The Effect of L-DOPA and Natural Products on Age-Related Motor Deficits and Dopaminergic Cell Survival

Erika Nicole Allen

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THE EFFECT OF L-DOPA AND NATURAL PRODUCTS ON AGE-RELATED
MOTOR DEFICITS AND DOPAMINERGIC CELL SURVIVAL

A Dissertation
Submitted to the Graduate School of Pharmaceutical Sciences
Mylan School of Pharmacy
Duquesne University

In partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By
Erika Nicole Allen

December 2013
THE EFFECT OF L-DOPA AND NATURAL PRODUCTS ON AGE-RELATED MOTOR DEFICITS AND DOPAMINERGIC CELL SURVIVAL

By

Erika Nicole Allen

Approved June 12, 2013

Jane E. Cavanaugh, Ph.D.
Assistant Professor of Pharmacology
Graduate School of Pharmaceutical Sciences
Duquesne University, Pittsburgh, PA
(Committee Chair)

Jelena Janjic, Ph.D.
Assistant Professor of Pharmaceutics
Graduate School of Pharmaceutical Sciences
Duquesne University, Pittsburgh, PA

Agnes M. Rimando, Ph.D.
Research Chemist
Natural Products Utilization Research
ARS, USDA
University, MS

Christopher K. Surratt, Ph.D.
Professor of Pharmacology
Graduate School of Pharmaceutical Sciences
Duquesne University, Pittsburgh, PA

Paula Witt-Enderby
Professor of Pharmacology
Graduate School of Pharmaceutical Sciences
Duquesne University, Pittsburgh, PA

J. Douglas Bricker, Ph.D.
Dean
Mylan School of Pharmacy and the Graduate School of Pharmaceutical Sciences
Duquesne University, Pittsburgh, PA
ABSTRACT

THE EFFECT OF L-DOPA AND NATURAL PRODUCTS ON AGE-RELATED MOTOR DEFICITS AND DOPAMINERGIC CELL SURVIVAL

By
Erika Nicole Allen

December 2013

Dissertation supervised by Jane E. Cavanaugh, Ph.D

With age, there is a loss of motor coordination leading to an increase in falls. Currently, there are limited therapeutic regimens for age-related motor deficits. However, L-DOPA is used for motor dysfunction in Parkinson’s disease (PD). Therefore, we investigated the effect of L-DOPA on motor deficits in young (2 mo) and old (20 mo) mice. We found that L-DOPA reversed age-related motor decline.

Chronic L-DOPA use produces involuntary movements or dyskinesias. Interestingly, natural foods, such as wild blueberries (WBB), and isolated compounds, such as resveratrol and pinostilbene, have beneficial effects on several physiological processes with few side effects. Therefore, to examine the effect of these natural products on motor function, mice (2, 10 and 20 mo) were fed resveratrol, pinostilbene, or WBB
containing diet. The age-related loss of motor coordination was attenuated in animals fed resveratrol and WBB diets, while pinostilbene diet increased spontaneous activity.

To investigate the mechanism that may underlie this reversal of motor deficits, striatal dopamine (DA) and DA metabolite levels and the number of DA neurons in the substantia nigra were examined. None of these parameters changed with age or in animals fed resveratrol or pinostilbene diets. However, WBB increased the number of DA neurons in the substantia nigra in aged animals.

To further investigate the mechanism that may underlie the alleviation of motor deficits, the effects of resveratrol or pinostilbene on oxidative stress-induced cell death and the expression and activation of ERK1/2 and ERK5, kinases known to protect from oxidative stress, were examined in DA cells. Resveratrol and pinostilbene decreased DA-induced cell death and activated ERK1/2, but not ERK5. Pretreatment with U0126, an inhibitor of the ERK1/2 pathways, blocked the resveratrol- and pinostilbene-mediated neuroprotection.

Together, these data suggest that resveratrol, pinostilbene, and/or WBB may decrease age-related motor deficits by protecting DA neurons from oxidative stress via activation of the ERK1/2 pathway. As the use of natural supplements is on the rise, it is important to understand the therapeutic and physiological effects of these compounds. Moreover, this research may lead to the use of these natural compounds as novel therapies for age-related motor deficits.
DEDICATION

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1. INTRODUCTION

1.1 Aging and society

The percentage of people aged 65 years and older has increased from 12.4% of the United States (US) population in 2000 to 13.0% of the US population in 2010 with a projected increase to 20.2% by 2050 (US Administration on Aging, 2011). Moreover, the number of people aged 65 and older is projected to more than double from 41.4 million in 2011 to 92 million in 2060 (US Administration on Aging, 2011). The significance of this increase in the aged population, along with a predicted deficit in Medicare and Social Security by 2016 (GAO, 2002), is that the incidence of age-related injuries and diseases will rise leading to a concomitant increase in health care costs.

Due to an increase in injuries, illness, and disease, the elderly population over 65 years of age averages more visits to doctor’s offices and longer, more frequent hospital stays than any other age group. This is problematic because older adults averaged out-of-pocket health care expenditures of $4,769 in 2011, an increase of 46% since 2000 (US Administration on Aging, 2011). Therefore, with an increase in the aging population and a concurrent decrease in the amount of funding for insurance, there is a concern about quality of life for the aging population.

1.2 Aging and motor coordination

Aging is associated with a decline in motor coordination, an increase in cognitive deficits, chronic illnesses, and diseases, such as Parkinson’s disease (PD) or Alzheimer’s disease (AD; Fozard et al., 1994; Prettyman, 1998; Hof and Mobbs, 2001). Of these
ailments, a loss in motor coordination becomes increasingly debilitating with age because the risk of injury from falling may lead to long-term disability, pain, and untimely death (Allum et al., 2002). For example, falls are the leading cause of unintentional injury and injury-related death in the elderly, and this increase in falls leads to an increase in the risk of fractures, with 20% of falls requiring admission to the hospital (Uusi-Rasi et al., 2012).

Currently, this age-related decline in motor skills is not treated clinically unless the loss of motor coordination is linked with diseases, such as PD or AD. When linked with PD, symptoms of motor deficits such as postural tremor, muscular rigidity, postural abnormalities, bradykinesia, and impaired balance are treated with drugs that increase the actions of the neurotransmitter dopamine (DA). The DA neuronal system and its role in motor function are reviewed below.

1.3 The dopamine system

1.3.1 Dopamine

Dopamine (DA) is a catecholamine that until the 1950’s was only known as a precursor for the neurotransmitter norepinephrine (NE; Carlsson, 1957; 1958; 1959). Currently, DA neurotransmission is known to play a role in regulating voluntary movement, motor coordination (Pijnenburg and Van Rossum, 1973; Jackson et al., 1975; Costall and Naylor, 1975; Costall et al., 1976), and higher cognitive functions, such as learning and memory (Ploeger et al., 1991; Cools et al., 1993; Coccurello et al., 2000; Mele et al., 2004). However, elucidation of specific signaling pathways regulated by DA is challenging because DA modulates many cellular proteins, such as kinases,
phosphatases, transcription factors, ion channels, and membrane receptors (Tritsch et al., 2012).

DA is synthesized from the amino acid tyrosine. Tyrosine is converted to levodopa (L-DOPA) by tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis. L-DOPA is then converted into DA by aromatic L-amino acid decarboxylase. DA is then packaged into vesicles and stored until a stimulus prompts release from the presynaptic neuron. Once released into the synaptic cleft, DA can be metabolized by catechol-O-methyltransferase (COMT), recycled into the presynaptic neuron by the dopamine transporter (DAT), or act on pre- or postsynaptic receptors.

DA mediates its actions by interacting with a group of G-protein coupled receptors (GPCRs) termed DA receptors 1 through 5 (D1-D5). D1 and D5 receptors act in a similar manner and are considered D1-like receptors. Similarly, D3, and D4 act in similar manner to D2 receptors and are therefore considered D2-like receptors. The binding of DA to D1-like receptors increases adenylyl cyclase (AC) activity, which leads to the production of cyclic adenosine monophosphate (cAMP) and the activation of protein kinase A (PKA). In contrast to D1-like receptors, when activated, D2-like receptors inhibit AC to limit PKA activation. Research has indicated that the motor symptoms associated with PD, and possibly aging, are linked to a decrease in striatal D2 and D3 receptors (Joyce, 2001).

Dopamine cell bodies are located in the midbrain, specifically the substantia nigra (SN) and ventral tegmental area (VTA). These cell bodies have projections that lead to other parts of the brain, such as the cortex and other parts of the midbrain (striatum; Fig. 3.
1). DA cells bodies and the projections have been grouped into three different tracts: mesocortical, mesolimbic, and nigrostriatal (Fig. 1). The mesolimbic and mesocortical tracts are important for reward, learning, and memory (van Domburg and ten Donkelaar, 1991). This study will focus on the nigrostriatal tract, which mediates motor coordination and behaviors (van Domburg and ten Donkelaar, 1991).

1.3.2 Basal Ganglia

The basal ganglia are a collection of nuclei involved in directed movement, planning and execution of goal-directed movement. The caudate, putamen, and nucleus accumbens nuclei in the basal ganglia process information received from the central nervous system, particularly the cortex. Within the basal ganglia are groups of dopaminergic, γ-aminobutyric acid (GABA)-ergic, norepinephrine (NE) and serotonin (5-HT) neurons. GABA neurons are not only in the basal ganglia but project to the substantia nigra to help regulate release of other neurotransmitters (Sian et al., 1999). Interactions between neurons in the basal ganglia, more specifically the striatum, and the substantia nigra help to regulate voluntary movement.
Figure 1. Dopamine system in the brain.
Dopamine cell bodies originate in the substantia nigra and project to the mesocortical, mesolimbic, and nigrostriatal tracts.
1.3.3 Substantia Nigra

DA cell bodies originate in the substantia nigra (SN) and project to the striatum in the midbrain. This DA system, known as the nigrostriatal dopamine tract, is one of the major control centers of motor coordination in the brain, and dopaminergic cell death in the SN leads to a loss of motor coordination. With age, there is approximately a 5% loss of neurons in this region per decade (Gao et al., 2013). However, depending on disease state and species examined, this loss of DA neurons with age is controversial. For example, some groups have noted a significant loss of DA neurons with age (McGeer et al., 1977; Fearnley and Lees, 1991), while other groups have not observed an age-related loss of DA neurons (Pakkenberg et al., 1991; Muthane et al., 1998) in elderly people. Therefore, a connection between an age-related loss of DA neurons from the SN and motor deficits is not universally supported by the literature. In contrast, it is widely supported that PD patients show a loss of DA neurons in the SN leading to the motor symptoms seen with this disease.

The DA neurons of the SN are particularly susceptible to oxidative stress and inflammation, both of which are known to increase with age (Jenner and Olanow, 1996; Joseph et al., 2000). There have been many reasons proposed as to why this occurs. One reason may be that with aging there is an increased expression of pro-oxidative and proinflammatory states in the nigra of aged animals, such as NADPH oxidase complex, and an increase in inflammatory cytokines, IL-1β and TNF-α (Labandeira-Garcia et al., 2011; Viller-Cheda et al., 2012). An increase in inflammation that is mediated by IL-1β has been shown to increase the susceptibility of DA neurons to degredation (Koprich et
al., 2008). Other studies, in both humans and rodents, have shown that SN neurons are sensitive to neuroinflammation (Viller-Cheda et al., 2012) and toxicity related to iron metabolism (Zecca et al., 2004) with age. Therefore, an imbalance in the homeostatic environment of the substantia nigra may lead to a loss of DA neurons with age.

1.3.4 Striatum

The striatum is located in the midbrain and is part of the basal ganglia. The striatum is broken into two subdivision, dorsal striatum that contains most of the caudate and putamen, and ventral striatum, which contains the nucleus accumbens, ventromedial parts of the caudate and putamen, as well as part of the olfactory tubercle. There are multiple types of neurons contained in the striatum, including GABAergic and cholinergic neurons (Ferre et al., 1997). GABAergic neurons make up 90-95% of the neurons in the striatum (Ferre et al., 1997). DA signaling in the striatum, via complicated connections with GABAergic interneurons and the rest of the basal ganglia plays a large role in the control of voluntary movements and, thereby, motor coordination. To this end, a loss of D2-like receptors in the striatum has been linked to motor decline seen in the elderly (Kaasinen et al., 2000). However, as treatments for motor deficits are limited to those that occur with a neurodegenerative disease, such as PD, there is a need for a treatment for motor deficits that occur with normal, non-diseased aging.

1.4 Dopamine system in vitro

Cell culture models have been shown to be important tools for the analysis of specific signaling pathways. To study cell signaling in DA neurons, primary cultures of mesencephalic DA neurons are the model, which most closely mimics the in vivo
environment. However, these cultures contain GABA neurons, glia cells, and DA neurons (Prochiantz et al., 1979, O’Malley et al., 1992) making it difficult to isolate the signaling occurring only in the DA neurons. These cultures are also difficult to maintain and give variable results depending on the DA neuronal content of each culture. Therefore, a cell-line model of DA neurons, such as SH-SY5Y cells is widely utilized (reviewed by Xie et al., 2010). SH-SY5Y cells are a dopaminergic-like cell model, which were derived from a neuroblastoma. These cells express TH and DAT and exhibit dopamine-ß-hydroxylase activity (Xie et al., 2010).

1.5 Treatment of motor deficits

Motor deficits that are associated with a disease, such as PD or AD, are most often treated with L-3,4-dihydroxyphenylalanine (L-DOPA). This treatment was first used following the discovery that striatal DA levels are decreased in PD patients as compared to age-matched individuals (Hornykiewicz, 1966). L-DOPA is administered in conjunction with a peripheral inhibitor of L-aromatic amino acid decarboxylase (AADC; carbidopa), which blocks peripheral metabolism of L-DOPA. DA is not able to cross the blood brain barrier (Pardridge, 2003). Therefore, the use of an AADC inhibitor improves the efficacy of L-DOPA by allowing it to penetrate the brain before it is metabolized to DA. Once in the brain, L-DOPA increases the synthesis and release of DA in the SN and striatum, respectively (Rinne et al., 1971; Lloyd et al., 1975; Navailles et al., 2013).

In stroke patients, treatment with L-DOPA improved the function of affected limbs (Rosser et al., 2008). Additionally, Floel and colleagues (2008) showed that treatment with L-DOPA improved fine motor hand function in elderly patients. However,
there are limited clinical studies with L-DOPA as a treatment for conditions other than PD.

1.6 Behavioral tests for motor function

1.6.1 Challenge beam task

The challenge beam test was developed as a modification of traditional beam tests to provide a more sensitive measurement of skilled walking and motor coordination (Fleming et al., 2004) and is used to measure time, steps, and errors while crossing the beam towards the home cage. These parameters have been shown to be reliable measurements of sensorimotor coordination (Ogawa et al., 1985; Goldberg et al., 2003; Fleming et al., 2005). Mice are trained to traverse an elevated beam (4 segments, 25 cm long by 3.5 cm at the widest segment, narrowing to 0.5 cm in 1 cm increments) and enter their home cage on 2 separate days 24 hours apart. One week after training, wire mesh with 1 cm² openings is placed over the beam segments and the animals are videotaped and timed during their traversals. Videotapes are then viewed and rated in slow motion for the number of steps, time to cross, and errors by an investigator blinded to the treatment group.

1.6.2 Spontaneous activity test

The cylinder test measures spontaneous activities through the examination of movements that may be indicative of exploration, such as forelimb and hind-limb steps, rears, and grooming. Mice will display exploratory behaviors in the cylinder by rearing and placing their forelimbs on the wall of the cylinder (Schallert et al., 2000). This test
has been used by others to show changes in forelimb and hind-limb movement and rearing activity in mice and rats (Fleming et al., 2004; Kumar et al., 2009; Glajch et al., 2012).

For these experiments, a small, transparent, Plexiglas cylinder (height 15.5 cm, diameter 12.7 cm) is placed on a piece of glass that was elevated by four pedestals (6 in). A mirror is placed underneath the glass and angled towards the video camera so that the mice could be viewed from below. Animals are videotaped in the cylinder for 3 min and videos analyzed for forelimb steps, hind-limb steps, rears, and time spent grooming by an investigator blinded to the treatments. A step is counted when the animal moves both paws across the glass and a rear is considered as a vertical movement with both front paws leaving the glass floor.

1.6.3 Rotarod

The Rotarod machine consists of a rotating drum that a mouse can be placed on and is expected to remain on the rod as it turns. These machines can be controlled to increase the speed at which the mouse must walk in order to remain on the rod.

1.6.4 Gait walking

A gait analysis system, such as Catwalk 7.1 (Noldus IT, the Netherlands), can be used to analyze gait parameters, such as walking speed, stride length, standing time, swing length of limbs (Abada et al., 2013). A Catwalk system consists of an enclosed walkway with a glass plate and a speed video recording camera. Gait performance was
assessed and recorded using the catwalk analysis software. Rodents are habituated to the walkway on the first day and the following day the runs are recorded by the software.

1.6.5 Balance beam

The balance beam is similar to the challenging beam; however there is not a mesh grid that is placed on the beam. Mice are expected to traverse a narrow beam to their home cage. The mice are then assessed for time it takes to traverse the beam and paw slips (Luong et al., 2011).

1.7 L-DOPA, Parkinson’s disease, and dyskinesias

Motor symptoms associated with PD include bradykinesia, resting tremor, rigidity, and postural instability. The appearance of these motor symptoms is thought to evolve from the progressive degeneration of DA neurons in the SN that give rise to the nigrostriatal tract (Hof and Mobbs, 2001). However, degeneration is advanced (50% of SN neurons and 70% of striatal DA projections) by the time these symptoms manifest (Joyce and Millan, 2007). Although L-DOPA is the most common treatment for motor symptoms associated with PD, it has been shown to have what is considered a “wearing-off” phenomenon, which leads to L-DOPA-induced dyskinesias, or abnormal involuntary movements, in a majority of PD patients that are on this therapy for a few years (Ahlskog and Muenter, 2001; Mazzella et al., 2005). Once these dyskinesias have developed, they are irreversible, even with cessation of L-DOPA treatment (Iderberg et al., 2012).

