatal 6-OHDA on the loss of TH-ir and IgG-ir. *A*. Striatal sections were examined for TH-ir 8h, 2d, and 1w following an intrastriatal injection of 6-OHDA (10µg/2µl). *B*. Effect of 6-OHDA on integrity of the BBB was examined by evaluating infiltration of IgG into striatum at 8h, 2d, and 1w following treatment. *C*. Optical density of TH-ir and IgG-ir was determined in the injected striatum and compared to the non-injected, contralateral (control) striatum. Data are expressed as percent of control, (Mean ± SEM; n=4-6), *p<0.05, significantly different from control.

2.3.3   Neurotoxin-induced BBB disruption.

To further evaluate immunohistochemical findings that showed increases in striatal IgG-ir following dopaminergic neurotoxin-induced BBB damage, protein levels of IgG-ir in striatum were examined at 2d following treatment with DA, saline, or 6-OHDA by Western blot analysis. Striatal IgG protein levels increased over the contralateral, non-injected control striatum following intrastriatal DA, 6-OHDA, or saline (Fig. 8A). The density of light chain IgG bands were determined, normalized to the GAPDH loading control, and expressed as a ratio of light chain IgG/GAPDH. Both intrastriatal DA and 6-OHDA resulted in significantly increased IgG-ir over the non-injected control striatum (Fig. 8B), providing additional evidence for dopaminergic neurotoxin-induced BBB disruption.
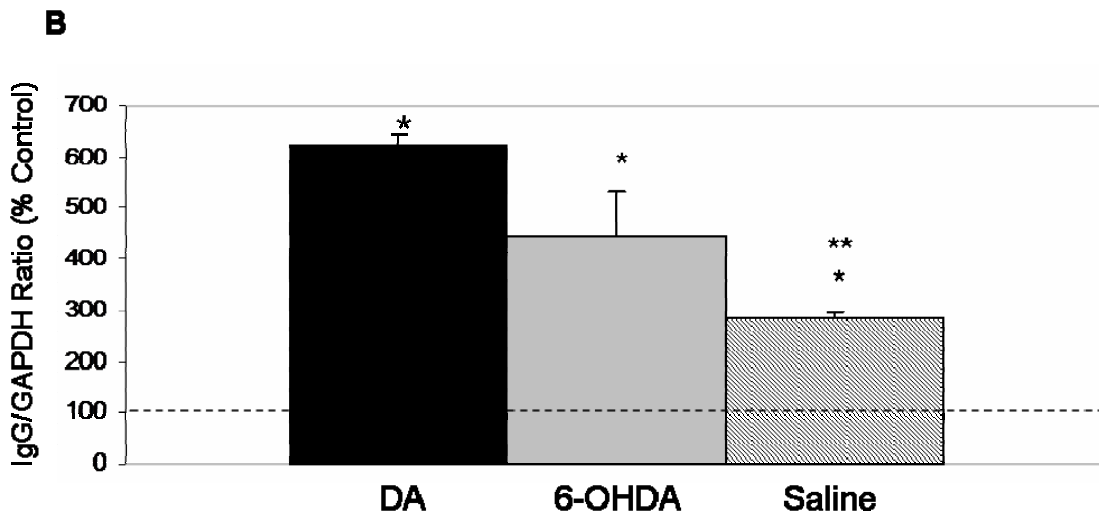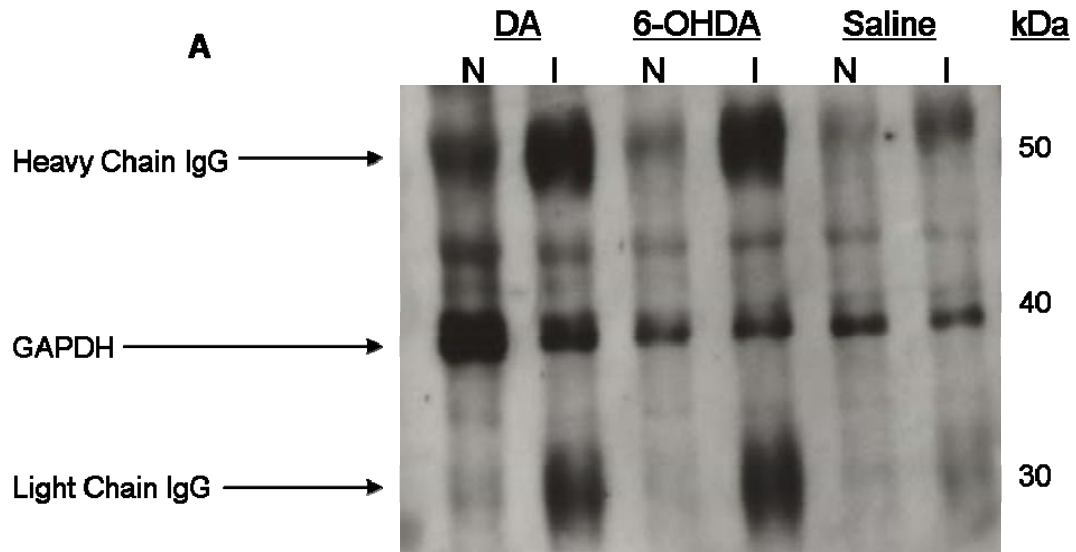
Figure 8: Effect of DA, 6-OHDA, or saline on striatal IgG protein levels. Striatal tissue (3mm) was taken from injected and non-injected striata 2d following an intrastriatal injection of DA (0.4μmol), 6-OHDA (10μg), or saline (2μl) and was examined for changes in IgG protein levels via Western blot. *A*. Representative blot for IgG (light chain and heavy chain) is shown for striatal tissue from the DA-, 6-OHDA-, or saline-injected striatum and the contralateral, uninjected striata. *B*. Light-chain IgG/GAPDH ratios from injected striata were quantified and compared to uninjected, control striatum. Data are expressed as percent of control for all treatment group, (Mean ± SEM; n=4), *p<0.05, significantly different from uninjected, contralateral control; **p<0.05, significantly different from DA- or 6-OHDA-injected striatum.

In addition to measuring IgG, Evans blue extravasation into striatum was examined at various time points. Evans blue is an azo dye that when injected i.v., binds to plasma albumin and fluoresces. Because neither Evans blue nor plasma albumin are able to cross an intact BBB, the presence of Evans blue fluorescence in the striatum is indicative of BBB disruption. Evans blue was administered i.v. into the tail vein of anesthetized rats 4h prior to brain collection for examination 8h, 2d, and 1w following an intrastriatal injection of DA or saline. Similar to IgG, Evans blue leakage into striatum was observed as early as 8h following an intrastriatal DA injection (Fig 9A). Evans blue extravasation was increased further at 2d following DA and still was present at 1w (Fig 9A). There was minimal Evans blue observed in striatum following saline at the 8h and 2d time points, and no observable striatal Evans Blue was present at 1w (Fig. 9B). No extravasation of Evans blue was observed in any of the contralateral, non-injected striata in any treatment group (data not shown).

To examine more precisely where the disruption of the BBB was occurring following a DA injection, immunoreactivity for EBA, a protein lining the brain microvessels which disappears following pathological disruption of the BBB (Cassella et al., 1996; Kalinin et al., 2006), was examined. A normal staining pattern for EBA in the non-injected (contralateral) striatum was observed, indicating a fully intact lining of brain microvessels (Fig. 10A). However, staining for EBA in the DA-injected striatum showed a patchy loss of EBA-ir at the site of the lesion, indicating damage to the BBB at the injection site (Fig. 10B).
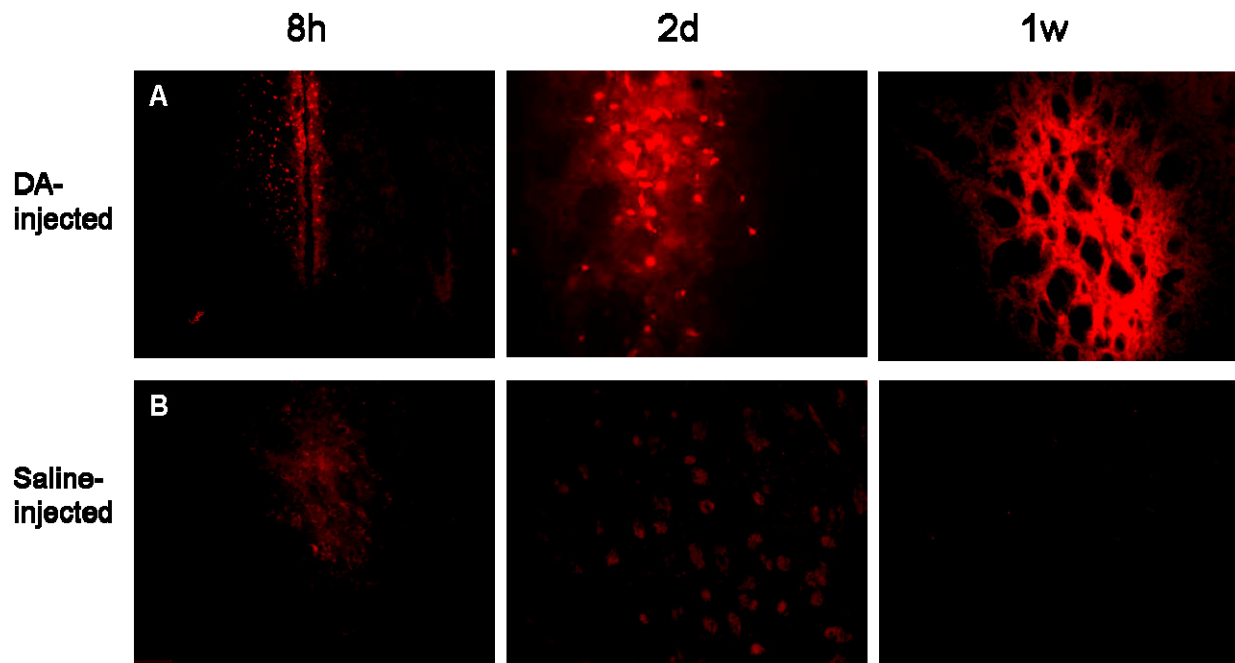
Figure 9: Evans blue extravasation following DA or saline injection. Presence of Evans blue-bound albumin was examined in the striatum following an intrastriatal DA injection (0.4μmol) or a saline injection (2μl) at 8h, 2d, and 1w following treatment. *A*. Extravasation of Evans blue is observed as red fluorescence as early as 8h and persisting for 1w in the DA-injected striatum, indicating disruption of the BBB at all time points. *B*. Extravasation of Evans blue is seen in the saline-injected striatum at 8h and 2d. There was no indication of BBB disruption at 1w following saline.
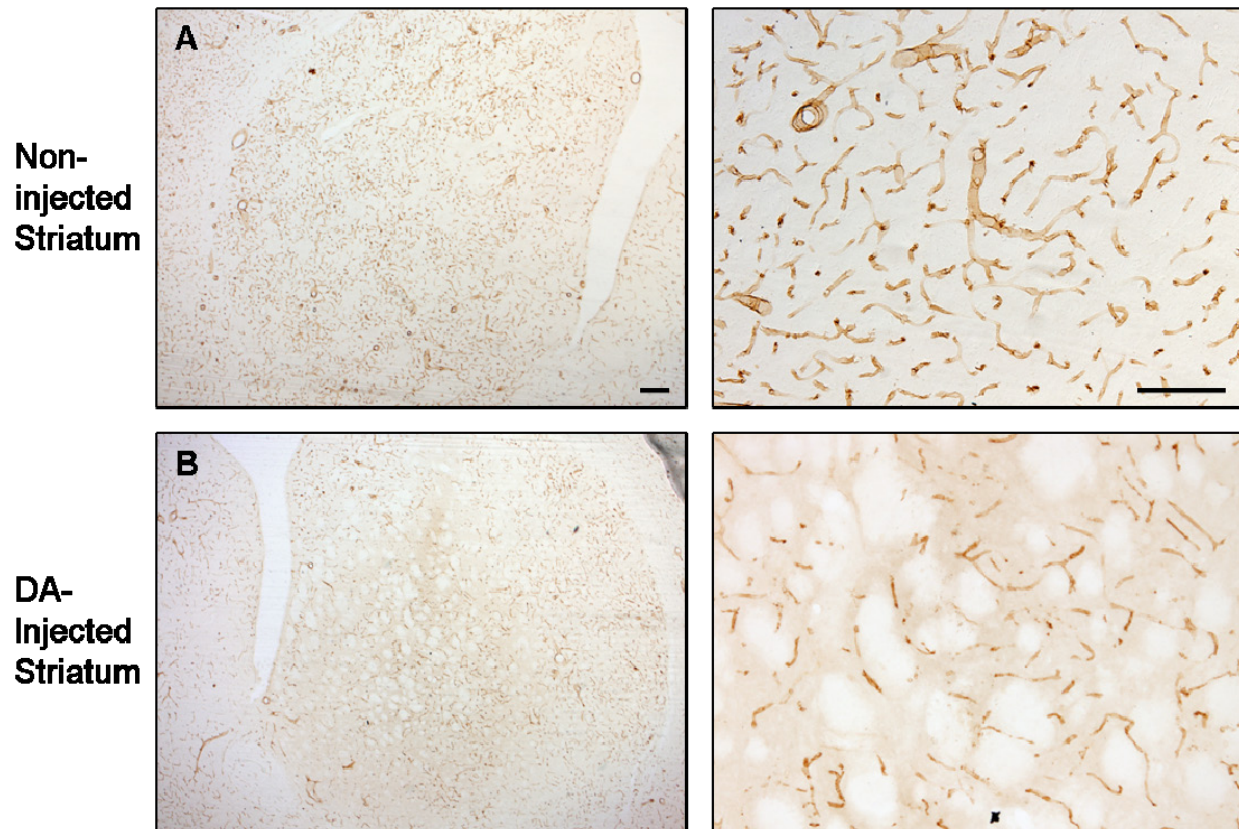
Figure 10: Examination of intrastriatal DA on EBA-ir. At 2d following an intrastriatal DA injection (0.4μmol) disruption of the BBB was evaluated by examining EBA-ir (n=3) in *A*. the non-injected, contralateral striatum and in *B*. the DA-injected striatum. Panel A shows uniform, fluid EBA-ir at the lining of the brain microvessel in the non-injected striatum, indicating no disruption of the BBB. Panel B shows a loss of EBA-ir at the injection site, indicating disruption of the BBB. Bar = 100μm.

2.3.4   Intrastriatal DA leads to neuroinflammatory effects.

Because neuroinflammation has been linked to disruption of the BBB, we also examined the effect of an intrastriatal DA injection on the neuroinflammatory response in striatum by examining microglial activation. Activation was determined by a.) an increase in OX-42-ir (CD11b) and b.) morphological criterion including swollen cell bodies with retracted processes or hypertrophic cell bodies and processes (Streit et al., 1999). Microglial activation was observed

in the DA-injected animals as early as 8h following injection (Fig. 11A). At this time point, microglial activation was localized to the site of the DA injection. Proliferation at this time point was minimal and at higher magnification it can be seen that microglia appear to have enlarged processes. By 2d following intrastriatal DA, there was likely proliferation and recruitment of microglia at the site of and peripheral to the injection site, forming a pattern for microglial activation that was similar to the area of dopaminergic terminal degeneration. Morphologically, the microglia exhibited enlarged processes and swollen cell bodies, indicative of activation. At 1w following intrastriatal DA, there was extensive hyperplasia, recruitment, and proliferation. Microglia, having lost contact inhibition, were packed into the site of the injection as well as peripheral to the injection. Microglia at this time point were clearly activated and morphologically phagocytic with bulbous cell bodies and retracted processes (Fig. 11A). Increases in OX-42-ir were observed in the DA-injected striatum at all time points when compared to the contralateral, non-injected striatum (Fig. 11B). The density of OX-42-ir in the contralateral, non-injected striatum did not differ at any time point (data not shown). Increases in OX-42-ir in the saline-injected striatum were localized specifically to the needle track (data not shown). Therefore, an intrastriatal DA injection resulted in an increase in microglial activation that was temporally and spatially correlated with the resulting BBB disruption, suggesting that DA-induced neuroinflammatory mechanisms may be involved.
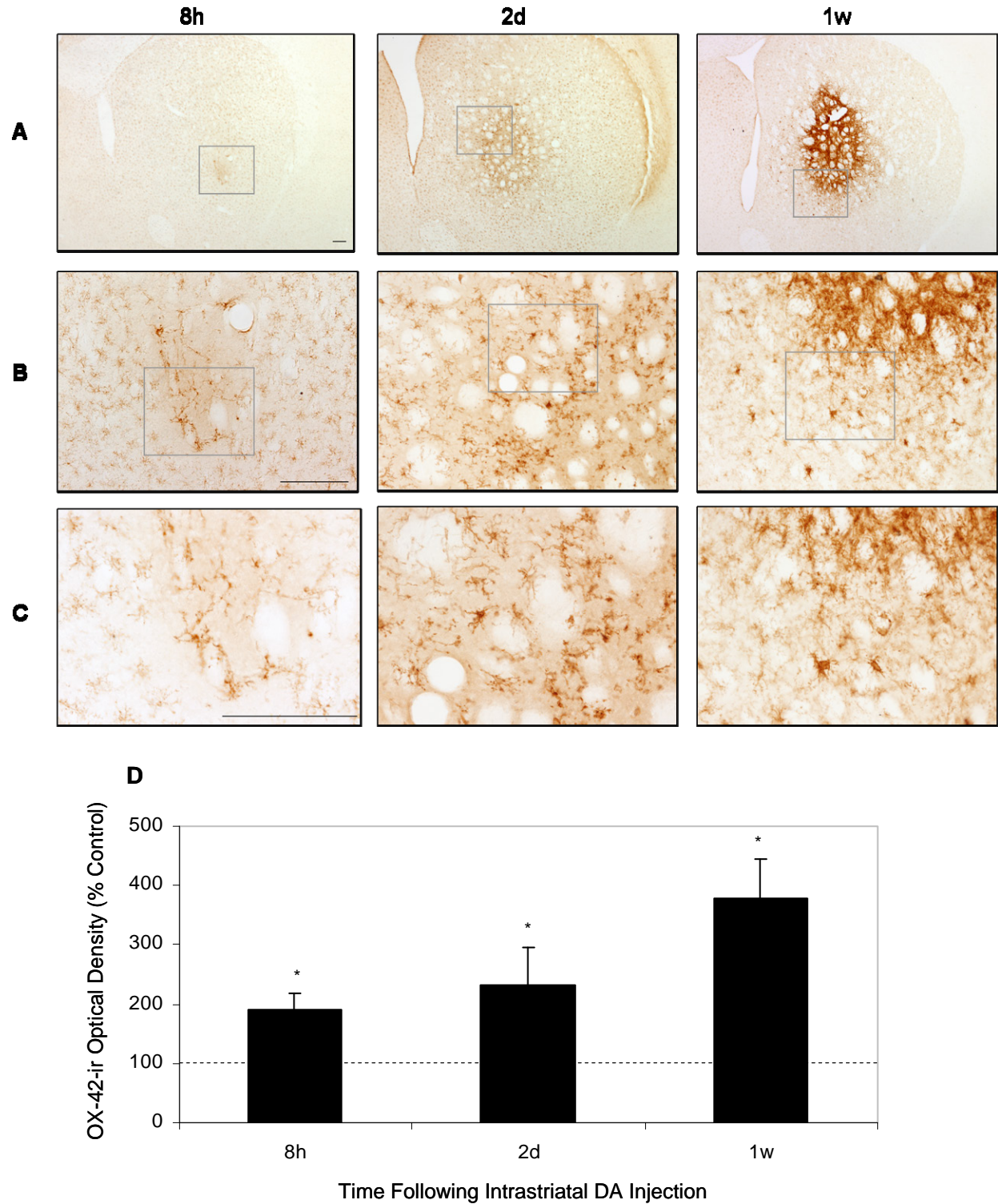
Figure 11: Effect of DA on microglial activation. Microglial activation was evaluated by examining OX-42-ir 8h, 2d, and 1w following an intrastriatal DA injection (0.4μmol). *A-C.* **Examination of microglial morphology showed activated microglia with a loss of contact inhibition, swollen or retracted processes, and**

bulbous cell bodies. By 8h, microglial activation is localized to the site of the intrastriatal DA injection. By 2d, an increase in microglial activation extends out to the peripheral areas surrounding the injection site in striatum and progresses over 1w. *D*. Density of OX-42-ir was measured in the DA-injected striatum and compared to the density of OX-42-ir in the contralateral, non-injected (control) striatum. Data are expressed as percent of control, (Mean ± SEM; n=4),* $p<0.05$, significantly different from control. Bars = 100μm.

2.3.5   Disruption of the BBB following rotenone-induced toxicity.

To evaluate the consequence of another dopaminergic neurotoxin on BBB integrity, the effect of systemically administered rotenone on the BBB was examined. Rotenone is a chronic, rather than an acute toxicity model that does not require physical disruption of the BBB for administration. Systemic administration of rotenone to rats has previously been shown to lead to dopaminergic nigrostriatal degeneration (Betarbet et al., 2000), which was examined in the current study by examining TH-ir in coronal brain sections at the level of the striatum and SN (See Appendix C for mapping of anatomical regions).   Rotenone-induced lesions of dopaminergic terminals, defined by a focal loss of striatal TH-ir, were observed in 28% (n=9) of the rotenone-treated animals (Fig. 12A). This group was defined as the rotenone-treated, lesioned group. The remaining rotenone-treated animals (n=23) showed no visible loss of TH-ir in striatal (Fig. 12A) or nigral (Fig. 12B) regions. This group was defined as the rotenone-treated, non-lesioned group. Likewise, animals infused with vehicle only for 28d displayed a uniform pattern of TH-ir throughout the striatum, olfactory tubercle, and nucleus accumbens (Fig. 12A). There was also prominent TH-ir in the ventral tegmental area, SN nigra pars compacta and the SN pars reticulata indicating no gross loss of dopaminergic neurons in these regions (Fig. 12B). The outcome variability in this model has been previously described (Betarbet et al., 2000).
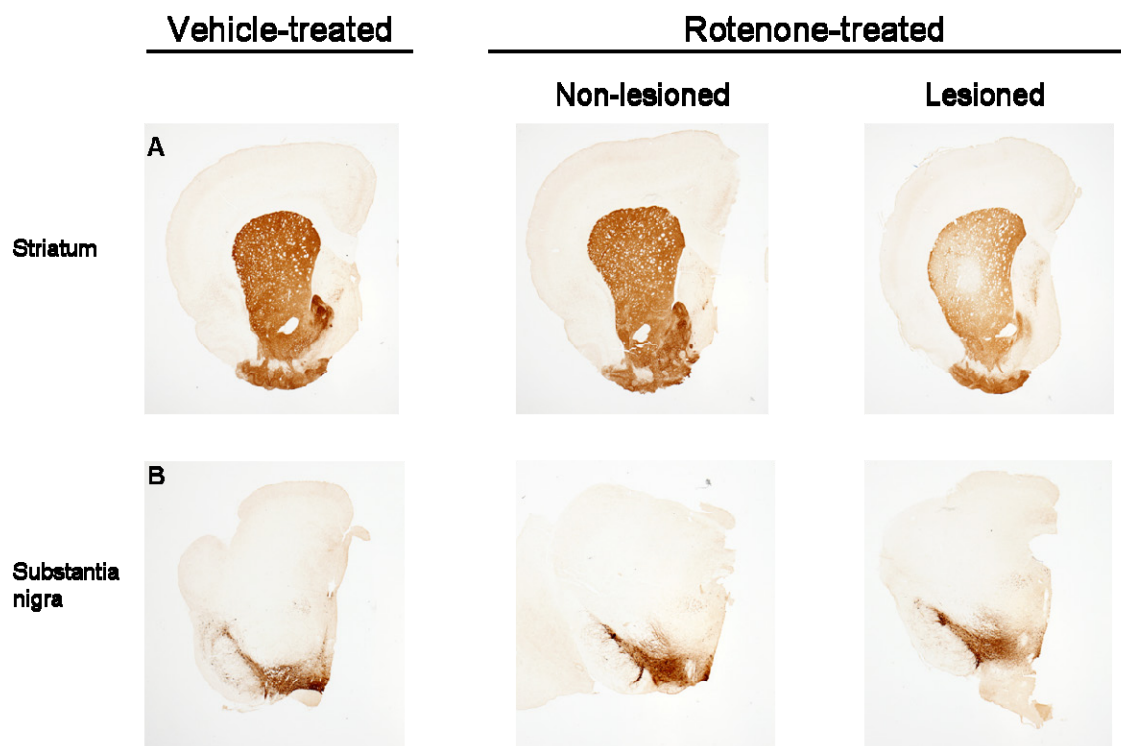
Figure 12: Striatal and nigral TH-ir following systemic rotenone or vehicle treatment. Tissue sections of *A*. striatum and *B*. substantia nigra (SN) were stained for TH following vehicle (DMSO/PEG), or rotenone (3mg/kg/day) treatment. Vehicle-treated animals (n=8) and rotenone-treated, non-lesioned rats (n=23) displayed uniform TH-ir throughout striatum and SN. Rotenone-treated, lesioned rats displayed a focal loss of TH-ir in the striatum (n=9), while loss of TH-ir from the SN was not apparent in the majority of rotenone-treated, lesioned rats (n=8), unless complete depletion of striatal TH-ir was observed (n=1; not shown).

Rats were grouped into 3 different categories based on the results from TH immunostaining of the striatum following treatment with either vehicle or rotenone. To examine changes in BBB integrity in this model IgG-ir was evaluated for each animal and is presented according to which of the 3 categories the animal was grouped based on TH-ir (1. rotenone-treated, lesioned; 2. rotenone-treated, non-lesioned; 3. vehicle-treated). Striatal and nigral areas from the vehicle-treated animals displayed no IgG-ir throughout the examined brain regions (Fig.

13A, B). Similarly, rotenone-treated, non-lesioned rats showed no IgG-ir throughout the examined brain areas (Fig. 13A, B), suggesting an intact BBB in these animals.

Animals from the rotenone-treated, lesioned group (n=9) displayed varying intensities of IgG-ir in several anatomical regions. For example, one rotenone-treated, lesioned animal displayed low-intensity IgG-ir at the lateral portion of the striatum and cortex (data not shown). However another rotenone-treated, lesioned animal displayed much more robust IgG immunostaining at the ventrolateral region of striatum, the lateral cortex, the olfactory tubercle, and the medial septum (data not shown). Other rotenone-treated, lesioned animals had IgG-ir throughout the striatum, cortex, olfactory tubercle, and medial septum (Fig. 13A), as well as throughout the ventral tegmentum area, SN and central tegmental tract (Fig 13B). Despite the rotenone-treated, lesioned group showing different anatomical areas immunoreactive for IgG, all 9 rotenone-treated, lesioned animals displayed an increase in IgG-ir at the striatum and SN. The increase in IgG-ir observed in all rotenone-treated lesioned rats indicated that disruption of the BBB is also a feature of rotenone-induced toxicity.
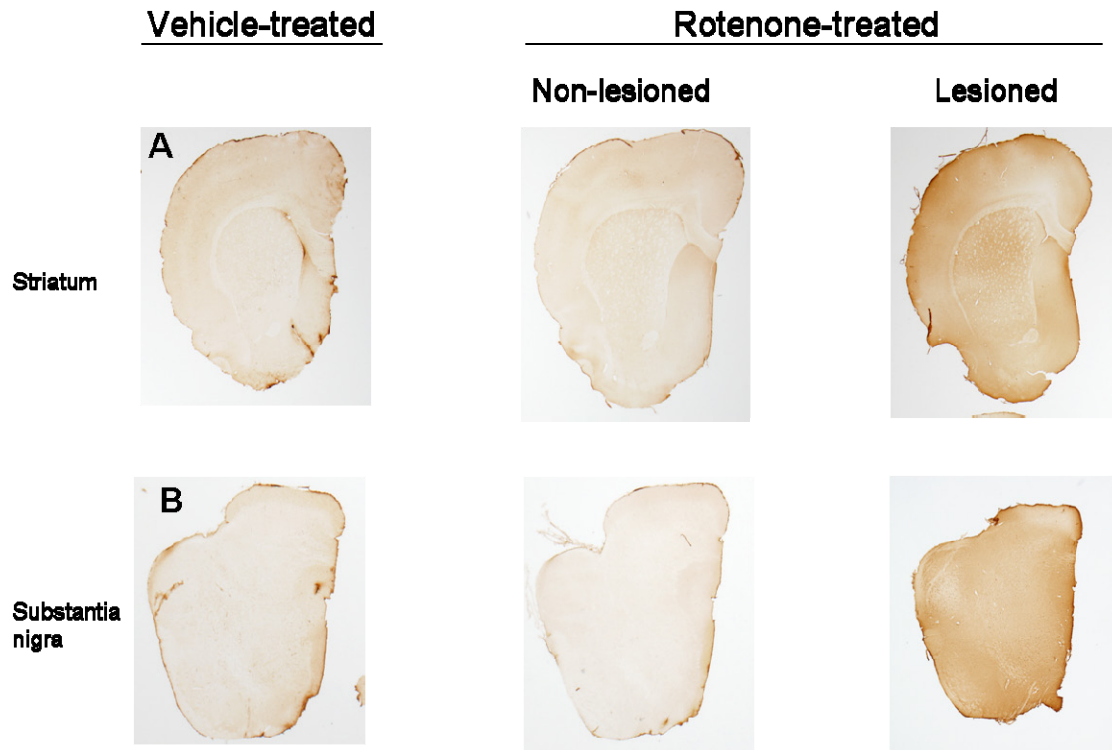
Figure 13: Striatal and nigral IgG-ir following systemic rotenone or vehicle treatment. *A.* Striatal and *B.* nigral sections were stained for IgG following vehicle (DMSO/PEG), or rotenone (3mg/kg/day) treatment. There was no observable IgG-ir in vehicle-treated animals (n=8) or the rotenone-treated, non-lesioned animals (n=23) in any region examined. Rotenone-treated, lesioned rats (n=9) displayed immunoreactivity for IgG in the striatum and SN, indicative of BBB disruption.

