POTENTIAL EFFECTS OF BLUEBERRIES ON MARKERS OF NEURODEGENERATIVE DISEASE

By

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Abstract

One of the main mechanisms contributing to neurodegenerative diseases (NDD) is oxidative stress, caused by the buildup of reactive oxygen species (ROS) in the brain. Chronic neuroinflammation is also involved with the pathology of NDD; when microglia are activated in response to the dysfunction and aggregation of alpha-synuclein and amyloid-beta, they release inflammatory mediators. NDD are linked to glutamate-mediated excitotoxicity as well, where cells are damaged by the excessive stimulation from glutamate via inadequate uptake by glial cells. Polyphenols, found in very high levels in blueberries, have antioxidant properties which may offer protection and intervention from neurological disorders by managing ROS and reducing the inflammatory response. Biochemical analysis was performed on extracts from frozen wild Newfoundland fruits and leaves, *Vaccinium angustiform spp*. Mouse-pup derived brain cultures, and pure cultures of rat neurons and microglia were exposed to 100μM glutamate, 100ng/ml alpha-synuclein or 7.5μM amyloid-beta for 24-hours, which decreased cell viability. Overall, there was an increase in the number of viable cells once blueberry extract was added, effectively reducing neuronal and microglial cell death, showing that compounds in berries are beneficial when cell death and damage is influenced by glutamate-mediated excitotoxicity or insoluble alpha-synuclein and amyloid-beta aggregates. The blueberry extracts have high antioxidant activity and therefore may be able to help combat oxidative stress and neuroinflammation in the brain.

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List of Abbreviations and Symbols

Ab: Antibody

ACh: Acetylcholine

AD: Alzheimer's disease

APP: Amyloid-beta precursor protein

BBB: Blood-brain-barrier

Ca^{2+}: Calcium

CNS: Central nervous system

CO_2: Carbon dioxide

DAPI: 4', 6-diamidino-2-phenylindole

dH_2O: Distilled water

DA: Dopamine

DI: Discrimination index

DNA: Deoxyribonucleic acid

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

G: Gravitational force

GAE: Gallic acid equivalents

GFAP: Glial fibrillary acidic protein

Glut: Glutamate

HPLC-MS: High performance liquid chromatography-mass spectroscopy

IACC: Institutional Animal Care Committee

IGF-1: Insulin-like growth factor-1

LRRK2: Leucine-rich repeat kinase 2

MUN: Memorial University of Newfoundland

mRNA: Messenger ribonucleic acid

NADPH: Nicotinamide adenine dinucleotide phosphate

NDD: Neurodegenerative disease

NF-κB: Nuclear factor kappa B

Nrf2: Nuclear factor f2

NMDA: N-Methyl-D-aspartate

NO: Nitric oxide

NOR: Novel object recognition

O_2: Oxygen

PBS: Phosphate buffer solution

PD: Parkinson's disease

PFA: Paraformaldehyde

PLO: Poly-L-Ornithine

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RPM: Revolutions per minute

SE: Standard error

SNpc: Substantia nigra pars compacta

TNF-α: Tumour necrosis factor alpha

Chapter 1: Introduction

1.1 General

We are now in an era where the world's population is living longer than ever, so age-related diseases such as Parkinson's (PD), Alzheimer's (AD), and Huntington's disease (HD) are becoming more and more common (Figueira *et al.*, 2017; Cirmi *et al.*, 2016). There is no cure for these neurodegenerative diseases (NDD), as the drugs and other therapies available only relieve the symptoms associated with the disease and have no effect on its pathology (Ataie *et al.*, 2016; Strathearn *et al.*, 2014). Only a small portion of NDD cases are caused by environmental or genetic factors, as the genetic basis of the majority of NDD is poorly understood. Most cases are sporadic or idiopathic and are very difficult to diagnose (Aarsland & Kurz, 2010; Ataie *et al.*, 2016). Neurodegeneration is the progressive loss of neuronal structure and function and is common even when a NDD is not present. Over time, as we age the body loses its ability to maintain and produce new nervous tissue, which can lead to many cognitive and motor impairments.

One of the main mechanisms contributing to NDD is oxidative stress, which is caused by the buildup of harmful reactive oxygen species (ROS) in the brain

(Ataie *et al.*, 2016). Neuroinflammation, which is the inflammation of nervous tissue, is also associated with the development and pathology of many NDD. For example, when microglia, the immune cells in the CNS, become over-activated this promotes the release of excess pro-inflammatory mediators which can lead to chronic neuroinflammation resulting in tissue damage (Morales *et al.*, 2014). NDD are linked to glutamate-mediated excitotoxicity as well, where cells are damaged by the excessive stimulation from glutamate, an excitatory neurotransmitter, via inadequate uptake by glial cells (Figueira *et al.*, 2017).

Identifying a potential drug or compound which is able to prevent further neuronal loss and/or reverse some of the damage is extremely difficult but very important as neurons are not as actively replaced by the body compared to other cell types, especially in an aging brain. Polyphenols are a natural antioxidant product associated with minimal risk in terms of toxicity and adverse side effects (D'Archivio *et al.*, 2008). Natural polyphenols have been shown to be preferred by individuals compared to synthetic antioxidant products (Papas, 1999).

1.2 Taxonomy

The goal of this study is to examine the neuroprotective role of low-bush wild blueberries, *Vaccinium angustifolium*, which are native to Newfoundland and Labrador. *Vaccinium* species belong to the family Ericaceae and are most

commonly found in eastern and central North America (Kloet, 1983). They are a perennial shrub which grow to be about 6-inches to 2-feet tall (Kloet, 1983). The buds produce a small white bell-shaped flower which is then followed by a blue fleshy fruit. The branches are small and thin, with glossy foliage that is green/blue in the spring and summer, and turns green/red in the fall (see Figure 1.1). *V. angustifolium* are very tolerant to a wide range of soils and climates (Kloet, 1983). They can be found growing in acidic, moist or droughty soils, which are generally very challenging for other plant species. They typically grow on alpine or subalpine zones, cliffs, ledges, grasslands, and mountain summits (Morrison *et al.*, 2000), where they are exposed to harsh winds and little shade. *V. angustifolium* are grown commercially in Canada, mainly in Nova Scotia and Quebec, as well as areas across the United States (Morrison *et al.*, 2000).

1.3 Blueberries as Nutraceuticals

Nutraceuticals are commonly defined as any substance that is considered a food, a part of food, a vitamin, a mineral or an herb that provides health benefits, including disease prevention and/or treatment (Raskin *et al.*, 2002; Vyas *et al.*, 2013). The use of botanical nutraceuticals for their antioxidant properties and overall health benefits have been investigated not only within the *Vaccinium* genus but also all across the plant kingdom (Vyas *et al.*, 2013). For example, grape

seeds (Strathearn *et al.*, 2014), walnuts (Orhan *et al.*, 2011), plums (Strathearn *et al.*, 2014), and myrtle (Tumen *et al.*, 2012) have been investigated as potential neuroprotective agents. Berries however, are the most researched plant species for phenolic studies and have been proven to contain very high levels of polyphenols compared to other plant species (Papandreou *et al.*, 2012; Balk *et al.*, 2006). Blueberries, in particular, contain the highest amount of polyphenols, anthocyanins and antioxidant activity among other berries studied (Poiana *et al.* 2010; Joseph *et al.*, 1999; Vyas *et al.*, 2013). Blueberries contain several different kinds of polyphenols that can work together and have a synergistic effect in the CNS (Tavares *et al., 2013*). This is important, particularly with regard to the localization of a particular antioxidant in the brain (Joseph *et al.*, 1999), and for activating neuroprotective response pathways (Strathearn *et al.*, 2014). Berries are very promising because they have more than one mechanism of action when it comes to neuroprotection. For example, the polyphenols found in blueberries have antioxidant properties that may offer protection and intervention from neurological disorders by managing ROS (Cirmi *et al.*, 2016; Strathearn *et al.*, 2014; Gao *et al.*, 2012), reducing the inflammatory response (Figueira *et al.*, 2017; Morales *et al.*, 2016), and providing protection in the brain from further degeneration (Fuentealba *et al.*, 2011; Takahashi *et al.*, 2015; Macedo *et al.*, 2015).

Vaccinium angustifolium

Figure 1.1: ***Vaccinium angustifolium.*** Lowbush wild blueberry species native to Newfoundland and Labrador.

1.4 Phenolics and Polyphenols

Polyphenols found in several species of plants, particularly blueberries, are botanical nutraceuticals with antioxidant properties. Polyphenols are known to have other health benefits as well such as anti-inflammatory, anti-cancer, and anti-infective properties (Vyas *et al.*, 2013; Virmani *et al.*, 2013). Polyphenols may have indirect peripheral effects in the brain (Figueira *et al.*, 2017) such as improving cardiovascular health resulting in increased blood flow to the brain (Slemmer & Weber, 2014). Some antioxidants are found in the body naturally while others need to be obtained in the diet (Vyas *et al.*, 2013).

There are more than 8,000 known phenolic structures (Dai & Mumper, 2010), composed of one or more aromatic rings with several hydroxyl groups attached (see Figure 1.2). Different classes of polyphenols exist based on their number of phenol rings and what structural element is present for binding them

together (D'Archivio *et al.*, 2007). It is important to determine the levels of polyphenols and the free radical scavenging ability of a particular sample to better understand the factors that influence phenolic content. Polyphenol content in a particular sample depends on several environmental factors such as soil composition, temperature, exposure to ultra-violet (UV) radiation, wind, rainfall and elevation (Chu *et al.*, 2011; Manach *et al.*, 2004). Ripeness also plays a role in the levels of phenolic compounds present in a berry sample (Manach *et al.*, 2004). Generally, the concentration of most phenolic compounds tend to decrease during ripening, however the concentration of anthocyanins (see section 1.4.2) increase. After the collection of berries, the type of storage can affect the polyphenol concentration as well, as polyphenols are easily oxidized (D'Archivio *et al.*, 2007). However, there is no significant difference between the antioxidant activity of extracts from fresh blueberries and berries that are frozen (Lohachoompol *et al.*, 2004). Berries can maintain high phenolic levels when frozen for a long period of time (Lohachoompol *et al.*, 2004), and therefore this type of storage can maintain the integrity of the compounds of interest. A study out of Italy in 2013 examined the difference between the polyphenol content in wild versus cultivated blueberries (Braga *et al.*, 2013). They determined that polyphenol levels were significantly greater in wild extracts, particularly

anthocyanin levels. They also determined through COMET assay, a technique used to detect deoxyribonucleic acid (DNA) damage in a cell, that there was strong DNA protection with wild blueberry samples compared to the cultivated samples.

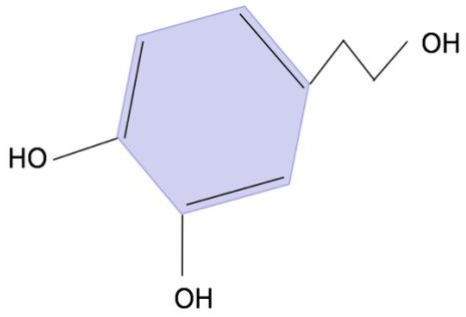


Figure 1.2: Basic phenolic structure. Polyphenols are composed of one or more aromatic rings with several hydroxyl groups attached.

Polyphenols can be classified into five major groups: diferuloylmethanes, stilbenes, flavonoids, phenolic acids and tannins (Pandareesh *et al.*, 2015). In a previous study in Dr. Weber's lab (here and after referred to as "our lab") (Vyas *et al.*, 2013), it was determined that in addition to the fruits containing high levels of phenolic compounds in both blueberries and lingonberries, the leaves exhibited much higher levels and proved to be more neuroprotective in an *in vitro* model of glutamate-mediated excitotoxicity. Thus, studies should include other plant components instead of just the fruits.

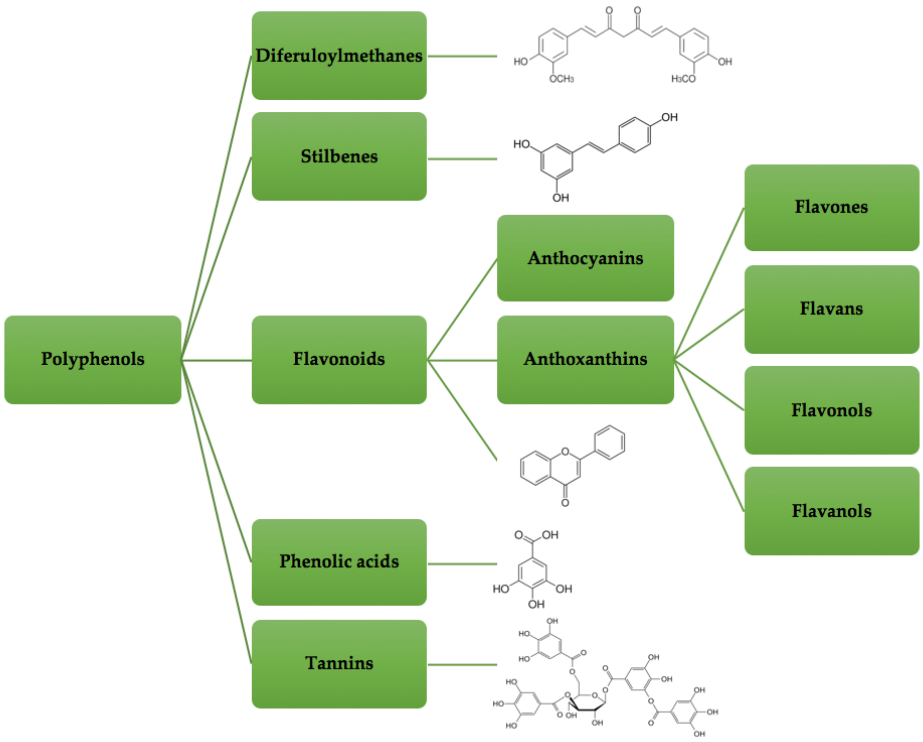


Figure 1.3: Phenolic family classification.

Adapted from "B Vitamins and Berries and Age-Related Neurodegenerative Disorders" by Balk *et al.* (2006) and "Dietary Polyphenols and Their Biological Significance" by Han *et al.*, 2007.

1.4.1 Tannin

A tannin is an astringent, polyphenol that has a bitter taste (Han *et al.*, 2007). Tannins are water-souble and are particularly prominent in unripened or bitter fruits and vegetables (Han *et al.*, 2007). Wines contain high levels of tannins

and are responsible for the "drying" feeling in the mouth when consumed. Wine can have strong or weak tannins depending on how long the juice sits with the grapes after they have been pressed; red is usually higher than white. Tannins also play a role in plant defense; because they are highly astringent and concentrated in unripened fruits this often results in a reduction in consumption by animals. This is beneficial for the plant because the fruits are not consumed until the seeds are mature and ready for dispersal, which is important for seed survival (Mehansho *et al.*, 1987).

1.4.2 Flavonoids

Flavonoids are the largest naturally occurring group of polyphenols, and are the most abundant polyphenols in our diets (Han *et al.*, 2007). They are composed of two aromatic rings which are bound together by three carbon atoms to form an oxygenated heterocycle (Manach *et al.*, 2004). Flavonoids can be further divided into two groups: anthoxanthins, which are colourless and composed of four subgroups, and the anthocyanins, which are responsible for the colour seen in many fruits and vegetables (Han *et al.*, 2007). The major flavonoids found in berries are anthocyanins and flavonols (Cho *et al.*, 2004), which are also responsible for the blue and red colours present in most berries (Hossain *et al.*, 2016). Generally, the darker the berry the higher the antioxidant activity. Berries

store the majority of their anthocyanins in the skin, which help protect the fruit from harmful interactions with the sun and other environmental factors (Hossain *et al.*, 2016). Blueberries contain at least five different anthocyanins, catechin being the most common. Catechin is often used as a standard (catechin equivalent) for quantifying levels of polyphenols in samples (D'Archivio *et al.*, 2007).

Flavonoids are the main compounds responsible for the major antioxidant activity of berries (Balk *et al.*, 2006; Pandareesh *et al.*, 2015). This is consistent with results from Strathearn *et al.* (2014) where they determined that anthocyanins and pro-anthocyanins were better able to suppress the neurodegenerative effects of rotenone in a cell model of PD compared to other phenolic compounds found in their blueberry extract.

1.5 Bioavailability

The overall health benefits of consuming polyphenols are directly related to the bioavailability and the amount consumed (Manach *et al.*, 2004). The absorption of polyphenols is highly dependent on the rate of metabolism and the microflora present within the body. The body is highly complex and there are a several steps that affect bioavailability including initial ingestion, absorption, plasma transport, elimination, cellular uptake, and crossing the blood-brain-barrier (BBB)

(Manach *et al.*, 2004; Kelly *et al.*, 2017). Polyphenols are not absorbed with equal efficacy; the bioavailability of polyphenols is largely concerned with the structure of active phenolic metabolites crossing the BBB, which depends on a number of factors such as size and lipophilicity (Weber *et al.*, 2012; Tavares *et al., 2013*).

Polyphenols are not taken up equally into all brain areas, for example, vitamin E uptake is lower in the striatum compared to other brain areas (Joseph *et al.*, 1999). Papandreou *et al.* (2012) found that different phenolic compounds present in blueberries had differences in the underlying neuroprotective mechanisms based on the characteristics of a specific cell line. For example, some cell lines required a higher concentration of berry extract than others to elicit protection against hydrogen peroxide-mediated cell death. These differences could be the chemical structure of the phenolic compound, and how it interacts with different cell surface receptors, or the unique cellular membrane composition of a particular cell type (Papandreou *et al.*, 2012).

Previous studies have detected the presence of particular polyphenols in the CNS showing that they are able to cross the BBB (Kelly *et al.*, 2017; Krikorian *et al.*, 2010; Andres-Lacueva *et al.*, 2005). Anthocyanins have been identified in several brain areas and have been associated with neuroregeneration and protection (Krikorian *et al.*, 2010). Specifically, Talavera *et al.* (2005) showed that

anthocyanins were detected in the CNS after oral administration of a blackberry-enriched diet to rats. Similarly, Andres-Lacueva *et al*. (2005) showed that after eight weeks of feeding with a 2% blueberry-enriched diet, anthocyanins from the blueberry extracts were found in the cerebellum, cortex, hippocampus and striatum in 19-month-old rats.

1.6 *In vitro* and *in vivo* studies

There are many advantages and disadvantages to studying the neuroprotective effects of polyphenols with both *in vitro* and *in vivo* experiments. The majority of experiments are conducted *in vitro* (Han *et al.*, 2007; Fuentealba *et al.*, 2011; Takahashi *et al.*, 2015; Macedo *et al.*, 2015), as they are more accessible, provide faster results, can use specific cell lines, and are generally less expensive than *in vivo* studies (Kelly *et al.*, 2017; Slemmer & Weber, 2014). *In vivo* models, which use oral administration to administer polyphenol-enriched diets (Slemmer & Weber, 2014), are generally more expensive and time consuming but are very important because they imitate the bioavailability and concentration of active metabolites in particular brain areas (Kelly *et al.*, 2017; Slemmer & Weber, 2014). With *in vivo* studies, different motor and cognitive tests can be performed to see if the addition of a particular compound can be beneficial. Motor and cognitive deficits accompany many NDD, such as PD and AD. *In vitro* studies, although

very important, only show the effects on a cellular level, i.e. the increase or decrease of cell death or levels of toxic protein. Therefore, being able to perform cognitive and motor tests in an *in vivo* model allows the researcher to observe significant behavioral changes over time. Previous *in vivo* studies (Atcha *et al.*, 2010; Grace *et al.*, 2009; El-Mohsen *et al.*, 2006), including those conducted in our lab, primarily use oral gavage as the main route of administration for polyphenol-enriched diets. This method is very invasive however, and has been shown to potentially cause extensive internal damage such as throat and oral damage, lung perforation and aspiration, and restraint-associated injuries (Vandenberg *et al.* 2014). Oral gavage can be very stressful for the animals, resulting in an increase in plasma levels of corticosterone, glucose, growth hormone, and prolactin (Balcombe *et al.* 2004). Other routes of administration such as voluntary consumption of these special diets has not yet been thoroughly investigated. Therefore, it would be beneficial to explore these other options which would reduce injury and mortality of the animals involved in the study, and reduce their overall stress levels so that results, particularly regarding behavioral tests, are not influenced. It would also be beneficial to look at the bioavailability of an alternate route of administration. When an animal is fed via oral gavage the compounds are deposited directly into the stomach. Therefore,

the compounds are not chewed or swallowed resulting in a decrease in absorption and metabolite breakdown from the esophageal mucosa and epithelia (Vandenberg *et al.* 2014). A study conducted by Atcha *et al.* (2010) showed that those animals fed by oral gavage compared to those animals that freely consumed and swallowed the pharmaceutical donepezil had lower blood and brain concentrations of the drug.

1.7 Antioxidants and Oxidative Stress

One of the main mechanisms of neurodegeneration is from the increased and long-term effects of oxidative stress. All cells need to utilize O_2 for cellular respiration, however it is important that oxygen is managed correctly and efficiently to prevent cell damage. Oxidative stress in the CNS is caused when ROS build up faster than the endogenous redox reactions can eliminate them (Ataie *et al.*, 2016). Disruption of the sensitive regulation of ROS and balance of antioxidants can lead to cell death and damage (Cirmi *et al.*, 2016). In addition, nitrosative stress, similar to oxidative stress, occurs when reactive nitrogen species (RNS) build up in the cell and additively contribute to the free radical load. Free radicals, ROS and RNS, are missing one or more electrons so they attack other molecules, such as cell structures, membranes, and DNA to replace their missing charge. This results in extensive cell damage and eventual cell

death. Healthy cells are capable of performing redox reactions to keep ROS and RNS in check, however cell damage and death occurs when these systems are overloaded (Cirmi *et al.,* 2016).

Redox reactions involve the transfer of electrons, and are very important in many biological processes such as cellular respiration and photosynthesis (Cirmi *et al.,* 2016). Oxidation is a process that occurs in all aerobic organisms, which refers to the loss of electrons. Free radicals can be produced during redox reactions when an electron detaches from a molecule. If the electron does not reattach to the molecule or an antioxidant it can cause extensive damage in the cell and surrounding area (Ataie *et al.*, 2016). NADH dehydrogenase is an enzyme in the mitochondria responsible for the transfer of electrons. Many NDD affect this site in the mitochondrial electron transport chain which allows electrons to escape, leading to an increase in ROS (Strathearn *et al.*, 2014). Interference with the mitochondria, which is responsible for generating energy, reduces the overall productivity of the cell as well (Virmani *et al.*, 2013). Polyphenols displace rotenone, a mitochondrial complex I inhibitor (Sanders & Greenamyre 2013), from its binding site on NADH dehydrogenase, resulting in the reduction of ROS produced (Strathearn *et al.*, 2014; Gao *et al.*, 2012). When the

levels of ROS are decreased, this reduces the amount of damage caused by oxidative stress.

The brain is particularly susceptible to oxidative stress because of its high demand for oxygen (Ataie *et al.*, 2016). Neurons have a high cellular respiration rate and low antioxidant defense systems and hence, are highly vulnerable to oxidative stress particularly in an aging brain (Cirmi *et al.*, 2016). Antioxidants are essential to scavenge free radicals and protect the CNS from further degradation (Ataie *et al.*, 2016). Moreover, antioxidants may reverse some of the oxidative damage. Antioxidants are electron donors and are able to neutralize these ROS without becoming free radicals themselves (Slemmer & Weber, 2014). The efficacy of antioxidants comes from their chemical structure, composed of one or more aromatic rings with several hydroxyl groups attached. These hydroxyl groups trap excess electrons balancing ROS (Pandareesh *et al.*, 2015). Certain antioxidants based on their chemical structure, i.e. the number of rings and the chemical element that binds them together (Manach *et al.*, 2004), may be better at trapping excess electrons and be a stronger antioxidant (Tavares *et al.*, 2013).

