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Modeling Music Therapy in an Alzheimer's Disease Cell Culture System through the use of BDNF and LM11A-31

A Thesis in Neuroscience

by

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Abstract

Alzheimer's disease (AD) is a form of dementia that impairs memory and mental functioning by disrupting neuronal metabolic processes (Selkoe 2001). One molecular system which is downregulated in the brain of AD patients is the neurotrophin, BDNF, and its main receptor tyrosine kinase, TrKB (Arancibia et al. 2008). In contrast, the p75 receptor, which is normally stimulated by proBDNF (uncleaved form of BDNF) and can lead to neuronal toxicity, is upregulated in AD patients and is one of the targets for amyloid beta (Dechant and Barde 2002). Recently, scientists have begun to explore potential therapies for AD such as the use of music which has the potential to stimulate cognitive functioning of the elderly (Simmons-Sterna 2010). Research in animal models suggests that BDNF can be enhanced by music exposure (Angelucci et al. 2007b). In addition, scientists have synthesized an exogenous p75 receptor ligand, LM11A-31, which may be able to shift neurons toward a survival pathway (Yang et al. 2008). In the present study, an Alzheimer's disease primary cortical neuronal culture model was created via the addition of amyloid beta or excess glutamate in order to test the potential interaction between BDNF and LM11A-31. Cell survival rates were based on an MTS Assay and neuronal morphology was characterized by tubulin staining. Cells were treated with BDNF and LM11A-31 in order to test the effects of these compounds on percent cell survival and relative tubulin staining of the neurons. The interaction between BDNF and LM11A-31 was also tested. The combined effects of BDNF and LM11A-31 decreased cell survival rates in most experiments, but this was not consistent across all testing conditions and therefore the data was inconclusive. Future studies will need to examine BDNF and LM11A-31 separately and in combination to gain a more complete understanding of these compounds as potential therapies for Alzheimer's disease.

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Introduction

Overview of Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease which causes pronounced memory deficits and decreased cognitive functioning predominantly in the elderly population (Selkoe 2001). It affects one in ten Americans over the age of 65. The largest risk factor is age as one's risk doubles every five years after age 65 (Alzheimer's Association 2016). Consequently, Alzheimer's disease is one of the top ten causes of death in America (Nauert 2015). With an increasingly elderly population, the number of individuals with Alzheimer's is expected to rise exponentially. As of 2014, the prevalence of AD has been estimated to be 19% of those ages 75 - 84 and 47% of those age 85 and older (Han and Han 2014). Unfortunately, it is predicted that by the year 2040, over 80 million people worldwide will have this disease (Bali et al. 2010).

Our understanding of the pathologies of Alzheimer's disease originate from a translation of an original case report from Dr. Alois Alzheimer, a German psychiatrist, who first described the condition in 1906 (Strassnig and Ganguli 2005). One of his patients, a fifty-one-year-old woman named Auguste D, started to show signs of memory loss and abnormal fluctuations in her emotional state (Strassnig and Ganguli 2005). For example, during observations she would sometimes hallucinate and call out for her husband. She also had difficulty forming sentences and answering questions. An autopsy on her brain later revealed abnormal dense neurofibrillary bundles which formed the basis for what is today known as the main hallmarks of Alzheimer's disease: plaques and tangles. The condition was later named Alzheimer's disease in 1910. During the 1960s, Alzheimer's research became more prevalent, further exploring the cellular and molecular basis of the condition (Bali et al. 2010). This increase in AD research was partly due

to a reclassification of elderly with "senile dementia" who were now getting diagnosed with Alzheimer's disease.

With an increasing elderly population, the cost of Alzheimer's disease continues to create significant economic consequences. In fact, it is estimated that by the year 2017, Alzheimer's disease as well as other dementias will cost the United Stated $259 billion dollars (Alzheimer's Association 2016). Aside from costs associated with research and insurance, AD has become a major issue for individual families as well. In addition to money, family members often need to spend time providing daily care for their loved ones and attending doctor's appointments. Other expenditures include caregivers, medications, adult day care services, and assisted living or nursing home facilities. According to the Alzheimer's Association, a private room in a nursing home costs approximately $250 a day or over $90,000 a year (2016).

In January 2011, President Obama signed the National Alzheimer's Project Act (NAPA) (National Institute on Aging 2013). Its basis was to create a national plan to combat Alzheimer's disease and improve nationwide care. This was then followed by the U.S. Department of Health and Human Services (HHS) creation of the National Plan to Address Alzheimer's Disease. Its goals were broad including furthering scientific research and expanding patient care. In total, the goals of these legislative acts are to "prevent and effectively treat Alzheimer's disease by 2025" (National Institute on Aging 2013). It is anticipated that with continued funding and support from the federal government to discover a treatment and cure, perhaps Alzheimer's may one day become a disease of the past.

Pathology of Alzheimer's Disease

In order to further research potential treatments for this condition, it is important to understand how AD can affect one's everyday life. Many people can live years with AD and

often times once a probable Alzheimer's diagnosis has been pronounced, the disease has already degraded brain areas and affected neuronal structure. Most people live four to eight years after diagnosis, but can also live much longer depending on the severity (Alzheimer's Association 2016). There are many stages of the disease but the main progression begins by affecting one's hippocampus, the main brain region responsible for memory formation (Selkoe 2001). Common effects of this preliminary memory loss may include misplacing items, forgetting names of family members, and having trouble performing simple everyday tasks. Individuals in this early stage of AD can often still be independent. This is then followed by language and problem-solving impairment. A common occurrence may include having trouble recalling a familiar word. During the moderate stages of Alzheimer's, emotional regulation becomes harder and hallucinations may form. Individuals may exhibit more frustration, confusion, and anger during this period. Personality changes can take place as well as increased difficulty to do daily routines such as getting dressed. Further, nerve cell damage can make expression of thoughts increasingly difficult. Information transmission at synapses decline and neurons die (Selkoe 2001). During the late stages of Alzheimer's disease, one's oldest memories can be disturbed as well as balance and coordination. Individuals lose awareness of their surroundings and have trouble with physical abilities such as walking. Full-time care is usually necessary. Lastly, regulation of breathing ceases which can ultimately lead to death. However, at this stage, individuals can also become more susceptible to other diseases such as pneumonia which can cause death even before breathing regulation is affected. The entire disease progression is long and can last for an average of eight to ten years.

Although the exact cause of Alzheimer's disease is still unclear, evidence strongly indicates that the formation of plaques and tangles (two main hallmarks of AD) and the loss of

neurons contribute to this disease (Selkoe 2001). Although these are the two main formations that occur in the brain of an Alzheimer's patient, they can only be confirmed with a post-mortem examination. Plaques are formed due to amyloid beta peptide aggregation, while tangles are created via hyper-phosphorylation of tau which is a microtubule associated protein (Bali et al. 2010). Plaques are due to an accumulation of protein which can disrupt cell signaling and synapses. For example, in a morphometric analysis study conducted by Davies et al. (1987), it was found that there was a greater loss in synapses in individuals with Alzheimer's disease compared to individuals without AD such that there was a 25% decrease in layers II-III of the temporal cortex, 36% decrease in layer V of the temporal cortex, and 27% decrease in layer V of the frontal cortex (1987).

In contrast, tangles are likely to play a role in disrupting cell transport systems in axons. Tau assists in helping molecules travel in an orderly fashion within the transport system. However, when tangles form, the transport system loses its organization and begins to collapse, inhibiting nutrients from passing through the cell body toward the axon terminals. Microtubule-associated abnormalities were seen in a recent early stage AD mouse model whereby a large accumulation of tau caused axonal swelling and inhibited normal axonal transport (Stokin et al. 2005). The study authors proposed that inhibition of microtubule transport can stimulate the formation of beta-amyloid precursor protein thus leading to the formation of plaques as well. Although development of plaques and tangles can vary, origination usually begins within the cortex, hippocampus, and basal forebrain (Stokin et al. 2005).

Scientists are still debating whether plaques and tangles are the cause of Alzheimer's disease or if they are a byproduct of the condition. Glutamate dysregulation may be one of the pathways by which the pathologies of the disease cause neurons to degenerate (Gray and Patel

1995). Glutamate is an excitatory neurotransmitter that plays a role in neuronal communication especially in learning and memory. However, if glutamate signaling is not regulated, over-excitation of brain cells can lead to damage and decreased cell viability especially in areas such as the hippocampus, a main brain region for memory (reviewed in Lin et al. 2003). Excessive activation of glutamate receptors (NMDA) can also cause an increase normal calcium levels in the brain. In a clinical study with three AD patients and three controls, scientists measured GNT levels (glutamate neurotransmission) via ^{13}C magnetic resonance spectroscopy (Lin et al. 2003). They determined that a decrease in GNT levels in AD individuals could directly correlate with memory and cognitive deficits although a larger sample size would be beneficial in providing further support for this claim (Lin et al. 2003).