2.4 DISCUSSION

Results from this study suggest that BBB disruption is a pathological feature of dopaminergic neurotoxins such as DA, 6-OHDA, and rotenone. An intrastriatal DA injection leads to early disruption of the BBB, as evidenced by both striatal IgG and Evans blue infiltration, prior to dopaminergic degeneration. Additionally, examination of EBA-ir 2d following intrastriatal DA

indicates that disruption of the BBB occurs at the site of the DA injection and dopaminergic terminal degeneration. Moreover, microglial activation was observed in the DA-injected striatum that correlated temporally and spatially with BBB disruption, suggesting that a neuroinflammatory mechanism may play a role in DA-induced BBB disruption. The complex I inhibitor rotenone, which leads to dopaminergic degeneration and a neuroinflammatory response, also had an effect on BBB integrity. Systemically administered rotenone led to infiltration of IgG in rotenone-treated animals that had a focal loss of striatal TH-ir, indicating that rotenone also leads to BBB disruption. Together, results from this study provide evidence that dopaminergic neurotoxins lead to disruption of the BBB.

In the present study we confirmed that intrastriatal DA acts as a dopaminergic neurotoxin. However, the effects of DA on BBB integrity have not been previously documented and therefore were investigated. One technique used to examine disruption of the BBB was the presence of striatal IgG since IgG is not detected in rat striatum under normal conditions (Yoshimi et al., 2002). Striatal IgG was observed as early as 8h following intrastriatal DA suggesting that BBB permeability occurs prior to a measureable loss of striatal TH-ir. Previous reports of dopaminergic neurotoxin-induced BBB disruption examined extravasation of serum proteins several days following intrastriatal injection of 6-OHDA (Carvey et al., 2005), however current findings reveal that BBB disruption in our model is an early event that precedes 6-OHDA- and DA-induced dopaminergic neurodegeneration.

IgG-ir was examined at the same time points following an intrastriatal saline injection to examine the effect of needle trauma on the BBB. Intrastriatal saline did lead to BBB damage and some IgG infiltration into the striatum was observed at 8h, 2d and 1w post-injection, however levels of IgG-ir were not significantly different from non-injected control striata. In contrast,

IgG-ir was significantly increased at 2d following intrastriatal DA and this increase over non-injected, control striatum persisted for 1w. Western blot analysis confirmed the presence of increased IgG protein in striatum 2d following intrastriatal DA and 6-OHDA, supporting a role for toxin-induced BBB disruption.

Extravasation of Evans blue is another well-documented technique to examine BBB disruption (Kim et al., 2003). While IgG-ir in striatum is a clear indication of BBB permeability, this technique may not allow for temporal determination of BBB permeability because the clearance of IgG from the striatum is not known. Evans blue is an azo dye that fluoresces when bound to plasma albumin and cannot cross the BBB unless integrity has been compromised. Therefore, the observation of Evans blue extravasation into the brain parenchyma indicated permeability of the BBB at specific time points following intrastriatal DA. Striatal Evans blue extravasation was apparent at 8h and up to 1w following injection. It has been well-documented that the disappearance of EBA-ir is also indicative of disruption of the BBB (Cassella et al., 1996; Abdel-Rahman et al., 2002; Kalinin et al., 2006). Alternatively, when components that comprise the BBB are fully intact, there is fluid staining of the vascular lining (Adayev et al., 1998; Hawkins and Davis, 2005). A loss of EBA-ir was observed in the DA-injected striatum compared to the contralateral, non-injected striatum at 2d.

Results from this study also demonstrate that systemic rotenone, a dopaminergic neurotoxin, leads to disruption of the BBB. Chronic, systemic rotenone leads to a selective loss of the dopaminergic nigrostriatal projections neurons (Betarbet et al., 2000), and microglial activation in the affected areas (Sherer et al., 2003b). The rotenone model also recapitulates other pathological features of PD (Betarbet et al., 2000; Greenamyre et al., 2003; Sherer et al., 2003b; Sherer et al., 2003a; Testa et al., 2005), making it ideal for examining BBB disruption in an

animal model for PD. Subcutaneous administration of rotenone inhibits complex I systemically (Betarbet et al., 2000), likely including in the endothelial cells that comprise the BBB. Endothelial cells contain high concentrations of mitochondria (Ribatti et al., 2006) and are extremely vulnerable to damage upon mitochondrial dysfunction (Kim et al., 2003). Therefore, rotenone-induced damage to the BBB could be the consequence of systemic complex I inhibition in the endothelial cells. Inhibition of mitochondrial complex I activity also increases ROS formation (Cassarino et al., 1997; Votyakova and Reynolds, 2001). Increases in oxidized proteins *in vivo* and *in vitro*, as well as the observation that antioxidant treatment attenuates rotenone-induced neurotoxicity *in vitro* (Sherer et al., 2003c), provide evidence that oxidative stress is a main mechanism by which rotenone leads to dopaminergic neurotoxicity. Since integrity of the BBB is also affected by oxidative stress, it is likely that this is a mechanism by which rotenone leads to BBB disruption.

As mentioned previously, DA forms the electron-deficient DAQ, increases cysteinyl catechol formation following intrastriatal administration (Hastings et al., 1996), leads to mitochondrial dysfunction (Berman and Hastings, 1999) and causes selective dopaminergic neurotoxicity (Hastings et al., 1996; Rabinovic et al., 2000). Because a main mechanism of DA-induced toxicity is via oxidative stress, it is likely that oxidative stress also contributes to DA-induced BBB disruption. Several studies have implicated oxidative stress in increased BBB permeability. Following systemic 3-nitropropionic acid administration, which induces ROS-dependent damage to striatal neurons, there was BBB disruption which was shown to be associated with a ROS-induced increase in matrix metalloproteinase-9 activity (Kim et al., 2003). In addition, superoxide radical production following menadione exposure of cerebrovascular endothelial cells increased cell permeability which was reversed upon addition

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of the antioxidant enzymes superoxide dismutase and catalase (Lagrange et al., 1999). It has also been well documented that ROS contribute to early damage of BBB endothelial cells following ischemia/reperfusion injury (Kontos, 1985; Asahi et al., 2001). Finally, Krizbai, et al. (2005) showed that exposure of cultured brain endothelial cells to 2,3-dimethoxy-1-naphthoquinone led to dysfunction and permeability of the brain endothelium. It is possible DA oxidation to the DAQ has a similar effect on the BBB *in vivo*, leading to the observed permeability of the BBB.

A recent study demonstrated that exposure of cultured mouse microglial cells to DAQ resulted in activation of microglia and a proinflammatory gene profile (Kuhn et al., 2006), linking DAQ formation and neuroinflammation. It is possible that exogenous DA can also lead to BBB disruption via activation of microglia, which is associated with BBB dysfunction. The current study showed that an intrastriatal DA injection leads to activated microglia within the striatum that correlates with both DA terminal degeneration (loss of TH-ir) and areas of BBB disruption (loss of EBA-ir), supporting a role for inflammation in DA-induced permeability of the BBB. These factors, neuroinflammation and oxidative stress, are currently being examined as mechanisms by which an intrastriatal DA injection leads to BBB disruption. Rotenone has also been shown to cause microglial activation and some reactivity of astrocytes when administered chronically *in vivo* (Sherer et al., 2003b), indicating the occurrence of neuroinflammatory processes during rotenone-induced toxicity, which may also contribute to BBB disruption in this model.

Previous studies have shown a dysfunctional BBB in animal models for PD. Intrastriatal and medial forebrain bundle injections of the neurotoxin 6-OHDA in rats leads to BBB disruption in rat (Carvey et al., 2005). Results from the current study, using other methods to examine BBB integrity, support these findings and add that BBB disruption following an

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intrastriatal DA injection occurs prior to apparent dopaminergic terminal degeneration. Dysfunction of the BBB was also observed in 6-OHDA-lesioned rats that were subsequently treated with L-DOPA to induce dyskinesia (Westin et al., 2006). Extravasation of FITC-bound albumin (indicative of BBB disruption) was observed in the SN and striatum of mice treated acutely with 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP; Zhao et al., 2007). Protection by minocycline in this model suggests a role for neuroinflammation in BBB disruption (Zhao et al., 2007). There is also evidence that supports a dysfunctional BBB in PD. Support for dysfunction of the poly-glycoprotein (P-gp) transporter comes from data correlating functional polymorphisms of the multi drug resistant 1 (*MDR1)* gene with PD, indicating a genetic susceptibility for PD due to the increased transport of environmental neurotoxins into the CNS (Lee et al., 2004), though this remains controversial (Furuno et al., 2002). A separate study involving PD patients observed increased brain levels of injected [11C]verapamil, a substrate for P-gp, compared to age-matched control subjects, suggesting dysfunction of this BBB efflux transporter in PD (Kortekaas et al., 2005). Additionally, vascular alterations that may be associated with BBB disruption have been observed in PD tissue post-mortem (Desai et al., 2007).

Studying the BBB in neurodegenerative diseases is of high importance because the BBB serves as an interface between the CNS and the periphery. Receptors, transporters, and junctional proteins of the BBB work to protect the CNS and maintain proper functioning. Disruption or dysfunction of the BBB can lead to a lack of nutrient transport, infiltration of peripheral blood and immune factors, and/or increase of environmental toxin transport into the brain leading to cellular damage and toxicity and potentially to neurodegenerative disease states. Maintaining BBB integrity may alter or diminish brain exposure to environmental toxins or peripheral blood

factors that may propagate neurodegeneration as occurs in PD. However, it is possible that permeability of the BBB may allow for the transport of therapeutic drugs to the CNS that ordinarily would not be able to cross the BBB from the periphery. Therefore, the disruption observed in the current models suggests that potentially therapeutic compounds that normally cannot penetrate in intact BBB are able to have brain access to exert a beneficial effect. Presently, discrepancies exist between pharmaceutical agents that protect against dopaminergic degeneration in animal models for PD and those which are actually clinically protective in patients. Therefore, understanding of the differences in the functional status of the BBB between animal models and PD may elucidate more effective treatments and therapies.

2.5   SUMMARY AND CONCLUSIONS

We observed disruption of the BBB in dopaminergic neurotoxicity models. An intrastriatal injection of DA or 6-OHDA led to BBB disruption in the injected striatum which preceded the observation of dopaminergic terminal degeneration, but was concordant with the observed increases in toxin-induced microglial activation. Additionally, systemically administered rotenone led to disruption of the BBB in the striatum and SN of rotenone-treated, lesioned rats. Results from this study are summarized in Appendix D. Together, these results suggest that BBB disruption is a feature of animal models of PD, which could have therapeutic implications.

3.0   THE ROLE OF DOPAMINE-INDUCED OXIDATIVE STRESS IN BLOOD-BRAIN BARRIER DISRUPTION

3.1   INTRODUCTION

The blood-brain barrier (BBB) is comprised of highly specialized endothelial cells that are packed tightly together and work in concert with tight junction proteins to form a physical and chemical barrier between the brain and the peripheral blood stream. The BBB is physically supported in the CNS parenchyma by astrocyte end feet. Highly specific influx transporters allow vital nutrients to enter the brain from the blood stream while efflux transporters excrete waste and environmental toxins from the brain into the peripheral blood stream (Schinkel, 1999; Terasaki and Ohtsuki, 2005; Abbott et al., 2006; Kim et al., 2006; Syvanen et al., 2006; Bechmann et al., 2007; Galea et al., 2007). These components are essential for proper functioning of the BBB and for maintenance of brain homeostasis by preventing potentially harmful environmental toxins from entering the brain and causing damage.

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by a loss of dopaminergic neurons from the nigro-striatal projection. While the causes in most PD cases are not known, substantial evidence suggests that oxidative stress plays a key role in PD pathogenesis (Fahn and Cohen, 1992; Schulz et al., 2000; Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Greenamyre and Hastings, 2004). One factor that may contribute to

oxidative stress and dopaminergic neuron susceptibility in PD is the neurotransmitter dopamine (DA). Normal metabolism of DA by monoamine oxidase results in the production of hydrogen peroxide which can then form highly reactive, toxic radical species such as the hydroxyl radical (Halliwell, 1992). Additionally, DA can oxidize to the DA quinone (DAQ), forming reactive oxygen species (ROS) which can increase oxidative stress and lead to cellular damage (Graham et al., 1978; Hastings et al., 1996). The electron-deficient DAQ is susceptible to attack by nucleophiles such as thiol groups on glutathione (GSH), free cysteine, and cysteinyl residues on proteins. Because cysteines are often found at the active sites of proteins, protein modification by DAQ could lead to inactivation of critical proteins and subsequent cellular damage. Previous studies have shown that an intrastriatal injection of DA into rat is selectively toxic to dopaminergic terminals and results in a loss of striatal tyrosine hydroxylase (TH)-immunoreactivity (-ir), which is prevented by treatment with antioxidants (Hastings et al., 1996; Rabinovic et al., 2000), indicating that DA leads to oxidative stress-induced cellular damage.

Endothelial cells, tight junctions, and transport systems of the BBB are particularly vulnerable to the effects of oxidative stress. A compromised BBB is a pathological feature of several neurodegenerative diseases in which oxidative stress is presumed to be a contributing factor, including Alzheimer's disease, acquired immune deficiency syndrome encephalitis, multiple sclerosis and stroke (Skoog et al., 1998; Dallasta et al., 1999; Schulz et al., 2000; Zlokovic, 2005; Poulet et al., 2006; Deane and Zlokovic, 2007; Leech et al., 2007; Matsumoto et al., 2007). There is also evidence for a dysfunctional BBB in PD (Lee et al., 2004; Kortekaas et al., 2005; Desai et al., 2007). Additionally, several animal models for PD that elicit oxidative stress-induced dopaminergic neurotoxicity are associated with BBB disruption including medial forebrain bundle and intrastriatal injection of the dopaminergic neurotoxin 6-hydroxydopamine

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(Carvey et al., 2005), intrastriatal injection of DA (see Chapter 2), systemic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration (Zhao et al., 2007), and systemic administration of the mitochondrial complex I inhibitor, rotenone (see Chapter 2).

Previous results (Chapter 2) suggested that BBB disruption occurs following an intrastriatal DA or 6-OHDA injection, and systemic rotenone treatment. In all cases, BBB disruption was noted in models where toxicity to dopaminergic terminals also occurred. Although evidence for BBB disruption appeared prior to the loss of striatal TH-ir following intrastriatal DA or 6-OHDA, it is not known whether toxicity to the dopaminergic terminals is required for BBB disruption. On the other hand, BBB disruption could be the result of toxin-induced oxidative stress (possible extracellularly), independent of dopaminergic toxicity. Therefore, in this study we examined the effect of an intrastriatal dihydroxyphenylacetic acid (DOPAC) injection to evaluate the role of oxidative stress as a mechanism of BBB disruption independent of dopaminergic terminal degeneration. We also evaluated the effects of a thiol antioxidant, N-acetylcysteine (NAC) on DA-induced BBB disruption to further examine the role of oxidative stress in BBB disruption.

3.2   METHODS

Animals.

Male Sprague-Dawley rats (300g; Hilltop Laboratories, Allison Park, PA), were used throughout the current study, and were kept in standard caging in a temperature- and light-controlled room on a 12h light/dark cycle. Food and water were available *ad libitum*. All experimental protocols were in accordance with the guidelines developed by the NIH Guide to

the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Surgical Procedures

Rats were anesthetized with Equithesin (3.0mg/kg i.p.). An injection cannula was placed stereotaxically into the right striatum (Coordinates from Bregma: -2.7 mm medial/lateral and +0.8 mm anterior/posterior. Coordinates from dura: -5.0 mm dorsal ventral). DOPAC (0.4μmol/2μl sterile water), NAC (0.4μmol/2μl sterile water), or DA (0.4 μmol DA/2 μl sterilized water) was unilaterally injected into the right striatum over 20 min via microfusion injection pump (model 341B; Sage Instruments, Boston, MA). All injected solutions were made immediately prior to surgery and kept on ice with minimal light exposure.

At indicated time points following surgery, animals were deeply anesthetized and perfused with ice cold 1% paraformaldehyde followed by 4% paraformaldehyde at a steady flow rate. Brains were rapidly removed, placed in 4% paraformaldehyde at 4°C for 24h, and then hydrated in 30% sucrose at 4°C for 48h. Fixed rat brains were sliced coronally (40μm) using a cryostat at -20°C. Tissue sections were placed serially into a 24-well plate with anti-freezing solution and stored at -20°C until further use for immunohistochemical analysis. Animals sacrificed for biochemical analysis were perfused with saline at 4°C. Brains were rapidly removed and striatal tissue surrounding the injection site as well as tissue from the contralateral, non-injected striatum were dissected using a 3mm biopsy punch, as described in Chapter 2. Tissue was then weighed, snap-frozen on dry ice, and stored at -80°C until analyzed.

Systemic N-acetylcysteine treatment.

A group of animals received systemic treatment with NAC (American Regent, Shirley, NY). Rats were treated i.p. with NAC at 500mg/kg (n=6), 1000 mg/kg (n=4), or with saline (n=4). For immunohistochemical analyses, rats were treated with NAC 1d and 30min prior to an intrastriatal DA injection followed by NAC at 30min, 3h, and then once daily for 7d (at the same dose as the initial injection) until sacrifice. For biochemical analyses, rats were treated with NAC 1d and 30min prior to and 30min following a DA injection.

Immunohistochemistry.

Mouse anti-CD11b (OX-42; 1:1000; Serotec, Raleigh, NC), rabbit anti-rat IgG (1:1000; Vector Laboratories, Burlingame, CA), and mouse anti-rat tyrosine hydroxylase (TH; 1:2000; Chemicon International, Temecula, CA) were used as primary antibodies for immunohistochemical detection. Specificity for each of these antibodies has been described (see Chapter 2). Immunohistochemical processing was standardized by processing tissue sections from each animal within an experiment simultaneously to reduce variability of staining.

Striatal sections were immersed in 1% H_2O_2 (15 min) to reduce endogenous peroxidase, followed by incubation with normal serum to reduce non-specific binding. Sections were then incubated with the primary antibody for 24h or 48h at 4°C, followed by the appropriate biotinylated secondary antibody and then processed by the avidin-biotin method (ABC method) using the Vectastain kit (Vector Laboratories, Burlingame, CA). Processed sections were visualized with diaminobenzidine (DAB), mounted onto slides, and coverslipped. Tissue sections were examined under a bright field microscope (BX-51, Olympus) or stereoscope (SX-61, Olympus America, Inc. Center Valley, PA).

Optical Density Measurements.

Optical density measurements of tissue sections processed for immunohistochemistry were obtained by outlining the entire striatum using a computer analysis system (MCID M5 5.1, Imaging Research, Inc.) to quantify immunoreactivity of TH, IgG, or OX-42 from a tissue section within each animal (n). To quantify the extent of dopaminergic terminal loss following an intrastriatal injection of DA, 6-OHDA, or saline TH-ir was quantified by measuring the adjusted optical density at the site of injection. Adjusted optical density was calculated using the following equation:

Adjusted Optical Density = log [density of background / density of striatum]

The section containing the injection site was viewed on a microscope and displayed on a monitor linked to a computer equipped with an MCID M5 image analysis system (Imaging Research Inc., Ontario, Canada). The background was obtained by measuring the optical density in corpus callosum, an area devoid of TH labeling. The density of TH-ir in striatum was obtained by outlining the entire striatum and measuring the optical density of the delineated area. Quantification of the extent of BBB disruption by examination of IgG-ir obtained in a similar manner. To quantify the extent of microglial activation, a density measurement was obtained by outlining the area of increased OX-42-ir. All density measurements were calibrated to an arbitrary scale of 0-255 with 0 being black and 255 being white. Measurements were then expressed as a percent of control based on comparison of optical density readings from the injected striatum to the contralateral, non-injected striatum within the same tissue section and animal. A Student's *t*-test was used to determine significance at $p < 0.05$.

Free thiol measurements.

Tissue from a 3mm striatal punch was homogenized in 1ml of 0.1M perchloric acid. An aliquot of the homogenate (250µl) was diluted 1:1 with 6.5% trichloroacetic acid and was mixed for 10min, followed by centrifugation at 35,000 g at 4°C for 15 min. The supernatant was separated from the pellet and added to 1.3ml 1M Tris-HCL (pH 7.6) buffer. Finally, 200µl 5,5´-dithio-bis(2-nitrobenzoic acid) (1.0 mM solution) was added to each sample and allowed to react for 20min. Absorbance was then determined with a spectrophotometer at 412nm. Total thiol levels in each sample were calculated using a GSH standard (1-40nmol) curve. Values were expressed as µmol of free thiols per g of tissue.

Protein cysteinyl catechol measurements.

Protein cysteinyl catechol measurements were obtained from striatal tissue samples following treatment. Tissue was homogenized in 1ml of 0.1M perchloric acid and centrifuged at 35,000g for 15min at 4°C. The resulting protein pellet was separated from the supernatant, washed, hydrolyzed in 6N HCl, and catechol-modified amino acids were extracted with alumina prior to analysis by HPLC as previously described (Hastings and Zigmond, 1994). Cysteinyl-DA, -DOPAC, and -DOPA levels were determined by comparison to cysteinyl catechol standards synthesized in the laboratory according to published procedures (Rosengren et al., 1985).

3.3   RESULTS

3.3.1   Effects of intrastriatal DOPAC injection on integrity of the BBB.

The DA metabolite DOPAC can oxidize to form the DOPAC quinone which can covalently modify cysteinyl residues on proteins, potentially rendering the proteins inactive (Fornstedt et al., 1990b). Unlike exogenous DA, however, exogenous DOPAC administered via intrastriatal injection is not a substrate for the DA transporter (DAT) and therefore induces oxidative stress extracellularly, likely via oxidation (Fig. 14). To examine the effects of DOPAC on dopaminergic terminals, TH-ir from the DOPAC-injected striatum was compared to TH-ir from the DA-injected striatum. An intrastriatal injection of DA led to a 30% loss of TH-ir in the striatum (Fig. 15A, C), as previously described (see Chapter 2). This specific loss of dopaminergic terminals is due to DA oxidation and cysteinyl protein modification (Hastings et al., 1996; Rabinovic et al., 2000). Although the catechol ring of DOPAC also oxidizes similarly to DA and leads to quinone modification of cysteinyl residues, an intrastriatal injection of DOPAC did not lead to a focal loss of striatal TH-ir (Fig. 15B, C), demonstrating that high levels of extracellular DOPAC are not toxic to the dopaminergic terminals.

A

DA → DA Quinone → 5-Cys-DA

DOPAC → DOPAC Quinone → 5-Cys-DOPAC

B

Dopaminergic terminal

DAT

Synapse

Postsynaptic membrane
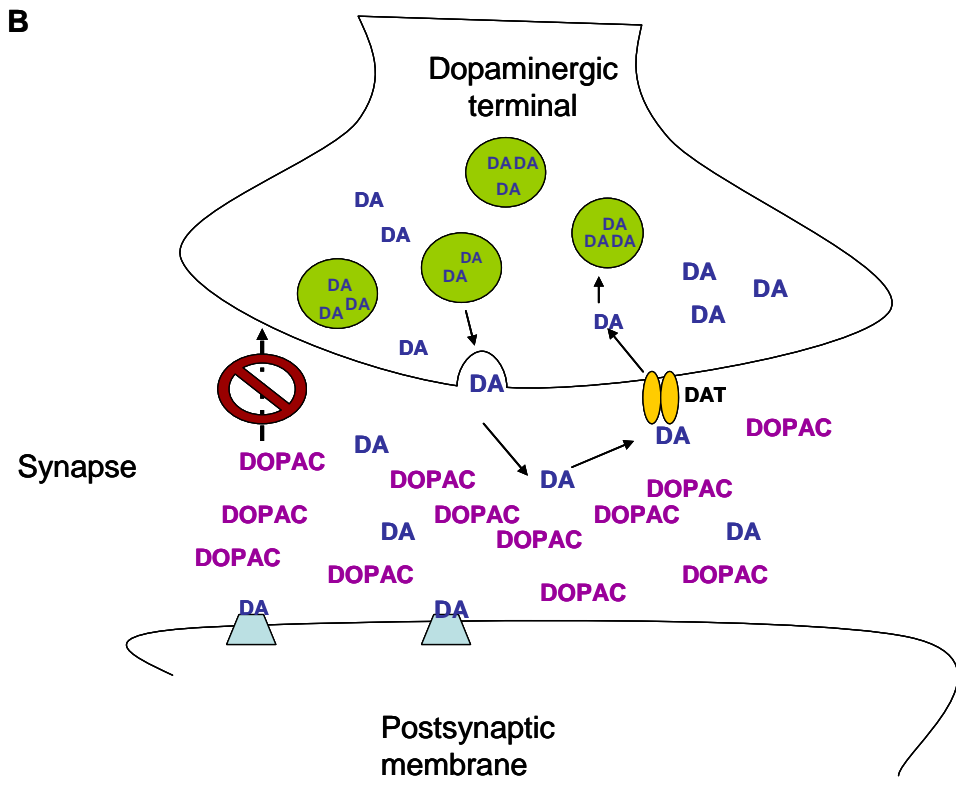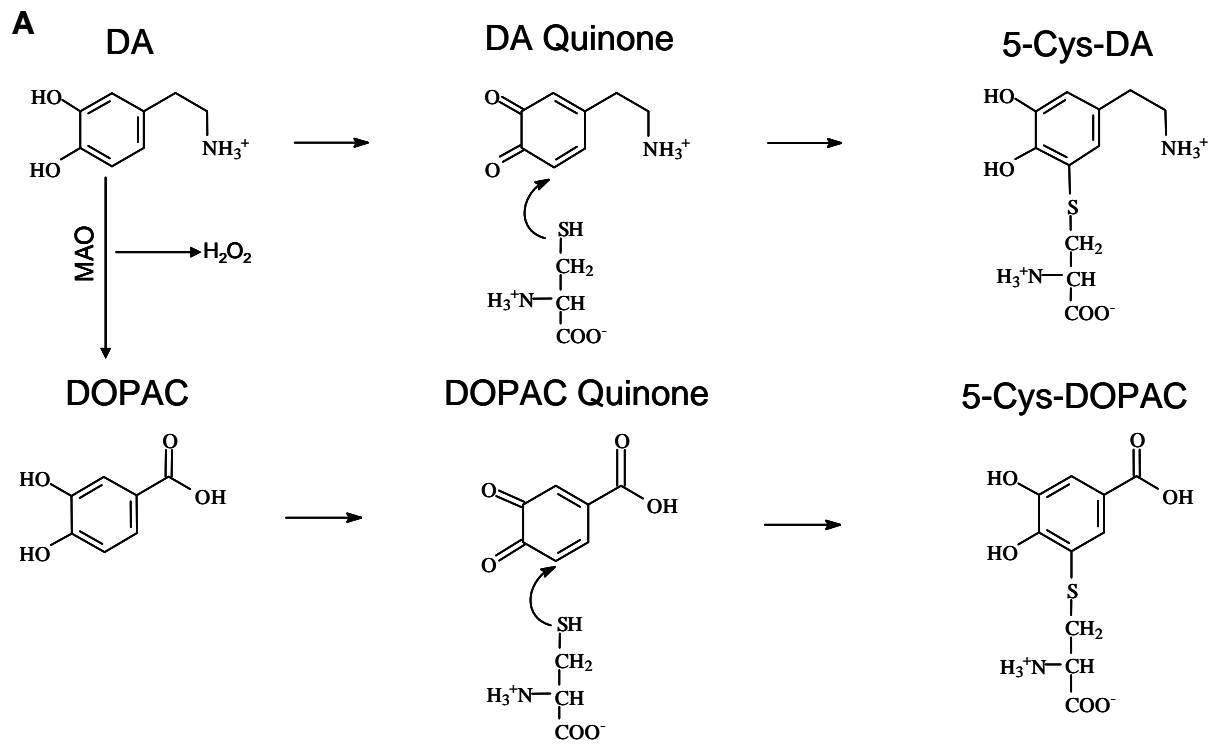
Figure 14: Pathways of DA and DOPAC oxidation. Normal metabolism of DA into DOPAC by MAO results in the production of H_2O_2, which itself is an ROS, but can also lead to the production of the highly reactive hydroxyl radical. *A*. Both DA and DOPAC can form the reactive quinone. The DA and DOPAC quinones are susceptible to attack by nucleophiles such as thiols which are normally found on cysteines. Covalent modification of cysteinyl residues on proteins results in 5-Cys-DA or 5-Cys-DOPAC, and can render the protein inactive, leading to cellular damage. *B*. While exogenous DA undergoes these reactions extracellularly when released and intracellularly when taken up by DAT, exogenous DOPAC is mainly oxidized extracellularly because it is not a substrate for DAT.