Polyphenols such as resveratrol, along with scavenging for free radicals, potentially stimulate genes that are responsible for the activation of endogenous

antioxidant pathways as well (Weber *et al.*, 2012). Furthermore, polyphenols may regulate the expression of cellular ROS scavenger enzymes (Tanigawa *et al.*, 2007). Nuclear factor f2 (Nrf2) is a transcription factor that regulates the gene expression of these detoxifying and antioxidant enzymes. A study conducted by Tanigawa *et al.* (2007) showed that the addition of polyphenols, specifically quercetin in this study, enhanced the upregulation of Nrf2 mRNA and protein. Quercetin was able to reduce the levels of Keap1 which is responsible for repressing Nrf2 activation. Polyphenols are also able to reduce cellular ROS concentrations through metal ion chelation to help detoxify the CNS (Ataie *et al.*, 2016; Kelly *et al.*, 2017). The hydroxyl groups within the molecule are the main site for metal ion chelation (Bernatoniene & Kopustinskiene, 2018). Detoxification through this process helps to minimize cell death and damage.

1.7.1 Excess Intracellular Ca^{2+} Levels

Oxidative stress can have an effect on lipids in the membrane of cells as well. Free radicals can build up in the membrane making it faulty (Ataie *et al.*, 2016; Adibhatla *et al.*, 2007). This results in the cell spilling its contents into the surrounding area causing further inflammation and damaging surrounding cells. Free radicals that build up in the membrane can also promote permeability to Ca^{2+} (Ataie *et al.*, 2016; Adibhatla *et al.*, 2007), which can cause changes in normal

cell function by over-activating Ca^{2+}-dependent enzymes, such as phospholipase A_2, a precursor to arachidonic acid which is responsible for mediating inflammatory responses (Adibhatla *et al.*, 2007). Excess Ca^{2+} can promote the production of free radicals in the cell and other harmful compounds such as peroxynitrate and hydrogen peroxide which are highly unstable and can cause extensive cellular damage as well as promote oxidative malfunction of the mitochondria in cells (Slemmer & Weber, 2014). Increased intracellular levels of Ca^{2+} can activate caspases, which are enzymes responsible for triggering apoptosis, and endonucleases which degrade DNA and proteins (Adibhatla *et al.*, 2007; Vyas *et al.*, 2013), resulting in further cell death and damage.

1.8 Polyphenols and Apoptosis Activation

Apoptosis, or programmed cell death, is a well-controlled process that is essential for the perpetuation of proper tissue function as it removes unwanted and damaged cells (D'Archivio *et al.*, 2008). The apoptotic pathway is characterized by a number of morphological changes within the cell. Dysfunction within the apoptotic pathway can play a role in NDD. The pathway is activated in response to several different agents such as oxidants, toxins, or ionizing radiation which can all increase cellular levels of ROS leading to oxidative stress (D'Archivio *et al.*, 2008). One of the most important steps in the pathway is the

activation of the caspase-3 enzyme which plays a key role in the cleavage of the cytoskeleton (Ndozangue-Touriguine *et al.*, 2008). Tumor protein 53 (p53) also plays an important role in apoptosis, and is activated in response to DNA damage in the cell acting as a pro-apoptotic signal.

Polyphenols influence different sections of the apoptotic pathway and help with the proper steps for shutting down a cell. For example, adding polyphenols to carcinogenic cells induces higher rates of apoptosis (Bernatoniene & Kopustinskiene, 2018; Curti *et al.*, 2017; D'Archivio *et al.*, 2008). Gupta *et al.* (2012) observed that the addition of polyphenols in prostate cancer cells induced apoptosis via p53 stabilization. In contrast, the addition of phenolic compounds to brain tissue results in reduced levels of apoptosis. This was shown in a study where the addition of polyphenols, mangiferin and morin, resulted in reduction of apoptosis by limiting the activation of the N-methyl-D-aspartate (NMDA) glutamate receptor responsible for activating caspase-3 neurons *in vitro* and *in vivo* (Gottlieb *et al.*, 2006; Virmani *et al.*, 2013). NMDA-receptor induced toxicity is highly influenced by the influx of Ca^{2+}. Mangiferin and morin were able to reduce oxidative stress, and lower Ca^{2+} concentration which limited NMDA glutamate receptor activation and therefore resulted in a decrease in caspase-3 activation. Similarly, Bureau *et al.* (2008) showed that the natural

polyphenols, resveratrol and quercetin, significantly decreased mRNA levels of pro-inflammatory protein interleukin 1-alpha and tumour necrosis factor alpha (TNF-α), in glial cells. They observed that the addition of these polyphenols reduced apoptotic cell death in neuronal cells induced by microglial activation.

1.9 Neurodegeneration and Neurodegenerative Disease

Neurodegeneration is the progressive loss of neuronal structure and function and eventual cell death. Neurons do not usually get replaced in the adult body, so chronic neurodegeneration can lead to incurable and debilitating conditions that cause problems with motor or cognitive functioning. The two most common age-related NDD are AD and PD (Balk *et al.*, 2006). NDD are often characterized with specific patterns of cell loss in the CNS. One of the main issues associated with NDD is that cellular dysfunction often occurs long before onset of the actual disease, making it very difficult to implement treatment in the early stages which is often the most beneficial (Dauer & Przedborski, 2003). The discovery of genes that may increase an individual's risk of developing a NDD has opened the door for research involving dysfunction and misfolding of proteins. It is hypothesized that mitochondrial dysfunction and oxidative stress play a large part in the accumulation of these insoluble proteins which cause extensive damage in the CNS (Dauer & Przedborski, 2003).

1.9.1 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease that causes irreversible damage to neurons which results in cognitive deficits (Balk *et al.*, 2006). AD is the most common type of dementia and is not part of normal aging. Symptoms associated with AD include disruption to short and long-term memory, poor judgement, and difficulty performing basic tasks (Balk *et al.*, 2006). The biggest risk factor for the development of AD, like many other NDDs, is age. Diagnosis is generally around the mid-60s; however, an individual can only be definitively diagnosed at autopsy. As mentioned previously, there are no drugs or therapies available to stop the progression of the AD. There are, however, drugs available to help treat the symptoms of AD. AD drugs work by regulating neurotransmitters, particularly cortical acetylcholine (ACh) (Balk *et al.*, 2006). Individuals with dementia usually have lower levels of ACh, which is important for the processes of memory, thinking, and reasoning (Richter *et al.*, 1980; Scarpini *et al.*, 2003). AD drugs work by increasing ACh production and presynaptic release, stimulating cholinergic receptors, such as muscarinic and nicotinic receptors, and reducing the degradation of ACh by cholinesterase inhibitors. The most common pharmacological drugs prescribed to treat AD are

donepezil, rivastigmine and galantamine, which are all acetylcholinesterase inhibitors (Scarpini *et al.*, 2003).

Amyloid-beta and Neurofibrillary Tangles

The pathological markers associated with the development of AD are extracellular amyloid-beta-peptide plaques and intracellular neurofibrillary tangles composed of tau protein (Balk *et al.*, 2006). They both cause inclusions in the nerve tissue leading to significant neuronal loss (Morales *et al.*, 2014). The plaques initially appear between nerve cells in the hippocampus, which is an area in the brain involved with the formation of new memories. The tau protein tangles are initially deposited in the nerve cells where they interrupt synaptic function (Morales *et al.*, 2014). As the disease progresses, the neurons in the hippocampus continue to die which eventually starts to affect other brain areas as well, resulting in significant tissue loss throughout the brain (Balk *et al.*, 2006). It is important to note that these proteins, particularly amyloid-beta, are also highly toxic when in high concentrations and are capable of causing neuronal injury and loss even if they are not in an aggregated form (Masliah *et al.*, 2001). Mutations in the genes encoding for amyloid-beta precursor protein (APP), and presenillin 1 and 2, are associated with the upregulation of cellular proteins and products that can be toxic to neurons (Balk *et al.*, 2006). Both mutations are

autosomal dominant which means they can be inherited (Singleton *et al.*, 2003), although these mutations only account for a very small percentage of AD cases as they are highly penetrant. Abnormal cleavage and increased production of APP can result in the aggregation and subsequent formation of insoluble amyloid-beta plaques which are one of the main pathological markers of AD (Balk *et al.*, 2006). Presenillin 1 and 2 are associated with the inheritance of early-onset familial AD. It is proposed that amyloid-beta plaques and tau-tangles are able to activate microglia, contributing to the inflammatory response which results in the overproduction of pro-inflammatory mediators (see section 1.8) (Morales *et al.*, 2014). Akiyama *et al.* (2000) showed that there was a higher concentration of inflammatory cytokines in the areas surrounding the amyloid-beta plaques and tau-tangles.

Aggregation and dysfunction of amyloid-beta protein is highly influenced by issues associated with the mitochondrial electron transport chain, and increased levels of ROS in the CNS (Balk *et al.*, 2006). In a study conducted by Ono *et al.* (2012), the addition of phenolic compounds, specifically myricetin and rosmarinic acid, inhibited the aggregation of amyloid-beta both *in vitro* and *in vivo*, as well as reduced cellular cytotoxicity. Similar results were observed by Fuentealba *et al.* (2011), where a neurodegenerative model induced by amyloid-

beta *in vitro* was supplemented with a blueberry extract. The addition of the blueberry extract was able to protect cells by reducing amyloid-beta protein aggregation, and reducing the inflammatory response of nuclear factor kappa B (NF-κB), which stimulates the release of inflammatory cytokines in the CNS (Pahl, 1999). Furthermore, Papandreou *et al.* (2012) noted that the addition of blueberry extract *in vitro* was able to reduce amyloid-beta aggregation by up to 70%.

1.9.2 Parkinson's Disease

Parkinson's Disease (PD) was first described in 1817 by James Parkinson where he referred to it as the "shaking palsy". PD is caused by neuronal cell death in the substantia nigra pars compacta (SNpc) in the midbrain (Braak *et al.*, 2003). This area is rich in dopamine neurons (DA) neurons which are responsible for sending chemical messages to parts of the brain that control movement and coordination. As the disease progresses the brain continues to lose DA neurons making it difficult for an individual to control their movements (Braak *et al.*, 2003). With normal aging, you start to lose DA, but PD accelerates this process. There is no single cause of PD, most cases are sporadic or idiopathic (Dauer & Przedborski, 2003). The biggest risk factor is age; it is rare to see anyone under the age of 60 with PD (Balk *et al.*, 2006). Only a very small portion of PD cases are

caused by genetic factors. Mutations in genes encoding for alpha-synuclein (duplications) and leucine-rich repeat kinase 2 (LRRK2) (multiple point mutations; code for a protein called dardarin) can increase an individual's risk of developing PD (Singleton *et al.*, 2003). Both mutations, similarly to those involved with AD, are highly penetrant and autosomal dominant. These two mutations are the most common, and most penetrant, but there is a long list of other mutations that may increase an individual's risk of developing PD (Singleton *et al.*, 2003).

There are currently no medications that help with the pathology of PD (i.e. depletion of DA neurons in the SNpc) (Dauer & Przedborski, 2003). However, the most common drugs available for PD are those that increase DA levels in the CNS and ease the motor symptoms such as tremor, rigidity and akinesia. The most common pharmacological drugs prescribed to treat PD are levodopa, which is a precursor to DA, and ropinirole which mimics DA in the CNS (Grosset, 2007).

Alpha-Synuclein and Lewy Bodies

Alpha-synuclein is a presynaptic protein abundant in the brain, however, its function is poorly understood (Strathearn *et al.*, 2014). Disruption to the electrons in the mitochondrial electron transport chain and chronic oxidative stress (Macedo *et al.*, 2015) promotes the abnormal aggregation of α-synuclein

(Dauer & Przedborski, 2003; Strathearn *et al.*, 2014). These insoluble aggregates, referred to as Lewy Bodies, are associated with extensive neuronal damage and death, and are one of the pathological markers contributing to the underlying mechanisms associated with PD. When the dopamine-producing cells in the SNpc begin to die this gives rise to the movement-related symptoms of PD (Strathearn *et al.*, 2014). Alpha-synuclein can contribute to neurodegeneration when Lewy Bodies are released from dead or dying neurons, where they can activate surrounding microglia and promote the release of inflammatory mediators leading to neuroinflammation (Block *et al.*, 2007). It is impossible to measure the presence of Lewy bodies in an individual with PD until that person is deceased. However, we can measure their presence with *in vitro* experiments, and in the brains of rodents. Lewy bodies are not exclusive to PD either, they are found in the brains of patients with AD and other kinds of dementia. Since its discovery, alpha-synuclein has been the focus of intensive efforts to determine the protein's role in PD and its potential as a target for neuroprotective therapies.

Previous studies have determined the effect polyphenols may have on the presence of Lewy bodies in the CNS. Both Takahashi *et al.* (2015) and Macedo *et al.* (2015) showed that the addition of polyphenols lowered cellular ROS levels which subsequently reduced cytotoxicity associated with the presence of alpha-

synuclein *in vitro* and reduced alpha-synuclein aggregation. Caruana *et al.* (2011) had similar results with the addition of several phenolic compounds, including tannic acid found in berry species, to cells *in vitro*. They observed a reduction in the oligomerization of alpha-synuclein and the prevention of further neurodegeneration. This is consistent with data showing that the consumption of berry extracts with high levels of anthocyanin and proanthocyanin provides protection against mitochondrial damage and reduces the risk of PD development (Gao *et al.*, 2012).

1.10 Neuroinflammation

Neuroinflammation is inflammation of nervous system tissue which can be caused from trauma, ischemic stroke, toxic metabolites, chronic oxidative stress and prolonged neurodegeneration (Morales *et al.*, 2014). Neuroinflammation is believed to be involved in many neurological disorders, including AD and PD. An inflammatory response is normally acute in which there is some sort of stimulus that promotes the repair of damaged tissue. However, during neurodegeneration with AD and PD the stimulus remains persistent over time which constantly increases the levels of inflammatory cytokines contributing to chronic inflammation in the CNS, as well as increased ROS cytotoxicity (Morales *et al.*, 2014; Morales *et al.*, 2016).

Microglia are the innate immune cells in the CNS which are activated in response to neuroinflammation and are mainly found in the hippocampus and the SNpc in individuals with AD or PD (Venneti *et al.*, 2009; Morales *et al.*, 2014). They usually remain in a resting state but can be activated by different cues such as brain trauma, environmental stimuli, Lewy Bodies (Block *et al.*, 2007), or high concentrations of amyloid-beta (soluble and aggregated forms) are present (Morales *et al.*, 2014). When microglia become activated they transform morphologically and are responsible for regulating brain function and cell survival (Block *et al.*, 2007). However, microglia can become over-activated which induces the overproduction of compounds that in high levels can become toxic and cause extensive damage. For example, inflammatory proteins such as TNF-α, which is also a marker of microglia activation, can be upregulated and released into the surrounding area causing other microglia to become over-activated as well, further contributing to neurodegeneration (Morales *et al.*, 2014). Targeting these microglia cells is believed to potentially offer new ways to combat neurodegenerative diseases.

Polyphenols are proposed to be able to target and inhibit microglia signaling pathways before they become activated and release excess inflammatory mediators which in turn could slow down the inflammatory

cascade and progression of the disease. The proposed mechanism is that polyphenols are able to increase levels of IκBα, which binds to and inhibits NF-κB, so NF-κB remains in the cytoplasm, therefore, preventing the transcription of DNA associated with inflammatory mediators (Figueira *et al.*, 2017). Polyphenols can inhibit microglia activation via antioxidant scavenging to reduce oxidative stress (Morales *et al.*, 2016). A study conducted by Zhu *et al.* (2008), found that blueberry extract significantly reduced amyloid-beta aggregation, enhanced the microglial clearance of amyloid-beta, and reduced microglia activation.

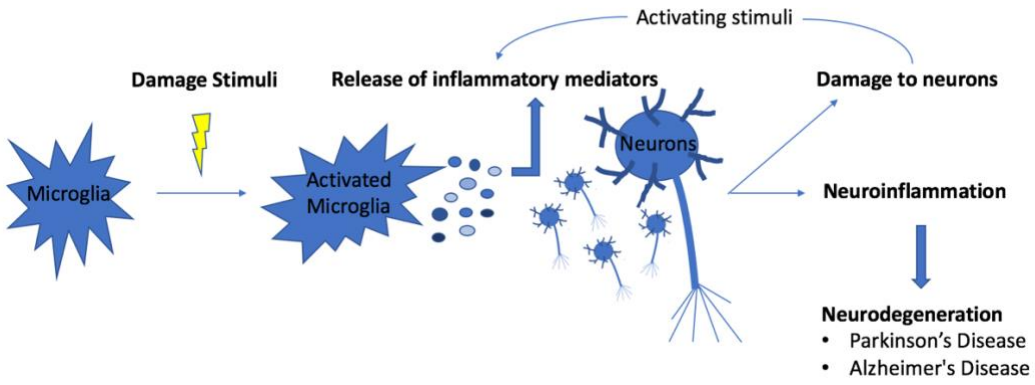


Figure 1.4: Neurodegeneration associated with neuroinflammation in the central nervous system.

Adapted from "Neuroinflammation and Neurodegeneration" by Morales *et al.* (2016) and "Neuroinflammation in the pathogenesis of Alzheimer's disease" by Morales *et al.* (2014).

1.11 Glutamate-mediated Excitotoxicity

Glutamate is an excitatory neurotransmitter, essential for sending electrical impulses from one neuron to another. Glutamate-mediated excitotoxicity is a pathological process by which cells are damaged and killed by the excessive stimulation of glutamate receptors which leads to oxidative stress (Figueira *et al.*, 2017). This abnormal process can be caused from excessive glutamate release, but is mainly influenced by inadequate uptake by glial cells though transporters (Gottlieb *et al.*, 2006). When cells are damaged, this results in a loss of ATP which inhibits the function of glutamate transporters on glial cells that are essential for removing excess glutamate from the extracellular space. Injured and damaged neural cells within the lesion site can spill glutamate into the extracellular space where glutamate can stimulate presynaptic glutamate receptors to enhance the release of additional glutamate.

Pro-inflammatory stimuli are also thought to provoke the release of glutamate by microglia. This can happen over the course of many years, like in NDD and brain aging, or very rapidly during TBI or stroke. Recent studies have found that glutamate receptors are found on glia in the brain, not just neurons. It has been suggested that these receptors may play a role in the activation of microglia in inflammation. Microglia are very sensitive to changes in their

environment, such as an increase in glutamate. Excess glutamate stimulation to glutamate receptors on microglia, can lead to over-activation of microglia (Morales *et al.*, 2016). When over-activated the microglia release excess inflammatory mediators which over time cause damage to neurons by promoting a chronic neuroinflammatory response and increasing cellular levels of ROS. An increase in intracellular Ca^{2+} levels can influence excitotoxicity as well, by causing changes in normal cellular processes and over-activating several Ca^{2+}-dependent enzymes (Adibhatla *et al.*, 2007) causing free radicals to be produced in cells (see section 1.6.1).

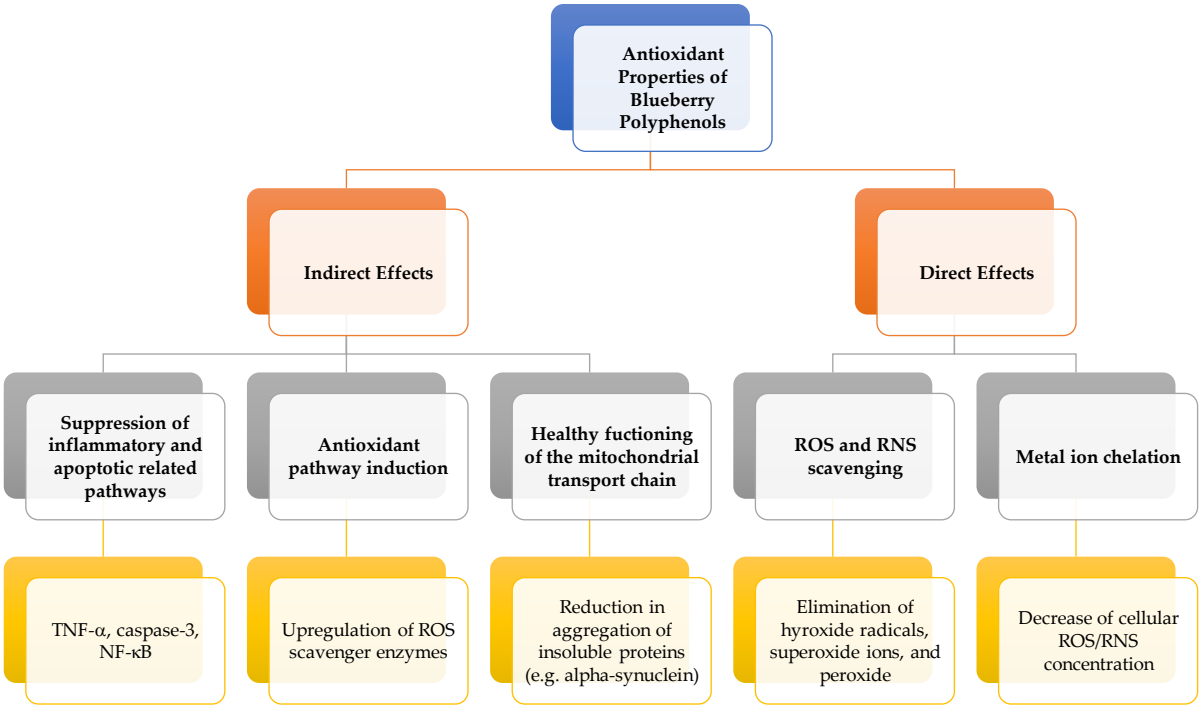


Figure 1.5: Overview of the antioxidant properties and mechanisms associated with polyphenols found in blueberry species.

Adapted from "The Role of Catechins in Cellular Responses to Oxidative Stress" by Bernatoniene and Kopustinskiene (2018).

1.12 Hypothesis

Considering blueberry extracts contain high levels of phenolic compounds which are known to have significant antioxidant activity and free radical scavenging ability, I predict that the addition of these extracts to cellular cultures *in vitro* may help combat oxidative stress and neuroinflammation in brain tissue. Secondly, I predict that the blueberry extracts will provide significant protection against glutamate-mediated excitotoxicity, and alpha-synuclein and amyloid-

beta toxicity. I predict the blueberry extracts will effectively reduce neuronal and microglial cell death, demonstrating that their presence is protective when cell death and damage is influenced by glutamate-mediated excitotoxicity, or the addition of alpha-synuclein and amyloid-beta protein.

1.13 Objectives

The objectives of this research project are:

- To conduct a biochemical analysis of wild blueberry fruits and leaves from four different locations in Newfoundland.
- To further investigate the potential neuroprotective effects of wild blueberry extracts on neurons and glial cells exposed to glutamate, alpha-synuclein, and amyloid-beta *in vitro*.
- To determine the palatability of a berry-enriched diet over the course of 5-weeks in a mouse model
- To perform a pilot study to determine the effects of wild blueberry extracts during motor and cognitive testing on C57BL/6 wild type mice.

Chapter 2: Methods and Materials

2.1 Plant collection and storage

Lowbush wild blueberries, *Vaccinium angustifolium*, were collected from two different locations in central St. John's, including two samples from Pippy Park and one Fort Amherst sample, as well as one sample from Keels, located in northcentral Newfoundland. Both blueberry fruits and leaves were collected from these locations in September 2016. After collection, the samples were immediately placed into a cooler with icepacks, then put into a -20°C freezer within 1.5 hours of collection and later transferred to a -80°C freezer within 24-hours.

2.2 Blueberry fruit and leaf extraction

2.2.1 Fruit extraction

Solvent was prepared consisting of 80% (v/v) acetone, 0.2% formic acid, and 19.8% distilled water (dH_2O). Blueberry fruits were removed from the -80°C freezer and thawed at room temperature (~20°C). Debris, such as vines and dirt were removed, as well as extensively damaged berries. The blueberry fruits were placed in a mortar and pestle and mashed. Weight was measured, and then solvent was added to dilute the ground blueberry fruits to a concentration of 1:2. The solution was then mixed by vortex for 20 seconds, placed in a cooler with ice

and shaken for 30 minutes. The solution was then centrifuged at 4°C for 20 minutes at 10 000 G (gravitational force). Supernatant was then drained in a separate container, and the whole procedure was repeated using the remaining residue. The final concentration was 250 mg/ml solvent of fresh weight, which is the weight of the specimen including its water content.