Aside from the formation of plaques and tangles and increased glutamate levels, there are other potential reasons for the development of AD. For example, acetylcholine breakdown is a common contributor to neuronal health decline due to over activity of the cholinesterase enzyme (McGleenon et al. 2009). Acetylcholine is a necessary neurotransmitter which plays a role in memory formation. Other approaches still being studied in the lab include reducing inflammation and preventing tau formation (Park 2016). Immune cells can respond to amyloid beta using specific immune receptors TREM22 and CD33 (Heneka et al. 2015). However, the result is often too intense, causing damage to the nerve cells and inflammation. Neuroinflammation may arise due to the high activity of microglia which are considered immune based glial cells within the brain. Furthermore, an increase in β- or γ-secretase activity can cause an increase in amyloid beta protein leading to plaque formation (Davies and Koppel 2009).

While AD is a condition that mainly affects the elderly population, there is also another form of the condition termed early-onset (Selkoe 2001). This type of Alzheimer's is mainly

genetic. Individuals with early onset will often begin to show signs of dementia as early as their 30s and 40s. Early-onset AD accounts for less than five percent of all Alzheimer's disease patients. The early-onset form of this condition is rare and autosomal dominant. Three genes that lead to early-onset AD include APP (amyloid precursor protein), PS-1 (Presenilin-1), and PS-2 (Presenilin-2.) PS-1 and PS-2 help cut APP in order to form amyloid beta (Bali et al. 2010). A variation in the PS-2 gene is one of the most common causes of inherited Alzheimer's disease.

One particular late-onset genetic risk factor is APOE (apolipoprotein) which comes in various forms: APOE2, APOE3, APOE4 (Alzheimer's Association 2015). Differences among these forms are due to the presence of cysteine or arginine in amino acids 112 and 158 (Liu et al. 2013). In regular circumstances, APOE assists in lipid transport and injury repair within the brain. Astrocytes produce APOE which transports cholesterol to neurons via specific receptors. APOE2 lowers one's risk of developing AD while APOE3 is relatively neutral. Everyone receives a copy of some form of the apolipoprotein. Risk for AD is associated with either one or two copies of APOE4, but increases further when two copies are present. APOE4 is the most dangerous form (Liu et al. 2013). It is associated with increased formation of amyloid beta plaques. However, even if one does inherit APOE4, Alzheimer's disease will not necessarily develop. This is because the presence of the APOE4 gene is not the direct cause of AD. The basis for how APOE4 can increase one's risk of AD is still being researched.

At present, there is currently no direct cure for Alzheimer's disease. Therefore, rather than researching a direct treatment, some pharmaceutical companies have shifted their lab research either towards lessening the effects of memory decline or symptom therapy (Bali et al. 2010). There are very few drugs approved by the U.S. Food and Drug Administration and they are all symptomatic drugs. The two main classes of drugs include cholinesterase inhibitors and

NMDA receptor blockers (Selkoe 2001). Cholinesterase inhibitors, such as Aricept and Razadyne, prevent the breakdown of acetylcholine in order to slow down memory loss. NMDA receptor blockers like Namenda regulate the activity of glutamate. One of the main reasons that so few drugs are available is because there are several causes of AD and not just one disease-causing pathology. Drugs usually target only one mechanism. Some strategies have been to inhibit the formation of amyloid beta plaques using small compounds that can cross the blood-brain barrier, decreasing β- or γ-secretase activity (Park 2016). Other labs are focused on researching AD anti-inflammatories. Additionally, since tau formation often appears later in AD disease development, some scientists propose that anti-tau treatments could potentially stop disease progression. For example, a vaccine with antibodies against the tau protein is presently being researched and developed. Aside from these approaches, other scientists are taking a more therapeutic approach to decrease the effects of symptoms or prevent AD from occurring in the first place rather than researching drugs for a direct cure. Some examples include using one's own immune system to clear beta-amyloid peptide, increasing vitamin E as an antioxidant to protect brain cells from deteriorating, and cognitive therapies such as puzzles and music.

Music Therapy and Memory

Music has become a new potential therapy for Alzheimer's patients. Neurologist Oliver Sacks once said that, "music evokes emotion, and emotion can bring with it memory… it brings back the feeling of life when nothing else can" (Rosatto-Bennett 2014). As Oliver Sacks alludes to, music is considered a universal language (Sacks 2007). It is now being used to help AD patients conjure old memories, even perhaps if they are unable to vocalize these memories with words (Simmons-Sterna et al. 2010). Interestingly, music processing is often spared even as neurodegeneration is robbing other cognitive skills. AD patients have an easier time recognizing

lyrics that are sung rather than spoken as words. Further, music is able to increase arousal in AD individuals and improve overall memory (Simmons-Sterna et al. 2010). There have been cases whereby individuals with Alzheimer's disease are able to comprehend and learn current events if the words are arranged into a song. In the Simmons-Sterna et al. study, AD patients and healthy adults of comparable age participated in the experiment which involved being presented with spoken words or musical lyrics with accompanying melody (2010). Participants were then asked if the words (lyrics or spoken) were repeated before or were novel. This involved memory and recall of what was heard prior. Following the tests, there was a clear difference between AD patients and regular individuals in that AD patients had greater mean recognition accuracy during songs. One possibility for this is that music is encoded differently than words. Music processing is more widespread than language processing in that it involves the basal ganglia, accumbens, hypothalamus, cerebellum, and prefrontal cortex (Simmons-Sterna et al. 2010). For example, the basal ganglia play a role in beat processing (Grahn 2009). The Grahn study measured activation of the basal ganglia during beat-based and non-beat sequences using functional magnetic resonance imaging (2009). Although this study focuses on Parkinson's disease patients and is not directly related to AD, perhaps this type of model can be a platform for AD music therapy research. The Grahn study also gives credence to the idea that music is processed not only by the auditory system but also within the nervous system.

The same brain areas involved in music are also the parts of the brain that are affected more slowly by AD (Simmons-Sterna et al. 2010). Since brain areas such as the cerebellum, prefrontal cortex, and basal ganglia are not as vigorously affected by AD, it leaves more opportunity for association and coding to be made compared to simply spoken words. Music interpretation also involves both hemispheres of the brain. For example, the left hemisphere

mostly processes rhythm and pitch while melody and tone of the music is processed in the right hemisphere (Jenkins 2001). Simmons-Sterna et al. determined that music can improve recognition memory encoding, a form of declarative memory, for AD patients (2010). Although the duration of time of improved memory was not specified, the mean recognition accuracy of lyrics was greater in sung rather than spoken word. For example, in AD patients, the mean recognition accuracy was 28% for spoken lyrics and 40% for sung lyrics (Simmons-Sterna et al. 2010). However, interestingly, healthy adults did not show any benefit of music with regard to memory in this particular experiment. This is perhaps because in healthy adults, the recognition task was very simple. It did not require recruitment of information from other areas of the brain as shown in AD patients. Yet, in other studies, music did improve memory in healthy individuals as well. For instance, background music has been shown to increase performance on verbal memory tasks, particularly categorical fluency (Thompson et al. 2005) and context-dependent memory (Standing et al. 2008). Therefore, presenting information via song to patients may be a therapy to reduce accompanying memory loss while also increasing overall comprehension (Kumar et al. 2009). Kumar et al. shows that music exposure can increase melatonin levels in Alzheimer's patients, creating a more soothing state for the AD individual.

The potential for music to be beneficial for health was first written about by Aristotle and Plato (as reported in American Music Therapy Association 2016). They argued that music may have a healing influence which could ultimately affect one's health and overall behavior. Music therapy began to take place during World War I and II when musicians would travel to veterans' hospitals to provide entertainment for patients. Following this, patients began to show signs of enhanced emotional and physical responses which later became the basis for music therapy, and hospitals soon after began to hire musicians. Years later in 1983, The Certification Board for

Music Therapists (CBMT) was created to ensure a standard of credentials for all music therapists.

In recent years, music has been used for its positive effects on memory and mood in the elderly. In particular, a non-profit organization based in New York, Music and Memory, founded by Dan Cohen, is dedicated to using music to improve quality of life for the elderly and those with dementia (Cohen 2017). The organization does this by training elder care workers as well as family members to create music playlists to assist individuals who have Alzheimer's and other cognitive deficits. The music is able to help individuals via music-triggered memories. The program has seen results especially with patients in nursing homes who once were not very responsive. Yet, when music is played they become more engaged and show signs of mental processing such as clapping to the beat and smiling. Patients are also more relaxed, attentive, and motivated (Clark et al. 1998). Aside from the patient, music also reduces the stress of the caregiver (Rosatto-Bennett 2014). In April 2014, a documentary based on the work of this nonprofit, *Alive Inside: A Story of Music and Memory*, was created. One of the many touching stories in the film was about a gentleman named Henry with Alzheimer's disease who was a nursing home resident and unresponsive and isolated. However, when given an iPod containing his favorite music, he began to sing and his facial expressions demonstrated happiness. Once the headphones were taken off, he was better able to respond to questions by his caregivers following this period of music exposure. He accurately responded to questions about his favorite music and recalled certain lyrics. Oliver Sacks explains that in a sense he "reacquires his sense of identity for a while through the power of music" (Rosatto-Bennett 2014). During the documentary, Dr. Bill Thompson, a geriatrician, explains that deep structures in the brain which hold the identity of a person often are undamaged when one has dementia. Yet, when analyzing

the importance of music for dementia patients, it is important to understand the value of individual examples versus scientific studies. Although Henry's story is compelling, it is only the experience of one individual. Individuals vary on multiple levels such as genetics and prior experiences. Therefore, the benefit of music on memory cannot be considered causational based on one particular case study or individual example. Scientific studies allow for more verifiable results and conclusions due to a greater sample size. They incorporate many individuals and can usually be repeated by future researchers. However, the use of both individual examples and scientific studies in tandem provides a broader view of the effect of music therapy on memory in AD patients.