The cause of L-DOPA induced dyskinesias is unknown. One hypothesis is that L-DOPA-induced dyskinesias develop due to the dosing regimen and mode of
administration of L-DOPA, which do not allow for consistent levels of DA to be sustained in the striatum (Smith et al., 2005; Cenci, 2007). Other studies suggest that L-DOPA, and its product DA, are toxic to dopaminergic cells. The toxicity of L-DOPA and/or DA is thought to occur due to the oxidation of DA into free radicals, quinones, hydrogen peroxide, and lipid peroxides (Mytilineou et al., 1993; Hastings et al., 1996).

1.8 Oxidative stress and aging

Normal cellular metabolism generates reactive oxygen species (ROS), such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^-$). Cells have natural defense mechanisms against ROS, such as glutathione (GSH), catalase, and superoxide dismutase (SOD; Long, 1999). With age, increases in the rate of production of ROS (Harman, 1992; Ames et al., 1993; Joseph et al., 1996), decreases in GSH and SOD levels (Mo et al., 1995; Papadopoulos et al., 1998; Sasaki et al., 2001), and an increased sensitivity of neurons to ROS (Ovadia et al., 1995; Desai et al., 1996; Ling et al., 2000; Stadtman, 2001) have been reported. In addition to an increase in reactive oxygen species (ROS), increases in reactive nitrogen species (RNS) have also been noted with age (Ladiges et al., 2010).

High levels of ROS and RNS lead to neuronal death (Farooqui and Farooqui, 2008). Specifically, dopaminergic cell death has been shown to occur concomitantly with an increase in levels of oxidative stress indicators (i.e., protein oxidation and lipid peroxidation) in the ventral midbrain (Lopez-Real et al., 2005; Munoz et al., 2006; Rey et al., 2007). Therefore, antioxidant compounds may protect neurons from oxidative stress-mediated toxicity leading to a preservation of motor function with age.
1.9 Natural products as antioxidants

Antioxidant compounds isolated from natural resources are available as supplements in pharmacies throughout the US and, therefore, their use by the general public is on the rise (Das et al., 2012; Chauhan et al., 2013). As the idea of using a natural compound is attractive to many, one of the goals of the current study was to examine the effect of natural compounds on motor function in an aging animal model.

While there are several known natural antioxidants, this study focused on isolated compounds derived from nuts and berries, resveratrol and pinostilbene, an analog of resveratrol. Additionally, we chose wild blueberry powder as an example of a whole fruit that has been shown to have antioxidant properties. The following sections provide a review of these compounds.

1.9.1 Resveratrol

Resveratrol (3, 4’, 5-trihydroxystilbene) is a polyphenol found in plants and fruits, such as grapes, mulberries, and peanuts (Baur et al., 2006). It is produced in response to pathogen exposure, such as fungal infection or UV irradiation, and when plants are stressed (Chao et al., 2010). Therefore, grapes that are grown in a warm, moldy, Southern climate have significantly higher levels of resveratrol in their skins than grapes grown in milder climates (Goldberg et al., 1994; Kolouchova et al., 2004; Lekli et al., 2010).

Isolated resveratrol has antioxidant (Olas, 2001; Chao et al., 2010), antibacterial (Zhong et al., 2012; Nawrocki et al., 2013), anti-inflammatory (Donnelly et al., 2004; Das et al., 2006), and anticancer properties (Nakagawa et al., 2001; Jang et al, 2001; Wang et
al., 2010) in vivo and in vitro. Moreover, resveratrol increases the lifespan of yeast, nematodes, *Drosophila melanogaster*, and mice on a high-fat diet (Baur and Sinclair, 2006; Baur et al., 2006).

1.9.2 Resveratrol analogs: Pinostilbene

Resveratrol has been shown to have poor bioavailability, in animal, human, and cellular studies (Andlauer et al., 2000; Soleas et al., 2001). The poor bioavailability of resveratrol is attributed to its rapid metabolism into glucuronides and sulfate conjugates within 15 minutes of absorption (Marier et al., 2002; Goldberg et al., 2003). Therefore, researchers have isolated or synthesized analogues of resveratrol, which may have less absorption issues and more potent effects in natural foods than resveratrol due to increased lipophilicity (Table 1). Figure 2 shows resveratrol and 8 analogs of resveratrol that have been synthesized in the laboratory of our collaborator, Dr. Agnes Rimando (Joseph et al., 2008). Chemical modifications to resveratrol, including methoxylation, have been made to increase its bioavailability (Wilson et al., 2008), and thereby its distribution throughout the body. One analog, pinostilbene (3,4'-dihydroxy-5-methoxystilbene), has a substitution of methoxy in place of the hydroxyl group on the 5 position of resveratrol. Methoxylated stilbenes, such as pinostilbene, are metabolized more slowly than hydroxylated stilbenes and, thereby, may have increased bioactivity (Wilson et al., 2008). The methoxylated group may also increase lipophilicity of the molecule, thereby increasing the permeation into cells and the brain. For example, Chao and colleagues (2010) found that pinostilbene penetrated into SH-SY5Y cells more effectively than resveratrol.
<table>
<thead>
<tr>
<th>Source</th>
<th>Resveratrol concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Natural peanut butter</td>
<td>0.65µg/g</td>
</tr>
<tr>
<td>Blueberries</td>
<td>32ng/g</td>
</tr>
<tr>
<td>Boiled peanuts</td>
<td>5.1µg/g</td>
</tr>
<tr>
<td>Cranberry raw juice</td>
<td>0.2mg/L</td>
</tr>
<tr>
<td>Dry grape skin</td>
<td>24.06µg/g</td>
</tr>
<tr>
<td>Grapes</td>
<td>0.16-3.54µg/g</td>
</tr>
<tr>
<td>Red wine blend</td>
<td>20.33µM; 0.1-14.3mg/L</td>
</tr>
<tr>
<td>Concord grape juice</td>
<td>1.56nmol/g</td>
</tr>
<tr>
<td>Pistachios</td>
<td>0.09-1.67µg/g</td>
</tr>
<tr>
<td>White grape juice</td>
<td>0.05mg/L</td>
</tr>
<tr>
<td>White wines</td>
<td>&lt;0.1-2.1mg/L</td>
</tr>
</tbody>
</table>

Table 1. Amount of resveratrol in foods and beverages
Figure 2. Structures of resveratrol and eight analogs.
1.9.3 Whole natural products: Wild Blueberries

Natural products are a fantastic resource for the discovery of new compounds and potential new therapeutics. A number of plants and foods have been shown to have beneficial effects on cardiovascular health, cognition, inflammation, stroke, cancer, and neuronal health. These foods contain compounds, such as anthocyanins and polyphenols, have been shown to reduce cancer risk and improve neuronal survival (Prior et al., 2008). Most of these natural products are available in our everyday diet in foods such as walnuts, spinach, strawberries, grapes, peanuts, and wild blueberries. Of these, wild blueberries are an excellent source of antioxidants and have the highest antioxidant capacity per serving when compared to more than 20 other fruits (oxygen radical absorbance capacity (ORAC) of Selected Foods, May 2010).

Wild blueberries are found growing in glacial soil, Northeastern United States, and Canada. Wild blueberries are dissimilar to cultivated blueberries in that they are smaller, darker in color. The dark skin of wild blueberries is an indication of the amount of anthocyanins in their skins, which are observed as a dark purple/blue color (Prior et al., 2008). Cultivated berries have more pulp and less skin as a percentage of the volume of the blueberry, which decreases the antioxidant properties. Moreover, wild blueberries are genetically diverse and offer a wide variety of protective properties, while cultivated berries offer a limited spectrum of antioxidants due to the narrow genetic profile. However, the question remains that when compounds are isolated from their natural environments will they be as effective as they are in their natural state? Joseph and colleagues (2010) showed that isolated compounds from wild blueberries, including
anthocyanins, proanthocyanins, chlorogenic acid, and low and high molecular weight proanthocyanins, were less effective at protecting primary hippocampal neurons from oxidative stress than wild blueberry powder itself. Together these data suggest that synergistic activity between the compounds may be necessary for full beneficial effects to occur.

1.10 In vitro studies with natural products

1.10.1 Resveratrol in vitro

Resveratrol has been shown to decrease the release of inflammatory cytokines (Tome-Carneiro et al., 2013; Zaky et al., 2013), improve neuronal cell survival following antioxidant exposure (Peritore et al., 2012; Wu et al., 2012; Zhang et al., 2012), and decrease cancer cell proliferation in vitro (Rigolio et al., 2005; Yin et al., 2013). For example, in a cell culture model of prostate cancer, resveratrol was shown to decrease cell proliferation, possibly by arresting these cells at the G1/S phase through the induction of cyclin dependent kinase inhibitor 1A and B (Wang et al., 2010). Resveratrol administration has also been shown to exhibit neuroprotective properties, possibly through direct radical scavenging or through the induction of antioxidant pathways. Resveratrol-mediated induction of heme oxygenase-1, an enzyme that catabolizes heme to the antioxidant biliverdin (Zheng et al., 2010), has been shown to decrease oxidative stress and, subsequently, cell death in primary neuronal cultures and smooth muscle cells (Zhuang et al., 2003; Juan et al., 2005). In SH-SY5Y cells, Wu and colleagues (2011) showed that resveratrol protected from rotenone-induced apoptosis by activating adenosine monophosphate-activated protein kinase/sirtuin-1 (AMPK/SIRT1) autophagy.
Sirtuins are also known as silent information regulator 2 (Sir2) proteins that are nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylases (Donmez and Outeiro, 2013). Sirt1 binds and deacetylates transcriptions factors, such as tau, retinoic acid receptor beta, and NF-κB in the brain and also can lead to mitochondrial biogenesis and fatty acid oxidation (Donmez and Outeiro, 2013). Wu and colleagues (2012) showed that resveratrol preconditioning increased the catalytic antioxidant system, methionine sulfoxide reductases A (MsrA), and protected against chloramine-T and 1-methyl-4-phenyl-pyridium induced cell death (MPTP). Resveratrol treatment has also been shown to protect against DA-induced oxidative stress in SH-SY5Y cells by the reduction in the cleavage of PARP, an increase in Bcl-2, and activation of caspase-3 (Lee et al., 2007).

1.10.2 Pinostilbene in vitro

Pinostilbene has been shown to have anticancer properties in a cell culture model of prostate cancer by arresting cells in the G1/S phase and prohibiting cell growth (Wang et al., 2010). Additionally, Chao and colleagues (2010) correlated that pinostilbene – mediated decreases of of JNK-1, JNK-2, and c-jun-1 activation in SH-SY5Y cells protected these cells from 6-OHDA-mediated neurotoxicity. The limited studies involving pinostilbene increases to the novelty of the current study.

1.10.3 Wild blueberries in vitro

Wild blueberries have been shown to inhibit cancer cell proliferation (Adams et al., 2011; Kausar et al., 2012; Rahal et al., 2013), decrease inflammation (McAnulty et al., 2011; Gustafson et al., 2012), and protect cells from oxidative stress (Senevirathne et
Wild blueberries have a significant amount of antioxidant potential, as measured by oxygen radical absorbance capacity (ORAC; Bagchi et al., 2006; Atala et al., 2009). The ORAC method of testing antioxidant capacity of natural products is the most popular method and assess antioxidant capacity by measuring the ability of a compound (antioxidant) to inhibit the consumption of a target molecule (Lopez-Alarcon and Denicola, 2013). The antioxidant potential of wild blueberries has been shown to protect against DNA damage via a decrease in gene and protein expression of p53, phospho-p53 and p21 in hepatocarcinoma, HepG2 cells (Liu et al., 2013). Blueberries have also been shown to improve vascular health through the activation of the phosphatidylinositol-3-kinase (PI3K) pathway, which resulted in cell migration and angiogenesis (Tulio et al., 2012). Wild blueberry treatment in vitro has been shown to attenuate neuronal sensitivity to Ca2+ fluctuations, which can occur with age and cause a loss of function of cells (Joseph et al., 2008; 2010).

1.11. In vivo studies with natural products

1.11.1 Resveratrol and animal studies

Resveratrol has been shown to have protective effects in animal models of cardiovascular disease (Lee and Moon, 2005; Das et al., 2006), diabetes (Zhang et al., 2012; Guschlbauer et al., 2013), viral infections, and lung disease. For example, Lu and colleagues (2008) found that resveratrol treatment attenuated acute pneumonia symptoms and lung bacterial load through a reduction in lung inflammation and the activation of cytokines, as well as an increase in natural killer (NK) cell activity. Topical application of resveratrol inhibits herpes simplex virus from spreading in mice through inhibition of
NF-κB in the nucleus (Docherty et al., 2005; Faith et al., 2006). Faith and colleagues (2006) showed that resveratrol inhibits NF-κB in the nucleus by providing data that the unactivated form of NF-κB in the cytoplasm is unaffected by resveratrol treatment.

Relevant to the current study, resveratrol has been shown to be neuroprotective in models of AD (Kumar et al., 2007), PD (Blanchet et al., 2008; Jin et al., 2008), Huntington’s disease (HD; Kumar et al., 2006), ischemia (Wang et al., 2002; Das et al., 2006; Della-Morte et al., 2009), traumatic brain injury (Ates et al., 2007) and epilepsy (Wu et al., 2009). Neuroprotection mediated by resveratrol in the above mentioned disease states is due to multiple factors, such as antioxidant and anti-inflammatory effects.

1.11.2 Wild blueberries and animal studies

Dietary supplementation with wild blueberries has been shown to improve cognition and motor coordination in a rodent model of Alzheimer’s disease as measured by the Y-maze and Morris water maze (Joseph et al., 2003). Moreover, rats that were fed a blueberry supplemented diet showed improvement in motor function as measured by the rod walking, accelerod, and Morris water maze tests (Joseph et al., 1999). Joseph and colleagues (2003) also showed that a blueberry supplemented diet improved cognitive deficits in a rodent model of AD, as well as increased hippocampal levels of extracellular signal regulated kinase (ERK) 1/2. Other studies have shown that antioxidant properties of blueberries protect against oxidative stress and improve memory (Casadesus et al., 2004; Andres-Lacueva et al., 2005; Duffy et al., 2008; Acosta et al., 2010).
1.12 Clinical studies with natural products

1.12.1 Resveratrol and clinical studies

Resveratrol has been shown to have many beneficial effects, both in vitro and in vivo, as described above. Red wine is considered the best source of resveratrol with concentrations ranging from 4-40µM (Goldberg et al., 1996). However, clinical studies with resveratrol have focused on the use of Concord grape juice or fruits to circumvent deleterious effects of alcohol administration (Table 1; Constant, 1997). Pace-Asciak and colleagues (1996) found that healthy male subjects that consumed grape juice had reduced risk of atherosclerosis, as measured by reduced rates of plasma thromboxane B2, and the IC50 (concentration required for 50% aggregation) for ADP and thrombin-induced platelet aggregation after four weeks of daily consumption. Additionally, one study showed the Concord grape juice improved verbal learning and enhanced verbal and spatial recall in older adults with mild cognitive impairment (Krikorian et al., 2010). Resveratrol supplementation (250mg/day) improved glycemic control in patients with type 2 diabetes mellitus via improving hemoglobin A1c and systolic blood pressure (Bhatt et al., 2012). Current clinical trials have not investigated resveratrol supplementation with respect to motor coordination. However there have been clinical trials that have analyzed the anti-inflammatory and antioxidant capabilities of resveratrol. These studies have shown a decrease in ROS generation, as well as a decrease of IL-6, NF-κB, and TNF-α (Culpitt et al., 2003; Ghanim et al., 2010; Karlsen et al., 2010). These changes in ROS generation and suppression of cytokine activation may suggest that
resveratrol could exhibit neuroprotective abilities in humans and thereby reduce motor function decline.

1.12.2 Wild blueberries and clinical studies

Dietary supplementation with wild blueberries has been shown to have many positive effects in one clinical trial that assessed mild cognitive decline associated with verbal memory and visual-spatial memory (Krikorian et al., 2010; Malin et al., 2011; Devore et al., 2012), motor coordination with age (Joseph, et al., 1999; Schrager et al., 2012) and vascular health (Kalt et al., 2008; Kristo et al., 2011; Xie et al., 2011). For example, two studies have shown that wild blueberry supplementation, juice or fruit, improved memory in older adults (Krikorian et al., 2010; Joseph et al., 2010). Related to the current study, elderly persons who ingested 2 cups of wild blueberry juice per day showed improvements on multiple motor tasks, such as an increase in self-selected walking speed, an increase in forced walking speed, a decrease in step errors during a challenging walking task, and improvements in reaction times (Schrager et al., 2012). In contrast, dietary supplementation of carrot juice had little to no effect on these parameters. These data suggests that anthocyanins in wild blueberries may be responsible for the positive effects in these motor tasks because carrots do not contain anthocyanins (Schrager et al., 2012).

Several clinical studies that explored possible mechanisms for the effect of wild blueberries on these behavioral and cognitive tasks have been conducted. Six weeks following daily ingestion of a wild blueberry drink for six weeks (25g powder/drink) levels of oxidized DNA bases and levels of H$_2$O$_2$-induced DNA damage were
significantly reduced (Riso et al., 2013). Surprisingly, another study found that one portion (300g) of blueberries has been shown to increase protection against H$_2$O$_2$-induced DNA damage in healthy male subjects (Del Bo et al., 2013).

### 1.13 MAPK signaling

Mitogen-activated protein kinases (MAPKs) are enzymes that mediate a multitude of processes, such as cell survival, differentiation, proliferation and apoptosis (Turjanski et al., 2007). Three members of the MAPK family are extracellular signal-regulated protein kinases 1/2 and 5 (ERK1/2 and ERK5). A protein cascade of phosphorylation by upstream kinases activates ERK1/2 and ERK5. The upstream kinases for ERK1/2 and ERK5 are MEK1/2 and MEK5, respectively.