To determine whether extracellular oxidative stress from an intrastriatal DOPAC injection led to BBB disruption despite not causing dopaminergic neurodegeneration, striatal IgG content was examined immunohistochemically. Since IgG is not normally found in rat striatum under basal conditions (Yoshimi et al., 2002), infiltration of IgG is indicative of a break in the BBB. An intrastriatal DA injection led to an 8.8-fold increase in IgG-ir over the contralateral, non-injected striatum (Fig. 16A, C). Similarly, an intrastriatal DOPAC injection led to a 9.5-fold increase in IgG-ir over the non-injected (control) striatum (Fig. 16B, C), indicating that intrastriatal DOPAC caused disruption of the BBB to a similar extent as an intrastriatal DA injection, despite the absence of dopaminergic terminal damage.
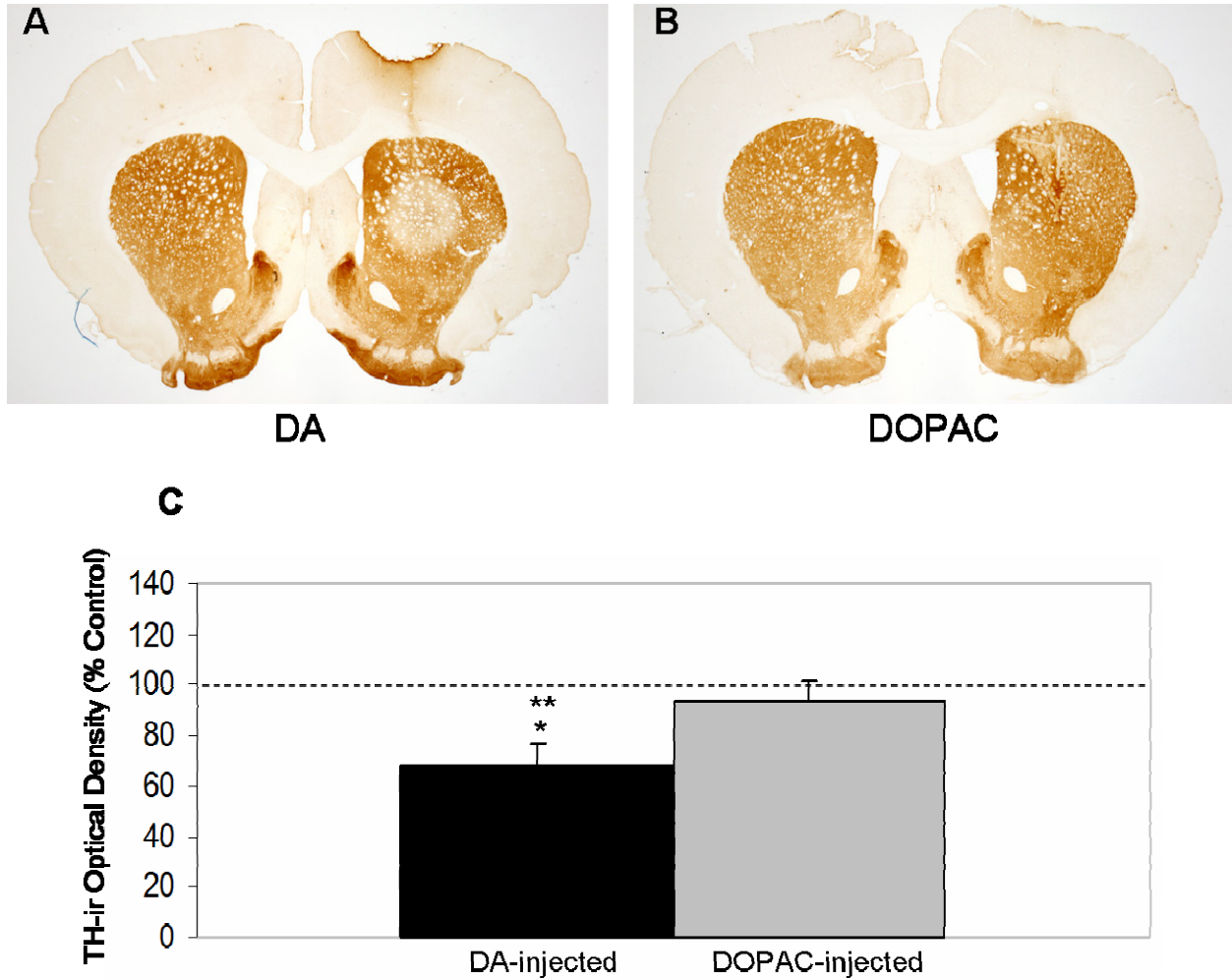
Figure 15: The effect of an intrastriatal DOPAC injection on TH-ir. At 1w following an intrastriatal *A.* **DA injection (0.4μmol) or** *B.* **DOPAC injection (0.4μmol), TH-ir was evaluated in striatum.** *C.* **Optical density measurements from the injected striatum (right) were compared to the contralateral, uninjected (control) striatum (left). Comparisons were also made between DA-injected and DOPAC-injected striata. Data are expressed as percent of control, (Mean ± SEM; n=4-6), *p<0.05, significantly different from non-injected striatum; **p<0.05, significantly different from DOPAC-injected striatum.**
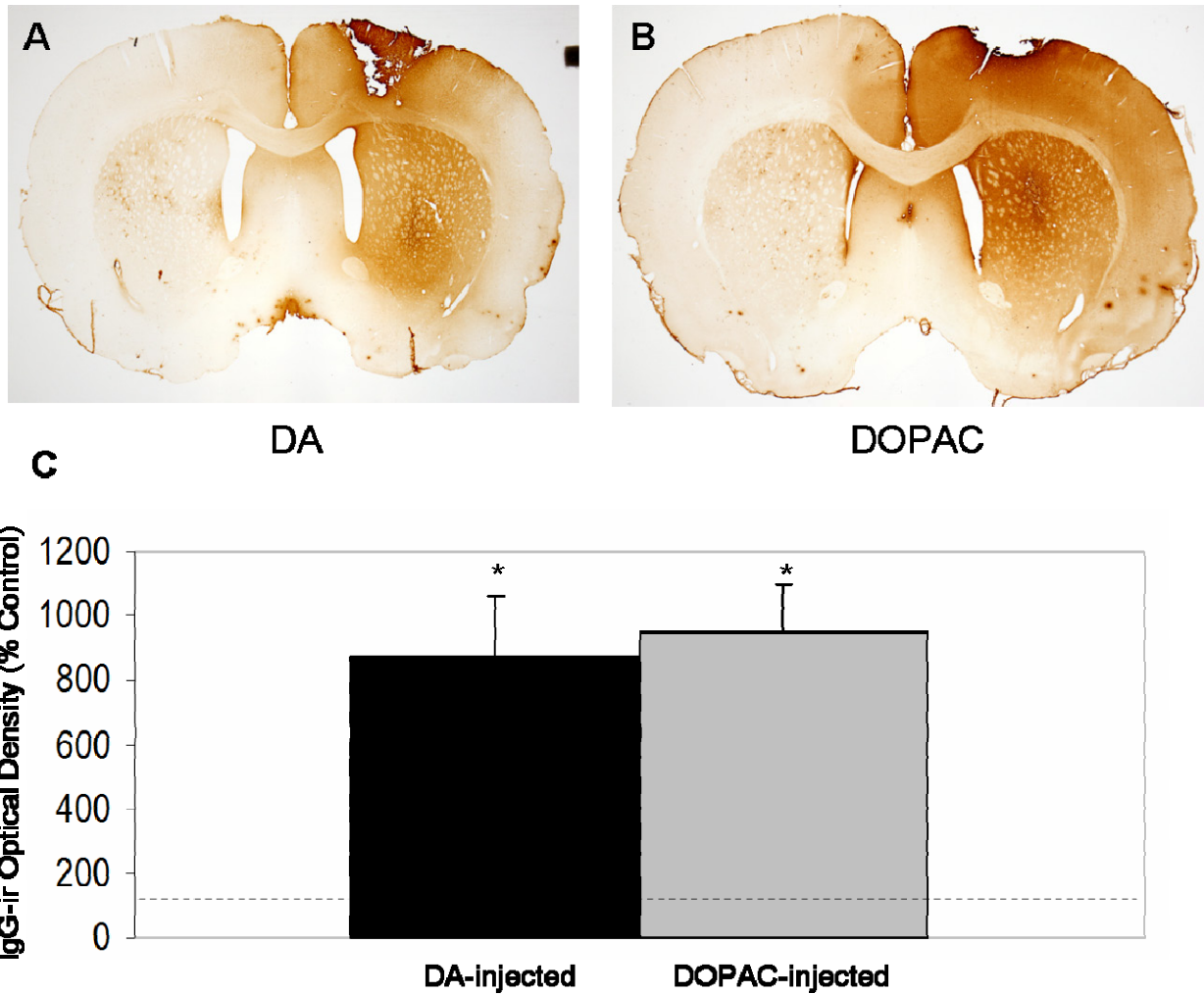
Figure 16: Effect of an intrastriatal DOPAC injection on BBB integrity. At 1w following an intrastriatal *A.* **DA injection (0.4μmol) or** *B.* **DOPAC injection (0.4μmol), IgG-ir was evaluated in striatum.** *C.* **Optical density measurements from the injected striatum (right) were compared to the non-injected, contralateral (control) striatum (left) to determine differences in IgG-ir. Optical density measurements were also compared between DA- and DOPAC-injected striata. Data are expressed as percent of control, (Mean ± SEM; n=4-6), *p<0.05, significantly different from non-injected striatum; **p<0.05, significantly different from DA-injected striatum.**

Microglial activation can occur in response to oxidative stress and exposure to the DAQ (Raivich et al., 1999; Kuhn et al., 2006). Despite the absence of dopaminergic terminal degeneration following an intrastriatal DOPAC injection, we examined whether DOPAC would result in microglial activation. As shown previously in Chapter 2, there was an increase in microglial activation in the striatum 1w following a DA injection (Fig. 17A). This occurred at the site of the TH-ir lesion, and resulted in a loss of microglial contact inhibition, bulbous cell bodies, and retracted processes. Intrastriatal DOPAC also led to an increase in microglial activation, but increased OX-42-ir occurred primarily at the injection site (Fig. 17B). Optical density measurements revealed an increase in OX-42-ir of 10.1-fold in the DA-injected striatum when compared to the non-injected striatum (Fig. 17C), whereas an intrastriatal DOPAC injection resulted in an increase in OX-42-ir 3.3-fold above the non-injected (control) striatum (Fig. 17C). Despite a significant difference in the overall density of OX-42-ir following a DOPAC injection, microglial morphology was still indicative of activation with bulbous cell bodies and enlarged processes (data not shown). Results from this study indicate that a DOPAC injection leads to an increase in IgG-ir and OX-42-ir but not to a loss in TH-ir, indicating that BBB disruption and microglial activation are not dependent on dopaminergic terminal degeneration.
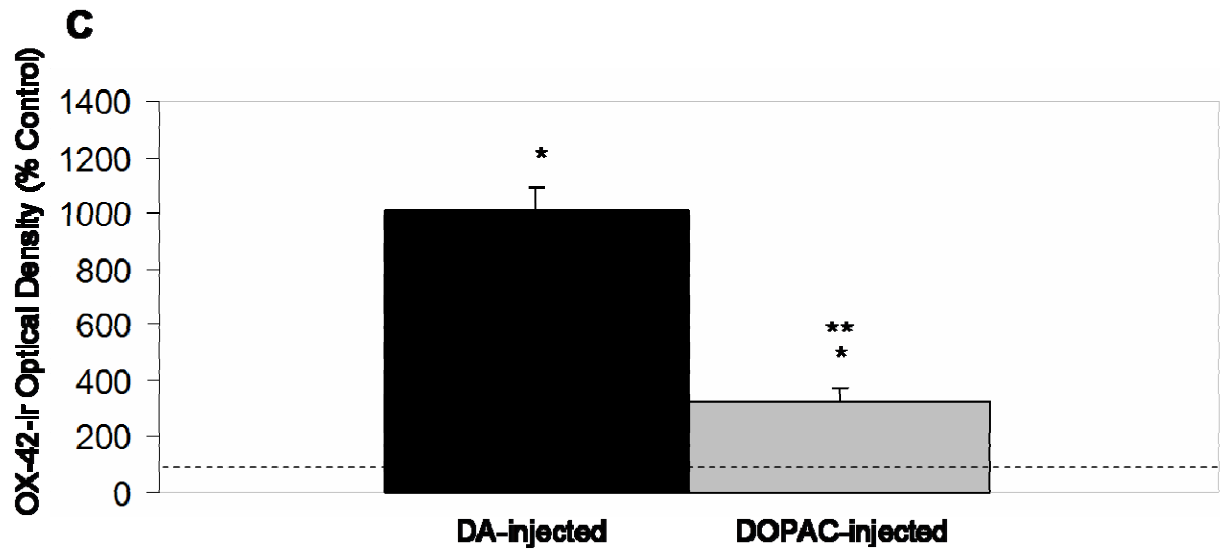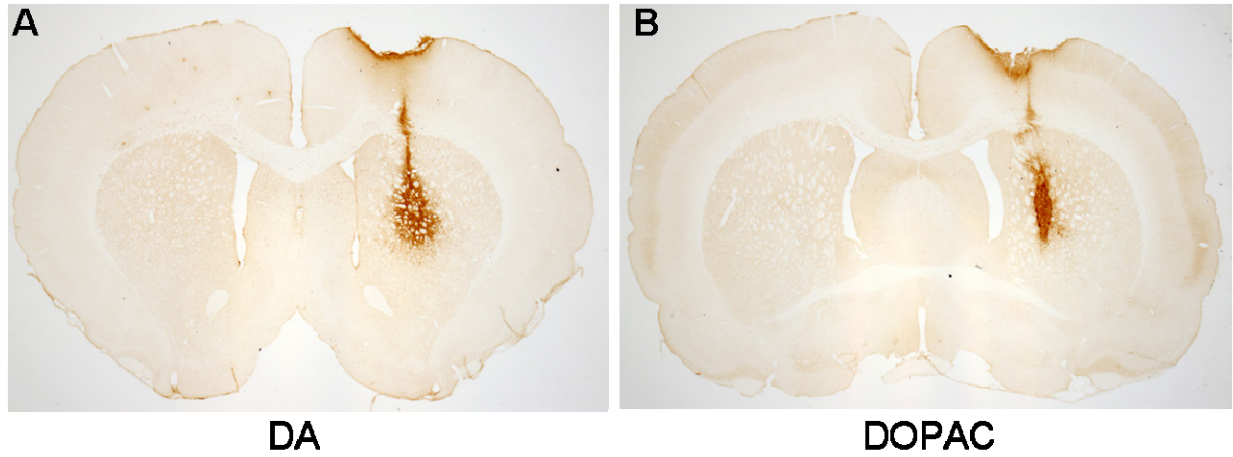
Figure 17: At 1w following an intrastriatal *A*. DA injection (0.4μmol) or *B*. DOPAC injection (0.4μmol), OX-42-ir was evaluated in striatum. *C*. Optical density measurements from the injection striatum (right) were compared to optical density measurements from the contralateral, non-injected (control) striatum (left) to determine differences in OX-42-ir. Optical density measurements were also compared between the DA- and DOPAC-injected striata. Data are expressed as percent of control (Mean ± SEM; n=4-6), *p<0.05, significantly different from non-injected striatum; **p<0.05, significantly different from DA-injected striatum.

3.3.2 The effect of systemic NAC on intrastriatal DA-induced BBB disruption.

An intrastriatal DOPAC injection led to increased IgG-ir but no loss of TH-ir, indicating that extracellular oxidation is a sufficient stimulus to cause BBB disruption regardless of dopaminergic terminal degeneration. This suggests that a mechanism by which intrastriatal DA may contribute to BBB disruption via oxidative stress. Protection by systemic NAC administration is dependent on brain penetration to increase free thiol levels. Systemic NAC (500mg/kg/i.p.) had been previously shown in the Hastings lab to increase free thiol levels in the striatum 2-fold above control at 2h following an intrastriatal DA injection. However, this occurred only in the injected striatum, suggesting disruption of the BBB which would allow NAC to enter the brain in this specific region. Therefore, using the same paradigm, we examined the effects of systemic NAC (500mg/kg/i.p.) on DA-induced disruption of the BBB. There was no difference in the loss of TH-ir between the DA and DA + systemic NAC groups (Fig. 18). Additionally, there was no difference in striatal IgG-ir between the DA and DA + systemic NAC groups (Fig. 19), indicating that systemic treatment with NAC had no effect on DA-induced dopaminergic terminal degeneration or BBB disruption.

Striatal free thiol levels were evaluated to determine if there was adequate brain penetration of NAC in this model. Free thiol levels were examined 2h following a DA injection because previous data from our lab revealed increased NAC-induced free thiol levels at this time point. Treatment with NAC (500mg/kg/i.p.) did not affect free thiol levels in the DA-injected or non-injected striatum compared to the saline-treated group (Fig. 20). It was possible that NAC was not adequately penetrating the brain due to extensive first-pass metabolism. Therefore, in an attempt to bypass metabolic interference, striatal free thiol levels were evaluated 2h following an intrastriatal DA injection in rats treated with an increased dose of NAC (1000mg/kg/i.p.). Again,

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free thiol levels did not differ between the DA-injected and non-injected striata in this treatment group and there were also no differences in free thiol levels from DA-injected striatum between saline- and NAC-treated groups (Fig. 20). These data suggested that 1000mg/kg NAC administered i.p. may not have been a high enough dose to bypass first-pass metabolism and achieve adequate brain penetration necessary for NAC to increase striatal free thiol levels. To examine whether or not NAC was present at a high enough concentration in striatum to exert an effect, DA oxidation products, protein cysteinyl catechols, were measured 2h following a DA injection. Systemic treatment with NAC (500mg/kg/i.p. or 1000mg/kg/i.p.) also did not change protein cysteinyl catechol levels in the DA-injected striatum 2h following a DA injection (data not shown).
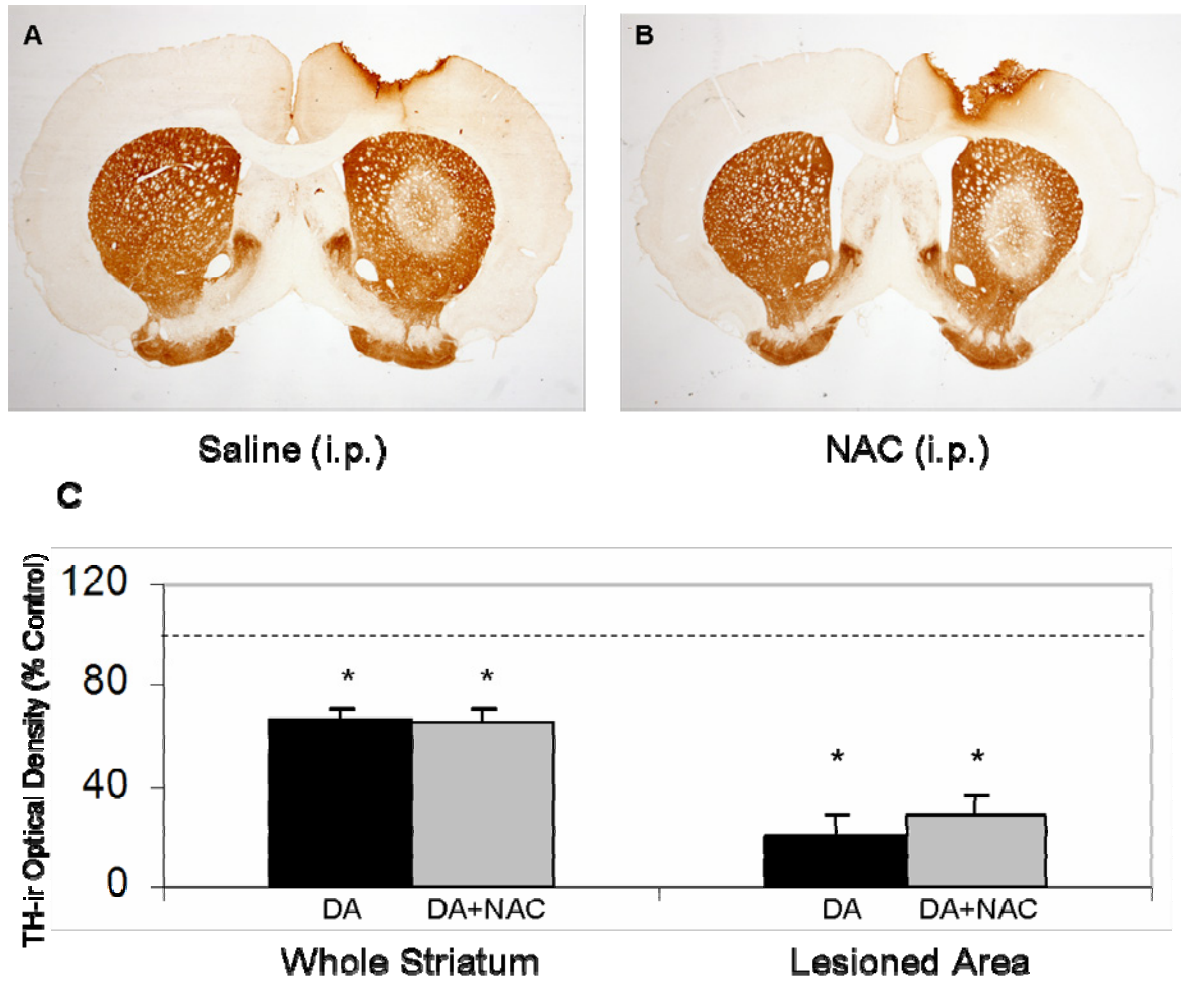
Figure 18: The effect of systemically administered NAC on DA-induced dopaminergic terminal degeneration. At 1w following an intrastriatal DA injection (0.4μmol), striatal TH-ir was evaluated in *A*. DA + saline- and *B*. DA + NAC-treated groups. *C*. Optical density measurements from either the whole striatum or the lesioned area from the DA-injected striatum (right) were compared to contralateral, non-injected (control) striatum (left) to determine differences in TH-ir. Optical density measurements from the injected striatum were also compared between DA + saline and DA + NAC treatment groups. Data are expressed as percent of control, (Mean ± SEM; n=4), *p<0.05, significantly different from non-injected striatum.
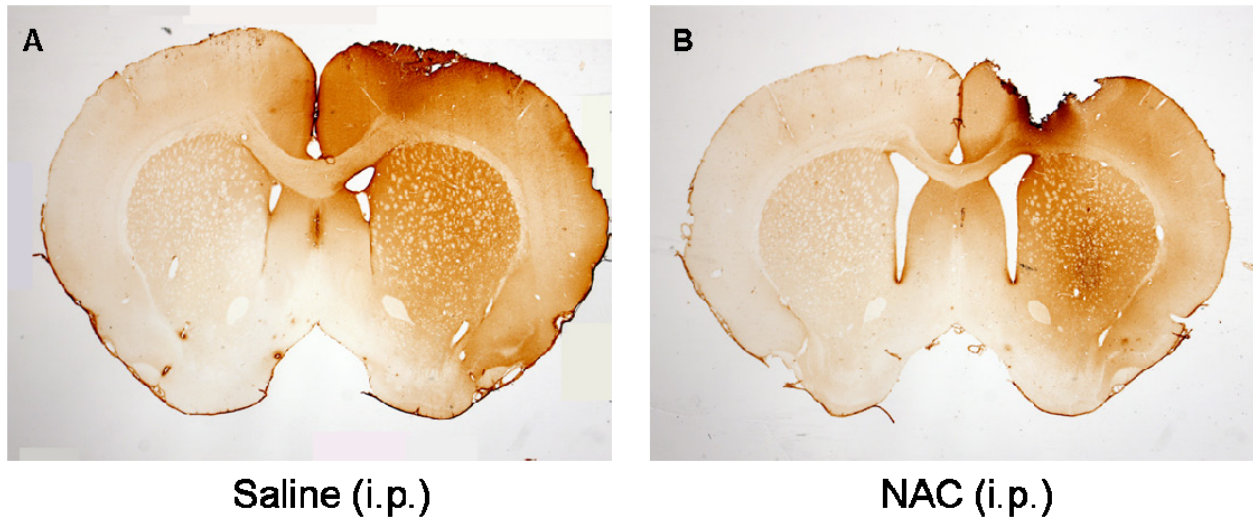
Figure 19: The effect of systemically administered NAC on DA-induced BBB disruption. At 1w following an intrastriatal DA injection (0.4μmol), striatal IgG-ir was evaluated in *A*. DA + saline and *B*. DA + NAC treatment groups. *C*. Optical density measurements from DA-injected striatum (right) were compared to contralateral, non-injected (control) striatum (left). Optical density measurements from the DA-injected striatum were also compared between DA + saline and DA + NAC groups. Data are expressed as percent of control, (Mean ± SEM; n=4), *p<0.05, significantly different from non-injected striatum.
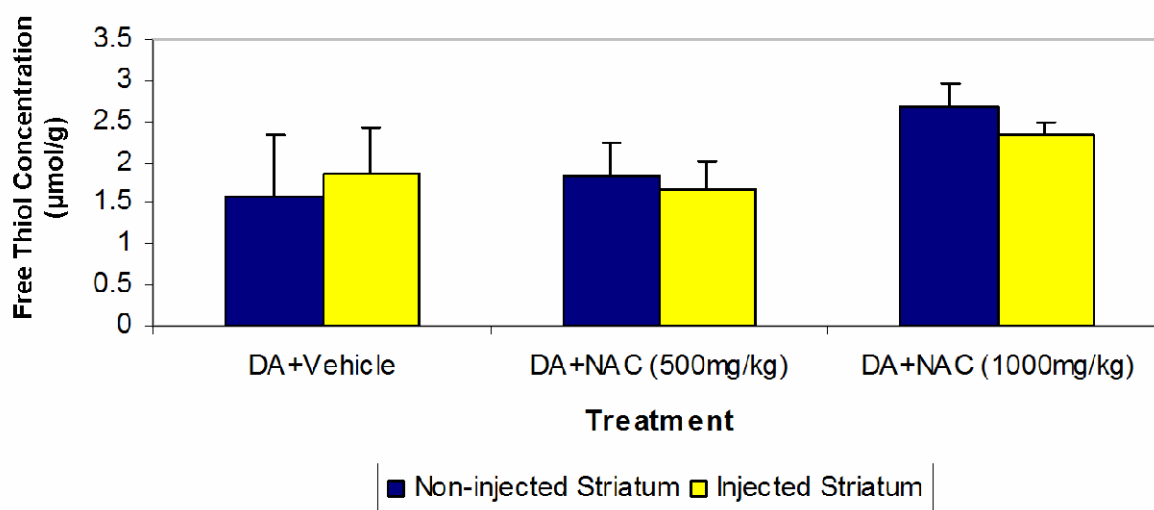
Figure 20: The effect of systemically administered NAC on free thiol levels. Free thiol levels were measured 2h following an intrastriatal DA (0.4μmol) injection in rats treated systemically with saline (i.p.), 500mg/kg NAC (i.p.), or 1000mg/kg NAC (i.p.). Differences were determined by comparing free thiol level concentrations from the DA-injected and non-injected striatum. Free thiol concentrations were also compared between treatment groups. Data are expressed as Mean ± SEM (n=4-6), p<0.05.

3.3.3 The effect of intrastriatal co-injection of DA+NAC on BBB integrity.

Because NAC administered systemically at either dose did not increase striatal free thiol levels or decrease protein cysteinyl catechol formation 2h following a DA injection, it was likely that NAC in this paradigm did not achieve adequate brain concentrations to exert protection. Therefore, an alternative approach was taken to examine the role of DA-induced oxidative stress on BBB disruption. Co-injection of equimolar DA+NAC was used to examine the effects of a thiol antioxidant on DA-induced damage. An intrastriatal DA injection led to a 35% loss of TH-ir in the DA-injected striatum when compared to the contralateral, non-injected striatum (Fig. 21A, D). However, at 1w following intrastriatal co-injection of DA+NAC, no focal loss of TH-ir

was observed and no significant difference in density of TH-ir between DA+NAC-injected and non-injected striatum was detected (Fig. 21B, D). An intrastriatal NAC injection (0.4µmol) alone also did not significantly alter TH-ir as compared to the non-injected striatum (Fig. 21C, D).

Figure 21: The effect of intrastriatal NAC on DA-induced dopaminergic terminal degeneration. Rats were injected intrastriatally with *A*. DA (0.4µmol), *B*. DA+NAC (0.4µmol), or *C*. NAC (0.4µmol) and striatal TH-ir was examined at 1w. *C*. Optical density measurements from the DA-, DA+NAC-, or NAC-injected striatum (right) were compared to the contralateral, non-injected (control) striatum (left) to determine differences in TH-ir. Differences in optical density of TH-ir were also examined between DA-, DA+NAC-, and NAC-injected striata. Data are expressed as percent of control, (Mean ± SEM; n=4),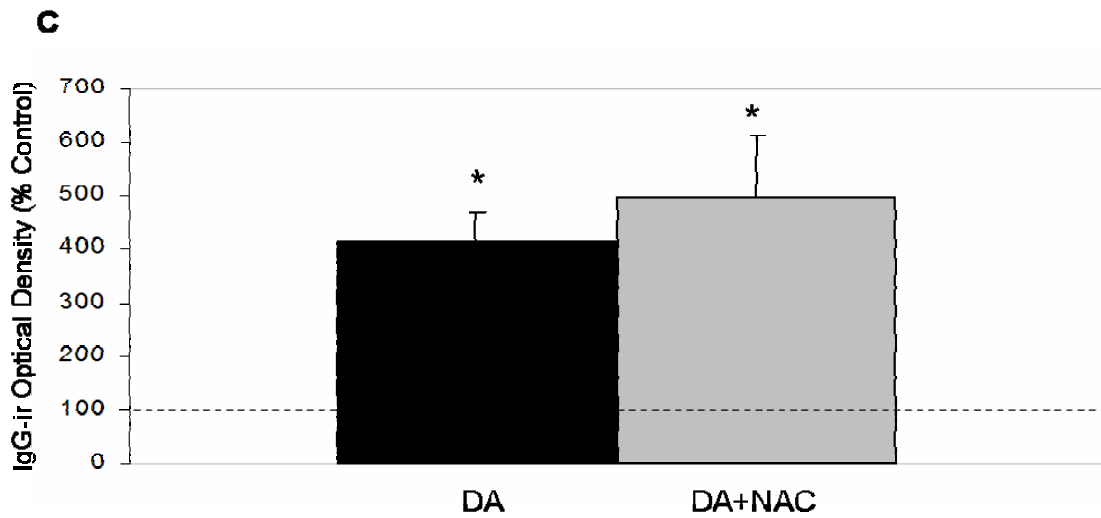 *p<0.05, significantly different from non-injected striatum; **p<0.05, significantly different from DA+NAC- and NAC-injected striatum.