Music therapy is a developing new option for those with Alzheimer's disease. Music can help put both the AD individual and family more at ease while also assisting with dementia and AD symptom reduction. Additionally, music could potentially decrease costs associated with dementia and Alzheimer's disease. Medications such as anti-psychotics are often used to sedate those in nursing homes (Beers et al. 1991). Rather, if music therapy is used more frequently, it could potentially improve quality of life and health of residents while also decreasing costs of medical care (Romo and Gifford 2007).

Although music therapy, either through the use of a musical device such as an iPod or via a music therapist session, is beneficial, some researchers have disputed its practicality (Götell et al. 2002). This is because music therapy is often very structured and conducted methodically with certain music and a trained musical therapist. However, it is not integrated into one's everyday routine and therefore may seem somewhat foreign to the AD individual. Therefore, the Götell et al. study explains that music therapy may be more beneficial if it is incorporated into one's daily activities and conducted with familiar individuals such as a caregiver or family

member (2002). One particular study piloted in a dementia care unit in Sweden sought to gain an understanding of how caregivers incorporating music into daily activities can affect one with dementia. The study consisted of three routine conditions for each patient: a control condition (i.e., the usual caring situation), a caring routine done with recorded music playing in the background, and a caring routine in which the caregiver sang to and/or with the patient. Caregivers were not given musical training prior to the session and thus the music they sang or hummed relied on their inherent musical knowledge and skill. This made the session more realistic. Following the completion of the study, it was found that when caregivers engaged in singing, the amount of explanation of activities and narration decreased and compliance increased. However, when the caregiver did not engage in singing, more explanation for the patient was necessary and they were more resistant to care. Therefore, this is yet another example of how incorporating music into the everyday lives of dementia patients can increase understanding of the world around them and lessen certain memory loss symptoms.

Music therapy is often deployed once an individual shows signs of dementia. Additionally, music therapy is not limited to memory loss patients but has been used for other conditions such as post-traumatic stress disorder individuals (Sorensen 2015). However, rather than use music as a type of therapy, some researchers are suggesting that learning a musical instrument at a younger age can be used as a preventive measure against dementia at a later age (Balbag et al. 2014). This particular study used twins to determine the effects of playing a musical instrument at an older age. Twins were a viable population to study because dementia is usually affected by genetic influences. By using twins, researchers were able to reduce confounds due to genetic and hereditary differences. It was found that the twin who played an instrument in older adulthood had a sixty-four percent lower chance of developing dementia

compared to the nonmusical twin. This allows music to be perceived as a potential protective agent against cognitive impairment aside from simply being a therapy after one develops dementia.

In a particular case study of an 82-year-old male who was a musicologist, the gentleman was still able to play particular piano pieces even though he could not recall the name of the composition or its composer (Crystal et al. 1989). Another example of music's ability to affect memory is seen in Clive Wearing (Sacks 2007). Wearing had amnesia. Even though he lost his memory for everyday facts and activities, he still recalled a memory for music. One reason for this is due to the anatomy of the brain and the coding of various types of memory. Procedural memory, such as playing the piano by memory, uses the striato-prefrontal circuits of the brain (Crystal et al. 1989). These particular areas tend to be unharmed by AD which is one of the reasons why procedural memory for playing a musical instrument or conducting a choir, such as Wearing did, may be conserved.

There are multiple benefits of music for AD patients such as enhancing memory while potentially protecting against cognitive decline (Simmons-Sterna et al. 2010). The basis for hearing music begins with vibrations of hair cells within the ear creating a nerve impulse, which causes the brain to respond (Purves et al. 2012). The cellular basis for how music is able to protect the brain from neurodegeneration is still an area of scientific study. Therefore, my study focuses on testing one of the potential processes that underlie music therapy in a cell culture model of AD. It also explores how certain brain chemicals such as brain derived neurotrophic factor (BDNF), shown in other models to increase during music exposure, in conjunction with LM11A-31, can lessen the effects of neurodegeneration.

Brain Derived Neurotrophic Factor (BDNF)

Certain neurotrophic factors can be enhanced by cognitive stimulation such as music. Neurotrophic factors are necessary for the health and regeneration of neuronal populations within the brain (Allen et al. 2013). Neurotrophins are proteins involved in the growth, survival, and overall function of neurons. There are various types of neurotrophic factors which have been implicated in cell survival. BDNF can be enhanced by engaging in auditory stimulation such as hearing music (Angelucci et al. 2007b). Angelucci et al. used a mouse model demonstrating that specific neurotrophins are affected by music exposure, including brain derived neurotrophic factor (BDNF) within the hypothalamus (2007b). The hypothalamus is an area of the brain relating to body homeostasis as well as overall regulation of the neuroendocrine and immune system. Additionally, Angelucci et al. performed a similar study focusing on the hippocampus (2007a). The study involved exposing mice to periods of music for twenty-one days followed by a passive avoidance test to measure learning. Mice exposed to music had higher scores on the test as well as increased levels of BDNF post-mortem. These results were similar to the Angelucci et al. study that looked at BDNF levels in the hypothalamus in that BDNF levels rose, while NGF decreased when mice were exposed to music (Angelucci et al. 2007b)

The experimental method employed in the Angelucci et al. hypothalamus study is an *in vivo* mouse model (2007b). A total of twenty male mice were exposed to six hours of slow rhythm instrumental music per day for a total of twenty-one days. On the twenty-second day, the animals were sacrificed and hypothalamus dissected. Using a two-site enzyme immunoassay, the concentrations of BDNF and NGF were obtained. Those mice exposed to music had an up-regulation of BDNF and down-regulation of NGF. Both Angelucci studies demonstrate that music can affect brain chemistry and, more specifically, that altered levels of BDNF and NGF

can correlate with physiological consequences, speculating an effect on HPA axis regulation as well as the hippocampus and memory (Angelucci et al. 2007a,b).

Since this particular study only used soothing, New Age music without lyrics, in future studies it may be interesting to determine how songs with lyrics as well as different music genres affect neurotrophic levels. The experiment's strength is that it shows that BDNF and NGF levels in the hypothalamus can be altered due to music exposure. The researchers suggested that altered levels of these neurotrophins can affect normal functioning of the hypothalamus such as regulating homeostasis, although this is only speculation. The hypothalamus is not generally involved in memory formation. Other studies in the field of music therapy have focused on the hippocampus, the central area for memory. One particular study by Kim et al. showed that exposure of fetal rats to music aided their spatial memory learning once born (2006). Although the methodology partially differs from the Angelucci et al. study (2007b), in that music exposure occurred before the mice were born, results of the Kim et al. study demonstrates that music exposure can have effects on overall behavior. When rat pups were exposed to stressful noise, neurogenesis within the hippocampus decreased, which ultimately affected the pup's cognitive abilities as measured through spatial learning tasks (Kim et al. 2006). The antithesis of these results were seen when the rat pups were exposed to music. Neurogenesis was enhanced as shown by brain slice images from the hippocampus using a photomicrograph. Spatial memory abilities also increased due to music exposure when pups participated in the radial-arm maze test. However, when speaking about the therapeutic use of neurotrophins, it is important to understand that BDNF cannot cross the blood brain barrier. If it were to be used as a potential AD therapy, it would need to be infused directly into the brain (Allen at el. 2013). One option may be developing an agonist for BDNF to bind to certain receptors.