#### 1.13.1 ERK1/2

ERK1 and ERK2 are 42 and 44kDa proteins, respectively. They have phosphorylation sites on the Thr–Glu–Tyr (TEY) in the protein kinase domain (Roskowski Jr., 2012). ERK1/2 has been shown to be important for cell survival, differentiation, cell adhesion, and migration (Nishimoto and Nishida, 2006; Krishna and Narang, 2008). However, levels of phosphorylated (activated) ERK1/2 have been shown to decline with age (Simonyi et al., 2003; Tortorella et al., 2004). As stated above, oxidative stress is known to increase with age and the ERK1/2 signaling pathway is activated in response to cellular stress (Roskowski Jr., 2012). H$_2$O$_2$-induced ERK1/2 activation is thought to play a critical role in cell survival in response to oxidative stress in rat PC12 cells and primary cortical neurons (Guyton et al., 1996; Crossthwaite et al., 2002). ERK1/2 activation was also shown to protect against hypoxia-induced cell death.
(Jin et al., 2002) and H$_2$O$_2$-mediated cell death (Crossthwaite et al., 2002). However, some studies indicated the ERK1/2 activation plays a role in apoptosis associated with oxidative stress (Ruffels et al., 2004; Kwon et al., 2011). The role of ERK1/2 is dependent on cell-type, toxin, or tissue. Conflicting data exists, even within SH-SY5Y cells. Some studies show that activation of ERK1/2 increases apoptosis (Arun et al., 2013) and others show neuroprotection (Jantas and Lason, 2009; Decressac et al., 2012; Minarini et al., 2012). Therefore, an aim of this study was to investigate the role of ERK1/2 in a model of DA cell death and protection by natural compounds.

1.13.2 ERK5

ERK5, also known as big mitogen-activated protein kinase-1 (BMK-1), is the most recently identified member of the MAPK family. ERK5 has a similar TEY activation motif; however, what distinguish ERK5 from ERK1/2 are a large C-terminal and a unique loop-12 sequence (Cavanaugh et al., 2000). The size of ERK5 is more than double that of ERK1/2 at approximately 102kDa (Nithianandarajah-Jones, et al., 2012). ERK5 has been shown to be highly active throughout prenatal neuronal development with a sharp decrease in activity after birth (Liu et al., 2003). It has been suggested that ERK5 plays a critical role in cortical development. ERK5 is also activated by stress, so it is an important pathway to examine in oxidative stress (Nishimoto and Nishida, 2006). Recent data from Dr. Jane Cavanaugh’s laboratory suggests the ERK5 activation declines with age, predominately in DA regions of the brain. Due to an increase in oxidative stress with age and a decrease in ERK5, this may indicate that ERK5 is important for DA cell survival with age. As mentioned above, ERK5 may have distinct roles from ERK1/2, but
due to a lack of specific inhibitors, their roles were not well understood. Recently, XMD8-92 has been shown to inhibit ERK5 phosphorylation in cancer cells (Yang and Lee, 2011; Yang et al., 2010). BIX02189 has also recently been synthesized and shows ERK5 specific inhibition in PC12 cells (Obara et al., 2009), cardiac myocytes (Kimura et al., 2010), and C6 glioma cells (Obara et al., 2011).
Figure 3. MAP Kinase Signaling cascade.
1.13.3 Inhibitors of ERK1/2 and ERK5

While the ERKs have been widely studied, the distinct roles of ERK1/2 versus ERK5 are not yet well defined. MAPKs are highly conserved enzymes and available pharmacological inhibitors of ERK1/2, such as U0126 and PD98059, were not specific for the ERK1/2 pathway in all cell types (Nishimoto and Nishida, 2006). For example, Yu and colleagues (2012) found that in a rodent model of cystitis, U0126 and PD98059 inhibited both MEK1/2 and MEK5. Another study showed that PD98059 and U0126 specifically inhibited ERK1/2 while ERK5 activity unaffected in multiple cell lines (kidney cells: RCC4, breast cancer cells: MCF-7; and human osteosarcoma cells: U2OS-HRE-luc; Sutton et al., 2007). More recently, MEK5 specific inhibitors, BIX02189 (Obara and Nakahata, 2010; Yang and Lee, 2011) and XMD8-92 (Yang and Lee, 2011), have been synthesized, that have been shown to be more specific for ERK5 in several cell culture models.

1.13.4 Resveratrol and ERK signaling

In natural killer, NK2 cells, resveratrol was shown to increase phosphorylation of ERK1/2 possibly through the natural killer receptor, NKG2D, and treatment with ERK1/2 specific inhibitors blocked NK cell cytotoxicity (Lu and Chen, 2010). In SH-SY5Y cells, resveratrol activated (1pM-10µM) and inhibited (50-100µM) ERK1/2 in a concentration-dependent manner (Miloso et al., 1999).

1.13.5 Pinostilbene and ERK signaling
As mentioned above, there is little research on pinostilbene, in vivo and in vitro. However, Chao and colleagues (2010) showed that pinostilbene has been shown to decrease activation of JNK-1, JNK-2, and c-jun-1 in SH-SY5Y cells. This data indicates that pinostilbene is able to modulate the JNK MAPK signaling pathways and, therefore, may possibly affect other MAPK pathways, including the ERK signaling cascades.

1.13.6 Wild blueberries and ERK signaling

A few groups have shown that wild blueberry supplementation improves memory in rodents via the activation of ERK1/2 in the hippocampus (Williams et al., 2008; Rendeiro et al., 2012). However, Vuong and colleagues (2010) have shown that blueberry juice protects neurons from H$_2$O$_2$-induced oxidative stress through inhibition of ERK1/2 mediated cell death. Therefore, although a hypothesis of this study is that wild blueberry supplementation will increase ERK1/2 activation and thereby protect DA cells from oxidative stress-induced cell death. However, it is possible that wild blueberry treatment will decrease or have no effect on ERK1/2 activity.

In conclusion, the first two aims of this research are to evaluate a widely used pharmaceutical product, L-DOPA, and natural products, including resveratrol, pinostilbene, and wild blueberries, as effective treatments for age-related motor deficits using behavioral tests of motor coordination and spontaneous activity and to investigate if there is a difference in the effectiveness of the isolated natural compound, resveratrol, and whole foods, such as wild blueberries. Finally, using the SH-SY5Y dopaminergic cell line, the third aim is to examine the effect of these natural products on DA neuronal survival and ERK signaling. Together these aims contribute to the overall goals of this
study, which are to begin to identify possible treatments for age-related motor deficits and to elucidate some of the mechanisms by which these compounds may produce beneficial effects.
Chapter 1

The Effect of L-DOPA on age-related motor deficits in mice

2. RATIONALE AND HYPOTHESIS

2.1 RATIONALE

Aging is usually accompanied by a decline in motor function, a phenomenon that can be observed in laboratory animals as well as in humans. For example, rodents typically exhibit a decline in spontaneous motor activity and motor coordination with age (Cantuti-Castelvetri et al., 2003; Fleming et al., 2004; Boger et al., 2006; Colebrooke et al., 2006). Such motor deficits have been shown to arise following alterations in the dopamine (DA) neuronal system (Packard and Knowlton, 2002). Indeed, as seen in studies of Parkinson's disease (PD), many of the motor deficits are strongly correlated with the loss of the DA neurons of the nigrostriatal projections (Eriksen, et al., 2009). L-DOPA, which increases the availability of DA in striatum, is able to temporarily improve motor function in PD patients (Obesa et al., 2008; Olanow et al., 2009).

The efficacy of L-DOPA in PD suggests the possibility that the drug may also be effective in the treatment of motor dysfunction that occurs with normal aging; a clinical problem for which there is no standard treatment. To test this hypothesis, the effects of L-DOPA on balance, motor coordination, and spontaneous activity were examined in young and old male mice using challenge beam and cylinder tests, respectively (Fleming et al., 2006; 2004a; 2004b). Following behavioral testing, DA and DA metabolite content were determined in the striata of these animals. Finally, in a separate group of animals, the
expression of tyrosine hydroxylase (TH) and the high affinity DA transporter (DAT) were determined in the striata one week following behavioral testing.

2.2 HYPOTHESIS

L-DOPA administration will attenuate age-related motor deficits in C57Bl/6 mice.

3. MATERIALS AND METHODS

3.1 Animals

C57Bl/6 mice were chosen as an aging mouse model. These mice display less co-morbidities than other mouse models. Young (2.5 mo) and old (20.5 mo) male C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) were housed 3 per cage and maintained on a 12 h light:dark cycle with ad libitum access to food and water. The animals were utilized in compliance with all applicable laws and regulations as well as principles expressed in the National Institutes of Health, University of Pittsburgh, and the Guide for the Care and Use of Laboratory Animals.

3.2 Treatment Groups

Mice were given benzerazide (12.5 mg/kg; i.p., cat # B7283, Sigma, St Louis, MO) to block peripheral decarboxylation of L-DOPA, followed 10 min later by L-DOPA (15 mg/kg, i.p., cat # D1507, Sigma) or an equivalent volume of saline vehicle.

3.3 Behavioral Analysis

3.3.1 Challenge beam task
Animals were tested on the challenge beam to assess motor performance as previously described (Fleming et al., 2004; Fleming and Chesselet, 2005; Hwang et al., 2005). Briefly, mice were trained to traverse an elevated beam (4 segments, 25 cm long by 3.5 cm at the widest segment, narrowing to 0.5 cm in 1 cm increments) and enter their home cage on 2 separate days 24 hours apart. One week after training, wire mesh with 1 cm² openings was placed over the beam segments and the animals were videotaped and timed during their traversals. Videotapes were viewed and rated in slow motion for the number of steps, time to cross, and errors by an investigator blinded to the treatment group.

3.3.2 Spontaneous activity

For these experiments, a small, transparent, Plexiglas cylinder (height 15.5 cm, diameter 12.7 cm) was placed on a piece of glass that was elevated by four pedestals (6 in). A mirror was placed underneath the glass and angled towards the video camera so that the mice could be viewed from below. Animals were videotaped in the cylinder for 3 min and videos were analyzed for forelimb steps, hind-limb steps, rears, and time spent grooming by an investigator blinded to the treatments. A step was counted when the animal moved both paws across the glass and a rearing was considered as a vertical movement with both front paws leaving the glass floor. Values were calculated as the mean ± SEM.

3.4 Tissue processing

Mice were decapitated one week following the last behavioral test. Striata were rapidly dissected, frozen on dry ice, and stored at -80°C until assayed. Striatal samples
were sonicated in 0.1N perchloric acid (20μl/mg wet tissue weight) for analysis of DA levels or in ice cold lysis buffer (20mM Tris, pH 6.8, 137mM NaCl, 25mM β-glycerophosphate, pH 7.14, 2mM NaPPI, 2mM EDTA, 1mM Na3VO4, 1% Triton X-100, 10% glycerol, 5μg/ml leupeptin, 5μg/ml aprotinin, 2mM benzamidine, 0.5mM DTT and 1mM PMSF) for analysis of two phenotypic markers of DA neurons, TH and DAT.

3.5 Western Blot analysis for DAT and TH

Protein content was assessed by bicinechonic acid assay (Pierce, Rockford, IL) and 20μg of protein was loaded on an SDS PAGE gel and transferred to an Immobilon-FL PVDF membrane (Millipore, Billerica, MA). Membranes were washed in Tris-buffered saline with 0.1% Tween (TTBS) and blocked for 1 hr in a fish-serum based blocking solution (LI-COR Biosciences, Lincoln, NB) at room temperature. Membranes were then incubated overnight at 4°C in monoclonal rat anti-DAT (1:2000; Chemicon, # MAB369, Temecula, CA), monoclonal mouse anti-TH (1:20,000, Chemicon, # MAB318), or monoclonal mouse anti-α-tubulin (1:50,000, Sigma Clone B512 T5168). Binding was visualized with infrared secondary antibodies fluorescing at 700 nm (goat anti-rat for DAT; goat anti-mouse for tubulin) or 800 nm (goat anti-mouse for TH; Odyssey Imaging; LI-COR) for 1 hr at room temperature (1:10,000) in blocking solution. Blots were scanned on an Odyssey Imager and quantified with Odyssey software, using background subtraction above and below each band. Grayscale images were used for quantification and the images were pseudo-colored red (700 nm) and green (800 nm) to delineate DAT and TH using distinct fluorescent wavelengths.

3.6 HPLC analysis for dopamine and metabolites, DOPAC and HVA
Striata were sonicated in 0.1N perchloric acid (20µL/mg of tissue). After filter centrifugation, an aliquot was analyzed by HPLC for DA, 3,4- dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) content as previously described (Smith et al., 2002). An aliquot of each sample was injected onto an ESA C18 column (2.1 × 150 mm, ESA, Inc., Chelmsford, MA). The mobile phase consisted of 75mM H2NaPO4, 1.7mM octanesulfonic acid, 25mM Na2EDTA 0.00001% triethylamine (v/v), and 10% acetonitrile (v/v), pH 3.0 and was pumped through the system at a rate of 0.3 ml/min using 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 E1 = −0.26 V, E2 = +0.28 V, guard cell = +0.4 V. The limits of detection were in the femtomole range. Quantification was based on standard curves for DA, DOPAC, and HVA.

3.7 Statistical analysis

All statistical tests were performed using Graph Pad Prism. The data in figures 1 and 2 were analyzed using a two-way ANOVA (p<0.05) followed by post hoc comparison using a Bonferroni multiple comparison test. The data in figures 3, 4, and 5 were analyzed using an unpaired t-test with a 95% confidence interval.
4. RESULTS

4.1 L-DOPA reverses the age-related decline in motor coordination

The challenge beam task was used to measure motor coordination in this aging mouse model. Aged (20.5 mo) C57Bl/6 male mice made significantly more errors on the challenge beam task than young (2.5 mo) mice (Fig. 4). Treatment with L-DOPA decreased the number of errors made by the old mice to that of young, saline treated animals (Fig. 4). L-DOPA had no effect on the motor coordination of young animals.
Figure 4. L-DOPA decreases the number of errors made by old animals on the challenge beam task. Results are presented as mean ± SEM, n = 6-9 per group. Old animals made significantly more errors as compared to their young counterparts (*p<0.05 in comparison to young saline). L-DOPA treatment (15mg/kg) reversed age-related motor deficits in old animals (**)p<0.05 in comparison to old saline). Data were analyzed using a two-way ANOVA followed by post hoc comparison using a Bonferroni multiple comparison test.
4.2 L-DOPA does not alter spontaneous activity

In order to determine if age has an effect on spontaneous activity, the cylinder test was used to measure four parameters for exploration used to determine spontaneous activity were rearing, forelimb and hind-limb steps and grooming. Each mouse was placed in a Plexiglas cylinder and videotaped the mouse for three minutes. Aged mice did not display deficits in any of these parameters as compared to young mice (Fig. 5). Moreover, L-DOPA treatment did not affect any of these behaviors in young or old mice.
Figure 5. Number of rears did not significantly change with age or L-DOPA treatment.
Results are presented as mean ± SEM, n = 6-9 per group. Young and old animals made similar numbers of rears in the cylinder test. L-DOPA had no effect on rears in young or old animals. Data were analyzed using a two-way ANOVA followed by post hoc comparison using a Bonferroni multiple comparison test.
4.3 DA, DOPAC, and HVA levels are not changed with age

To determine if age altered the DA neuronal system, DA, DOPAC, and HVA levels were measured by HPLC in the striatum of young (2.5 mo) and old (20.5) mice. The levels of DA, DOPAC, and HVA were not altered in an age-related manner in this mouse model (Fig. 6A). Administration of L-DOPA did not change the amount of DA or metabolites in young or old mice (Fig. 6B).

Western blot analysis was performed to determine if the protein expression of the dopamine transporter (DAT) or tyrosine hydroxylase (TH) was altered with age in the striatum. Neither DAT nor TH expression were altered with age (Fig. 7A and 7B). Administration of L-DOPA did not change the expression of TH or DAT in the striatum of young or old mice (Fig. 7).
Figure 6: DA, DOPAC, and HVA levels are unchanged with age. Results are presented as mean ± SEM. (A) Striatal dopamine levels and metabolites are unchanged with age. Accordingly, dopamine turnover was also unchanged with age. (B) L-DOPA administration did not alter striatal DA levels or metabolites. Data were analyzed using a two-way ANOVA followed by post hoc comparison using a Bonferroni multiple comparison test.
Figure 7: Phenotypic markers of DA neurons are unchanged with age or L-DOPA administration.
Results are presented as mean ± SEM. (A) The dopamine transporter, DAT, was unchanged with age. Administration of L-DOPA did not alter striatal DAT (B) Tyrosine hydroxylase (TH), the rate limiting enzyme in DA synthesis, was unchanged with age or with L-DOPA administration. Data were analyzed using an unpaired t-test with a 95% confidence interval.
5. DISCUSSION

A loss of motor coordination, which can lead to an increase in slips and falls (Schoene et al., 2013), has been noted with age (Potvin et al., 1980). With the current and projected increase in the percentage of the population 65 years and older, this loss of motor coordination creates a national health care crisis. Currently, there are no mainstay therapies for motor deficits that are associated with non-diseased, normal aging. However, in one clinical trial, L-DOPA, a first-line therapy in the treatment of motor symptoms associated with PD, was shown to improve fine motor control in elderly patients (Floel et al., 2008). The current study examined the effect of L-DOPA on age-related motor deficits in a mouse model of aging.

The challenge beam task was used to measure the effect of aging on motor coordination. The use of the challenge beam is a novel behavioral test that measures the number of slips or errors, steps, and amount of time it takes to cross the beam (Fleming et al., 2005; Fleming et al., 2006; Hwang et al., 2005). Aged animals made significantly more errors on the beam than young animals (Fig. 4). Administration of L-DOPA decreased the number of errors to that of the young saline group (Fig. 4). However, L-DOPA treatment did not affect the number of errors made by young animals. These data suggest that L-DOPA treatment reverses age-related motor deficits in coordination as measured by the challenge beam task. This study is the first to use the challenging beam task to analyze age-related motor deficits. Other studies have shown a decrease in motor performance with age using a multitude of other behavioral tests such as, accelerating rotarod test (Colebrooke et al., 2006; Esteban et al., 2010; Parameshwaran et al., 2010),
wire hanging (Thiruchelvam et al., 2003; Takahashi et al., 2009), balance beam (Emerich et al., 2008), and hind-limb extension (Takahashi et al., 2009). However, the challenge beam task is a more sensitive test and can detect changes in fine motor coordination before traditional behavioral tests and, importantly, before a significant loss of DA neurons in the substantia nigra (SN; Fleming et al., 2004).

