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The Effect of Resveratrol on Lipopolysaccharide-induced Dopaminergic Deficits and BV-2 Cell Activation

Katherine Marie Rose

THE EFFECT OF RESVERATROL ON LIPOPOLYSACCHARIDE-INDUCED DOPAMINERGIC DEFICITS AND BV-2 CELL ACTIVATION

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By

Katherine Marie Rose

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ABSTRACT

THE EFFECT OF RESVERATROL ON LIPOPOLYSACCHARIDE-INDUCED DOPAMINERGIC DEFICITS AND BV-2 CELL ACTIVATION

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Thesis supervised by Jane E. Cavanaugh, Ph.D

Neuroinflammation is a common pathology found in patients with Parkinson's disease (PD). PD involves a loss of dopamine (DA) neurons and an increase in activated microglia with subsequent proinflammatory cytokine secretion in the substantia nigra (SN) and striatum. A loss of DA neurons is found in the offspring of animals exposed prenatally to the bacteriotoxin, lipopolysaccharide (LPS) (Ling et al., 2002). Activation of the extracellular regulated kinases, ERK1/2 and ERK5, the downstream targets in the mitogen-activated protein kinase (MAPK) pathway, has been shown to be involved in the dysregulation of the inflammatory process (Cuschieri and Maier, 2005). Consequently, LPS-induced activation of ERK1/2 and ERK5 may cause an increase in production and secretion of proinflammatory cytokines in activated microglia. LPS-mediated activation of ERK1/2 has been shown to be decreased by the phytochemical resveratrol (Zhang et

al., 2010). However, the effect of LPS or resveratrol on ERK5 signaling has not been explored. The purpose of this study was to determine (1) the effect of resveratrol on LPS-induced dopaminergic deficits in pups exposed prenatally to LPS, (2) the impact of resveratrol on LPS-induced BV-2 microglial cell activation and (3) the roles of ERK1/2 and ERK5 in resveratrol mediated inhibition of LPS-induced BV-2 cell activation. To test our hypothesis, pregnant rats received an intraperitoneal (i.p.) injection 10,000 EU/kg LPS at gestational day 10.5 (E10.5) and were fed a resveratrol-enriched diet for 20 days (E3 – E22.5). LPS-induced dopaminergic deficits in pups exposed prenatally at postnatal day 21 (P21), but not at P10 or P40. These deficits were exhibited by a loss of striatal 3,4-dihydroxyphenylacetic acid (DOPAC) and DA content and tyrosine hydroxylase (TH) expression in the P21 animals. However, dietary resveratrol supplementation increased TH expression, DA and DOPAC levels in the P21 pups following prenatal exposure to LPS. Thus, these data suggest that resveratrol treatment may restore the homeostasis of the DA neuronal system *in vivo*. However, contrary to previous reports it was determined *in vitro* that LPS-mediated BV-2 activation and ERK1/2 phosphorylation was not inhibited by resveratrol pretreatment. Interestingly, at 6 hours the MEK inhibitor U0126 decreased LPS-mediated ERK1/2 activation and TNF-α release. ERK5 was not activated by LPS, but preliminary data suggest that the MEK5 inhibitor BIX02189 inhibited LPS-induced TNF-α release. Therefore, BIX02189 may be inhibiting a distinct pathway in our model. Overall, these studies suggest that the use of dietary resveratrol supplementation may be protective against LPS-induced loss of striatal dopaminergic deficits in a time-dependent manner and inhibition of ERK signaling may reduce LPS-mediated microglial activation.

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1. INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disease that causes deficits in movement and coordination. Nearly 1 million people in the United States are diagnosed with PD. The cause of PD is unknown, but several factors are thought to play a role in its onset (Sulzer, 2007). The development of PD may be due to the exposure to various insults, such as early life exposure to brain injury, chemicals, or infection. These events can cause oxidative damage, excitotoxicity or mitochondrial dysfunction. In PD, the DA neurons of the substantia nigra (SN) are the main targets (Sulzer, 2007). As the disease advances, dopamine (DA) neurons progressively degenerate and the clinical symptoms worsen. Exposure to various insults during development may predispose an individual to becoming more vulnerable to subsequent exposures, resulting in cumulative neurotoxicity to the dopaminergic system. Thus, PD is a multifactorial disease and may be the result of multiple exposures to various risk factors throughout life (Sulzer, 2007).

PD patients exhibit a loss of DA neurons and an increase in activated microglia, subsequently leading to chronic neuroinflammation. Activated microglia release proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), in regions of the brain that contain an abundant amount of microglia, including the SN and striatum (Dutta et al., 2008, Choi et al., 2011). Thus, it is believed that neuroinflammation is associated with the development and progression of PD.

Neuroinflammation develops after an exposure to endotoxins, such as lipopolysaccharide (LPS). LPS is found in the outer membrane of gram-negative bacteria. LPS is an endotoxin that induces a strong immune response through the activation of microglia (Dutta et al., 2008). *In utero* exposure to LPS causes a loss in the number of DA neurons in the postnatal rat midbrain. This DA neuronal loss remains stable throughout life. LPS also significantly decreases the expression of tyrosine hydroxylase (TH), a phenotypic marker of DA neurons, in the SN (Ling et al., 2004a).

The interest in developing studies that target the release of the proinflammatory cytokines and other neurotoxic factors has increased over the past decade. These potential therapies have focused on natural supplements and their anti-oxidant and anti-inflammatory properties. Many studies have demonstrated the protective properties of fruits and vegetables that contain high levels of polyphenols against diseases of the central nervous system (CNS) (Poulose et al., 2012). Specifically, the phytochemical resveratrol, which is found in peanuts and in the skin of grapes, has been shown to protect against brain disorders in many different experimental models (Baur and Sinclair, 2006). The exact role that resveratrol plays in microglial activation in neurodegenerative disease models has not been fully elucidated.

Recent data has shown that resveratrol plays a role in several signaling cascades that are involved in endotoxin (LPS) exposure. Resveratrol inhibited the LPS-induced activation of the NF-kB pathway and, therefore, reduced the expression and activation of downstream proinflammatory genes (Zhang et al., 2010). Activation of the extracellular regulated kinases (ERKs), including ERK1/2 and ERK5, has been shown to be involved in the modulation of the inflammatory process (Cuschieri and Maier, 2005). LPS-induced

activation of ERK1/2 causes an increase in production and secretion of proinflammatory cytokines in activated microglia. Interestingly, Zhang and colleagues (2010) demonstrated that resveratrol inhibits LPS-induced activation of ERK1/2. Therefore, resveratrol may prevent neuroinflammation by inhibiting ERK1/2 and ERK5 activation in microglia.

The objective of this study was to examine the effects of resveratrol on LPS-induced microglial activation, the subsequent increase in dopaminergic deficits and if these changes were, in part, due to the modulation of the ERK1/2 and ERK5 signaling pathways (Fig. 1). It was hypothesized that resveratrol inhibits LPS-induced deficits in the developing dopaminergic neuronal system and BV-2 cell activation by inhibiting the activation of the ERK1/2 and ERK5 signaling cascades. Our rodent model was unique in that a resveratrol-enriched diet was administered to pregnant dams as an early intervention against LPS-induced toxicity *in utero*. The diet was administered before LPS exposure and continued throughout the rat's pregnancy. Intervening early, during prenatal development, may inhibit the long term consequences of a neurotoxin to the DA system. Also the role that ERK5 plays in the LPS-induced activation in BV-2 microglial cells has not been elucidated. It is crucial to first understand the signaling pathway involved in microglial activation and then begin to develop strategies that inhibit the activation of the pathways involved in the inflammatory response. A possible treatment that could inhibit ERK5 is resveratrol. Targeting specific kinases with a compound such as resveratrol in overactivated microglia may lead to the development of therapies that treat neuroinflammation and the dopaminergic deficits associated with it.

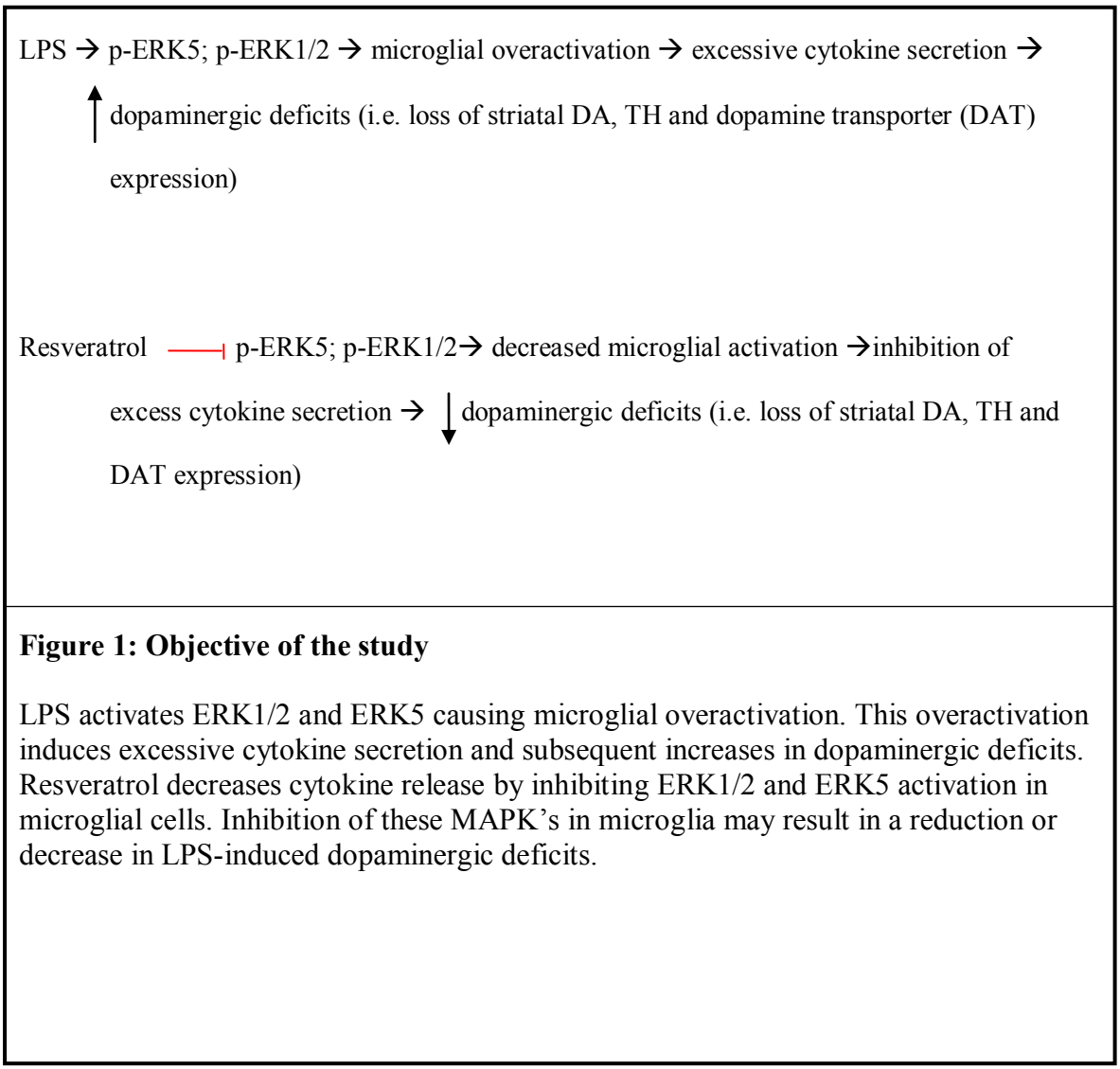


Figure 1: Objective of the study

LPS activates ERK1/2 and ERK5 causing microglial overactivation. This overactivation induces excessive cytokine secretion and subsequent increases in dopaminergic deficits. Resveratrol decreases cytokine release by inhibiting ERK1/2 and ERK5 activation in microglial cells. Inhibition of these MAPK's in microglia may result in a reduction or decrease in LPS-induced dopaminergic deficits.

2. LITERATURE REVIEW

2.1 LPS and Microglial Activation

Chronic inflammation accompanies a variety of pathophysiologies that are present in many neurodegenerative diseases, such as PD. Brain inflammation occurs when microglia, the mediators of the innate immune response, become activated (Choi et al., 2011). Once activated, microglia act as phagocytes by clearing cellular debris found in the CNS. Following activation, proinflammatory cytokines, chemokines, proteinases, prostaglandins and reactive oxygen or nitrogen species are released (Dutta et al., 2008). The release and accumulation of these cytotoxic factors produce an enhancement of the inflammatory response and subsequently cause neuronal damage. This is achieved through a cyclic response involving an increase in neuronal cell death followed by the continued activation of microglia (Zhang et al., 2010).

Mouse microglial cells (BV-2) are commonly used as a model for *in vitro* studies of activated microglia. This cell line was derived from raf/myc-immortalized murine neonatal microglia and expresses functional nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme that has been associated with microglia-triggered neuronal damage. BV-2 cells share more than 90 % of their genes that are induced by LPS with primary microglia (Henn et al., 2009). This microglia model is well established, for BV-2 cells contain morphological, phenotypical and functional properties found in primary microglia cultures (Blasi et al., 1990).

A highly activated microglial state has been noted following LPS exposure. LPS causes an increase in the release of reactive oxygen species (ROS), nitric oxide (NO) and

proinflammatory cytokines, such as TNF-α and IL-β (Laurenzi et al., 2001, Park et al., 2007, Poulose et al., 2012). Alone, LPS is not directly toxic to neurons as its toxic properties are achieved through the activation of microglia (Shih et al., 2009). For example, *in vitro* studies have demonstrated that LPS alone does not affect DA uptake or cell death in neuron-enriched cultures or cultures with microglial depletion. However, when microglia are added to the cultures, a 52 % loss of DA uptake and DA cell death was observed (Qin et al., 2005, Qian et al., 2006b, Block et al., 2007, Battes et al., 2012). Thus, LPS acts as an endotoxin and increases the release of cytokines, which in turn causes a toxic immune response (Gao et al., 2008).

Application of LPS is one of the most common experimental techniques utilized to activate microglia and is accomplished *via* prenatal exposure, intracerebral injection or systemic injection. Mechanistically, LPS binds to toll-like receptor 4 (TLR-4) on the microglia, which enables regulatory molecules within the cell (Mal, MyD88, Tram, and Trif) to trigger reactions that activate NF-kB. Activated NF-kB enters the cell nucleus and switches on genes coding for proinflammatory cytokines (Gao et al., 2008). Prenatal exposure of LPS is believed to activate the immune system of the pregnant dam causing subsequent alterations in the CNS of the developing fetus (Oskvig et al., 2012). LPS increases TNF-α, IL-1β and IL-6 levels in the amniotic fluid, choriamnion and placenta of pregnant dams (Urakubo et al., 2001, Gayle et al., 2004, Ashdown et al., 2006, Chlodzinska et al., 2011, Oskvig et al., 2012). Radiolabeled LPS (^{125}I-LPS) was detected in the maternal placenta, blood and tissues of the liver and kidneys (Ashdown et al., 2006) after i.p. LPS injection. Thus, LPS indirectly affects the CNS of the offspring

prenatally exposed to LPS through the use of maternal immune mediators such as TNF-α and IL-1β.

2.2 LPS and Neurodegenerative Diseases

Patients with PD exhibit an increase in activated microglia, subsequently leading to chronic neuroinflammation. Microglia release proinflammatory cytokines, such as TNF-α, in regions of the brain that play a role in inflammation-mediated neurodegeneration (Dutta et al., 2008, Choi et al., 2011). A loss of DA neurons is found in the offspring of animals exposed prenatally to the bacteriotoxin LPS and this reduction in DA neurons is thought to occur through the activation of the inflammatory process (Ling et al., 2002).

Neurodegenerative diseases such as PD are believed to develop from neurotoxic exposure and genetic factors that cause oxidative damage and cell death (Ling et al., 2004a). The DA neurons in the SN are the most sensitive to such damage (Barlow et al.). Once the inflammatory process is initiated, subsequent oxidative damage and excitotoxicity later in life may cause additional SN neuronal death (Sulzer, 2007). Thus, *in utero* LPS exposure may predispose an individual to developing PD by acting as an early insult which could, in turn, lead to a more detrimental loss of DA neurons when exposed to a second toxin later in life.

2.3 LPS and bacterial vaginosis (BV)

Gram-negative bacterial infections, such as BV, cause various complications related to early pregnancy and subsequent fetal death (Aroutcheva et al., 2008). BV is

caused by a replacement of the normal vaginal flora by an overgrowth of anaerobic gram-negative microorganisms and some gram-positive facultative anaerobes. Specifically, an overgrowth of *Gardnerella vaginalis* and *Escherichia coli* in the vaginal environment is associated with BV (Ling et al., 2002).

Gram-negative bacterial endotoxins, such as LPS, have been found to be considerably damaging to preimplantation stage embryos. The most common period of infection occurs during the preimplantation and implantation stages of embryonic development. Both are early stages of pregnancy, usually resulting in the pregnancy being unnoticed by the mother. Undiagnosed BV in a pregnant woman places both the life of the fetus and of the mother at risk (Deb et al., 2004).

The LPS model is used to replicate BV in animals. Loss of DA neurons is found in the offspring of animals prenatally exposed to LPS (Ling et al., 2002). It is believed that BV may cause a decrease in DA neuronal development and is associated with low birth weight, prenatal complications and fetal mortality. Patients with BV have increased levels of LPS, IL-1β, IL-6 and TNF-α in their chorioamniotic environment (Deb et al., 2004). Prenatal exposure has been linked to neurological diseases such as periventricular leukomalacia, intraventricular hemorrhage and cerebral palsy (Ling et al., 2002).