Striatal IgG-ir was evaluated to determine the effect of NAC on DA-induced disruption of the BBB. At 1w following an intrastriatal DA injection, striatal IgG-ir was increased 9.1-fold over contralateral, non-injected striatum (Fig. 22A, D). In contrast, an intrastriatal co-injection of DA+NAC resulted in only a 4.3-fold increase in IgG-ir compared to non-injected striatum (Fig. 22B, D). Thus striatal IgG-ir following intrastriatal DA+NAC was decreased by 53% compared to an intrastriatal injection of DA alone (Fig. 22D). Intrastriatal injection of NAC alone also led to a 4.9-fold increase in IgG-ir in the injected striatum compared to non-injected striatum (Fig. 22C, D), similar to the DA+NAC group. Therefore co-injection of NAC with DA significantly reduced damage to the BBB resulting from a DA injection, but intrastriatal NAC also caused damage of the BBB on its own, perhaps accounting for the presence of IgG-ir following an intrastriatal DA+NAC injection.
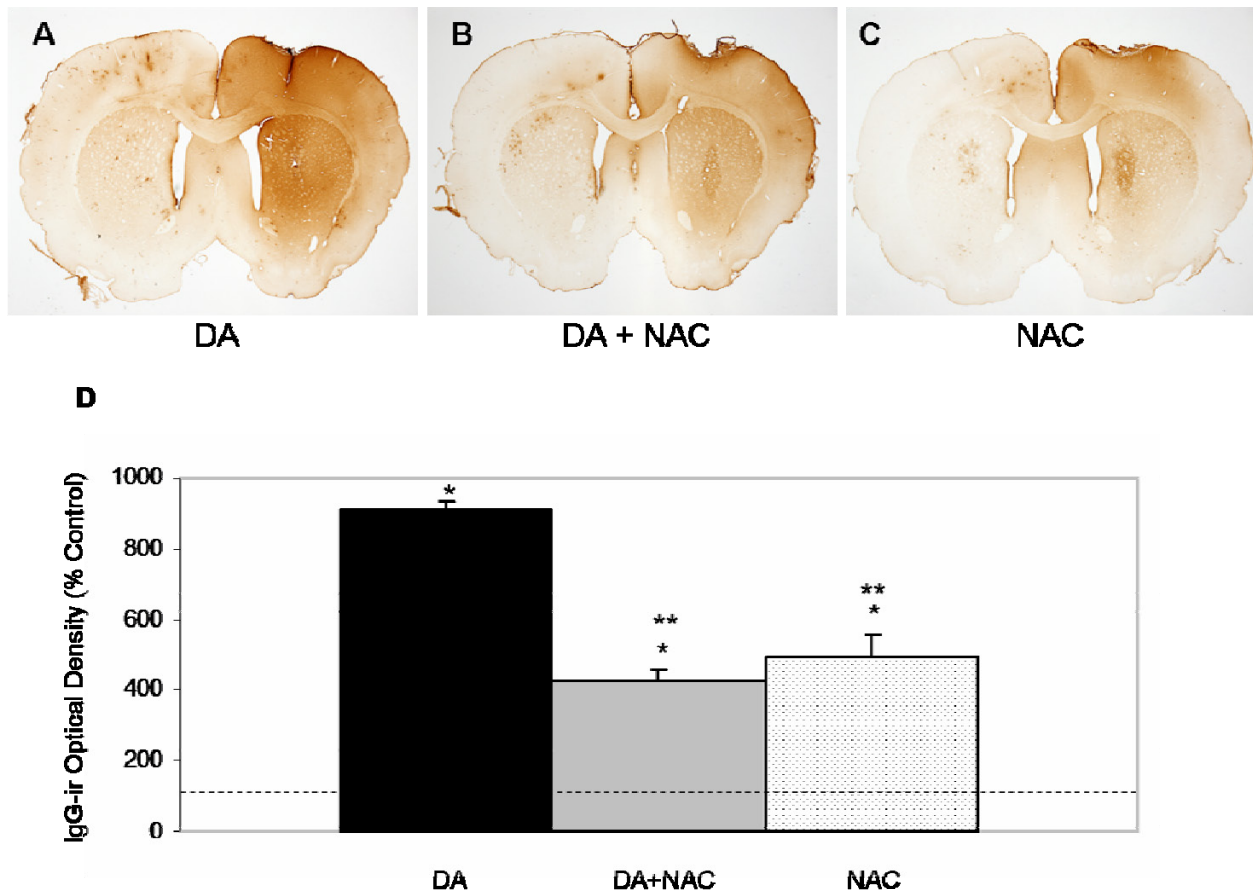
Figure 22: The effect of intrastriatal NAC on DA-induced BBB disruption. Rats were injected intrastriatally with *A.* **DA (0.4μmol),** *B.* **DA+NAC (0.4μmol), or** *C.* **NAC (0.4μmol) and striatal IgG-ir was examined at 1w.** *C.* **Optical density measurements from the injected striatum (right) were compared to the contralateral, non-injected (control) striatum (left) to determine differences in IgG-ir. Optical density measurements from the DA-, DA+NAC-, and NAC-injected striata were also compared between groups. Data are expressed as percent of control (Mean ± SEM; n=4), *p<0.05, significantly different from non-injected striatum; **p<0.05, significantly different from DA-injected striatum.**

Because oxidative stress can lead to neuroinflammation, which may further enhance the production of ROS, microglial activation was examined at 1w following intrastriatal co-injection of DA+NAC. An intrastriatal DA injection led to a 7.3-fold increase in OX-42-ir compared to the non-injected striatum (Fig. 23A, D) and the area of OX-42-ir encompassed the area associated with TH-ir loss. Intrastriatal DA+NAC resulted in a 4.7-fold increase in OX-42-ir as compared to the non-injected striatum (Fig 23B, D), which was significantly attenuated (-37%) compared the DA-injected striatum (Fig. 23D). An intrastriatal NAC injection alone also led to increased OX-42-ir (5.1-fold) compared to the non-injected striatum (Fig. 23C, D). Again, this may account for the increase in OX-42-ir observed with the DA+NAC co-injection and is consistent with the previous observation showing NAC-induced damage to the BBB. These results indicate that NAC partially protects against DA-induced microglial activation.
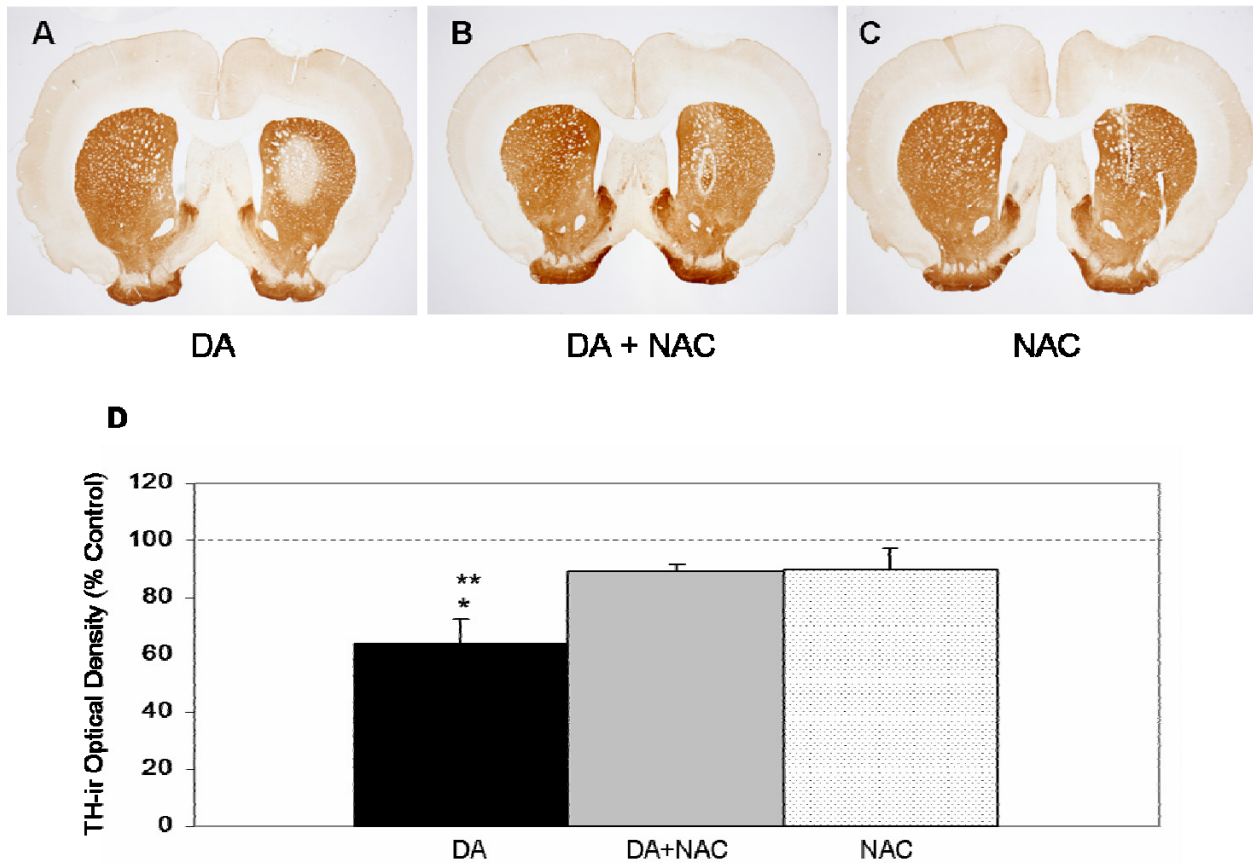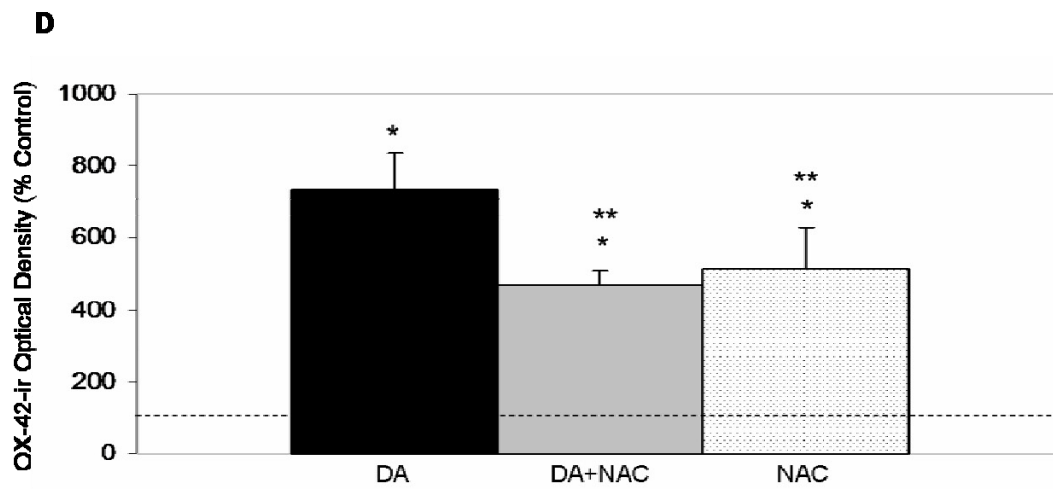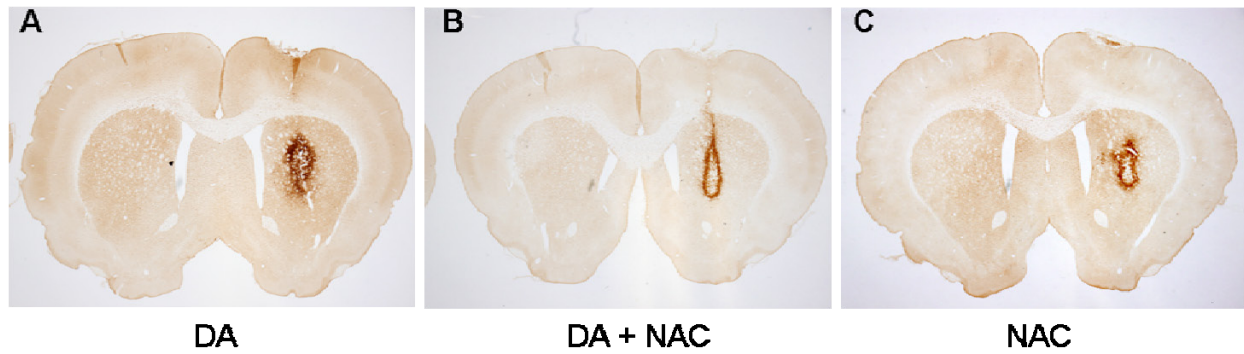
Figure 23: The effect of intrastriatal NAC on DA-induced microglial activation. Rats were injected intrastriatally with *A.* **DA (0.4μmol),** *B.* **DA+NAC (0.4μmol), or** *C.* **NAC (0.4μmol) and striatal OX-42-ir was examined at 1w.** *D.* **Optical density measurements from injected striatum (right) were compared to contralateral, non-injected (control) striatum (left). Optical density measurements were also compared between DA-, DA+NAC-, and NAC-injected striata to determine differences in OX-42-ir. Data are expressed as percent of control, (Mean ± SEM; n=4), *p<0.05, significantly different from non-injected striatum; p<0.05, significantly different from DA-injected striatum.**

3.4   DISCUSSION

Previous studies have shown that an intrastriatal DA injection leads to oxidative stress-induced degeneration of dopaminergic terminals in striatum (Hastings et al., 1996; Rabinovic et al., 2000). It was also previously shown that intrastriatal DA also leads to BBB disruption and microglial activation (Chapter 2). However, the roles of oxidative stress and dopaminergic terminal degeneration in DA-induced BBB disruption have yet to be examined. Therefore, we examined the effects of intrastriatal DOPAC, which leads to extracellular DOPAC oxidation (Fig. 14). Results revealed that while extracellular oxidation does not lead to dopaminergic neurotoxicity, it may lead to disruption of the BBB, supporting a possible role for oxidative stress-induced BBB disruption. Data from this study also indicated that the antioxidant NAC is partially protective against DA-induced disruption of the BBB, further supporting the hypothesis that oxidative stress is a mechanism of DA-induced BBB disruption.

DOPAC is not a substrate for the DAT and does not lead to dopaminergic terminal degeneration. Therefore an intrastriatal injection of DOPAC was used as a tool to separate oxidative stress-induced BBB disruption from the loss of dopaminergic terminals. An intrastriatal injection of DOPAC, unlike DA, did not lead to a striatal lesion, determined by no loss of TH-ir. This finding supports evidence from our lab that intracellular, as opposed to extracellular, DA oxidation is primarily responsible for DA-induced dopaminergic neurotoxicity. In DAT knockout mice, an intrastriatal injection of DA was not toxic to dopaminergic terminals and did not lead to a loss of striatal TH-ir (unpublished observations by J.G. of the Hastings Lab). Additionally, inhibition of both DAT and the norepinephrine transporter prevented DA-

induced toxicity to PC-12 and SH-SY5Y cells (unpublished observations from the Hastings Lab), supporting a role for intracellular oxidation leading to DA-induced degeneration of the dopaminergic terminals.

Despite the observation that DOPAC was not toxic to striatal terminals, an intrastriatal injection of DOPAC did lead to disruption of the BBB similarly to an intrastriatal DA injection. Damage to the integrity of the BBB from an intrastriatal DOPAC injection was likely due to extracellular oxidative stress. Since oxidative stress and quinone formation (Streit et al., 1999; Qin et al., 2004; Kuhn et al., 2006) as well as BBB disruption (Nimmerjahn et al., 2005) can lead to a neuroinflammatory response, microglial activation was examined following a DOPAC injection. Intrastriatal DOPAC led to an increase in microglial activation at the site of the injection cannula, indicating that an inflammation response was elicited by the DOPAC injection.

To further examine the role of oxidative stress in dopaminergic neurotoxin-induced BBB disruption, the effects of a thiol antioxidant on DA-induced BBB disruption were evaluated. NAC (500mg/kg/i.p.) had been previously observed in our lab to increase free thiol content in the injected striatum 2h following intrastriatal DA (unpublished observation by M.G.S. of the Hastings Lab). For NAC to exert its function and increase free thiol levels, it must have access to the brain, suggesting BBB disruption at this time point following intrastriatal DA since NAC does not readily cross an intact BBB. However in the current paradigm, systemic administration of NAC at 2 different doses (500mg/kg/day and 1000mg/kg/day) did not increase free thiol levels in striatum and also did not reduce protein cysteinyl-catechol formation, indicating that adequate brain penetration was not achieved for NAC to exert its antioxidant effect in the current paradigm. Not surprisingly, systemic administration of NAC also did not lead to a reduction in

either the DA-induced striatal lesion size (as measured by TH-ir) or DA-induced disruption of the BBB (as measured by IgG-ir). It has been previously reported that NAC (100mg/kg/s.c.) treatment protected against intraventricular 6-OHDA-induced loss of striatal and nigral TH-ir (Munoz et al., 2004) and a previous unpublished study from our lab found a reduction in striatal lesion size following intrastriatal DA with systemic treatment of NAC (500mg/kg/i.p.; unpublished observations from M.G.S. of the Hastings lab). However, this neuroprotection could not be replicated in the current experiment, possibly due to differences in rat vendors from the previous experiment compared to the current experiment and/or differences in the compound ingredients. NAC undergoes extensive first-pass metabolism and therefore achieving adequate brain concentration for NAC to exert its antioxidant effect was unlikely. In an attempt to address this issue, a dose of 1000mg/kg was administered but this was also not successful.

Thus, a different approach to the administration of NAC was taken and NAC was delivered directly to the striatum by intrastriatally co-injecting equimolar concentrations of DA+NAC. This route of administration, while having little clinical application, allowed NAC to act locally at the site of damage to evaluate the role of oxidative stress in BBB disruption following an intrastriatal DA injection. Co-injection of intrastriatal DA+NAC resulted in a protection against DA-induced dopaminergic terminal degeneration. To evaluate whether oxidative stress was also a mechanism by which DA leads to BBB disruption, IgG-ir was examined in striatum following a co-injection of DA+NAC. The co-injection resulted in partial protection against DA-induced BBB disruption by reducing IgG-ir by 53% compared to a DA-injection alone. These results further support the notion that DA-induced oxidative stress contributes to BBB disruption following a DA injection.

In endothelial cells that comprise the peripheral vasculature, oxidative stress from ROS such as superoxide and hydrogen peroxide as well as organic pesticides led to endothelial cell injury and death (Moutet et al., 1998). There is evidence that this occurs in endothelial cells that comprise the BBB, as well. BBB permeability was demonstrated *in vitro* following brain endothelial cell exposure to xenobiotics that promote ROS generation. This effect was dose-dependent and blocked when endothelial cells were pre-incubated with the antioxidants superoxide dismutase and catalase suggesting that ROS contributed to the BBB disruption (Lagrange et al., 1999). Furthermore, following exposure of endothelial cells to 2,3-dimethyoxy-1,4-naphthoquinone *in vitro*, endothelial cell function was altered and eventually lead to a decrease in cell viability (Krizbai et al., 2005). One of the factors that may render the endothelial cells of the BBB more sensitive to oxidative stress is that they have substantially increased mitochondrial content as compared to endothelial cells that make up the peripheral vasculature (Hawkins & Davis, 2005). Both oxidative stress and mitochondrial dysfunction has been implicated in PD pathogenesis (Beal, 2003; Dawson and Dawson, 2003; Greenamyre and Hastings, 2004) which may contribute to BBB permeability upon endothelial cell damage. Therefore, based on previous findings and results from the current study, it is likely that DA-induced oxidative stress plays a key role in DA-induced BBB disruption.

The fact that a co-injection of DA+NAC and injection of NAC alone led to increased IgG-ir compared to the non-injected striatum but did not result in dopaminergic terminal loss, provides further evidence for BBB disruption occurring separately from dopaminergic terminal degeneration. The mechanism by which intrastriatal DA+NAC and NAC alone leads to BBB disruption is not known, but it is possible that low pH of the intrastriatally injection solutions contributed to this observation. Systemically administered NAC does not cross the BBB and

intrastriatally administered NAC caused nominal damage to the BBB on its own, suggesting that the use of NAC to examine the role of oxidative stress in DA-induced BBB disruption may not be ideal. However, another thiol antioxidant that is known to cross the BBB, AD4, has been shown to be protective against dopaminergic neurotoxin-induced insults (Bahat-Stroomza et al., 2005), and may be an alternative to NAC for this examination.

Interestingly, co-administration of DA+NAC and intrastriatal NAC alone also led to increases in OX-42-ir. Microglial activation is an important component of the neuroinflammatory response which has been implicated in contributing to damage of several brain structures including neurons and the BBB. Oxidative stress can lead to microglial activation (Raivich et al., 1999; Kuhn et al., 2006) which then further contributes to oxidative stress via the release of ROS and reactive nitrogen species. Because DOPAC also led to an increase in microglial activation despite no dopaminergic terminal loss, it is possible that another mechanism by which dopaminergic neurotoxins lead to BBB disruption is via neuroinflammation. Several of the dopaminergic neurotoxins that have been reported in animal models to lead to disruption of the BBB also have an inflammatory component (Akiyama and McGeer, 1989; Sherer et al., 2003b; LaVoie et al., 2004; Thomas et al., 2004; Zhao et al., 2007), including the findings from this chapter and Chapter 2, which has been implicated in disruption of the BBB (Lynch et al., 2004). Further examination of neuroinflammation as a contributory factor is will be discussed in Chapter 4.

3.5   SUMMARY AND CONCLUSIONS

An intrastriatal DOPAC injection led to disruption of the BBB, likely due to extracellular oxidative stress. In addition, intrastriatal co-administration of DA and the antioxidant NAC led to partial protection of BBB disruption compared to disruption from an intrastriatal DA injection alone. Additionally, intrastriatal DOPAC and DA+NAC led to disruption of the BBB but did not cause dopaminergic neuronal degeneration, which indicates that BBB disruption likely occurs independently of dopaminergic terminal degeneration in these models. These lines of evidence support a role for oxidative stress in dopaminergic neurotoxin-induced BBB disruption. The findings are summarized in Appendix E.

4.0 THE ROLE OF DOPAMINE-INDUCED NEUROINFLAMMATION IN BLOOD-BRAIN BARRIER DISRUPTION

4.1 INTRODUCTION

Neuroinflammation is a pathological characteristic of several neurodegenerative disorders such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease (PD). Evidence for neuroinflammation in these disorders comes from observations of reactive glia, activated microglia in particular, in the affected areas (Woodroofe et al., 1986; McGeer et al., 1988). Microglial activation results from a variety of insults and is the primary immune response in the brain. Upon activation, microglia lose contact inhibition, proliferate, undergo morphological changes, migrate to the site of damage, and release cytotoxic and beneficial factors (Streit et al., 1999; Streit, 2002; Nimmerjahn et al., 2005).

Microgliosis in the substantia nigra has been observed in PD, which is characterized by a loss of nigrostriatal dopaminergic projection neurons (McGeer et al., 1988; Teismann et al., 2003; Hirsch et al., 2005; Nagatsu and Sawada, 2005; Sawada et al., 2006). Increased microglial activation is associated with an increase in nitric oxide production, proinflammatory cytokine production, reactive oxygen species (ROS) production, and increases in other harmful mediators of inflammation-induced toxicity. Evidence exists for increases in such factors when compared to age-matched controls in the substantia nigra and caudate/putamen of post-mortem PD tissue

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(Boka et al., 1994; Nagatsu et al., 2000; Hunot and Hirsch, 2003; Perry, 2004; Imamura et al., 2005; Nagatsu and Sawada, 2005). Additionally, i*n vivo* positron emission tomography studies of PD patients revealed changes in microglial activation which correlated with dopaminergic terminal loss in the midbrain (Ouchi et al., 2005). Thus, evidence suggests that microglial activation plays a role in degeneration of dopaminergic neurons in PD. Support for an aberrant role of microglial activation in dopaminergic neurodegeneration is demonstrated by the protective effect of the anti-inflammatory agent, minocycline. As an anti-inflammatory compound, minocycline acts to decrease microglial activation, likely through inhibition of the inflammation promoter, poly(ADP-ribose) polymerase-1 (Alano et al., 2006). Systemically administered minocycline decreased microglial activation and increased dopaminergic cell survival following treatment with the dopaminergic neurotoxins MPTP (Du et al., 2001; Wu et al., 2002), rotenone (Casarejos et al., 2006), and lipopolysaccharide (LPS) (Tomas-Camardiel et al., 2004).

Activated microglia may contribute to cellular toxicity via ROS production, particularly through NADPH oxidase activation. The microglial NADPH oxidase is comprised of cytosolic subunits (p47^{phox}, p67^{phox}, p40^{phox}, and the G-protein Rac) and a membrane protein complex known as cytochrome b$_{558}$ (p22^{phox} and gp91^{phox}). Upon microglial activation from pro-inflammatory stimuli, signaling events occur to promote phosphorylation of p47^{phox} and p67^{phox} and subsequent binding of the cytosolic components. The cytosolic complex then translocates to the membrane where p47^{phox} binds to gp91^{phox}, initiating an electron flow, catalyzing the generation of superoxide. Apocynin is a methoxy-substituted catechol that is known to inhibit NADPH oxidase oxidation, possibly by interfering with binding of p47^{phox} to cytochrome b$_{558}$ (Stolk et al., 1994; Johnson et al., 2002; Kanegae et al., 2007). Treatment with apocynin is

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protective following ischemia- (Wang et al., 2006) and traumatic brain injury-related (Lo et al., 2007) neuronal degeneration *in vivo*, as well as following toxin-induced dopaminergic degeneration both *in vivo* and *in vitro* (Gao et al., 2003a; Gao et al., 2003b; Choi et al., 2005; Anantharam et al., 2007; Rodriguez-Pallares et al., 2007). ROS production, in turn, can also lead to the activation of microglia (Raivich et al., 1999; Kuhn et al., 2006).

In PD, increased oxidative stress, possibly by dopamine (DA) oxidation to the DA quinone (DAQ), is a factor that may contribute to the vulnerability of dopaminergic neurons (Graham et al., 1978; Hastings et al., 1996). The DAQ is electron-deficient and therefore is susceptible to attack by nucleophiles, including thiol groups found on glutathione (GSH), free cysteine, and cysteinyl residues on proteins. Cysteines are often found at the active sites of proteins, and therefore, modification of proteins by DAQ could lead to protein inactivation/impairment and subsequent cellular damage. An intrastriatal injection of DA into rat results in the loss of TH-positive dopaminergic terminals of the striatum (Hastings et al., 1996; Rabinovic et al., 2000). The DA-induced lesion is dose-dependent and correlates with increases in free and protein cysteinyl catechols (Hastings et al., 1996; Rabinovic et al., 2000). Further, loss of dopaminergic terminals is prevented by co-injection with antioxidants (Hastings et al., 1996), providing evidence that oxidative stress is a mechanism by which DA acts as a neurotoxin. A recent study also showed that intrastriatal DA leads to microglial activation at the site of dopaminergic terminal degeneration (Chapter 2), providing evidence for neuroinflammation.

Many neurodegenerative diseases, as well as animal models for dopaminergic neurodegeneration, not only have a neuroinflammatory component, but also show evidence for blood-brain barrier (BBB) disruption. While neuroinflammation may contribute to

neurodegeneration, evidence also suggests that neuroinflammation may also be harmful to components of the BBB (Brooks et al., 2005; de Boer and Gaillard, 2006; Krizanac-Bengez et al., 2006). Suppression of microglial activation by minocycline was protective against neurotoxin-induced BBB disruption following ischemic injury (Yenari et al., 2006), MPTP treatment (Zhao et al., 2007), and intranigral LPS injection (Tomas-Camardiel et al., 2004) in rodents. Apocynin treatment prevented damage-induced BBB disruption following hypoxic and ischemic insult (Yenari et al., 2006; Kuhlmann et al., 2007) and traumatic brain injury (Lo et al., 2007) in rats, providing support for the contribution of neuroinflammation to BBB.

Previous data indicated that an intrastriatal DA injection led to increased microglial activation, which occurred concurrently with DA-induced BBB disruption and preceded dopaminergic neurodegeneration (see results from Chapter 2). To examine the effect of DA-induced microglial activation on the integrity of the BBB and dopaminergic neurodegeneration, rats were treated with either the anti-inflammatory agent, minocycline, or the NADPH oxidase inhibitor, apocynin. Results from this study provide evidence for neuroinflammation playing a role in dopaminergic neurotoxin-induced BBB disruption.

4.2   METHODS

Animals.

Male Sprague-Dawley rats (300g; Hilltop Laboratories, Allison Park, PA) were used throughout all experiments. The rats were kept in standard caging in a temperature- and light-controlled room that was on a 12h light/dark cycle. Food and water were available *ad libitum*. All experimental protocols were in accordance with the guidelines developed by the NIH Guide

to the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Surgical procedures.

Rats were anesthetized with Equithesin (3.0ml/kg/i.p.). An injection cannula was placed stereotaxically into the right striatum (Coordinates from Bregma: -2.7 mm medial/lateral and +0.8 mm anterior/posterior. Coordinates from dura: -5.0 mm dorsal ventral), and DA (0.4μmol DA/2μl sterilized water) was unilaterally injected into the right striatum over 20 min via microfusion injection pump (model 341B; Sage Instruments, Boston, MA). All injected solutions were made immediately prior to surgery and kept on ice with minimal light exposure.

At indicated time points following surgery, rats were perfused with ice cold 1% paraformaldehyde followed by 4% paraformaldehyde at a steady flow rate. Brains were rapidly removed, placed in 4% paraformaldehyde at 4°C for 24h, and then hydrated in 30% sucrose at 4°C for 48h. Fixed rat brains were sliced coronally (40μm) using a cryostat at -20°C. Tissue sections were placed serially into a 24-well plate with anti-freezing solution and stored at -20°C until further use. Animals sacrificed for biochemical analysis were perfused with saline at 4°C. Brains were rapidly removed and striatal tissue surrounding the injection site, as well as tissue from the contralateral, non-injected striatum were dissected using a 3mm biopsy punch, as previously described in Chapter 2. Tissue was then weighed, snap-frozen on dry ice, and stored at -80°C until analyzed.

Minocycline treatment.

Minocycline was made up fresh in sterile saline (vehicle) prior to each treatment. Animals (n=6) treated with minocycline (50mg/kg, i.p.) received treatment every 12h, 1d prior to and each day following an intrastriatal DA injection up until sacrifice, 1w following intrastriatal DA. The vehicle-treated control group (n=4) received an intrastriatal DA injection and vehicle (i.p.) on the same schedule as the minocycline-treated group. Within each treatment group, the contralateral striatum served as the within animal non-DA-injected control.

Apocynin treatment.

Apocynin was made up fresh daily in 0.01% dimethylsulfoxide and sterile saline (vehicle). The group of animals treated with apocynin (10mg/kg, i.p.; n=4) received treatment 30min prior to and every 24h following an intrastriatal DA injection until the day of sacrifice at 1w following DA. The vehicle-treated control group (n=4) received an intrastriatal DA injection and vehicle treatment (i.p.) on the same schedule as the apocynin-treated group. Within each treatment group, the contralateral, non-injected striatum served as the within-animal control.

Immunohistochemistry.