Brain derived neurotrophic factor is often seen in brain areas with high plasticity such as the hippocampus (Tapia-Arancibia et al. 2004). BDNF plays a role in maintaining cell survival, health, and growth of the neuron (Allen et al. 2013). When aging occurs, BDNF expression is often altered as is the expression of its main receptor, TrkB. The receptor, TrkB, is part of a family of Trk receptors, including neurotrophin receptors TrkA and TrkC (Longo and Massa 2013). Patterson et al. was the first to explore the link between TrkB, BDNF, and long term potentiation (1992). It was further researched by collaborative scientists at the Max Planck Florida Institute of Neuroscience and Duke University (Harward et al. 2016). Scientists began by monitoring TrkB activity in live rat brain tissue in regions involved with memory. When excitatory glutamate was added, there was an increase in dendritic spine size and activation of the TrkB receptors. However, when BDNF was inactivated via the use of an antibody, TrkB signaling was impaired which negatively impacted long term memory potentiation (LTP). Therefore, this helped further demonstrate that both BDNF and TrkB were necessary for learning and memory formation. When BDNF was released post-synaptically from the cell, it helped activate TrkB during functional and structural LTP, creating a BDNF-TrkB signaling loop necessary for synaptic plasticity. Recently, scientists have discovered that providing more BDNF or stimulating its receptors could offset or decrease the speed of age related brain changes and prove to be a therapeutic alternative for aging. For example, in the Cirulli et al. study, rats receiving intrahippocampal administration of BDNF had increased short-term behavioral plasticity (2004). However, rats lacking the BDNF gene had decreased long-term potentiation. Memory retention abilities were evaluated by measuring latency and path length to reach the platform using a Morris water maze. Therefore, there is evidence for a potential relationship between BDNF and its abilities to aid in maintaining cognitive abilities as one ages.

BDNF levels can also be enhanced by physiological and physical actions such as diet restriction, exercise, and cognitive stimulation (Zuccato and Cattaneo 2009). The number of newly generated neurons in the hippocampus can increase via cognitive stimulation such as listening to music (Fukui and Toyoshima 2008). Fukui and Toyoshima explain that music causes regeneration and repair of neurons by modulating hormone secretion, causing cerebral plasticity (2008). Specifically, music affects levels of cortisol (C), testosterone (T) and estrogen (E). The nervous system is a large target for these steroids which regulate brain development, neurogenesis, neuroprotection, cognition, and memory (Fukui and Toyoshima 2008). Just as music can alter steroid levels affecting neuronal growth, BDNF levels, which can be enhanced by music exposure, can also affect neuronal morphology and neurogenesis at the level of the dendritic spine within specific brain areas (Tapia-Arancibia et al. 2008).

LM11A-31

Individuals with Alzheimer's disease often have an upregulation of p75 receptors specifically in the cortical and hippocampal regions (Hu et al. 2002). The p75 receptor binds multiple neurotrophins including BDNF, NGF, NT-3, and NT-4, and is part of the TNF (tumor necrosis factor) receptor superfamily (Casaccia-Bonnefil et al. 1999). The p75 receptor has been shown to function as a co-receptor for Trk tyrosine kinases (Casaccia-Bonnefil et al. 1999). Depending on the presence of particular receptor ligands, cells expressing the p75 receptor can be pushed towards either a survival or death signaling pathway (Hempstead 2002). The Yang et al. study was conducted using primary neuronal cultures of cortical, hippocampal, and septal cultures followed by incubation with amyloid beta (2008). Following a period of incubation, neurons were viewed under a fluorescent microscope and categorized based on morphology. Neurite dystrophy was confirmed with immunostaining. Hippocampal slice cultures were used to

measure induced LTP via electrophysiological recordings. Scientists in the Yang et al. research lab explored how p75 ligands can potentially decrease neurodegeneration, maintain synaptic functioning, and enhance cell signaling (2008). This study relates to broader questions in AD as it demonstrates that there is a probable interaction between amyloid beta, a hallmark of Alzheimer's disease, and p75 signaling, in that these ligands can inhibit amyloid beta-induced cell death. The p75 ligands can assist in protecting neurons via three main methods, including blocking interaction between p75 and amyloid beta, down-regulating certain protein signaling pathways, and up-regulating survival signaling. The experimental method of the Yang et al. study included primary neuronal cell cultures of E16 mice (2008). Neuronal survival was quantified with Syto 13 or TUNEL/DAPI staining (TUNEL negative indicates cells were protected). Cells were characterized based on shape of soma and presence of beaded neurites to quantify torturosity or how much stress the neurons encountered. The results of this study exhibited how protective effects of compounds such as LM11A-31 occur via the p75 receptor (Yang et al. 2008). This particular ligand prevents amyloid beta neurite dystrophy or induced cell death.

The implications of these results indicate that LM11A-31 could be a potential new component of Alzheimer's drug development and therapeutics. This p75 receptor ligand also has a low nanomolar potency, which means that only a small amount is required to have a strong influence. Also, LM11A-31 drugs displace NGF since they have the same binding site. Additional experiments may include creating an *in-vivo* model experiment in an Alzheimer's induced rat to determine if p75 ligands can assist in decreasing neuronal degeneration. One particular review paper by Elizabeth Coulson further explores the complexity of p75 signaling, explaining that the pathway does not always promote cell death (2006). There could be various

p75-mediated signaling pathways that may contribute to AD. Therefore, further research on the topic of p75 ligands is necessary in order to gain a broader understanding of their cellular mechanisms in Alzheimer's disease.

Scientists have begun experimenting with *in vivo* models to determine the efficacy of LM11A-31 as an effective Alzheimer's therapy. Researchers have shown that neurons of brain areas that typically degenerate in AD tend to exhibit p75 (Knowles et al. 2013). Some examples of these include the hippocampal, basal forebrain cholinergic, entorhinal, and locus coeruleus neurons (Knowles et al. 2013). LM11A-31 is a molecule that is a derivative of water-soluble isoleucine containing a morpholino group, which is a relatively new type of oligomer molecule being applied to medicine. LM11A-31 decreases neurodegeneration caused by amyloid beta by blocking amyloid beta-induced pathways such as GSK3B (Knowles et al. 2013). Furthermore, this compound also prevents excess tau phosphorylation. In the Knowles et al. study, an APP $^{L/S}$ mouse model was used to mimic the effects of Alzheimer's disease and determine if early treatment with LM11A-31 could slow down the disease process. The effectiveness of the compound was quantified based on behavioral deficits and neurite degeneration.

The methodology of the experiment involved dissolving LM11A-31 in sterile water and administering it at various doses either orally or via intraperitoneal injections to the APP $^{L/S}$ mice, which is a standard AD mouse model (Knowles et al. 2013). The mice were treated for two and a half months prior to beginning the novel object recognition test. The goal of the test was to determine if mice returned to familiar objects they were exposed to twenty-four hours prior by measuring if they made contact with the object with their whiskers, paws, or nose. A spatial memory task was also performed using a Y maze test. Aside from behavioral experiments, the experimenters also measured the length of neurites.

The findings from the Knowles et al. study indicate that the LM11A-31 ligand can decrease AD pathology and consequential memory deficits by acting through p75 (2013). For example, rats treated with the compound had similar performance on the number of entries in the Y-maze compared to the wildtype control condition. Additionally, dystrophic neurite clusters, characteristic of AD, increase with age, but is reduced by the LM11A-31 treatment. For instance, eight-month-old rats had approximately 2300 um^2 mean cluster area exhibiting neurite dystrophy while rats treated with LM11A-31 had about 1700 um^2 mean cluster area. In the novel object recognition test, wildtype mice treated with LM11A-31 explored new objects more frequently and showed increased recognition as opposed to control mice. Therefore, this research validates that p75 ligands can potentially be used as neuroprotective compounds for Alzheimer's disease (Knowles et al. 2013).

Relationship between BDNF and LM11A-31

Research has shown that there may be a protective interaction between BDNF and p75 receptor ligand, LM11A-31, for individuals with Alzheimer's disease. Dr. Frank Longo of Stanford University explains that scientists have the ability to cure Alzheimer's in mice, but are not able to see the same results in humans (Simmons et al. 2014). Longo describes that scientists have been able to degrade amyloid beta in rat models using various compounds. However, similar results in humans may be possible with the use of the compound LM11A-31 (Knowles et al. 2013). Rather than degrade accumulated amyloid beta in the brains of AD patients, its goal is to increase the strength of neurons against the assaults of neurological factors such as amyloid beta and excess tau phosphorylation. In addition to LM11A-31, scientists at Rush Alzheimer's Disease Center have shown evidence for a correlation between high levels of BDNF and protection from cognitive decline associated with amyloid beta plaque formation (Buchman et al.

2016). Based on this post-mortem study, high levels of BDNF were associated with a fifty percent increase in mental functioning based on set cognitive tests, demonstrating that BDNF may be a sort of natural brain factor that can offset cognitive decline.