2.2.3 Leaf extraction

Healthy green leaves were selected, removed from their vines, and placed in a mortar and pestle. Liquid nitrogen was slowly added to the blueberry leaves with constant stirring. This helped with the breakdown of cellulose resulting in the leaves turning into a fine dry powder. Then the solvent was added to the powdered blueberry leaves at a concentration of 1:2. The solution was mixed by vortex for 20 seconds, placed in a cooler with ice and shaken for 30 minutes. The solution was then centrifuged at 4°C for 20 minutes at 10 000 G. Supernatant was drained in a separate container, and the whole procedure was repeated using the remaining residue. The final concentration was 250 mg/ml solvent of fresh weight, which is the weight of the specimen including its water content.

2.3 Biochemical assays

All stock blueberry fruit and leaf extracts were used for the biochemical assays within one week of extraction, and all experiments were repeated three

times for each assay. All assays followed the same methods as Vyas *et al.* (2013). Further dilution was necessary for the stock extracts at 1:10 (stock:solvent) for the fruit and 1:100 for the leaves, to a final concentration of 25 mg/ml of fruit and 2.5 mg/ml of leaf fresh weight. Distilled H_2O was used for the blank and used to rinse out the quartz cubes between samples.

2.3.1 Total antioxidant capacity

Total antioxidant capacity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, which is a stable free radical. DPPH is violet in colour, but when it is neutralized a colourimetric reaction occurs that makes the solution lighter in colour. This method allows for the quantification of free radical scavenging ability of the sample, as antioxidants are able to neutralize the radical DPPH. The method was adapted from Vyas *et al.* (2013), and Brand-Williams *et al.* (1995), where 100 μl of blueberry extract was added to 1.9 mL of 0.04 mM stock DPPH. The solution was then mixed by vortex for 20 seconds, protected from light for 20 minutes and measured with the spectrometer at 517 nm. Total antioxidant capacity was expressed as '% of inhibition of DPPH consumption', and the results were expressed as 'Gallic Acid Equivalents' (GAE) using the Gallic acid standard curve.

2.3.2 Total phenolic content

In order to determine the total phenolic content of the blueberry fruit and leaf extracts, Folin-Ciocalteu reagent was used. The method for this assay was adapted from Chandrasekara and Shahidi (2011). Folin-Ciocalteu is a redox reagent that is blue in colour; the higher the phenolic content the darker the blue. 0.5 mL of blueberry extract was added to 0.5 mL of Folin-Ciocalteu reagent. Then, 1 mL of sodium carbonate (Na_2CO_3; 25 g of Na_2CO_3 into 250 mL dH_2O) and 8 mL of dH_2O was added to the solution and mixed by vortex for 30 seconds. The solution was protected from light for 35 minutes, and then centrifuged at room temperature for 10 minutes at 1000 G. Absorbance was measured at 725 nm using the spectrometer. Total phenolic content was then determined using a Gallic acid standard curve, and the results were expressed as milligrams of GAE per gram of fresh weight.

2.3.3 Total flavonoid content

The method was adapted from Zhishen *et al.* (1999), and the results were expressed as μmol of catechin equivalents (CE) per gram of sample. To determine the total flavonoid content of the samples, 1 mL of extract was added to 4 mL of dH_2O, and 0.3 mL of 5% sodium nitrite ($NaNO_2$) and left to sit for 5 minutes. Then, 0.3 mL of 10% aluminum chloride ($AlCl_3$) was added, and one minute later

2 mL of sodium hydroxide (NaOH) and 24 mL of dH_2O was mixed in. The solution was protected from light for 15 minutes, and then centrifuged at room temperature for 4 minutes at 4000 G. The solution was measured at 510 nm with the spectrometer.

2.3.4 Total tannin content

Total tannin content was determined using vanillin-hydrogen chloride reagent (0.5 g of vanillin added to 96 mL methanol [MeOH], plus 4 mL of HCl). For this assay, 1 mL of blueberry extract was added to 5 mL of 9.5% vanillin-HCl reagent and the resulting colourimetric reaction produced a bright red colour. The solution was then incubated and protected from light for 20 minutes and the absorbance was measured at 500 nm using the spectrometer. The results were expressed as micromoles (μmol) of CE per gram of sample. The method for total tannin content was adapted from Chandrasekara and Shahidi (2011).

2.3.5 Total anthocyanin content

This assay required two solutions:

i. Potassium chloride buffer

   1.86 g KCl + 980 mL dH_2O → pH=1, 0.025 M

ii. Sodium acetate buffer

   54.43 g $NaCO_2CH_3$ + 3 dH_2O → pH=4.5, 0.4 M

The two solutions were mixed together and incubated at room temperature for 40 minutes. 1 mL of blueberry extract was added to 4 mL of $KCl/NaCO_2CH_3$ buffer for a total volume of 5 mL, and placed in the dark for 15 minutes. The absorbance was measured at both 510 nm and 700 nm using the spectrometer. The corrected absorbance result was calculated as ([A520 – A700] pH=1.0 – [A520 – A700] pH=4.5). The method was adapted from Foley and Debnath (2007), and the results are expressed as GAE

2.4 Cell culture experiments

For the cell culture experiments the Pippy Park (2/09/16) blueberry fruit sample was used. This was determined based on the high quantity and quality of this sample, as it contained the highest total antioxidant activity compared to samples from the other locations. Samples from Pippy Park (2014) were selected for previous studies in our lab where high-performance liquid chromatography-mass spectrometry (HPLC-MS) was performed. All chemicals and reagents used for cell culturing were filtered through a syringe or bottle filter to ensure sterility, and 70% ethanol was used as the disinfectant. All steps for the cell culture experiments were conducted under a biosafety cabinet, with exception of the actual dissection, and media was always placed in a water bath at 37°C for 10 minutes before the media was changed. Mixed cultures of neurons and glia were

produced from dissected C57BL/6 strain mouse-pup cortical brain tissue, while pure cultures of either cortical neurons or microglia were purchased from a commercial source (ScienCell Research Laboratories, Carlsbad, CA). Mixed cultures were used to represent the conditions present in the brain where both neurons and many different types of glia reside and can influence the concentration of excess glutamate, alpha-synuclein, and amyloid-beta. Pure cultures of neurons and microglia were purchased because the breeding pairs from our colony were not producing enough pups for us to produce our own pure cultures at an adequate level. Pure cultures of microglia and neurons allowed us to determine the direct effect the addition of glutamate and high concentrations of alpha-synuclein and amyloid-beta had on specific cell types. The sensitivities could then be analyzed among the cell types to see how microglia and neurons specifically reacted to glutamate, alpha-synuclein, or amyloid-beta. Mouse-derived neurons and microglia were not available from ScienCell when the experiments were being conducted so rat cultures were used instead.

2.4.1 Dissection plates

The animals used for the dissection process were C57BL/6 mouse pups aged one to three days old. They were from breeding pairs located in our mouse

lab colony in the Health Sciences Centre (HSC) Animal Care facility. All protocols and procedures were approved by the Institutional Animal Care Committee (IACC) of Memorial University of Newfoundland (MUN). Twenty-four hours prior to the dissection, 24-well Costar® culture plates or 16-well glass chamber slides were coated with 500 μg/mL poly-L-ornthine (PLO) solution to ensure cell adherence to the flat bottom wells. PLO solution was prepared by dissolving 100 mg of PLO in 10 mL of dH_2O, which was then aliquoted into 1.5 mL centrifuge tubes, with 300 μL per tube and stored at -80°C. These aliquots were diluted before plating by mixing 5.7 mL dH_2O to the 300 μL aliquot, resulting in a PLO concentration of 500 μg/mL. For the Costar® plates each well received 250 μL of PLO and the 16-well glass slides received 100 μL of PLO per well. Plates were then incubated overnight at 37°C and 5% carbon dioxide (CO_2). In the morning of the dissection each well was washed twice with dH_2O before cells were plated.

Dissection procedure:

All tools were first rinsed in 70% ethanol. Two small petri dishes and two 15 mL test tubes were placed on ice and filled with 10 mL dissection media, which consisted of 100 mL of Hank's Buffered Salt Solution (HBSS) and 1 mL penicillin/streptomycin (PenStrep). The mouse-pup was quickly decapitated with

large scissors, and the body discarded. The skin on the skull was cut posterior to anterior using small scissors. A lateral cut was made by going into the foramen magnum on both sides of the skull and cutting towards the front of the head. The skin was then peeled back, where the skull was visible and then cut medially. The brain was carefully removed and then placed in a petri dish with ice cold dissection media. A scalpel was used to remove the cerebellum, before using a dissection scope to carefully remove the meninges with fine forceps. The whole brain (minus the cerebellum) was then placed in one of the 15 mL tubes containing fresh dissection media.

The tissue was then washed by centrifuge at 1000 revolutions per minute (rpm) at 4°C for 3 minutes. Supernatant was removed, and replaced with 10 mL of fresh dissection media, and washed again with the same conditions as above. Supernatant was removed, and replaced with 0.8 mL of 0.25% Trypsin-EDTA (see Table 2.1) diluted in 9.2 mL fresh dissection media, and incubated at 37°C for 15 minutes. Supernatant was again discarded and the tissue was washed with 10 mL growth media at 1200 rpm at 4°C for 5 minutes. Cells were triturated with 4 mL fresh growth media using a 10 mL pipette, and then a 100 μL pipette tip once large aggregates were broken down. The solution was filtered through a 70 nm nylon strainer into a 50 mL test tube, and diluted with growth media for a total

volume of 12 mL. For the 24-well Costar® plates each well received 250 μL of solution and for the 16-well glass slides, each well received 100 μL. Plates were then incubated at 37°C and 5% carbon dioxide (CO_2) for 10 to 16 days. The plates were undisturbed for 24-hours after initial plating, followed by a complete media change. This entailed drawing off all of the dead cells and adding 500 μL fresh culture media to the Costar® plates and 150 μL for the 16-well glass slides. Subsequently, the media was changed every 2 to 3 days, where 90% of the media was drawn from each well and then replaced with fresh culture media. For the Costar® plates 450 μL was drawn from each well and 500 μL of fresh culture media added. For the 16-well glass slides 135 μL was taken out and 150 μL fresh culture media was added to the wells.

Table 2.1: List of chemicals and reagents used for cultured cells.

Chemical/Reagent	**Recipe**
Dissection media	100 mL HBSS + 1 mL PenStrep
0.1% Trypsin-EDTA Solution	100 mL Trypsin-EDTA + 100 mL HBSS + 1 mL PenStrep
32.5% Glucose Solution	32.5 g Glucose + 67.5 mL dH_2O

Sodium Pyruvate Solution	550 mg Sodium pyruvate + 50 mL dH_2O
Growth Media	500 mL sterile basal medium + 5 mL PenStrep + 8 mL 32.5% glucose solution + 5 mL sodium pyruvate + 5 mL N2 supplements + 5 mL amphotericin B + 50 mL horse serum
Culture Media	500 mL sterile basal medium + 5 mL PenStrep + 8 mL 32.5% glucose solution + 5 mL sodium pyruvate + 5 mL N2 supplements + 10 mL B27 supplements

2.4.2 Rat neuron plates

Neurons were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) which were isolated from postnatal day 2 CD® IGS rat brains,

cryopreserved after purification and frozen at -80°C prior to use. Plates were incubated at 37°C overnight with 500 μg/mL PLO solution. Specific neuronal medium (ScienCell) was prepared at our lab, which consisted of 500 mL sterile neuronal medium, 5 mL Neuronal Growth Supplement, and 5 mL of penicillin/streptomycin solution stored at 4°C. The optimal growth period for the ScienCell rat neurons was 7-10 days. Each vial contains >1 x 10^6 cells in 1 mL volume, with an optimal density between 20,000-25,000 cells/cm^2:

- Total μL of cells/number of wells= X μL per well

  X μL per well/SA of well= cells/cm^2 (density)
- With 24-well Costar® plates well SA=1.9 cm^2, results in ~41,700 cells per well per plate and 21,929 cells/cm^2 with 1 mL of media
- With 16-well glass slides well SA=0.4 cm^2, results in ~8928 cells per well and 22,321 cells/cm^2 with 200 μL of media

2.4.3 Rat microglia plates

Microglia were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) which were isolated from postnatal day 2 CD® IGS rat brains, cryopreserved after purification and frozen at -80°C prior to use. Specific microglia medium (ScienCell) was prepared at our lab, which consisted of 500 mL of sterile microglia medium, 5 mL of penicillin/streptomycin solution, 25 mL

fetal bovine serum, and 5 mL Microglia Growth Supplement stored at 4°C. The optimal growth period for the ScienCell rat microglia was 3 to 6 days. The rat microglia were plated with the same densities as the ScienCell neurons, however PLO was not used for the microglia plates, as previous studies in our lab found adequate adherence of microglia without the application of PLO.

2.4.4 Cell treatments

Once cells were plated and started to differentiate during their optimal growth (10 to 16 days for dissection plates; 7 to 10 days for ScienCell rat neurons; 3 to 6 days for ScienCell rat microglia) the cells were given a particular treatment and then placed back into the incubator at 5% CO_2 and 37°C for 24-hours. After treatment, the cells were then fixed, stained, and analyzed. Stock solutions of glutamate, alpha-synuclein, amyloid-beta and berry extract were produced, aliquoted, sterilized, and stored in a -80°C freezer. The control treatment was composed of 10 μL sterile dH_2O/ mL of media.

Aqueous Berry:

This stock was made by thawing 10 to 20 frozen blueberry fruits from the Pippy Park (2/09/16) sample at room temperature and then mashing them in a mortar and pestle. The mixture was filtered through a 70 nm nylon strainer into a 50 mL test tube, sterilized, aliquoted in 1.5 mL centrifuge tubes, and stored in a

-20°C freezer. This solution was used alone, to see if the extract had any effect on the viability of cells, or with glutamate, alpha-synuclein, or amyloid-beta to see if the extract provided any cellular protection. This concentration was previously determined by the research group to be the optimal concentration to elicit a neuroprotective effect.

- 1 μL blueberry extract stock/ mL of media

Glutamate:

A stock solution of 10 mM glutamate was made, where 10 ml of sterile dH_2O was added to 14.7 mg of L-glutamic acid (Research Biochemicals Inc.) and sterile filtered. This concentration was previously determined by our lab to be the optimal glutamate concentration causing neuro-inflammation.

- 10 μL of 10 mM glutamate stock/ mL of media for a final concentration of 100 μM glutamate

Glutamate + aqueous berry:

- 10 μL of 10 mM glutamate stock/ mL of media for a final concentration of 100 μM glutamate
- 1 μL blueberry extract stock/ mL of media

Alpha-synuclein:

A stock solution of alpha-synuclein was made by dissolving 100 μg/ml of alpha-synuclein into 5 mL of dH_2O. This concentration was previously determined by our lab to be the optimal alpha-synuclein concentration causing neuro-inflammation.

- 1 μL alpha-synuclein stock/ mL of media for a final concentration of 100 ng/mL alpha-synuclein

Alpha-synuclein + aqueous berry:

- 1 μL alpha-synuclein stock/ mL of media for a final concentration of 100 ng/mL alpha-synuclein
- 1 μL blueberry extract stock/ mL of media

Amyloid-beta:

Amyloid-beta (rPeptide®) from recombinant DNA encoding for the human beta-amyloid sequence (1-42) expressed in E. coil, was prepared by first diluting 0.5 mg of amyloid-beta in 0.5 mL of 1% ammonium hydroxide (NH_4OH) to a stock concentration of 221 μM. This solution was then sonicated for 1 minute. The second dilution was with cell culture media when amyloid-beta was put into the individual wells to achieve a final concentration of 7.5 μM as described by

Nafar and Mearow (2014). The solution was then sterilized with a syringe filter, aliquoted in 1.5 mL centrifuge tubes and stored in a -80°C freezer.

- 33.86 μL amyloid-beta stock/ mL of media for a final concentration of 7.5 μM amyloid-beta

Amyloid-beta + aqueous berry:

- 33.86 μL amyloid-beta stock/ mL of media for a final concentration of 7.5 μM amyloid-beta
- 1 μL blueberry extract stock/ mL of media

2.5 Immunohistochemistry and imaging

After 24-hours of treatment the cells were fixed and then labeled/stained. Media was removed from each well and put into a 1.5 mL centrifuge tube. The tube was then dipped in liquid nitrogen for 30 seconds and immediately put into a -80°C freezer. The following steps use 250 μL of reagent for the 24-well plates and 150 μL of reagent for the 16-well glass slides unless otherwise specified.

Once the media was removed the wells were washed twice with Phosphate Buffer Solution (PBS), and fixed for 20 minutes with 4% Paraformaldehyde Fixative (PFA), cultures were then permeabilized with 0.2% Triton X for 10 minutes, and washed twice with PBS.

Table 2.2: Chemicals and reagents used for staining and fixing cultured cells.

Chemical/Reagent	**Recipe**
Phosphate Buffer Solution (PBS)	500 mL 0.2 M phosphate buffer + 500 mL dH_2O + 9 g sodium chloride pH=7.4
4% Paraformaldehyde Fixative (PFA)	400 mL dH_2O raised to 70°C + 40 g paraformaldehyde + 10 drops of sodium hydroxide added slowly + 500 mL 0.2 M PBS + dH_2O until total volume reaches 1000 mL
0.2% Triton X	2 mL 10% Triton X + 98 mL PBS
Blocking Solution	2g 2% bovine serium albumin + 2 mL 2% fetal calf serum + 200mg 0.2% fish skin

	gelatin + 98 mL PBS + mixed with heat

2.5.1 Labeling and staining of cells

After the fixing procedure the cultured cells were labeled with antibody (Ab) solutions. First the cultures were incubated with blocking solution for 30 minutes to inhibit non-specific binding, and then incubated for 1 hour with primary antibody diluted with PBS (90%) and blocking solution (10%). After the incubation period the wells were washed twice with PBS, and then incubated for an hour with the appropriate secondary antibody diluted with PBS (90%) and blocking solution (10%).

Table 2.3: Primary and secondary antibodies used for labeling and their concentrations.

Primary Antibodies:	**Secondary Antibodies:**
Neurons: • Microtubule-associated protein 2 (MAP-2) Ab (1:500) • Neuronal nuclei (NeuN) Ab (1:500) Microglia	• Alexa 488 goat anti-mouse (green; 1:300) • Alexa 488 goat anti-rabbit (green; 1:300) • Alexa 488 goat anti-rat (green; 1:200)

• IbA-1 Ab (1:500) Astrocytes • Glial fibrillary acidic protein (GFAP) Ab (1:500) Apoptosis • Caspase 3 Ab (1:1000)	• Alexa 594 goat anti-rabbit (red; 1:300)

After labeling with the secondary Ab the cultures were washed twice with PBS, and the wells were then dehydrated with 70% ethanol, followed by further dehydration with 100% ethanol. Once each well dried completely, 10 μl of VectaShield mounting medium with the fluorescent stain 4', 6-diamidino-2-phenylindole (DAPI) was added to all the Costar® wells and then sealed with a 15 mm glass coverslip. This stain is able to pass through an intact cell membrane and therefore labels the nucleus of both live and fixed cells. For the 16-well glass slides once fixing was finished, the plastic well chambers were snapped off and a scalpel was used to scrape off the remaining silicon on the glass slide. Approximately 5 to 8 μl of VectaShield was added to the slide where each well used to be, and the slide was completely covered with one glass coverslip. Clear nail polish was put around the edges to ensure the coverslip was stuck in place and prevented the slide from drying out. All plates and slides were wrapped in

tinfoil to protect the fluorescence from light, stored in a -20°C freezer and then imaged within a few days.

2.5.2 Imaging

All Costar® cell culture images were captured with a Zeiss A1 Observer inverted microscope with a PCO.Pixelfly qe CCD camera at a magnification of 40X using AxioVision and PCO Camware software. A total of five images were taken from separate locations in each well, then cells were counted manually by two individuals and the counts were averaged. The 16-well glass slides were imaged by Dr. Noriko Daneshtalab using an inverted Olympus Fluoview FV 1000 confocal microscope at 40X, 3.5x zoom, and 65 to 79% light intensity for DAPI and Alexa 488 fluorescence.

Figure 2.1: Zeiss Axio Observer. A1 microscope, Sensicam PCO. Pixelfly V1.101.0 qe CCD camera connected to AxioVision and PCO. Camware software.

2.5.3 Apoptotic counts

Apoptotic counts were conducted manually for the cell culture plates, however, due to calibration issues with the Sensicam PCO. Pixelfly camera the counts were not reliable or consistent and are therefore not included. Images were not of sufficient quality to reliably determine if cells were potentially apoptotic.

2.6 Pilot blueberry diet study

This study was conducted in August to September of 2017, and took place over 34 days (start date: Aug 8, 2017; end date: Sept 11, 2017). This diet study was mainly to determine the palatability of using a blueberry and pellet-powder "mash" over an extended period of time. Up until this point other research groups, including our own, mostly focused on oral gavage for administration of a special diet (Atcha *et al.*, 2010; Grace *et al.*, 2009; El-Mohsen *et al.*, 2006). This method can be very invasive and has been shown to induce significant stress in the animal, including increased plasma concentrations of corticosterone, glucose, growth hormone, and prolactin (Balcombe *et al.* 2004). As behavioral experiments were being conducted during the study it was important to minimize stress that could confound the results. The animals were able to freely eat the mash just as they would freely eat their regular pellets, which minimized stress and the

potential for severe internal damage. Oral gavage when not conducted by someone highly experienced can cause extensive throat and oral damage, lung perforation and aspiration, and restraint-associated injuries (Vandenberg *et al.* 2014). Also, oral gavage bypasses the oral and esophageal mucosa and epithelia which can lead to potential differences with the absorption, breakdown of metabolites and bioavailability of the compounds of interest.

There were 18 C57BL/6 mice used in this study from our breeding colony in the HSC Animal Care facility at MUN. All protocols and procedures were approved by the IACC MUN. Conditions included a standard 12-hour light and 12-hour dark cycle. There were two groups, nine animals in each consisting of a control group and an experimental group that received the berry-enriched diet. The animals in the colony were both male and female and varied in age. The animals were assigned to a group by a member of the lab not involved in the study, based on these characteristics and not their starting body weight or performance during rotarod training (see Table 2.4 below). Animals were housed individually overnight when the control or blueberry-enriched diet was presented to them. During the day, any animals that were housed together were put back into a single cage (1-3 animals) to socialize in hopes to reduce anxiety and depression-like behaviors (Martin & Brown 2010). The main focus of this

study was to ensure that the mice would voluntarily eat the prepared mash, and ensure that there were no weight fluctuations or overt behavioral changes associated with its consumption.

Table 2.4: Age and sex of control and experimental C57BL/6 wild type mice used in palatability diet study.