Mouse anti-CD11b (OX-42; 1:1000; Serotec, Raleigh, NC), rabbit anti-rat IgG (1:1000; Vector Laboratories, Burlingame, CA), and mouse anti-rat tyrosine hydroxylase (TH; 1:2000; Chemicon International, Temecula, CA) were used as primary antibodies for immunohistochemical detection. Specificity for each antibody has been previously described (see Chapter 2). Immunohistochemical processing was standardized by processing tissue sections from each animal within an experiment simultaneously to reduce variability of staining.

Striatal sections were immersed in 1% H_2O_2 to reduce endogenous peroxidase, followed by incubation with normal serum to reduce non-specific binding. Sections were then incubated with the primary antibody for 24h or 48h at 4°C, followed by the appropriate biotinylated secondary antibody and then processed by the avidin-biotin method (ABC method) using the Vectastain kit (Vector Laboratories, Burlingame, CA). Processed sections were visualized with diaminobenzidine (DAB), mounted onto slides, and coverslipped. Tissue sections were examined under a bright field microscope (BX-51, Olympus) or stereoscope (SX-61, Olympus America, Inc. Center Valley, PA).

Optical density measurements.

Optical density measurements of tissue sections processed for immunohistochemistry were obtained by outlining the entire striatum using a computer analysis system (MCID M5 5.1, Imaging Research, Inc.) to quantify immunoreactivity of TH, IgG, or OX-42 from a tissue section within each animal (n). To quantify the extent of dopaminergic terminal loss following an intrastriatal injection of DA, 6-OHDA, or saline TH-ir was quantified by measuring the adjusted optical density at the site of injection. Adjusted optical density was calculated using the following equation:

Adjusted Optical Density = log [density of background / density of striatum]

The section containing the injection site was viewed on a microscope and displayed on a monitor linked to a computer equipped with an MCID M5 image analysis system (Imaging Research Inc., Ontario, Canada). The background was obtained by measuring the optical density

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in corpus callosum, an area devoid of TH labeling. The density of TH-ir in striatum was obtained by outlining the entire striatum and measuring the optical density of the delineated area. Quantification of the extent of BBB disruption by examination of IgG-ir obtained in a similar manner. To quantify the extent of microglial activation, a density measurement was obtained by outlining the area of increased OX-42-ir. All density measurements were calibrated to an arbitrary scale of 0-255 with 0 being black and 255 being white. Measurements were then expressed as a percent of control based on comparison of optical density readings from the injected striatum to the contralateral, non-injected striatum within the same tissue section and animal. A Student's *t*-test was used to determine significance at p<0.05.

Western blotting and analysis.

At 1w following intrastriatal DA administration, animals were deeply anesthetized and perfused transcardially with ice-cold saline, brains were immediately removed, and 3mm striatal punches were removed from both injected and uninjected striata, as previously described (Rabinovic et al., 2000). Striatal samples were homogenized in lysis buffer (9M urea, 2% w/v CHAPS, 10mM Tris-base, and protease inhibitor cocktail [2.5µl/mg protein], pH 8.5), kept on ice for 20 min, centrifuged, and protein amounts in the supernatant were assayed (Bradford, 1976). Samples (50µg protein) were loaded onto a 12% SDS-polyacrylamide gel and electrophoresed, followed by transfer onto a nitrocellulose membrane. Non-specific staining was blocked with LICOR blocking buffer and the blot was then incubated with rabbit anti-p47^{phox} antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-actin (1:10,000; Millipore, Bedford, MA), followed by incubation with anti-rabbit IgG secondary antibody (IRDye 800, LI-COR biosciences, Lincoln, NE) and anti-mouse IgG secondary antibody (IRDye

680, Licor, Lincoln, NE). Protein bands were visualized by fluorescent detection using the Odyssey Infared Imaging System (LI-COR Biosciences, Lincoln, NE) and actin was used as a loading control. The density of the band corresponding to p47^{phox} was expressed as a ratio of the actin band. Significance was set at $p<0.05$ using a Student's *t*-test.

4.3 RESULTS

4.3.1 Effect of minocycline on DA-induced inflammation and damage.

Previous studies provided evidence for neuroinflammation contributing to DA-induced BBB disruption (see Chapters 2 and 3). This was examined by treating rats with the anti-inflammatory agent minocycline prior to and following an intrastriatal DA injection for 1w. The effect of minocycline on DA-induced neuroinflammation was evaluated by examining striatal OX-42-ir, a marker for microglia, in rats treated with minocycline compared to vehicle-treated rats. Intrastriatal DA led to a 14.4-fold increase of OX-42-ir (Fig. 24A, C), whereas intrastriatal DA + systemic minocycline treatment resulted in an 8-fold increase in OX-42-ir (Fig 24B, C). Therefore, minocycline treatment resulted in a reduction (-45%) in density of OX-42-ir compared to vehicle treatment (Fig. 24C). While striatal tissue from DA-injected vehicle-treated rats revealed densely-packed OX-42-ir microglial cells with activated morphology (Fig. 24A), tissue from DA + minocycline-treated rats revealed a lower density of OX-42-ir (Fig. 24B).
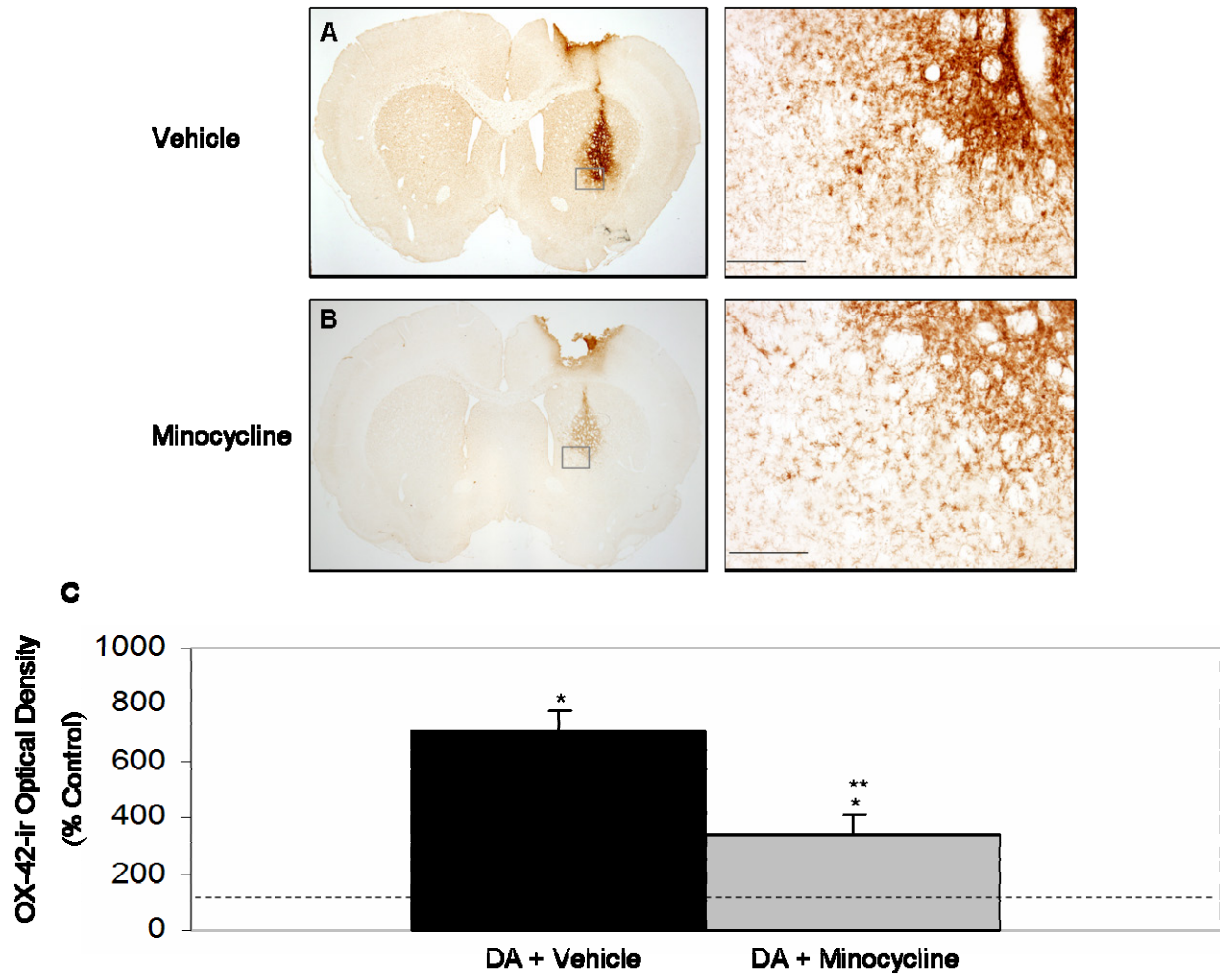
Figure 24: The effect of minocycline treatment on DA-induced microglial activation. OX-42-ir was evaluated in the striatum at 1w following an intrastriatal DA injection (0.4μmol) + *A.* **vehicle treatment (i.p.) or** *B.* **minocycline treatment (50mg/kg/i.p.).** *C.* **Optical density measurements from the DA-injected striatum (right) were compared to the contralateral, non-injected (control) striatum (left). Optical density measurements were also compared between DA-injected striata from the vehicle- and minocycline-treated groups. Data are expressed as percent of control (Mean ± SEM; n=4-6), *p<0.05, significantly different from non-injected (control) striatum; **p<0.05, significantly different from DA + vehicle. Bar = 100μm.**

To determine if DA-induced neuroinflammation contributed to BBB disruption, IgG-ir was examined following DA + vehicle and DA + minocycline treatment. IgG-ir is not detected in the rat striatum under basal conditions and therefore the presence of IgG is indicative of BBB disruption (Yoshimi et al., 2002). At 1w following an intrastriatal DA injection, IgG-ir was increased 9.5-fold over the contralateral, non-injected striatum in the vehicle-treated group (Fig. 25A, C). DA + minocycline treatment resulted in only a 3.9-fold increase in IgG-ir over the non-injected striatum (Fig. 25B, C), resulting a significant attenuation in IgG-ir following DA + minocycline compared to the DA + vehicle-treated group (Fig. 25C), suggesting that minocycline treatment was partially protective against DA-induced BBB disruption.
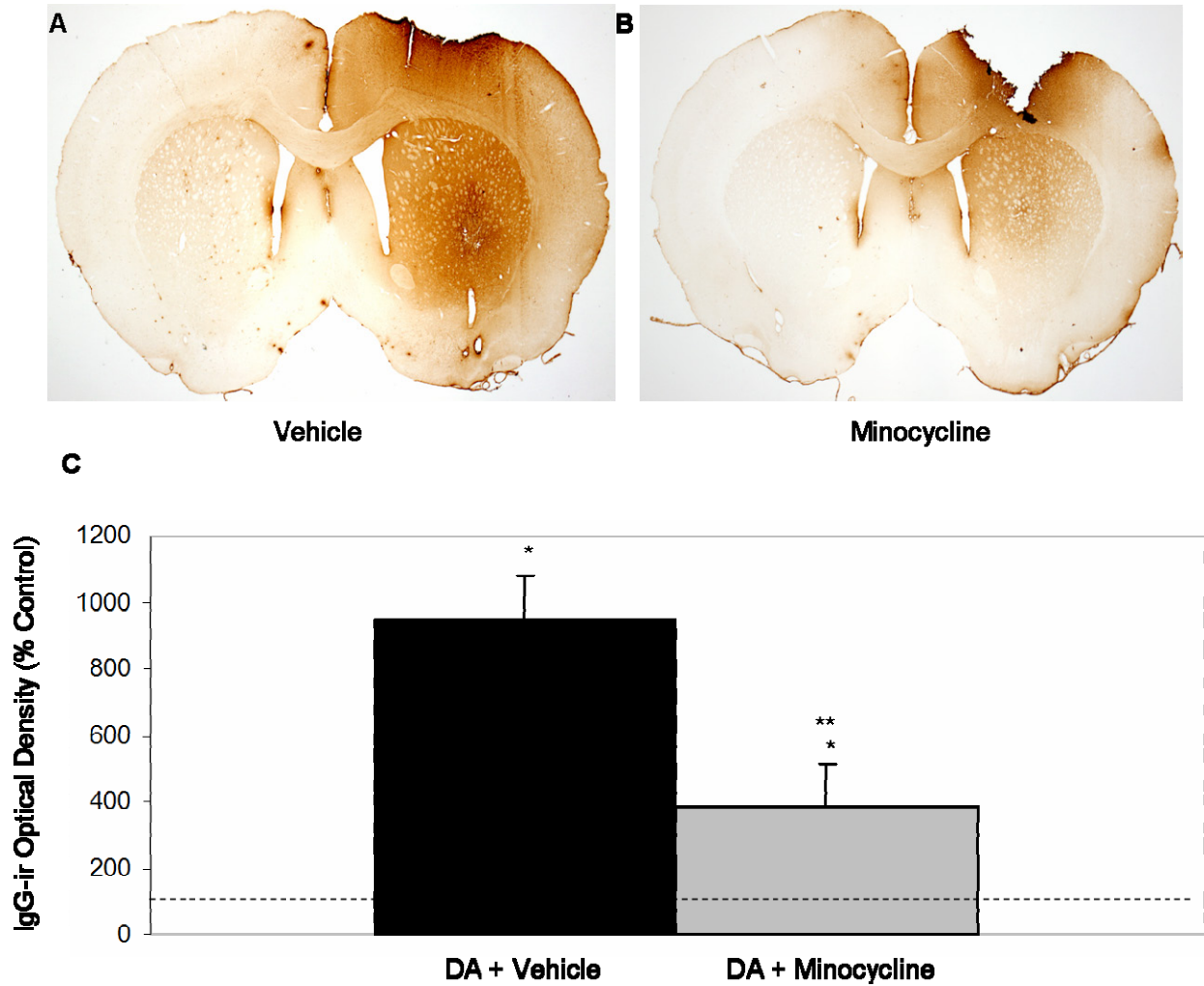
Figure 25: The effect of minocycline treatment on DA-induced BBB disruption. IgG-ir was examined 1w following an intrastriatal DA injection (0.4μmol) + *A.* **vehicle (i.p.) or** *B.* **minocycline (50mg/kg/i.p.) treatment.** *C.* **Optical density measurements from the DA-injected striatum (right) were compared to measurements from the contralateral, non-injected (control) striatum (left) for both treatment groups. Comparisons of the injected striatum were also made between the DA + vehicle and DA + minocycline treatment groups. Data are expressed as percent of control (Mean ± SEM; n=4-6), *p<0.05, significantly different from non-injected (control) striatum; **p<0.05, significantly different from DA + vehicle.**

An intrastriatal DA injection led to a 28% loss of TH-ir compared to the non-injected striatum in the vehicle-treated group (Fig. 26A, C). Examination of the DA + minocycline group also revealed a focal loss of striatal TH at 1w (-26%) which was a significant loss when compared to the non-injected striatum (Fig. 26B, C). Loss of striatal TH-ir did not differ between the DA + vehicle-treated and DA + minocycline-treated groups. Therefore, minocycline did not have an effect on DA-induced dopaminergic terminal degeneration.

Figure 26: The effect of minocycline treatment on DA-induced dopaminergic terminal degeneration. At 1w following an intrastriatal DA injection (0.4μmol), TH-ir was evaluated in striatum in *A.* vehicle-treated and *B.* minocycline-treated groups. *C.* Optical density measurements from DA-injected (right) striatum was compared to contralateral, non-injected (control) striatum (left). Measurements from DA-injected striata were also compared between vehicle- and minocycline-treated groups. Data are expressed as percent of control (Mean ± SEM; n=4-6), *p<0.05, significantly different from non-injected (control) striatum; **p<0.05, significantly different from DA + vehicle.

4.3.2   Effect of apocynin of DA-induced inflammation and damage

Apocynin is an NADPH oxidase inhibitor that has been shown to protect against BBB disruption in other animal models (Yenari et al., 2006; Kuhlmann et al., 2007). To examine the effects of apocynin in the DA-injection model, protein levels of p47^{phox}, a cytosolic NADPH oxidase subunit, were evaluated 1w following an intrastriatal DA injection. An intrastriatal DA injection did not lead to changes in p47^{phox} protein levels compared to the contralateral, non-injected striatum at 1w (Fig. 27A, B). Treatment with apocynin led to a decrease (-17.1%) in p47^{phox} protein levels in the DA-injected striatum (Fig 27B), indicating that apocynin exerted an effect on NADPH oxidase in the DA-injection model at the current dosing regimen. Therefore, apocynin was used as a tool to evaluate the role of NADPH oxidase activation in DA-induced toxicity.
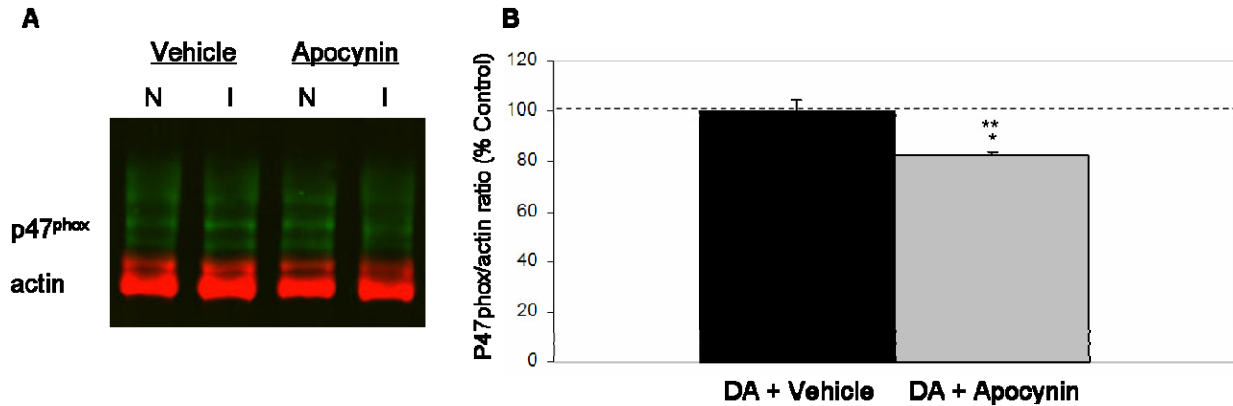
Figure 27: Effect of apocynin on p47^{phox} protein expression levels following an intrastriatal DA injection. At 1w following an intrastriatal DA injection (0.4μmol), striatal tissue was examined from the injected (I) and non-injected (N) striata from the DA + vehicle and DA + apocynin treatment groups to determine differences in p47^{phox} protein levels via Western blot. *A.* A representative blot for p47^{phox}. *B.* p47^{phox}/actin ratios from injected striatum were quantified and compared to non-injected (control) striatum and were expressed as average DA-injected striatal p47^{phox} /actin immunoblot intensity in percent of control (Mean ± SEM; n=4), *p<0.05, significantly different from non-injected (control) striatum; **p<0.05, significantly different from DA + vehicle.

To examine the effect of apocynin treatment on DA-induced microglial activation, striatal OX-42-ir was evaluated. Intrastriatal injection of DA led to an 11.2-fold increase in OX-42-ir over the contralateral, non-injected striatum after 1w in the vehicle-treated group (Fig. 28A, C). Apocynin treatment resulted in an 8-fold increase in OX-42-ir 1w following intrastriatal DA when compared to the non-injected striatum in the DA + apocynin group (Fig. 28B, C). Optical density measurements revealed that apocynin treatment significantly attenuated the increase in OX-42-ir (-28.25%) when compared to OX-42-ir in the vehicle treated group (Fig 28C).

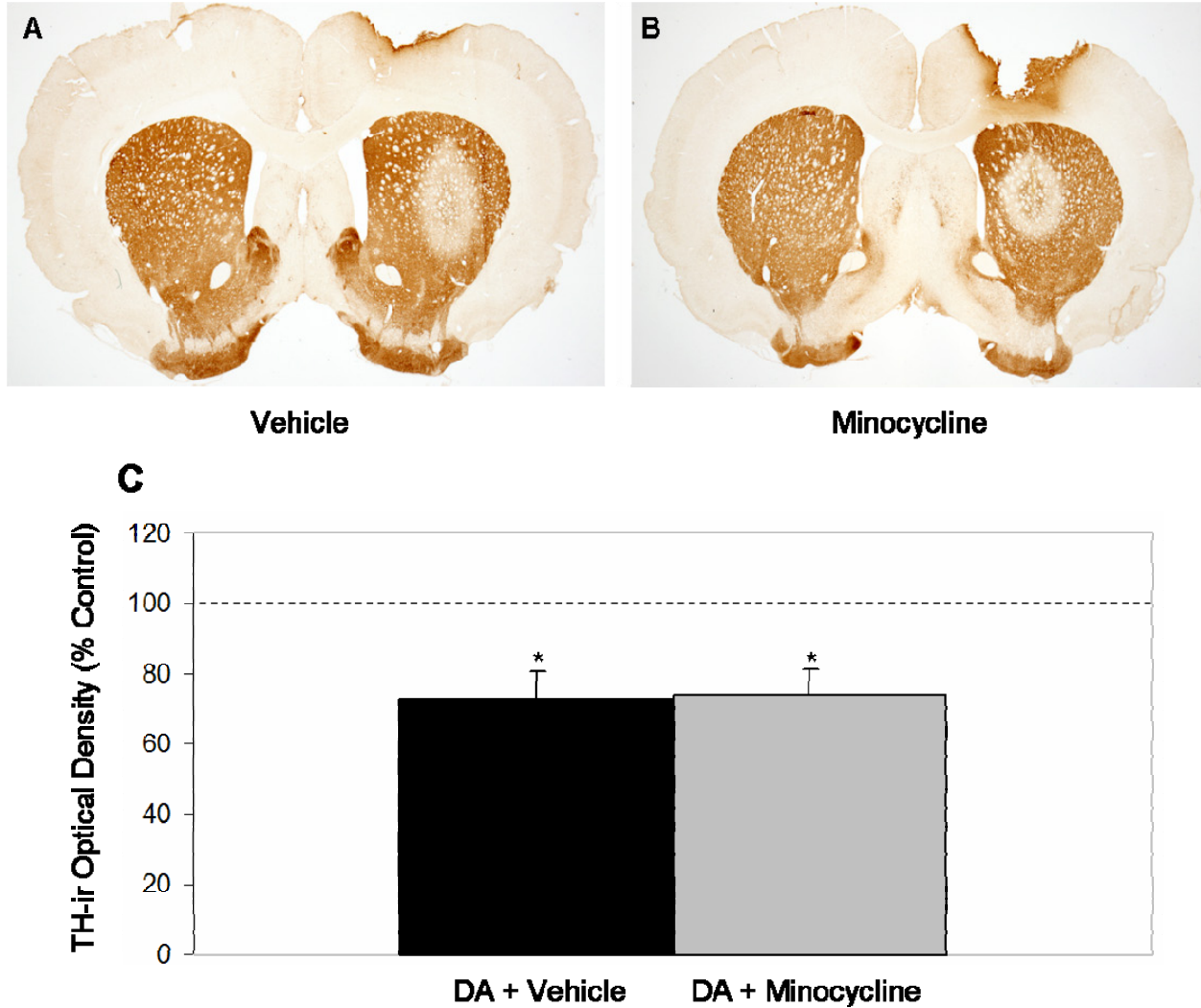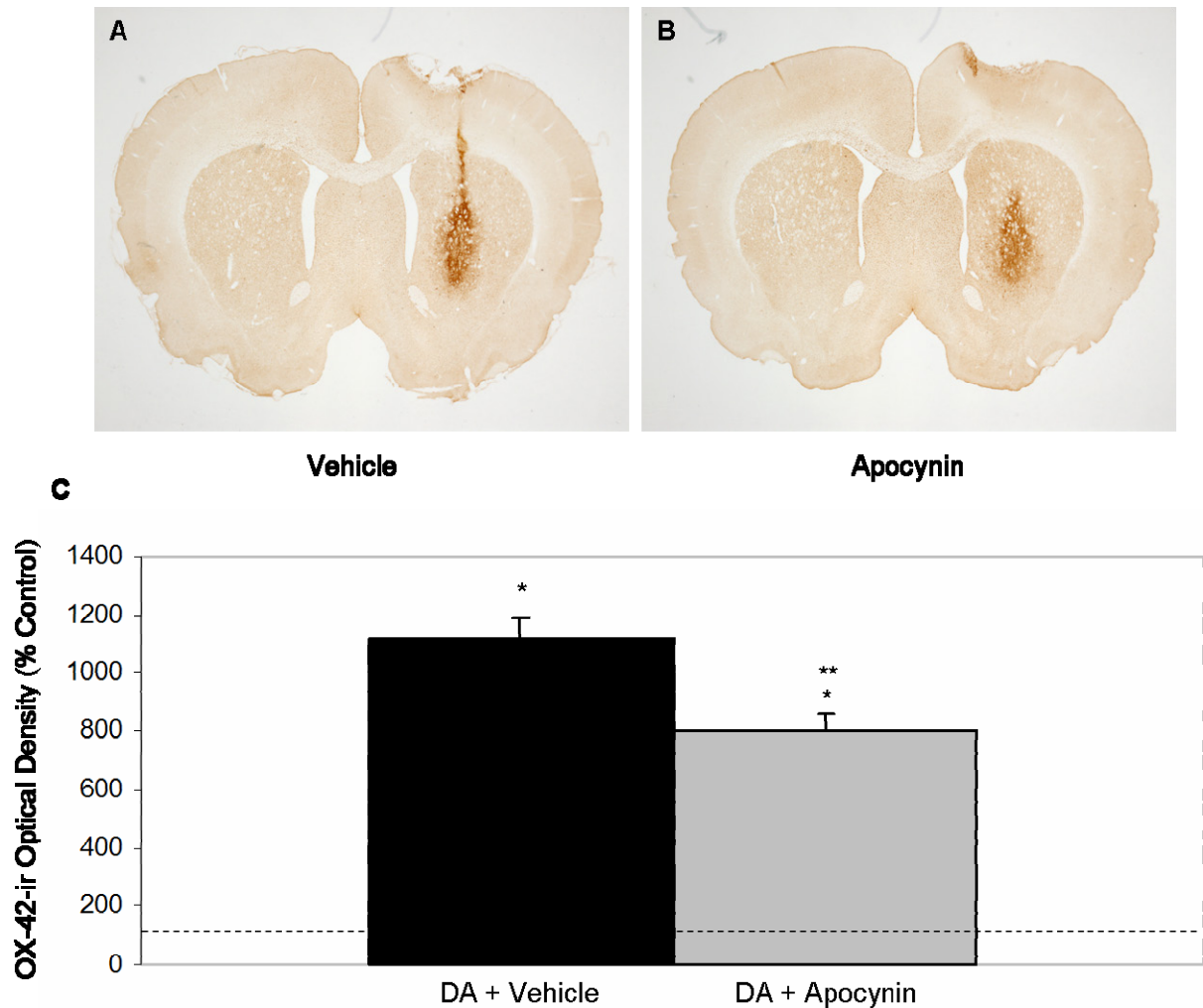**Figure 28: The effect of apocynin treatment on DA-induced microglial activation. At 1w following an intrastriatal DA injection (0.4µmol), OX-42-ir was examined in the striatum in *A*. vehicle- (i.p.) or *B*. apocynin- (10mg/kg/i.p.) treated groups. *C*. Optical density measurements from the DA-injected striatum (right) were compared to the contralateral, non-injected (control) striatum (left). Measurements from the DA-injected striata were also compared between vehicle- and apocynin-treated groups. Data are expressed as percent of control (Mean ± SEM; n=4), *p<0.05, significantly different from non-injected (control) striatum; **p<0.05, significantly different from DA + vehicle.**

To determine the role of NADPH oxidase activation in DA-induced BBB disruption, the effects of apocynin treatment on DA-induced IgG-ir were evaluated at 1w following an intrastriatal DA injection. DA-injected, vehicle-treated rats revealed an increase in IgG-ir by 10.1-fold over contralateral, non-injected striatum (Fig. 29A, C). In contrast, apocynin treatment resulted in a 4.4-fold increase in IgG-ir over the non-injected striatum at 1w following intrastriatal DA (Fig. 29B, C). DA + apocynin treatment led to an attenuation in IgG-ir (-56.7%) compared to DA + vehicle treatment (Fig. 29C), suggesting that apocynin provided significant protection against DA-induced BBB disruption.
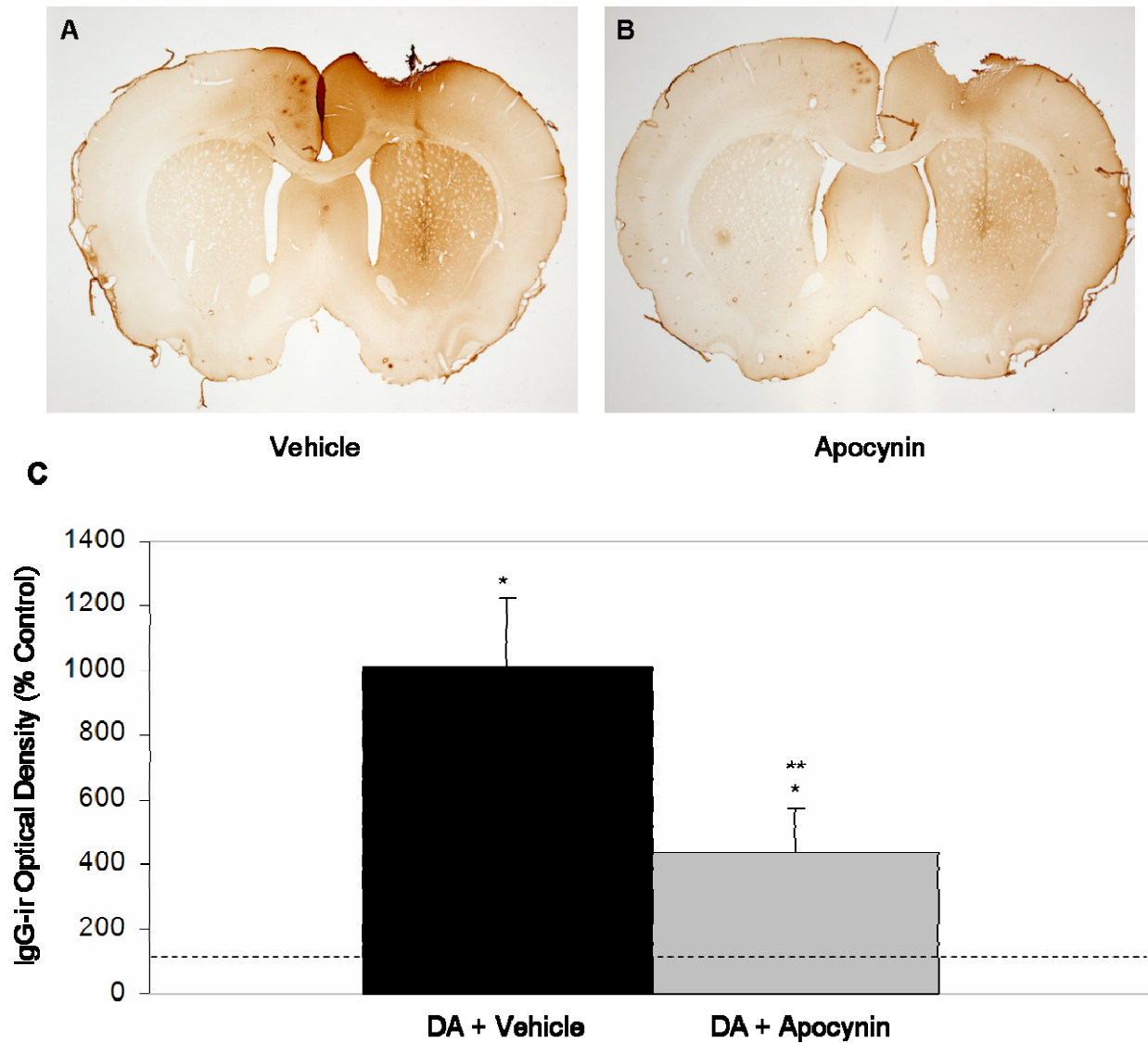
Figure 29: Effect of apocynin treatment on DA-induced BBB disruption. At 1w following an intrastriatal DA injection (0.4μmol) + either *A*. vehicle (i.p.) or *B*. apocynin (10mg/kg/i.p.) treatment, IgG-ir was examined. *C*. Optical density measurements from DA-injected striatum (right) were compared to contralateral, non-injected (control) striatum. Differences in IgG-ir between DA-injected striata following vehicle or apocynin treatment were also examined. Data are expressed as percent of control (Mean ± SEM; n=4), *p<0.05, significantly different from non-injected control; **p<0.05, significantly different from DA + vehicle.