2.4 Resveratrol protection against LPS-induced toxicity

The polyphenolic compound resveratrol is produced naturally by many plants as a defense against disease and fungal infections. It is found in peanuts, cocoa powder and the skin of grapes and is believed to have cardioprotective, anti-oxidant and anti-cancer effects. The activation of microglia and the subsequent release of proinflammatory

factors have been attenuated through resveratrol treatment. This reduction is believed to occur through inhibition of the transcription factor NF-kB (Ling et al., 2004a, Zhang et al., 2010).

Several *in vitro* and *in vivo* studies have been conducted to determine the absorption, bioavailability and metabolism of resveratrol. After oral administration resveratrol is rapidly absorbed by the small intestine. Glucuroconjugates and sulfoconjugates of resveratrol are then formed. The two main metabolites of resveratrol are *trans*-resveratrol-3-O-glucuronide and *trans*-resveratrol-3-sulfate (Amri et al., 2012). Resveratrol either enters mesenteric circulation leading to hepatic uptake and systemic distribution or it is excreted via the kidneys (Abraham and Johnson, 2009, Amri et al., 2012). Despite previous reports stating that resveratrol's metabolism in the intestine and liver results in poor oral bioavailability, recent studies have shown resveratrol and/or its metabolites can cause biological effects in the brain (El Mohsen et al., 2006, Abraham and Johnson, 2009, Vingtdeux et al., 2010, Pasinetti et al., 2011).

Previous studies demonstrated that resveratrol administration 7 days prior to LPS exposure in an adult mouse model reversed LPS-induced depletion of superoxide dismutases, catalase and peroxidase (Sebai et al., 2009). Dietary supplementation of resveratrol reduced LPS-induced IL-1β production and locomotor deficits (Abraham and Johnson, 2009). Pre-treatment with resveratrol also improved the survival rate of LPS treated mice (Sebai et al., 2009, Sebai et al., 2010). Thus, resveratrol neuroprotection of DA neurons against LPS-mediated neurotoxicity may be achieved through its anti-oxidant properties, inhibition of microglial activation and the release of proinflammatory factors.

In vitro studies have shown the neuroprotective effects of resveratrol. In mixed neuron-glia cultures, resveratrol protected DA neurons against LPS-induced toxicity, decreased LPS-induced ROS production and attenuated the LPS-induced decrease in DA uptake (Zhang et al., 2010). Resveratrol decreased TNF-α and NO production, caused the inhibition of the transcription factor NF-kB and the gene expression and the release of many other proinflammatory cytokines in primary mouse and rat microglial cultures (Bi et al., 2005, Baur and Sinclair, 2006, Lu et al., 2010, Zhang et al., 2010). In N9 murine microglial cells and primary microglial cultures, resveratrol inhibited LPS-induced TNF-α, iNOS expression, NO production and activation of the mitogen activated protein kinase (MAPK) p38 (Bi et al., 2005, Baur and Sinclair, 2006, Lu et al., 2010, Zhang et al., 2010, Choi et al., 2011). Other signaling cascades, P13K/Akt, SIRT1 and glycogen synthase kinase-3β, have been associated with resveratrol-mediated inhibition of LPS-induced microglial activation. In addition, resveratrol inhibited the activation of NADPH oxidase, an enzyme complex involved in the production of ROS (Lu et al., 2010, Zhang et al., 2010, Choi et al., 2011). In both primary microglia and N9 cells, resveratrol inhibited LPS-induced expression and production of IL-6, IL-1β and MCP-1 (Lu et al., 2010). Thus, resveratrol differentially modulates the production and expression of proinflammatory cytokines in activated microglia. These inhibitory properties of resveratrol are specific to different cell lines and primary microglial cultures.

2.5 ERK1/2 and ERK5

The MAPK signaling pathways have been associated with many cellular functions. The MAPK family of proteins includes the p38, c-*Jun* N-terminal kinase

(JNK) and extracellular signal-regulated kinases (ERKs) (Obata et al., 2007). ERK1/2 and ERK5 activation has been associated with the misregulation of the inflammatory process (Cuschieri and Maier, 2005). Previous literature has shown that in primary microglia and BV-2 cells, LPS activated ERK1/2 and this activation was inhibited by PD98059, a non-specific inhibitor of the upstream activator of ERK, MAPK kinase kinase (MEK) (Bhat et al., 1998, Park et al., 2007, Wang et al., 2011). In addition, LPS-induced NO production, iNOS gene expression, TNF-α and IL-1β release and expression were inhibited by PD98059 in BV-2 cells and primary microglia (Bhat et al., 1998, Park et al., 2007, Lu et al., 2010). These data suggest that ERK1/2 plays an important role in LPS stimulated microglial activation. However, it has been shown that PD98059 also inhibits the upstream kinase of ERK5, MEK5 (Kamakura et al., 1999). Therefore, the ERK5 signaling pathway may be involved in LPS-induced cytokine production and release in microglial cells. Further studies are needed to determine the role of ERK5 in neuroinflammation.

Various studies have been conducted that look at the effect of resveratrol on MAPK signaling. Resveratrol inhibited LPS-induced activation of ERK1/2 in primary microglia-enriched cultures (Zhang et al., 2010). However, in N9 murine microglia cells, resveratrol had no effect on LPS-induced phosphorylation of ERK1/2 (Lu et al., 2010). These findings suggest that resveratrol has varying effects on ERK1/2 activation depending on the cell line or culture. Therefore, it is possible that LPS induces activation of ERK1/2 and ERK5 in other microglial cell lines, such as BV-2. An increase in the production and secretion of proinflammatory cytokines in activated BV-2 cells may occur, in part, due to the activation of ERK1/2 and ERK5. Resveratrol treatment may

inhibit these signaling pathways which inhibit the release of these cytokines leading to a decrease in neuroinflammation caused by overactive microglia. Therefore, elucidating the effect of resveratrol on ERK1/2 and ERK5 activation in BV-2 cells may lead to potential therapies that target overactivated microglia.

2.6 Clinical Significance

Neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, PD and Huntington's disease have been shown to involve many factors that promote the inflammatory process (Yang et al., 2008). Neuroinflammation that results from exposure to a neurotoxin may be inhibited by resveratrol, resulting in a lower risk of developing PD and other neurodegenerative diseases later in life. Moreover, elucidating the mechanisms associated with the loss of DA neurons in individuals with a preexisting proinflammatory state may lead to the development of effective preventive strategies and therapies.

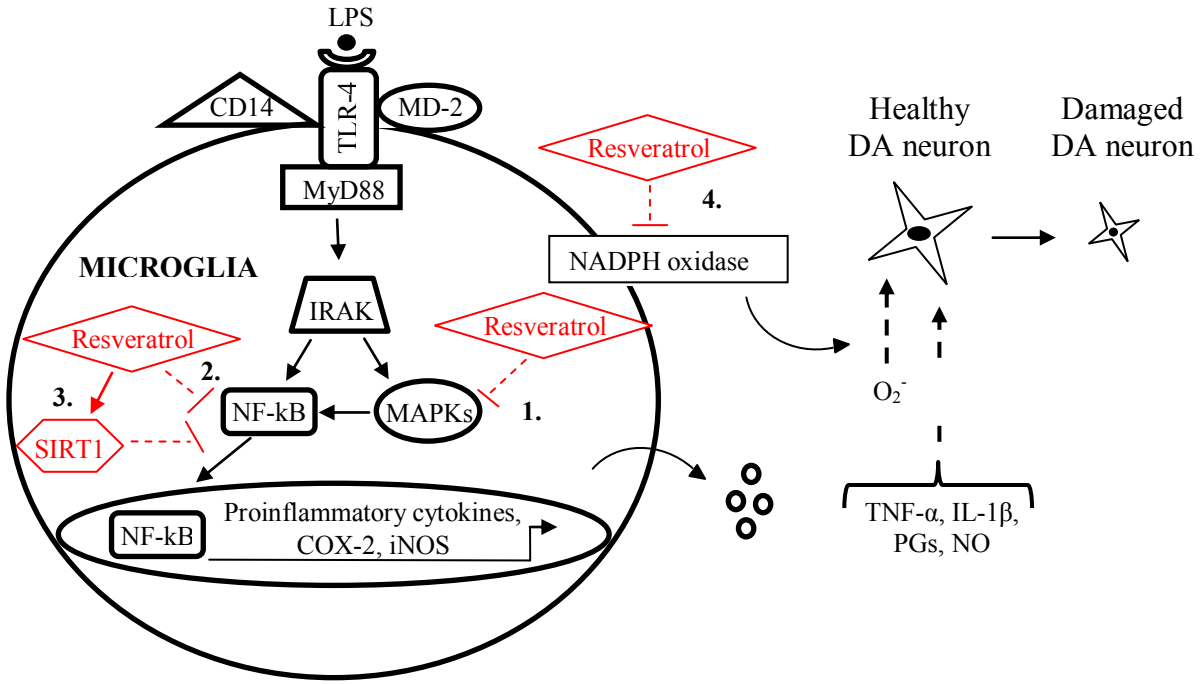


Figure 2: Schematic representation of the possible mechanisms of resveratrol on LPS-induced microglial activation and the subsequent DA neuronal damage

Adapted and modified from Dutta et al., 2008. LPS binds to toll-like receptor 4 (TLR-4), the intermediate receptor (CD14) and the accessory adaptor protein (MD-2) to activate regulatory molecules within microglia. This initiates the activation of the downstream signaling cascades such as interleukin-1R-associated kinase (IRAK), mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-kB). Activated NF-kB enters the cell nucleus and switches on genes coding for proinflammatory cytokines (TNF-α and IL-1β), COX-2 and iNOS. COX-2 and iNOS expression releases prostaglandins (PGs) and nitric oxide (NO). NADPH oxidase generates O_2^- that combines with NO to form the free radical ($ONOO^-$). The proinflammatory cytokines, ROS and lipid metabolites collectively damage the DA neuron, consequently leading to DA neurodegeneration (Gao et al., 2008).

Several possible mechanisms of resveratrol protection against LPS- induced toxicity include:

1) Inhibition of the activation of the MAPK pathway, specifically ERK1/2 and/or ERK.

2) Inhibition of NF-kB activation that consequently inhibits gene transcription for proinflammatory cytokines, COX-2 and iNOS.

3) Activation of Sirtuin 1 (SIRT1) which, in turn, suppresses LPS-induced NF-kB activation.

4) Resveratrol acts as an antioxidant agent by inhibiting the activation of NADPH oxidase, leading to a decrease in the production of ROS.

3. MATERIALS AND METHODS

3.1 Animals

Twenty timed-gravid Sprague Dawley rats at embryonic day two (E2) were obtained from Hilltop Lab Animals Inc. (Scottdale, PA) and were housed 1 per cage. All animals were maintained on a 12 hour light/dark cycle. Water and food were supplied *ad libitum*. All procedures were conducted in strict accordance with the guidelines for the National Institutes of Health Care and Use of Laboratory Animals and approved by the Duquesne University Institutional Animal Care and Use Committee.

3.2 LPS Administration

LPS (*Escherichia coli* O26:B6, product # L8274) was purchased from Sigma (St. Louis, MO) and dissolved in normal saline at 10,000 endotoxin units (EU)/mL (Jann et al., 1975). At E10.5, eight females were administered an i.p. injection of 10,000 EU/kg LPS. Vehicle-treated animals received a normal saline (1 mL/kg) i.p. injection and control animals did not receive an injection. All animals were observed daily for signs of distress. The rats were allowed to give birth and the pups were weaned at P21 and housed with same sex partners. Two LPS-treated females failed to give birth.

3.3 Resveratrol Administration

Gravid females in both the resveratrol and LPS+ resveratrol treatment groups were provided with 40 g of a pelleted resveratrol-enriched diet (120 mg of resveratrol per kg of regular rodent diet) prepared by Harlan Teklad (Madison, WI). Vehicle treated and

control animals received a regular rodent diet (Labdiet 5001; PMI Nutrition International, Brentwood, MO). The rats were fed either the resveratrol-enriched or control diet beginning at E3 and ending after pup delivery (E22.5).

3.4 Preparation of Tissues

At postnatal day 10 (P10), P21 and P40 the pups were sacrificed during the light phase by decapitation under aseptic conditions. At P10 in each treatment group, 8-12 pups were taken from 2-4 dams; at P21 in each treatment group, 10-18 pups were taken from 2-3 dams; at P40 in each treatment group, 9-21 pups were taken from 2-3 dams. In total, 179 pups from 15 dams were obtained. The left and right striatum, prefrontal cortex and hippocampus were dissected, frozen on dry ice and stored at -80 ºC. The hindbrains were removed quickly and placed in a 4 % paraformaldehyde fixing solution for a maximum of seven days, then transferred to 30 % sucrose in a 0.1M PBS solution and stored at 4 °C. The experimental design is illustrated in Fig. 3.

3.5 HPLC Analysis

The left striatum of P10, P21 and P40 pups was sonicated in 0.1N perchloric acid. After filter centrifugation, aliquots of the samples were analyzed by HPLC. Levels of DA and its metabolites, DOPAC and homovanillic acid (HVA), and serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were measured. Sandra Castro Scheirer ran the tissue samples at the University of Pittsburgh, Pittsburgh, PA. An aliquot of each sample was injected into an ESA C18 column (2.1 X 150 mm, ESA, Inc., Chelmsford, MA). The mobile phase consisted of 75 mM H_2NaPO_4, 1.7 mM octanesulfonic acid, 25

mM Na_2EDTA 0.00001 % triethylamine (v/v) and 10 % acetonitrile (v/v), pH 3.0. The reagents were pumped through the system at a rate of 0.3 mL/min with an ESA LC-10AD pump (ESA Inc., Chelmsford, MA). Samples were detected coulometrically using an ESA Coulochem Model 4100A detector, an ESA Model 5010 conditioning cell and an ESA Model 5014B microdialysis cell (ESA Inc., Chelmsford, MA). The settings for detection were E_1 = 0.26 V, E_2 = +0.28 V, guard cell = +0.4 V. The limits of detection were in the femtomole range. Quantification was done using standard curves for DA, DOPAC, HVA, 5-HIAA and 5-HT. Data were analyzed at Duquesne University, Pittsburgh, PA.

3.6 Western blot Analysis for DAT and TH

The right striatum of P10, P21 and P40 pups was sonicated in cold lysis buffer. Protein (60 μg) was separated on a 10 % SDS/PAGE gel at 100v for 2 hours. The gel was transferred to a PVDF membrane and blocked for 1 hour in casein blocking buffer (Sigma, St. Louis, MO*)*. The membranes were incubated in rabbit monoclonal anti-DAT (1:3,000; catalog # AB2231; lot # NG7853750); Millipore, Billerica, MA) or mouse monoclonal anti-TH (1:2,000; catalog # MAB318; lot # LV1594176; Millipore, Billerica, MA) antibodies in casein blocking buffer (Sigma, St. Louis, MO*)* overnight at 4 °C. The membranes were washed four times with 10X PBS/0.1 % Tween20/0.1 % NaN_3 and incubated for 1 hour at room temperature in the dark with IR680-labeled goat anti-rabbit (lot # C00617-05) or IR800-labeled goat anti-mouse (lot # B90608-03; Li-Cor; Lincoln, NE) both at 1:20,000 dilutions secondary antibodies. Mouse anti-GAPDH (1:5,000; lot # 078K4781; Millipore, Billerica, MA) antibody was used as a loading control. The

membranes were scanned with LI-COR Odyssey™ Infrared Imaging System for protein visualization.

3.7 BV-2 Cell Culture

BV-2 murine microglial cells (generously provided by Dr. Jonathon Godbout, The Ohio State University, Columbus, OH) were maintained at 37 °C and 5 % CO_2. Cells were grown and passaged in Dulbecco's modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10 % fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 250 µg/mL Amphotericin B and 3.7 g/L sodium bicarbonate (Sigma; St. Louis, MO). The cells were grown until they were nearly 80 % confluent in 162 cm^2 cell culture flasks, incubated with 2 mL TrypLE™ Express (Life Technologies, Grand Island, NY) for 5 minutes then plated at desired densities for various assays.

3.8 SH-SY5Y Cell Culture

SH-SY5Y human neuroblastoma cells (CRL-2266) were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10 % fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 100 mM µg/mL sodium pyruvate and uridine (Sigma; St. Louis, MO) at 37 °C and 5 % CO_2. SH-SY5Y cells were plated in 10 cm cell culture plates, incubated with 2 mL TrypLE™ Express (Life Technologies, Grand Island, NY) for 5 minutes then plated at a density of $1.5x10^4$ cells per well in 96-well sterile plates.

3.9 Cell Viability Assay

In mono-culture, BV-2 or SH-SY5Y cells were seeded in 96-well culture plates ($1.5x10^4$ per well/200µl) and treated with various concentrations (100-1000 ng/mL) of LPS (*Escherichia coli* O26:B6, product# L8274; Sigma; St. Louis, MO) for 24 and 48 hours. H_2O, DMSO and DA (100 µM) were used as controls. Thirty minutes prior to LPS treatment, BV-2 cells were treated with 1, 3 or 5 µM resveratrol (Sigma, St. Louis, MO*)*. The number of viable cells in culture was measured and based on the quantitation of ATP by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI).