Subject Number	**Condition**	**Birthday**	**Age**	**Sex**
1	Control	23-Feb-17	<6mo-≦3mo	M
2	Control	26-Jan-17	≧6mo	M
3	Control	26-Jan-17	≧6mo	M
4	Control	03-Apr-17	<6mo-≦3mo	F
5	Control	03-Apr-17	<6mo-≦3mo	F
6	DIED FROM SEZIURE DAY 10			F
7	Control	12-Jun-17	<3mo	F
8	Control	03-Apr-17	<6mo-≦3mo	M
9	Control	03-Apr-17	<6mo-≦3mo	M
1	Experimental	26-Jan-17	≧6mo	F
2	Experimental	26-Jan-17	≧6mo	F
3	Experimental	03-Apr-17	<6mo-≦3mo	M

4	Experimental	03-Apr-17	<6mo-≦3mo	M
5	Experimental	12-Jun-17	<3mo	F
6	Experimental	12-Jun-17	<3mo	F
7	Experimental	26-Jan-16	≧6mo	M
8	Experimental	26-Jan-17	≧6mo	M
9	Experimental	03-Apr-17	<6mo-≦3mo	F

2.6.1 Preparation of mouse feed with blueberry extract

Fresh mash was made daily in the afternoon from frozen Pippy Park (2/09/16) blueberry fruits that were stored in -80°C and then thawed at room temperature. The berries were mashed in a mortar and pestle until they were grinded into a liquid state, including all parts of the berry (i.e. skins, seeds). The normal pellets given to the mice in the animal care facility at MUN were then ground into a very fine powder and mixed with distilled water at 1:1 (eg. 50 g of powder: 50 mL of dH_2O). Each mouse in the experiment was given 5 g of mash every day (Bachmanov *et al.* 2004). The control group just received mash that consisted of the pellet powder mixed with dH_2O as the solvent. For the experimental group 0.1 g (2% of 5 g) of the grinded blueberries were measured and then mixed directly with 5g of the mash. The mash was presented to both

groups in a small petri dish, which were labeled and washed daily and the mice were always given access to water. For the blueberry-enriched diet, 2% fruit extract was chosen based on previous studies including Andres-Lacueva *et al*. (2005) and Kalt *et al*. (2008) where 2% berry extract yielded the best results.

2.6.2 Daily procedures

Every day the mice were weighed in the morning, and those housed together before the experiment began were allowed to spend some time together in the same cage, if they were in the same group. At this time, regular food pellets from Animal Care were returned to the cages and any tests, such as rotarod or novel object recognition were performed. Later in the afternoon the animals were placed back in their individual cages for the night and the pellets were removed from the cage. The mice were then given the control mash, or the 2% blueberry mash. The next morning, the empty dishes that held the mash were removed during weighing.

2.6.3 Rotarod

The rotarod is a tool used to measure motor coordination, balance, and endurance of an animal by measuring how long they are able to stay on a rotating rod. The animal will try to avoid falling off of the rod as best they can, but the rod is not high enough to harm the animal even if they do fall off.

Training was conducted over the course of three days where animals were acclimated to the process of rotarod testing and were placed on the rotarod at 4rpm for 5 minutes. Training was conducted on August 1 to 3 2017, five days before the beginning of the diet study. For this study, fixed-speed rotarod testing was used where the rod was set at a constant speed, either 5 rpm, 10 rpm, or 15 rpm without acceleration for 120 seconds. Once the animal was securely on the rotating rod, the timer was set and would continue until the animal fell and triggered the time-stop or was able to stay on the rod for the full 120 seconds. All animals were tested 3x at each fixed speed (5 rpm, 10 rpm and 15 rpm) during each session. Rotarod testing was performed twice a week, with a total of 8 sessions over the duration of the study.

2.6.4 Novel Object Recognition

Novel object recognition (NOR) is used to assess non-spatial memory. The mice were first habituated to the box for 5 minutes on two days prior to testing, there were no objects in the box at this time. On test day, the mice were placed into the box with two identical objects for 5 minutes (AA or BB). An hour later mice were placed back in the box with the familiar object and a novel object (A/B) for five minutes. Then, 24-hours later, the mice were placed back in the box with the familiar object and novel object (A/B) for five minutes. The videos were

scored based on the animals exploration of the novel object, exploration of the familiar object, total exploration time and discrimination index ([exploration of novel - exploration of familiar]/[total exploration time]). When the discrimination index (DI) is greater than zero it indicates that the animal explored the novel object more than the familiar object and retained a memory of the familiar object (as delay is increased memory retention should become more difficult). Videos were scored by two individuals and reliability statistics were determined.

2.6.5 Blood collection

Blood was collected from three subjects from each of the groups halfway through the study (week of August 21st, 2017), however, there were insufficient amounts to conduct any analysis and were therefore discarded. Further attempts were not made in order minimize any stress for the animals considering the small sample size.

2.6.6 Brain collection

All brains from the mice in the pilot diet study (except for control 6) were collected at the end of the study. Animals were placed into a container where a small amount of halothane/isoflurane was present for about 1 minute. Anesthetization was confirmed when there was no blink reflex after lightly touching the animals eye. Mice were then decapitated using an animal guillotine

and brains were collected (see cell culture dissection procedure). The brains were then dipped in liquid nitrogen for 1 minute, placed in a 15 mL test tube, and stored in a -80°C freezer. Samples are currently awaiting analysis.

2.7 Statistical analysis

For the biochemical analysis of the blueberry samples from Pippy Park, Fort Amherst, and Keels, each experiment was repeated three times and statistically significant results between the blueberry fruits and leaves for the samples were determined via two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$) using GraphPad: Prism-7, data represented as mean ± standard error (SE). Significant differences between the fruits from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$) using SPSS-39, data represented as mean ± SE. Significant differences between the leaves from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$) using SPSS-39, data represented as mean ± SE. For the cell culture experiments, statistically significant results between the different treatment conditions was determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$) using GraphPad: Prism-7, data represented as mean ± SE. Weight, rotarod, and NOR results for the diet study including differences between the control and experimental group were analyzed via two-way ANOVA and *post hoc* Bonferroni

test ($p<0.05$) using GraphPad: Prism-7, data represented as mean ± SE. For the NOR testing, reliability statistics were also conducted for the NOR video scoring using Cronbach's Alpha in SPSS-39. Cronbach's Alpha measures consistency and reliability by comparing the scores from the two individuals that scored the NOR videos.

Chapter 3: Results

3.1 Biochemical Assays

Figures 3.1 to 3.15 show the results of biochemical assays conducted for blueberry fruits and leaf samples from Pippy Park (2/09/16) and (21/09/16), Keels (17/09/17), and Fort Amherst (25/08/17). Overall, the leaves were higher for total antioxidant activity (Figure 3.1), total phenolic content (Figure 3.4), and total flavonoid content (Figure 3.7). Total anthocyanin content (Figure 3.13) was the exception where the fruits were significantly higher than the leaves. Total tannin content (Figure 3.10) had mixed results where both Pippy Park samples had higher anthocyanin levels in the leaves, and Keels and Ft. Amherst had higher levels in the fruits. Even though the locations were geographically close together, there were significant differences between the different samples for both fruit and leaves which varied based on the assay. All biochemical assays were repeated three times for each sample, and all samples were collected in 2016. Significant differences between the fruits and leaves of the sample were determined via two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$), represented as 0.033(*), 0.002 (**), and <0.0001(***). Significant differences between the fruits from the four samples and the leaves from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$).

Different letters (a, b, c, d) indicate statistical significance between locations at $p<0.05$.

3.1.1 Total antioxidant activity

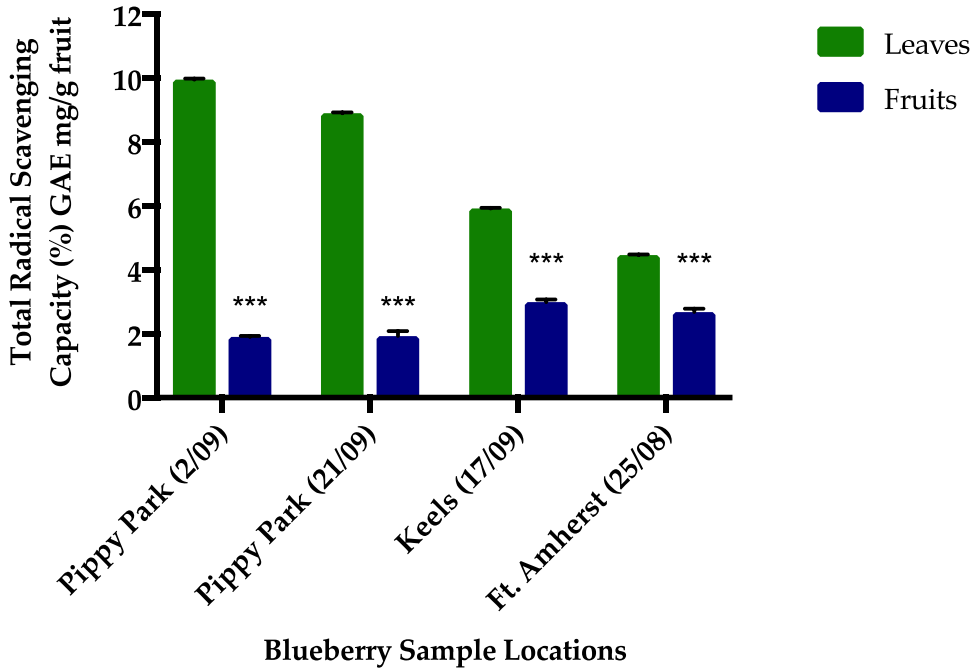


Figure 3.1: Total antioxidant activity of blueberry fruit vs leaf extracts.

Significant differences between the fruits and leaves of the sample were determined via two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$), represented as 0.033(*), 0.002 (**), and <0.0001(***). Significant difference between the fruits and the leaves are observed for all four locations, where leaves had a higher total antioxidant activity than the fruits ($p=<0.0001$, DF=16). Leaves from Pippy Park (2/09/16) and fruits from Keels had the highest total antioxidant activity. The data are expressed as the mean ± SE.

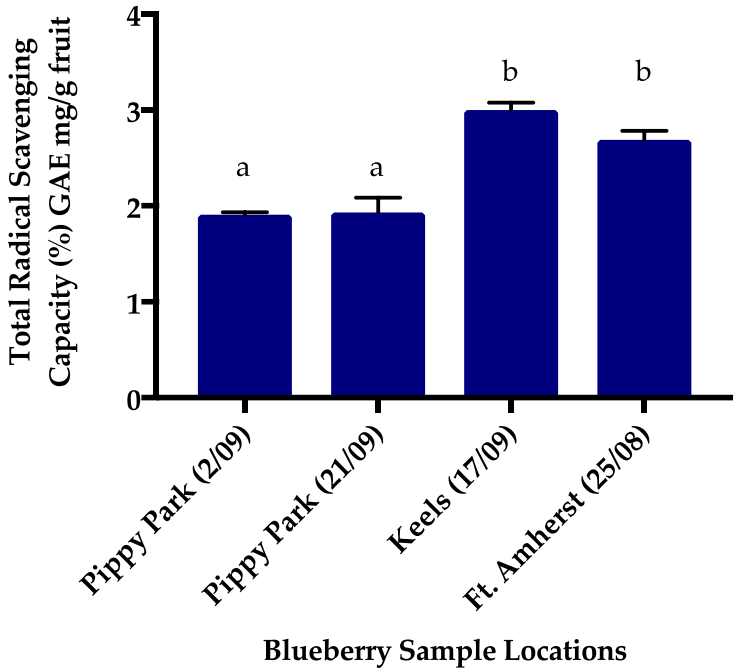


Figure 3.2: Total antioxidant activity of the blueberry fruit extracts.

Significant differences between the fruits from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences for the total antioxidant activity of the fruits are between the Pippy Park samples and Keels, and the Pippy Park samples and Ft. Amherst ($p=<0.0001$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.1.

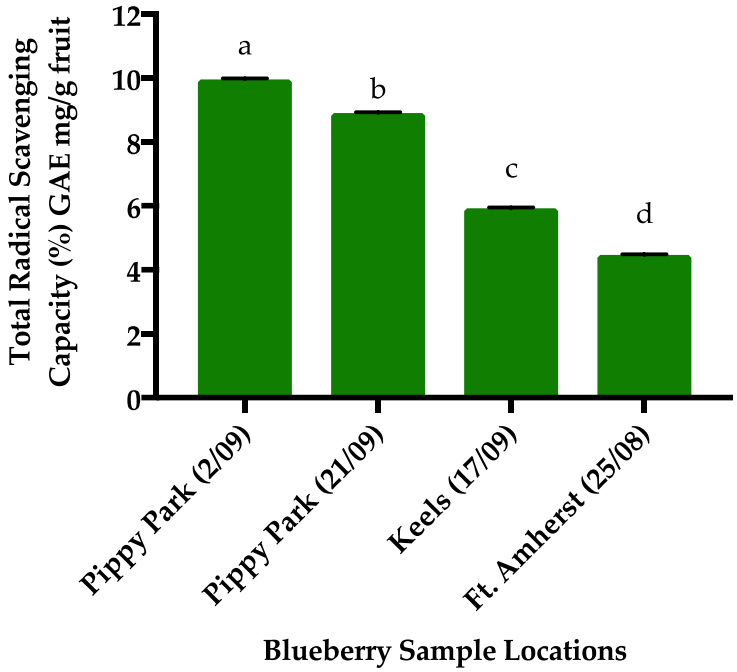


Figure 3.3: Total antioxidant activity of the blueberry leaf extracts.

Significant differences between the leaves from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences for the total antioxidant activity of the leaves are between all four samples ($p=<0.0001$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.1.

3.1.2 Total phenolic content

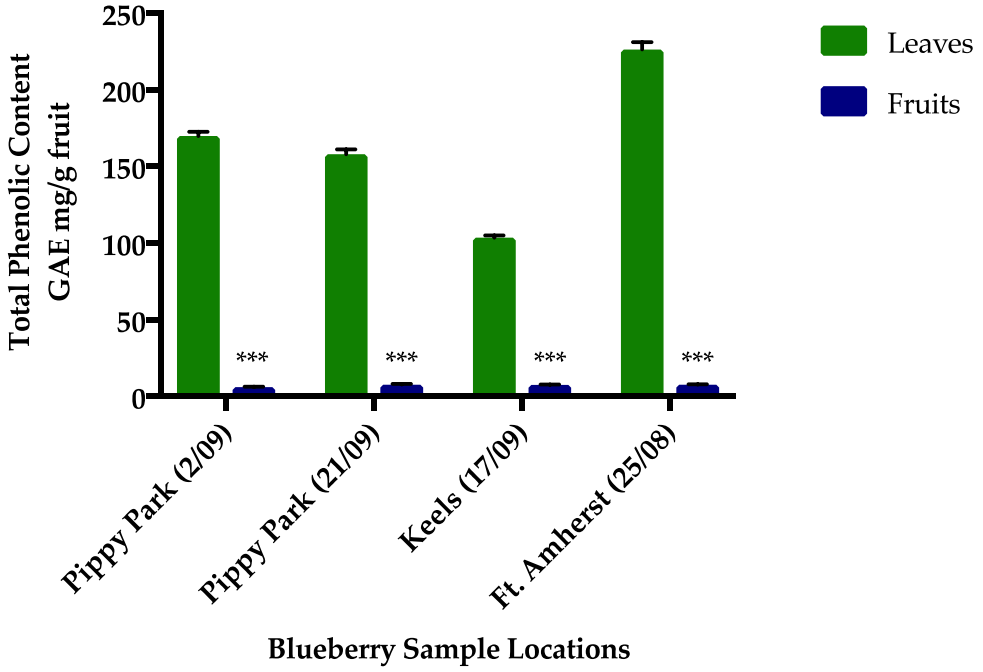


Figure 3.4: Total phenolic content of blueberry fruit vs leaf extracts.

Significant differences between the fruits and leaves of the sample were determined via two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$), represented as 0.033(*), 0.002 (**), and <0.0001(***). There is a significant difference between the fruits and the leaves observed for all four locations where leaves had a higher total phenolic content than the fruits (p=<0.0001, DF=16). Leaves from Ft. Amherst and fruits from Pippy Park (21/09/16) had the highest total phenolic content. The data are expressed as the mean ± SE.

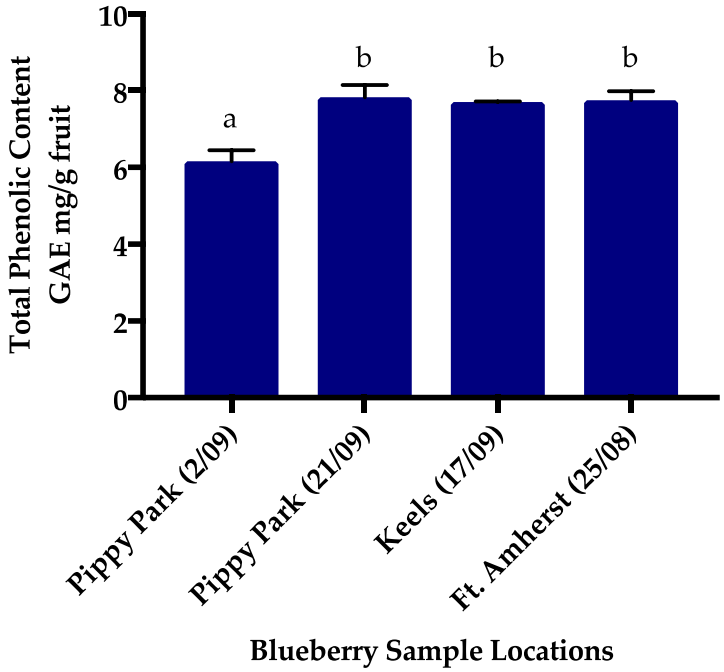


Figure 3.5: Total phenolic content of the blueberry fruit extracts.

Significant differences between the fruits from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences for the total phenolic content of the fruits are between Pippy Park (2/09/16) and Pippy Park (21/09/16), Pippy Park (2/09/16) and Keels, and between Pippy Park (2/09/16) and Ft. Amherst ($p=0.003$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.4.

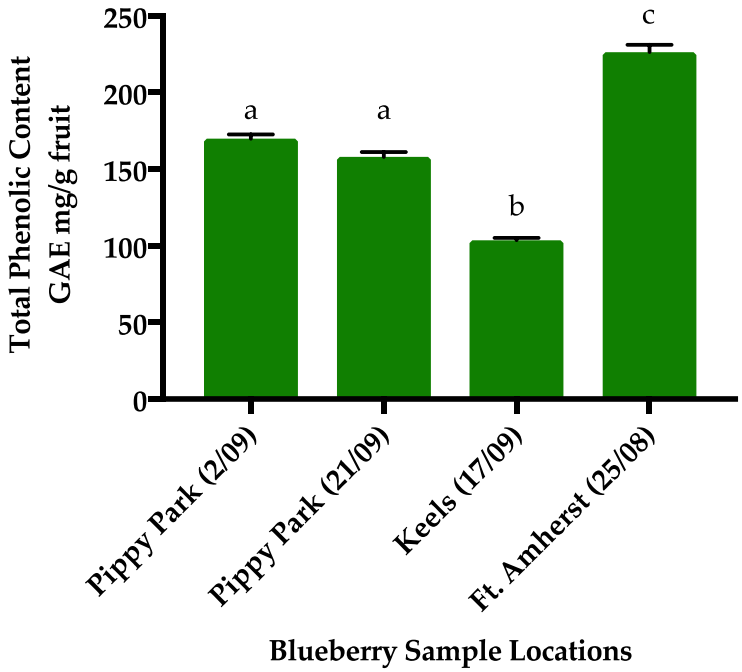


Figure 3.6: Total phenolic content of the blueberry leaf extracts.

Significant differences between the leaves from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences for the total phenolic content of the leaves are between the Pippy Park samples and Keels, the Pippy Park samples and Ft. Amherst, and between Keels and Ft. Amherst ($p=<0.0001$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.4.

3.1.3 Total flavonoid content

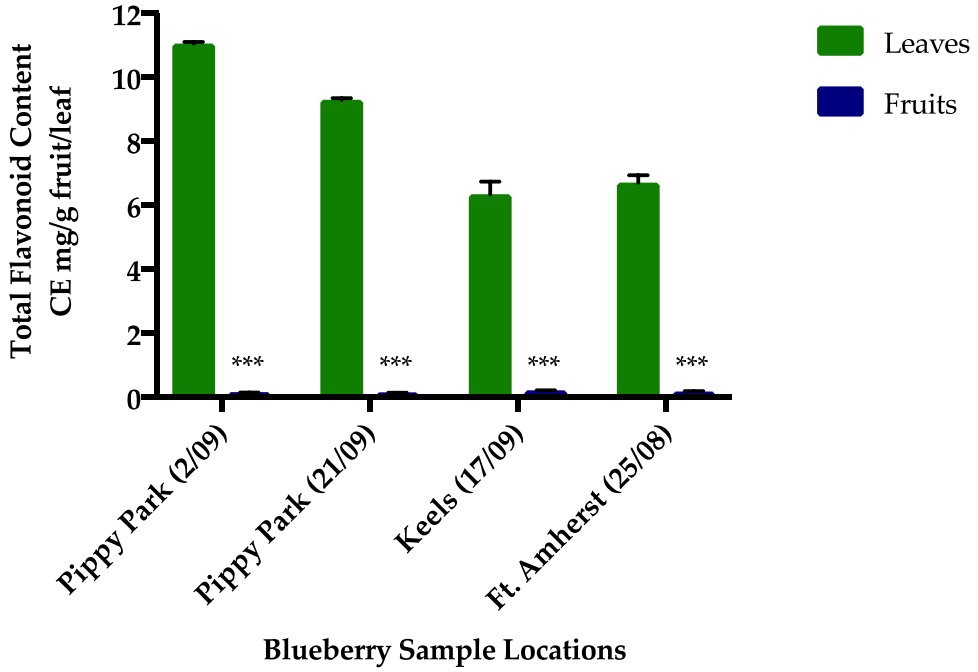


Figure 3.7: Total flavonoid content of blueberry fruit vs leaf extracts.

Significant differences between the fruits and leaves of the sample were determined via two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$), represented as 0.033(*), 0.002 (**), and <0.0001(***). Significant difference between the fruits and the leaves are observed for all four locations, where leaves had a higher total flavonoid content than the fruits ($p=<0.0001$, DF=16). Leaves from Pippy Park (2/09/16) and fruits from Keels had the highest total flavonoid content. The data are expressed as the mean ± SE.

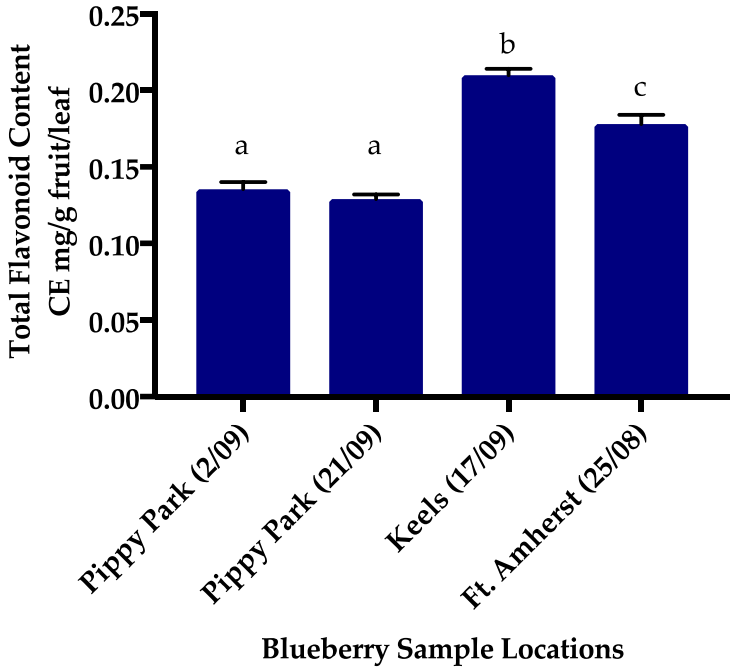


Figure 3.8: Total flavonoid content of the blueberry fruit extracts.

Significant differences between the fruits from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences for the total flavonoid content of the fruits are between the Pippy Park samples and Keels, the Pippy Park samples and Ft. Amherst, and between Keels and Ft. Amherst ($p=<0.001$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.7.