The effects of apocynin on DA-induced dopaminergic neurotoxicity were evaluated by comparing TH-ir between DA + vehicle- and DA + apocynin-treated groups. A 31.1% loss of TH-ir compared to the contralateral, non-injected striatum was observed in the DA + vehicle-treated group (Fig. 30A, C). A 20.3% loss of TH-ir in the DA-injected striatum compared to the non-injected striatum was observed in the DA + apocynin treatment group (Fig. 30B, C). A significant difference in loss of TH-ir was observed between the DA + vehicle and DA + apocynin groups (Fig. 30C), indicating that apocynin treatment attenuated the DA-induced dopaminergic terminal degeneration.
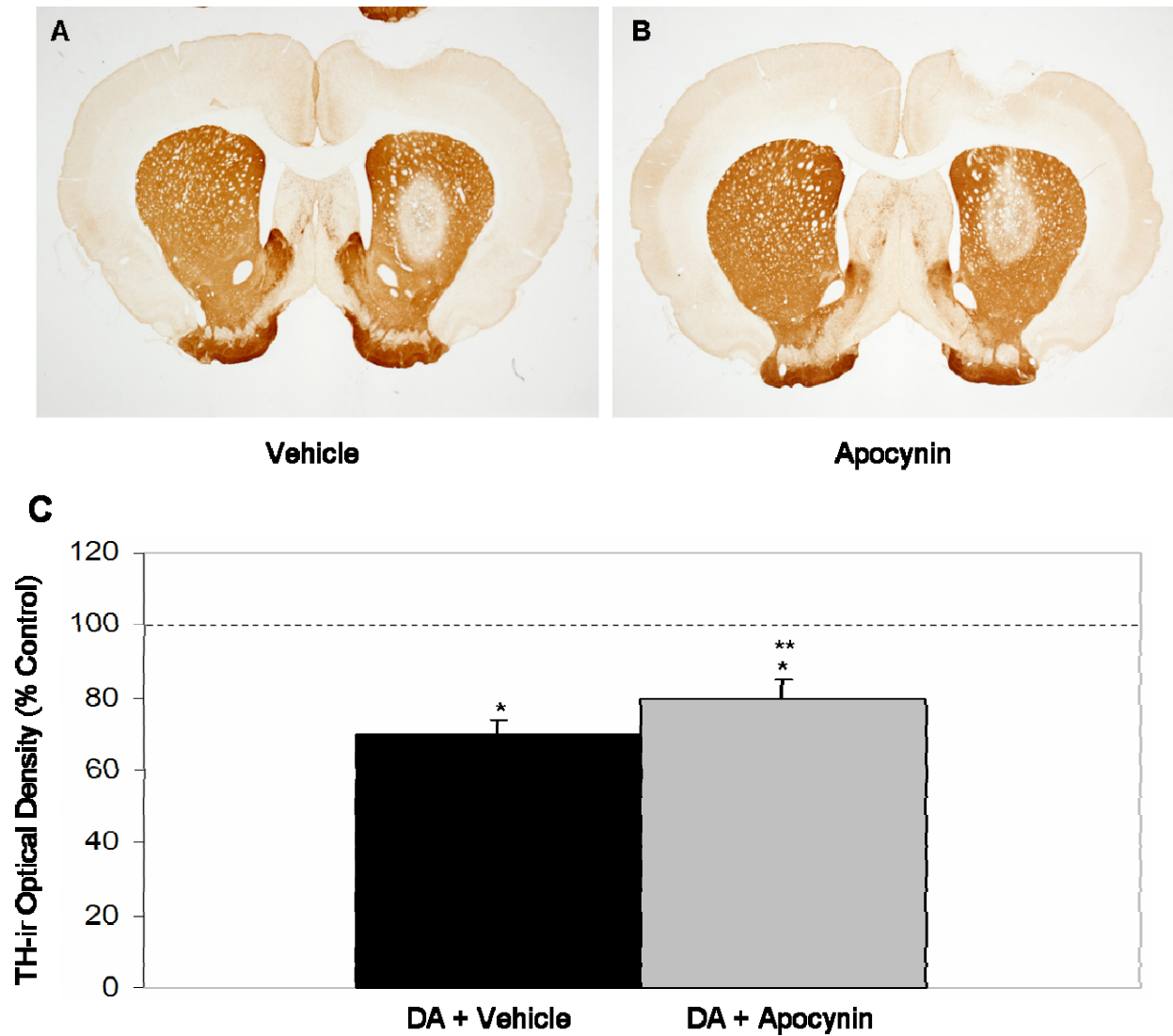
Figure 30: Effect of apocynin treatment on DA-induced dopaminergic terminal degeneration. At 1w following an intrastriatal DA injection (0.4µmol) + *A.* vehicle (i.p.) or *B.* apocynin (10mg/kg/i.p.) treatment, striatal TH-ir was examined. *C.* Optical density measurements from DA-injected striatum (right) were compared to contralateral, non-injected (control) striatum (left). Differences in TH-ir were also examined between DA-injected striata in the vehicle- and apocynin-treatment groups. Data are expressed as percent of control (Mean ± SEM; n=4), *p<0.05, significantly different from non-injected (control) striatum; **p<0.05, significantly different from DA + vehicle.

4.4   DISCUSSION

The contribution of DA-induced neuroinflammation on integrity of the BBB was examined in the current study. Systemic treatment of the anti-inflammatory agent minocycline attenuated both microglial activation and BBB disruption at 1w following an intrastriatal DA injection, indicating that microglial activation may contribute to DA-induced BBB disruption. Additionally, systemic treatment with apocynin, an NADPH oxidase inhibitor, attenuated DA-induced BBB disruption, suggesting that a mechanism by which DA leads to disruption of the BBB is via activated microglia-induced NADPH oxidase activation and subsequent ROS production.

Minocycline has been shown to reduce toxin-induced microglial activation in several animal models of dopaminergic neurodegeneration (Du et al., 2001; He et al., 2001; Wu et al., 2002; Tomas-Camardiel et al., 2004; Choi et al., 2005; Casarejos et al., 2006; Sriram et al., 2006; Zhao et al., 2007). To examine whether minocycline had an effect on neuroinflammation in the DA-injection model, microglial activation was evaluated 1w following an intrastriatal DA injection in minocycline-treated versus vehicle-treated rats. While striatal tissue from DA + vehicle-treated rats revealed densely-packed OX-42-ir microglial cells with activated morphology, tissue from DA + minocycline-treated rats revealed a decrease in the density of activated microglia which likely indicates less cellular proliferation and less activation. Activated microglia proliferate and migrate to the site of damage following loss of contact inhibition, upon which microglia pack tightly together, intertwining their processes (Geisert et al., 2002; Koguchi et al., 2003). Evidence for a less reactive state of the observed activated microglia is based on the comparison of cellular morphology of the OX-42-ir cells from the DA + vehicle- and DA + minocycline treated groups at 1w. Microglia from the vehicle-treated, DA-injected striatum

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displayed phagocytic, reactive, and hyper-ramified states of activation, according to the classification by Streit et al., 1999 (see Appendix A). However, the incidence of phagocytic microglia 1w following a DA injection was reduced with minocycline treatment.

Minocycline treatment also led to significant attenuation of the increase in striatal IgG-ir following DA compared to the DA-injected vehicle-treated group, indicating protection against DA-induced BBB disruption. Previous studies have also found minocycline to be protective against BBB disruption following LPS (Tomas-Camardiel et al., 2004) and MPTP (Zhao et al., 2007) treatment in rats. Results from the present study suggest that a mechanism by which minocycline may contribute to the observed attenuation of DA-induced BBB disruption was by reducing the DA-induced inflammatory response, specifically DA-induced microglial activation.

A mechanism by which microglial activation may lead to BBB disruption is via activation of NADPH oxidase and subsequent ROS production. Previous evidence (discussed in Chapter 3) supports a role for oxidative stress in DA-induced BBB disruption. To examine the effect of NADPH oxidase activation on DA-induced neuroinflammation and BBB disruption, animals were treated with an NADPH oxidase inhibitor, apocynin, and striatal tissue was subsequently examined. Treatment with apocynin led to attenuation of DA-induced OX-42-ir at 1w when compared to the vehicle-treated group, indicating decreased microglial activation. It is possible that the apocynin-induced reduction in OX-42-ir was the result of decreased microglial proliferation since a previous study revealed that apocynin treatment prevented cell proliferation *in vitro* (Stolk et al., 1994).

Treatment with apocynin also significantly attenuated striatal IgG-ir in the DA-injected striatum as compared to the DA-injected vehicle-treated group, indicating significant protection against DA-induced BBB disruption by apocynin. Other studies using have also reported

protective effects of apocynin on integrity of the BBB. For example, hypoxia-induced damage to the BBB and its components was prevented with apocynin treatment both *in vivo* and *in vitro* (Yenari et al., 2006; Kuhlmann et al., 2007; Xian et al., 2007). Protection in the current study suggests that a mechanism by which DA-induced neuroinflammation may contribute to BBB disruption is via NADPH oxidase activation and perhaps subsequent superoxide production, though this was not examined directly in the current study.

Previous studies have found apocynin to be protective not only against BBB disruption, but also against neurodegeneration. NADPH oxidase has been observed to play a role in MPTP-(Wu et al., 2003), rotenone- (Gao et al., 2003a), thrombin- (Choi et al., 2005), 6-OHDA-(Rodriguez-Pallares et al., 2007), and α-synuclein- (Zhang et al., 2007) induced dopaminergic neurodegeneration. The current study supports the findings that apocynin is neuroprotective in the DA-injection model. At 1w following DA + vehicle treatment, there was a 31.1% loss of striatal TH-ir from the injected striatum. This loss was reduced to 20.3% with DA + apocynin treatment, which was a significant decrease in TH-ir when compared to the contralateral, non-injected striatum, but was a significant attenuation of the loss of TH-ir (+10.8%) compared to the vehicle-treated group. This finding suggests that apocynin is partially protective against dopaminergic terminal degeneration, and further supports a role for NADPH oxidase activation in DA-induced neurotoxicity.

Despite the protective effects of apocynin on the DA-induced BBB disruption and loss of TH-ir terminals, an intrastriatal DA injection did not lead to a change in protein expression of NADPH oxidase subunits at 1w, indicating that an intrastriatal DA injection does not lead to increases in NADPH oxidase subunits, as do a variety of other neurotoxic stimuli which result in NADPH oxidase activation (Wu et al., 2002; Gao et al., 2003a; Choi et al., 2005; Anantharam et

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al., 2007). However, a study showing involvement of NADPH oxidase in MPTP-induced dopaminergic neurodegeneration reported increased protein levels of cytosolic and membrane-bound subunits that peaked at 2d following treatment, but returned to basal levels by 1w (Wu et al., 2003). Therefore, it is possible that changes to $p47^{phox}$ protein levels occurred in response to DA, but had returned to basal levels by 1w. Further, apocynin treatment did not lead to a decrease in $p47^{phox}$ in the contralateral, non-injected striatum but did so in the DA-injected striatum. Again, it is possible that this observation is a result of the time point at which $p47^{phox}$ was examined. However, apocynin treatment occurred every 24h until the day of sacrifice, and therefore an effect on protein levels of $p47^{phox}$ should have been retained 1w following the intrastriatal injection. Apocynin crosses the BBB, and therefore brain penetration of the drug is not a factor. Thus in the current paradigm it appears that apocynin-induced inhibition of $p47^{phox}$ occurs in the presence of a neurotoxin (DA) but not systemically without a stimulus, such as a DA injection.

The current study supports the finding that neuroinflammation contributes to DA-induced BBB disruption. There is also substantial evidence from other animal models that neuroinflammation may contribute to dopaminergic degeneration (Du et al., 2001; He et al., 2001; Wu et al., 2002; Choi et al., 2005; Casarejos et al., 2006; Zhang et al., 2007). However, microglial activation does not appear to be associated with dopaminergic terminal degeneration in the current model. Minocycline treatment led to a significant decrease in DA-induced OX-42-ir, compared to the vehicle-treated group, but did not lead to any protection against the loss dopaminergic terminals. While minocycline has been found to be neuroprotective in some cases, several studies have reported contradicting evidence. For example, minocycline was not found to be neuroprotective in the 3-nitropropionic model for Huntington's disease (Diguet et al., 2004)

nor in the R6/2 transgenic mouse model for Huntington's disease (Smith et al., 2003). Treatment with MPTP or methamphetamine in mice led to selective nigrostriatal degeneration that was not affected by minocycline treatment, despite minocycline-induced decreases in microglial activation (Sriram et al., 2006). Other studies have reported that minocycline treatment may even exacerbate toxin-induced neuronal injury. Mice treated with MPTP + minocycline suffered greater loss of dopaminergic neurons than those treated with MPTP + vehicle, despite the reduction in microglial activation (Yang et al., 2003). Increased loss of nigral neurons was observed in MPTP-treated primates with treatment of minocycline compared to animals treated with MPTP + vehicle (Diguet et al., 2004). The current study provides evidence that minocycline does not protect against DA-induced dopaminergic neurodegeneration. Additionally, since neuroprotection was not observed following DA + minocycline treatment despite a decrease in microglial activation, it is possible that inflammatory mechanisms do not contribute greatly to DA-induced dopaminergic terminal degeneration.

Dopaminergic neurodegeneration does not appear to cause BBB disruption, since minocycline treatment was protective against DA-induced BBB disruption but not DA-induced dopaminergic neurodegeneration. These findings also suggest that BBB disruption alone does not lead to dopaminergic neurodegeneration. This is also supported by previous experiments from Chapter 3 which showed that an intrastriatal DOPAC injection led to similar BBB disruption as an intrastriatal DA injection, despite no loss of TH-ir dopaminergic terminals. Therefore, observed BBB disruption may be a marker of damage, but does not necessarily indicate dopaminergic neuronal degeneration within the time-frame and parameters examined in this study. However, substantial evidence exists for vascular dysfunction in neurodegenerative diseases and disorders of the central nervous system that involve neuroinflammation. For

example, disruption of the BBB is common in stroke patients (Gasche et al., 1999; Asahi et al., 2001), relapse-remitting multiple sclerosis (Leech et al., 2007), and Alzheimer's disease (Skoog et al., 1998; D'Andrea, 2003; Zlokovic, 2005). In addition, there is evidence to suggest that BBB disruption precedes pathology and therefore may contribute to neurodegeneration in these examples. Previous data from the DA-injection model (presented in Chapter 2) also revealed that disruption of the BBB preceded neurodegeneration. Correspondingly, dysfunction of the BBB may be a pathological characteristic in PD (Lee et al., 2004; Kortekaas et al., 2005; Desai et al., 2007), however the role that BBB disruption plays in this disease remains unclear.

4.5   SUMMARY AND CONCLUSIONS

The present study provides evidence that neuroinflammation contributes to DA-induced BBB disruption. An intrastriatal DA injection led to increases in striatal microglial activation, which was prevented by treatment with the anti-inflammatory agent minocycline. An intrastriatal DA injection also led to disruption of the BBB which was attenuated with minocycline treatment, suggesting that neuroinflammation plays a role in DA-induced BBB disruption. Intrastriatal DA-induced increase in IgG-ir, indication of BBB disruption, was also attenuated by treatment with the NADPH oxidase inhibitor apocynin, indicating that a mechanism by which activated microglia may contribute to DA-induced BBB disruption is through ROS production.

5.0   CONCLUDING REMARKS

The current study examined the effects of dopaminergic neurotoxins on integrity of the BBB. Examining BBB disruption in these models for PD is important to further understand contributing factors to the disease and explore more effective treatment options. The causes and consequences of BBB disruption in PD are not known, however the novel findings reported from this study provide insight into mechanisms by which disruption of the BBB may occur.

5.1   SUMMARY OF RESULTS

The first study, presented in Chapter 2, provided evidence for disruption of the BBB in three animal models for dopaminergic neurodegeneration, the DA-injection model, the 6-OHDA-injection model, and the chronic rotenone treatment model. It was found in the toxin injection models that BBB disruption occurred early, preceding observable loss of striatal TH-ir, representing dopaminergic terminal degeneration. This raises the possibility that disruption of the BBB may contribute to neurodegeneration. Furthermore, systemically administered rotenone resulted in the disruption of the BBB in the striatum and SN of rotenone-treated lesioned animals; while the BBB of rotenone treated non-lesioned animals appeared to remain intact. This finding provides further evidence that BBB disruption may contribute to neurodegeneration.

Alternatively, it is also possible that BBB disruption in this model may result from rotenone-induced neurodegeneration, as discussed later.

The second study, presented in Chapter 3, provided evidence that oxidative stress plays a role in dopaminergic neurotoxin-induced BBB disruption. Previous studies from our lab found that an intrastriatal injection of DOPAC resulted in quinone formation, leading to increases in oxidative stress but not dopaminergic neurodegeneration in this model. Since DOPAC is not a substrate for the DAT, and is therefore not taken up into the dopaminergic terminals, oxidation from intrastriatal DOPAC occurs primarily extracellularly. The current data provide evidence that intracellular oxidation mediates dopaminergic neurotoxin-induced degeneration of the dopaminergic terminals. However, an intrastriatal DOPAC injection resulted in BBB disruption to a similar extent as an intrastriatal DA injection, suggesting that 1) dopaminergic neurodegeneration does not necessarily lead to BBB disruption and 2) that extracellular oxidative stress is likely harmful to BBB integrity. To examine the role of oxidative stress in DA-induced BBB disruption, DA-injected animals were treated with the antioxidant NAC. An intrastriatal co-injection of DA+NAC partially protected against DA-induced BBB disruption, adding further support for a role for oxidative stress in dopaminergic neurotoxin-induced BBB disruption. Implications and alternative interpretations of these findings will be discussed.

The third study, presented in Chapter 4, provided evidence that neuroinflammatory mechanisms are involved in DA-induced BBB disruption. Treatment of DA-injected animals with the anti-inflammatory agent, minocycline, resulted in a decrease in DA-induced microglial activation with concordant attenuation of DA-induced BBB disruption but no protection against DA-induced dopaminergic neurodegeneration. These data suggest the following possibilities in this model: 1) microglial activation contributes to disruption of the BBB, 2) microglial activation

is not a main mechanism by which intrastriatal DA leads to dopaminergic neurodegeneration, 3) dopaminergic degeneration does not contribute to intrastriatal DA injection-induced BBB disruption, and 4) DA-induced BBB disruption does not lead to neurodegeneration. This study also provided evidence that NADPH oxidase activation contributes to DA-induced BBB disruption. Treatment with the NADPH oxidase inhibitor apocynin protected against DA-induced BBB disruption. Additionally, apocynin treatment attenuated DA-induced microglial activation and dopaminergic degeneration, providing evidence for NADPH oxidase activation as a mechanism by which an intrastriatal DA injection results in neurotoxicity, propagation of the inflammatory response, and disruption of the BBB. The important issues raised regarding the role of neuroinflammation and cellular toxicity in this model will be discussed further.

5.2 DISRUPTION OF THE BBB FOLLOWING INTRASTRIATAL DA AND 6-OHDA

Chapter 2 reported that an intrastriatal injection of DA or 6-OHDA led to disruption of the BBB in agreement with other studies that reported similar findings in response to dopaminergic neurotoxin administration. Carvey, et al. (2005) reported that both an intrastriatal and a medial forebrain bundle (MFB) injection of 6-OHDA led to disruption of the BBB specifically in the striatum and SN. Evidence for BBB disruption was observed 10d following intrastriatal or MFB 6-OHDA injection and disruption persisted for 34d following a MFB injection. BBB disruption was evaluated by perfusing the rats with heparin, followed by infusion with FITC-bound albumin, or by perfusing rats with horseradish peroxidase (HRP), neither of which normally cross an intact BBB. Therefore evidence of FITC or HRP extravasation into the brain parenchyma from the blood vessels was reported as indicative of BBB disruption in this

study (Carvey et al., 2005). Examination of the integrity of the BBB occurred differently in the current study, but with the same underlying theories as in Carvey et al. (2005). First, disruption of the BBB following either an intrastriatal 6-OHDA or DA injection was evaluated by examining extravasation of Evans blue into the brain parenchyma. Rats were intrastriatally injected with a neurotoxin, followed by a tail-vein injection of Evans blue 4h prior to sacrifice by transcardial perfusion with paraformaldehyde. Evans blue is an azo dye which does not cross the BBB and when injected intravenously, binds to plasma albumin and fluoresces. This technique for examining BBB disruption has been well-characterized and used in a variety of models (Asahi et al., 2001; Ujiie et al., 2003; Sharma and Ali, 2006). Extravasation of Evans blue was observed in the striatum 8h following intrastriatal DA or 6-OHDA (data not shown), indicating disruption of the BBB prior to the observed dopaminergic degeneration.

Another technique used to examine BBB disruption in this study was immunohistochemical staining for presence of IgG. When the BBB is intact, the rat striatum is not endogenously immunoreactive for IgG (Yoshimi et al., 2002). Additionally, infiltration of IgG upon disruption of the BBB has been shown to correlate with infiltration of albumin (Rabchevsky et al., 1999), suggesting that the examination of IgG-ir in rat striatum is indicative of BBB disruption. Accordingly, we reported similar results detecting IgG-ir and extravasation of Evans blue in the striatum. Striatal IgG-ir was increased following an intrastriatal injection of either 6-OHDA or DA at 8h and remained elevated in the injected striatum at 1w following toxin injection. To further evaluate the location of toxin-induced BBB disruption, we examined the presence of endothelial barrier antigen (EBA), a BBB-specific marker that is present when the BBB is intact but not present upon disruption of the BBB (Cassella et al., 1996; Abdel-Rahman et al., 2002). In this study, loss of EBA was apparent in the DA-injected striatum, further

confirming that BBB disruption occurred in the striatum 2d following an intrastriatal DA injection. Therefore, because presence of Evans blue, IgG-ir, and lack of presence of EBA-ir all corresponded, immunoreactivity for IgG was used throughout the remaining experiments to determine disruption of the BBB following the administration of dopaminergic neurotoxins.

5.3   DISRUPTION OF THE BBB FOLLOWING SYSTEMIC ROTENONE

Results from Chapter 2 also indicated that systemically administered rotenone leads to BBB disruption. Despite the fact that an intrastriatal injection of saline resulted in significantly less Evans blue extravasation and IgG-ir compared to an intrastriatal injection of DA or 6-OHDA at all time points, intrastriatal injections led to some BBB disruption due to the insertion of the injection cannula. Therefore, we examined BBB integrity following rotenone, a chronic, systemically administered complex I inhibitor that is a model for dopaminergic neurotoxicity (Betarbet et al., 2000). A previous study reported disruption of the BBB following systemic administration of the complex I inhibitor and dopaminergic neurotoxin, MPTP (Zhao et al., 2007). MPTP is able to cross the BBB in mice and primates and upon brain entry is metabolized into MPP+, a toxic compound that is a substrate for the DAT, therefore leading to selective dopaminergic neurotoxicity (Langston et al., 1983; Nicklas et al., 1985; Ramsay et al., 1986). Zhao, et al. (2007) administered MPTP systemically (i.p.) and effects on the BBB were examined 72h following treatment with the toxin, revealing that MPTP led to disruption of the BBB in the striatum and SN at this time point (Zhao et al., 2007). We reported that systemically administered rotenone (s.c.) also led to disruption of the BBB in rotenone-treated lesioned rats. While rotenone is also a dopaminergic neurotoxin that leads to complex I inhibition, it does so

systemically, unlike MPTP which is selectively toxic to dopaminergic neurons due to affinity of MPP+ for the DAT. Rotenone, however, is a lipophilic molecule that crosses membranes readily and therefore affects all cells. Therefore, the selective neurotoxicity for dopaminergic neurons is intriguing and one hypothesis proposed is that rotenone leads to increased oxidative stress in an already oxidative environment in dopaminergic neurons (Sherer et al., 2003c).

In the current study, rotenone-treated non-lesioned rats did not show any observable BBB disruption. This suggests 2 possibilities: 1) in the rotenone model, disruption of the BBB is due to dopaminergic neurotoxicity and 2) there may be metabolic differences between rats that result in adequate brain concentrations of rotenone to cause cellular toxicity in the CNS. Variability of dopaminergic lesions resulting from this route of administration has been well-documented (Betarbet et al., 2000; Sherer et al., 2003b; Sherer et al., 2003a), but the reason for this is unknown. It appears that the variability of the lesion could result in the variability of BBB disruption as well. To our knowledge, the results obtained were not due to mini-pump malfunctions since rotenone-treated non-lesioned animals displayed similar behavioral abnormalities and reductions in weight gain throughout the study as did rotenone-treated, lesioned animals. One way in which rotenone-induced dopaminergic degeneration could contribute to BBB disruption is through the activation of microglia. While we did not report increased microglial activation in the current study, rotenone administration (s.c.) has been shown to lead to microglial activation in the striatum and SN, which was increased in rotenone-treated non-lesioned animals over vehicle-treated rats (Sherer et al., 2003b). However this activation was dramatically increased in rotenone-treated, lesioned rats, which could be a response to neuronal injury (Streit et al., 1999). Therefore, since microglial activation can lead to BBB disruption (Lossinsky and Shivers, 2004; Yenari et al., 2006), it is possible that

dopaminergic terminal degeneration-induced microglial activation contributed to BBB disruption suggesting that in this model BBB disruption occurs as a consequence of dopaminergic degeneration. However, data from Chapter 3, showing that an intrastriatal DOPAC injection leads to BBB disruption without leading to dopaminergic terminal degeneration, suggests that dopaminergic terminal degeneration is not necessary for dopaminergic neurotoxin-induced BBB disruption.

Alternatively, the observed increases in microglial activation following rotenone administration (Sherer et al., 2003b) could be due to disruption of the BBB since it has been observed that disruption of the BBB leads to microglial activation (Nimmerjahn et al., 2005). It has also been reported that microglial activation is harmful to dopaminergic neurons and that rotenone-induced toxicity is dependent upon NADPH oxidase activation (He et al., 2001; Wu et al., 2002; Gao et al., 2003a; Gao et al., 2003b; Wu et al., 2003; Tomas-Camardiel et al., 2004; Choi et al., 2005; Hunter et al., 2007; Rodriguez-Pallares et al., 2007). Therefore it is possible that rotenone-induced BBB disruption contributes to the observed dopaminergic degeneration in rotenone-treated lesioned animals through activation of microglia.

Endothelial cells that comprise the BBB contain more mitochondria than endothelial cells at any other barrier in the body to sustain energy demands (Ribatti et al., 2006; Ueno, 2007), and therefore mitochondrial dysfunction may result in endothelial cell toxicity. Because rotenone is a complex I inhibitor (Fang and Casida, 1999; Betarbet et al., 2000; Votyakova and Reynolds, 2001), it is possible that rotenone causes mitochondrial dysfunction in endothelial cells, leading to disruption of the BBB. However this is unlikely a primary mechanism by which rotenone leads to BBB disruption since rotenone-treated non-lesioned rats showed no evidence for

disruption of the BBB. The cause and consequence of BBB disruption in this model require further examination to determine the role of BBB disruption in rotenone-induced toxicity.

5.4   A ROLE FOR OXIDATIVE STRESS IN BBB DISRUPTION

Oxidative stress has been implicated as a contributing factor in BBB disruption throughout the literature. Particularly in stroke models it has been shown that reduction of oxidative stress-induced damage resulted in decreased BBB disruption and improved neurological outcome (Gasche et al., 1999; Asahi et al., 2001; Chen et al., 2006; Poulet et al., 2006; Davis et al., 2007; Dinapoli et al., 2007; Dong et al., 2007; Haorah et al., 2007). Data presented in Chapter 3 suggest that oxidative stress may contribute to BBB disruption in the dopaminergic neurodegeneration models, as well. An intrastriatal DOPAC injection was used as a tool to distinguish between oxidative stress-induced BBB disruption and BBB disruption as a result of dopaminergic terminal degeneration. Previous, unpublished results from our lab have shown that an intrastriatal injection of DOPAC leads to increases in extracellular DOPAC quinone formation, but is not toxic to dopaminergic terminals as intracellular, but not extracellular, oxidation has been implicated in dopaminergic toxicity (unpublished observations from our lab). At 1w following a DOPAC injection, we also observed no loss of TH from the injected striatum, confirming that intrastriatal DOPAC did not lead to dopaminergic terminal degeneration. Examination of striatal IgG-ir did however reveal that a DOPAC injection leads to an BBB disruption to a similar extent as an intrastriatal DA injection. This provides evidence that BBB disruption in the injection models occur independently of dopaminergic terminal

degeneration. Additionally, it is likely that extracellular oxidation of DOPAC contributed to BBB disruption, supporting a role for oxidative stress.