Scientists have started to determine a beneficial link between BDNF and p75 receptor ligands, specifically LM11A-31 (Longo and Massa 2013). Neurotrophin receptors are often shown in neurons susceptible to AD, and neurotrophins, such as BDNF, can interact with different receptors, one of which is the p75 receptor. When neurotrophins bind to p75 receptors, the pathway can lead to either cell death or cell survival. Yet, when the cells are treated with the p75 receptor ligand, LM11A-31, the addition of this synthetic chemical causes a shift towards the survival pathway (Longo and Massa 2013). Research has also shown that LM11A-31 activity can cause cell death through apoptosis signaling pathways since LM11A-31 can act as an agonist competing with BDNF for the same binding site on the p75 receptor (Nykjaer et al. 2005). As explained by Nykjaer et al., proNGF and sortilin (proNGF receptor) can inhibit cell survival. The receptor complex p75/sortilin works with the proneurotrophin ligand and lead to cell death (Lu et al. 2005). Sortilin acts as a type of coreceptor with the p75 receptor to induce a cell death pathway. Therefore, some scientists have been developing types of antagonists for sortilin to inhibit the cell death pathway (Teng et al. 2005). The p75/Trk receptor complex works with matured neurotrophins to enhance overall cell survival. Thus, presumably proBDNF can bind with p75 (proBDNF receptor) and cause cell death. Yet, p75 and Trk receptor activity leads to enhanced cell survival. These variances in pathways are important in determining whether cells live or die, and may be a potential reason for the narrow use of the combination of BDNF and LM11A-31 for pharmaceutical drugs. Furthermore, the present limited use of neurotrophins in medicine include plasma instability, difficulty crossing the blood brain barrier, and its pleiotropic

nature, meaning that they can have multiple effects from a single gene, some of which could cause harmful consequences (Longo and Massa 2013). Additionally, short half-lives and low oral availability (if administered as a drug, only a small amount reaches the blood circulation), make neurotrophins an increasingly difficult option to use and are some of the reasons for little success in early clinical trials. Therefore, one possibility to correct these difficulties involves combining neurotrophins with small molecule receptor ligands to provide a therapeutic option for those with Alzheimer's disease. By using neurotrophic receptor ligands, it may allow for more selective, beneficial effects of neurotrophins like BDNF (Longo and Massa 2013).

To gain a clearer understanding of how BDNF and LM11A-31 interact, first one must understand how neurotrophins and their receptors are synthesized. BDNF is produced in the endoplasmic reticulum as a pre-proprotein. It is then processed by PCSK enzymes (proprotein convertase subtilisin kexin) into a mature form and released from the cell (Longo and Massa 2013). The neutrophins then bind to a specific neurotrophic receptor such as p75. This receptor takes part in specific binding of neurotrophins while modulating TRK signaling. When neurotrophins bind Trk receptors, there is an increased possibility for cell survival (Table 1). Yet, precursor neurotrophins in their proBDNF form can bind to p75 receptors and have the potential to increase the probability of cell death pathways (Lu et al. 2005). For example, p75 receptors have low nanomolar selectivity for proNGF and BDNF and a greater affinity for propeptide forms. Since the p75 receptors exhibit nonselective binding, it can cause a wide array of effects including either cell survival or cell death, depending on TRK receptors present or other intracellular signaling factors. However, it is believed that if LM11A-31 is given exogenously and binds to the p75 receptor, the neuronal survival rate will increase. Based on prior *in vitr*o studies, LM11A-31 can inhibit death of hippocampal neurons at 100-1000pM (Longo and Massa

2013). It has the ability to penetrate the blood brain barrier, decrease demyelination, and improve cognitive functioning in mice (Longo and Massa 2013). Moreover, BDNF and TrkB-mediated pathways have been shown to enhance synaptic plasticity, which may help decrease cognitive decline in Alzheimer's patients (Longo and Massa 2013). Amyloid beta, a hallmark of Alzheimer's disease, interacts with the p75 receptor and can result in change in structure and function of brain regions. Thus, modifying neurotrophic receptor signaling allowing a synergistic effect between the p75 receptor ligand, LM11A-31, and brain derived neurotrophic factor (BDNF) may be a viable therapeutic option for AD patients.

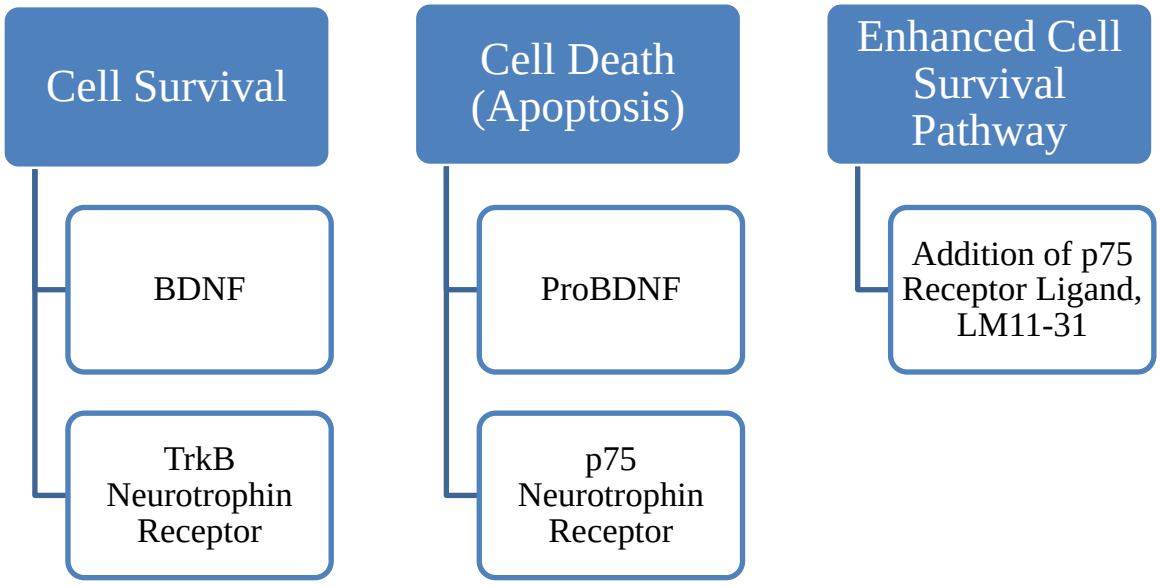

Table 1: Major Molecular Components of *In-Vitro* Model Brain derived neurotrophic factor (BDNF) binds the TrkB Neurotrophic Receptor which leads to a cell survival pathway for neurons (Lu et al. 2005). When proBDNF (precursor of BDNF) binds the p75 neurotrophin receptor, it can induce an increased rate of cell death (apoptosis). It has been hypothesized that the addition of the p75 receptor ligand, LM11-31, to the p75 neurotrophin receptor, can enhance overall cell survival and thus may have the ability to temper neurodegeneration.

As scientists explore the benefits of neurotrophic receptor ligands, it is important to be mindful of potential limitations of their use as well (Longo and Massa 2013). Some of these

include the possibility for nonspecific binding and required re-dosing. As discussed by Longo et al., BDNF mimics NGF in that certain areas of the peptide are specific to aiding cell survival through binding to a p75 receptor (Longo et al. 1997). Such NGF peptide derivatives (region 29-35) can be synthesized. There can also be side effects since neurotrophic receptors are not often controlled well by homeostatic mechanisms (Longo and Massa 2013). However, beyond their possible side effects, it is expected that neurotrophic receptors, along with specific ligands, will play a large role in therapies for neurological disorders such as Alzheimer's disease, as p75 receptor and neurotrophin interactions have already shown promising results in some clinical studies. Perhaps one day "designer ligands" may become a modern approach for not only battling Alzheimer's disease but other diseases as well through the use of agonists and antagonists at specific receptors.

Research Goals

This study concentrates on testing the potential protective interaction between BDNF and LM11A-31 in the presence of Alzheimer's induced associated cellular stressors such amyloid beta and glutamate. BDNF has been shown to promote cell survival and can be enhanced by cognitive stimulation such as music (Angelucci et al. 2007b). When neurotrophins such as BDNF bind to p75 receptors, the pathway can lead to either cell death or survival, depending on the form of the peptide. Mature BDNF binds TrkB enhancing cell survival, while proBDNF binds p75 increasing the risk of cell death (Nykjaer et al. 2005). Yet cells exposed to LM11A-31, a p75 receptor ligand, tend to shift more towards the survival pathway (Yang et al. 2008). Pro-BDNF preferentially binds to p75 receptors whereby mature BDNF works via TrkB receptors (Je et al. 2012). Therefore, three main hypotheses were tested whereby compounds were tested alone and in conjunction with each other. BDNF is protective against glutamate and amyloid beta

toxicity. LM11A-31 is protective against glutamate and amyloid beta toxicity. BDNF and LM11A-31 in combination are protective against glutamate and amyloid beta toxicity. In addition to these main hypotheses, other premises that were tested as part of this study were as follows: Glutamate has a main effect on percent cell viability. Amyloid beta has a main effect on percent cell viability.

The first component of the project involved creating a cell culture model exhibiting neuronal toxicity due to components of Alzheimer's disease such as amyloid beta accumulation and excess glutamate. Both of these manipulations were predicted to decrease cell viability. Neurons were harvested from prenatal mice following a protocol to reduce the amount of contamination from meninges, as blood vessels may course through meningeal layers (Wooten and Seibenhener 2012). The cells matured for approximately two weeks to allow for cell growth, and media was changed every two to three days (Yang et al. 2008). Concentration curves were used to determine the proper amount of glutamate that would provide appropriate neurotoxicity. Amyloid beta was also added to various neuronal cell cultures. An amyloid beta suspension was created in regular growth media to model Yang et al.'s method which used a sodium hydroxide suspension (2008). It was expected that this cell culture model would decrease cell viability and inhibit normal cell development and morphology.