Some years ago, a report appeared in which the effects of \( L \)-DOPA were examined in 10 healthy elderly volunteers (Newman et al., 1985). In this study, \( L \)-DOPA had no effect on movement velocity, reaction time or tremor (Newman et al., 1985). However, the number of people in this study was very limited and the investigators did not measure motor coordination per se. In agreement with the results of the current study, a more recent study showed that \( L \)-DOPA improves upper limb function in otherwise normal aged rhesus monkeys (Grondin et al., 2000). Furthermore, in a study of motor memory performance in the elderly, \( L \)-DOPA enhanced the effects of training on motor memory formation, in part via an increase in DA release in the caudate nucleus (Floel et al., 2008). Therefore, one possible explanation for the effect of \( L \)-DOPA on motor coordination in the C57Bl/6 aged mouse model is that it reverses age-related losses of striatal DA or turnover.

In the present study, the decline in motor function was not accompanied by age-related alterations in DA, DOPAC, or HVA striatal content or DA turnover as determined by the ratio of DA metabolite levels to DA levels (Fig. 6A). This is in contrast to research that has shown that there are age-related decreases in striatal DA (Emborg, 1998; Colebrooke et al., 2006; Cruz-Muros et al., 2007), and decreases (Esquifino et al., 2002;
Purdom et al., 2003) or increases (Yurek et al., 1998) in DA turnover. However, in agreement with the results in these aged C57Bl/6 mice, others have reported no change in striatal DA content with age (Bowenkam et al., 2000). Together, these data suggest that there may be species differences in alterations of the DA system that occur with age.

Treatment with L-DOPA did not affect the amount of striatal DA, DOPAC or HVA (Fig. 6B). While these results may seem surprising, the animals were sacrificed one week after L-DOPA treatment. The half-life of L-DOPA is 60-90 min (Poewe and Mahlkne, 1997). Therefore, the effect of L-DOPA on DA and DA metabolites may be transient and unable to be observed one week following treatment.

In addition to age-related losses of striatal DA, striatal TH and DAT (Troiano et al., 2010; Yue et al., 2012) content have been reported to decline with age (Meng et al., 1999; Salvatore et al., 2003). However, in the present study there was not an age-related decrease in either striatal TH or DAT expression (Fig. 7). Similar to these results, Salvatore and colleagues (2009) did not observe alteration in total striatal DAT or DA content with age; however, they noted an age-related decrease in DAT protein recovered from the plasma membrane. Together with the data from the current study, these results suggest that internalization of DAT, but not decrease in total DAT, may occur with age. It is also possible that in these mice, male C57Bl/6, there is no change in DAT with age.

Other alterations in the DA neuronal system that have been observing in aging models include decreases in D₁ (Wang et al., 1998) and D₂ (Hara et al., 1992; Roth and Joseph, 1994; Volkow et al., 1998) and DA receptor expression. Therefore, it is possible that a decrease in DA receptors, and thereby a decrease in DA signaling, occurred in our
animal model and contributed to the age-related decline in motor function. L-DOPA, in turn, may have increased DA neurotransmission and/or signaling to alleviate the loss of motor coordination noted with age (Boger et al., 2010; Hoekzema et al., 2010; Yue et al., 2012).

Striatal dopamine release has also been shown to decline with age (Yurek et al., 1998; Gerhardt and Maloney, 1999; Stanford et al., 2000). Again, it is possible that DA release was decreased with age in our model to produce motor deficits and L-DOPA treatment increased DA release to restore motor function.

Finally, DA cell bodies that reside in the SN have been shown to degenerate with age (McNeill et al., 1984a; 1984b; Boger et al., 2006). Therefore, age-related degeneration of these DA neurons may have occurred in our model even though we did not observe a loss of DA content in the striatum. Future studies will explore these possibilities.

In addition to possible effects of L-DOPA on DA neuronal parameters that were not measured in this study (i.e. DA receptor levels, DAT internalization, DA release), a possible explanation for the effect of L-DOPA on motor coordination in these aged animals is that it increases striatal noradrenergic function. Rommelfanger and colleagues (2007) found that dopamine β-hydroxylase knockout mice (Dbh−/−), which lack norepinephrine (NE) but have normal DA levels in the striatum, have a significant loss of motor coordination as compared to heterozygous litter mates (Dbh+/−) as measured by the same challenge beam test used in this study. Therefore, it is possible that in our animals there was an age-related decline in NE levels that contributed to the motor function decline.
dysfunction and L-DOPA increased NE levels and signaling in the aged animals to restore coordination.

One other possibility is that there was a loss of equilibrium between DA and acetylcholine (Ach) in the striatum of our aged animals (Di Chiara et al., 1994; Calabresi et al., 2000) and L-DOPA treatment restored the balance between these neurotransmitters. Some of the motor symptoms associated with PD can be reversed by inhibiting Ach activity, which suggests that DA-Ach interactions are important for motor coordination (Berg et al., 1987). It is possible that there was a decrease in Ach that changed the DA-Ach balance and contributed to the age-related motor deficits in these C57Bl/6 mice, however, this parameter was not investigated.

In contrast to the loss of motor coordination noted with age on the challenge beam test, spontaneous activity as measured by number of rears during the cylinder test was not significantly decreased in C57Bl/6 aged male mice (Fig. 5). Interestingly, rearing or vertical movement has been shown to be mediated by DA neurotransmission (Kalivas et al., 1984; Koene et al., 1993; Swanson et al., 1997). The data in the current study support a role for DA in rearing behavior in this aging mouse model as age-related deficits in rearing behavior or striatal DA and DA metabolites were not observed. Moreover, the fact that there was no loss in spontaneous activity in this aging model allows for interpretations of the challenge beam test without the confounding factor of a loss of spontaneous movement in old animals.

In contrast to the effect of L-DOPA on motor coordination, L-DOPA treatment did not increase spontaneous activity in the young or old mice. The cylinder test was
performed within 30 min following the L-DOPA injection and there was no change in the average number of rears with age or L-DOPA treatment. As mentioned above, when measured one week following L-DOPA treatment, striatal DA or DA metabolites levels were unchanged as compared to the vehicle treated animals. As L-DOPA is known to increase extracellular DA in the striatum (Obesa et al., 2008; Olanow et al., 2009), we hypothesized that L-DOPA would increase spontaneous activity.

L-DOPA therapy, which has been used for more than 40 years in the treatment of PD (Mercuri and Bernardi, 2005) is well tolerated in early PD and the side effects that typically appear in the later stages of PD are likely to be a consequence of the progressive loss of DA neurons that accompanies the disease and thus may not be a factor in normal aging. Therefore, on the basis of the data from the current study, the few related findings that have appeared in the literature, and the apparent lack of side effects of the drug in otherwise normal adults, we suggest that with further investigation L-DOPA could be considered as a useful treatment for aged-related motor decline.
Chapter 2

The effect of natural products on age-related motor deficits in mice

6. RATIONALE AND HYPOTHESIS

6.1 RATIONALE

L-DOPA only treats the motor symptoms of PD and does not halt the progressive degeneration of nigrostriatal tract. For these reasons, L-DOPA works optimally early in the treatment of the PD (for the first 2 years); however, as the progression of PD continues (5–10 years), the efficiency of L-DOPA decreases, such that many patients develop motor fluctuations (‘wearing-off’ phenomenon) and involuntary movements or dyskinesias (Marsden and Parkes, 1976; Shimizu and Ohno, 2012). Importantly, once these dyskinesias have developed, they are irreversible, even with cessation of L-DOPA treatment (Iderberg et al., 2012). Consequently, while L-DOPA was an effective treatment for motor deficits in our aged mouse model, our study was short-term and did not address the long-term effects of L-DOPA. Therefore, a literature was done to investigate other possible treatments for age-related motor decline that may not have such detrimental side effects. One such treatment is the natural compound, resveratrol.

Resveratrol is a compound found in many whole foods, including grapes and blueberries. These whole foods and many others, including walnuts and spinach, are natural sources of antioxidants. One hypothesis for the pathology that underlies motor deficits that occur with age is that there is an increase in oxidative stress with age leading to an increase in neuronal cell death (Stadtman, 1992; Harman, 1992; Ames et al., 1993).
DA neurons are particularly sensitive to oxidative stress (Foley and Riederer, 2000). Therefore, administration of exogenous antioxidants could provide an effective treatment for this imbalance of ROS.

A caveat for using resveratrol is that it has limited bioavailability (Andlauer et al., 2000; Soleas et al., 2001) and high doses may actually be toxic to neurons (Johnson et al., 2011). Therefore, pinostilbene, a resveratrol analog, that has also been shown to protect neurons against oxidative stress in vitro and in vivo (Chao et al., 2010) became of interest in the current study.

Finally, an intriguing theory in natural product research is that the isolated compounds are more effective in their natural environment, such as a whole fruit. Wild blueberries are one of these whole foods that contain resveratrol (Rimando et al., 2004) and have been shown to have neuroprotective properties (Kumar et al., 2006; Kumar et al., 2007; Blanchet et al., 2008; Jin et al., 2008). Therefore, the current study was to examine the effect of these natural products, either as isolated compounds or as the whole food, on age-related motor deficits in the aged C57Bl/6 mouse model.

**6.2 HYPOTHESIS**

Dietary supplementation with resveratrol, pinostilbene, or wild blueberries will attenuate age-related motor deficits. Wild blueberries will be more effective than resveratrol and pinostilbene at attenuating the loss of motor function noted with age.
7. MATERIALS AND METHODS

7.1 Animals

Young (2 mo), middle-aged (10 mo), and old (22 mo), male, C57BL/6 mice were obtained from the NIA aging colony (Charles River, New York, NY) and were housed singly. All animals were maintained on a 12 hr light/dark cycle. Water and food were supplied *ad libitum* until the study began. All procedures were conducted in accordance with the guidelines for the NIH Care and Use of Laboratory Animals and approved by the Duquesne University Institutional Animal Care and Use Committee.

7.2 Resveratrol or pinostilbene dietary supplementation

In order to determine the average amount of food consumption for mice, we discussed rodent behavior and feeding habits with the Duquesne University animal facilities manager, Denise Butler-Buccilli. Mice were given 6g per day of resveratrol- or pinostilbene-supplemented diet (120 mg/kg of diet) for 8 weeks. The diets were prepared at Harlan Teklad (Madison, WI) by adding crystalline resveratrol (TCI America, Waltham, MA) or pinostilbene (synthesized by Dr. Cassia Mizuno, ARS USDA facility, University, MS) to the control diet, which is a modification of the standard rodent chow supplied at the Duquesne University Animal Facility (Purina Rodent Chow; 6 gm/day, 0.004% w/w). Food consumption was measured daily and mice ate ~4.5 grams/daily. Therefore, calculating the amount of resveratrol and pinostilbene consumed, we determined that the mice ate 0.54 mg/kg of body weight daily. The average weight of the mice during the study was 30 g, therefore, calculating for the average human male (70
kg), the dose that a human would have to eat to be equivalent to this study would be 1.26 g.

7.3 Wild Blueberry Dietary Supplementation

Mice were fed a wild blueberry powder supplemented diet (2%; Harlan Teklad) for 8 weeks. The wild blueberry powder was a generous gift from the Wild Blueberry Association (Nutraceuticals, Momence, IL). The diet was prepared at Harlan Teklad by adding wild blueberry powder to the control diet, which is the standard rodent chow used in the Duquesne University animal facility. The amount of corn in an additional control diet was adjusted to account for the added quantity of wild blueberries and make an iso-caloric control. Mice on the wild blueberry diet ate approximately 4.5 grams of diet daily. We calculated the amount of wild blueberries consumed daily, each mouse ate approximately 90 mg. The average weight of the mice over the course of the study was 30 g. Therefore, calculating for the average human male (70 kg), the equivalent dose would be 210 g, which would be approximately 1.5 to 2 cups of wild blueberries daily.

7.4 Behavioral analysis (see Chapter 1 Methods pg 30-31)

7.4.1 Challenge beam test

7.4.2 Spontaneous Activity

7.5 Tissue processing

Mice were decapitated 24 hrs after the cylinder test for spontaneous activity, and the striatum was rapidly dissected, frozen on dry ice, and stored at -80°C until assay. The
hindbrain was stored in fix solution (4% formaldehyde/4% NaF in PB) for one week and then changed to a 30% sucrose solution in 1XPBS. Samples were sonicated in 0.1N perchloric acid (20μl/mg wet tissue weight) for analysis of DA levels or in ice-cold lysis buffer for analysis of two key phenotypic markers of DA neurons, TH and DAT. The lysis buffer consisted of 20mM Tris, pH 6.8, 137mM NaCl, 25mM β-glycerophosphate, pH 7.14, 2mM NaPPi, 2mM EDTA, 1mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 5μg/ml leupeptin, 5μg/ml aprotinin, 2mM benzamidine, 0.5mM DTT and 1mM PMSF.

7.5 Western Blot Analysis

One half of the striatum from each animal was sonicated in ice-cold lysis buffer. Protein (60μg) was run on an 8% SDS-PAGE minigel at 100V for 2 hrs. The gels were then transferred to nitrocellulose membranes (Li-Cor Biosciences; Lincoln, NE) and were blocked for 1h at room temperature with shaking. The membranes were incubated in mouse anti-ERK1/2 (1:1000, catalog # 9107; Cell Signaling Technology, Danvers, MA), rabbit anti- phospho-ERK1/2 (1:1000, catalog # 9101; Cell Signaling Technology) rabbit anti-ERK5 (1:1000, Sigma, catalog # 1523), rabbit anti-phospho-ERK5 (1:1000, catalog # 3371; Cell Signaling Technology), rabbit monoclonal anti-DAT (1:3000, catalog # AB2231, Millipore), mouse anti-TH (1:2000; catalog # MAB318, Millipore), or GAPDH (1:1000, Millipore) antibodies overnight in casein blocking buffer (Li-Cor Biosciences). The membranes were washed four times with 1XPBS/0.1%Tween-20/0.01%NaN₃ followed by a one-hour incubation with IR680-labeled goat anti-rabbit or IR800-labeled goat anti-mouse secondary antibody (1:10,000, Li-COR Biosciences). Blots were scanned on an Odyssey Imager and quantified with Odyssey software, using background
subtraction above and below each band. Grayscale images were used for quantification, and the images were pseudo-colored red (700 nm, goat anti-rabbit) and green (800 nm goat anti-mouse) to delineate using distinct fluorescent wavelengths.

7.6 HPLC Analysis (see Chapter 1 Methods, pg 32-33)

7.7 Gas chromatography-Mass spectrometry (GC-MS)

7.7.1 Compounds

Resveratrol was a commercial sample (ChemPacific, Baltimore, MD). Pinostilbene was synthesized by Dr. Cassia Mizuno in Dr. Agnes Rimando’s laboratory following published procedures (Polunin and Schmalz 2004). Briefly, pterostilbene was methylated with methyl iodide to obtain trimethoxystilbene. Trimethoxy stilbene was then demethylated with lithium thioethoxide in dimethylformamide (160°C for 2h; 67% yield). The compound identity and configuration was established by nuclear magnetic resonance spectroscopy.

7.7.2 Analysis of stilbenes by gas chromatography-mass spectrometry (GC-MS)

Tissue samples were kept in -80°C freezer until used for analysis. Tissues were thawed on ice, then homogenized in 150µl of sodium phosphate buffer (0.2M NaHPO₄: 0.2M Na₂HPO₄; 80:20), pH 7.4, and centrifuged for 15 mins at 7000g, 4°C. The pellet was homogenized a second time with 150µl of phosphate buffer. The supernatants were combined; half-volume was treated with β-glucuronidase (5000 U/ml potassium phosphate buffer, 75mM, pH 6.8; 50 µl/125µl extract) and incubated at 37°C with shaking at 750 rpm for 20 hrs. Potassium phosphate buffer was added to the other half-
volume as control. The mixture was partitioned with ethyl acetate (200µl x 3). The combined ethyl acetate extracts was dried under a stream of nitrogen, and derivatized with 30µL of a 1:1 mixture N,O-bis[trimethylsilyl]trifluoroacetamide and dimethylformamide (Pierce Biotechnology, Inc., Rockford, IL, USA), heated at 70°C for 40 min, and used for analysis of pinostilbene and resveratrol.

Analysis of pinostilbene and resveratrol in the HPC tissues by GC-MS (JEOL GCMate II Instrument; JEOL USA Inc., Peabody, MA, USA) was performed using a J&W DB-5 capillary column (0.25 mm internal diameter, 0.25µm film thickness, and 30 m length; Agilent Technologies, Foster City, CA, USA). The GC temperature program was: initial 190°C, increased to 242°C at 30°C/min rate, increased to 248°C at the rate of 0.4°C/min, then finally increased to 300°C at the rate of 30°C/min and held at this temperature for 0.5 min. The carrier gas was ultrahigh purity helium (1 mL/min flow rate). The injection port, GC-MS interface, and ionization chamber were kept at 250, 230, and 230°C, respectively. The volume of injection was 2µL (splitless injection). The mass spectrum was acquired in the positive, selected ion-monitoring mode; electron impact 70 eV. The retention time for pinostilbene was 10.4 min (monitored with m/z 388, 373, and 356). The retention time for resveratrol was 11.2 min (monitored with m/z 446, 431, and 373). GC-MS analyses were done in duplicates. Quantitation of pinostilbene and resveratrol was done using external standards. These analyses were done in the laboratory of our collaborator, Dr. Agnes Rimando.