3.10 TNF-α ELISA

Quantification of TNF-α release in BV-2 cell culture media was performed using an ELISA kit (R&D Systems, Minneapolis, MN) and completed according to the manufacturer's instructions. Cells were plated in 24-well culture plates ($2x10^5$ per well/mL) and treated with 500 ng/mL LPS for 6 or 24 hours. Thirty minutes before LPS treatment the cells were treated with 3 µM resveratrol (Sigma, St. Louis, MO). Prior to resveratrol pretreatment, the cells were treated with 10 µM U0126 (catalog # 9903; Cell Signaling Technology, Inc.; Boston, MA) or BIX02189 (catalog # S1115; Selleck Chemicals; Houston, TX) for 1 hour. The cell supernatants were collected and centrifuged at 1200 xg for 10 minutes at 4 °C. Samples were stored at -80 °C, then thawed and diluted (60x) with calibrator diluent RD5K (R&D Systems, Minneapolis, MN). 50 µL of sample was added to a 96-well plate coated with a monoclonal antibody specific for mouse TNF-α and incubated for 2 hours. Wells were washed and a TNF-α conjugate was added to each well and incubated for an additional 2 hours. Each well was

washed again and incubated with substrate solution for 30 minutes, followed by a stop solution. The optical density was determined using a microplate reader set at 450 nm. The TNF-α concentration in the samples was calculated from the linear equation that was derived from a standard curve and expressed in pg/mL. All samples and standards were tested in duplicate.

3.11 Western blot Analysis for ERK1, ERK2 and ERK5

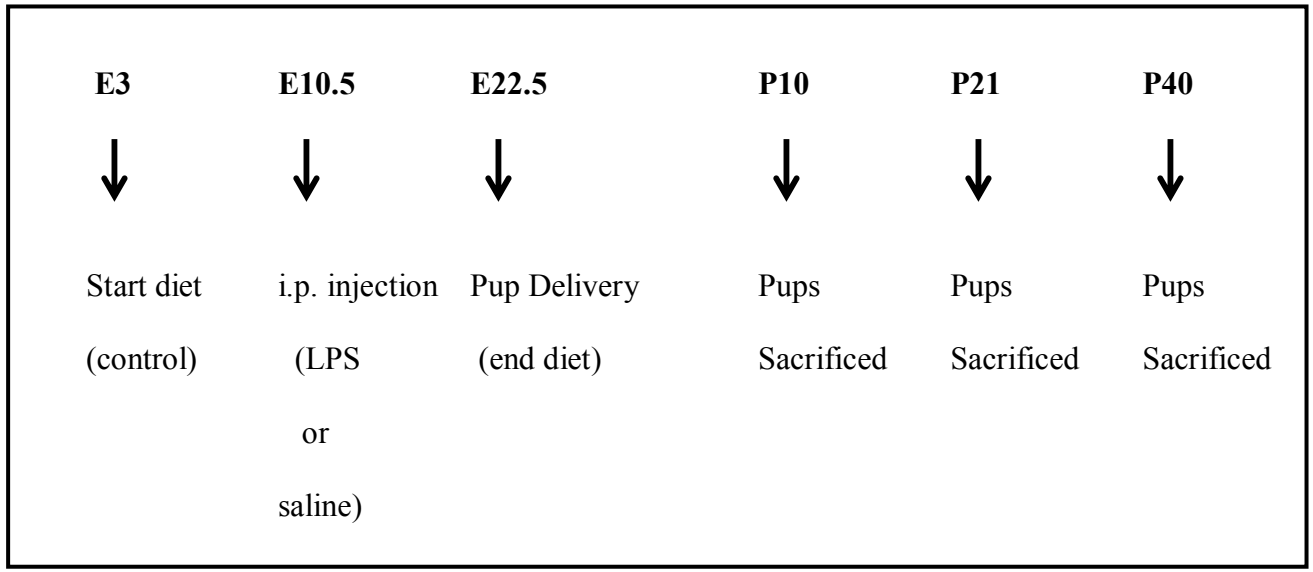
BV-2 cells were plated in 35mm ($6x10^5$ cells / 2mL) and 60 mm ($1x10^6$ cells/ 4mL) plates, and treated with 500 ng/mL LPS for various time points. Thirty minutes before LPS treatment the cells were treated with 3 μM resveratrol (Sigma, St. Louis, MO). Prior to the resveratrol pretreatment the cells were treated with 10 μM U0126 (catalog # 9903; Cell Signaling Technology, Inc.; Boston, MA) or 10 μM BIX02189 (catalog # S1115; Selleck Chemicals; Houston, TX) for 1 hour. The cells were sonicated in cold lysis buffer. Protein was separated by a 10 % SDS/PAGE gel at 100 for 2 hours. The gel was transferred to a PVDF membrane and blocked for 1 hour in casein blocking buffer (Sigma, St. Louis, MO*)*. The membranes were incubated in monoclonal rabbit anti-pERK1/2 (1:1,000; catalog # 9101L; lot#26; Cell Signaling Technology, Inc.; Boston, MA), monoclonal mouse anti-ERK1/2 (1:2,000; catalog # 9107S; lot # 7; Cell Signaling Technology, Inc.; Boston, MA), monoclonal rabbit anti-pERK5 (1:1,000; catalog # 33171S; lot # 4 Cell Signaling Technology, Inc.; Boston, MA) or monoclonal mouse anti-ERK5 (1:1,000; catalog # H0510) antibodies in casein blocking buffer (Sigma, St. Louis, MO*)* overnight at 4 °C. The membranes were washed four times with 1XPBS/0.1 % Tween20/0.1 % NaN_3 for 5 minutes and incubated for 1 hour at room

temperature in the dark with IR680-labeled goat anti-rabbit (lot # C00617-05) or IR800-labeled goat anti-mouse (lot # B90608-03) both 1:20,000 dilutions (Li-Cor; Lincoln, NE) secondary antibodies. The membranes were scanned with LI-COR Odyssey™ Infrared Imaging System for protein visualization.

3.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA). Grubbs' test (GraphPad Software, Inc., La Jolla, CA) was performed to determine if there were any significant ($p<0.01$) outliers in the experimental groups. Data were analyzed by unpaired t-test or one-way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison or Tukey's Multiple Comparison post hoc test. Significant statistical differences were defined as $p<0.05$.

3A. Vehicle and LPS groups



3B. Resveratrol and LPS+ resveratrol groups

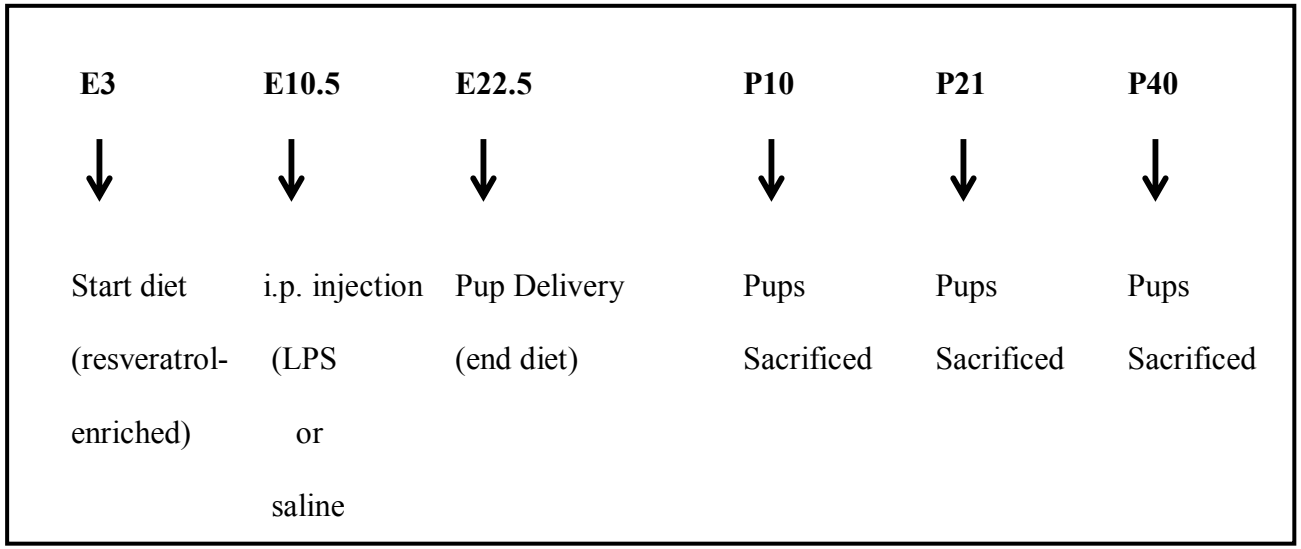


Figure 3: *In vivo* Experimental Design

Timeline 3A illustrates the vehicle and LPS-alone treatment groups. 3B illustrates the resveratrol-alone and the LPS+ resveratrol treatment groups. Gravid females received a control or resveratrol-enriched diet from E3-E22.5. They were administered an i.p. injection of either saline or LPS at E10.5. The rats delivered their pups normally at E22.5 and the pups were sacrificed at P10, P21 and P40.

4. RESULTS

4.1 Vehicle treatment did not affect DA, DOPAC or HVA levels

HPLC was performed to measure DA, DOPAC and HVA content in the striatal tissue of P10, P21 and P40 pups. There was no difference in DA, DOPAC or HVA levels in the vehicle treatment group when compared to the control group in the P10 and P40 pups. However, at P21 there was a significant decrease in DA, DOPAC and HVA content in the vehicle treatment group when compared to the control group. Based on the P10 and P40 data it was concluded that the vehicle treatment group had no effect on the DA, DOPAC or HVA levels. After reviewing our protocol it was determined that there was an experimental error in tissue preparation or homogenization. Human error might have occurred during manual dissection of the P21 vehicle treated group. This group was the first group of the P21 pups to be dissected. Therefore, this might have possibly resulted in inexperienced tissue identification which was corrected in the subsequent dissections. Also, experimental error might have occurred during tissue homogenization. Striatal tissue samples of the P21 vehicle treatment group were homogenized on a different day than other P21 treatment groups. Therefore, a variation in the perchloric acid buffer might have occurred on this day. The DA, DOPAC and HVA raw data values in the P21 vehicle treated group were extremely lower than the other treatment groups as well. Therefore, it is believed that there was not a true significant difference in DA, DOPAC or HVA levels between the vehicle or control group (Fig. 4). In the remaining figures the vehicle treatment group was excluded from graphs and analyses.

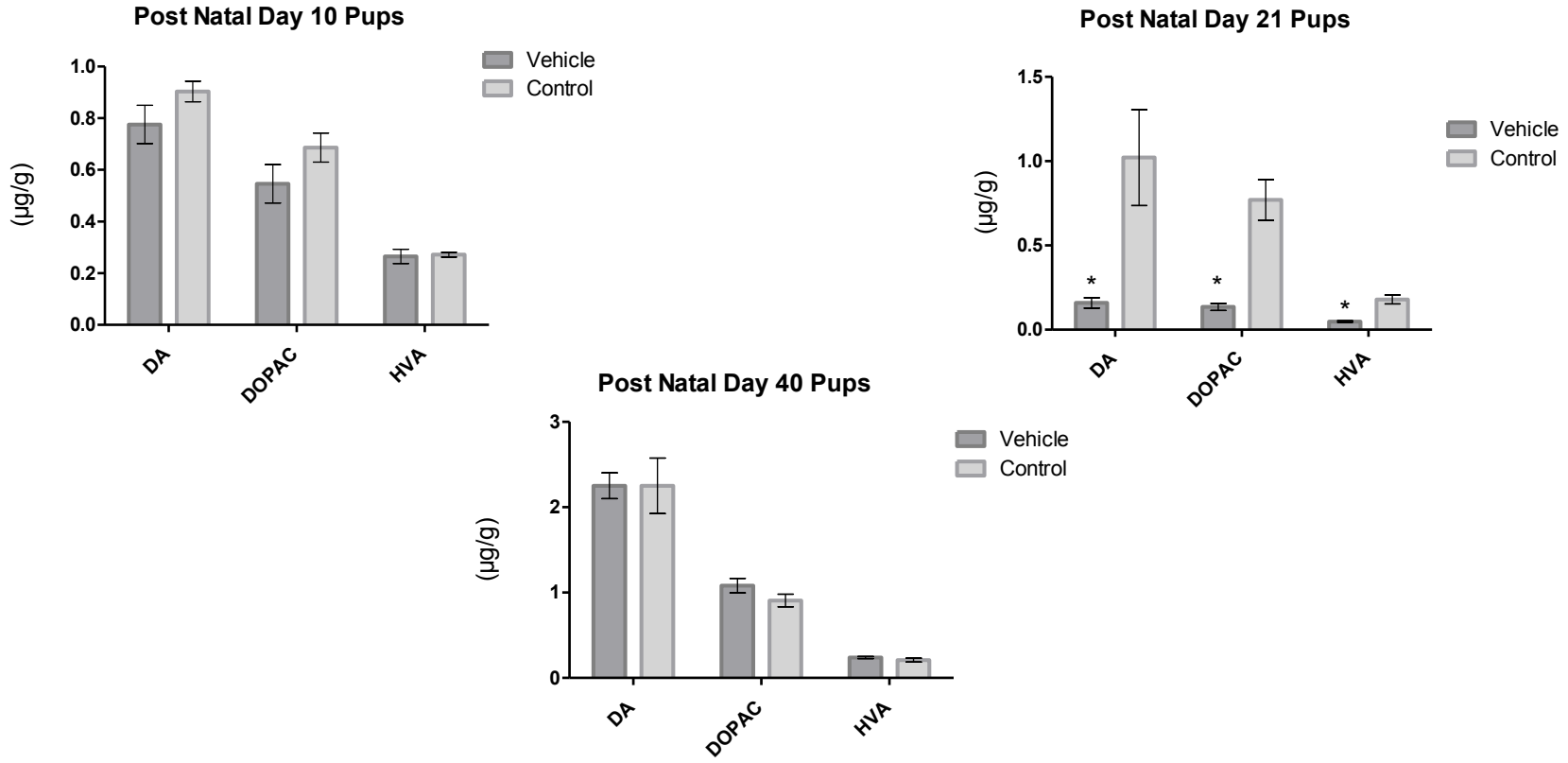


Figure 4: DA, DOPAC and HVA levels were not affected by vehicle treatment

Results are presented as mean ± SEM, n= 8-10 per group. P10, P40 and P21 striatal tissue was analyzed for DA, DOPAC and HVA content using HPLC. There was no significant difference in DA, DOPAC and HVA levels in the vehicle when compared to the control in P10 and P40 pups. A significant decrease in DA, DOPAC and HVA content in the vehicle treatment group when compared to the control group was observed at P21 However, it is believed that this difference was not a true representation of vehicle and control DA, DOPAC and HVA levels in the P21 pups after analyzing the P10 and P40 data. Data were analyzed by an unpaired t-test and significance (*) is defined as $p<0.01$ when compared to control.

4.2 LPS treatment did not alter striatal DA, DA metabolites or DA turnover when compared to control in P10 pups

DA turnover ([HVA/DA]), DA, DOPAC and HVA content were measured in striatal tissue of P10 pups by HPLC. DA turnover, DA and HVA levels in the resveratrol, LPS or LPS+ resveratrol groups were not significantly different than the control. DOPAC levels in the dietary resveratrol supplementation group were significantly lower when compared to the control and the LPS-treated groups (Fig. 5A). To determine whether the effects of LPS altered other neurotransmitters, 5-HT turnover ([5-HIAA/5-HT]), 5-HT and 5-HIAA content was measured. As shown in Fig. 5B, no change in 5-HT turnover, 5-HT or 5-HIAA content was observed in any treatment group.

4.3 Resveratrol protected against the LPS-induced loss of striatal DA and DOPAC in P21 pups

HPLC was performed to measure DA, DOPAC, HVA, 5-HT and 5-HIAA content in the striatal tissue of P21 pups. LPS significantly decreased striatal DA and DOPAC content when compared to control. Dietary resveratrol supplementation protected against the LPS-induced loss of DA and DOPAC levels. DOPAC and HVA levels in the resveratrol treatment group were significantly higher than in the LPS treated group (Fig. 6A). No change in DA turnover, 5-HT turnover, 5-HT or 5-HIAA content was observed in any of the treatment groups (Fig. 6A & 6B).