While extracellular DA oxidation has not been linked with dopaminergic neurotoxicity in our models, the effects of extracellular oxidation are relevant for both METH abuse and L-DOPA administration. METH is a DA agonist that is toxic to dopaminergic and serotinergic terminals in animal models, perhaps through ROS production (Yamamoto and Zhu, 1998) and DAQ formation (LaVoie and Hastings, 1999b). METH leads to an increase in DA release and a decrease in uptake at the dopaminergic terminals (Yamada et al., 1988; O'Dell et al., 1991; Stephans and Yamamoto, 1994), thus increasing extracellular levels of DA. The effects of METH on the BBB in abusers have not been examined, however METH administration in rats leads to microglial activation (LaVoie et al., 2004; Thomas et al., 2004) and increased activity of matrix metalloproteinases (Conant et al., 2004), both of which can contribute to disruption of the BBB. Mice treated with METH had increased leakage of albumin into the cerebral cortex (Sharma and Ali, 2006), providing further evidence that METH may be toxic to the BBB. The effect of a methamphetamine derivative, 3,4-methylenendioxymethamphetamine (MDMA) on the BBB has also been minimally examined in drug abusers. However exposure in rats suggests that MDMA may be deleterious to the BBB since acute treatment led to evidence of long-term disruption of the BBB in the striatum and hippocampus of rats (Bankson et al., 2005; Quinton and Yamamoto, 2006). Results from the current study suggest that mechanisms by which METH and MDMA may lead to disruption of the BBB are increases extracellular oxidative stress and increases in microglial activation, both of which have been shown to contribute to disruption of the BBB in our models.

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Extracellular oxidative stress may also play a role in toxicity of the BBB in PD patients treated with L-DOPA, though this has been minimally examined. Damage of the dopaminergic terminals during PD pathogenesis can lead to increases in free DA in the intracellular and extracellular space (Ogawa et al., 2005) and during later stages of PD, a dysregulation of uptake and release of DA by the remaining nigrostriatal neurons could increase extracellular DA (Olanow et al., 2004). The addition of L-DOPA during these pathological conditions could contribute to increases in extracellular oxidative stress because 1) impaired DA uptake mechanisms may allow DA and L-DOPA to remain in the extracellular space for an extended period of time and 2) reactivity of astrocytes can lead to astrocytic dysfunction and may therefore result in to the release of excess DA and L-DOPA into the extracellular space (Ogawa et al., 2005). We have shown that extracellular oxidation likely causes disruption of the BBB following an intrastriatal DOPAC injection (Chapter 3). In support of these findings, it was recently reported that rats lesioned with 6-OHDA and later treated with L-DOPA for various amounts of time had increased dysfunction of the BBB in the basal ganglia (Westin et al., 2006). This phenomenon, however, was primarily observed in L-DOPA-treated animals that had developed L-DOPA-induced dyskinesia, similar to occurrences often reported by PD patients (Thanvi et al., 2007). Tissue from these animals showed evidence for microvascular proliferation and remodeling which was associated with a loss of EBA-ir and extravasation of albumin (Westin et al., 2006), providing evidence for L-DOPA leading to disruption of the BBB. Treatment with L-DOPA, however, does not appear to be harmful to dopaminergic neurons in PD despite the development of dyskinesia, which suggests that L-DOPA-associated microvascular changes may not directly lead to increased neurotoxicity.

In addition to increases in extracellular oxidative stress, an intrastriatal DOPAC injection also led to increased microglial activation. Intrastriatal DOPAC resulted in less microglial activation than following an intrastriatal DA injection, however this may be due to the lack of degenerating dopaminergic terminals following the DOPAC injection since neurodegeneration has been shown to lead to microglial activation (Streit et al., 1999). Oxidative stress has also been shown to increase the neuroinflammatory response (Raivich et al., 1999) and DAQ formation induces activation of microglia (Kuhn et al., 2006), suggesting that a likely factor contributing to DOPAC-induced microglial activation is via DOPAC quinone formation. The observed microglial activation may contribute to DOPAC-induced BBB disruption, since microglial activation has been shown to alter functional components of the BBB and be detrimental to BBB integrity (for review see de Boer, et al., 2006; Lossinsky and Shivers, 2004).

To further examine a role for oxidative stress in DA-induced BBB disruption, animals were treated with the thiol antioxidant NAC, and IgG-ir was examined. We chose to examine the effects of a thiol antioxidant because DA has been observed to exert its toxicity through oxidation into the DAQ which leads to 5-cyteinyl catechol formation (Graham et al., 1978; Hastings et al., 1996). NAC competitively binds with the DAQ to prevent protein modification and DA-induced toxicity. An intrastriatal co-injection of DA+NAC prevented DA-induced loss of striatal TH-ir, providing evidence that NAC was an effective antioxidant in this model. Similar results have previously been reported following intrastriatal co-injection of either GSH or ascorbate, which were also protective against an intrastriatal DA (Hastings et al., 1996). Since NAC was found to be an effective antioxidant at protecting against DA-induced dopaminergic terminal degeneration, the effects of NAC on DA-induced BBB disruption were examined. A co-injection of DA+NAC led to significant attenuation of IgG-ir compared to a DA injection alone,

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although there was still some increase in IgG-ir, suggesting that NAC was partially protective. This provides further evidence that DA-induced oxidative stress contributes to BBB disruption.

The fact that BBB disruption was observed without loss of TH-ir provides additional evidence that dopaminergic degeneration does not lead to BBB disruption in this model. The fact that DA+NAC co-injection did not completely prevent DA-induced BBB disruption could possibly be due to 3 factors:

1) Although cysteinyl catechol levels were not measured in the current study following a co-injection of DA+NAC, it is likely that NAC does not completely abolish the DAQ protein modification and therefore leads to some oxidative stress. This is evidenced by results from a previous study which reported that co-injection of DA with either GSH or ascorbate led to a 50% reduction of cysteinyl catechol levels, as determined by biochemical analysis (Hastings et al., 1996);

2) Co-injection of DA+NAC led to decreased OX-42-ir, suggesting attenuation but not a complete blockage of microglial activation. The reduction in OX-42-ir possibly resulted from the lack of dopaminergic terminal degeneration or from NAC acting as an anti-inflammatory agent as it has been shown to do (Radomska-Lesniewska et al., 2006). Although there is a correlation between treatment with DA+NAC and decreased microglial activation, the partial but not complete abrogation may have contributed to the observed DA-induced BBB disruption. In addition, although biochemical analyses were not performed for this paradigm, it is possible that microglial activation was due to the only partial inhibition of cysteinyl-catechol formation by NAC, as reported previously and

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discussed above (Hastings et al., 1996), since DAQ formation has been shown to lead to microglial activation (Kuhn et al., 2006);

3) It is possible that the low pH of the DA+NAC solution contributed to BBB disruption. This possibility is supported by the fact that an intrastriatal injection of NAC alone led to a similar increase in IgG-ir as co-injection of DA+NAC. Additionally, injection of NAC alone led to increases in OX-42-ir. If the low pH of the injected solution led to disruption of the BBB, then it is possible that NAC-induced BBB disruption contributed to the increase in microglial activation since disruption of the BBB is a sufficient stimulus to result in microglial activation (Nimmerjahn et al., 2005).

While results following an intrastriatal co-injection of DA+NAC provided evidence for oxidative stress as a mechanism by which intrastriatal DA leads to BBB disruption, this method of administration has little relevance clinically. However administration of NAC systemically to protect against DA oxidation-induced BBB disruption has more practical utility. Protection by systemic administration of NAC is dependent upon brain penetration of NAC to increase free thiol levels. It was previously observed in the Hastings lab that systemic treatment with NAC (500mg/kg/i.p.) increased free thiol levels by 2-fold over control levels in the brain 2h following an intrastriatal DA injection, but only in areas where a toxin was injected. This indicated that toxin injection may lead to BBB disruption by allowing NAC to have brain access at the sites of disruption.

Therefore, using the same paradigm, we examined the effects of systemic NAC on DA-induced damage to the BBB. However, systemic NAC treatment (500mg/kg/i.p.) did not protect against either DA-induced dopaminergic terminal degeneration or DA-induced BBB disruption,

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as described in Chapter 3. Further examination also found that NAC at this dose and at a higher dose (1000mg/kg/i.p.) did not lead to an increase in free thiol levels 2h following a DA-injection, suggesting that there was not adequate brain penetration of NAC at the DA injection site. Metabolic differences in rats as well as changes to the NAC compound could have accounted for differences in results observed from this study and the study that was performed previously in the Hastings Lab. However, it is also possible that the BBB was not disrupted to a sufficient extent 2h following an intrastriatal DA injection to allow for adequate brain penetration of NAC. This presents a problem when trying to examine the effects of systemically administered NAC on DA-induced cellular toxicity because it has been previously reported that an intrastriatal DA injection led to the greatest increase in cysteinyl-catechol formation at 2h, suggesting that the greatest extent of DA-induced damage occurs at that time point. It is possible that systemic administration of NAC at a later time point, when it is known that there is adequate BBB disruption, may arrive to the area of DA oxidation too late to protect against DA-induced damage to either the dopaminergic terminals or to the BBB. Therefore, it may be more useful to take advantage of a thiol antioxidant that crosses the BBB, such as AD4. This compound has been protective against experimental autoimmune encephalomyelitis-induced demyelination (Offen et al., 2004) as well as DA, L-DOPA, 6-OHDA, MPP+, and rotenone-induced neuronal toxicity *in vitro* and rotenone-induced dopaminergic neuronal degeneration *in vivo* (Bahat-Stroomza et al., 2005), suggesting that systemically administered AD4 may be an ideal compound to examine the role of oxidative stress in DA-induced BBB disruption in future studies.

5.5   A ROLE FOR NEUROINFLAMMATION IN BBB DISRUPTION

Intrastriatal injection of DOPAC and co-injection of DA+NAC revealed that oxidative stress likely plays a role in dopaminergic neurotoxin-induced BBB disruption. However both injection paradigms led to increases in microglial activation, suggesting a role for neuroinflammation in dopaminergic neurotoxin-induced BBB disruption. To examine this possibility we chose to evaluate the effects of the anti-inflammatory agent minocycline on DA-induced BBB disruption. Minocycline is a tetracycline antibiotic that is used clinically as an anti-inflammatory agent and is therefore a safe and well-tolerated compound. Minocycline has been widely used to determine the effects of neuroinflammation on neurodegeneration in several animal models (Zemke and Majid, 2004; Elewa et al., 2006). When administered systemically (50mg/kg/12h/i.p.), we observed that minocycline reduced DA-induced microglial activation by 60%, suggesting that it is an effective anti-inflammatory agent in this model.

Minocycline treatment was protective against DA-induced BBB disruption, as discussed in Chapter 4, suggesting that DA-induced neuroinflammation contributes to BBB disruption. While minocycline did not offer complete protection, minocycline also did not completely prevent microglial activation which may contribute to the observed IgG-ir. Studies have reported evidence for neuroinflammation leading to disruption of the BBB in other animal models involving dopaminergic neurodegeneration. For example, intranigral injections of LPS led to BBB disruption which was prevented by treatment with minocycline (Tomas-Camardiel et al., 2004). Additionally, it has been observed that minocycline treatment prevented MPTP-induced BBB disruption in the striatum and SN (Zhao et al., 2007). MPTP also did not lead to disruption of the BBB in TNF-α knockout mice (Zhao et al., 2007), providing additional support for neuroinflammatory factors being involved in dopaminergic neurotoxin-induced BBB disruption.

Despite that minocycline was protective against DA-induced BBB disruption, it did not protect against DA-induced dopaminergic terminal degeneration. Several studies involving models of dopaminergic neurodegeneration have reported that minocycline treatment is protective against dopaminergic neurodegeneration (Du et al., 2001; He et al., 2001; Wu et al., 2002; Tomas-Camardiel et al., 2004; Choi et al., 2005; Casarejos et al., 2006; Quintero et al., 2006) and these findings have contributed to the idea that neuroinflammatory processes are involved in dopaminergic neurodegeneration. However, other studies have reported that minocycline does not protect against dopaminergic neurodegeneration (Sriram et al., 2006; Zhao et al., 2007) and may in fact increase dopaminergic neurotoxicity (Yang et al., 2003; Diguet et al., 2004). These reports support the results from this study which question the role microglial activation in DA-induced dopaminergic terminal degeneration.

While minocycline treatment did not lead to complete prevention of microglial activation, there has long been controversy about whether activated microglia are harmful or protective (Streit et al., 1999; Streit, 2002). When activated, microglia phagocytose cellular debris, release certain anti-inflammatory cytokines and chemokines, and produce neurotrophins that aid in the regrowth of fibers back into the injured area (Batchelor and Howells, 2003). With these properties, activated microglia are generally considered to be beneficial to the damaged area, as they appear to aid in the restoration of normal functioning. Activated microglia are also known, however, to release particular pro-inflammatory cytokines, such as TNF-α, increase oxidative stress possibly through NADPH oxidase activation (Qin et al., 2004), release NO and prostaglandins (Petrova et al., 1999), and synthesize C1q which is a component of the complement pathway (Lynch et al., 2004). The secretion of all such factors can cause further

damage to the affected area by promoting degeneration and perhaps impede regeneration (Wilhelmsson et al., 2004).

This evidence suggests that activated microglia have the capability to be neuroprotective, neurodegenerative, and neuroregenerative, depending on what is necessary at the time. However, the mechanism by which such "decisions" are made is unknown. Neurotrophin and cytokine production by activated microglia at different time intervals during degeneration has yet to be characterized, and therefore we currently rely on morphological evaluation to determine activation "states" of microglial (Streit et al., 1999; Appendix B). Elevation of OX-42-ir was observed in the DA-injected striatum following minocycline treatment, suggesting the possibility that microglial activation, even in very small amounts, can contribute to dopaminergic terminal degeneration. However, morphology of OX-42-ir cells were not as reactive in the minocycline-treated group as in the non-treated control group. While biochemical analyses were not performed in this experiment, it is likely that minocycline treatment did not prevent DA-induced increases in DAQ formation contributing to 1) dopaminergic terminal degeneration and 2) microglial activation. The lack of neuroprotection by minocycline treatment also suggests that not only is dopaminergic neurodegeneration not required for BBB disruption (as discussed in Chapter 3), but BBB disruption is not required for dopaminergic terminal degeneration. However, BBB disruption has been shown in other models to increase susceptibility to neurotoxicity, which will be discussed further in a later section.

Because it appeared that neuroinflammatory processes played a role in DA-induced BBB disruption, and data supported a role for oxidative stress in DA-induced BBB disruption, the effects of NADPH oxidase activation on BBB integrity were examined. Microglial activation leads to NADPH oxidase activation which results in the production of superoxide and the

subsequent increase in highly reactive and toxic ROS (see Appendix C). Treatment of rats with apocynin, an inhibitor of NADPH oxidase activation, resulted in protection against DA-induced BBB disruption in the present study, in agreement with evidence from stroke models that NADPH oxidase activation is involved in BBB disruption (Wang et al., 2006; Xian et al., 2007).

Additionally, apocynin led to attenuation of DA-induced microglial activation. This decrease could be due to 3 contributing factors in toxin-induced increases in microglial activation: 1) proliferation, 2) activation in response to neuronal degeneration, and 3) perpetuation of the neuroinflammatory response via ROS. When microglia become activated, they can proliferate, resulting in increased immunoreactivity for immunohistochemical markers for microglia (Streit et al., 1999). Apocynin has been shown to decrease activated cell proliferation (Stolk et al., 1994; Johnson et al., 2002). While the effect of apocynin on microglial cell proliferation has not been reported, this is a potential mechanism by which apocynin may lead to a decrease in DA-induced microglial activation. Additionally, neuronal degeneration can lead to microglial activation (Streit et al., 1999). Apocynin treatment led to a decrease in DA-induced dopaminergic terminal degeneration which may have resulted in less microglial activation in response to this stimulus. Finally, ROS have been shown to induce microglial activation (Raivich et al., 1999; Streit et al., 1999; Bellander et al., 2004; Kuhn et al., 2006). Since apocynin reduces NADPH oxidase activation and subsequent ROS production, the reduction in microglial activation may be due to a reduction in ROS. The extent to which apocynin inhibited ROS production was not examined in this study and requires further analysis.

As mentioned previously apocynin treatment offered slight protection against DA-induced dopaminergic terminal degeneration. NADPH oxidase activation has been reported to be a mechanism by which dopaminergic neurotoxins such as 6-OHDA, MPTP, and rotenone lead to

dopaminergic neuronal degeneration, and apocynin has been reported as neuroprotective in these models *in vitro* (Gao et al., 2003a; Gao et al., 2003b; Wu et al., 2003; Rodriguez-Pallares et al., 2007). Some of the mechanisms by which apocynin is neuroprotective in the current model have been discussed but include: 1) a decrease in ROS production, 2) and decrease in BBB disruption, and 3) a decrease in microglial activation. Data from the current experiments support a role for decreased ROS production as a main mechanism by which apocynin is neuroprotective against DA-induced dopaminergic terminal degeneration. A decrease in BBB disruption following minocycline treatment did not lead to a decrease in dopaminergic terminal degeneration suggesting that BBB disruption may not play a primary role in DA-induced neurotoxicity. Additionally, a decrease in microglial activation following minocycline treatment was not correlated with a decrease in dopaminergic terminal degeneration, suggesting that microglial activation is not detrimental to dopaminergic neurons in the DA-injection model. However, co-injection of DA+NAC resulted in complete protection against DA-induced dopaminergic terminal degeneration, supporting a role for ROS production, possibly through NADPH oxidase activation, contributing to DA-induced neurotoxicity.

The fact that minocycline treatment, a broad-spectrum anti-inflammatory agent that led to reduced microglial activation, was not neuroprotective but that apocynin, an NADPH oxidase inhibitor that reduces activated microglial-induced ROS production, was partially neuroprotection against an intrastriatal DA injection was somewhat perplexing. Although this was not measured, it is very likely that an overall reduction in microglial activation, as occurred with minocycline treatment, would lead to an overall reduction in microglia-induced ROS production. However this did not correlate with protection against dopaminergic terminal loss following a DA injection, as treatment with apocynin did. It has long been reported that

endothelial cell- and neuronal-induced NADPH oxidase activation was not particularly toxic, while activated microglial-induced NADPH oxidase activation was responsible for NADPH oxidase-induced cellular toxicity (see Johnson et al., 2002). However a recent report demonstrated that neuronal NADPH oxidase was responsible for hypoglycemia-induced neurodegeneration (Suh et al., 2007), suggesting that neuronal NADPH oxidase activation following an intrastriatal DA injection could contribute to dopaminergic terminal degeneration, lending insight to the discrepancy between results following minocycline and apocynin treatment.

5.6   WHAT ROLE DOES BBB DISRUPTION PLAY IN NEURODEGENERATION IN THESE MODELS?

The role that BBB disruption plays in dopaminergic neurodegeneration in the current models is unclear. On the one hand, systemic rotenone administration led to BBB disruption, but only in animals that developed striatal lesions, suggesting that perhaps dopaminergic degeneration contributes to BBB disruption. However, intrastriatal DOPAC, DA+NAC, and NAC injections did not lead to dopaminergic neurodegeneration but resulted in BBB disruption, suggesting that dopaminergic terminal degeneration does not lead to BBB disruption. On the other hand, minocycline treatment protected against DA-induced BBB disruption but did not protect against dopaminergic terminal degeneration, suggesting that BBB disruption does not lead to dopaminergic neurotoxicity. In the DA- and 6-OHDA-injection models, BBB disruption preceded dopaminergic terminal degeneration, however, suggesting that BBB disruption may play a role in neurotoxicity. It has been previously reported that disruption of the BBB

contributes to neurodegeneration. For example, ischemic and hemorrhagic stroke lead to BBB disruption followed by neurodegeneration. Prevention of BBB disruption in these models protected against neurodegeneration and improved neurological outcome in these models (Asahi et al., 2001; Kim et al., 2003; Chen et al., 2006; Dong et al., 2007). Further, mice with a dysfunctional BBB that lacked the efflux transporter P-gp had increased susceptibility to environmental neurotoxin-induced neurodegeneration than mice with a fully functional BBB (Schinkel et al., 1994), providing additional evidence for BBB disruption contributing to neuronal toxicity.

In the rotenone experiment, 89% of the rotenone-treated lesioned rats had to be sacrificed early due to the development of illness, as previously described (Chapter 4). Illness and infection can lead to disruption of the BBB in severe cases where neuroinflammation occurs (Langford and Masliah, 2001; Lossinsky and Shivers, 2004). Therefore, it is possible that BBB disruption in these animals was not due to rotenone as a primary factor but was due to the development of illness. However, one of the rotenone-treated, lesioned animals survived the 4w treatment of rotenone administration without the development of life-threatening illness yet the BBB was disrupted in this animal, suggesting that it is indeed rotenone itself that is harmful to the BBB. Again, it is likely that dopaminergic terminal degeneration is not responsible for rotenone-induced BBB disruption, but instead that rotenone-induced BBB disruption contributes to dopaminergic neuronal toxicity and perhaps also the development or progression of illness in the lesioned animals. However the role that BBB disruption plays in neurotoxicity in these models is unclear from the current studies and requires further examination.

5.7   THE RELEVANCE OF BBB DISRUPTION IN PD: CONTRIBUTIONS OF FINDINGS FROM DOPAMINERGIC NEUROTOXIN MODELS

As mentioned throughout this document, there is evidence for BBB disruption in PD. A functional polymorphism of the multi drug resistant (*MDR1*) gene which produces the BBB efflux transporter P-gp, is associated with PD (Lee et al., 2004). P-gp functions to remove waste and foreign material from the brain into the bloodstream (Bauer et al., 2005; Terasaki and Ohtsuki, 2005). Dysfunction of this transporter has been found to be detrimental to neurons by allowing the influx of microorganisms and environmental toxins into the brain as well as preventing efflux of accumulated waste from the CNS (Schinkel et al., 1994; Cirrito et al., 2005). A study involving Chinese patients discovered three single nucleotide polymorphisms of the *MDR1* gene which were associated significantly with PD patients when compared to control patients (Lee et al., 2004). Another study involving PD patients found increased P-gp-substrate uptake via positron emission tomography and supports the hypothesis that BBB dysfunction is a clinical feature of the disease (Kortekaas et al., 2005). These data suggest a genetic susceptibility for PD due to increased access by environmental toxins to the CNS. This conclusion is somewhat controversial since a study involving Italian PD patients found no dysfunction of P-gp (Furuno et al., 2002) and cerebral spinal fluid samples taken from PD patients and analyzed for serum albumin content revealed no difference between PD patients and controls, indicating normal function of the blood-cerebrospinal fluid barrier (Haussermann et al., 2001). It is important to note however, that the blood-cerebrospinal fluid barrier is comprised of epithelial cells and is therefore structurally and functionally separate from the BBB (Zheng, 2001), suggesting that CSF biomarkers may not be indicative of a dysfunctional BBB.

Evidence for microvessel angiogensis was observed in the SN of PD patients (Barcia et al., 2004). Interestingly, evidence for angiogensis was observed in the SN in rats in which there was BBB disruption following an intrastriatal 6-OHDA-injection (Carvey et al., 2005), suggesting that microvascular changes observed in PD may indicate BBB disruption. Increases in IgG and β-integrin (a marker for microvessel angiogenesis) has recently been reported in the SN of PD patients compared to age-matched controls (Desai et al., 2006), providing additional support for BBB disruption as a pathological feature as it is in other neurodegenerative diseases such as Alzheimer's disease (Skoog et al., 1998; Zlokovic, 2005; Algotsson and Winblad, 2007; Deane and Zlokovic, 2007; Matsumoto et al., 2007) and multiple sclerosis (Banks, 1999; Langford and Masliah, 2001; Lossinsky and Shivers, 2004; Engelhardt, 2006; Leech et al., 2007). Additionally, evidence for microvascular angiogensis was observed in rats which developed L-DOPA-induced dyskinesia (Westin et al., 2006), suggesting that L-DOPA treatment in PD could contribute to the observations of microvascualr changes in PD patients.

The findings from the current sets of experiments suggest that disruption of the BBB may be a pathological feature of PD. Intrastriatal injection of DA or 6-OHDA and systemic rotenone administration lead to dopaminergic terminal degeneration as well as key features of PD that may also be detrimental to the BBB such as microglial activation and oxidative stress (see Chapter 2). Additionally, protection against dopaminergic neurotoxin-induced BBB disruption was observed when oxidative stress and neuroinflammation were mitigated. Antioxidants and anti-inflammatory agents have also shown to be neuroprotective in animal models for PD (for review, see Beal, 2003) and have been examined as potential therapeutic agents in PD patients with variable results (Singh et al., 2007). A recent study reported that chronic use of non-steroidal anti-inflammatory agents were associated with a decrease in risk for PD (Wahner et al.,

2007), suggesting neuroprotection via reduction of inflammation, which has been associated with protection against BBB disruption in the current studies. The role of BBB disruption in the risk for and development of PD has not been thoroughly examined, although several lines of evidence exist which suggest that BBB disruption and PD may be associated. In addition to similar molecular mechanisms that may contribute to BBB disruption and PD pathogenesis, there are also similar risk factors for PD and a dysfunctional BBB.

5.7.1   The Association of Age with BBB Disruption and Risk for Development of PD

The association between aging and PD has long been noticed. James Parkinson described this relationship in the early nineteenth century and to this day, PD is known to mainly affect the aged population (Knapowski et al., 2002). Incidence rates of PD increase over the age of 50 and increase even further over the age of 75 (de Lau et al., 2004). Along with age being associated with the development of the disease, clinical progression of PD is positively correlated with age such that the later the age of onset, the faster the progression of the disease (Levy, 2007). These data indicate that aging is an important risk factor for PD.

It is currently not known exactly how aging leads to the development of PD, but there is evidence for known contributing factors of PD as a consequence of aging such as oxidative stress, mitochondrial dysfunction, and the occurrence of dystrophic microglia. Additionally, evidence from post-mortem tissue suggests that dopaminergic pathways and projection sites are particularly vulnerable to the effects of aging (McGeer et al., 1977; Stark and Pakkenberg, 2004). Age-induced alterations in function and expression of components of the BBB have also been observed that can increase BBB permeability (Mooradian and McCuskey, 1992; Mooradian et al., 2003), including alterations in transport mechanisms (Mooradian, 1988, 1990; Shah and

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Mooradian, 1997), suggesting reduced transport of nutrients to the endothelial cells and the rest of the CNS. There is also evidence that with aging, the BBB becomes more vulnerable to insult (Dinapoli et al., 2007).

Oxidative stress, itself, may be a primary culprit in aging (Harman, 1956). Oxidative stress occurs when there is an imbalance between ROS production and antioxidant activity. Age-related increases in oxidative stress in the brain such as lipid peroxidation, protein oxidation, and DNA oxidation (Droge and Schipper, 2007) and a decrease in endogenous antioxidants and their activity, including GSH, SOD, and catalase (Fornstedt et al., 1990a; Schulz et al., 2000; Halliwell, 2001) have been correlated with normal aging. Brain mitochondria also become dysfunctional with age (Toescu and Verkhratsky, 2003; Lin and Beal, 2006; Droge and Schipper, 2007), possibly contributing to increases in mitochondrial derived ROS and making the mitochondria-enriched endothelial cells of the BBB increasingly vulnerable to cellular toxicity. Additionally, age-induced changes to the vasculature of the BBB occur that can weaken the ability of endothelial cells to detoxify and inhibit repair functions of the BBB (Zheng, 2001; Donato et al., 2007). While the consequence of aging on the BBB was not directly assessed in the current study, results presented in Chapter 3 provide additional evidence for increased oxidative stress contributing to disruption of the BBB as well as to dopaminergic degeneration. Therefore, the present data support the hypothesis that age-related increases in oxidative stress may be harmful to integrity of the BBB which may contribute to age-related increases in the risk for development of PD.

In addition to oxidative stress, evidence supports that neuroinflammation increases with age. Increases in neuroinflammation have also been implicated in PD and disruption of the BBB. Basal reactivity of microglia increases in very old age (Galea et al., 2007) and the presence of

dystrophic microglia throughout the brain increases with age (Streit et al., 2004). It is possible that aging microglia lose some of their original, intended function and this may have implications for age-related neurodegenerative disorders, such as PD (Dawson & Dawson, 2003). For example, activated microglia play a role in repair following brain insult through the release of growth factors, which normally functions to aid in the maintenance of DA neurons (Imamura et al., 2003). However, dystrophic microglia may not have the same capacity for generation of such growth factors and it is notable that certain growth factors are reduced in the SN of PD patients (Mogi et al., 1999), despite increased microglial activation. There was also a positive correlation observed with age and MPTP-induced increases in neuroinflammation, which contributed to increased neurotoxicity (Sugama et al., 2003). Additionally, age increases have corresponded with increases in vascular permeability (Donato et al., 2007). Other changes including decreases in growth factors and chemokines for repair, and weakened interactions between endothelial cells and astrocytes have been reported with old age, as well (Zheng, 2001; Dinapoli et al., 2007). Results from the current study support a role for neuroinflammation in BBB disruption, which may contribute to increasing risk for the development of PD. This possibility may be supported by the observation that chronic use of anti-inflammatory supplements was linked to a decrease in incidence of PD (Wahner et al., 2007), although the effect of non-steroidal anti-inflammatory agents on the BBB was not reported.