7.8 Hindbrain analysis

7.8.1 Tissue processing
Brains were stored at 4°C in cryoprotectant (30% sucrose in 1XPBS) until processing. Approximately, six separate series of 30μm coronal brain sections were obtained from each hindbrain with a sliding microtome (Microm HM 450, Thermo Scientific, Asheville, NC). After the sections were collected, the slices were analyzed for TH⁺ using a protocol established and verified to be as accurate as stereology by Tapias and colleagues (2012). The immunostaining and analysis of these hindbrain sections were done in collaboration with Dr. Victor Tapias in Dr. Timothy Greenemyre’s laboratory in the Pittsburgh Institute for Neurodegenerative Disease at the University of Pittsburgh, Pittsburgh, PA. Selected sections (3–4 sections per well of a 24-well plate) were washed 3 times in 1XPBS, pH 7.6, for 10 min and incubated with 1% Triton X-100 in PBS solution for 5 hr at 4 °C. Next, sections were washed 3 times for 10 min in 1XPBS and blocked with 10% normal donkey serum and a permeabilizing reagent (0.3% Triton X-100) in 1XPBS solution for 30 min at room temperature. Subsequently, sections were incubated in 1XPBS/ 0.3% Triton X-100 (to facilitate antibody access to the epitope) for 72 hr at 4°C with the following primary antibodies directed against the protein of interest: 1) mouse monoclonal antibody for MAP2 (1:2000; #MAB378, Millipore), a cytoskeletal protein that binds to tubulin and stabilizes microtubules and is essential for the development and maintenance of neuronal morphology, was used for neuron staining and 2) sheep polyclonal antibody for TH (1:2000; #AB1542, Millipore), the rate-limiting enzyme in DA synthesis, was used to visualize DA neurons. After an additional incubation in primary antibody solution for 1 hr at RT, the sections were washed 3 times for 10 min each in 1XPBS to remove unreacted primary antibodies and were then incubated with secondary antibodies: Cy3-conjugated anti-sheep antibody (1:500; #713-
165-003, Jackson-ImmunoResearch) and Alexa Fluor-conjugated 647 anti-mouse antibody (1:500; #A31571, Invitrogen) for 2 hr at RT. Tissue sections were then washed twice in 1XPBS for 10 min and H 33342 (1:3000; #B2261, Sigma-Aldrich) reagent was used as a nuclear counterstain for 5 min at RT. Finally, after the sections were washed 3 times with 1XPBS for 10 min each, the sections were mounted onto plus-coated slides and cover-slipped using Gelvatol mounting media.

7.8.2 Motorized stage imaging analysis

The microscope used for these studies was an automated Nikon 90i upright fluorescence microscope equipped with 5 fluorescent channels (blue, green, red, far red and near IR), and high N.A. plan fluor/apochromat objectives which is housed in the Center for Biologic Imaging facilities at the University of Pittsburgh. The studies described here were all performed using 40X objective (0.75 N.A.). Images were collected using Nikon NIS-Elements software and a Q-imaging Retiga cooled CCD camera. The stage was scanned using a Renishaw linear encoded microscope stage (Prior Electronics). Neuronal counting was performed by a single trained investigator (Dr Victor Tapias, PhD). All slides were scanned under the same conditions for magnification, exposure time, lamp intensity and camera gain. Quantitative analysis was performed on fluorescent images generated in 3 fluorescent colors (stained for MAP2, TH⁺, and H 33342). Images were stitched with NIS-Elements, following background subtraction and thresholding for each individual channel. Once stitched together, colocalization and subsequent exclusion was performed on the images.
Figure 8. Representative images from immunohistochemistry of aged substantia nigra.
7.9 Statistical Analysis

All statistical tests were performed using Graph Pad Prism 5 (Graphpad Prism, Inc., La Jolla, CA). The data were analyzed using a two-way ANOVA (p < 0.05) followed by post hoc comparison using an uncorrected Fisher’s LSD test.

8. RESULTS

8.1 Weight and food intake over course of the study

8.1.1 Middle-aged animals fed the control diet gained weight and weighed more than young and old animals over the eight week study

The animals were weighed and the amount of food consumed was measured daily to determine the effect of different diets on body weight and food intake. Young and old mice on the control diets maintained their approximate starting weight and food intake throughout the entire study (Fig. 9A). However, middle-aged mice on the control diet alone gained a significant amount of weight starting at day 29 through the end of the study (Fig. 9A). Middle-aged mice also ate significantly more at days 15, 22, 43, 50, and 57 (Fig. 9C).

Weight and food intake was also analyzed between age groups. Young mice weighed significantly less than middle-aged or old mice throughout the duration of the study (Fig. 9A). Moreover, middle-aged mice weighed significantly more than aged mice over the eight-week period.
Figure 9. Age-related changes in weight and food intake.
Results are presented as a mean ± SEM. n = 5. (A) Body weight (in grams) was measured daily. Middle aged mice gained a significant amount of weight over an eight week period (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs middle-aged day 0). (B) Food intake was monitored daily by administration of 6g/day and weight of food (in grams). (C)Middle-aged mice ate significantly more throughout the eight week study as compared to the beginning (*p<0.05, **p<0.01, ***p<0.001 vs middle-aged day 0). Data were analyzed using a one-way ANOVA followed by a post hoc comparison using a Dunnett’s test.
8.1.2 Young animals fed the pinostilbene diet gained weight, while the middle-aged and old animals weighed the most, over the eight-week study

In order to determine if dietary supplementation with resveratrol, pinostilbene, or wild blueberries increased weight over the course of the study, food intake and body weight was measured periodically. Interestingly, young animals on the pinostilbene, but not resveratrol, diet gained a significant amount of weight, as compared to their starting weight, starting at day 50 through the end of the study (Fig. 10A). In contrast, middle-aged and old animals in the three diet groups (control, resveratrol, and pinostilbene) did not gain or lose a significant amount of weight as compared to their starting weights (Fig. 10B and C).

The difference in weight gain between age groups was also investigated. On the resveratrol diet, middle-aged mice weighed significantly more than young mice on days 8, 15, and 36 through the end of the study (Fig. 11A). Old animals weighed significantly more than young and middle-aged animals on resveratrol-supplemented diet, but only towards the end of the eight-week study (days 36-57; Fig. 11A). Middle-aged and old mice on pinostilbene diet also weighed significantly more than young mice (days 8, 15, and 37-57 and days 36-57, respectively; Fig. 11B).
Figure 10. Weight changes between diet groups with age.
Results are presented as a mean ± SEM; n= 5. (A) Body weight (in grams) was measured daily. (A) Young mice on the pinostilbene diet gained a significant amount over weight over the course of the study as compared to the starting weight (*p<0.05). (B) Middle aged mice did not display a significant change in weight. (C) Old mice did not gain a significant amount of weight throughout the course of the study. Data were analyzed using a one-way ANOVA followed by a post hoc comparison using a Dunnett’s test.
Figure 11. Dietary supplementation with resveratrol or pinostilbene does not change food intake between age groups.
Results are presented as a mean ± SEM; n= 5. Food consumption (in grams) was measured daily. There was no change in food consumption by either the resveratrol (A) or pinostilbene (B) supplemented mice. Data were analyzed using a one-way ANOVA followed by a *post hoc* comparison using a Dunnett’s test.
8.3 Aged mice make more errors than young and middle-aged animals on the challenge beam task

In the current study, age-related motor deficits were measured using two behavioral tests. The first test, the challenge beam task was used to measure motor coordination by examining three parameters: 1) the number of errors made, 2) steps taken while crossing the beam, and 3) the time it takes to transverse the length of the beam. These parameters measure motor coordination (errors) and activity (steps taken and time to cross). Aged (22 mo) C57Bl/6 male mice made significantly more errors on the challenge beam than young mice (2 mo; Fig. 12A). Additionally, while it was not statistically significant, old mice made more errors than middle-aged mice (10 mo; p value = 0.053, Fig. 12A).

In regards to number of steps taken to cross the beam, middle-aged mice take significantly fewer steps than young mice (Fig. 12B). When analyzing the data as errors/step rather than just the number of errors, old mice still made significantly more errors than young mice, however the trend towards significance was lost when comparing middle and old-aged mice (p=0.17; Fig. 12C).

Another parameter that might affect the errors made on the beam, is the time taken to cross the beam. Although the hypothesis was that old animals would take longer to transverse the beam, the time to cross the beam was not significantly different between the age groups (Fig. 12D). Taken together, these data suggest that the number of steps or the time they take to cross the beam does not affect the increase in the number of errors made by the old animals.
The challenge beam consists of four segments (25 cm each, 1 m total length) that get progressively narrower (3.5 cm to 0.5 cm by 1 cm increments) as the mouse moves towards its home cage. Therefore, we examined the number of errors the mice made on each segment of the beam to determine if the age-related changes in motor coordination are dependent on the width of the beam. Middle-aged and old mice made significantly more errors than young mice on the narrowest part of the beam (Fig. 13). Young, middle-aged, and old mice made significantly more errors on the fourth, narrowest, segment of the beam than the first segment (Fig. 13). Old mice make significantly more errors on the fourth segment of the beam than either the second or third segments of the challenge beam.
Figure 12. Age-related changes in motor coordination as measured by the challenge beam in C57Bl/6 male mice.
Results are presented as mean ± SEM. n = 37-40. C57Bl/6 male mice display age-related motor deficits as measured by the challenge beam task. (A) Old mice made significantly more errors than young mice (**p < 0.05) and more errors than middle-aged mice (p=0.053). (B) Middle-aged mice took significantly less steps while traversing the challenge beam than young mice. (C) Old mice make significantly more errors on the challenge beam than young mice (***p<0.0001). (D) Time to traverse the beam was not different between the different age groups. Data was analyzed using a two-way ANOVA followed by a post hoc analysis with an uncorrected Fisher’s LSD test.
Figure 13. Age-related increases in the number of errors of aged C57Bl/6 male mice on the challenge beam task.
Results are presented as mean ± SEM. n = 37-40. C57Bl/6 male mice display age-related motor deficits as measured by the challenge beam task. The beam becomes progressively narrower as the mice traverse to their home cage. Middle-aged and old mice make significantly more errors on the narrowest segment of the beam than young mice (**p<0.01 and ****p < 0.0001). Old mice make significantly more errors than middle-aged mice on the fourth segment of the beam (****p<0.0001). Data was analyzed using a two-way ANOVA followed by a post hoc analysis with an uncorrected Fisher’s LSD test.
8.4 Dietary supplementation with resveratrol or WBB alleviate age-related motor deficits

In order to determine if four weeks of dietary supplementation would improve motor coordination on the challenge beam task, mice were retested on the challenge beam. Similar to the results obtained before the start of the diets, old mice on control diet made significantly more errors than young or middle-aged mice at the four week time point (Fig. 14). While dietary supplementation with wild blueberry powder did not alter the number of errors made by young or middle-aged animals, this diet significantly decreased the number of errors made by the old mice as compared to old mice fed control diet (Fig. 14A). Moreover, old mice fed resveratrol and wild blueberry diets, no longer made significantly more errors than the young or middle-aged animals in the same groups. In contrast to resveratrol, pinostilbene supplementation did not alter the number of errors made by any age group (Fig. 14A). After eight weeks of dietary supplementation, old mice still made significantly more errors than young or middle-aged mice on the control diet (Fig. 15A). However, middle-aged and old mice on a pinostilbene supplemented diet made significantly more errors than young mice (Fig. 15A).

Similar to the comparison of the control only groups, the narrowest segment (segment 4) of the beam was analyzed after four weeks to determine if dietary supplementation would improve fine motor control. Aged mice made more errors than young or middle-aged mice (14B). Interestingly, old mice on the wild blueberry supplemented diet made significantly less errors than their old counterparts on control
diet (14B). Resveratrol and pinostilbene supplemented diets did not improve motor coordination on the narrowest segment of the beam. After eight weeks, old animals still make more errors on the narrowest, most difficult, segment of the beam as compared to young animals on the control diet (15B). However, old animals on the resveratrol-supplemented diet now make significantly more errors than young mice on the resveratrol diet (15B). While at four weeks aged mice on the resveratrol diet make an average of 3.6 errors/segment 4 by eight weeks they are making 4.15 errors/segment 4. Also at eight weeks wild blueberry supplementation no longer improves motor coordination on the narrowest segment of the beam.
Figure 14. Wild blueberry dietary supplementation attenuates age-related motor deficits after four weeks.
Results are presented as mean ± SEM. n = 5-10. Mice were retested on the challenge beam task after 4 weeks. (A) Aged mice made significantly more errors than young (***p<0.001) and middle-aged (^p<0.05) mice. Old mice on wild blueberry diet made significantly less errors than old control mice (**p<0.01). (B) Wild blueberry supplementation improves motor coordination in aged mice on the fourth and most difficult segment of the beam (p<0.05 vs old control). Data was analyzed using a two-way ANOVA followed by a post hoc analysis with an uncorrected Fisher’s LSD test.
Figure 15. Old mice continue to make more errors on the challenge beam task after eight weeks on natural supplemented diets.

Results are presented as mean ± SEM. n = 5-10. Mice were retested on the challenge beam task after 8 weeks. (A) After 8 weeks, there is no difference in errors between middle-aged and old mice. However, old mice made more errors than young mice (*p<0.05). (B) Old mice make significantly more errors on the most narrow segment of the beam (*p<0.05 vs young controls; ^p<0.05 vs middle aged pinostilbene). Data was analyzed using a two-way ANOVA followed by a post hoc analysis with an uncorrected Fisher’s LSD test.
8.5 Age related changes with spontaneous activity

To determine whether age had an effect on spontaneous activity, each animal was measured at the end of the eight week study by placing each mouse in a Plexiglas cylinder and videotaping their movements for three minutes. The videotapes were then analyzed for rearing behavior, forelimb and hind-limb steps, and time the mice spent grooming by an investigator blind to the age and treatment group. Age did not alter the average number of rears (16A), the average number of forelimb steps (16B), or the amount of time that each mouse spent grooming (16D). However, old mice made significantly less hind-limb steps than young mice (16C).
Figure 16. Hindlimb stepping is decreased in aged C57Bl/6 male mice.
Spontaneous activity was measured by counting rears, forelimb and hind-limb steps, and grooming over three minutes. Age did not alter the amount of rears (A) or forelimb steps (B) or time spent grooming (D) made by the mice. However, old mice made significantly less hind-limb steps than young animals (C, *p<0.05vs young). Data was analyzed using a one-way ANOVA followed by post hoc analysis using an uncorrected Fisher’s LSD test.
8.6 Pinostilbene diet increases rearing activity in middle-aged and old mice

In order to determine in dietary supplementation with natural products would alter exploratory or spontaneous activity, each animal was measured at the end of the eight-week study by placing each mouse in a Plexiglas cylinder and videotaping their movements for three minutes. The videotapes were then analyzed for rearing behavior, forelimb and hind-limb steps, and time the mice spent grooming by an investigator blind to the age and treatment group. Pinostilbene supplementation significantly increased the number of rears made by middle-aged and old animals (Fig. 17A). Moreover, old animals treated with pinostilbene made more rearing movements than old control animals (Fig. 17A). Neither resveratrol nor wild blueberry powder supplementation significantly increased the number of rears made by young, middle, or old aged animals. Forelimb and hind-limb steps and time spent grooming were not changed with age or dietary supplementation of resveratrol, pinostilbene, or wild blueberry powder (Fig. 17).
Figure 17. Pinostilbene diet increases rearing activity in middle-aged and old mice.

Results are presented as mean ± SEM, n = 5-10. Spontaneous activity was measured by counting rears, forelimb and hind-limb steps, and grooming. Age did not alter the amount of rears (A) or forelimb steps (B) or time spent grooming (D) made by the mice. (A) Pinostilbene diet increased rearing significantly from young pinostilbene (*p<0.05) and old control (^p<0.05). Neither age nor dietary supplementation altered forelimb steps (B) or time spent grooming (D). However, pinostilbene diet decreased hind-limb steps in young mice (**p<0.05). Data were analyzed using a two-way ANOVA with a post hoc analysis with an uncorrected Fisher’s LSD test.
8.7 Resveratrol and pinostilbene are present in the hippocampus after eight weeks of dietary supplementation

To determine if pinostilbene or resveratrol penetrated the blood brain barrier the hippocampi of each group of mice were analyzed by GC-MS after the eight weeks of dietary supplementation. Resveratrol was detected in trace amounts in both middle-aged and old animals; however it was not detected in young animals (Table 2). Pinostilbene was present in the hippocampus of all the age groups (Table 2). The amount of pinostilbene found in middle and old-aged animals was double the amount found in young animals.
Table 2. Pinostilbene and resveratrol penetrate the blood-brain barrier in aged animals.
Results are presented as ng/mg fresh tissue, n = 2. Hippocampi were analyzed by GC-MS for the presence of pinostilbene and resveratrol.
NA = not applicable; pinostilbene or resveratrol was not analyzed
nd = not detected
trace = peaks are below limit of quantitation.

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Pinostilbene (ng/mg tissue)</th>
<th>Resveratrol (ng/mg tissue)</th>
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</thead>
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<tr>
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<td>0.0365</td>
<td>NA</td>
</tr>
<tr>
<td>Middle</td>
<td>0.0772</td>
<td>NA</td>
</tr>
<tr>
<td>Old</td>
<td>0.0776</td>
<td>NA</td>
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<tr>
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<tr>
<td>Old</td>
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</table>
8.8 DA and DA metabolite levels and TH and DAT expression in the striatum are not altered with age

In order to determine if the amount of striatal dopamine system was altered with age, HPLC was used to measure DA and metabolite (DOPAC and HVA) content in the striatum of young, middle-aged, and old animals. There was not an age-related change in DA or metabolite content in the striatum (Fig. 18). Tyrosine hydroxylase and DAT in the striatum were analyzed by Western blot. Similar to DA and DA metabolite levels, TH and DAT expressions were unchanged with age (Fig. 18A and B).
Figure 18. DA, DOPAC and HVA levels are unchanged with age.