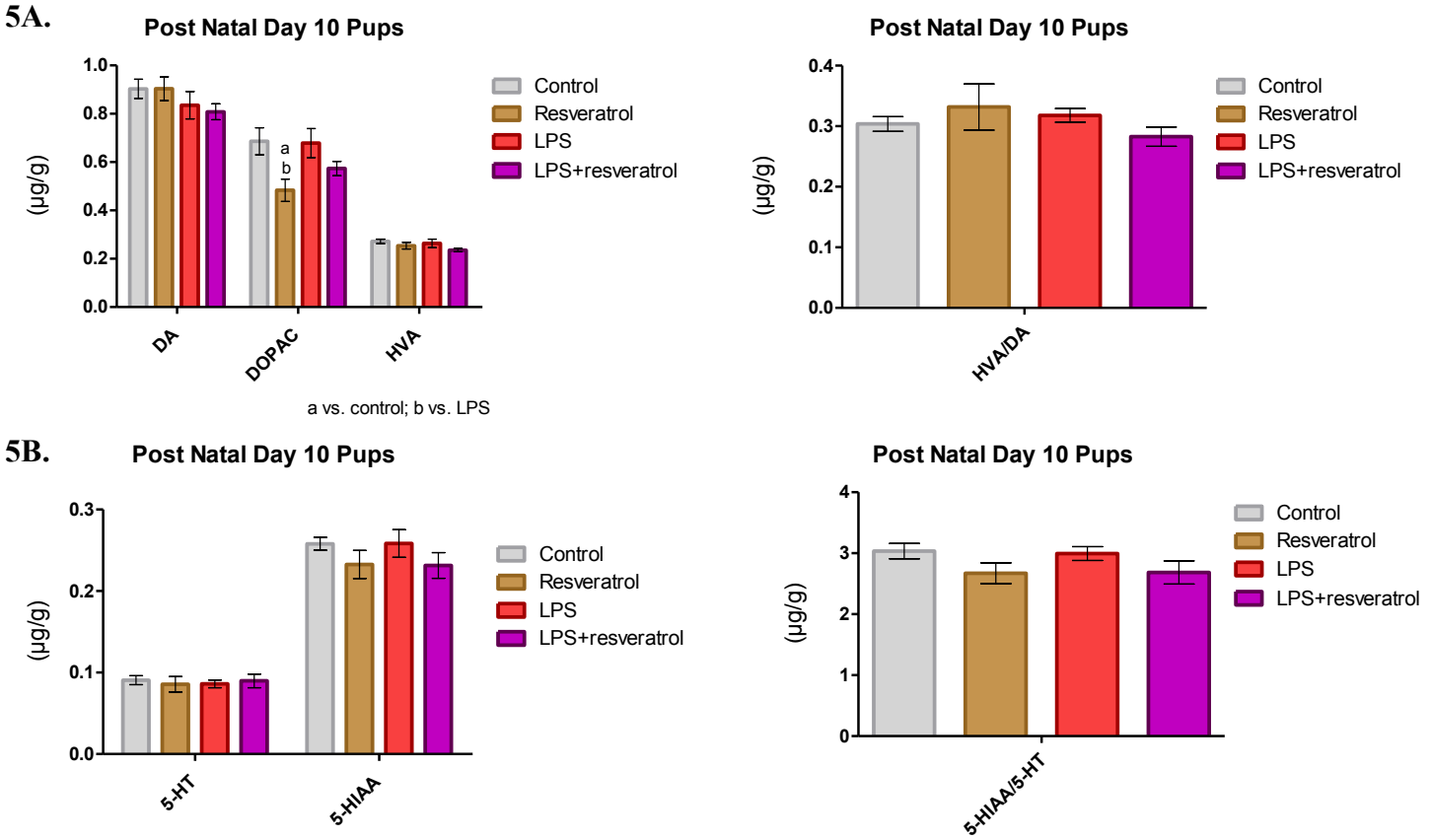


Figure 5: Striatal DA, DA metabolites or DA turnover were not altered by LPS treatment at P10

Results are presented as mean ± SEM, n= 8-10 per group. P10 striatal tissue was analyzed for DA, DOPAC, HVA, 5-HT and 5-HIAA content using HPLC. DA turnover, DA and HVA levels in all treatment groups were not significantly different than the control. DOPAC content in the resveratrol treated group was significantly lower than the control and LPS treatment groups (Fig. 5A). 5-HT turnover, 5-HT and 5-HIAA content were not altered in any treatment group (Fig. 5B). Data were analyzed by 1-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$).

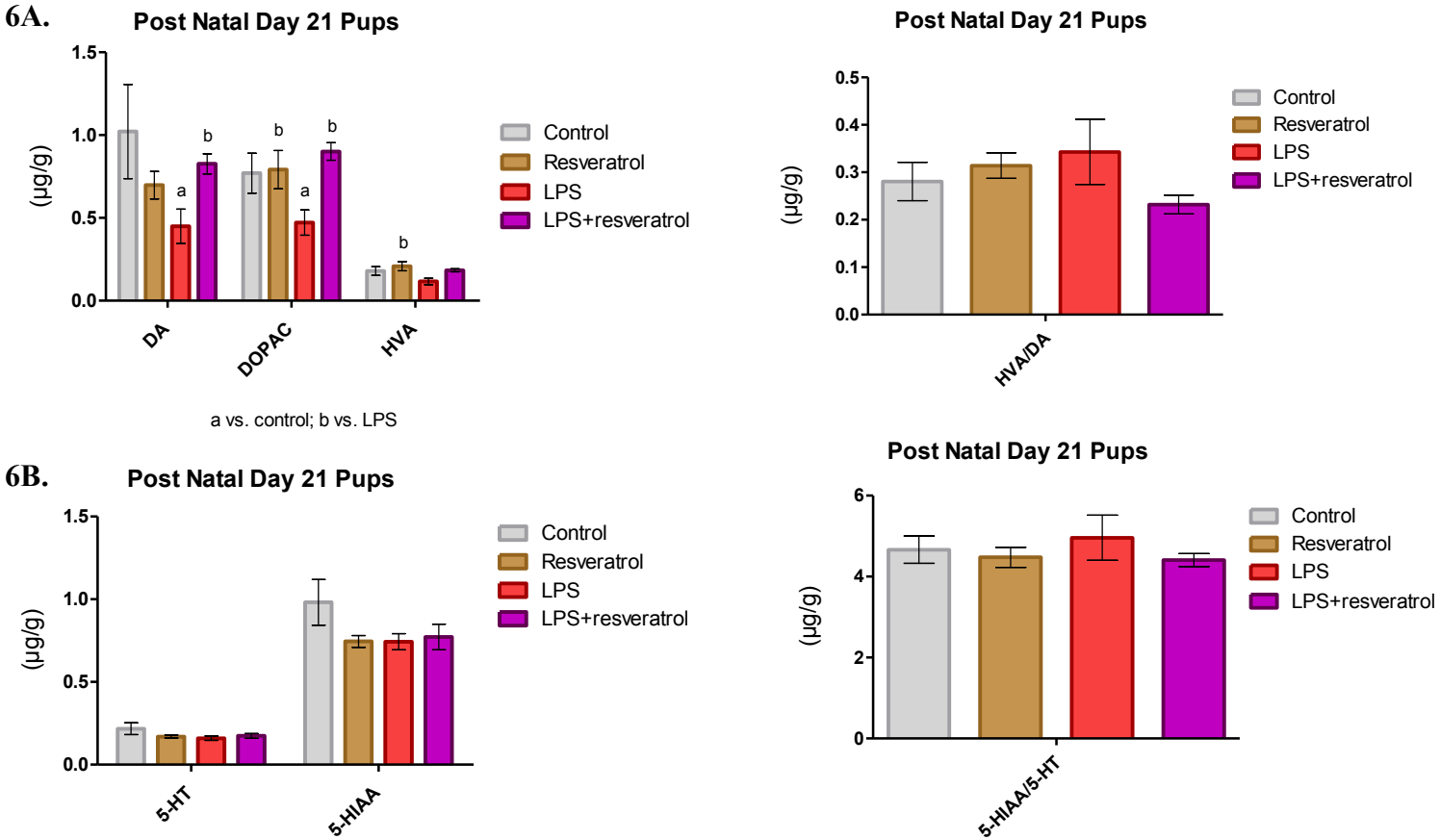


Figure 6: Dietary resveratrol supplementation protected against LPS-induced loss of DA and DOPAC at P21

Results are presented as mean ± SEM, n= 8-10 per group. Striatal DA and DOPAC levels were significantly lower in the LPS treated group when compared to the control. Resveratrol treated groups had higher DOPAC and HVA levels when compared to the LPS treated groups. The LPS-induced decrease of DA and DOPAC levels was inhibited by resveratrol dietary supplementation. No change in DA turnover, 5-HT turnover, 5-H or 5-HIAA levels was observed. Data were analyzed using an unpaired t-test and 1-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$).

4.4 LPS did not change DA, DOPAC or HVA levels in P40 pups

DA, 5-HT and their metabolites were analyzed using HPLC. DA, DOPAC, HVA content and DA turnover were not significantly altered in any of the P40 treatment groups. Dietary resveratrol supplementation significantly increased 5-HT levels at P40 when compared to the control and LPS treatment groups. LPS increased 5-HIAA levels when compared to control. Resveratrol treatment significantly reduced the LPS-induced increase in 5-HT turnover (Fig. 7A & 7B).

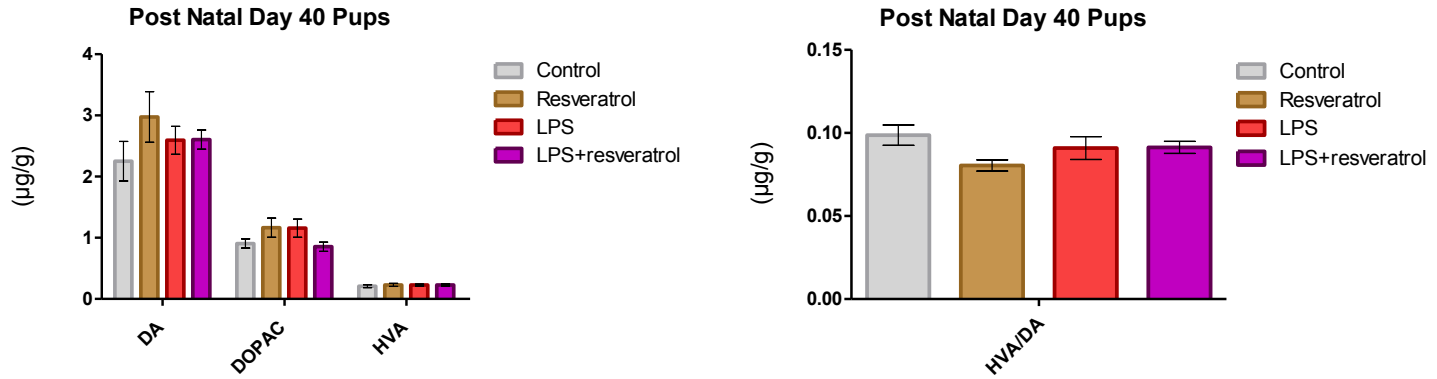
4.5 Vehicle treatment did not affect TH or DAT expression

DAT and TH expression in P10, P21 and P40 pups were measured using western blot analysis. DAT and TH expression in the vehicle group were not significantly different than the control for all ages (Fig. 8). Therefore, the vehicle treatment groups were eliminated from the remaining western blot graphs.

4.6 LPS increased TH expression but did not alter DAT expression at P10

Western blot analysis was performed to determine DAT and TH expression in the striatal tissue of P10 pups. LPS significantly increased TH expression when compared to the control group. DAT expression was not altered in any of the treatment groups (Fig. 9A & 9B).

7A.



7B.

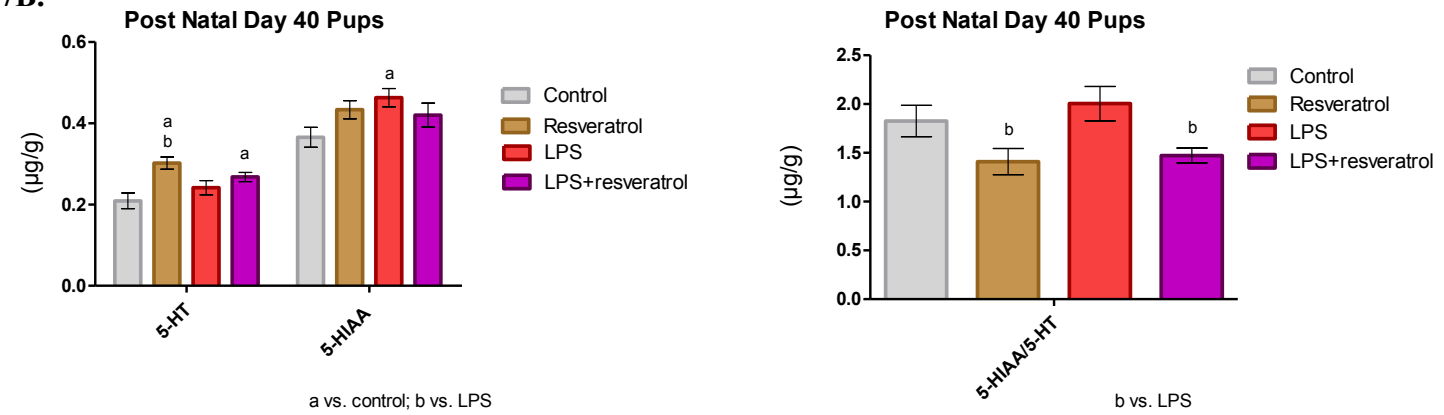
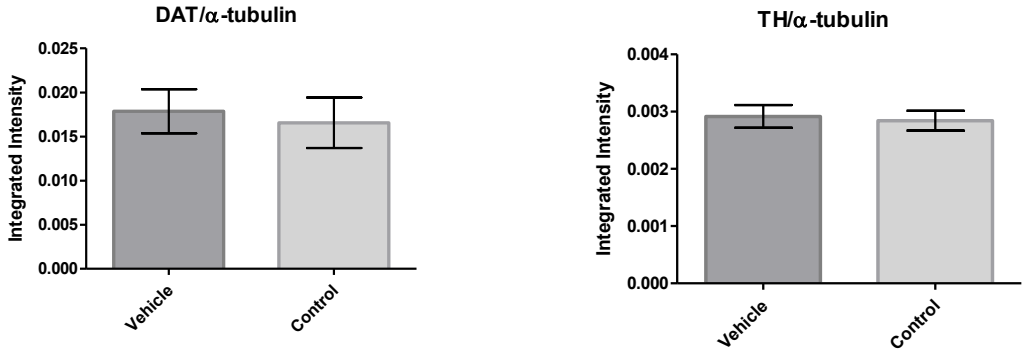


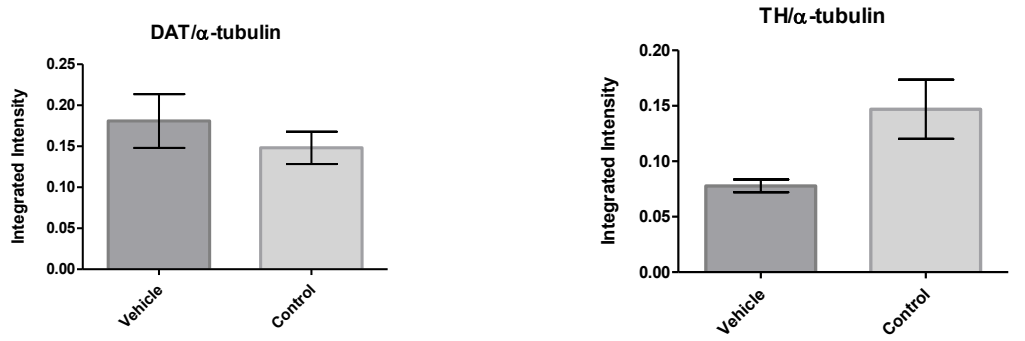
Figure 7: LPS did not alter DA, DOPAC or HVA levels in P40 pups

Results are presented as mean ± SEM, n= 9-10 per group. LPS did not alter DA turnover, DA, DOPAC or HVA levels. Resveratrol treated animals had significantly higher 5-HT content when compared to control and LPS groups. 5-HIAA levels in the LPS group were significantly higher than the control. Resveratrol treatment significantly reduced the LPS-induced increase in 5-HT turnover. Data were analyzed using 1-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$).

8A.



8B.



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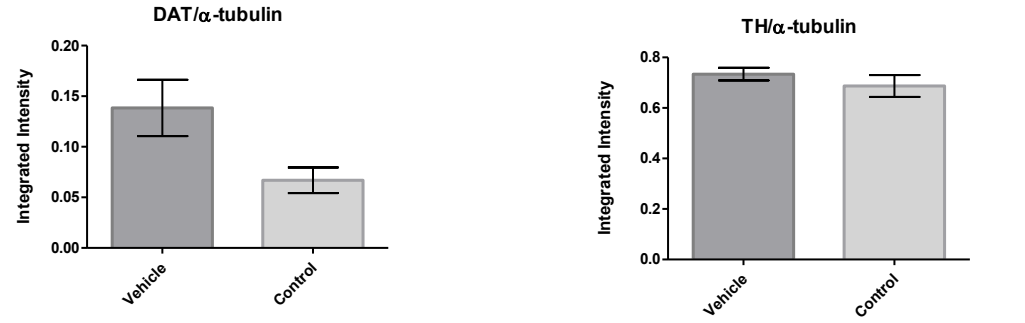
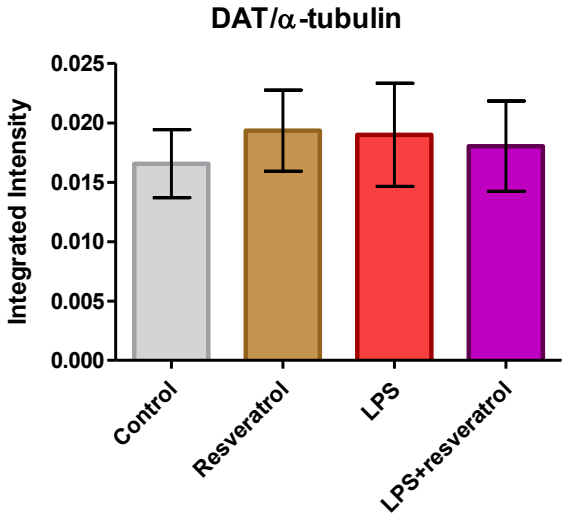


Figure 8: Vehicle treatment did not alter DAT or TH expression

Results are presented as mean ± SEM, n= 8 per group. There was no significant difference between the vehicle and control treatment groups at any age P10 (A), P21 (B) or P40 (C). Data were analyzed using 1-way ANOVA followed by Tukey post hoc test.

9A.



9B.

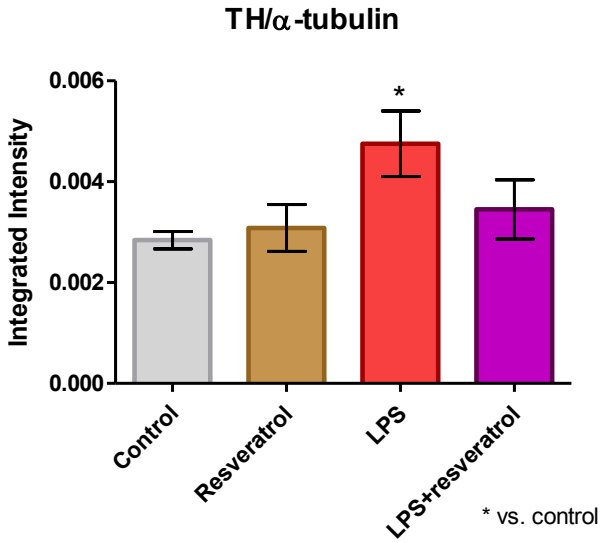


Figure 9: LPS increased TH expression but did not alter DAT expression in P10 pups

Results are presented as mean ± SEM, n= 8 per group. LPS significantly increased TH expression when compared to control, but did not alter DAT expression at P10. Data were analyzed using 1-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$).