Once cells were in an Alzheimer's disease state (96 well: based on percent cell viability threshold set at less than 80%, 24 well: morphologically altered), various concentrations of BDNF and LM11A-31 were added to create different testing conditions and study their effects on this *in vitro* model. Varying concentrations and exposure time periods were tested. For example, based on prior studies, LM11A-31 added to AD cell cultures had the ability to inhibit the effects of amyloid beta's harmful signaling properties, causing activation of the CDK5 pathway (cyclin-

dependent kinase 5), excess tau phosphorylation, neurite beading and dystrophy, and ultimately death of cultured neurons (Longo and Massa 2013). Furthermore, elevated amounts of BDNF were added to the neuronal cultures to mimic the effects of music exposure, which has been shown to increase the levels of this neurotrophic factor (Angelucci et al. 2007b). Although Angelucci et al. (2007b) used a mouse model, the experiment helped to outline my own research as my current study is modeling music therapy exposure within a cell culture via an *in vitro* model and exposing cells to the compound BDNF to determine if cell viability is enhanced. Equilavency between the Angelucci et al. (2007b) *in vivo* study and our cell culture experiments was based on devising concentrations of BDNF and LM11A-31 appropriate for cell culture models. BDNF has been shown to decrease in AD individuals, yet exogenous administration of BDNF can potentially offset brain changes by activating the receptor TrkB (Tapia-Arancibia et al. 2008). For this experiment, some cell culture conditions involved just one compound while others combined both BDNF and LM11A-31 to determine their joint effect. Initial experiments were used to gain knowledge of the effects of the compounds such as appropriate concentrations and exposure time periods to play a protective role and maintain cell viability compared to control conditions.

Following a specified exposure time ranging from 24 hours to three days, data was collected either via cell viability MTS assay or by studying the cell morphology using immunocytochemistry staining and image analysis. It was expected that BDNF and LMN11A-31 would help maintain neuronal health and survival rates within the AD cell culture model. The hypothesis was that LM11A-31 binding to the p75 receptor would enhance the neuron's survival rate. This, coupled with BDNF binding, could serve as a potential therapy for AD, as LM11A-31 can facilitate pro-BDNF binding to the same receptor (Longo and Massa 2013).

Materials and Methods

Primary Cortical Neuronal Cultures

In order to prepare the neuronal cell cultures, both 96 and 24 well plates were prepped using sterile Cellstar plates with a lid. To prepare the plates, within the hood of the sterile culture room, Poly-l-lysine (Sigma, P6282-5MG) was added to the bottom surface of each of the inner wells. Poly-l-lysine helps the cells adhere to the wells. Approximately twenty-four hours later, the poly-l-lysine was removed. Next, washes of the inner wells were performed with HBSS (Hank's Balanced Salt Solution, Gibco, REF: 24020-117). REF refers to all catalog numbers. Washes were also completed with autoclaved water. After fifteen minutes, the solution was aspirated from the wells. This process of washes was repeated a total of three times to make sure that all of the poly-lysine had been removed. Following the final wash, plating media was added to the inner wells. Plating media creates a suitable, nutritious environment for the cells as it contains neurobasal media (Gibco, REF: 21103-049), fetal bovine serum (FBS) (Gibco, REF: 16000-044), and penicillin-streptomycin antibiotics (Gibco, REF: 15140-122). HBSS or autoclaved water was also added to the wells of the perimeter of the plate to prevent evaporation of the inner wells. The plate was then placed in the incubator (37 degrees Celsius with 5% CO_2) for at least one hour prior to plating of cells so that the media became warm.

Cells were first obtained via a dissection of E18 rat embryonic cortical neurons. The dissection process began by first sacrificing the pregnant female Sprague Dawley rat in a CO_2 gas chamber. The embryos were surgically extracted. The fetal rats were then decapitated and heads were placed in an HBSS dish on ice. Under a microscope, the brain was removed by placing the forceps in the eye sockets, gently penetrating the skull, and pulling back the tissue. Red film areas designated as meninges were also detached even if brain tissue had to be

sacrificed, as the meninges could cause contamination to the cell cultures. The cerebellum and midbrain were also removed. The hemispheres were then placed in a separate HBSS dish and teased apart with forceps. The brain tissue was then pipetted into 3mL of trypsin（Sigma Aldrich, REF: T4424) at 37 degrees Celsius for a total of five minutes in the water bath. Trypsin helps the clumps of cells separate so they do not adhere to the side walls of the conical tube. The tissue was then transferred into another conical tube containing 3mL of HBSS at 37 degrees Celsius for three minutes in the water bath. This HBSS step was then repeated. Afterwards, the tissue was transferred to a conical tube containing 3mL of plating media. Cells were then fully dissociated via the use of a flame polished glass pipet (pipetted up and down about 50 times). Cells were counted using a Bright-Line Hemocytometer in order to calculate the proper concentration of cells desired. Cells were diluted into plating media to create a 1×10^6 cells/mL concentration. Prior to plating, the cells were again pipetted up and down to make sure they were evenly dispersed in the media. With the use of a micropipette, 100 ul of cell concentrate was added to each of the inner wells of the poly-l-lysine treated plates and placed in the incubator. Following one to four hours, all of the plating media was replaced with growth media and then placed back in the incubator. Growth media consists of neurobasal media, a B-27 supplement (Gibco, REF: 17504-044), and penicillin-streptomycin antibiotics. To ensure that the cells had enough nutrients and media within the wells, every two to three days, fifty percent of the media was replaced with new growth media. This allowed new nutrients to be added, while maintaining beneficial factors secreted by the cells during maturation. Neurons were grown for one to two weeks prior to beginning stimulations (Table 2).

Table 2: Neuronal Growth and Stimulation Timeline

Day 1	Day 4	Day 7	Day 9	Day 10-12	Day 14	Day 15-17
Dissection of fetal rat brains. Cells plated in 24 and 96 well plates.	Cells fed with growth media.	Cells fed with growth media.	1 week cells stimulated. 2 week cells fed with growth media.	1 week cells MTS Assay or Fixed/Stained (24-72 hour stimulation). 2 week cells fed with growth media on Day 11.	2 weeks cells stimulated.	2 weeks cells MTS Assay or Fixed/Stained (24-72 hour stimulation).

Glutamate and Amyloid Beta Conditions

Following one to two weeks of growth, cells were stimulated with glutamate or amyloid beta. To make a specified glutamate concentration, a stock of 50 mM glutamate was diluted in growth media to the desired concentration. Based on prior data from the Knowles Lab and an experimental concentration curve, glutamate concentrations tested ranged from 0.05 – 1.00 mM. To create an appropriate amyloid beta concentration, the stock solution of 100 uM was diluted into growth media to obtain a 100 nM concentration. This concentration mixture was then placed in a sterile 6-well plate in the incubator and grown for at least forty-eight hours so that oligomer formations developed.

BDNF and LM11A-31 Stimulations

Brain derived neurotrophic factor (BDNF, recombinant, expressed in *E. coli*, lyophilized powder, suitable for cell culture, Sigma, B3795-5UG) was aliquoted. To complete this process, 500 ng of powder was diluted into 250 uL of autoclaved water and then filtered in order to create a stock solution of 20 ng/uL. Stimulation concentrations tested ranged from 10 – 100 ng/mL diluted with growth media (Cardenas-Aguayo et al. 2013).

LM11A-31 dihydrochloride (2-Amino-3-methyl-N-[2-(4-morpholinyl) ethyl]-pentanamide dihydrochloride) (Sigma, SML0664-25MG) was diluted into DMSO (dimethyl sulfoxide, EMD Millipore, MX1458-6) to create a stock solution of 100uM. Controls were not exposed to DMSO-containing vehicle alone. Stimulation concentrations tested ranged from 25 – 150nM and were diluted in growth media.

Various combinations of the compounds were tested along with amyloid beta or glutamate to determine the effects of BDNF and LM11A-31 both separately and in conjunction with each other. Duration of stimulations ranged from 24 – 72 hours. All of the growth media was removed and then immediately replaced with the stimulation concentrations in order to eliminate confounding variables of external neuronal factors within the media. Each plate also contained a vehicle control containing solely growth media.

Compound Light Microscope Observations

Throughout the growth and stimulation periods, cells were periodically observed under a Nikon Eclipse TS100 Compound Light Microscope to check for contamination and observe neuronal health and growth.