Results are presented as mean ± SEM, n=7. Striatal DA and metabolites were unchanged with age in C57Bl/6 male mice. Data were analyzed using a two-way ANOVA with a *post hoc* analysis with an uncorrected Fisher’s LSD test.
8.9 DA and DA metabolite levels and TH and DAT expression in the striatum are not altered following 8 weeks of dietary supplementation with pinostilbene or wild blueberry powder; however, resveratrol decreases striatal DA

To determine if dietary supplementation with resveratrol, pinostilbene, or wild blueberries altered the striatal dopamine neuronal system, HPLC was used to measure DA and DA metabolite (DOPAC and HVA) content in the striatum of young, middle-aged, and old animals after eight weeks. DA content was decreased in the striatum of old mice fed the resveratrol-supplemented diet (Fig. 19C). In contrast, there was not a change in DA or DA metabolite content in the striatum in any age group fed a pinostilbene or wild blueberry powder supplemented diet (Fig. 19). Striatal TH and DAT were analyzed by Western blot. Dietary supplementation with resveratrol, pinostilbene, or wild blueberry powder did not alter TH or DAT expression in the striatum (Fig. 20).
Figure 19. Resveratrol decreases DA in the striatum in aged mice.
Results are presented as mean ± SEM, n=7. In aged mice, resveratrol significantly decreased striatal DA levels. However DOPAC and HVA were unchanged. Data were analyzed using a two-way ANOVA with a post hoc analysis with an uncorrected Fisher’s LSD test.
Figure 20. Age and dietary supplementation do not alter striatal TH or DAT.
Results are presented as mean ± SEM. (A) Tyrosine hydroxylase (TH), the rate limiting enzyme in DA synthesis, was unchanged with age or with dietary supplementation with resveratrol, pinostilbene or wild blueberries (B) The dopamine transporter, DAT, was unchanged with age. Dietary supplementation with resveratrol, pinostilbene or wild blueberries did not alter striatal DAT. Data were analyzed using an unpaired t-test with a 95% confidence interval.
8.10 The number of TH$^+$ neurons in the substantia nigra is not altered with age

To assess whether or not age decreased the number of DA neurons in the substantia nigra, immunohistochemistry of the hindbrain was performed to measure the number of TH$^+$ neurons in the striatum. Hindbrain slices were probed with TH and MAP-2 antibodies and nuclei were stained with Hoechst dye to quantify the number of DA neurons, the number of neurons in general, and the total number of cells, respectively. Our data suggests there was no change in the number of TH$^+$ neurons in the substantia nigra with age (Fig. 21). In future studies, the total number of neurons and cells will be quantified to determine if there are age-related changes in these parameters.

8.11 Dietary supplementation with pinostilbene or wild blueberries increases the number of TH$^+$ neurons visualized in the substantia nigra

To assess whether or not dietary supplementation with resveratrol, pinostilbene, or wild blueberries altered the number of DA neurons in the substantia nigra, immunohistochemistry was performed on the hindbrain to measure TH$^+$ neurons. Treatment with pinostilbene or wild blueberries, but not resveratrol, increased the number of TH$^+$ neurons visualized in the substantia nigra in old animals (Fig. 22).
Figure 21. Age does not alter the amount of TH+ neurons in the substantia nigra.
Results are presented as mean ± SEM, n=5. The number of TH+ neurons were measured by fluorescent microscopy. Age did not alter the amount of TH+ neurons in the SN. Data were analyzed using a two-way ANOVA with a post hoc analysis with Fisher’s LSD test.
Figure 22. Wild blueberry and pinostilbene supplementation increase the amount of TH+ neurons in the substantia nigra.
Results are presented as mean ± SEM, n=5. Dietary supplementation with resveratrol, pinostilbene, or wild blueberries in young (A) or middle-aged (B) mice. (C) Pinostilbene and wild blueberries significantly increased TH+ neurons in the SN of aged mice (*p<0.05). Data were analyzed using a two-way ANOVA with a post hoc analysis with an uncorrected Fisher’s LSD test.
9. DISCUSSION

A number of deficits, both behavioral and neurological, have been shown to increase with age. In this study, we used a mouse model of aging to investigate age-related alterations in the nigrostriatal DA system and motor coordination. Additionally, we examined the effect of natural supplements on motor function and, subsequently, the DA neuronal system.

While relatively little research has been done in animal models to examine changes occurring due to normal aging, the literature that does exist consistently demonstrates a decline in spontaneous motor activity and motor coordination in rodents (Cantuti-Castelvetri et al., 2003; Boger et al., 2006; Colebrook et al., 2006). Currently, most aging studies do not include a middle age group. It is the recommendation of the NIA that middle-aged animals be included in aging animals studies as any changes that occur may begin at mid-life and interventions may be more effective if started during this time period (JEC, personal communication). Therefore, the current study sought to assess age-related differences in young, middle-aged, and old C57BL/6 male mice using the challenge beam and cylinder behavioral tests to measure motor coordination and spontaneous activity, respectively.

Since the weight of the animals may affect their ability to cross the challenge beam, such that a significant weight gain could cause a decrease in mobility, body weights and food intake were recorded over the eight-week testing period. Metabolism is known to slow with age, in part, due to an age-related increase in fat mass and a concomitant decrease in lean muscle (Nassis and Geladas, 2003; Hughes et al., 2004;
Newman et al., 2005). Therefore, the current hypothesis was that the old animals would gain weight and weigh significantly more that the young and middle-aged animals during the course of our study. Surprisingly, the middle-aged, but not the old, animals gained a significant amount of weight throughout the study (Fig. 9A). The increase in body weight in middle-aged animals may be accounted for by the increased food consumption (Fig. 9C). Also, middle-aged mice weighed more than old mice throughout the eight week study. Because the challenge beam test is a beam that gets progressively narrower; weight may affect the amount of errors made by the animals. A different task, such as gait analysis or wire grasping may be more appropriate if weight is an issue. However, the amount of errors per weight was examined and even after taking weight into consideration, aged mice made significantly more errors on the challenge beam task, as compared to young and middle-aged mice in our study (data not shown). Weight may also alter the measures of spontaneous activity, such as time and steps taken to cross the beam, because larger animals may be more sedentary, and thereby slower, than smaller animals. However, as there was not a difference in spontaneous activity as noted by the number of steps or time taken to cross the beam, it appears that weight was not a confounding factor in our studies.

Also the number of steps that mice made while crossing the beam was examined. Steps are an important parameter because the number of steps taken by each mouse may contribute to the number of errors made by the animals on the challenge beam. In this study, young and old animals took the same number of steps while middle-aged mice took fewer steps than aged mice. Another parameter measured using the challenge beam task is the amount of time it takes the mouse to traverse the beam. Surprisingly, the mean
time that it took each group to cross the beam was not significantly different between the three age groups. Therefore, as the data did not note a decrease in the number of errors that correlated with the steps or time data, this concludes that the challenge beam test data reflects age-related motor decline unaffected by these other parameters.

The challenge beam gets progressively narrower as the mouse moves towards the home cage, getting more challenging for the mouse. Therefore, the errors per segment of beam were analyzed to show where the mice made the most errors while traversing the beam. Middle and old aged mice made significantly more errors than young mice on the fourth beam segment, the narrowest portion of the beam. This may indicate the middle-aged and old mice lose more fine motor control and/or balance, while young mice maintain those motor functions.

To analyze whether the challenge beam could be used for a longer period of time, we retested the mice after four and eight weeks on the supplemented diets in our animal facility. Aged mice made significantly more errors than young and middle-aged mice at four weeks. However, by eight weeks the difference between middle-aged and old mice was no longer significant. This is due to the fact that middle-aged mice made more errors at week eight than at week four ($\bar{X}$=5.6 and $\bar{X}$=3.8, respectively), while aged mice make approximately the same number of errors at weeks four and eight ($\bar{X}$=6.9 and $\bar{X}$=7.0, respectively). This increase in number of errors in the middle-aged group suggests that a decline in motor coordination may begin to occur at 12 months. As mentioned earlier, until recently, most studies using aged animals did not include a middle-aged group. Our data underscore the importance of including the middle-aged group as behavioral changes
Similar to the data at the beginning of the study and at four weeks, aged mice fed control diet still make more errors than young mice at eight weeks (p<0.05, Fig. 15A). These data suggest that the challenge beam test is an appropriate behavioral task for the measurement of age-related loss of motor coordination up to at least eight weeks following the first test. Therefore, this behavioral test was used to study the effect of 8 weeks of dietary supplementation with resveratrol, pinostilbene, and wild blueberry powder on motor coordination using 4 and 8 weeks as time points for testing.

To examine the effect of diet on body weight and food intake, animal weights and the amount of food intake was measured daily in the resveratrol, pinostilbene and WBB supplemented groups as we did for the control groups. A review of the literature shows that resveratrol supplemented diets suppresses body fat accumulation via fat oxidation, carbohydrate oxidation, or protein oxidation in rodents (Kim et al., 2011; Gomex-Zorita, 2012; Nagao et al., 2013). Therefore, the hypothesis was that animals fed the resveratrol diet might lose, rather than gain, weight. In support of this hypothesis, middle-aged and old animals did not gain a significant amount of weight on resveratrol or pinostilbene diet, as opposed to the same age groups on the control diet (10B). Surprisingly, young mice on the pinostilbene, but not resveratrol, diet gained a significant amount of weight by the end of the study as compared to their starting weight (10A). With an increase in weight in the young pinostilbene supplemented group, the hypothesis was that this group also increased food consumption; however, food intake between the different diets did not change. No other groups have measured dietary intake changes with resveratrol or
pinostilbene and nothing has been reported about the effect of pinostilbene on metabolic rate. Therefore, although this data suggests that pinostilbene decreased basal metabolism in the young animals, the current data cannot make this conclusion without further studies.

After four weeks of dietary supplementation with resveratrol, old animals made fewer errors than old mice on control diet (14A). Moreover, there was no longer a significant difference in the number of errors between young, middle-aged, and old mice fed the resveratrol diet, indicating that dietary resveratrol may attenuate age-related motor deficits. In contrast to resveratrol, pinostilbene had no effect on motor function. However, similar to results with resveratrol, wild blueberry powder supplementation significantly decreased the amount of errors made by old animals, when compared to the old control group and there was no longer significance between age groups (14B). Concurrently, after four weeks on a wild blueberry supplemented diet, aged animals make significantly less errors as compared to their aged counterparts (14B). These data suggest that resveratrol and wild blueberry diets decrease age-related motor deficits, in as little as four weeks. In accordance with this data, Galli and colleagues (2002) showed that 2% wild blueberry powder dietary supplementation increased motor performance on the rod walk task and accelerating rotorod and improved cognitive memory as measured by the Morris water maze in Fisher 344 rats. Pearson and colleagues (2008) also showed that resveratrol-supplemented diet improved performance on the accelerating rotorod in C57Bl/6 mice. This is the first study to examine the effect of pinostilbene on motor coordination and to compare the effect of isolated compounds with a whole fruit on age-related motor decline.
At the eight-week time point, old mice continue to make significantly more errors than young mice on the challenge beam task (15A). However, the difference in errors made by aged mice on the control diet and aged mice on the wild blueberry supplemented diet is no longer significantly different (15A). Aged mice make approximately the same average number of errors between weeks four and eight ($\bar{x}=6.8$ and $\bar{x}=7.0$, respectively). However, aged mice on the wild blueberry supplemented diet have an increase in the average amount of errors from weeks four to eight ($\bar{x}=3.1$ and $\bar{x}=5.5$, respectively). The rise in the average number of errors in the aged wild blueberry supplemented mice may account for the loss of protection from age-related motor deficits.

Spontaneous activity, as measured by the cylinder test, was also examined in this study as a measure of exploratory behavior. This test can be used to analyze four parameters for exploration as described above. There was not a significant difference in the average amount of rears, forelimb steps, or time spent grooming by the three different ages fed control diet (16A, C, and D). Aged mice did take significantly less hind-limb steps as compared to the young mice (16C). Interestingly, pinostilbene supplementation increased the amount of rears made by middle-aged and old mice, as compared to young mice on the pinostilbene diet (17A). Young mice on the pinostilbene supplemented diet made significantly less hind-limb steps than their young control counterparts (17C). As weight gain is often accompanied with a sedentary lifestyle, this result may be due to the fact that young mice gained a significant amount of weight towards the end of the study. Alternatively, this result may be due to the fact that these animals spent more time on their hind legs rearing on the side of the cylinder rather than exploring on the floor of the
cylinder. None of the other parameters showed a significant difference either in the control or treatment groups.

Motor coordination is largely controlled by the dopamine system (Pijnenburg and Van Rossum, 1973; Jackson et al., 1975; Costall and Naylor, 1975; Costall et al, 1976). Loss of dopamine neurons in the substantia nigra and striatum are seen with age and in Parkinson’s disease with a concurrent loss of motor coordination (McGeer et al., 1977; Fearnley and Lees, 1991). Interestingly, resveratrol and wild blueberries have been shown to protect neurons in vivo and in vitro through a variety of antioxidant signaling mechanisms. Therefore, the antioxidant properties of these compounds may decrease oxidative stress and thereby protect DA neuronal function in the striatum or substantia nigra to improve motor coordination. For these reasons, after the conclusion of the behavioral portion of this study, the DA neuronal system was analyzed to determine whether age-related motor deficits were associated with a loss of DA or DA neurons.

Before examining the effect of the natural products on the DA system, there was a need to determine if the natural compounds were able to penetrate the blood brain barrier. To this end, the hippocampus from each age group was analyzed for the presence of pinostilbene or resveratrol. Resveratrol was found in trace amounts in the hippocampi of middle-aged and old animals; however it was not detected in the hippocampus of young animals (Table 2). Pinostilbene was found at detectable levels in young, middle-aged, and old animals. Twice the amount of pinostilbene was detected in the hippocampi of middle-aged and old as compared to the young animals (Table 2). As seen with the resveratrol animals, young mice showed less penetration of each of the compounds. The lack of
resveratrol and the lower levels of pinostilbene in the hippocampi of the young animals may be due to the fact that resveratrol and pinostilbene were rapidly metabolized in the periphery in the young animals. Also, while this lack of or decreased absorption across the blood brain barrier in the young animals may be due to rapid metabolism of the compounds, it may also be due to differences in the integrity of the blood brain barrier in the different age groups. Farrall and Wardlaw (2009) have shown that there is a breakdown of the BBB integrity with age. Therefore, the breakdown of this barrier, while detrimental for toxic insults to the brain, may have led to an increased absorption of these two compounds in middle-aged and old animals.

As previously mentioned changes in the DA system with age are controversial and strain specific. It has previously been reported that DA and DA metabolite levels, as well as TH and DAT expression were unchanged with age in the striatum (Allen et al., 2011). Similarly, in a separate group of animals used in this study, there were no changes in DA or DA metabolites (DOPAC and HVA) with age in the striatum (Fig. 18). TH and DAT expression were also unchanged with age. Surprisingly, resveratrol treatment decreased the amount of DA in the striatum of aged animals (Fig. 19). While resveratrol has antioxidant properties, it has also been shown to have pro-oxidant activities (Ahmad et al., 2003; de la Lastra and Villegas, 2007). DA is quickly oxidized once it is released into the synaptic cleft; therefore, an increase in a pro-oxidant compound, such as resveratrol, may further decrease DA content in the striatum.

As with the previous study, there was no change in TH or DAT expression in the striatum of animals in any of the treatment groups (Fig. 20). In contrast, in humans, it has
been reported that there is a loss of DA neurons with age (McGeer et al., 1977; Fearnley and Lees, 1991). Other groups have reported a loss of TH, indicating a loss of DA neurons and/or projections, with age in monkeys (Emborg et al., 1998). Moreover, a loss of DAT expression with age has been noted in monkeys (Yue et al., 2012) and humans (Emborg et al., 1998; Troiano et al., 2010). Therefore, as there were not alterations in TH or DAT these DA neuronal markers with age may be species specific.

Age-related decreases in the expression of the D1 (Henry et al., 1987; Wang et al., 1998) and D2 (Roth and Joseph, 1994; Volkow et al., 1998) receptors have also been noted in rodents and humans. Therefore, future studies will examine D1 and D2 receptor expression in our aging mouse model. Others have shown that in addition to changes in the striatal DA system, there are alterations at the level of the substantia nigra, including a loss of DA neurons, which may affect motor function (Fleming et al., 2006). Therefore, this study examined the number of DA neurons in the SN across ages and treatment groups using immunostaining to quantify the number of TH+ neurons. Analysis of hindbrain sections containing the SN indicated that there was not a decrease in the number of TH+ neurons with age (Fig. 21). In young and middle-aged animals, dietary supplementation with resveratrol, pinostilbene, or wild blueberry powder did not alter the number of TH+ neurons (Fig. 22A/B). However, in the aged group, dietary supplementation with pinostilbene or wild blueberry powder increased the number of TH+ neurons visualized (Fig. 22C). One explanation for this increase is that DA neurons become quiescent with age, losing expression of TH as a phenotypic marker, and wild blueberry powder or pinostilbene treatment returns the neurons to a more functional state restoring TH levels. Another possibility is that there is an increase in oxidative stress in
the SN in this aging mouse model and these antioxidant compounds restore homeostasis and allow these cells to function normally. The increase in TH+ neurons may be an indication of how wild blueberries alleviate age-related motor deficits. Similar results were seen in the SN of aged animals treated with pinostilbene; however pinostilbene did not change age-related motor deficits. Although resveratrol did slightly decrease age-related motor deficits, there was not an increase in TH+ neurons in the SN with resveratrol dietary supplementation; this may be due to the limited penetration of resveratrol in the brain. Future studies will explore these hypotheses.

In conclusion, aged C57Bl/6 male mice make significantly more errors on the challenge beam test as compared to young mice without a significant contribution of weight, food intake, average number of steps, or time to cross the beam. These data suggest that C57Bl/6 male mice and the challenge beam test are a good model and behavioral measurement of age-related motor deficits, respectively. Moreover, these data suggest that age-related motor deficits may not be due to a loss of DA or DA metabolite content in the striatum, but rather, a loss of functional DA neurons in the SN. Finally, dietary supplementation with resveratrol or wild blueberry powder alleviated the motor deficits observed on the challenge beam test in as few as four weeks and pinostilbene diet increased the spontaneous activity of the middle-aged and old animals. Interestingly, resveratrol diet had no effect on the DA parameters measured, while pinostilbene and wild blueberry supplemented diet increased the number of TH+ neurons visualized in the SN. Although the mechanism of protection against motor decline with age is not entirely clear and more studies need to be performed, the data thus far suggest that natural products may be effective therapies for motor deficits that are associated with aging.
Chapter 3

The effect of isolated natural products on dopamine-induced oxidative stress in SH-SY5Y cells

10. RATIONALE AND HYPOTHESIS

10.1 RATIONALE

Natural products have been shown to alleviate motor deficits associated with age and neurodegenerative diseases (Joseph et al., 1999; 2003) and in our own previous studies, we showed that dietary supplementation with resveratrol or wild blueberries decreased age-related motor deficits. Therefore, we sought to investigate the possible mechanisms by which these natural products may produce the beneficial effects seen in vivo.