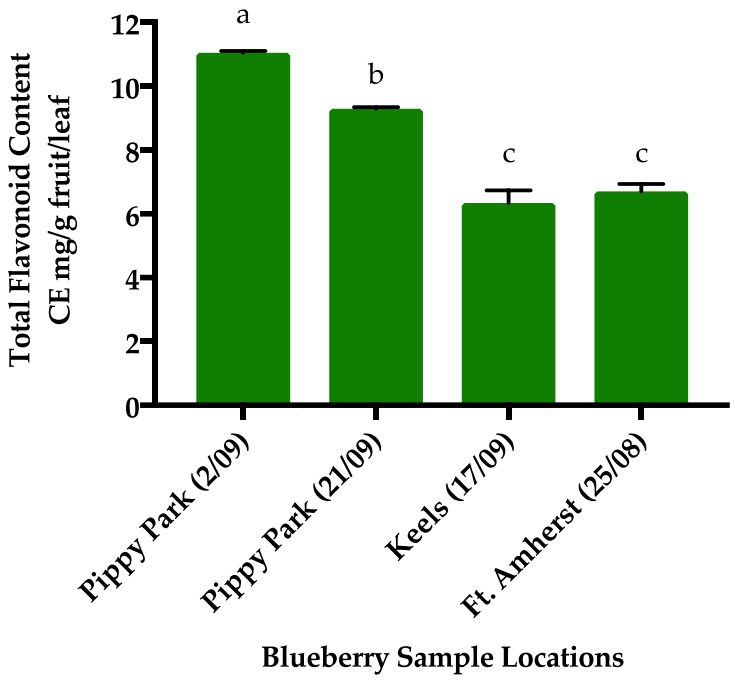


Figure 3.9: Total flavonoid content of the blueberry leaf extracts.

Significant differences between the leaves from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences between the total flavonoid content of the leaves are between Pippy Park (2/09/16) and Pippy Park (21/09/16), Pippy Park (2/09/16) and Keels, Pippy Park (2/09/16) and Ft. Amherst, Pippy Park (21/09/16) and Keels, and Pippy Park (21/09/16) and Ft. Amherst ($p=<0.0001$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.7.

3.1.4 Total tannin content

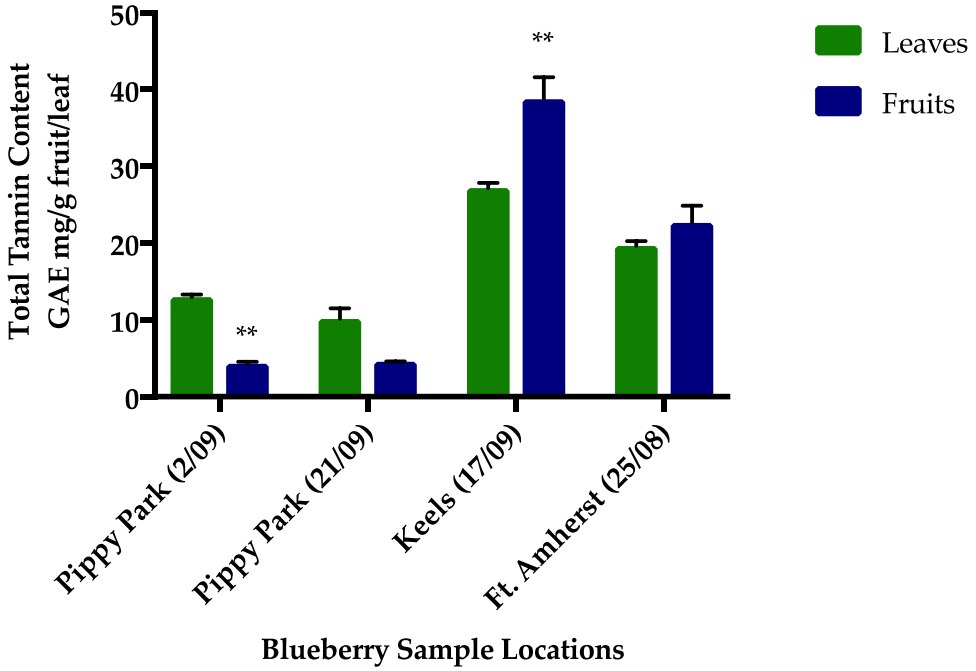


Figure 3.10: Total tannin content of blueberry fruit vs leaf extracts.

Significant differences between the fruits and leaves of the sample were determined via two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$), represented as 0.033(*), 0.002 (**), and <0.0001(***). A significant difference between the fruits and leaves are observed for the Pippy Park (2/09/16) sample where leaves had a higher total tannin content than the fruits, and for the Keels sample where the fruits had a higher total tannin content ($p=0.0004$, DF=16). Both leaves and fruits from Keels had the highest total tannin content. The data are expressed as the mean ± SE.

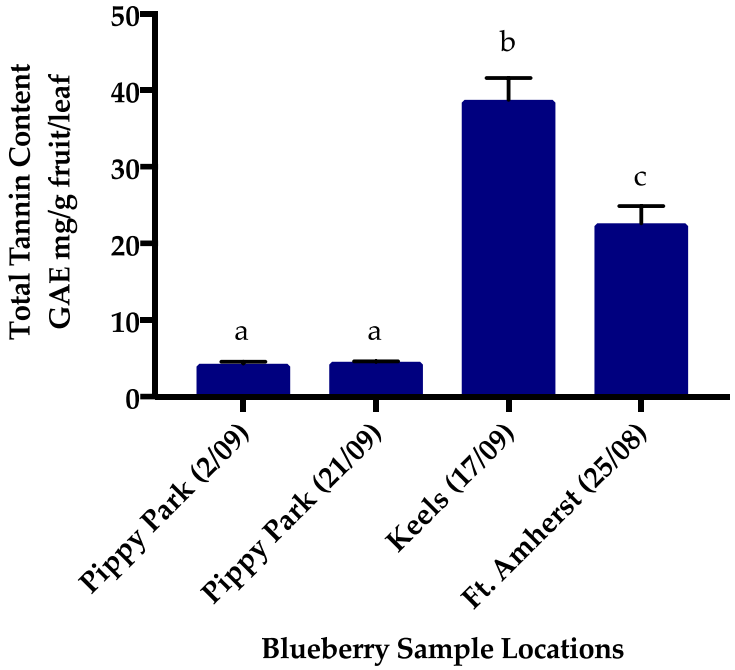


Figure 3.11: Total tannin content of the blueberry fruit extracts.

Significant differences between the fruits from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences between the total tannin content of the fruits are between the Pippy Park samples and Keels, the Pippy Park samples and Ft. Amherst, and between Keels and Ft. Amherst ($p=<0.0001$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.10.

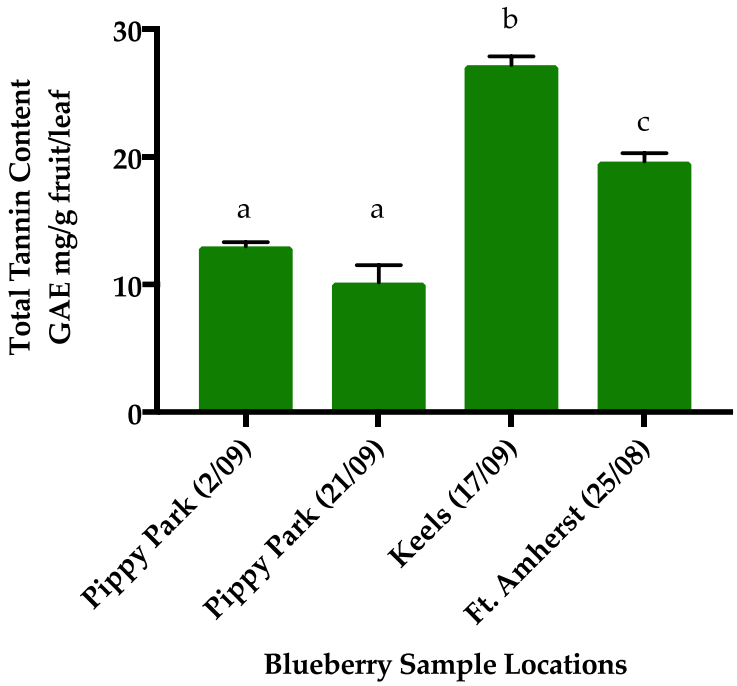


Figure 3.12: Total tannin content of the blueberry leaf extracts.

Significant differences between the leaves from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences between the total tannin content of the leaves are between the Pippy Park samples and Keels, the Pippy Park samples and Ft. Amherst, and Keels and Ft. Amherst ($p=<0.0001$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.10.

3.1.5 Total anthocyanin content

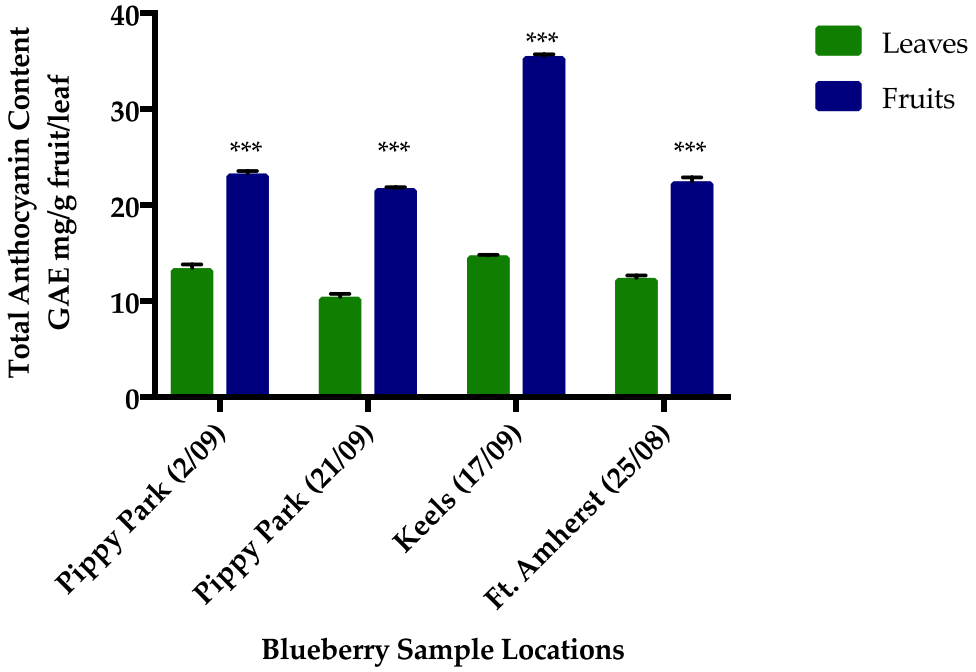


Figure 3.13: Total anthocyanin content of blueberry fruit vs leaf extracts.

Significant differences between the fruits and leaves of the sample were determined via two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$), represented as 0.033(*), 0.002 (**), and <0.0001(***). Significant difference between the fruits and the leaves are observed for all four samples where fruits had a higher total anthocyanin content than the leaves ($p=<0.0001$, DF=16). Both fruits and leaves from Keels contained the highest total anthocyanin content. The data are expressed as the mean ± SE.

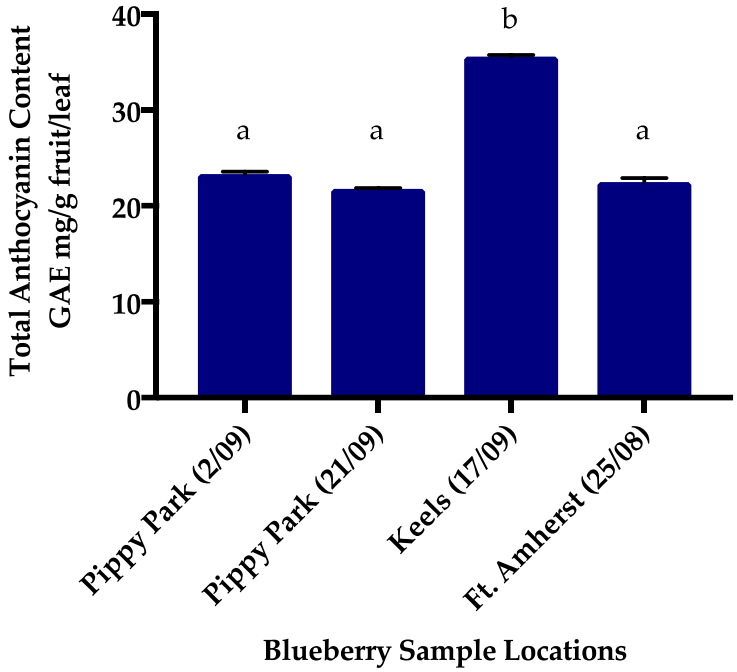


Figure 3.14: Total anthocyanin content of the blueberry fruit extracts.

Significant differences between the fruits from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences for the total anthocyanin content of the fruits are between the Pippy Park samples and Keels, and Keels and Ft. Amherst ($p=<0.0001$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.13.

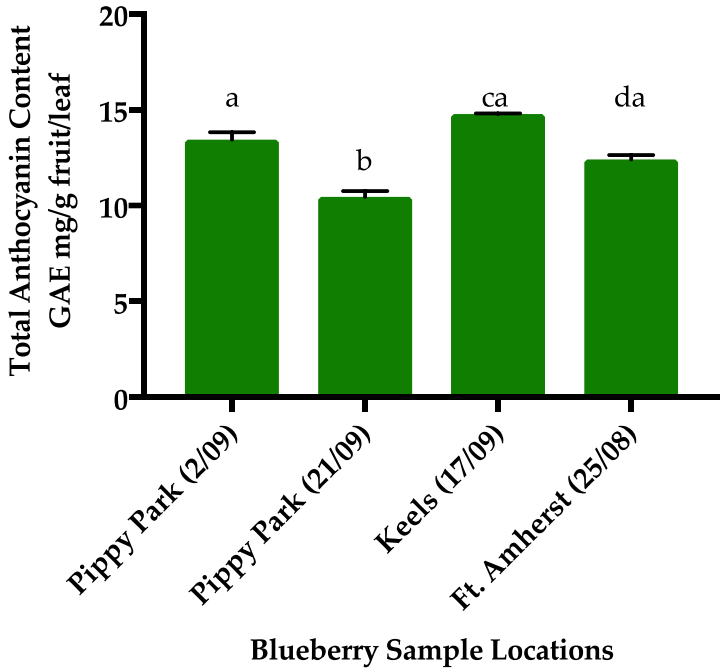


Figure 3.15: Total anthocyanin content of the blueberry leaf extracts.

Significant differences between the leaves from the four samples were determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$). Significant differences for the total anthocyanin content of the leaves are between Pippy Park (2/09/16) and Pippy Park (21/09/16), Pippy Park (21/09/16) and Keels, Pippy Park (21/09/16) and Ft. Amherst, and Keels and Ft. Amherst ($p=0.002$, DF=11). Values represented by the same letter indicate there are no significant differences within samples from those locations. The data are expressed as the mean ± SE, and are represented in Figure 3.13.

3.2 High-performancy liquid chromoatography-mass spectrometry

HPLC-MS is a technique used to separate, detect and potentially identify chemicals of particular masses in the presence of other chemicals. HPLC-MS was conducted by Michelle Debnath-Canning and Scott Unruh in our research group in 2015. The method was adapted from Cho *et al.*, (2004) and literature values were obtained from Cho *et al.*, (2004) and Esposito *et al.*, (2014). There were nine different major anthocyanins detected using HPLC-MS from blueberry fruit extracts from Pippy Park collected in 2014.

Table 3.1: Anthocyanin compounds identified in blueberry fruit extracts from Pippy Park using HPLC-Mass Spectrometry detection at positive ESI.

Peak number	HPLC retention time (minutes)	Compound Identified	m/z value in literature	m/z value
1	20.2	Delphinidin-3-galactoside	465	464.9
2	21.7	Delphindin 3-glucoside	465	464.8
3	22.4	Cyanidin-3-galactoside	449	448.8
4	23.2	Delphinidin-3-arabinoside	435	434.8
5	24.2	Cyanidin-3-glucoside	449	448.8
6	26.6	Petunidin-3-galactoside	479	479.1
7	28.8	Peonidin 3-galactoside	463	462.9
8	29.2	Malvidin-3-galactoside	493	492.8
9	31.9	Peonidin-3-glucoside	463	462.9

*literature value obtained from Cho *et al.* (2004) and Esposito *et al.* (2014)

*HPLC-Mass Spectrometry conducted by Michelle Debnath-Canning and Scott Unruh in our lab in 2015.

Through HPLC-MS, nine major anthocyanin compounds were identified including: Delphinidin 3-galactoside, Delphinidin 3-glucoside, Cyanidin 3-galactoside, Delphinidin-3-arabinoside, Cyanidin 3-glucoside, Petunidin-3-galactoside, Peonidin-3-galactoside, Malvidin-3-galactoside and Peonidin-3-glucoside.

3.3 Cell culture experiments

After determining the phenolic content of the blueberry samples using biochemical assays, and analyzing previous HPLC-MS data, I investigated the potential neuroprotective effects of these extracts on brain tissue. This was done using mixed culture plates (C57BL/6 mouse-pup dissections), and pure rat neurons and microglia (ScienCell). Mixed cultures were used to represent the conditions normally found in the brain where neurons and many types of glia are found. Pure cultures of neurons and microglia were used to determine the direct effects of the addition of glutamate, alpha-synuclein, and amyloid-beta on the specific cell types. The Pippy Park (21/09/16) sample was chosen based on the results of the biochemical assays, as this extract contained high levels of phenolic compounds, as well as the quantity and quality of the sample. Cells were

quantified by counting the number of DAPI-stained nuclei (% control) after 24-hours of treatment. All counts were done by two individuals and then averaged. After the addition of the blueberry extract to wells treated with 100 μM glutamate, 100 ng/ml alpha-synuclein or 7.5 μM amyloid-beta there was an increase in the average percent of control for all of the cell types. Statistically significant results between the different treatment conditions was determined via one-way ANOVA and *post hoc* Tukeys test ($p<0.05$), represented as mean ± SE. Statisitically significant results in Figure 3.16 were determined via unpaired t-test, represented as mean ± SE. Each condition within each cell type had at least three independent cultures, and n=the number of wells for a given condition.

3.3.1 Mixed cultures of neurons and glia (dissection plates)

Figure 3.16 represents the control vs wells treated with the blueberry extract. There is no significant difference between the two, and the percent control was 98.44 (± 1.56) for the blueberry extract, showing that the addition of the blueberry extract did not affect the viability of the cells. Figure 3.17 represents the number of DAPI-stained nuclei (% control) for the control, 100 μM glutamate treated cells, and 100 μM glutamate plus blueberry extract treated cells. Figure 3.18 represents the number of DAPI-stained nuclei (% control) for the control, 100 ng/ml alpha-synuclein treated cells, and 100 ng/ml alpha-synuclein plus

blueberry extract treated cells. And Figure 3.19 represents the number of DAPI-stained nuclei (% control) for the control, 7.5 μM amyloid beta treated cells, and 7.5 μM amyloid beta plus blueberry extract treated cells. Although not significant, there appeared to be a decrease in the number of viable cells after wells were treated with 100 μM glutamate, 100 ng/ml alpha-synuclein or 7.5 μM amyloid-beta.

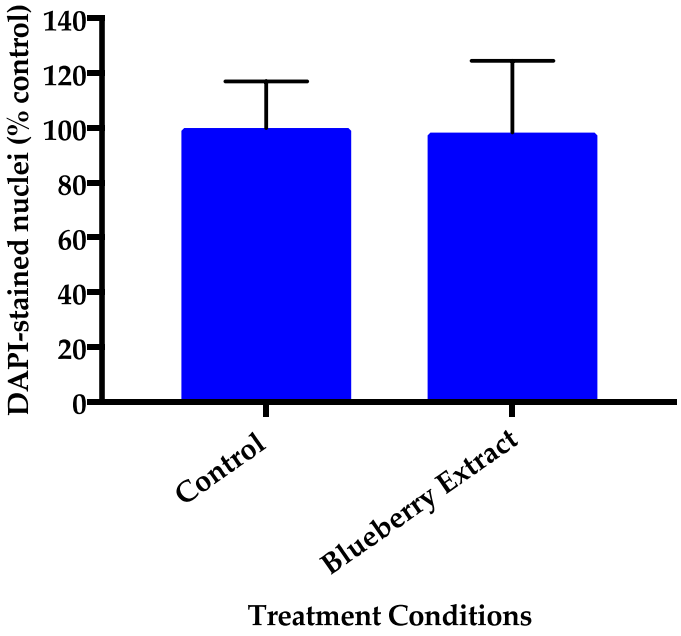


Figure 3.16: Dissected mouse neurons and glia treated with pure blueberry extract.

The % of control was determined by quantifying the number of DAPI-stained nuclei. The n=22 for the control and n=20 for the blueberry extract. There

was no significant difference between the control and the blueberry extract treated wells (unpaired t-test p=0.96). The data are expressed as the mean ± SE.

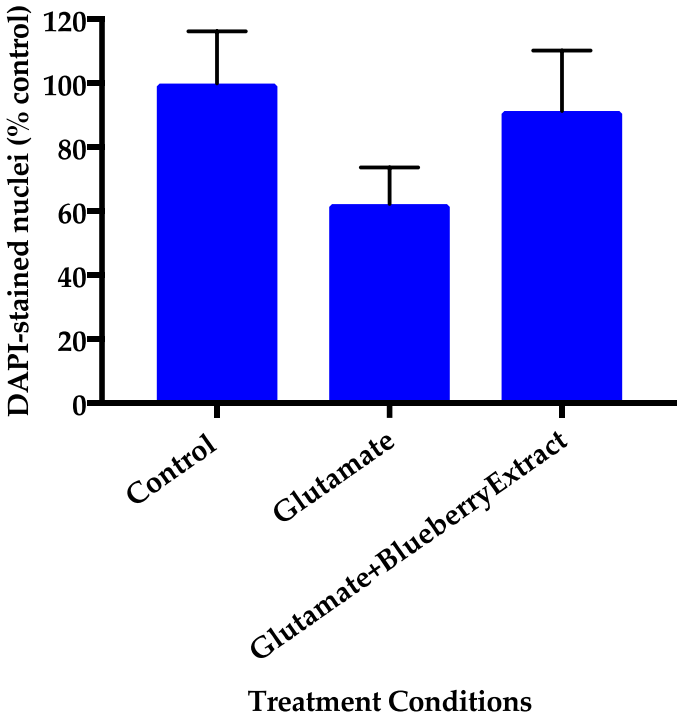


Figure 3.17: Dissected mouse neurons and glia treated with 100 μM glutamate.

The % of control was determined by quantifying the number of DAPI-stained nuclei. The n=20 for the control, n=18 for glutamate, and n=18 for glutamate plus blueberry extract. The one-way ANOVA had a p=0.19 (DF=54). Although not statistically significant, the control vs. 100 μM glutamate *post hoc* Tukeys had a p=0.25, 100 μM glutamate vs. 100 μM glutamate plus blueberry extract *post hoc* Tukeys had a p=0.59, and control vs. 100 μM glutamate plus

blueberry extract *post hoc* Tukeys had a p=>0.99. The data are expressed as the mean ± SE.

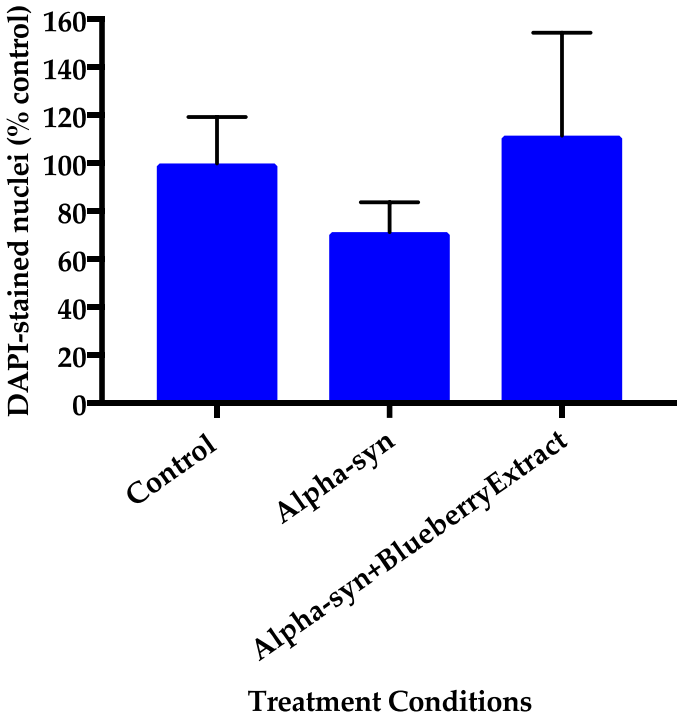


Figure 3.18: Dissected mouse neurons and glia treated with 100 ng/ml alpha-synuclein.