4.7 Resveratrol-enriched diet significantly increased TH expression when compared to LPS treatment group at P21

DAT and TH expression in P21 pups were measured using western blot analysis. DAT expression was not altered in any of the treatment groups (Fig. 10A). However, dietary resveratrol supplementation significantly increased TH expression when compared to the LPS-treated group (Fig. 10B)

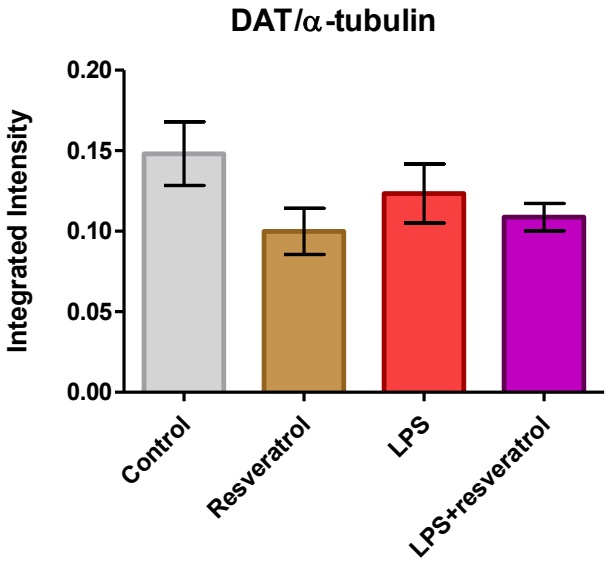
4.8 LPS did not alter DAT and TH expression in the striatum of P40 pups

LPS did not alter striatal DAT or TH expression in any of the P40 treatment groups (Fig. 11A & 11B).

4.9 SH-SY5Y cell viability was not changed after 24 hour LPS treatment

SH-SY5Y cells were plated in 96-well culture plates ($1.5x10^4$ per well/200µl) and treated with 100, 500 or 1000 ng/mL of LPS for 24 hours. DA (100 µM) was used as a positive control for the induction of cell death. The number of viable cells in culture was based on the quantitation of ATP by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). LPS at all doses did not significantly decrease cell viability after 24 hours when compared to vehicle (Fig. 12).

10A.



10B.

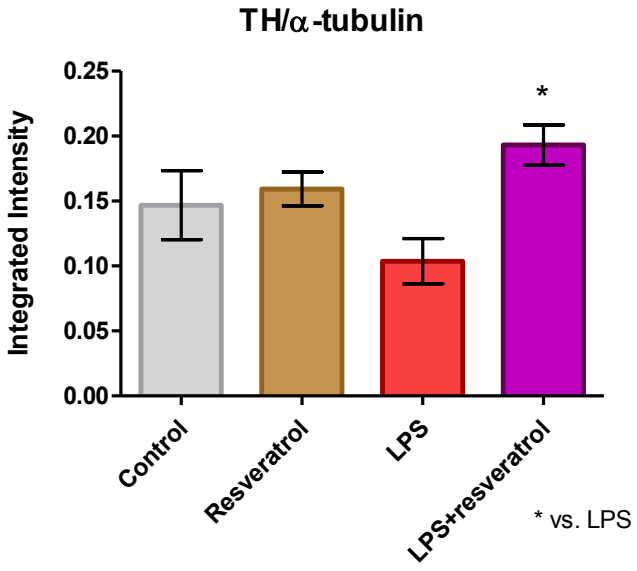
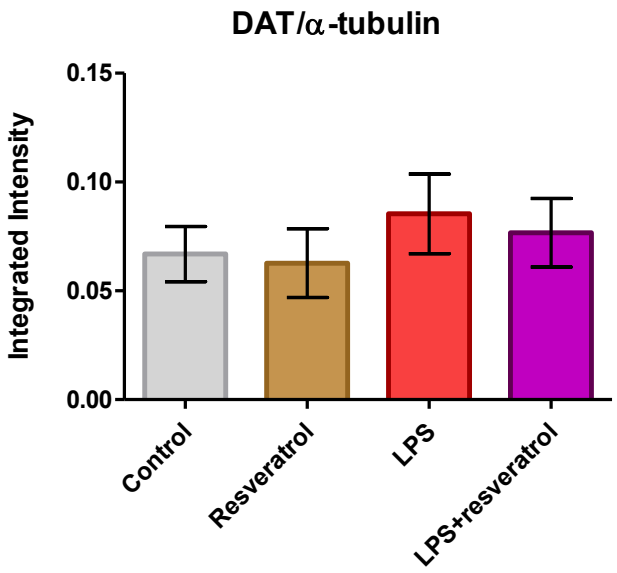


Figure 10: Dietary resveratrol supplementation increased TH expression when compared to the LPS treatment group at P21

Results are presented as mean ± SEM, n= 8 per group. At P21 DAT expression was not altered in any of the treatment groups. The resveratrol-enriched diet significantly increased TH expression when compared to the LPS-treated group. Data were analyzed using 1-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$).

11A.



11B.

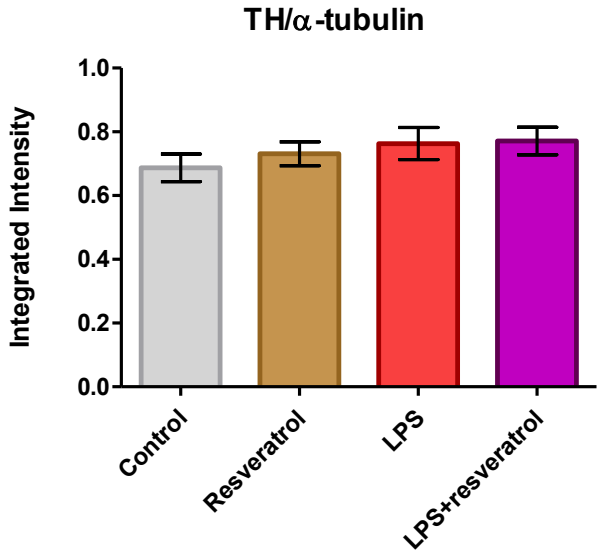


Figure 11: LPS did not change striatal DAT or TH expression at P40

Results are presented as mean ± SEM, n= 8 per group. At P40 DAT and TH expression was not altered in any of the treatment groups. Data were analyzed using 1-way ANOVA followed by Newman-Keuls post hoc test.

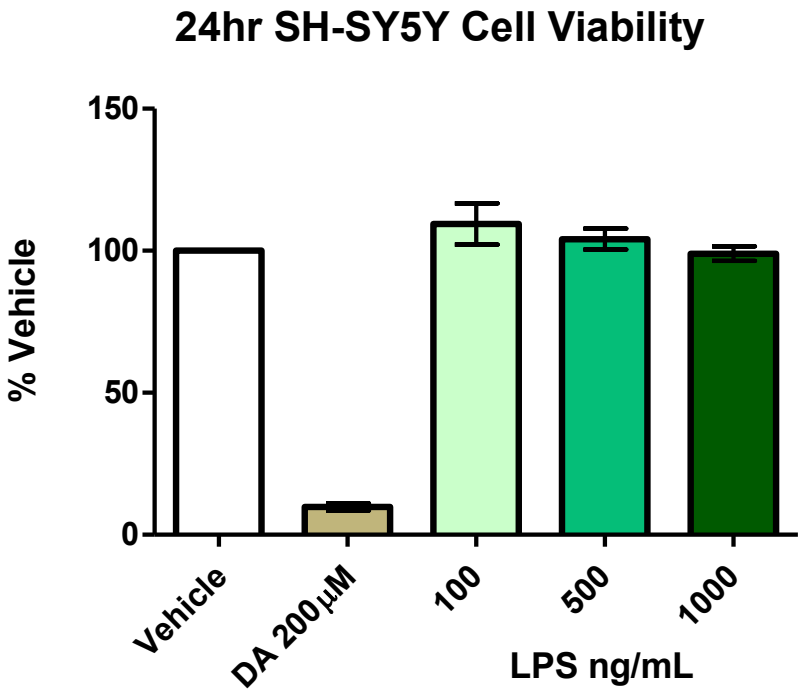


Figure 12: LPS did not alter SH-SY5Y cell viability

Results are presented as % vehicle ± SEM of 3-7 individual experiments performed in triplicate. LPS treatment at all doses (100, 500 and 1000 ng/mL) did not decrease SH-SY5Y cell viability after 24 hours when compared to vehicle. DA (200μM) was used as a positive control for the induction of cell death. Data were analyzed by unpaired t-test and 1-way ANOVA followed by Tukey's Multiple Comparison post hoc test.

4.10 Resveratrol pretreatment did not change BV-2 cell viability after 24 hour LPS treatment

BV-2 cells were plated in 96-well culture plates ($1.5x10^4$ per well/200µl) and treated with 100, 250, 500 or 1000 ng/mL of LPS for 24 hours. The cells were pretreated with resveratrol (1, 3, or 5 µM) for 30 minutes prior to the LPS treatment. The number of viable cells in culture was based on the quantitation of adenosine-5'-triphosphate (ATP) by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). LPS at all doses did not decrease BV-2 cell viability after 24 hours when compared to vehicle (Fig. 13). Resveratrol pretreatment or alone did not change cell viability at any dose when compared to vehicle. However, the resveratrol-treated alone group had a significant increase in cell viability when compared to the LPS treated groups.

4.11 The LPS-induced decrease in BV-2 cell viability after 48 hours was not inhibited by resveratrol pretreatment

BV-2 cells were treated with 100, 250, 500 or 1000 ng/mL of LPS for 48 hours. The cells were pretreated with resveratrol (1, 3, or 5µM) for 30 minutes prior to the LPS treatment. The number of viable cells in culture was based on the quantitation of ATP by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). LPS at all doses significantly decreased BV-2 cell viability after 48 hours when compared to vehicle. Resveratrol pretreatment was not protective against the LPS-induced decrease in cell viability (Fig. 14).

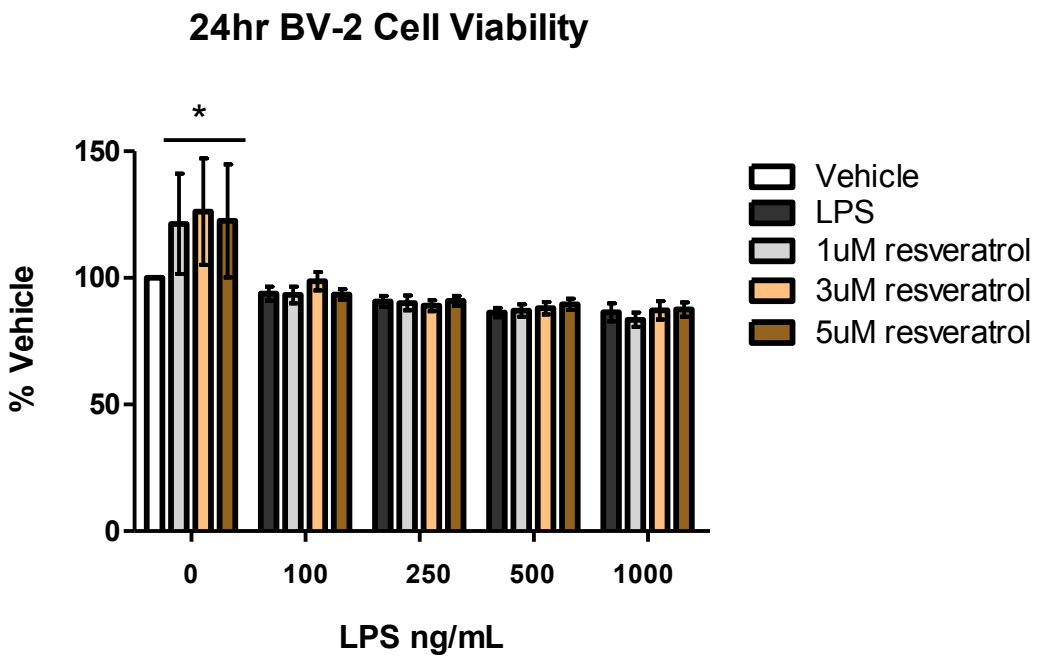


Figure 13: LPS and resveratrol did not change BV-2 cell viability after 24 hours when compared to vehicle

Results are presented as % vehicle ± SEM of 8-10 individual experiments performed in triplicate. LPS treatment at all doses (100, 250, 500 and 1000 ng/mL) did not decrease BV-2 cell viability after 24 hours when compared to vehicle. Thirty minute resveratrol pretreatment and resveratrol treatment alone did not alter BV-2 cell viability in comparison to vehicle. Resveratrol-alone treatment significantly increased cell viability when compared to the LPS-treated groups. Data were analyzed by unpaired t-test and 1-way ANOVA followed by Tukey's Multiple Comparison post hoc test. Significance (*) is defined as $p<0.05$ when compared to LPS treatment groups.

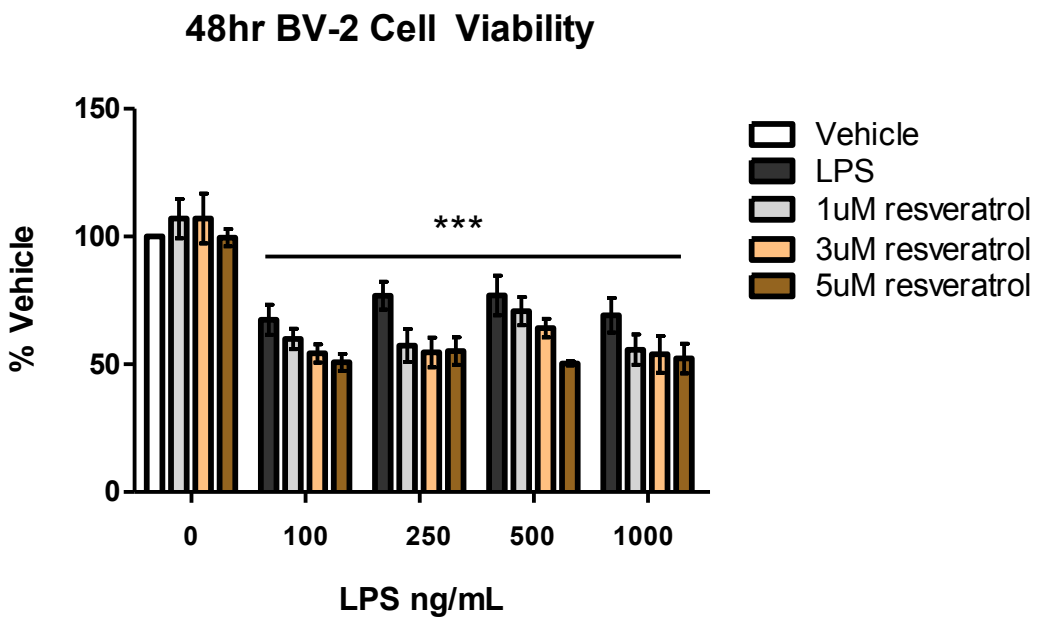


Figure 14: Resveratrol did not protect against the LPS-induced decrease in cell viability after 48 hours

Results are presented as % vehicle ± SEM of 4-6 individual experiments performed in triplicate. LPS treatment at all doses (100, 250, 500 and 1000 ng/mL) significantly decreased BV-2 cell viability after 48 hours when compared to vehicle. Thirty minute resveratrol pretreatment at all doses (1, 3, or 5 μM) did not cause or inhibit the LPS-induced cell death. Data were analyzed by unpaired t-test and 1-way ANOVA followed by Tukey's Multiple Comparison post hoc test. Significance (*) is defined as $p<0.0001$ when compared to vehicle.

4.12 TNF-α release induced by LPS was inhibited by U0126 and BIX02189, but not resveratrol pretreatment

BV-2 cells were plated in 24-well culture plates ($2x10^5$ per well/1mL) and treated with 500 ng/mL LPS for 6 or 24 hours. Thirty minutes before LPS treatment the cells were treated with 3 μM resveratrol. Prior to resveratrol pretreatment, the cells were treated with 10 μM U0126 or BIX02189 for 1 hour. TNF-α release in cell supernatants was measured through ELISA. LPS significantly increased TNF-α release at 6 and 24 hours when compared to the vehicle treated cells. U0126 inhibited TNF-α release induced by LPS and significantly inhibited TNF-α release induced by LPS in the resveratrol pretreated cells. Preliminary data demonstrated BIX02189 inhibited LPS-induced TNF-α release (Fig. 15)

4.13 Resveratrol did not inhibit LPS-induced activation of ERK1 or ERK2 in BV-2 cells

BV-2 cells were plated in 35 mm ($6x10^5$cells/ 2mL) and 60mm ($1x10^6$cells/ 4mL) plates and treated with 500 ng/mL LPS at various time points. Thirty minutes before LPS treatment the cells were treated with 3 μM resveratrol. LPS activated ERK1/2 at 15 minutes and the time points thereafter. Resveratrol pretreatment did not activate ERK1/2 or inhibit LPS-induced activation of these kinases (Fig. 16A & 16B).