As mentioned previously, oxidative stress is known to increase with age in the brain (Harman, 1992; Ames et al., 1993; Joseph et al., 1996). A concurrent decrease in antioxidant mechanisms causes an environment that may be toxic to neurons and lead to neurodegeneration. DA neurons are especially vulnerable to oxidative stress, possibly due to the increased exposure to ROS produced by the by-products of DA metabolism. Many cell signaling pathways are activated in response to oxidative stress; however, this research is specifically focused on the MAPK pathways, ERK1/2 and ERK5. ERK1/2 activation is thought to play a critical role in cell survival in response to oxidative stress in rat PC12 cells and primary cortical neurons (Guyton et al., 1996; Crossthwaite et al., 2002). Moreover, ERK1/2 activation protects against hypoxia-induced cell death in
primary cortical cultures (Wang et al., 2001) and H_{2}O_{2}-mediated cell death in primary striatal cell cultures (Crossthwaite et al., 2002).

Interestingly, wild blueberry treatment has been shown to activate ERK1/2 in primary hippocampal cells that were exposed to DA (Joseph et al., 2010). However, Vuong and colleagues (2010) found that inhibition of ERK1/2 protected cells from H_{2}O_{2}-induced cell death in N2A neuroblastoma cell culture. Resveratrol research is divided when it comes to ERK signaling. Some groups show resveratrol-mediated protection from oxidative stress is through activation of ERK1/2 (Maher et al., 2011; Simao et al., 2012) and others find that inhibition of ERK1/2 is protective (Arun et al., 2013).

In the current study, the dopaminergic-like SH-SH5Y cells were used as an in vitro model of DA neurons and DA treatment to produce oxidative stress to examine the effect of resveratrol, pinostilbene, and wild blueberry powder on DA-induced toxicity. Furthermore, the effect of these compounds on ERK activation and explored a role for the MAPK pathways in protection from oxidative stress were examined.

10.2 HYPOTHESIS

Natural products protect against dopamine-induced oxidative stress via activation of the ERK1/2 and/or ERK5 signaling pathways
11. MATERIALS AND METHODS

11.1 SH-SY5Y Cell Culture

SH-SY5Y (catalog # CRL- 2266; ATCC, Manassas, VA) cells were grown on 10cm cell culture plates (Sarstedt, Newton, NC) in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biological, Lawrenceville, GA), 0.1% Uridine (Sigma, St. Louis, MO), 0.1% Pyruvate (Sigma), and 1% penicillin/streptomycin (Gibco). Cells were maintained at 37°C with 5% CO₂. After cells reached 85-90% confluency in 10cm plates, they were incubated with 1.5mL TrypLE (catalog # 12604; Invitrogen; Grand Island, NY) and plated at a density of 1.5 x 10⁴ cells per well for 96 well plates and 6.0 x 10⁶ cells per 35mm plate.

11.2 Resveratrol and Analogues

The structures of resveratrol and analogs are shown in Figure 2. Resveratrol and the analogs were gifts from Dr. Agnes Rimando at the USDA facility in University, Mississippi. For further derivations, see Joseph et al., 2008.

11.2 Treatment of Cell Cultures

SH-SY5Y cells were plated in 96-well plates (Becton Dickinson, Franklin Lakes, NJ), 35mm plates (Becton Dickinson) and 60 mm culture plates (Sarstedt, Newton, NC) and allowed to grow for 48 hrs.

11.2.1 Resveratrol or pinostilbene treatment
Resveratrol was purchased from a commercial source (TCI America, Portland, OR). Pinostilbene was a gift from Dr. Agnes Rimando. A stock solution of each compound was dissolved in DMSO (Sigma Aldrich). For each treatment, a fresh dilution of resveratrol or pinostilbene was made in serum-free media. SH-SY5Y cells were treated with 1, 3 or 5µM resveratrol or pinostilbene. SH-SY5Y cells were treated with DMSO (2µL, the total volume of resveratrol or pinostilbene treatment) as a vehicle control.

11.2.2 Dopamine treatment

Dopamine (Sigma Aldrich, cat # H8502, lot # 1381581) was dissolved in sterile water to an initial concentration of 100mM. The dopamine solution was further diluted with sterile water to treat. Treatment with dopamine (50, 100 or 200µM) followed 30 min after resveratrol or pinostilbene treatment and lasted for 24 hrs. SH-SY5Y cells were also treated with H₂O alone as a vehicle control.

11.2.3 U0126 treatment

U0126 (Cell Signaling, cat # 9903, lot #11), the ERK1/2 specific inhibitor, was dissolved in DMSO. The initial concentration was 10mM. U0126 was further diluted to 1mM in DMSO for treatment of SH-SY5Y cells. Cells were treated with U0126 (10µM) 1 hr prior to resveratrol treatment (90 min prior to dopamine treatment). SH-SY5Y cells were also treated with DMSO (2µL; the total volume of U0126 treatment) as a vehicle control.

11.3 Cell Death Assays

11.3.1 Cell Titer Glo®
Following treatment in 96 well plates, SH-SY5Y cells were analyzed by Cell Titer Glo® (Promega, Madison, WI) for cell viability at 24 and 48 hours. The Cell Titer Glo® reagent uses luciferin, which gives off a luminescent signal after interacting with ATP, Mg$^{2+}$, and mono-oxygen (Promega Technical Bulletin, 2012). SH-SY5Y cells were removed from the 5% CO$_2$ incubator and equilibrated to room temperature for 30 min. From each well, 150µL of media was removed, leaving a final volume of 50µL. Then 50µL of Cell-Titer Glo reagent was added and the contents were vigorously mixed for 2 min on a shaker. The plates were incubated for 10 min at room temperature. Following incubation, 50µL of cells and Cell-Titer Glo® reagent was transferred to a black 96-well microplate. Luminescence was recorded using a microplate reader (Perkin Elmer, Waltham, MA).

### 11.3.2 Hoechst Staining

SH-SY5Y cells were plated into 96 well plates (Midi-Sci). Cells were pre-treated with 1 or 5µM resveratrol or pinostilbene. After 30 min pretreatment, 0, 50 or 100µM DA was added to each respective well for 24 hours. Media was removed from the wells and the cells were fixed with 500µL of 4% paraformaldehyde/4% sucrose in PBS for 15min. Fix was removed and cells were washed three times with 1XPBS/0.02%NaN$_3$. After the washes, 500µL of Hoechst stain (bis-benzimide, Sigma) was added to each well. Hoechst dye is cell membrane permeable and binds to double stranded DNA. After 20 min, the Hoechst stain was removed and the cells were stored in 1XPBS/0.02%NaN$_3$ at 4°C until they were visualized. Four pictures of each well was taken at 12, 3, 6, and
9’o’clock on an epifluorescent microscope (EVOS®, Advanced Microscopy Group, Bothell, WA). Each picture was then counted for live and dead cells.

### 11.4 Western Blot Analysis

SH-SY5Y cells were plated in 60mm plates and treated with resveratrol (RV1; 5µM) or pinostilbene (RV2; 5µM) over a time-course (15 min – 48 hours). The cells were washed with ice-cold 1XPBS and then lysed (1% Triton X-100 buffer containing 20mM Tris (pH 6.8), 137mM NaCl, 25mM beta glycerophosphate, 2mM NaPPi, 2mM EDTA, 1mM Na3VO4, 10% glycerol, 5µg/mL leupeptin, 5µg/mL aprotinin, 2mM benzamidine, 0.5mM DTT, and 1mM PMSF) at the times stated above. The lysates were then centrifuged at 10,000 rpm for 10 min at 4°C. Determination of protein content in the supernatant was done using a Bradford assay (Biorad, Hercules, CA).

Equal amounts of protein (30µg) from each treatment were separated on 8% SDS gels and transferred for 1h at 100 volts to nitrocellulose membrane (Li-Cor Biosciences, Lincoln, NE) for Western blot analysis. Membranes were blocked in casein blocking buffer (Li-Cor Biosciences) for 1 hr and then incubated overnight at 4°C in primary antibody: mouse anti-ERK1/2 (1:1000, catalog # 9107; Cell Signaling Technology), rabbit anti-phospho-ERK1/2 (1:1000, catalog # 9101; Cell Signaling Technology) rabbit anti-ERK5 (1:1000, Sigma), rabbit anti-phospho-ERK5 (1:1000, catalog # 3371; Cell Signaling Technology) or mouse anti-GAPDH (1:1000, Millipore). Blots were washed with 1XPBS/0.1% Tween for 20 min. Binding was visualized with infrared secondary antibodies fluorescing at 700 nm (goat anti-rabbit for phospho-ERK1/2, ERK5, and phospho-ERK5) or 800 nm goat anti-mouse for phospho-ERK1/2 and GAPDH; Odyssey
Imaging; LI-Cor Biosciences) applied for 1 hr at room temperature (1:10,000 in casein blocking solution; Li-Cor Biosciences). Blots were scanned on an Odyssey Imager and quantified with Odyssey software, using background subtraction above and below each band. Grayscale images were used for quantification, and the images were pseudo-colored red (700 nm) and green (800 nm) to delineate anti-rabbit and anti-mouse antibodies using distinct fluorescent wavelengths.

**11.5 Statistical Analysis**

All statistical tests were performed using Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA). Grubb’s test (GraphPad Software, Inc., La Jolla, CA) was used to determine if there were any significant outliers (p<0.05) in the data. The data were analyzed using a one-way or two-way ANOVA (p<0.05) followed by *post hoc* comparison using an uncorrected Fisher’s LSD test.
12. RESULTS

12.1 Resveratrol and resveratrol analogs protect against DA-induced oxidative stress

In order to determine if dopamine was an appropriate toxin SH-SY5Y cells were treated with increasing concentrations of DA (50, 100, or 200µM). DA treatment significantly decreased SH-SY5Y cell viability in a concentration dependent manner as measured by loss of ATP (data not shown). For this experiment, one concentration of DA (100µM) was chosen to induce a loss of cell viability. Further, SH-SY5Y cells were pretreated with 5µM of resveratrol (RV1), pinostilbene (RV2), desoxyrhapontigenin (RV3), pterostilbene (RV4), pterostilbene glucoside (RV5), resveratrol trimethylether (RV6), picetannol (RV7), or piceid (RV8) to determine if any of these compounds could prevent DA-induced cell death. Resveratrol (RV1), pinostilbene (RV2), desoxyrhapontigenin (RV3), and piceid (RV8) significantly inhibited DA-induced loss of SH-SY5Y cell viability (Fig. 23).
Figure 23. Resveratrol and analogs protect against DA-induced oxidative stress.

Results are presented as mean ± SEM, n = 3. Dopamine treatment (100µM; 24hr) significantly decreased cell viability in SH-SY5Y cells, measured by ATP luminescence. Pretreatment (30 min) with 5µM of resveratrol (RV1; *p<0.05 vs 100µM DA), pinostilbene (RV2; **p<0.01 vs 100µM DA), desoxyrhapontigenin (RV3; *p<0.05 vs 100µM DA), or piceid (RV8; **p<0.01 vs 100µM DA) increased cell viability after DA treatment. Data were analyzed using a two-way ANOVA with a post hoc analysis with Fisher’s LSD test.
12.2 Resveratrol and pinostilbene protect against DA-induced oxidative stress

In order to determine if resveratrol or pinostilbene would protect against DA-induced oxidative stress at lower concentrations, SH-SY5Y cells were pretreated with varying concentrations of resveratrol or pinostilbene (1, 3, or 5µM) followed by DA treatment (50, 100 or 200µM). DA treatment significantly decreased SH-SY5Y cell viability at 50µM (85% of control) and 100µM (57% of control; Fig. 24A, white bars) as measured by loss of ATP luminescence. Pretreatment with resveratrol (RV1; 1, 3, or 5µM) or pinostilbene (RV2; 1, 3, or 5µM) inhibited the DA-induced loss of SH-SY5Y cell viability (Figs. 24A and 25A).

In support of the cell viability data measured by ATP levels, treatment with DA significantly decreased the amount of live cells (24B and 25B). However, resveratrol pretreatment (1µM) preserved SH-SY5Y cell nuclear integrity, as measured by Hoechst staining, following 50 and 100µM DA exposure (Figs. 24B). However, pinostilbene pretreatment did not protect against the DA-induced loss of nuclear integrity (Fig. 25B).
Figure 24. Resveratrol protects against DA-induced oxidative stress.
Results are presented as mean ± SEM, n = 3. (A) DA significantly reduced ATP luminescence in SH-SY5Y cells (^p<0.05, ^^p<.01 vs H2O control). Pretreatment (30min) with resveratrol (RV1; 1-5µM) protected against loss of ATP luminescence at 100 and 200µM DA. (B) DA treatment significantly decreased the average number of intact nuclei as measured by Hoechst stain (^p<0.05 vs H2O control). Pretreatment with resveratrol (1µM) protected nuclear viability from DA-induced oxidative damage (100µM; *p<0.05). Data were analyzed using a two-way ANOVA with a post hoc analysis with Fisher’s LSD test.
Figure 25. Pinostilbene protects SH-SY5Y cells from DA-induced oxidative stress.

Results are presented as mean ± SEM, n = 3. (A) DA significantly reduced ATP luminescence in SH-SY5Y cells (^p<0.05, ^^p<0.01 vs H2O control). Pretreatment (30min) with pinostilbene (RV2; 1-5µM) protected against loss of ATP luminescence at 100µM DA (*p<0.05, **p<0.01, ***p<0.001 vs 100µM DA). (B) DA treatment significantly decreased the average number of intact nuclei as measured by Hoechst stain (^p<0.001 vs H2O control). Pretreatment with pinostilbene did not protect nuclear viability from DA-induced oxidative damage. Data were analyzed using a two-way ANOVA with a post hoc analysis with Fisher’s LSD test.
12.3 Resveratrol treatment increases ERK1/2 activation in SH-SY5Y cells

To assess whether resveratrol or pinostilbene treatment could activate the MAP kinases, ERK1/2 or ERK5, SH-SY5Y cells were treated with 5µM or resveratrol (RV1) or pinostilbene (RV2) over a time course (5min-48hr). Resveratrol treatment (5µM) transiently increased ERK1/2 and 5 activation in SH-SY5Y cells, such that activation was returned to basal levels by 24 hr (Fig. 26A, B, and C). In contrast to resveratrol treatment, pinostilbene treatment (5µM) increased activation of ERK1 and ERK2 by 15 min and was sustained through 48 hr (Fig. 27B). Due to the large variability in the phosphorylation of ERK5 following pinostilbene treatment, it was unclear whether there was any significant activation of ERK5 by pinostilbene in these cells (Fig. 27C).
Figure 26. Resveratrol treatment activates ERK1/2 in a time-dependent manner. Results are presented as mean ± SEM. n = 3 SH-SY5Y cells were treated with resveratrol (5µM) over a time course and assessed for ERK1/2 and ERK5 activation. Resveratrol treatment increased pERK1 (A) and pERK2 (B) in a time-dependent manner (*p<0.05 vs no treatment; NT). ERK5 (C) was not activated by resveratrol treatment. Data were analyzed using a one-way ANOVA with a post hoc analysis with Fisher’s LSD test.
Pinostilbene treatment activates ERK1 and ERK2 in a time-dependent manner.