5.7.2 Environmental Contributors to BBB Disruption and Risk for Development of PD

In the 1970's a student trying to cook a synthetic form of heroin accidentally produced and self-administered MPTP. In mere days following this incident, the student developed parkinsonian motor symptoms. Upon brain autopsy, it was found that there was selective

neurodegeneration of the nigrostriatal pathway, suggesting that MPTP was capable of mimicking clinical symptoms and pathological manifestations of PD (Langston et al., 1983). As a result MPTP and its toxic metabolite, MPP+, are widely used as models (Nicklas et al., 1985; Ramsay et al., 1986) to examine potential causes of and therapeutics for PD. Acute treatment of MPTP also leads to disruption of the BBB in mice (Zhao et al., 2007). Nicotine has been shown to inhibit MPTP transfer across the BBB and also inhibits uptake of MPTP and MPP+ into brain endothelial cells (Liou et al., 2007), suggesting a protective role for nicotine against brain penetration of MPTP. Interestingly, heavy smoking is associated with a decreased likelihood for the development of PD (Morens et al., 1996; Hernan et al., 2003). The reported decrease in risk for developing PD is also dose-dependent in that the more packs of cigarettes smoked, the less the prevalence of PD (Gorell et al., 1999a; Tanner and Aston, 2000). The mechanism by which neuroprotection occurs is unclear, however it may be possible that the effect of nicotine on inhibiting toxin uptake into the endothelial cells that comprise the BBB plays a role.

Other toxins, including some pesticides such as rotenone, are also known complex I inhibitors. Correspondingly, PD is significantly associated with 10-20 years or greater of occupational exposure to pesticides (Gorell et al., 1998; Fall et al., 1999; Gorell et al., 1999b; Tanner and Aston, 2000). Rotenone and paraquat are pesticides that have been identified as dopaminergic neurotoxins and are used in animal as models of PD to evaluate disease onset, progression, and potential therapeutics (Koller, 1986; Rajput et al., 1987; Betarbet et al., 2000). The incidence of PD is higher in rural populations than in urban or suburban areas and frequent exposure to these pesticides may be at least partially responsible. The link between pesticide exposure and dysfunction of the BBB, however, has not been thoroughly examined. Therefore, the novel findings of this study described in Chapter 2, provide evidence for systemic rotenone

exposure leading to disruption of the BBB in rotenone-treated lesioned rats, indicating that BBB disruption may be a pathological feature due to this pesticide.

There are also specific dietary behaviors associated with either an increased or decreased risk for BBB disruption and the development of PD. For example, increased dietary fat from animal sources and obesity is associated with the development of PD (Hellenbrand et al., 1996; Anderson et al., 1999; Lai et al., 2002; Hu et al., 2006; Ikeda et al., 2007) and there is a lower incidence of PD in cultures which consume mainly vegan foods (McCarty, 2001), suggesting that obesity may be a risk factor for the development of PD. Additionally, insulin and leptin transporters at the BBB are inhibited as a result of obesity in rats (Banks, 2006), providing evidence for obesity also leading to a dysfunctional BBB, as well. Polyphenols are antioxidants found in such food items as berries, nuts, wine and grapes, beer, tea, and chocolate (Arts and Hollman, 2005). In animals and cell models for PD, polyphenol compounds have been protective against toxin-induced dopaminergic neurodegeneration and inflammation (Levites et al., 2001; Levites et al., 2002; Nie et al., 2002; Li et al., 2004; Mercer et al., 2005; Guo et al., 2007; Tomobe et al., 2007). Interestingly, the consumption of tea is associated with decreased risk for developing PD (Hellenbrand et al., 1996; Fall et al., 1999; Hu et al., 2007). It was also observed that tea-derived polyphenols resulted in decreased BBB disruption from ischemic stroke which was associated with a decrease in neurodegeneration (Dong et al., 2007), suggesting that anti-oxidant protection against BBB disruption may be neuroprotective. We have also reported that antioxidant treatment reduces dopaminergic terminal degeneration and neuroinflammation from intrastriatal DA, which was associated with decreased BBB disruption (Chapter 3), providing additional support for the reduction of oxidative stress for protection against BBB disruption.

These examples of environmental factors that increase or decrease the risk for development of PD also affect integrity of the BBB. The fact that PD and BBB disruption share identified environmental and molecular risk factors suggests mechanisms by which BBB disruption may occur in PD. Whether or not a dysfunctional BBB contributes to the development of PD or is associated with PD-induced damage is unknown. Results from the current study suggest that BBB disruption does not occur as a result of dopaminergic terminal degeneration and it is possible that BBB disruption may contribute to increased susceptibility for neuronal damage, although this requires further investigation. However, it is possible that a loss in integrity of the BBB leads to neurological problems which could contribute to an increased risk for PD because a dysfunctional BBB is unable to maintain brain homeostasis which is vital for health and maintenance of the CNS.

5.7.3 Implications of BBB Disruption in Animal Models for PD

While BBB disruption may be a pathological feature in PD, an examination of the literature provides evidence that the extent to which the BBB is disrupted differs between animal models of dopaminergic neurodegeneration and PD cases. It is also possible that BBB components that are affected in animal models differ from those affected in PD. Of particular interest in PD is the dysfunction of the efflux transporter P-gp (Lee et al., 2004; Kortekaas et al., 2005) which could lead to brain increases in environmental toxins, thus contributing to PD pathogenesis. Alternatively, dysfunction of P-gp could be a result of the disease process, itself. While the effects of dopaminergic neurotoxins on P-gp function were not examined, ROS and neuroinflammation have been associated with alterations in P-gp expression and function (Schinkel et al., 1994; Schinkel, 1999; Hartz et al., 2004; Bauer et al., 2005; Hartz et al., 2006;

Deane and Zlokovic, 2007), providing evidence that molecular contributors to PD such as oxidative stress and neuroinflammation may lead to disruption of the BBB. Additionally, potential dysfunction of the BBB as a consequence of L-DOPA-induced dyskinesia should be examined further to determine if these alterations 1) occur in PD patients and 2) affect transport of potential therapeutic agents that have been found to be neuroprotective in animal models.

Therefore, differences between the effects of PD models on the BBB and BBB integrity in PD need to be elucidated in order to develop effective therapeutic agents because differences in BBB disruption between PD and animal models can lead to differences in drug delivery and transport into the brain. The findings by us and others that dopaminergic neurotoxins lead to disruption of the BBB could contribute to the discrepancy observed between therapeutics that are shown to be neuroprotective in animal models but have no measurable effect on disease progression in PD patients. A more complete understanding of the structural and functional changes that occur to the BBB during PD and in animal models may enhance the development of effective neuroprotective strategies in PD.

5.8   CONCLUSION

The novel findings described in this document provide evidence that the integrity of the BBB is compromised in animal models for dopaminergic neurodegeneration. Upon examination of mechanisms by which this occurs, it was found that oxidative stress and neuroinflammation are likely contributors, and that a dysfunctional BBB could be a contributing factor to neurotoxicity in these models. Findings from the current study may also have implications for the interpretation of previous animal studies that have shown the systemic administration of

antioxidants, anti-inflammatory agents, and other potential therapeutic agents to be protective against toxic insult, but without similar therapeutic results in PD. Therefore, it may be useful to further characterize disruption of the BBB in models for dopaminergic neurotoxicity to improve upon current understandings of PD models and to enhance treatment options.

APPENDIX A

MICROGLIAL ACTIVATION STATES

Resting Microglia

Microglial Activation Stimulus

BBB disruption   Neuronal damage

Baterium   ROS, DAQ

Pro-inflammatory cytokines

Hyper-ramified

Reactive
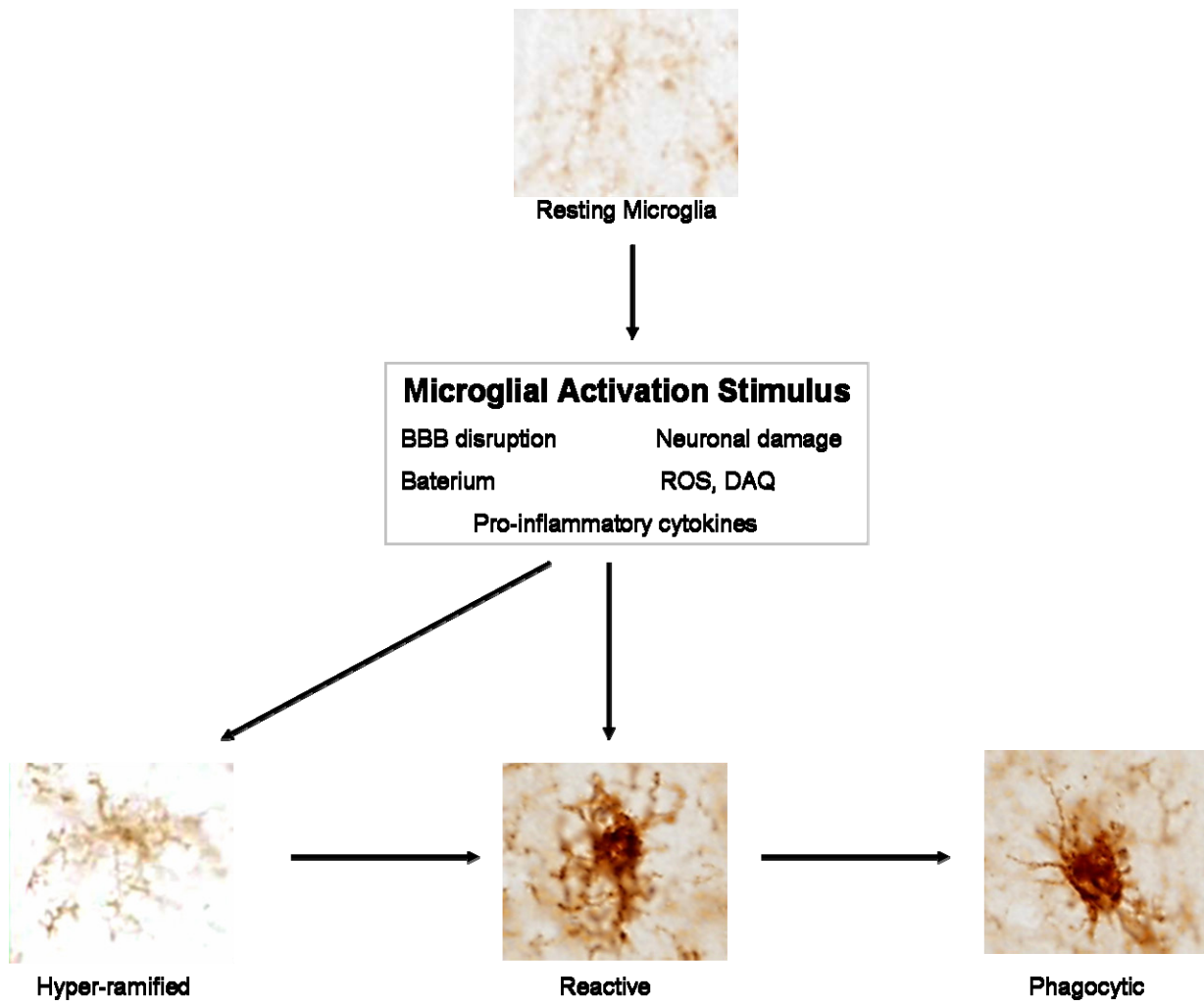
Phagocytic

APPENDIX B

THE PHAGOCYTIC NADPH OXIDASE
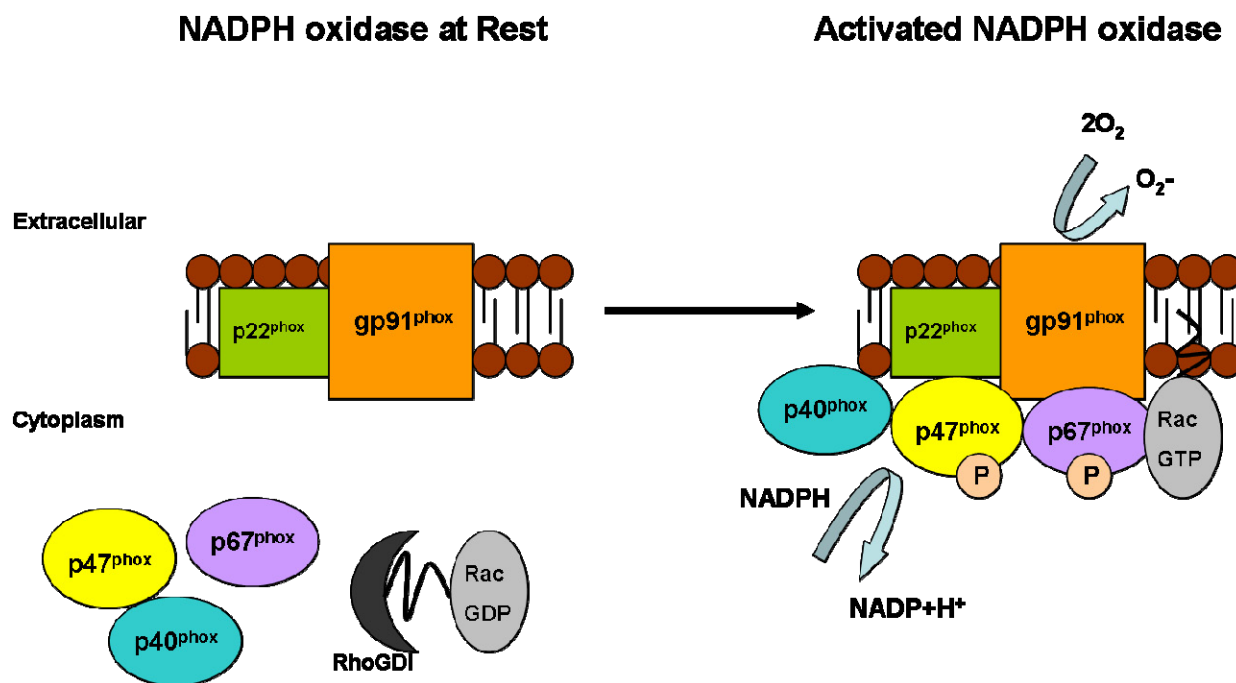
Upon an activation stimulus, there is a GDP/GTP exchange on Rac and phosphorylation of the cytosolic subunits p47^{phox} and p67^{phox}. The phosphorylated cytosolic subunits then translocate to the membrane where they interact with p22^{phox} and gp91^{phox}, resulting in NADPH oxidase activation and the production of O$_2$- via electron exchange.

ANATOMICAL REGIONS
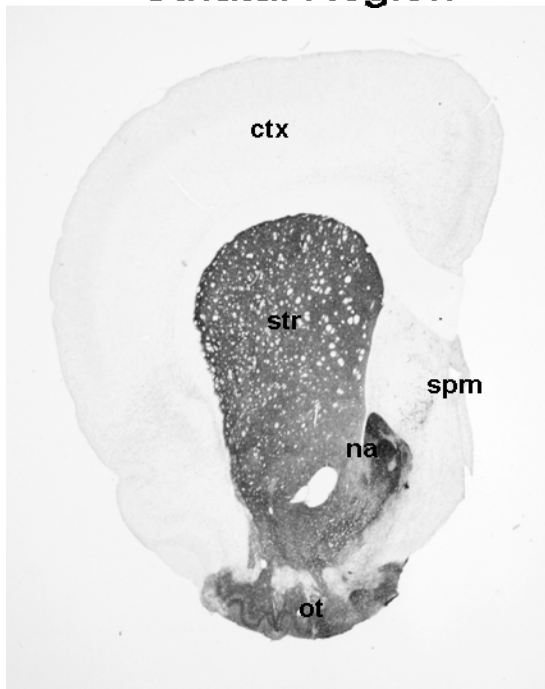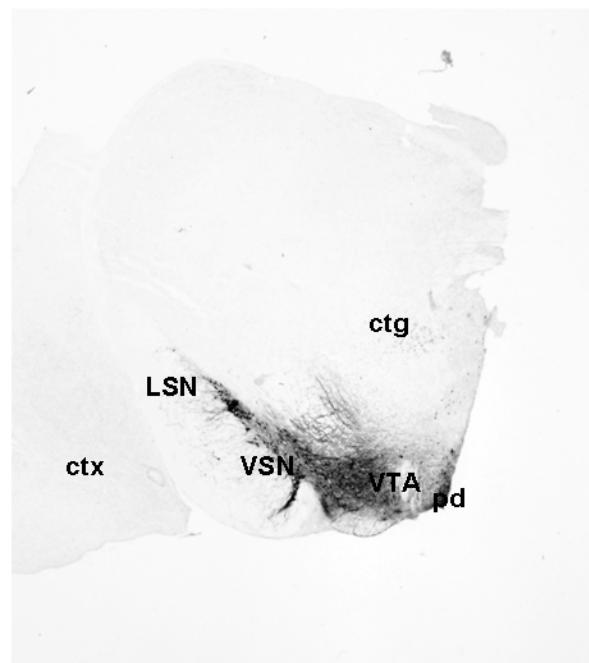
The above image represents anatomical regions discussed throughout this document. Abbreviations are as follows: **ctx** = cortex; **str** = striatum; **na** = nucleus accumbens; **ot** = olfactory tubercle; **spm** = septum; **LSN** = lateral substantia nigra; **VSN** = ventral substantial nigra; **VTA** = ventral tegmental area; **pd** = peduncle; **ctg** = central tegmental tract.

APPENDIX D

SUMMARY SCHEMATIC FOR CHAPTER 2
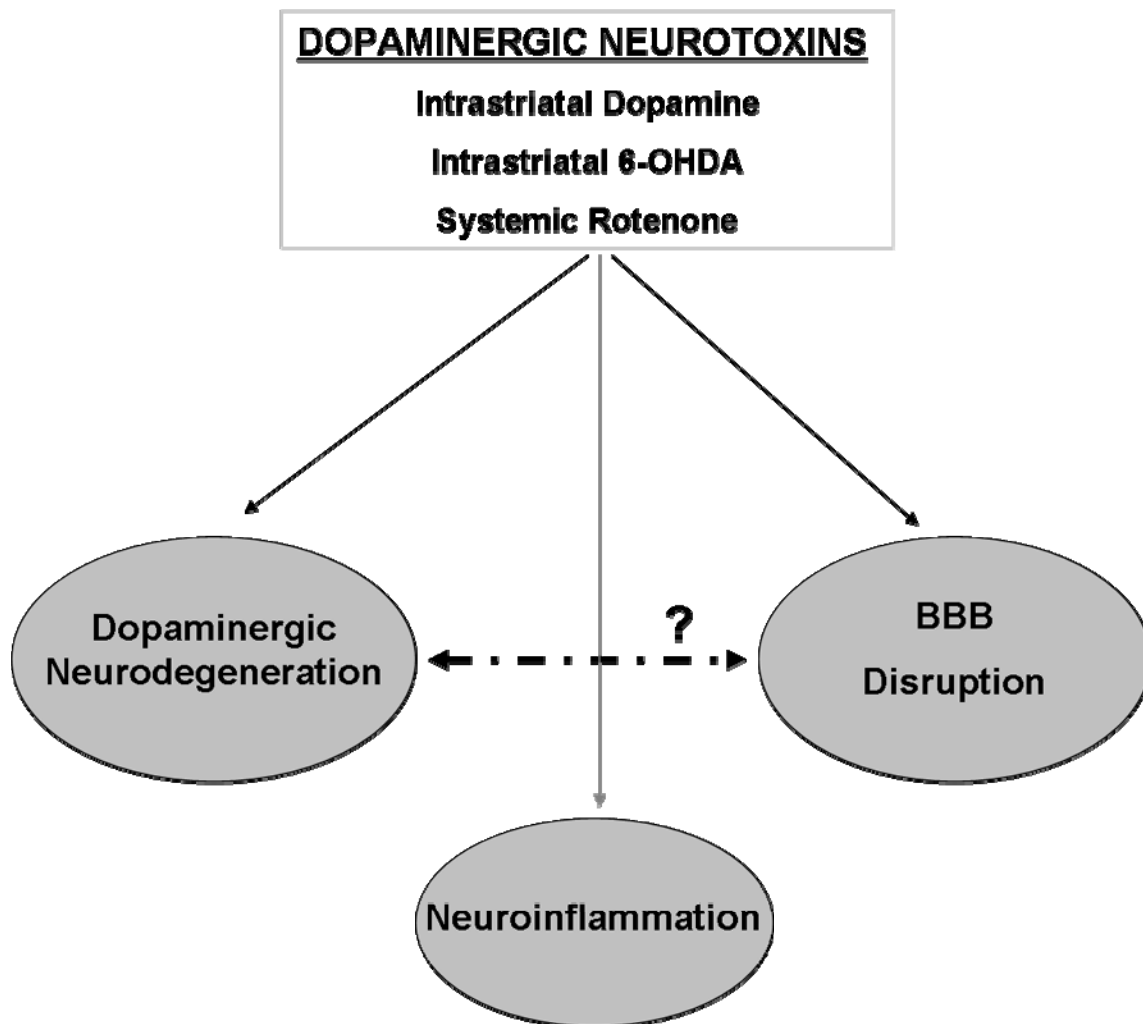
Schematic diagram summarizing results from Chapter 2. Dopaminergic neurotoxins were found to have led to dopaminergic terminal degeneration, increases in neuroinflammation, and BBB disruption. Disruption of the BBB occurred prior to dopaminergic terminal degeneration in the DA- and 6-OHDA-injection models, suggesting that BBB disruption may contribute to the observed loss in striatal TH-ir. However, in the rotenone model, BBB disruption only occurred in the rotenone-treated lesioned animals, suggesting that dopaminergic terminal degeneration may contribute to BBB disruption. Therefore, the role of BBB disruption and mechanisms by which BBB disruption occurs in these models needs to be elucidated and will be examined.

SUMMARY SCHEMATIC FOR CHAPTER 3
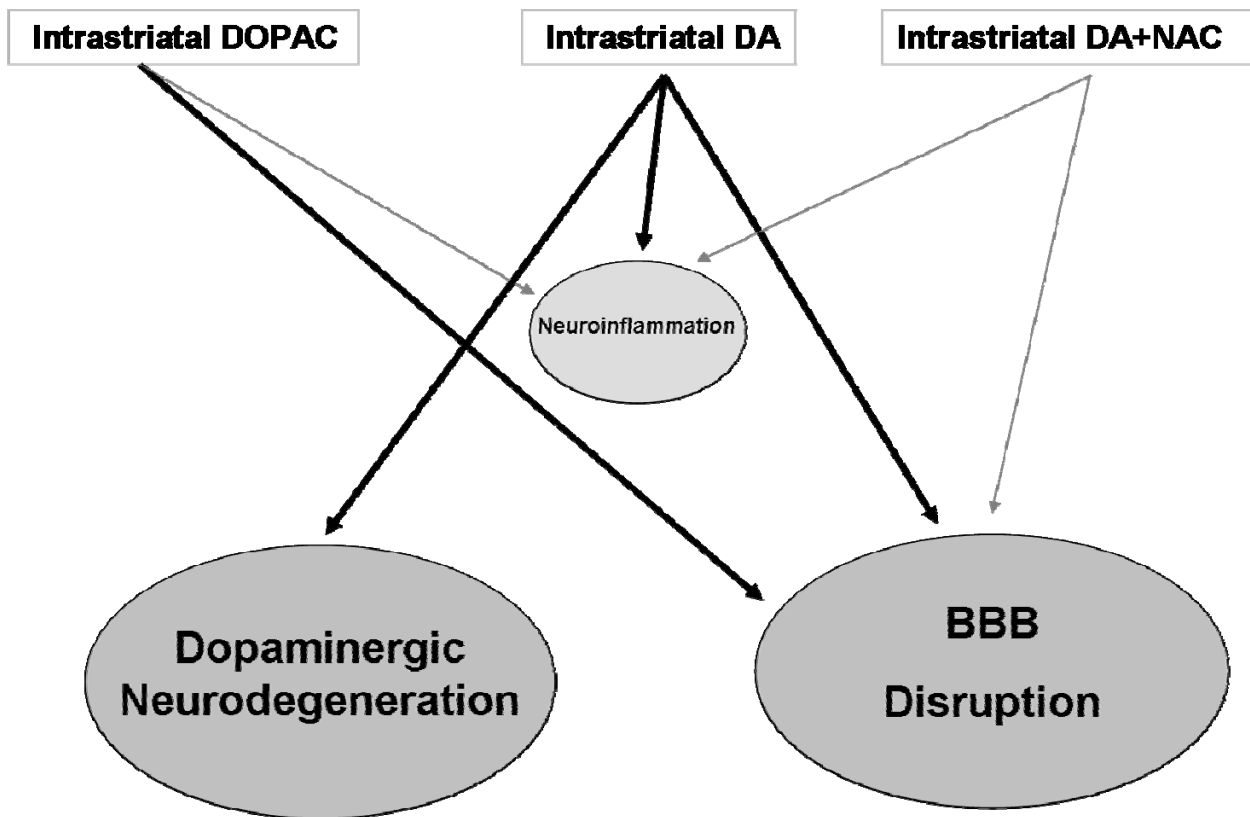
Schematic diagram summarizing results from Chapter 3. An intrastriatal DOPAC injection resulted in BBB disruption to a similar extent as an intrastriatal DA injection, but DOPAC did not lead to dopaminergic terminal degeneration. An intrastriatal co-injection of DA+NAC also did not lead to observable dopaminergic terminal degeneration, but did lead to BBB disruption,

although to a much less extent than DA. These findings support a role for oxidative stress in dopaminergic neurotoxin-induced BBB disruption and indicate that BBB disruption does not occur as a result of dopaminergic terminal degeneration. Intrastriatal DOPAC, DA, and DA+NAC injections also led to increased microglial activation, which may be a potential mechanism by which dopaminergic neurotoxins lead to BBB disruption.

SUMMARY SCHEMATIC FOR CHPATER 4

INTRASTRIATAL DA

+ Minocycline:
- ↓ DA-induced BBB disruption
- ↓ DA-induced microglial activation

+ Apocynin:
- ↓DA-induced BBB disruption
- ↓DA-induced microglial activation
- ↓DA-induced dopaminergic terminal degeneration

Microglial Activation ↔ NADPH oxidase activation

Dopaminergic Terminal Degeneration
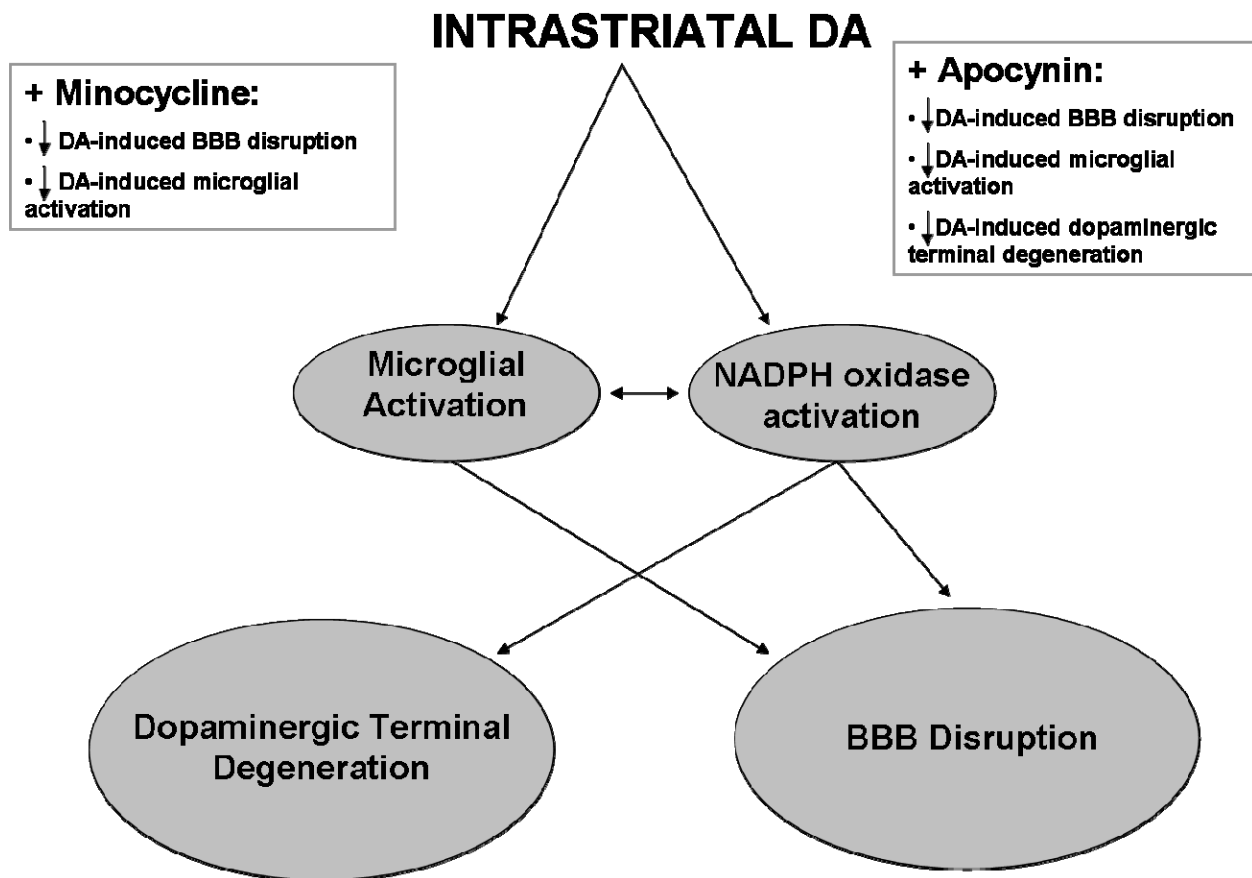
BBB Disruption

Schematic diagram summarizing results from Chapter 4. The contribution of DA-induced increases in microglial activation to BBB disruption and dopaminergic terminal degeneration in the striatum was examined utilizing the anti-inflammatory agent, minocycline. Treatment with

minocycline resulted in a reduction of DA-induced microglial activation and BBB disruption, but did not affect dopaminergic terminal degeneration, suggesting that neuroinflammation contributes to disruption of the BBB, but not to the loss of dopaminergic terminals in this model. This also provided additional evidence that dopaminergic terminal degeneration does not contribute to BBB disruption. Additionally, the effects of apocynin, an NADPH oxidase inhibitor, on DA-induced BBB disruption were examined. Apocynin treatment resulted in a decrease in DA-induced BBB disruption, microglial activation, and dopaminergic terminal degeneration, providing additional support for neuroinflammatory mechanisms in DA-induced BBB disruption.

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