The % of control was determined by quantifying the number of DAPI-stained nuclei. The n=15 for the control, n=16 for alpha-synuclein, and n=16 for alpha-synuclein plus blueberry extract. The one-way ANOVA had a p=0.58 (DF=44). Although not statistically significant, the control vs. 100 ng/ml alpha-synuclein *post hoc* Tukeys had a p=0.94, 100 ng/ml alpha-synuclein vs. 100 ng/ml alpha-synuclein plus blueberry extract *post hoc* Tukeys had a p=0.89, and control

vs. 100 ng/ml alpha-synuclein plus blueberry extract *post hoc* Tukeys had a p=>0.99. The data are expressed as the mean ± SE.

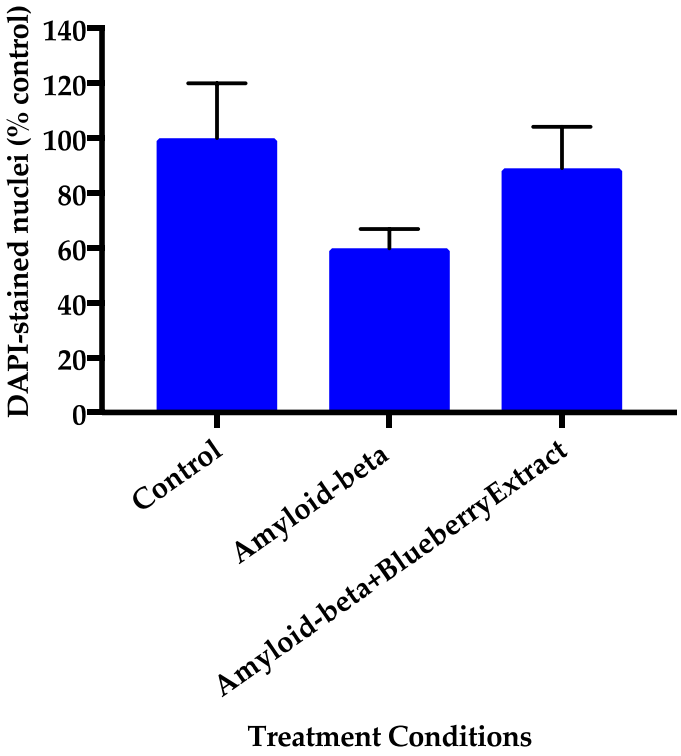


Figure 3.19: Dissected mouse neurons and glia treated with 7.5 μM amyloid-beta.

The % of control was determined by quantifying the number of DAPI-stained nuclei, and n=12 for all conditions. The one-way ANOVA had a p=0.16 (DF=33). Although not statistically significant, the control vs. 7.5 μM amyloid-beta *post hoc* Tukeys had a p=0.20, 7.5 μM amyloid-beta vs. 7.5 μM amyloid-beta plus blueberry extract *post hoc* Tukeys had a p=0.53, and control vs. 7.5 μM

amyloid-beta plus blueberry extract *post hoc* Tukeys had a p=>0.99. The data are expressed as the mean ± SE.

3.3.2 Rat neurons

Figure 3.20 represents the number of DAPI-stained nuclei (% control) for the control, 100 μM glutamate treated neurons, and 100 μM glutamate plus blueberry extract treated neurons. Figure 3.21 represents the number of DAPI-stained nuclei (% control) for the control, 100 ng/ml alpha-synuclein treated neurons, and 100 ng/ml alpha-synuclein plus blueberry extract treated neurons. And Figure 3.22 represents the number of DAPI-stained nuclei (% control) for the control, 7.5 μM amyloid beta treated neurons, and 7.5 μM amyloid beta plus blueberry extract treated neurons. A significant difference is observed between the control and 100 μM glutamate and 7.5 μM amyloid beta treated wells, although not statistically significant the addition of alpha-synuclein also resulted in a reduction in viable cells. This shows that the addition of excess glutamate and amyloid-beta resulted in a decrease in the number of cells present after 24-hours of treatment. There was an increase in the number of viable cells when blueberry extract was added to culture wells treated with 100 μM glutamate or 7.5 μM amyloid-beta, resulting in numbers closer to the number of cells in the

control wells. Pure blueberry extract alone was tested in the neurons (n=6) and was not statistically significant from the control.

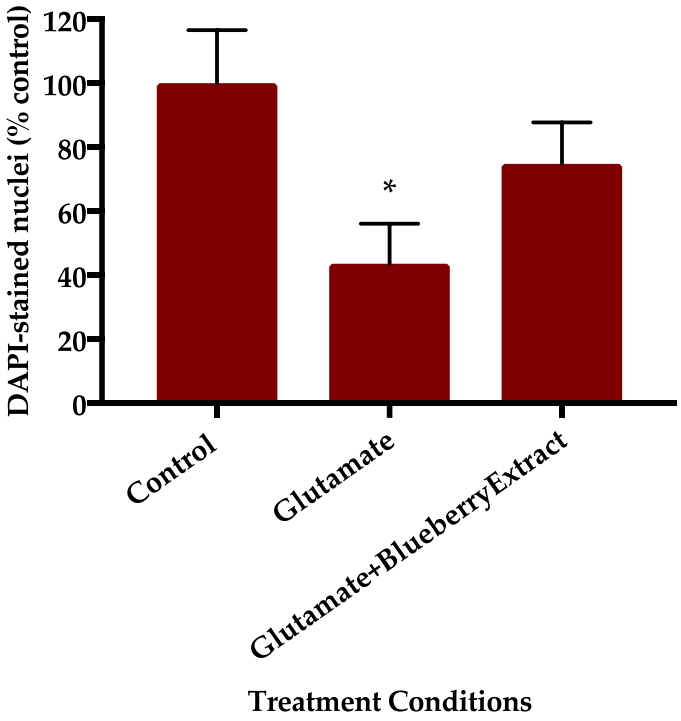


Figure 3.20: Rat neurons treated with 100 μM glutamate.

The % of control was determined by quantifying the number of DAPI-stained nuclei. The n=7 for the control, n=9 for glutamate, and n=9 for glutamate plus blueberry extract. The one-way ANOVA had a p=0.03 (DF=22). Statistical significance (* $p<0.05$) was determined between control and 100 μM glutamate treated wells (*post hoc* Tukeys p=0.03). Although not statistically significant, 100 μM glutamate vs. 100 μM glutamate plus blueberry extract *post hoc* Tukeys had a

p=0.33, and control vs. 100 μM glutamate plus blueberry extract *post hoc* Tukeys had a p=0.67. The data are expressed as the mean ± SE.

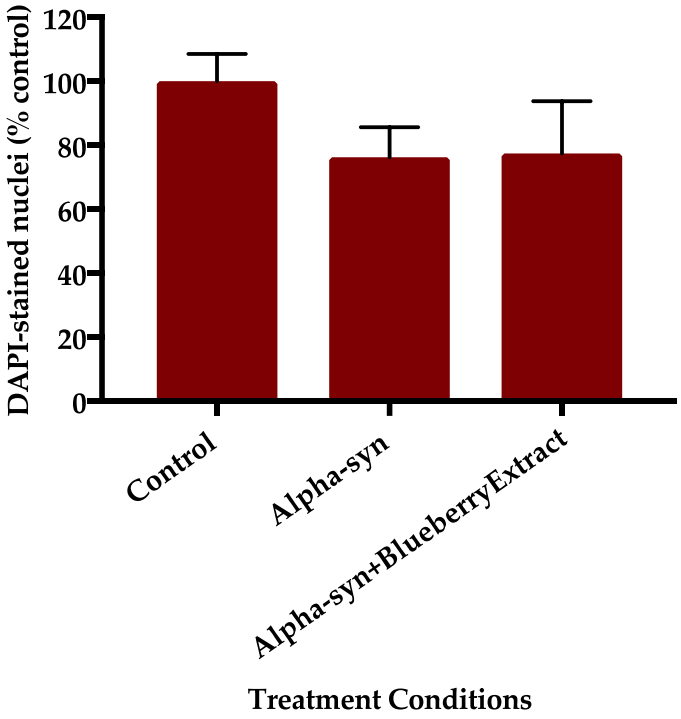


Figure 3.21: Rat neurons treated with 100 ng/ml alpha-synuclein.

The % of control was determined by quantifying the number of DAPI-stained nuclei. The n=10 for the control, n=8 for alpha-synuclein, and n=10 for alpha-synuclein plus blueberry extract. The one-way ANOVA had a p=0.31 (DF=25). Although not statistically significant, the control vs. 100 ng/ml alpha-synuclein *post hoc* Tukeys had a p=0.58, 100 ng/ml alpha-synuclein vs. 100 ng/ml alpha-synuclein plus blueberry extract *post hoc* Tukeys had a p=>0.99, and control

vs. 100 ng/ml alpha-synuclein plus blueberry extract *post hoc* Tukeys had a p=0.58. The data are expressed as the mean ± SE.

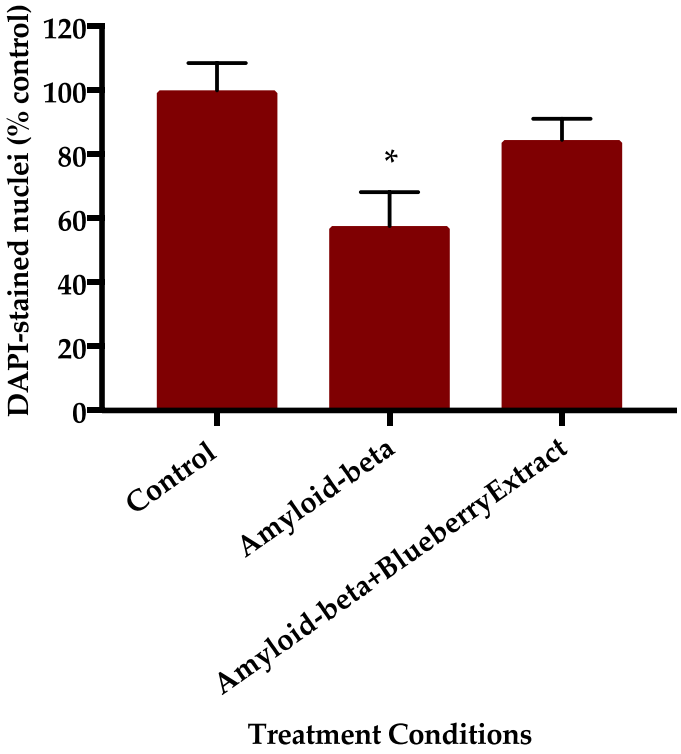


Figure 3.22: Rat neurons treated with 7.5 μM amyloid-beta.

The % of control was determined by quantifying the number of DAPI-stained nuclei. The n=10 for the control, n=8 for amyloid-beta, and n=8 for amyloid-beta plus blueberry extract. The one-way ANOVA had a p=0.008 (DF=23). Statistical significance (* $p<0.05$) was determined between control and 7.5μM amyloid-beta treated wells (*post hoc* Tukeys p=0.006). Although not statistically significant, 7.5 μM amyloid-beta vs. 7.5 μM amyloid-beta plus blueberry extract *post hoc* Tukeys had a p=0.14, and control vs. 7.5 μM amyloid-

beta plus blueberry extract *post hoc* Tukeys had a p=0.53. The data are expressed as the mean ± SE.

3.3.3 Rat microglia

Figure 3.23 represents the number of DAPI-stained nuclei (% control) for the control, 100 μM glutamate treated microglia, and 100 μM glutamate plus blueberry extract treated microglia. Figure 3.24 represents the number of DAPI-stained nuclei (% control) for the control, 100 ng/ml alpha-synuclein treated microglia, and 100 ng/ml alpha-synuclein plus blueberry extract treated microglia. Although not statistically significant, there appeared to be an increase in the number of viable cells when blueberry extract was added to culture wells treated with 100 ng/ml alpha-synuclein, resulting in numbers close to the number of cells under control treatment. A significant difference is observed between the control and 100 ng/ml alpha-synuclein treated wells, showing that the addition of alpha-synuclein to these cultures resulted in a significant decrease in the number of cells present.

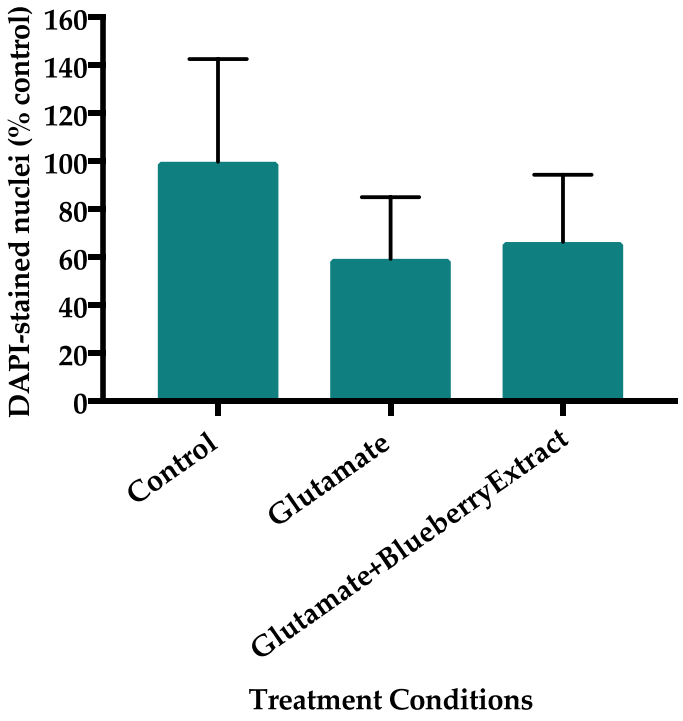


Figure 3.23: Rat microglia treated with 100 μM glutamate.

The % of control was determined by quantifying the number of DAPI-stained nuclei, and n=10 for all of the conditions. The one-way ANOVA had a p=0.19 (DF=27). Although not statistically significant, the control vs. 100 μM glutamate *post hoc* Tukeys had a p=0.77, 100 μM glutamate vs. 100 μM glutamate plus blueberry extract *post hoc* Tukeys had a p=0.98, and control vs. 100 μM glutamate plus blueberry extract *post hoc* Tukeys had a p=0.86. The data are expressed as the mean ± SE.

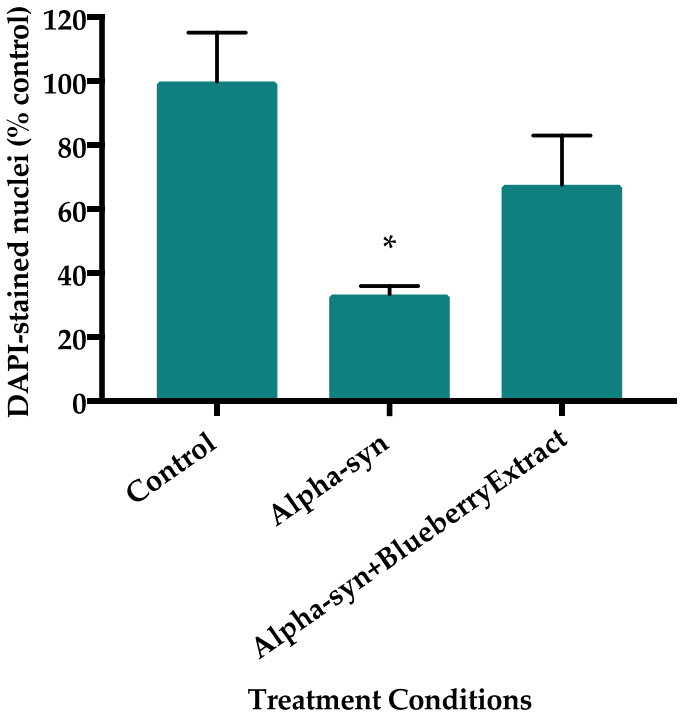


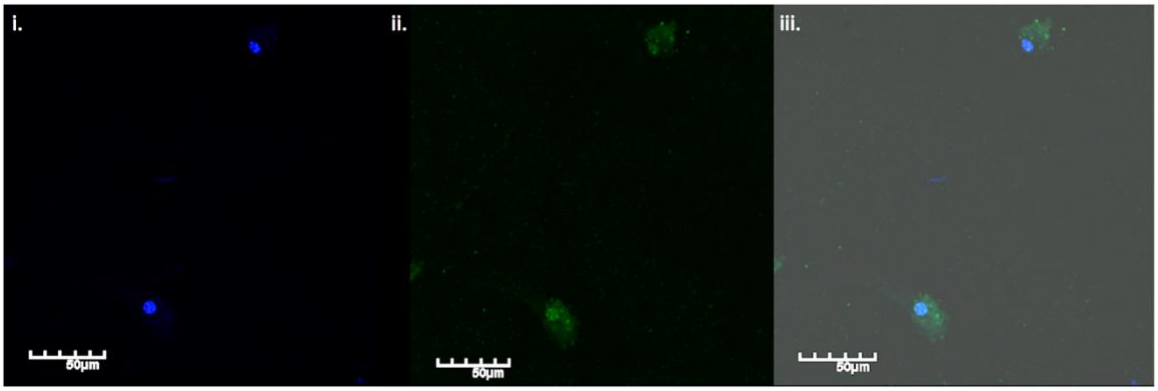
Figure 3.24: Rat microglia treated with 100 ng/ml alpha-synuclein.

The % of control was determined by quantifying the number of DAPI-stained nuclei. The n=14 for the control, n=12 for alpha-synuclein, and n=14 for alpha-synuclein plus blueberry extract. The one-way ANOVA had a p=0.007 (DF=20). Statistical significance (* $p<0.05$) was determined between control and 100 ng/ml alpha-synuclein treated wells (*post hoc* Tukeys p=0.006). Although, not statistically significant, 100 ng/ml alpha-synuclein vs. 100 ng/ml alpha-synuclein plus blueberry extract wells *post hoc* Tukeys had a p=0.29, and the control vs. 100 ng/ml alpha-synuclein plus blueberry extract *post hoc* Tukeys had a p=0.30. The data are expressed as the mean ± SE.

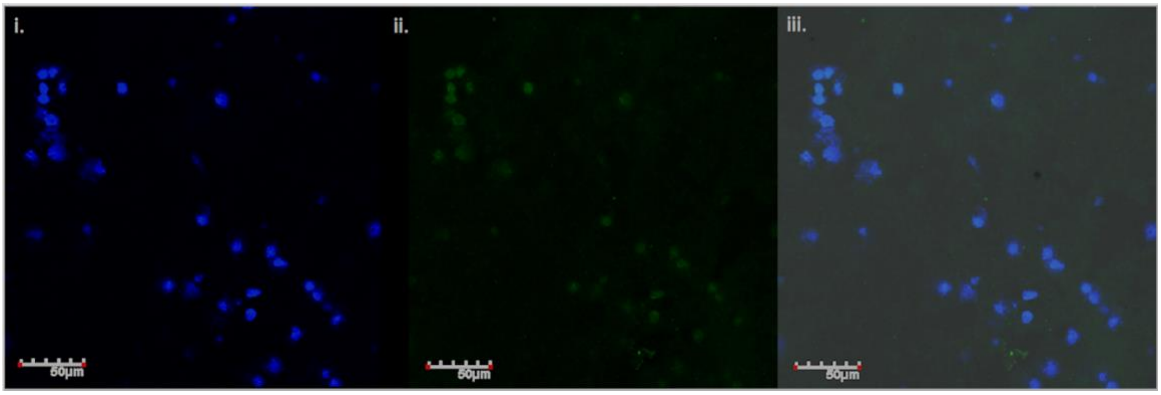
3.3.4 Confocal imaging of caspase-3 in mixed cultures

Caspase-3 is a marker of apoptosis and was used to determine the extent of programmed cell death in mixed cultures of neurons and glia treated with alpha-synuclein. Images captured by Dr. Noriko Daneshtalab show mixed culture morphology as well as cellular compartmentalization of activated caspase-3 in control wells (A), alpha-synuclein treated wells (B), and wells treated with alpha-synuclein plus the Pippy Park (21/09/16) blueberry extract (C). Each image includes the DAPI image (i) in blue, activated caspase-3 (ii) (Alexa 488 goat anti-rabbit) in green, and the merged image (iii). Images were taken at 8-micron thickness, 20X magnification, and 2.6X-3X zoom.

A)



B)



C)

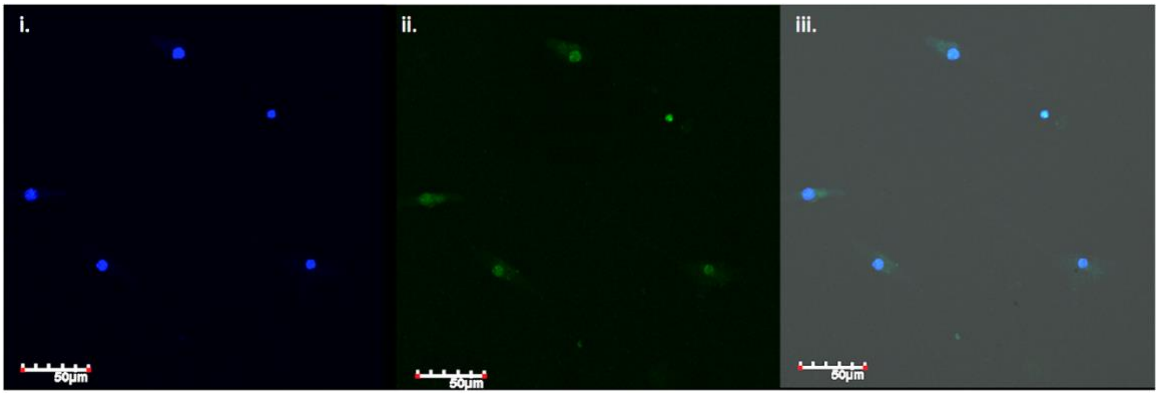


Figure 3.25: Confocal images of DAPI and activated caspase-3 in an 100 ng/ml alpha-synuclein treated dissection plate. (A) Control wells, (B) alpha-synuclein treated wells, and (C) wells treated with alpha-synuclein plus the blueberry extract. DAPI image (i) in blue, activated caspase-3 (ii) in green, and the merged image (iii). Scale bar, 50 μM.

When observed under the confocal microscope, as well as in the captured images in Figure 3.25, cells in the control wells (A) appear healthier, circular and more spread out, whereas cells in the alpha-synuclein treated wells (B) look damaged, with an uneven shape and border, and are clumped together. Cells in the control wells (A), and alpha-synuclein plus Pippy Park (21/09/16) blueberry extract wells (C) appear more elongated compared to those wells that were treated with just alpha-synuclein. There was a difference with regard to the cellular localization of activated caspase-3 among the different treatment conditions. The different subcellular compartmentalization of caspase-3 may play a role in apoptosis activation as translocation of caspase-3 is critical for the development of the apoptotic pathway (Kamada *et al.*, 2005). In the control wells (A) and the alpha-synuclein plus blueberry extract wells (C), activated caspase-3 appeared to be mainly in the cytoplasm. However, with the alpha-synuclein treated wells (B) activated caspase-3 appeared to be localized just in the nucleus.

3.3.5 Immunofluorescence imaging of neurons

The images in Figure 3.26 were captured with a fluoresce microscope at a magnification of 40X using PCO Camware software (see Figure 2.1). Images captured cell morphology and cellular differentiation of pure neurons (ScienCell)

in control (A), alpha-synuclein treated wells (B), and wells treated with alpha-synuclein plus the Pippy Park (21/09/16) blueberry extract (C).