Results are presented as mean ± SEM. n = 3 SH-SY5Y cells were treated with pinostilbene (5µM) over a time course and assessed for ERK1/2 and ERK5 activation. Pinostilbene treatment increased pERK1 (A) and pERK2 (B) in a time-dependent manner. ERK5 (C) was not activated by pinostilbene treatment. Data were analyzed using a one-way ANOVA with a post hoc analysis with Fisher’s LSD test.
12.4 U0126 treatment blocks ERK1/2 activation in SH-SH5Y cells

To assess the specificity of the ERK1/2 inhibitor, U0126, in SH-SY5Y cultures, cells were treated with 10, 30 or 50µM for 1hr. All doses of U0126 blocked basal ERK1/2 activation (Fig. 28). From these results 10µM U0126 was chosen for further studies. SH-SY5Y cells were then treated over a time course with 10µM U0126 and analyzed by Western blot. U0126 (10µM) effectively blocked ERK1/2 activation for 24 hr (Fig. 29). ERK5 expression and activation was also analyzed to determine the specificity of U0126. Basal ERK5 activation was unaffected by 10µM U0126 treatment in SH-SY5Y cells (Fig. 29).
Figure 28. U0126 treatment inhibits ERK1/2 activation in SH-SY5Y cells.
Results are presented as mean ± SEM. n = 3 SH-SY5Y cells were treated with pinostilbene (5µM) over a time course and assessed for ERK1/2 and ERK5 activation. Pinostilbene treatment increased pERK1 (A) and pERK2 (B) in a time-dependent manner. ERK5 (C) was not activated by pinostilbene treatment. Data were analyzed using a one-way ANOVA with a post hoc analysis with Fisher's LSD test.
Figure 29. U0126 treatment inhibits prolonged ERK1/2 activation in SH-SY5Y cells.
Results are presented as mean ± SEM. n = 3 SH-SY5Y cells were treated with U0126 (10µM) over a time course and assessed for ERK1/2 activation. Treatment with U0126 decreased pERK1 (A) and pERK2 (B) in a time-dependent manner and was sustained for 24hr. Total protein levels of ERK1 and ERK2 were unaffected by U0126 treatment. Western blot was performed by Mayur Parmar.
12.5 Treatment with U0126 blocks resveratrol activation of ERK1/2

In order to assess whether resveratrol or pinostilbene treatment would increase phosphorylation (activation) of ERK1 and ERK2, SH-SY5Y cells were treated with 5µM of resveratrol or pinostilbene for 30min. Cells were then lysed and assessed for activation of ERK1/2 by Western blot analysis. Resveratrol and pinostilbene treatment (5µM) increased ERK1 and ERK2 activation. Further, SH-SY5Y cells were treated with U0126 (10µM) prior to treatment with resveratrol or pinostilbene to determine if resveratrol or pinostilbene activation of ERK1/2 would be inhibited. Resveratrol and pinostilbene activation of ERK1 and ERK2 was significantly decreased by pretreatment with 10µM U0126 (Fig. 30A/B and 31A/B, respectively). Analysis of ERK5 expression showed no change in activation after treatment with resveratrol, pinostilbene, and/or U0126 (Fig. 30C and 31C).
Figure 30. Resveratrol mediated activation of ERK1/2 is inhibited by U0126 treatment in SH-SY5Y cells.
Results are presented as mean ± SEM, n = 3. (A) Resveratrol treatment (1µM) increases pERK1 (*p<0.05). U0126 (10µM) inhibits resveratrol mediated activation of ERK1 (*p<0.05). (B) Resveratrol treatment increases PERK2 and U0126 inhibits this activation of ERK2 (*p<0.05). ERK5 is not activated by resveratrol treatment and is unaffected by U0126 treatment. Data were analyzed using a two-way ANOVA with a post hoc analysis with Fisher’s LSD test.
Figure 31. Pinostilbene mediated activation of ERK1/2 is inhibited by U0126 pretreatment in SH-SY5Y cells.
Results are presented as mean ± SEM, n = 3. (A) Pinostilbene treatment (1µM) increases pERK1. U0126 (10µM) inhibits pinostilbene mediated activation of ERK1 (**) *p<0.01) and decreases basal activation of ERK1 (*p<0.05). (B) Pinostilbene treatment increases PERK2 and U0126 inhibits this activation of ERK2 (*p<0.05). ERK5 is not activated by resveratrol treatment and is unaffected by U0126 treatment. Data were analyzed using a two-way ANOVA with a post hoc analysis with Fisher’s LSD test.
12.6 Resveratrol-mediated protection from DA-induced oxidative stress in SH-SY5Y cells is blocked by U0126 pretreatment

To examine the role of ERK1/2 activation in resveratrol-mediated protection from DA-induced oxidative stress, SH-SY5Y cells were pretreated with 10µM U0126 prior to resveratrol (1, 3, and 5µM) and dopamine (100µM) treatments. In agreement with earlier data, 100µM DA treatment significantly decreased cell viability and pretreatment with resveratrol (1, 3, and 5µM) increased cell viability as measured by an increase in ATP luminescence (32A). Pretreatment with 10µM U0126 blocked this resveratrol-mediated protection at all three concentrations (32A; 1, 3, and 5µM).

Corroborating the data from the ATP viability assays, treatment with 100µM DA decreased SH-SY5Y cell viability by 40% as measured by Hoechst staining. Treatment with resveratrol (1µM) significantly increased cell viability as measured by counting live cells (Fig. 32B). Pretreatment with U0126 (10µM) inhibited resveratrol-mediated protection (32B).

Similarly, pinostilbene treatment (1, 3, and 5µM) increased cell viability after exposure to DA (100µM), as measured by ATP luminescence (33A). Pretreatment with U0126 (10µM) blocked pinostilbene-mediated protection of cell viability.

In agreement with the Cell Titer Glo data, treatment with 100µM dopamine significantly decreased nuclear integrity as measured by Hoechst stained nuclei (33B). Surprisingly, pinostilbene treatment (1, 3, or 5µM) did not protect SH-SY5Y cells from dopamine (100µM) - induced oxidative stress (Fig. 33B).
Figure 32. Resveratrol mediated protection from DA-induced oxidative stress is inhibited by U0126.

Results are presented as mean ± SEM, n = 3. (A) DA significantly reduced ATP luminescence in SH-SY5Y cells (^p<0.05 vs H2O control). Pretreatment (30min) with resveratrol (RV1; 1-5µM) protected against loss of ATP luminescence at 100µM DA (**p<0.01, **p<0.001 vs 100µM DA). Pretreatment with U0126 (1hr; 10µM) blocked resveratrol-mediated protection. (B) DA treatment significantly decreased the average number of intact nuclei as measured by Hoechst stain (^p<0.001 vs H2O control). Pretreatment with resveratrol (1µM) protected nuclear viability from DA-induced oxidative damage (*p<0.05 vs 100µM). Pretreatment with U0126 blocked resveratrol-mediated protection. Data were analyzed using a two-way ANOVA with a post hoc analysis with Fisher’s LSD test.
Figure 33. Pinostilbene mediated protection from DA-induced oxidative stress is inhibited by U0126.

Results are presented as mean ± SEM, n = 3. (A) DA significantly reduced ATP luminescence in SH-SY5Y cells (^p<0.05 and ^^p<0.01 vs H2O control). Pretreatment (30min) with pinostilbene (RV2; 1-5µM) protected against loss of ATP luminescence at 100µM DA (*p<0.05, **p<0.01, ***p<0.001 vs 100µM DA). Pretreatment with U0126 (1hr; 10µM) blocked pinostilbene-mediated protection. (B) DA treatment significantly decreased the average number of intact nuclei as measured by Hoechst stain (****p<0.0001 vs H2O control). Pretreatment with pinostilbene did not protect nuclear viability from DA-induced oxidative damage. Data were analyzed using a two-way ANOVA with a post hoc analysis with Fisher’s LSD test.
12.7 Preparation of wild blueberry powder affected SH-SY5Y cell viability

To assess whether wild blueberry powder would protect against DA-induced oxidative stress, SH-SY5Y cells were pretreated with two concentrations of dissolved WBB (250 and 500µg/mL) followed by treatment with DA (50, 100, and 200µM). DA treatment (100 and 200µM) significantly decreased cell viability, as measured by ATP luminescence (**p<0.01 and ***p<0.001, respectively). Wild blueberry powder did not show protection from DA-induced oxidative stress (Fig 34), however this may be due to our inability to fully dissolve the wild blueberry powder, despite several attempts using protocols that were successful in other laboratories (communications with Dr. Barbara Shukitt-Hale and Derek Fisher). The powder was suspended in serum-free media and incubated at 37°C for 15 min. The suspension was briefly sonicated and vortexed. After the suspension looked fully mixed by eye, it was centrifuged for 5 min at 2000rpm. The supernatant was then used to treat SH-SY5Y cells. Non-soluble particles were still left at the bottom of the centrifuge tube. Some particles remained in the supernatant, which may have affected cell viability.
Figure 34. Wild blueberry powder does not protect against DA-induced oxidative stress.

Results are presented as mean ± SEM, n = 3. DA significantly reduced ATP luminescence in SH-SY5Y cells (**p<0.01, ***p<0.001 vs H2O control). Pretreatment (30min) with wild blueberry powder (WBB) did not protect against loss of ATP luminescence by DA-induced oxidative stress. Data were analyzed using a two-way ANOVA with a post hoc analysis with Fisher’s LSD test.
13. DISCUSSION

In the current study, SH-SY5Y cells were used as a model of DA neurons to test the protective effect of the isolated natural compounds, resveratrol and pinostilbene, against oxidative stress. DA, which is an endogenous neurotransmitter, was used as a toxin that produces ROS. DA increased SH-SY5Y cell death in a concentration dependent manner. Initially a multitude of compounds, including resveratrol (see page 14) were used to determine which would protect against DA-induced oxidative stress. Two compounds that were chosen to move forward with were resveratrol (RV1) and pinostilbene (RV2) as they both showed significant protection against DA-induced oxidative stress. Further, pretreatment with even lower concentrations of resveratrol and pinostilbene (1-5µM) protected against DA-induced cell death. Resveratrol and pinostilbene activated ERK1/2 in these cells and the ERK1/2 inhibitor, U0126, blocked the resveratrol and pinostilbene-mediated protection from DA toxicity. These data suggest that resveratrol and pinostilbene protect DA neurons from oxidative stress via activation of the ERK1/2 pathways. These are the first studies to show activation of ERK1/2 by resveratrol and pinostilbene in DA cells and a role for these signaling cascades in resveratrol and pinostilbene-mediated protection.

Decreases in DA neurons and projections have been noted with aging and, as DA neurons are more susceptible to oxidative stress, these age-related alterations in the DA system may occur due to an increase in ROS with age. One reason that DA neurons may be more susceptible to oxidative stress is that DA metabolism produces reactive oxygen species, such as oxidative radicals and DA quinones. With age, it has also been shown
that there is a decrease in endogenous antioxidant systems, such as GSH, catalase, and NADPH (Mo et al., 1995; Papadopoulos et al., 1998; Sasaki et al., 2001). Therefore, DA neurons are in an environment high in ROS with reduced antioxidant systems that could lead to increased vulnerability. This increase in oxidative stress in the brain with aging has been shown to be either contributing to or be caused by several neurodegenerative diseases (Junqueira et al., 2004; Wang and Michaelis, 2010). With respect to age-related motor decline and DA neurodegeneration, oxidative stress has been shown to increase with age and to be toxic to DA neurons (Joseph et al., 1999). It follows that administration of antioxidants, such as those found in natural products, would be a viable treatment for age-related increases in oxidative stress.

Many dietary products have been shown to exhibit antioxidant properties, such as green tea, red wine, grapes, and blueberries. Additionally, some isolated compounds from these natural products have been shown to have anticancer, anti-inflammatory, cardioprotective and neuroprotective effects. One such compound is resveratrol. Resveratrol has been shown to have neuroprotective effects in SH-SY5Y cells that were exposed to 6-OHDA (Chao et al., 2008; 2010). However, the exact mechanism by which resveratrol protects from oxidative stress has not been fully elucidated. One pathway that is activated in response to stress that is specifically of interest in this project is the MAPK pathway, in particular ERK1/2.

ERK1/2 has been shown to be important for neuronal differentiation, proliferation, and cell fate (Nishimoto and Nishida, 2006; Krishna and Narang, 2008; Van Kolen et al., 2013). During embryonic development, ERK1/2 has been shown to be
particularly important and knockout models of ERK2 are embryonic lethal, possibly due to a retardation of growth of the placenta and heart of the embryos (Hatano et al., 2003). However, ERK1 knockout models are not embryonic lethal, indicating that ERK1 and ERK2 do have individual roles in embryonic development (Samuels et al., 2009). ERK1/2 deficiencies are also associated with mental retardation, indicating an importance in the regulation of neural progenitor cells (Samuels et al., 2009).

Another important role of the MAPK pathways, particularly ERK1/2, is activation in response to cellular stress, such as ROS. H2O2-induced ERK1/2 activation is thought to play a critical role in cell survival in response to oxidative stress in rat PC12 cells and primary cortical neurons (Guyton et al., 1996; Crossthwaite et al., 2002). ERK1/2 activation was also shown to protect against hypoxia-induced cell death (Wang et al., 2001). However, some studies indicated the ERK1/2 activation plays a role in apoptosis associated with oxidative stress (Ruffels et al., 2004). Therefore, this study investigated the role of ERK1/2 in a model of DA-induced oxidative stress to determine whether resveratrol-mediated protection of SH-SY5Y cells was via ERK1/2 activation.

Treatment with resveratrol (5µM) increased both ERK1 and ERK2 activity in a time-dependent manner (Fig. 26). ERK5 was also transiently activated following resveratrol treatment. Without further investigation these studies could not definitively determine whether resveratrol-mediated protection against DA-induced oxidative stress was due to ERK1/2 activation. Magliaro and Saldanha (2009) found the use of U0126, the ERK1/2 specific inhibitor, protected PC-12 cells from H2O2-induced cell death; however Gomez-Santos and colleagues (2002) found that activation of ERK1/2 protected
SH-SY5Y cells from MPP+ toxicity. Therefore, the ERK1/2 specific inhibitor, U0126, was used to block ERK1/2 activation. Treatment with resveratrol (1µM) increased both ERK1 and ERK2 activation, which was subsequently blocked by pretreatment with U0126 (10µM; Fig. 30). ERK5 was not activated with resveratrol treatment and pretreatment with U0126 did not alter ERK5 expression or activation.

Since, U0126 effectively inhibited resveratrol-mediated activation of ERK1 and ERK2; U0126 was used to examine the role of ERK1/2 in resveratrol-mediated protection against oxidative stress. SH-SY5Y cells treated with 100µM DA showed a significant decrease in cell viability, as measured by ATP luminescence. Pre-treatment with resveratrol (1, 3, and 5µM) increased cell viability significantly against DA-induced oxidative stress (Fig. 32). Further, treatment with U0126 prior to administration of resveratrol showed a reduction in cell viability as measured by ATP luminescence and nuclear integrity. These data suggest that resveratrol-mediated protection of SH-SY5Y cells is due to the activation of ERK1/2. In agreement with this data, Gu and colleagues (2013) found that propofol, an anesthetic agent, protected SH-SY5Y cells from H₂O₂-induced oxidative stress via ERK1/2 activation.

As stated previously, resveratrol has low bioactivity due to rapid metabolism into glucuronides and sulfate conjugates (Marier et al., 2002; Goldberg et al., 2003). Methylation of the hydroxyl groups of resveratrol has been shown to increase bioactivity, through prolonged bioavailability and increased permeability of the cell membrane (Chao et al., 2010). Therefore, this study sought to investigate the role of a resveratrol analog, pinostilbene, in the protection of SH-SY5Y cells from DA-induced cell death.
Similar to the resveratrol study, DA was used to induce oxidative stress. Increasing concentrations of DA reduced cell viability significantly in SH-SY5Y cells as measured by ATP luminescence and nuclear integrity. Pretreatment with pinostilbene (1, 3, and 5µM) protected against DA-induced oxidative stress, as measured by ATP luminescence (Fig. 25A). However, pinostilbene treatment did not protect against DA-induced loss of nuclear integrity as measured by Hoechst staining (25B). Chao and colleagues (2010) showed that pinostilbene decreased activation of JNK-1, JNK-2, and c-jun-1 in SH-SY5Y. This data indicates the pinostilbene is able to modulate signaling in the MAPK family. Therefore, further studies were done to investigate pinostilbene activation of the ERKs.

To investigate the role of the MAPKs, ERK1/2, SH-SY5Y cells were treated with pinostilbene (5µM). Pinostilbene transiently increased ERK1 and ERK2 activation. As seen with the resveratrol treatment, activation of ERK5 by pinostilbene treatment is inconclusive due to the large errors bar with ERK5 quantification (Fig. 27C). These data may indicate that there are distinct roles for ERK1/2 versus ERK5 in cell survival.

Currently, there is limited research on pinostilbene and no studies investigating pinostilbene and ERK1/2. Since the data of this study found that pinostilbene protected SH-SY5Y cells from DA-induced cell death and activated ERK1/2, we wanted to investigate the role of ERK signaling and pinostilbene-mediated protection of SH-SY5Y cells from DA-induced cell death. Therefore, SH-SY5Y cells were treated with U0126 prior to pinostilbene (1, 3 or, 5µM) and DA (100µM) treatment. As previously seen, DA treatment decreased cell viability by 54.6% and treatment with pinostilbene (1, 3 or,
5µM) increased cell viability, as measured by ATP luminescence. Pretreatment with U0126 inhibited approximately half of the cell viability increase mediated by pinostilbene treatment (Fig. 33A). These data suggest that pinostilbene-mediated protection of SH-SY5Y cells from oxidative stress may be modulated by ERK1/2 activation.

Joseph and colleagues (2010) have shown that treatment with wild blueberry powder attenuates Ca\(^{2+}\) signaling in primary hippocampal neurons, leading to cell survival. Therefore, the role of WBB in SH-SY5Y cells and oxidative stress was investigated in the current study. In this model, DA effectively decreased cell viability, as measured by ATP luminescence (Fig. 34). However, treatment with wild blueberry (WBB) powder did not protect against DA-induced oxidative stress (Fig. 34). The lack of protection may be due to the incomplete dissolution of the WBBs. After the SH-SY5Y cells were treated with the suspended WBBs, they were observed under a light microscope. There were distinct particles observed in the media that may have been insoluble fractions of the WBB powder. Therefore, the dissolution of the WBB may have been incomplete. Different techniques to dissolve WBB powder were discussed with the Joseph lab and, even with their guidance, WBB powder was not successfully dissolved. Non-soluble particles still remained in the solution, even after sonication, centrifugation, and filtration with a 0.45µm syringe filter. This solubility issue with the WBB powder may be why there was not protection with the WBB solution.

In conclusion, resveratrol and pinostilbene protected SH-SY5Y cells from DA-induced oxidative stress. This data suggests that activation of the ERK1/2 signaling
cascades may be involved due to a reduction in cell viability with the use of the ERK1/2 specific inhibitor, U0126. As these natural supplements are widely used, it is important to understand the mechanism by which they provide neuroprotection so as to avoid unwanted side effects and/or drug–drug interactions. Furthermore, elucidation of the mechanism of action of these compounds will allow for future design of compounds that target these same signaling pathways.
14. CONCLUSION

The aging population is increasing at a significant rate. There is a lack of treatments for age-related motor deficits. While L-DOPA effectively reversed age-related motor deficits, long-term treatment can cause dyskinesias. Therefore, there is a need for an effective treatment for motor deficits. Our study showed that natural products may fill this need.

In the present study, we found that L-DOPA, wild blueberries - and resveratrol — supplemented diets attenuated age-related motor deficits in an aging mouse model. We did not find any changes in the DA system with age, however wild blueberries increased the amount of TH+ neurons in the SN. These data indicate that natural products, such as wild blueberries may be effective at reversing age-related motor deficits.
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16. SUPPLEMENTAL FIGURES

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

Supplemental Figure 2. PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one)
Supplemental Figure 3. XMD 8-92 (2-[[2-Ethoxy-4-(4-hydroxy-1-piperidinyl)phenyl]amino]-5,11-dihydro-5,11-dimethyl-6H-pyrimido[4,5-b][1,4]benzodiazepine-6-one
Supplemental Figure 4. BIX02189 (3-[[3-[dimethylamino)methyl]phenyl]amino]phenylmethylene]-2,3-dihydro-N,N-dimethyl-2-oxo-1H-indole-6-carboxamide)