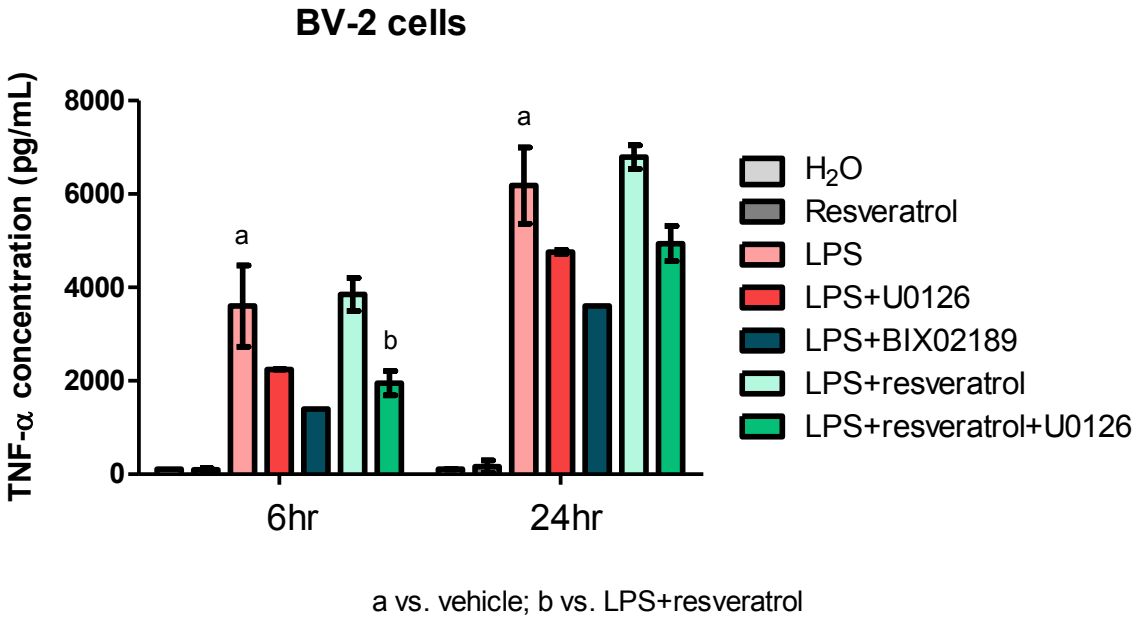
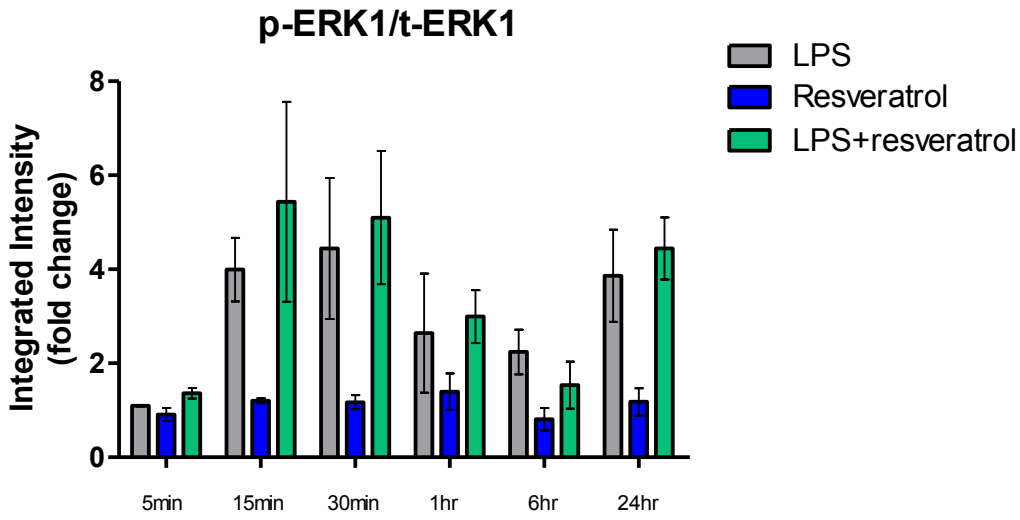


Figure 15: The LPS-induced release of TNF-α was inhibited by U0126 and BIX02189

Results are presented as TNF-α concentration (pg/mL) ± SEM of 4 individual experiments performed in duplicate; BIX02189 n=1. LPS significantly induced TNF-α release at 6 and 24 hours when compared to vehicle (H_2O). U0126 inhibited TNF-α release induced by LPS and significantly inhibited TNF-α release induced by LPS in the resveratrol pretreated cells. BIX02189 inhibited LPS-induced TNF-α release. Data were analyzed by unpaired t-test and 1-way ANOVA followed by Tukey's Multiple Comparison post hoc test ($p<0.01$).

16A.



16B.

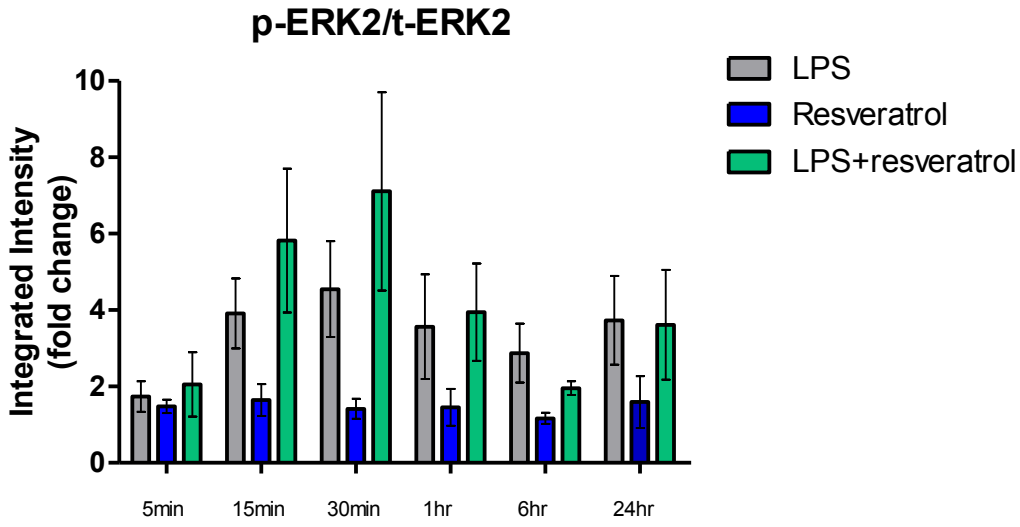


Figure 16: Resveratrol did not inhibit LPS-induced ERK1/2 activation

Results are presented as fold change; ± SEM of 4-5 individual experiments. LPS (500 ng/mL) activated ERK1/2 at 15 minutes and thereafter. Resveratrol pretreatment did not activate ERK1/2 or inhibit LPS-induced activation. Data were analyzed by unpaired t-test and 1-way ANOVA followed by Tukey's Multiple Comparison post hoc test.

4.14 U0126 significantly inhibited LPS-induced activation of ERK1 and ERK2 at 6 hours

BV-2 cells were treated with 500 ng/mL LPS for 30 minutes, 1, 6 and 24 hours. Thirty minutes before LPS treatment the cells were treated with 3 μM resveratrol. Prior to the resveratrol pretreatment the cells were treated with 10 μM U0126. There was a temporal pattern to ERK1/2 inhibition by U0126 in the LPS and resveratrol pretreatment groups, such that the inhibition was noted at 30 minutes and 6 hours (Fig. 17A & 17B). As expected, the putative MEK5 specific inhibitor, BIX02189, had no effect on ERK1/2 phosphorylation in any group (Fig. 18A & 18B).

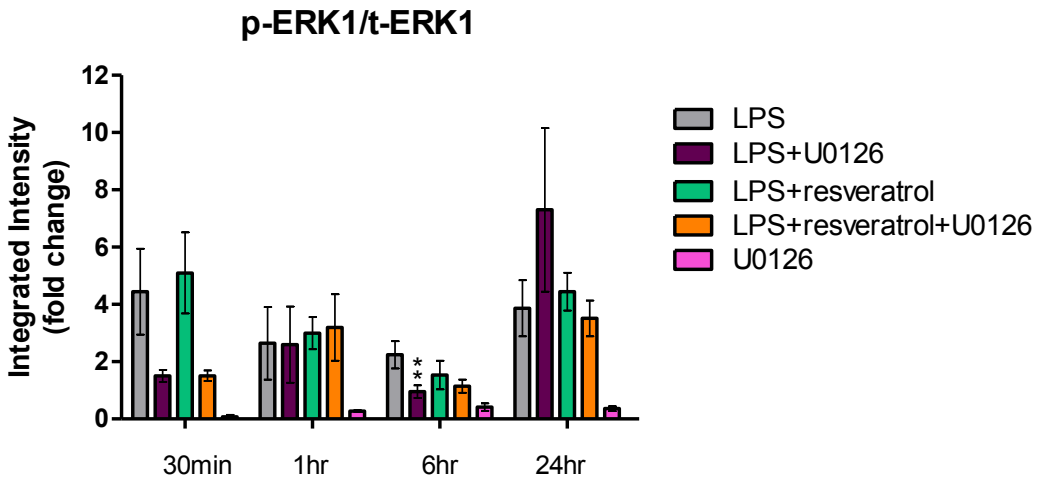
4.15 ERK5 was not activated by LPS or resveratrol in BV-2 cells

BV-2 cells were plated in 35 mm ($6x10^5$ cells/2mL) and 60 mm ($1x10^6$ cells/4mL) plates and treated with 500 ng/mL LPS at various time points. Thirty minutes before LPS treatment the cells were treated with 3 μM resveratrol. Neither LPS nor resveratrol activated ERK5 above basal levels at any of the time points (Fig. 19).

4.16 U0126 or BIX02189 did not inhibit basal ERK5 activation

BV-2 cells were treated with LPS (500 ng/mL) for 30 minutes, 1, 6 and 24 hours. Thirty minutes before LPS treatment the cells were treated with 3 μM resveratrol. Prior to the resveratrol pretreatment the cells were treated with 10 μM U0126 or BIX02189 for 1 hour. U0126 and BIX02189 did not inhibit basal ERK5 activation (Fig. 20A & 20B).

17A.



17B.

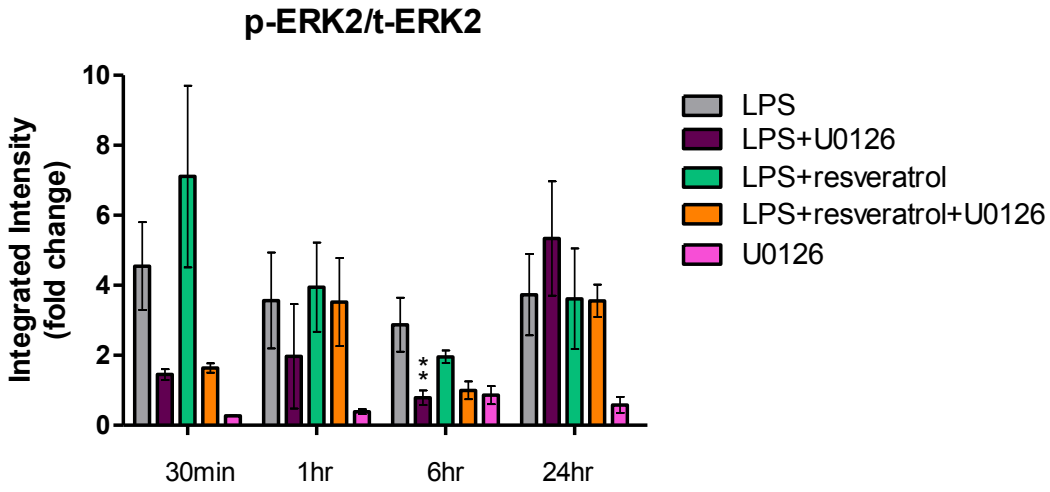
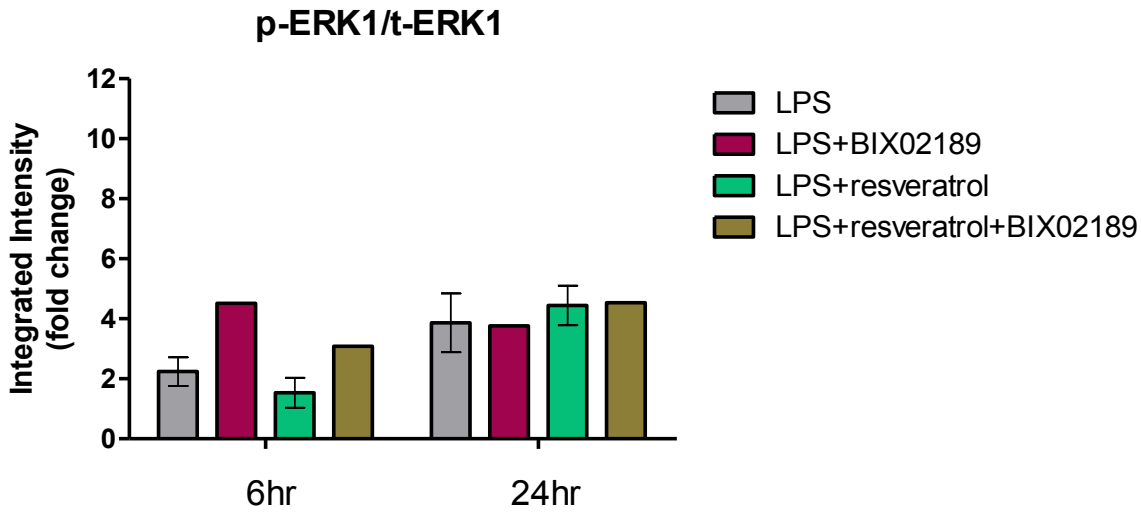


Figure 17: U0126 significantly inhibited LPS-induced activation of ERK1/2 at 6 hours

Results are presented as fold change; ± SEM of 4-5 individual experiments. LPS (500 ng/mL) activated ERK1/2 at all time points. U0126 inhibited LPS-induced ERK1/2 at 30 minutes and significantly inhibited ERK1/2 at 6 hours. Data were analyzed by unpaired t-test and 1-way ANOVA followed by Tukey's Multiple Comparison post hoc test. Significance (**) is defined as $p<0.01$ when compared to LPS.

18A.



18B.

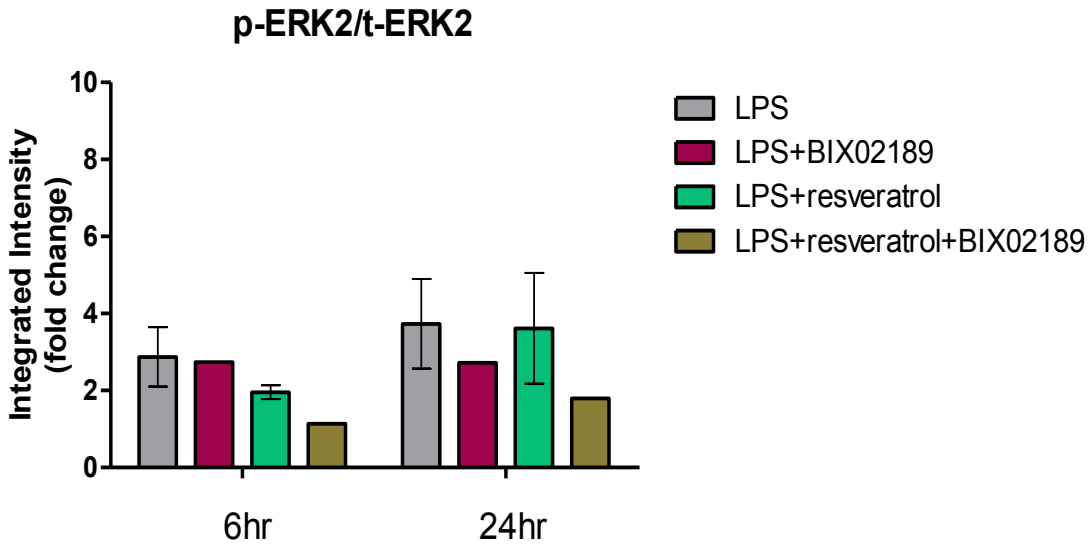


Figure 18: BIX02189 did not inhibit ERK1/2 activation at 6 or 24 hours

Results are presented as fold change; BIX02189 n=1. LPS (500 ng/mL) activated ERK1/2 at 6 and 24 hours. BIX02189 did not inhibit LPS-induced ERK1/2 activation alone or with resveratrol pretreatment at 6 or 24 hours.