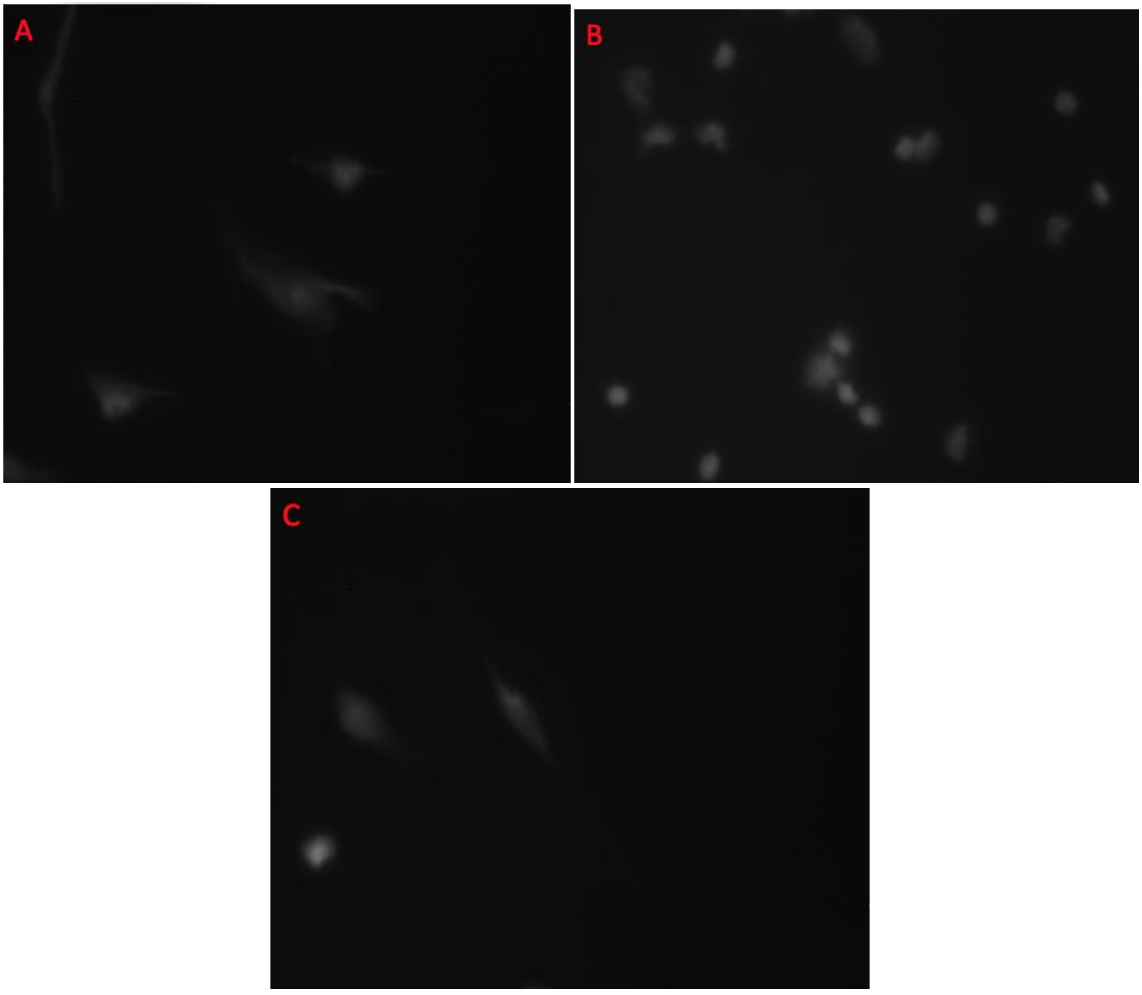


Figure 3.26: Immunoflorescence microscope imaging with DAPI staining in pure rat neurons treated with alpha-synuclein.

Neurons in the control wells (A) are very elongated and look healthy compared to cells in the alpha-synuclein treated wells (B). The cells in the alpha-synuclein treated wells are compact with little to no elongation. These cells were

observed to be very clumped together, where sections of the well would have no cells at all, but then other areas had cells layered on top of each other. The neurons in the wells treated with alpha-synuclein plus the Pippy Park (21/09/16) blueberry extract (C) contain both clumped/round and elongated neurons. Therefore, the addition of the blueberry extract appeared to promote the growth, and elongation of healthy neurons when in the presence of alpha-synuclein.

3.4 Pilot diet study

In order to determine the palatability of a 2% blueberry-enriched diet, a pilot feeding study was conducted. Blueberries collected from Pippy Park (21/09/16) were administered to C57BL/6 mice over a 5-week period. There were 18 mice involved in this study, however one mouse from the control group died during this study from a seizure on day 10. The Pippy Park (21/09/16) sample was chosen based on the results of the biochemical assays, because we had the highest quantity of it, and because it was previously used for the cell culture studies. Through two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$), results show that there was no significant weight change from the initial to the final date of the pilot study, showing that administration of the mash is palatable over time (Figure 3.27). Currently, the brains of these mice remain in a -80°C freezer

awaiting HPLC-MS analysis to detect and identify specific compounds from the blueberries present in the brain tissue.

Mice were tested on a rotarod apparatus in order to monitor motor function over the course of this study. For all speeds (5 rpm, 10 rpm, 15 rpm) of the fixed-speed rotarod testing, a slight incline was observed where the experimental group was able to stay on the rotarod for a longer period of time by the end of the study. Results showed no statically significant results between the control and experimental groups at any of the speeds, via two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$). For NOR testing, the experimental group had a higher DI in the 1-hour trails, showing they were able to make a concrete memory of the familiar object, because they explored the novel object more. The 24-hour trials saw opposite results, and animals in both groups had a very low DI. There were no significant differences between the control and experimental groups for NOR testing at 1-hour or at 24-hours, and no significant differences between the 1-hour and 24-hour trials determined via two-way ANOVA and *post hoc* Bonferroni test ($p<0.05$).

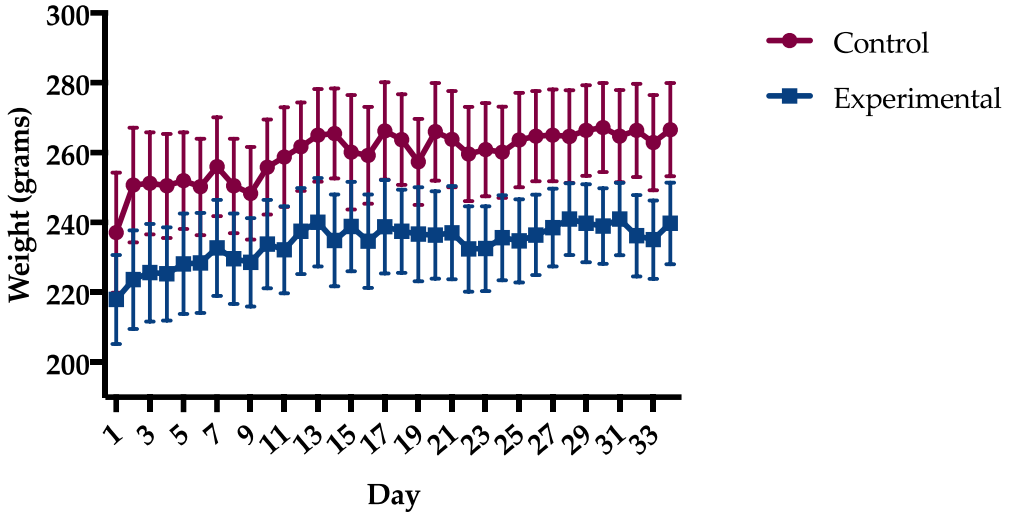


Figure 3.27: Daily mean weight measurements for the control and experimental groups.

All animals were able to maintain their weight, or gain weight when voluntarily consuming the blueberry mash, concluding that the berry-enriched diet is palatable over time. No statistically significant differences (two-way ANOVA and *post hoc* Bonferroni test, $p<0.05$) were observed between the groups, or between the individual animals starting and final weight ($p=0.997$, f-value=0.03, DF=510). The data are expressed as the mean ± SE.

3.4.1 Rotarod

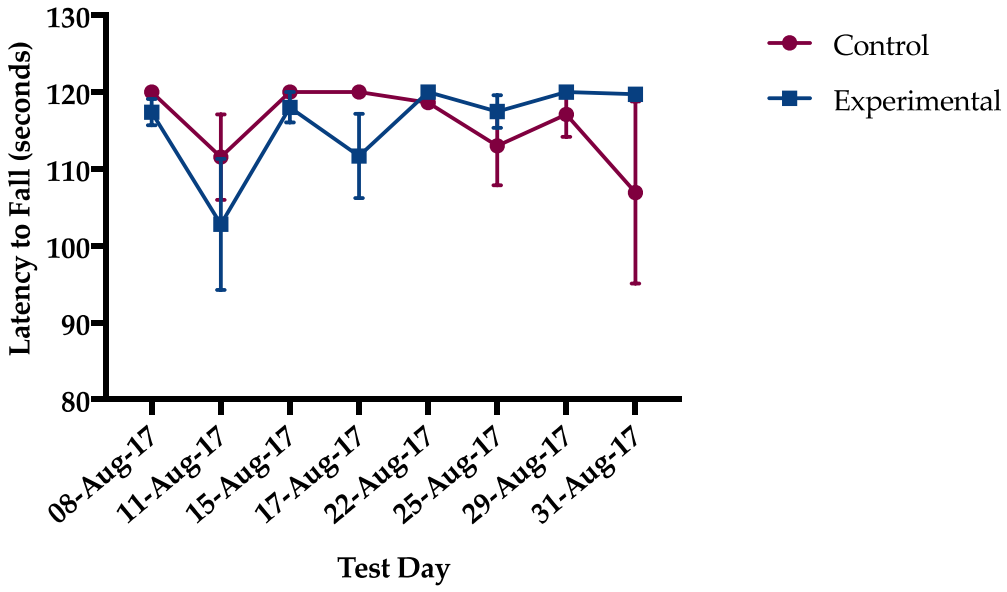


Figure 3.28: Rotarod test day means for the control and experimental groups at 5 rpm.

At 5 rpm there are no significant differences (two-way ANOVA and *post hoc* Bonferroni test, $p<0.05$) between the test day means for the control and experimental groups ($p=0.262$, f-value=1.28, DF=120). The experimental group initially had lower scores but were able to stay on the rotarod longer at 5 rpm by the end of the study. Animals in both groups were able to stay on the longest at this speed. The data are expressed as the mean ± SE.

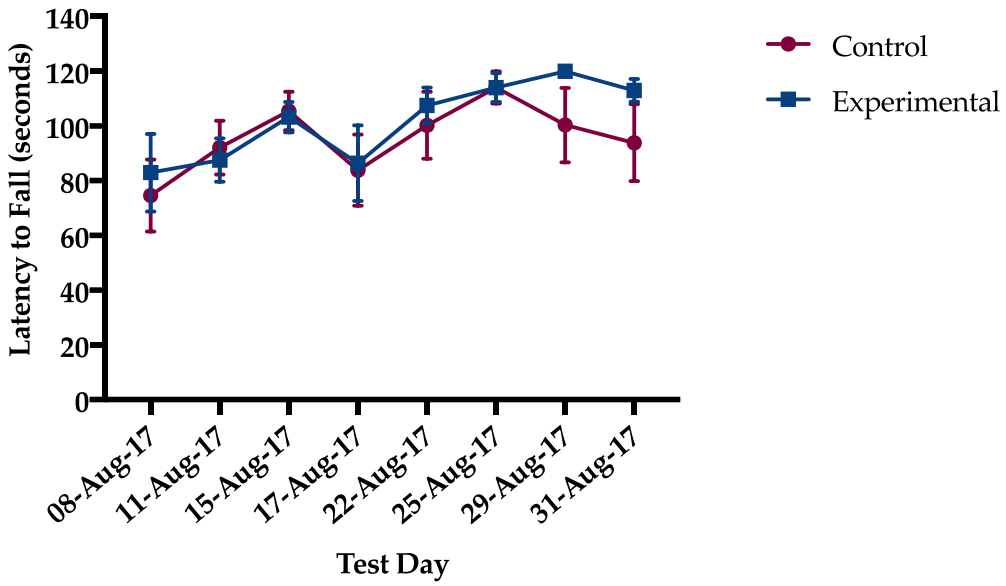


Figure 3.29: Rotarod test day means for the control and experimental groups at 10 rpm.

At 10 rpm there are no significant differences (two-way ANOVA and *post hoc* Bonferroni test, $p<0.05$) between the test day means for the control and experimental groups ($p=0.883$, f-value=0.43, DF=120). The experimental group continuously performed better on the rotarod (with the exception of August 17th), whereas the control group did not improve very much throughout the study. The data are expressed as the mean ± SE.

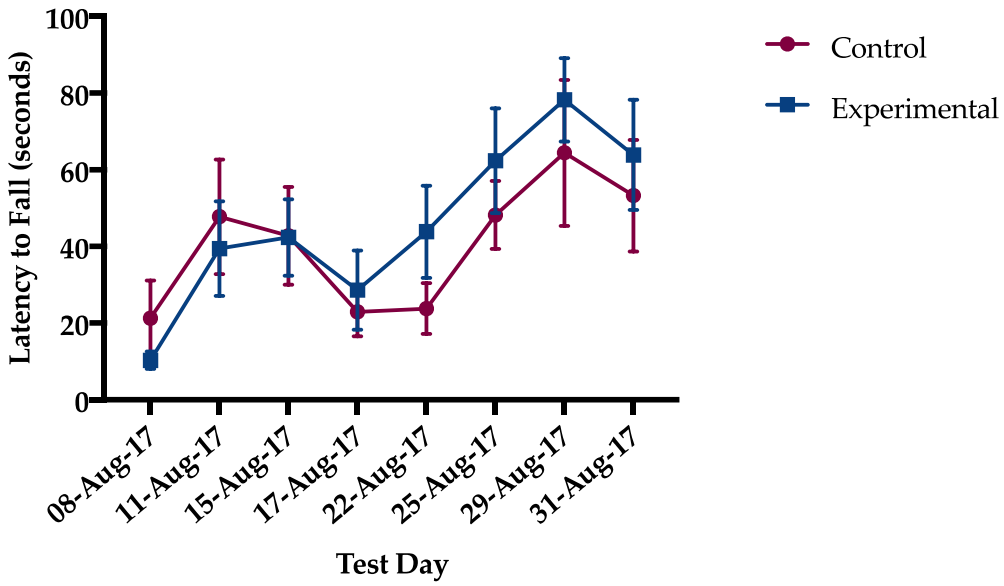


Figure 3.30: Rotarod test day means for the control and experimental groups at 15 rpm.

At 15 rpm there are no significant differences (two-way ANOVA and *post hoc* Bonferroni test, $p<0.05$) between the test day means for the control and experimental groups ($p=0.869$, f-value=0.45, DF=120). Both groups were able to gradually improve throughout the study. At the beginning of the study the control group was able to stay on the rotarod longer at 15 rpm, however by the end of the study the experimental group performed better. The data are expressed as the mean ± SE.

3.4.2 Novel object recognition

The discrimination index is above zero for both the 1-hour trials and 24-hour trials, indicating that the animals in both groups retained a memory of the

familiar object and preferred to explore the novel. The experimental group spent more time exploring the novel object compared to the control in the 1-hour trials, and the opposite was seen in the 24-hour trials. Reliability stats using Cronbach's Alpha were also determined (1-hour familiar: 0.93, 1-hour novel: 0.92 and 24-hour familiar: 0.89, 24-hour novel: 0.9).

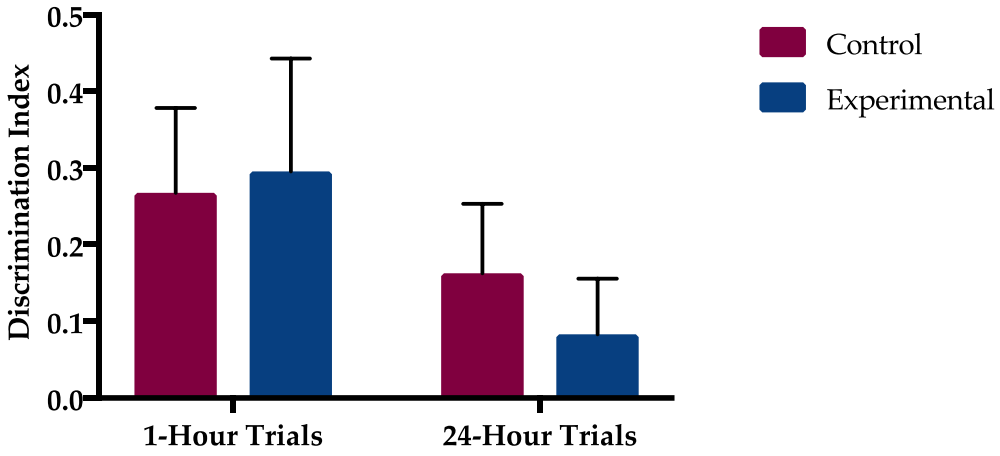


Figure 3.31: Novel object recognition for the 1-hour and 24-hour trials.

There are no significant differences (two-way ANOVA and *post hoc* Bonferroni test, $p<0.05$) between the control and experimental groups for the 1-hour or 24-hour trials ($p=0.629$, f-value=0.24, DF=30). The opposite is seen for the 24-hour trials, as the DI for both groups was lower, which is expected as time increases, however, the DI for the experimental group was low compared to testing the previous day. This could be due to a number of factors which will be examined in Chapter 4. The data are expressed as the mean ± SE.

Chapter 4: Discussion

4.1 Biochemical analysis

Blueberry (*Vaccinium angustifolium spp.*) fruits and leaves collected from Pippy Park, Keels and Ft. Amherst in Newfoundland and Labrador were analyzed using biochemical assays. It was determined that both the fruits and leaves contained high levels of phenolic compounds including flavonoids, anthocyanins, and tannins. These samples showed high antioxidant activity measured as the total radical scavenging capacity. For total phenolic content, total antioxidant activity, and total flavonoid content the leaves were significantly higher than the fruits. This is consistent with previous findings in our research group (Vyas *et al.*, 2013), where the leaves contained higher levels of phenolic compounds for both blueberries and lingonberries. Total tannin content saw mixed results where both Pippy Park samples had higher levels in the leaves and Keels and Ft. Amherst had higher levels in the fruits. For the total anthocyanin content, the fruits were significantly higher than the leaves for all the locations. Anthocyanins are responsible for the red and blue pigments found in the fruits of berries (Hossain *et al.*, 2016). Berries store the majority of their anthocyanins in the skin, which help protect the fruit from harmful interactions

with the sun and other environmental factors (Hossain *et al.*, 2016), so it makes sense that higher levels of anthocyanins are in the fruits.

Total antioxidant activity was the highest in the leaves collected from Pippy Park (2/09/16), and in the fruits collected from Keels (17/09/17). Total phenolic content was the highest in the leaves collected from Ft. Amherst (25/08/17), and the fruits collected from Pippy Park (21/09/16). Total flavonoid content was highest in the leaves collected from Pippy Park (2/09/16) and fruits from Keels (17/09/17). Total tannin content and total anthocyanin content was highest in the leaves and fruits collected from Keels (17/09/17). Even though samples were collected from the same geographical area there are significant differences observed among the samples depending on the biochemical assay. It is particularly interesting to see a significant difference between the two Pippy Park samples even though they were collected from the same location just a few weeks apart (Figures 3.3, 3.5, 3.9, and 3.15). Similar results were observed in a study conducted by Connor *et al.* (2002) which found differences between total antioxidant capacity, total phenolic content, and total anthocyanin content between samples collected from the same location. This gives us insight into the variety of phenolic compounds that can exist within a particular area based on environmental factors and different genotypes (Connor *et al*, 2002). Collection

times could have played a role in the difference, based on the ripeness and size of the berry, a decrease in temperature or changes in weather patterns.

The total tannin content for both Pippy Park samples is particularly low, as seen in Figure 3.10. Tannin content is highly dependent on the stage of development and ripening of the plant (Hanlin & Downey, 2009). The tannin content of plants tends to decrease after the plant is mature and the fruit is ripe (Hanlin & Downey, 2009). Wild blueberries ripen in early August into September, and the Pippy Park samples were collected in September. This could have influenced these results, but these samples were collected around the same time as the Keels and Fort Amherst samples which had a significantly higher total tannin content for both fruits and leaves. Therefore, the environmental factors associated with their location may have played a bigger role in the total tannin content. Plants growing in areas more exposed to sunlight have much higher levels of tannins compared to those that are in more shaded areas (Nichols-Orians, 1991). The Pippy Park samples were collected in a flat area on top of a peak. There were several tree and bush species growing in this area as well, which provided partial shading to most of the blueberry plants.

After determining the levels of polyphenols present in the samples, we examined the specific anthocyanin compounds detected in previous samples in

our lab. HPLC-MS was conducted on a Pippy Park sample collected in 2014, where a total of nine major anthocyanin compounds were found in the fruits of this sample (Table 3.1). Through HPLC-MS Michelle Debnath-Canning and Scott Unruh were able to identify Delphinidin 3-galactoside, Delphinidin 3-glucoside, Cyanidin 3-galactoside, Delphinidin-3-arabinoside, Cyanidin 3-glucoside, Petunidin-3-galactoside, Peonidin-3-galactoside, Malvidin-3-galactoside and Peonidin-3-glucoside. Previous HPLC-MS analysis was conducted in our lab on the leaves from the 2016 Pippy Park sample, resulting in the identification of only six major compounds. Although the leaves have higher activity in biochemical assays, they contain fewer distinct phenolic compounds. The HPLC-MS data for the fruits gives us insight into potentially active compounds responsible for neuroprotection and free radical scavenging ability of polyphenols as well as determining the difference between 2014 and 2016 samples. Further HPLC-MS data would be beneficial to our lab, not only to identify and quantify the levels of anthocyanins and other phenolic compounds but to determine the different compounds present based on geographical location, and determine if these specific compounds can be detected in brain tissue and other organs after the consumption of the extract. Andres-Lacueva *et al.* (2013) was able to detect 8 major anthocyanins, including six compounds (Delphinidin 3-galactoside,

Cyanidin 3-galactoside, Delphinidin-3-arabinoside, Cyanidin 3-glucoside, Peonidin-3-galactoside, and Malvidin-3-galactoside) found in Table 3.1, in the cerebellum, cortex, hippocampus and striatum of aged rats fed a 2% blueberry-enriched diet for 8 to 10 weeks.

4.2 Cell culture studies

After analyzing the biochemical properties of the blueberry samples, we determined the neuroprotective role of these blueberries *in vitro*. As previously mentioned the Pippy Park (21/09/16) sample was used for the cell culture experiments because this sample contained high levels of phenolic compounds and was of the highest quality and quantity, compared to the other three samples. The fruit extracts were chosen over the leaf extracts because the fruits are more palatable. The fruits are generally consumed more often than the leaves, and using the leaves, which are in a powder after extraction, would require the addition of solvent into the wells. We primarily wanted to focus on using a blueberry extract that was pure blueberries and contained no solvent at all, so it would mimic what an individual or an animal in an *in vivo* study would consume. Previous studies conducted by our research group have only tested blueberries diluted with solvent in cell culture. We were able to sterilize and add just pure blueberries after mashing up whole berries without the addition of any

potentially toxic compounds (i.e. acetone/formic acid). Mixed culture plates, including neurons and glia from dissected 1 to 3 day old C57BL/6 mouse brains were tested along with pure cultures of neurons and microglia isolated from postnatal day 2 CD® IGS rat brains. For each well, 5 images were taken and the number of DAPI-stained nuclei (% control) was quantified by two individuals.