MTS Assay

Once stimulations were completed, MTS cell viability assays were conducted for 96 well plates. A MTS Assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, REF: G3581) is a colorimetric assay used to assess metabolic activity of the following compound: 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H tetrazolium (MTS). When first placed on the cells, the dye appears a bright yellow, but once metabolized by mitochondrial reductase enzymes, the color changes to dark brown. Healthy cells metabolize the dye at a greater rate. To initiate the assay, 20ul of pre-warmed MTS dye was

added to each of the inner wells as well as one row of outer wells to act as blank, thus recording wells without plated cells. Following two to four hours, the 96 well plate was then placed in the Molecular Devices SpectraMax microplate reader. Using the SoftMax Pro Data Acquisition and Analysis Software, the plate configuration was labeled on the computer model for the various conditions. Absorbance data was recorded at 490 nm. Once the data was collected it was then exported as a Microsoft Excel file. All conditions were normalized and converted to percent cell survival by standardizing each condition to the growth media control so that various experiments could be compared and analyzed. Percent cell viability was normalized by dividing the mean value absorbance for the given condition by the mean value absorbance for the control and then multiplying by one hundred.

Immunocytochemistry

To analyze the 24 well plates, cells were fixed in 4% paraformaldehyde (Sigma, P6148) for twenty minutes at room temperature followed by three phosphate buffered saline (1 PBS tablet diluted into 200mL of deionized water, 0.01 M, Sigma, P4417) washes each for three minutes. Incubation at room temperature with 0.5% Triton-100 (Sigma, T8532) for ten minutes was then followed by a PBS wash. Triton penetrates the neuronal membranes, allowing the antibody to bind. Primary monoclonal anti-acetylated tubulin antibody (Sigma, T9450-200UL) was incubated for one hour followed by three PBS washes. Incubation for one hour with an anti-mouse in goat IgG fragment secondary antibody (FITC produced, Sigma, F0257) allowed for fluorescence and detection of neuronal structures. The addition of the secondary antibody was then followed by three PBS washes.

Dark Room Imaging

Using a Zeiss Axiovert 200M Fluorescent Microscope and NIS Elements imaging software, light intensity of the images was measured at a consistent exposure and neuronal morphology was analyzed. Images were taken at a magnification of 20x at 490nm wavelength on the FITC setting as the FITC dye was conjugated to the secondary antibody. Fields of analysis were selected systemically by separating each well into four quadrants and sampling the same number of images from each quadrant. To normalize the images, the level of black (minimum intensity) was subtracted from the mean intensity implementing the following equation: Relative Intensity = Mean Intensity – Minimum Intensity.

Statistical Analysis

Data was analyzed using the Statistical Package for the Social Sciences (SPSS) to test for statistical significance of the data. One-way and two-way ANOVAs were used to determine differences between groups. A post-hoc Tukey analysis tested for significant effects between conditions. Graphs with error bars representing standard error of the mean were created in Microsoft Excel.

Results

Experiments performed in this study were to test the potential effects of BDNF and LM11A-31, both separately and combination, on cell viability, tubulin staining, and overall morphology in the presence of glutamate and amyloid beta.

Effects of BDNF

To begin experiments with a glutamate stressor, a low concentration of glutamate (500uM) was tested with BDNF (Figure 1). All conditions were normalized to the control. A two-way ANOVA was run. There was a statistically significant main effect of glutamate

whereby glutamate decreased overall percent cell viability ($p<0.001$). BDNF also significantly decreased percent cell viability ($p<0.001$). There was no interaction between glutamate and BDNF ($p>0.05$).

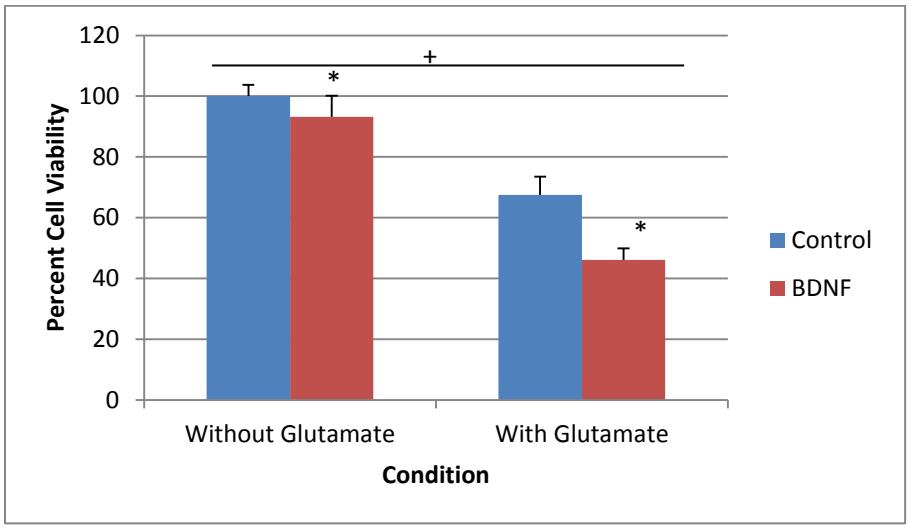

Figure 1: Effects of BDNF and Glutamate on Percent Cell Survival Two week cultured neurons in a 24 hour stimulation with 50ng/mL BDNF and 0.5mM Glutamate. All data are mean +/- SEM. (N=10 for each condition) The + symbol indicates a statically significant main effect of glutamate ($p<0.001$) and the * symbol shows a significant main effect of BDNF ($p<0.001$).

Effects of LM11A-31

The effect of 50uM glutamate and various concentrations of LM11A-31 (0nM, 25nM, 50nM, 100nM, 150nM) on percent cell viability gathered from an MTS assay was tested using a two way ANOVA (Figure 2). All conditions were normalized to the control. Two plates were eliminated from this test, one because each well exhibited high levels of variability and the other because the assay did not work (all negative values). There was a statistically significant main effect of glutamate ($p=0.009$). There was no statistically significant main effect of LM11A-31 ($p=0.279$). There was no interaction between glutamate and LM11A-31 ($p=0.719$). No significant pairwise comparisons were present.

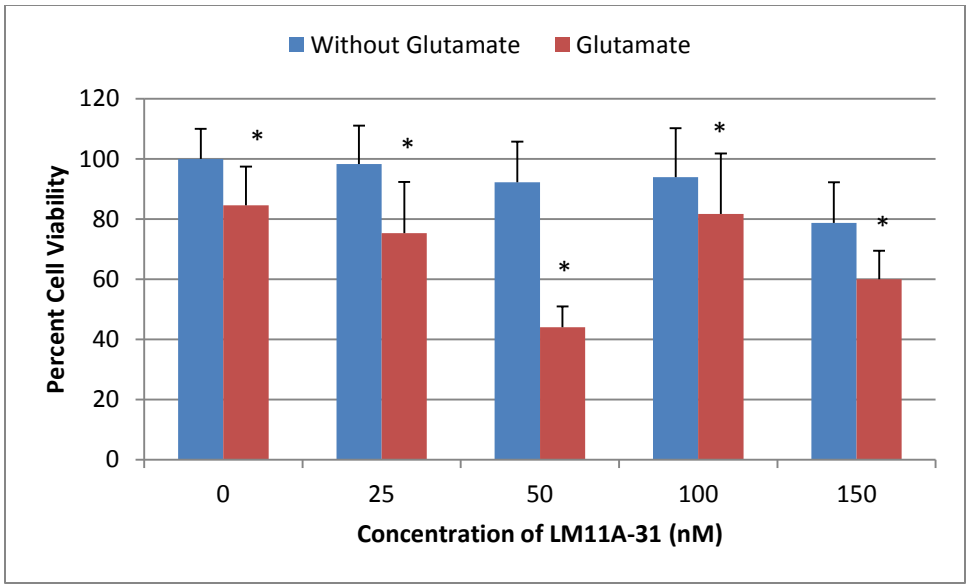

Figure 2: Varying LM11A-31Concentrations (nM) with and without Glutamate 24 hour stimulation of two week aged cortical neurons with LM11A-31 and 50uM glutamate. (N= 12 per condition aside from 0 nM without glutamate, 50 nM with glutamate, and 150 nM with glutamate whereby N=11) Data is presented as mean +/- SEM. The * symbol indicates that there was a significant main effect of glutamate ($p=0.009$) but no effect of LM11A-31 and no interaction ($p>0.05$).

Effects of BDNF and LM11A-31

For all of the experiments up until this point in the research, the reagents BDNF and LM11A-31 were added at the same time as glutamate-infused growth media. However, it was hypothesized that a pretreatment with these compounds may help reduce the effect of Alzheimer's as it develops later in life. Therefore, this particular test compared the effect of a seven-day pre-exposure to the compounds as opposed to no pretreatment (Figure 3 A and B). All conditions were normalized to the control. Using a one-way ANOVA, glutamate in the pretreatment conditions was shown to compromise overall cell survival (Figure 3A). There was a statistically significant effect of condition whereby the control was significantly greater than all conditions with glutamate ($p<0.001$). While the control exhibited 100% cell viability, each of the other conditions had less than 40% survival rate.