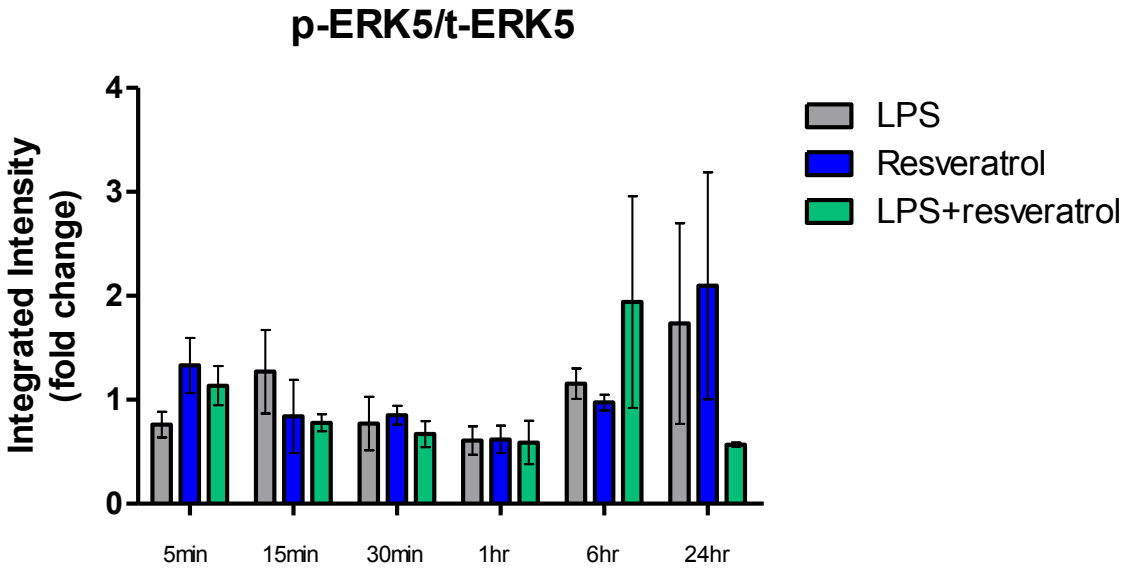
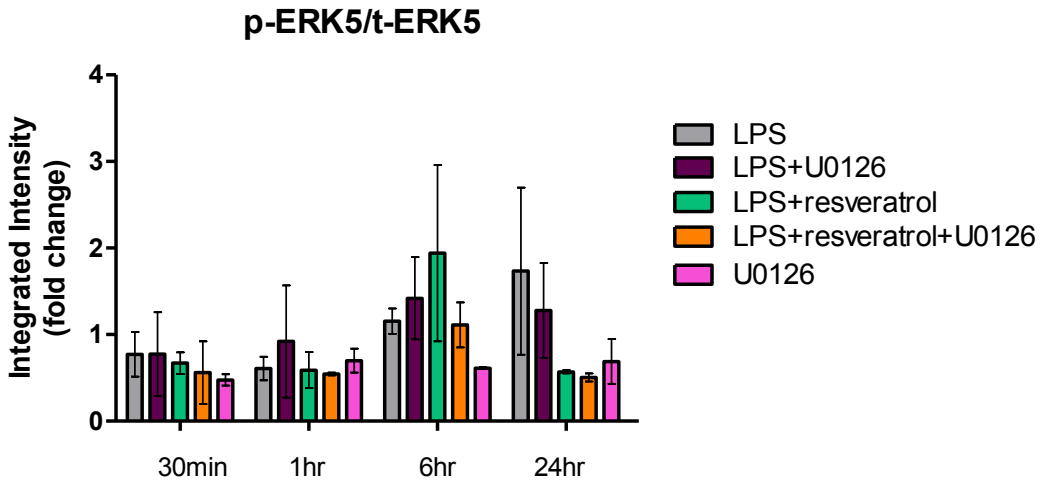


Figure 19: LPS and resveratrol did not activate ERK5 in BV-2 cells

Results are presented as fold change; ± SEM of 4-5 individual experiments. LPS (500 ng/mL) or resveratrol (3 μM) did not activate ERK5 at any time point. Data were analyzed by unpaired t-test and 1-way ANOVA followed by Tukey's Multiple Comparison post hoc test.

20A.



20B.

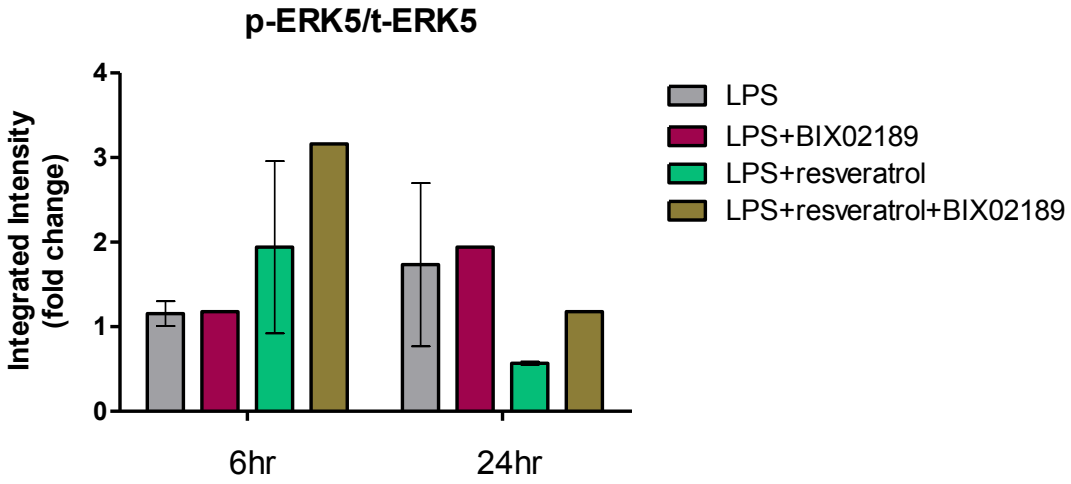


Figure 20: ERK5 activation was not inhibited by U0126 or BIX021890

Results are presented as fold change; ± SEM of 4-5 individual experiments; BIX02189 n=1. U0126 or BIX02189 did not inhibit basal ERK5 activation. Data were analyzed by unpaired t-test and 1-way ANOVA followed by Tukey's Multiple Comparison post hoc test.

5. DISCUSSION

The development of PD has been associated with an increase in activated microglia which, in turn, leads to chronic neuroinflammation. Prenatal exposure to LPS is used as a model to activate microglia and induce neuroinflammation. Normal cytokine levels, specifically IL-1β, are believed to be involved in regulating the differentiation of neural progenitor cells into mature neurons (Ling et al., 2002, Crampton et al., 2012). It has been shown that a loss in DA neurons can occur as early as *in utero* in the offspring of animals prenatally exposed to LPS (Ling et al., 2002). During normal embryonic development the rat dopaminergic fibers reach the striatum at E12.5. Postnatal DA ontogenic cell death in the SN is biphasic with a peak at P2 and another at P14. Complete maturation of DA neurons occurs during the third postnatal week to which the neurons have obtained adult morphology and functionality (Prakash and Wurst, 2006).

Our study examined the effect of dietary resveratrol supplementation on prenatal LPS exposure. A resveratrol-enriched diet was administered to pregnant rats at E3. At E10.5, slightly before neural progenitor cells mature into DA neurons the rats received an i.p. injection of LPS. The effects of resveratrol treatment were examined in their pups at different postnatal days. The possible differences in LPS and resveratrol treatment at P10, P21 and P40 were elucidated.

Previous studies have shown that prenatal LPS exposure significantly reduced the number of TH+ cells in the SN of P10 rat pups and this loss continued throughout adulthood. A reduction in TH+ cells in the SN of adult rats that received a supranigral infusion of LPS has also been observed (Ling et al., 2002, Ling et al., 2004a, Ling et al.,

2004b, Ling et al., 2006). A decrease in DA neurons in the SN was accompanied by a loss in striatal DA content and an increase in DA turnover. Striatal DA levels of animals prenatally exposed to LPS were reduced relative to control levels. Striatal ([HVA/DA]) content was measured and used as an index of DA activity or turnover. After birth and at different time points throughout adulthood, DA turnover was increased after prenatal exposure to LPS when compared to control animals (Ling et al., 2002, Ling et al., 2004a, Ling et al., 2004b, Ling et al., 2006). These studies demonstrated a specific LPS-induced toxicity to DA neurons in the SN.

Our data demonstrated at P10 and P40 there was no significant loss in striatal DA, DOPAC or HVA levels in the LPS treated animals when compared to control. This was also true for DA turnover measured by the ([HVA/DA]) ratio. The lack of a loss in striatal DA content observed at P10 might have been due to the pups being able to readily compensate for the LPS-induced loss of DA neurons. During this natural time of DA neuronal pruning both at P2 and P14, the pup's dopaminergic system might have been more efficient at returning striatal DA levels back to normal. Previous literature has shown differences in DA turnover after LPS exposure between ages. Significant decreases in DA content and increases in DA turnover ([HVA/DA]) were found at P10 and P21 (Ling et al., 2002). However, in other studies the significant increase in DA turnover was lost at 4 months, but observed again at 17 months (Ling et al., 2004a, Ling et al., 2004b). A similar discrepancy was observed in striatal DA content. At 4 months a significant decrease in DA levels was found, however at 16 months there was not a significant loss (Ling et al., 2004a, Ling et al., 2004b). The absence of a loss in striatal

DA levels at P40 might have been due to the pup's ability to restore the homeostasis of the DA neuronal system that was altered earlier during development.

Also, other studies have shown gender differences in striatal DA turnover at 4 months. Male rats had a significant increase of DA turnover when compared to the control, but the female rats did not (Ling et al., 2009). Thus, these animals showed a gender specific response to prenatal LPS exposure.

The contrasting data that was obtained in our study might have been due to a variety of factors. As previously mentioned, differences in DA turnover among male and female rats at 4 months were observed (Ling et al., 2009). Differences between male and female DA levels and DA turnover at P10 and P40 were not analyzed in our study. Furthermore, if the ratio of female pups to male pups was greater, then differences in DA levels and DA turnover might have varied between treatment groups. Also, there were small differences between our experimental design and Ling and colleagues (2002; 2004). These differences included the euthanasia that was performed on the pups. In the Ling and colleagues (2002; 2004) studies, the animals were perfused with ice cold saline and DA content was detected by HPLC from pooled left and right tissue punches that were taken from the center of each striatum. In our study the animals were put on ice and quickly decapitated. The whole left striatum was analyzed for DA content by HPLC. Possible differences between left and right striata could lead to the contrasting data. The discrepancy between our results and those of Ling and colleagues (2002; 2004) might also have been due to the use of distinct animal populations and/or dissimilar environments.

In our study, at P21 the pups exposed prenatally to LPS showed a significant decrease in striatal DA and DOPAC levels when compared to the control. These data support the results reported by Ling and colleagues (2002). This loss of striatal DA might have occurred due to a decrease in the number of DA neurons in the SN which, in turn, caused a decrease in the amount of DA produced by the neurons. In fact, a previous study has shown a loss of DA neurons was demonstrated by a decrease in TH+ cells in the SN of P21 pups exposed *in utero* to LPS (Ling et al., 2002). However, DA turnover measured by the ratio of ([HVA/DA]) was not changed in the P21 pups prenatally exposed to LPS. Therefore, this could suggest that at this age, the pups were unable to or did not significantly compensate for the DA neuronal loss, possibly due to the lack of achieving adult DA functionality at P21.

The effect of the resveratrol-enriched diet alone on DA, DOPAC and HVA content in the pups was different at all the postnatal days. At P10, resveratrol alone decreased DOPAC levels when compared to control and LPS groups, but DA content was not altered. This may suggest that the resveratrol-enriched diet inhibited the catabolism of DA. Interestingly, it has been shown that resveratrol inhibits monoamine oxidase (MAO) and aldehyde dehydrogenase, enzymes involved in the catabolism of DA into DOPAC (Mazzio et al., 1998, Gursoy and Buyukuysal, 2008, Xu et al., 2010). Therefore, resveratrol may be involved in DA catabolism directly. However, at P21 and P40, dietary resveratrol supplementation alone did not alter striatal DA, DOPAC or HVA levels when compared to the control. This data suggests that the resveratrol-enriched diet did not inhibit DA catabolism at P21 or P40. Thus, resveratrol treatment alone might be more profound in inhibiting DA catabolism at different developmental stages.

Dietary resveratrol supplementation in combination with LPS treatment did not alter DA, DOPAC or HVA levels in P10 or P40 pups when compared to the control. However, the decrease in DOPAC content that was observed in the P10 dietary resveratrol supplementation group was inhibited when treated in combination with LPS. This suggests that LPS may interfere with the effects of resveratrol on DA catabolism at P10.

At P21, the resveratrol-enriched diet significantly inhibited the LPS-induced loss of striatal DA and DOPAC content. The resveratrol-enriched diet might have protected the LPS-induced loss of DA neurons in the SN which, in turn, led to an increase in DA content in the striatum. Therefore, dietary resveratrol supplementation increased DA and DOPAC levels up to control levels, possibly suggesting its protection against LPS-induced DA toxicity.

Unanticipated results were found in the HPLC analysis of the P21 vehicle group. The striatal DA, DOPAC and HVA content was significantly lower in the vehicle treatment group when compared to all the other treatment groups. However, these results were not observed in the vehicle treatment of the P10 or P40 age groups. After careful review of our protocol it was determined this difference was due to an experimental error in tissue preparation or homogenization. Again, these might include human error during manual striatal dissection of the P21 vehicle treated group or changes in the perchloric acid buffer used in the tissue homogenization. The lower HPLC values were only observed in the P21 vehicle treatment group. The other treatment groups of all ages were in similar range to one another. Therefore, in reference to the P10 and P40 data, it was

concluded that the vehicle treatment had no effect on the striatal DA, DOPAC and HVA levels.

Previous studies have shown that LPS decreased 5-HT levels and increased 5-HT turnover in the striatum of 7 month old pups exposed prenatally to LPS (Ling et al., 2006). It has also been reported that LPS increases 5-HT turnover in adult rats subcutaneously injected with LPS (Hrupka and Langhans, 2001). Our data illustrated that at P10 and P21 there was no detectable change of striatal 5-HT and 5-HIAA content or 5-HT turnover after LPS exposure. However, at P40 LPS increased 5-HT turnover and striatal 5-HIAA levels when compared to control. The LPS-induced increase in 5-HT turnover was inhibited by dietary resveratrol supplementation. Also, 5-HT levels were greater in the group that was administered the resveratrol-enriched diet when compared to control. Resveratrol has been shown to inhibit 5-HT turnover (Yanez et al., 2006) and increase 5-HT levels (Xu et al., 2010). Thus, the resveratrol-enriched diet appears to have blocked the LPS-induced changes of serotonergic system at P40.

Neither LPS nor dietary resveratrol supplementation altered DAT expression in P10, P21 and P40 pups when compared to the control. Previous studies demonstrated that 1 hour after a systemic LPS injection striatal DAT expression was acutely decreased. However, this decrease was not seen at 3 or 7 days after the injection (Lai et al., 2009). Together with our current data, this suggests that the LPS-induced effect on striatal DAT was short lived. Therefore, although we noted a significant loss of striatal DA content at P21, DA neuronal function, as indicated by DAT expression, was not altered in our animals.

Striatal TH expression in the P40 pups was not altered in any of the treatment groups. This was supported with earlier data which demonstrated that striatal DA levels were not changed by LPS at P40. TH is the rate-limiting enzyme involved in the biosynthesis of the DA and is used as a marker for DA neurons and fibers (Gayle et al., 2002). Striatal TH expression was significantly increased in the P10 pups. This might indicate that at P10 a compensatory response occurred after LPS treatment. This increase in TH expression might have upregulated the biosynthesis of striatal DA. This response might have been responsible for the lack of striatal DA loss observed at P10.

At P21, striatal DA levels were significantly lower in the LPS treatment group as compared to the control. However, even though it was not significant, this trend was also noted for TH expression. Previous studies demonstrated that there was a LPS-induced loss of TH+ neurons in the SN of P21 pups exposed *in utero* to LPS (Ling et al., 2002). In the current study, a similar decrease in TH expression in the striatum was observed in the LPS treatment group and this decrease was significantly inhibited by dietary resveratrol supplementation. Thus, resveratrol demonstrated a protective mechanism against a possible decrease in DA projections to the striatum.

Previous literature has demonstrated that LPS alone is not directly toxic to primary DA neurons (Shih et al., 2009) or the SH-SY5Y dopaminergic cell line (Monaghan et al., 2008). Instead, LPS-induces the activation of microglia and the subsequent release of TNF-α and IL-1β. This release of proinflammatory cytokines leads to DA neuronal death (Qian et al., 2006a, Block et al., 2007). Therefore, LPS-induced toxicity *in vitro* has been achieved by using mixed primary neuron-glial cultures or co-cultures. Shih and colleagues (2009) demonstrated this type of LPS-induced toxicity

against SH-SY5Y cells by using BV-2 co-cultures. Our data demonstrated that LPS treatment did not alter SH-SY5Y cell viability after 24 hours. Thus, our data supports the findings that LPS is not directly toxic to DA cells.

Similar to data from SH-SY5Y cells, BV-2 cell viability was not decreased 24 hours following LPS, resveratrol, or LPS+ resveratrol treatment. LPS has been shown to activate BV-2 cells by inducing the release of TNF-α, IL-1β and NO without causing cytotoxicity (Laurenzi et al., 2001, Park et al., 2007). In primary microglia and N9 cells, resveratrol and LPS did not cause changes in cell viability when measured by a MTT assay after 24 hours or LDH release after 8 hours (Bi et al., 2005, Lu et al., 2010). Our data supports these findings in BV-2 cells. However, even though there was no significant difference in cell viability of the vehicle and resveratrol treatment groups, resveratrol significantly increased cell viability when compared to the LPS-treated group. This difference might have been due to the large error bars graphically represented in the resveratrol treatment group. Thus, this difference might not have been a true representation of the data, which ultimately caused this unexplained significant difference.

In contrast to the effect at 24 hours LPS caused a 33 % decrease in BV-2 cell viability after 48 hours. Miller and colleagues (2007) demonstrated LPS-induced toxicity in primary microglial cultures. Cell death was illustrated by a decrease in cell number and an increase in LDH release (Miller et al., 2007). The LPS-induced decrease in cell viability might have been due to BV-2 cell apoptosis or necrosis. Furthermore, LPS demonstrated a dose dependent loss in cell viability after 48 hours in BV-2 cells (Lee et

al., 2001). Lee and colleagues (2001) revealed that LPS decreased cell viability by 50 % after 48 hours. Moreover, this decrease in BV-2 cell viability was due to apoptosis.