First, the pure blueberry extract was tested by itself to see if it had any effect on cell viability. It was determined that the addition of the blueberry extract resulted in no significant differences in cell numbers (Figure 3.16). The neuroprotective role of the blueberry extract was then tested against glutamate-mediated excitotoxicity. Glutamate-mediated excitotoxicity is primarily caused by high levels of glutamate from the inadequate uptake of glutamate through transporters in glial cells. This results in extensive cell death and damage by overstimulating glutamate receptors leading to oxidative stress (Figueira *et al.*, 2017) and neuroinflammation (Morales *et al.*, 2016) in the CNS. Previous cell culture studies conducted in our lab have concluded that the addition of blueberry extracts to mixed cultures of rat brain cells showed neuroprotection against glutamate-mediated excitotoxicity (Vyas *et al.*, 2013). This study along with work conducted by Michelle Debnath-Canning set the parameters for the final 100 μM glutamate concentration that was used. For the rat neurons (Figure

3.20), there was a significant decrease in the number of cells present compared to the control, showing that the addition of excess glutamate to these wells resulted in cell damage and death. Wells treated with glutamate and the blueberry extract saw an increase in the number of DAPI-stained nuclei compared to the glutamate treated wells. For the rat microglia (Figure 3.23), a similar trend was observed, where the addition of excess glutamate resulted in a decrease in the number of viable cells compared to the control, however this result was not significant. There were no significant differences observed between the treatment groups for the dissection plates consisting of neurons and glia (Figure 3.17), however, there appeared to be a potential decrease in the number of cells when excess glutamate was added to the wells. For the dissection plates there appeared to be an increase in the number of cells when the blueberry extract was added to culture wells treated with 100 μM glutamate, although it was not significant, the trend suggests that the addition of the blueberry extract was able to increase cell numbers closer the numbers observed in the control wells.

As stated above, there were significant differences observed when excess glutamate was added to pure rat neurons but not the microglia. Neurons were likely more affected by glutamate because they use glutamate extensively as an excitatory neurotransmitter to transmit signals from one neuron to the other.

Therefore, they may have been more susceptible or sensitive to glutamate increase compared to the microglia which have fewer glutamate receptors (Matute *et al.*, 2006). Microglia are the least susceptible to excitotoxcicity compared to other cells in the CNS, and that glutamate receptors are generally only expressed when microglia are in their activated form. It is also suggested that microglia are able to uptake glutamate and metabolize it to glutamine, resulting in a clearance of extracellular glutamate and reduction in its excitotoxic effects (Howe & Barres, 2012). The ability of microglia to eliminate excess glutamate within the surrounding area would have led to a decrease in the amount of cell death and damage caused by overstimulation.

We also wanted to look at the neuroprotective role the blueberry extract may have on cells treated with alpha-synuclein. The final concentration for alpha-synuclein (100 ng/ml) was determined by the research team Dr. Weber was previously a part of at the University of Marburg, Germany (data not published). The abnormal accumulation of alpha-synuclein protein caused by chronic neuroinflammation and oxidative stress (Strathearn *et al.*, 2014), is one of the pathological markers associated with PD. These insoluble aggregates damage the DA neurons in the SNpc contributing to the painful motor dysfunctions associated with PD (Strathearn *et al.*, 2014). Aggregates of alpha-synuclein can

activate surrounding microglia, contributing to inflammation by promoting the release of inflammatory cytokines (Block *et al.*, 2007). In Figure 3.24, there is a significant difference between the control treatment and the alpha-synuclein treated wells for the microglia, where the addition of alpha-synuclein resulted in a significant decrease in the number of viable cells, showing that this protein *in vitro* was capable of causing extensive cell death and damage, similarly to the pathology seen in PD. For the microglia wells treated with alpha-synuclein and the blueberry extract, there appeared to be an increase in the number of DAPI-stained nuclei compared to the alpha-synuclein treated wells. There was no significant difference between the control and wells treated with alpha-synuclein for the neurons, even though there appeared to be a decrease in the number of cells present after alpha-synuclein was added for 24-hours. Similarly, to the glutamate treated dissection plates, the dissection plates treated with alpha-synuclein saw no significant difference between the treatment conditions. However, the trends suggest that there was a decrease in the number of cells in the conditions where alpha-synuclein was added, and the addition of the blueberry extract to these wells resulted in an increase in the number of cells present.

There were significant differences observed when alpha-synuclein was added to pure rat microglia but not the neurons. Microglia were much more sensitive to the addition of alpha-synuclein compared to the neurons, and responded better to the addition of the blueberry extract. Microglia are responsible for the immune response in the CNS, and can uptake excess or abnormally folded alpha-synuclein to reduce cell death and damage (Zhang *et al.*, 2005). The increased presence of this protein promotes the activation of microglia which leads to an inflammatory response and the release of cytokines. This activates other surrounding microglia, resulting in oxidative stress and cellular damage (Zhang *et al.*, 2005). Therefore, one of the potential neuroprotective mechanisms associated with blueberries is preventing the activation of microglia. Neurons were not as directly affected as the microglia following the addition of alpha-synuclein, as this protein might not be as readily taken up in the neurons during this 24-hour treatment period.

Finally, we looked at the neuroprotective role of the blueberry extract when cells are treated with amyloid-beta. The final concentration of amyloid-beta (7.5 μM) was determined by work conducted by Nafar and Mearow (2014). Amyloid-beta in high concentrations and the accumulation of insoluble amyloid-beta-peptide plaques is one of the underlying mechanisms associated with AD.

Amyloid-beta buildup in between nerve cells in the hippocampus causes extensive damage and prevents neurons from functioning properly resulting in cognitive issues, such as the formation of new memories (Balk *et al.*, 2006). Dysfunction of the mitochondrial electron transport chain and long-term oxidative stress is associated with the aggregation of amyloid-beta (Balk *et al.*, 2006). For the rat neurons, there was a significant difference observed between the control and amyloid-beta treated wells. The addition of blueberry extract to wells treated with amyloid-beta resulted in an increase in the number of neurons present. The addition of amyloid-beta in the dissection plates was not statistically significant from the control, however there appeared to be a decrease in the number of viable cells when amyloid-beta was added. There also appeared to be an increase in the number of cells present when the blueberry extract was added to wells treated with amyloid-beta in the dissection plates.

For the neurons, the addition of amyloid-beta was significant but the addition of alpha-synuclein was not. The neurons were more sensitive to amyloid-beta aggregation and responded more positively to the addition of the blueberry extract. Alpha-synuclein is a protein that is abundant in the brain, however its function is poorly understood. The pathological issues associated with alpha-synuclein only arise when the protein becomes misfolded and begins

to aggregate (Masliah *et al.*, 2001). Amyloid-beta is also present in the brain, just in much lower levels, however when amyloid-beta is in high concentrations it is very toxic whether it is aggregated or not. Therefore, for this reason the addition of excess amyloid-beta to the culture wells for the 24-hour period could have had a greater effect and resulted in extensive cell damage to neurons compared to alpha-synuclein. Masliah *et al.* (2001) observed that transgenic mice with neuronal expression of amyloid-beta developed motor deficits before transgenic alpha-synuclein mice. Furthermore, the authors show that amyloid-beta plaques are able to promote the aggregation of alpha-synuclein in transgenic mouse models showing the connected relationship between AD and PD (Chaves *et al.*, 2010).

There were significant differences observed between the treatment groups for the pure rat neurons and microglia, however not for the dissection plates, containing both neurons and glia. There was a greater extent of cell death observed in the pure rat neurons and microglia compared to dissection plates. For example, there was a decrease in the number of cells by 48.5% on average after the addition of glutamate in pure rat neurons and microglia. However, for the dissection plates there was only a decrease in the number of cells by 37.7% after the addition of glutamate. For the pure rat neurons and microglia, the

addition of alpha-synuclein resulted in a decrease in the number of cells by 45.2% on average, where the reduction was only 28.9% for the dissection plates. The presence of different types of glia in the dissection plates may have influenced this result. Glia, particularly astrocytes and oligodendrocytes, which are not present in the isolated neurons and microglia cultures, are able to take up excess glutamate or alpha-synuclein (Brück *et al.*, 2016; Matute *et al.*, 2006), which would reduce their overall presence in the well, resulting in a decrease in cell death and damage. Alpha-synuclein aggregates have been detected in the cytoplasm of astroglial and oligodendroglial cells *in vitro* (Wakabayashi *et al.*, 2000; Brück *et al.*, 2016). These types of glia present in the brain were able to provide additional protection for surrounding cells by reducing the levels of glutamate and alpha-synuclein. These plates are more representative of the actual human brain including all the cell types. However, it is still beneficial to study the pure cultures as one is able to observe the direct effects that these pathological markers have on specific cell types in the brain.

The concentration of blueberry extract used was determined by our research group, and by looking at similar studies conducted in the field. In the future, it would be beneficial to further investigate different concentrations of blueberry extract to determine the specific effects they would have on dose

response curves and morphology. It would also be interesting to conduct incubations with different time periods instead of only 24-hours to determine different stages/levels of of neuroinflammation. Insoluble alpha-synuclein and amyloid-beta aggregates may require longer exposure to neurons which could play a role in why we observed a limited response.

The caspase-3 protein plays a key role in triggering apoptosis, and we wanted to investigate whether the addition of the blueberry extract had any effect on the presence of caspase-3 in mixed culture plates. Although in the confocal images captured I was not able to clearly quantify the levels of activated caspase-3 between the three treatment conditions, it is evident in the confocal images in shown in Figure 3.25 that there are differences among the cellular compartmentalization of activated caspase-3. The different subcellular compartments may play a role in apoptosis activation as translocation of caspase-3 is critical for the development and regulation of the apoptotic pathway (Kamada *et al.*, 2005). For the control wells (Figure 3.25A) and wells treated with alpha-synuclein plus blueberry extract (Figure 3.25C), caspase-3 is primarily localized in the cytoplasm, which is particularly evident when looking at the merged images (ii). However, with the alpha-synuclein treated wells (Figure 3.25B) activated caspase-3 appears to be localized just in the nucleus. Similar

results were observed by Kamada *et al.* (2005) where activated caspase-3 was first observed in the cytoplasm and then in the nucleus in apoptotic cells. It is suggested that some cytoplasmic substrates, such as caspase-3, translocate into the nucleus from the cytoplasm following apoptosis induction, resulting in a nuclear accumulation of active caspase-3 during apoptosis (Kamada *et al.*, 2005). This could suggest that cells treated with alpha-synuclein are more likely to undergo apoptosis. Feng *et al.* (2005) initially saw the activation of caspase-3 on the inner surface of the cellular membrane, where it was then transported to the cytoplasm and then translocated into the nucleus. The authors suggest that the different subcellular localizations are directly related to the steps in the apoptotic pathway, which results in changes in cell morphology throughout the process. This could suggest that the cells treated with alpha-synuclein go through the apoptotic pathway faster than cells in the control wells, and the addition of alpha-synuclein could result in immediate induction of apoptosis. In wells treated with alpha-synuclein plus blueberry extract, caspase-3 is present in the both the nucleus and cytoplasm. Therefore, the addition of the blueberry extract may protect the cells by limiting the activation of apoptosis, or even slow the progression of the apoptotic pathway. The cells may be given more time to properly shut down or fix some of the damage caused by the addition of alpha-

synuclein. However, more research still needs to be conducted to determine the exact location of capase-3 within cells treated with our blueberry extract. This will require further caspase-3 labeling, as well at the determination of cell types in our dissection plates using specific antibody labeling or using pure cultures, as the translocation and compartmentalization may be dependent on the cell type and how it specifically regulates apoptosis.

Using confocal microscopy, there were differences in cell morphology observed between the conditions. Cells in the control wells looked healthier, stained better, and were more dispersed. Cells in the alpha-synuclein treated wells however, were clumped together. Cells treated with alpha-synuclein plus the blueberry extract were more dispersed, however did contain areas of clumped cells. This conclusion may not be entirely evident in all of the images that were captured but certainly was evident under the confocal microscope. This may likely be due to the fact that the alpha-synuclein treated cells were clumped and damaged resulting in faint Alexa 488 labeling.

The images in Figure 3.26 capture differences in cell morphology and cellular differentiation of pure neurons in control (A), alpha-synuclein treated wells (B), and wells treated with alpha-synuclein plus the Pippy Park (21/09/16) blueberry extract (C). Neurons in the control wells are very elongated and show

different levels of differentiation. Neurons in the alpha-synuclein treated wells however show no neuronal elongation and are very clumped together. In the wells treated with alpha-synuclein plus the blueberry extract there was some elongation seen in these wells, however, not nearly as much as in the control wells. There was clumping seen in these wells, but not to the extent it was observed in the wells without the blueberry extract. The presence of the extract was able to not only show protection in reducing the amount of cell death (Figure 3.20), but the extract was able to promote morphology associated with healthy neurons even in the presence of alpha-synuclein. Even though both morphologies were observed in these wells we can conclude that the addition of the blueberry extract was able to promote elongation and prevent the cells from clumping together. Axon growth is not a default action for neurons and must be properly and specifically signaled (Goldberg, 2004). Therefore, the addition of alpha-synuclein may interrupt these specific signaling pathways, and the blueberry extract may prevent alpha-synuclein from interfering. Sharada *et al.* (2015) found a similar result where hippocampal neurons were treated with alpha-synuclein, which resulted in defective axon elongation and blunted growth cone turning. Similarly, Takenouchi *et al.* (2000) showed that rat neurons transfected with human alpha-synuclein resulted in a decrease in neurite

extension. However, in this study the addition of antioxidants including catalase, superoxide dismutase and vitamin E, were not able to significantly affect neurite extension.

4.3 Pilot diet study

We wanted to determine the palatability of a 2% blueberry enriched diet in C57BL/6 mice, which took place over 5 weeks. As mentioned previously, most studies, including those conducted in our lab use oral gavage as the route of administration. Oral gavage has been shown to lead to differences in bioavailability (Atcha *et al.*, 2010), be very stressful for the animals (Balcombe *et al.*, 2004), and potentially cause extensive internal damage (Vandenberg *et al.*, 2014). To avoid these concerns, a plain mash (control) or a 2% blueberry-mash (experimental) was provided to the animals' *ad lib* overnight. Every morning the dishes were empty for both the control and experimental groups, showing that all 5 g of the mash was consumed by each animal without having to force them to consume it. There was no significant weight gain or loss for any of the animals or any adverse behavioral issues (aggression, anxiety, fighting, lack of grooming), showing that this method of feeding is palatable over time. Moving forward it might be worth looking at the amount of food (pellets during the day) and water consumed daily, to see if the blueberry extract had any effect on their intake. In

the future, it might be interesting to look at the different levels of stress-related hormones in the animals fed via oral gavage and those fed the blueberry-mash. Also, determining the palatability of a blueberry leaf-enriched diet using the mash would be very important to investigate. The leaves are much more bitter than the fruits, and it would be interesting to see if the animals would be interested in voluntarily consuming the mash with leaf extract added, and whether this may influence weight.

Fixed-speed rotarod testing at 5 rpm, 10 rpm, and 15 rpm was conducted throughout the study for a total of 8 sessions. There were no significant differences observed between the experimental and the control group at any of the speeds. However, there was a slight incline observed for all three speeds, suggesting that by the end of the study the experimental group was potentially able to stay on the rod slightly longer. This may suggest that the animals in the experimental group could have potentially had better motor coordination and balance, and were able to gradually improve over the course of the study. This observation would likely be more evident if the sample size was larger and the study had continued for longer than 5-weeks. For the 5 rpm trials there is not a lot of difference between the two groups, the animals in both groups were able to stay on the longest at 5 rpm, as it is the slowest and least difficult, however the

experimental group performed better than the control for the last three test days of the study. For the 10 rpm trials there is a slight difference observed between the two groups, where the controls were only able to stay on the rod for an average of 75 to 90 seconds by the end of the study, whereas the majority of the animals in the experimental group were able to stay on for the full 120 seconds by the end of the study. For 15 rpm a slight incline was observed where both groups showed gradual improvement throughout the study. Animals in both groups were not able to stay on the rod as long at this speed, as it was the fastest and most difficult. For the second half of the study, it appears that the experimental animals were able to stay on the rod longer than the control group at 15 rpm. Similar results were observed from a study conducted by Shukitt-Hale *et al.* (2013), where aged rats given a blackberry diet showed an improvement in motor performance, particularly balance and co-ordination for the accelerating rotarod, wire suspension and the small plank walk.

Novel object recognition for both the 1-hour and 24-hour trials had a DI above zero indicating that the animals in both groups were able to form a memory of the familiar object. The experimental group spent more time exploring the novel object suggesting that they may have better short-term memory compared to the control for the 1-hour trials. The DI for the 24-hour trials was substantially lower

than the 1-hour trials, which is expected as difficulty increases with time. A trend was observed for the 24-hour trials where the control group actually spent more time exploring the novel object compared to the experimental group. This fluctuation was likely influenced by the fact that two experimenters were involved with NOR testing during the 1-hour trials, making testing more efficient and coherent, whereas only one experimenter was present for the 24-hour trials resulting in the NOR testing taking twice as long. Shukitt-Hale *et al.*, (2013), saw similar cognitive results to our 1-hour trials, where animals fed a blackberry-enriched diet had significantly better short-term memory performance compared to the control group via Morris water maze testing.

There are limitations that are associated with the use of rodents including housing conditions, strain, gender, biological rhythms, and route of drug administration (Balk *et al.*, 2006). This particular study had a small sample size with both males and females of varying ages, which resulted in limitations regarding splitting up the animals into either the control or experimental group. The differences among the 18 C57BL/6 mice was due to the fact that those animals were the only wild-type mice we had in our colony at the time. It was decided that animals were to be split into the two groups based on their sex and their age, factors we thought would allow for the most accurate balance among

the two groups. However, even after dividing the animals evenly into the groups, the experimental group was slightly lighter overall than the control group (see Figure 3.27). This could have potentially skewed rotarod results as the lighter group may have been able to stay on the rod longer (Kovacs & Pearce, 2013). However, two-way ANOVA *post hoc* Bonferroni test was conducted and there were no significant differences between the performance of males vs females in the rotarod or significant differences between the ages (<3mo, 6mo-≦3mo, ≧6mo). Female animals and younger animals are lighter (Kovacs & Pearce, 2013), but they did not significantly perform better than the males or older mice on the rotarod. Therefore, this slight difference in the weights between the experimental groups may not have influenced the data, as weight did not affect the rotarod results. These insignificant results also show that the grouping based on age and sex, even though it was not exactly equal, did not significantly influence results. The groups were well balanced with each other, even though they differed.

The duration of the study could have been longer as well. Initially we wanted to do a full 8-week study but do to time constraints it was cut down to 5 weeks. It would be interesting to see the results for the rotarod and NOR testing had the study gone on longer, and if some of the baseline trends we began to see would

become more apparent. It would be beneficial to repeat this study using animals of the same sex, age, and relative weight to maintain consistency. As well as repeat the study with aging animals, or animal's models where pathological markers associated with AD or PD are present. A major goal of the pilot study was to verify palatability and weight gain so that we can take the next step and test in animal models of NDD. This way we could look at the levels of insoluble protein aggregates, such as alpha-synuclein and amyloid-beta, and determine if the addition of our blueberry extract to the diet is able to eliminate their presence and aggregation. This was seen in a study where transgenic AD mice were fed pomegranate juice, which is very high in polyphenols, or sugar water from 6-12.5 months of age (Hartman *et al*., 2006). Mice given the pomegranate juice had significantly reduced amyloid-beta accumulation in the hippocampus compared to the mice given the sugar water.

Determining the improvement of the symptoms associated with NDD such as memory loss with AD, and akinesia with PD, in NDD animal models would be beneficial as well. Casadesus *et al.* (2004) looked at the effect of a short-term blueberry diet administered to aging rats, where they saw an overall improvement of hippocampal plasticity and cognitive behaviors. Enhanced learning and memory, associated with age-related neurodegeneration was

improved by increasing the levels of insulin-like growth factor (IGF-1) and IGF-1 receptors, which mediate the rate of neurogenesis.

Chapter 5: Conclusions

In conclusion, through biochemical analysis, this study has shown that low-bush wild blueberry fruits and leaves have very high levels of phenolic compounds and high antioxidant activity. It was determined that the leaves had significantly higher antioxidant activity and total phenolic and flavonoid content compared to the fruits. The total anthocyanin content was significantly higher in the fruits. The total tannin content had mixed results, where one half of the samples had significantly higher levels in the fruits, and the other had significantly higher levels in the leaves. There was a significant difference between the phenolic levels in the fruits and leaves collected within and among the different locations. In the future, it may be beneficial to identify the genotypes of blueberry plants in particular areas that exhibit very high antioxidant scavenging abilities and breed them (Connor *et al.*, 2002) in order to improve the efficacy of blueberry samples.

Through *in vitro* experiments with mixed cultures, containing many different types of neurons and glia, as well as with pure rat neurons and microglia (ScienCell), a trend was observed where we saw an increase in the number of viable cells when blueberry extract was added to cells treated with 100 μM glutamate, 100 ng/ml alpha-synuclein, or 7.5 μM amyloid-beta, bringing them to

numbers close to the number of cells observed in the control wells. Even though these results are promising, replications of similar studies are needed in order to confirm these conclusions. There are several proposed mechanisms of action associated with the addition of phenolic compounds found in blueberries to help treat NDD and prevent neurodegeneration in the CNS. The diverse phenolic compounds present in this blueberry species may have additional mechanisms of action that could account for the potential neuroprotective role they exhibit. NDDs, such as PD and AD are having a major impact on the health of our aging population. Further enhancement and development of potential drugs and therapies to help treat these diseases will depend on further understanding of their underlying pathology. Through better understanding, we will be able to implement treatment in the early stages of these diseases by identifying potential biomarkers and high-risk individuals (Balk *et al.*, 2006). The identification of potential biomarkers is extremely important, as cellular dysfunction occurs long before a lot of the symptoms associated with these diseases (Dauer & Przedborski, 2003). Early treatment could prevent significant damage and improve quality of life for those living with a NDD.

Even though the addition and consumption of wild Newfoundland blueberries in this study has been shown to be beneficial and neuroprotective, it

is important not to overgeneralize or misinterpret data. For example, *in vitro* and *in vivo* rodent models are beneficial for investigating the mechanisms of action associated with the neuroprotective role of polyphenols, however they differ greatly with respect to clinical trials. *In vitro* and *in vivo* experiments can be strictly controlled (Balk *et al.*, 2006), however there are many factors contributing to the bioavailability of the active metabolites in the human body (see section 1.5). There have been very few clinical studies looking at the effects of a blueberry enriched diet and most are highly debatable due to low sample sizes and inconsistencies during the study. A preliminary study conducted by Krikorian *et al.* (2010) looked at the effects of wild blueberry juice consumption in aging adults experiencing early memory changes over the course of 12 weeks. The results showed that the group that received the wild blueberry juice performed better with paired associate learning and list recall compared to the placebo group. A recent clinical study looked at the cognitive effects of the addition of 24g/day of blueberries to the diet of adults aged 60-75 (Miller *et al.*, 2018). The study was a double-blind placebo-controlled trial, and the results showed that the group given the fresh blueberries daily had significantly fewer repetition errors in the California Verbal Learning test compared to the control group. Better knowledge of the bioavailability of the phenolic metabolites found

in blueberries is essential if their effects are to be fully understood so further comprehensive clinical trials can be conducted (Manach *et al.*, 2004). Further studies will need to be conducted in order to determine toxicity levels and proper 'dosing' for an individual to experience a beneficial effect. This dose will not only depend on the amount required to elicit some form of neuroprotection, but how this dose can be properly and effectively introduced into an individual's lifestyle.

This study provides an essential step for determining the nature, distribution, mechanisms, and neuroprotective role of polyphenols in our diet by investigating the relation between the intake of these substances and the risk of NDD development and prevention. A blueberry enriched diet consisting of fresh fruits and leaves, or a nutraceutical product such as tea, powder, or juice, may slow the progression of NDD such as AD and PD. To summarize, blueberry fruits and leaves have the potential to influence future treatment or prevention of NDD, and may have many mechanisms of action associated with slowing the pathology of these diseases.

Chapter 6: References

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