The variations in cell toxicity found in our study and previous studies might have been due to the type of assay performed and/or the time point at which the toxicity was measured. LPS-induced toxicity to DA neurons is believed to occur indirectly through the release of proinflammatory cytokines and ROS. BV-2 cell viability after resveratrol treatment alone and in combination with LPS was evaluated. This was done to verify that the possible decrease in proinflammatory cytokine release from the BV-2 cells was not due to resveratrol's toxicity. It was demonstrated that after 24 and 48 hours resveratrol alone or as a pretreatment did not change BV-2 cell viability. Our data demonstrates that after 48 hours LPS becomes toxic to BV-2 cells and therefore inhibition of BV-2 activation with resveratrol treatment was elucidated at earlier time points.

In BV-2 cells LPS significantly induced TNF-α release at 6 and 24 hours when compared to vehicle. Previous literature has shown that LPS-induces TNF-α release in primary microglial cultures and microglial cell lines (Bhat et al., 1998, Lee et al., 2001, Bi et al., 2005, Qian et al., 2006b, Miller et al., 2007, Park et al., 2007, Gao et al., 2008, Lu et al., 2010, Zhang et al., 2010, Choi et al., 2011). In our study, resveratrol pretreatment did not inhibit the LPS-induced release of TNF-α at 6 or 24 hours in BV-2 cells. Zhang and colleagues (2010) demonstrated that resveratrol pretreatment significantly reduced LPS-induced TNF-α release in primary microglial cultures after 3 hours. This inhibition was also observed after 24 hours of LPS treatment. However, in other studies at 24 and 48 hours in both primary rat microglia and N9 cells, resveratrol pretreatment did not inhibit LPS-induced TNF-α release. Instead, resveratrol co-treatment

with LPS inhibited the release of TNF-α (Bi et al., 2005, Meng et al., 2008). Resveratrol concentrations remained relatively similar in each of these studies. However, there were significant differences in LPS doses. The degree of sensitization to LPS-induced TNF-α release varies among doses. Our study analyzed a single dose of LPS (500ng/mL) and resveratrol (3μM) at two different time points (6 and 24 hours) in BV-2 cells. Thus, future studies using different doses of LPS and resveratrol in BV-2 cells are necessary to determine the effect of resveratrol at different time points.

The non-selective MEK inhibitor, U0126, inhibited TNF-α release induced by LPS and significantly inhibited TNF-α release induced by LPS in the resveratrol pretreated BV-2 cells. Resveratrol alone had no significant effect on TNF-α release. Thus, it can be suggested that the release of TNF-α in the resveratrol pretreated cells was mainly due to LPS treatment. In support of these data, Mir and colleagues (2008) have shown that U0126 decreased intracellular TNF-α levels. Moreover, previous studies have demonstrated that a different MEK inhibitor, PD98059, caused inhibition of TNF-α release and expression in BV-2 cells and primary microglial cultures (Bhat et al., 1998, Park et al., 2007, Lu et al., 2010). However, both PD98059 and U0126 have been reported to also inhibit ERK5 (Kamakura et al., 1999). A pilot study using a selective MEK5 inhibitor, BIX02189, demonstrated a greater decrease in LPS-induced TNF-α release in BV-2 cells at both 6 and 24 hours This may suggest that activation of ERK5 in addition to ERK1/2 promoted the release of TNF-α after LPS treatment. Additional BV-2 cell experiments using BIX02189 are needed to validate this observation.

LPS activated ERK1/2 at 15 minutes and at all the time points thereafter. The dose response and time course of LPS-induced activation of ERK1/2 illustrated that the

maximum phosphorylation of ERK1/2 occurred at 30 minutes. Such findings are similar with other reports of LPS-induced ERK1/2 activation in both primary microglia and BV-2 cells (Bhat et al., 1998, Park et al., 2007, Afonso-Oramas et al., 2009, Wang et al., 2011).

Resveratrol alone did not activate ERK1/2 and as a pretreatment resveratrol did not inhibit LPS-induced activation in BV-2 cells. A similar effect was observed in N9 cells for resveratrol did not inhibit LPS-induced activation of ERK1/2 (Lu et al., 2010). In contrast, Zhang and colleagues (2010) reported that resveratrol pretreatment inhibited LPS-induced ERK1/2 activation after 15 minutes in primary microglial cultures.

The diverse effects of resveratrol on LPS-induced TNF-α release and ERK1/2 activation might have been due to the various differences in the biological characteristics of microglia. The murine microglial cell lines BV-2 and N9 are two of the most commonly used models of primary microglia in culture. However, they were originally derived from different mouse strains and transformed with different oncogenes (Blasi et al., 1990, Corradin et al., 1993). The discrepancies in the response to resveratrol treatment in these cell lines may be due to the differences in LPS-induced activation. This could include the degree of the response to LPS treatment for it may be a less pronounced in BV-2 cells than in N9 cells or primary microglia. Thus, differences in the response to resveratrol treatment might have been due to cell origin or they might have been a product of selection and culture conditions.

U0126 transiently inhibited LPS-induced activation of ERK1/2. U0126 inhibited LPS-induced ERK1/2 activation at 30 minutes and 6 hours but not at 1 and 24 hours. Resveratrol alone did not affect ERK1/2 phosphorylation; therefore, the effect of resveratrol pretreatment on ERK1/2 activation was negligible. The pilot study using the

MEK5 inhibitor, BIX02189, demonstrated that it did not inhibit LPS-induced ERK1/2 activation at 6 or 24 hours. BIX02189 is reported to be selective for MEK5 in some paradigms and this lack of inhibition in ERK1/2 activation in the present study supports the selectivity of BIX02189 in our system (Tatake et al., 2008).

Previous literature has demonstrated that LPS induces ERK5 activation in RAW 264.7 murine macrophage cells (Zhu et al., 2000). Microglia are closely related to macrophages, for both act as CNS scavengers and release proinflammatory cytokines (Lee et al., 2001). However, in another murine macrophage cell line, BAC1.2F5, LPS did not induce ERK5 activation (Rovida et al., 2008). Our data demonstrated that ERK5 was not activated by LPS or resveratrol treatment in BV-2 cells at any time point. Neither U0126 nor BIX02189 inhibited basal ERK5 activation. Thus, MAPK signaling pathways are differentially involved in the response to inflammatory activators, such as LPS, in cell-mediated neuroinflammation.

6. CONCLUSION

The outcome of this study demonstrates that contrary to other findings LPS-induced dopaminergic deficits in P21, but not P10 and P40 pups exposed prenatally to LPS. This was demonstrated as a loss of striatal DA and DOPAC content and TH expression in the P21 animals. Resveratrol alone decreased DOPAC levels at P10, suggesting its direct involvement in the catabolism of DA into DOPAC. This might have been accomplished through inhibiting the MAO and aldehyde dehydrogenase enzymes involved in DA catabolism. Dietary resveratrol supplementation increased DA and DOPAC content and TH expression in the P21 pups following prenatal exposure to LPS. Together these data suggest that resveratrol may increase striatal DA content by inhibiting DA catabolism and increasing DA synthesis. Additionally, resveratrol induced an increase in striatal 5-HT content at P40, suggesting that resveratrol may also preserve 5-HT innervation to the striatum. The differences observed in the three postnatal age groups show an age-dependent specificity of LPS and dietary resveratrol supplementation. This study is the first to demonstrate that LPS-induced toxicity is more prominent at certain developmental stages, therefore suggesting that neurotoxin exposure may be more detrimental to DA neurons during different times of DA neuronal pruning.

In addition, *in vitro* data demonstrated that LPS-mediated activation of microglia occurs at earlier time points (6 and 24 hours) than LPS-induced toxicity (48 hours). Moreover, LPS increased ERK1/2, but not ERK5, phosphorylation. Resveratrol did not activate or inhibit LPS-mediated activation of ERK1/2 or ERK5. However, U0126 inhibited LPS-mediated ERK1/2 activation and TNFα release at 6 hours following

treatment. Although ERK5 was not activated by LPS, preliminary data suggest that BIX02189 inhibits LPS-induced TNFα release. Therefore, BIX02189 may be inhibiting a distinct pathway in our model. Together these data suggest that inhibition of ERK1/2 activation decreases LPS-induced TNFα release, and resveratrol may be ineffective in inhibiting TNFα release due to the lack of effect of resveratrol on ERK1/2 activation in BV-2 cells. As evidenced by the BIX02189 data, an unidentified pathway may also play a role in inhibiting TNFα release in LPS activated microglia.

These findings aide in the quest to determine the possible triggers that are responsible for the development of PD. The development of PD is unknown, but sporadic PD is thought to occur through various exposures to neurotoxic insults (Sulzer, 2007). The exposure to brain injury, pesticides or bacterial infections early in life may cause the developing CNS to become more vulnerable to future insults. These multiple exposures may cause a synergistic response resulting in a greater loss in the number and functionality of DA neurons. In fact, this has been postulated and is referred to as the "Multiple Hit Hypothesis" of PD (Sulzer, 2007).

A comparable human disease that exposures the developing fetus to LPS *in utero* is BV. It has not been elucidated in humans, but it has been shown in our studies and others that LPS exposure in the rat decreases striatal DA content and DA neurons (Ling et al., 2002, Ling et al., 2004a). Therefore, administration of a natural supplement such as resveratrol in capsule form or as dietary consumption can decrease the levels of LPS and the subsequent proinflammatory cytokine release in the pregnant mother. Decreasing the inflammatory response in the mother may lessen the risk for the fetus to become exposed

to the cytokines *in utero*. Therefore, exposure to a second insult may decrease the likelihood of the offspring to develop a neurodegenerative disease such PD later in life.

These studies allow for the development of a safe and natural remedy that may be administered to pregnant woman as a protective measure to aide against the common occurrence of bacterial infections such as BV. Also, development of such therapies may help to modulate ERK function in overactivated microglia. Targeting the source of neuroinflammation in the mother may inhibit the release of TNF-α and other cytokines from overactivated microglia. In addition to developing a protective treatment against LPS-induced neuroinflammation through resveratrol treatment, a preventive strategy that focuses on the inhibition of ERK activation induced by prenatal LPS exposure is crucial as well. Thus, the development of PD may be prevented as early as *in utero,* ultimately changing the focus of PD research from age-related factors to early interventions.

Future studies could be directed toward the study of ERK1/2 upstream activators or downstream targets in LPS-induced BV-2 cell activation. This could include IRAK or NF-kB activation. NF-kB has been shown to play an important role in LPS-induced microglial activation (Zhang et al., 2010). In BV-2 cells resveratrol may inhibit the activation of IRAK and NF-kB, which may lead to a decrease in proinflammatory cytokine release. Also, the measurement of other cytokines such as IL1-β or IL-6 and NO production may be conducted. It for it has been found that LPS activates these cytokines in microglia as well (Nakamura et al., 1999). Measurement of LPS-induced NADPH oxidase activation could lead to the understanding of resveratrol's possible role as an antioxidant agent. Therefore, measuring NADPH oxidase activation and expression in BV-2 cells might demonstrate the direct effect of resveratrol on ROS release.

In vivo the measurement of inflammatory markers such as TNF-α and IL-1β in the pup's blood may verify that an inflammatory response has occurred after prenatal exposure to LPS. Also, measurement of striatal TNF-content of the pups might demonstrate a direct LPS-induced inflammatory response occurred. Lastly, analysis of TH+ neurons, a phenotypic marker of DA neurons, in the SN of the pups might show that the loss of striatal DA content at P21 was due to LPS-induced DA neuronal toxicity. Thus, this could support the notion that at P21 resveratrol was protective against the toxic effects of LPS. Overall, these studies provide an insight into the use of dietary resveratrol supplementation as a protective agent against prenatal LPS-induced dopaminergic deficits and the possible strategies that focus on targeting the overactivated microglia responsible for these deficits.

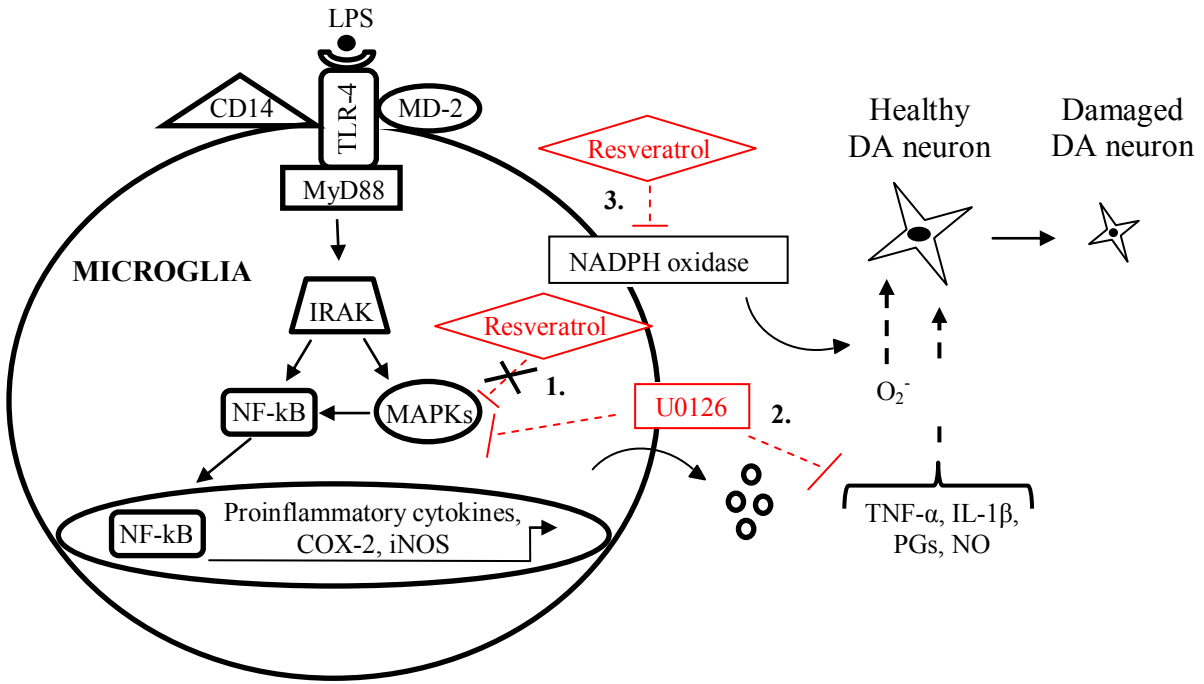


Figure 21: Schematic diagram showing the results of our study depicting the possible mechanisms of resveratrol and U0126 on LPS-induced microglial activation and subsequent DA neuronal damage

Adapted and modified from Dutta et al., 2008. LPS activates downstream signaling cascades IRAK, MAPK and NF-kB. Activation of these cascades causes the release of proinflammatory cytokines, ROS and lipid metabolites, which all collectively damage DA neurons. **1)** Resveratrol did not inhibit the activation of ERK1/2 or ERK5; however the results of our study demonstrated other possible mechanisms that might have been utilized to inhibit LPS-induced toxicity and proinflammatory cytokine release. These mechanisms might have involved:

2) U0126 inhibition of ERK1/2 activation and TNF-α release.

3) Resveratrol acted as an antioxidant agent by inhibiting the activation of NADPH oxidase, leading to a decrease in production of ROS.

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APPENDIX

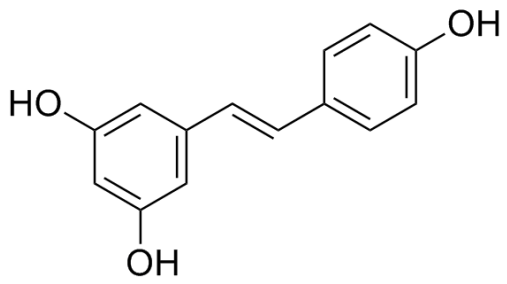

Figure 22: Resveratrol (3,5,4'-trihydroxy-trans-stilbene)

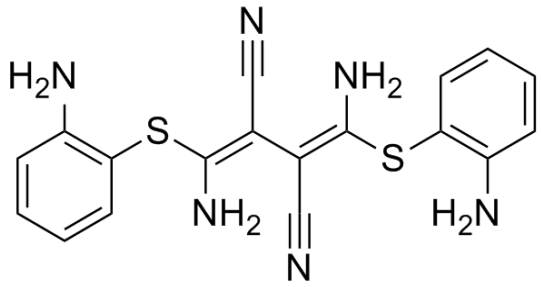

Figure 23: U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene)

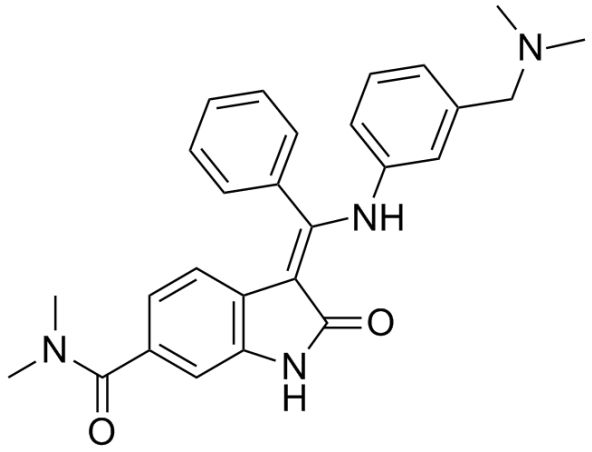

Figure 24: BIX02189 (3-[[[3-[(Dimethylamino)methyl]phenyl]amino]phenylmethylene]-2,3-dihydro-N,N-dimethyl-2-oxo-1H-indole-6-carboxamide)

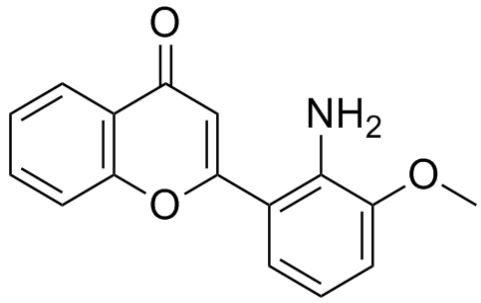

Figure 25: PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one)