The same conditions were also tested without a pretreatment with BDNF and/or LM11A-31 (Figure 3B). All conditions were normalized to the control. Using a one-way ANOVA, there was a statistically significant effect of condition ($p<0.001$). The control condition was statistically significantly different from every other condition, each of which included glutamate ($p<0.001$). This showed that the decrease in cell viability was due to glutamate alone. The overall mean percent cell survival for the control was 99%, while the glutamate condition showed 59% cell viability (Figure 3B). Therefore, BDNF and LM11A-31 alone or in combination did not increase cell viability whether they were added at the same time as glutamate or seven days prior to glutamate exposure.

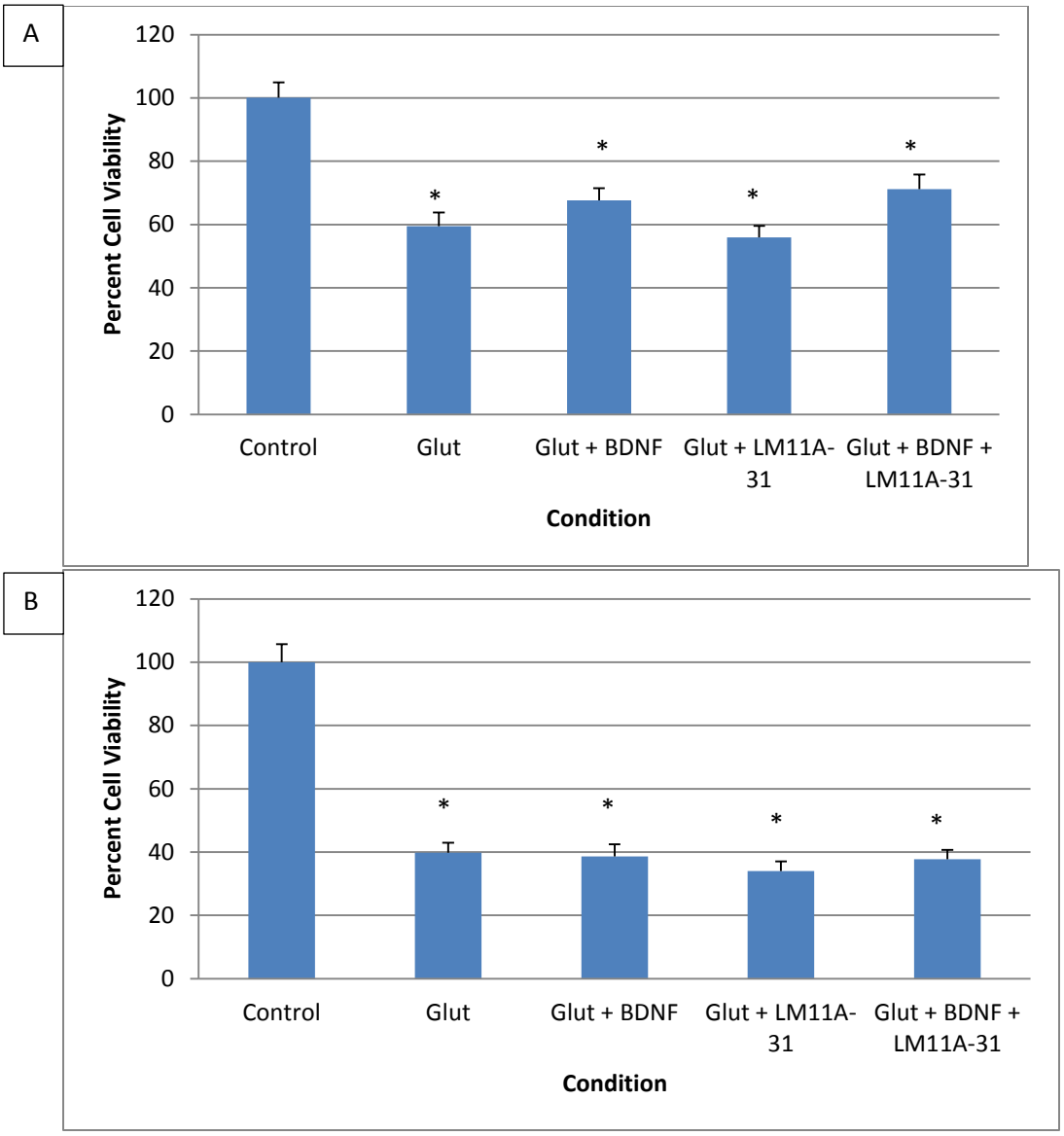

Figure 3: Effect of Pretreatment of Cells Prior to the Addition of Glutamate (1mM) Stimulation involved 50 ng/mL BDNF and 100nM LM11A-31. Data are presented as mean+/- SEM. (N=12 for each condition) The * symbol indicates a significant difference from the control ($p<0.05$). A: Pretreatment: 7 Days of Exposure to BDNF and LM11A-31 (Friday, Monday, Wednesday). B: No Pretreatment: BDNF and LM11A-31 added on same day as Glutamate (Wednesday).

BDNF and LM11A-31 were also tested under amyloid beta conditions (Figure 4). Aside from glutamate as a contributing factor in Alzheimer's, amyloid beta also poses a serious threat by causing plaque formation. Thus, 1 uM amyloid beta was infused into growth media to test the

potential roles of the compounds in maintaining cell viability. All conditions were normalized to the control and a one-way ANOVA was run. Amyloid beta significantly decreased cell viability ($p<0.001$). Without amyloid beta, mean percent cell viability was 79.951 +/- 0.5581 (SEM) for all conditions without amyloid beta and 53.344 +/- 0.667 (SEM) for all conditions with amyloid beta. Although there was no main effect of BDNF ($p>0.05$), there was an interaction between BDNF and amyloid beta ($p=0.009$). LM11A-31 also significantly decreased percent cell viability ($p=0.013$) Without LM11A-31, mean percent cell viability was 72.401 +/- 0.558 (SEM) while when LM11A-31 was present, percent cell viability was reduced to 60.893 +/- 0.667 (SEM). In contract to BDNF, LM11A-31 did not have an interaction with amyloid beta ($p>0.05$). There was also no 3-way interaction between BDNF and LM11A-31 ($p>0.05$). Therefore, BDNF may not promote survival on its own, but may reduce the toxic effects of amyloid beta.

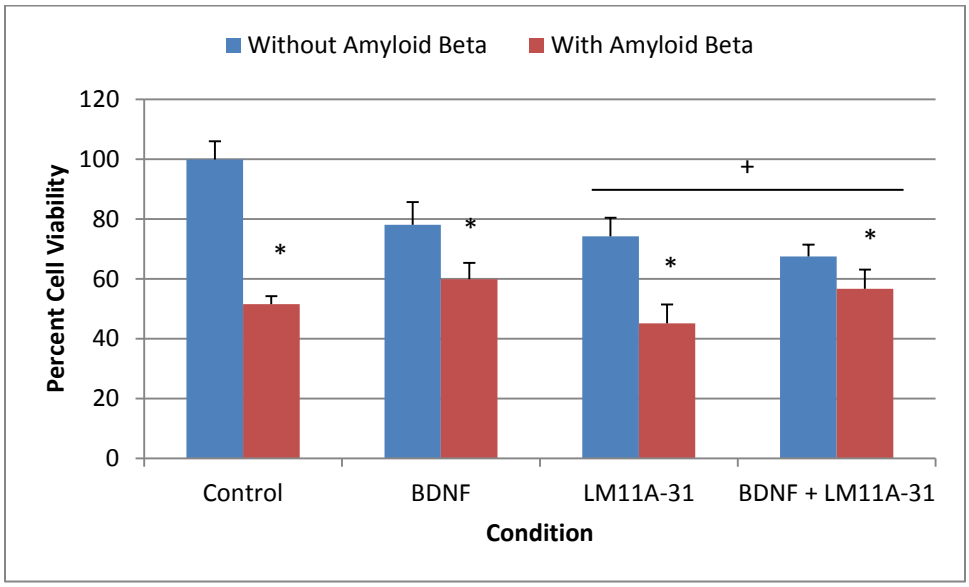

Figure 4: Amyloid Beta Stimulation 72 hour stimulation of one week aged cortical neurons with 50ng/mL, 100nM of LM11A-31, and 1uM amyloid beta (AB). Data are the mean +/- SEM (N=12 for the Control, N=6 for all other conditions). The * symbol indicates a main effect between conditions with and without amyloid beta ($p<0.001$). There is also a main effect between conditions with and without LM11A-31 as show by the + symbol ($p=0.013$).

In order to remove the confound of the effect of LM11A-31, Figure 5 presents the same data reanalyzed without the presence of LM11A-31. Only wells without LM11A-31 were

analyzed. As presented in Figure 4, there was a main effect of amyloid beta because conditions with amyloid beta were consistently lower than conditions without amyloid beta. Therefore, adding amyloid beta decreases cell viability. There was a statistically significant interaction between amyloid beta and BDNF ($p=0.009$). There is a trend for the control without amyloid beta to be greater than the BDNF without amyloid beta condition ($p=0.077$). Thus, when amyloid beta is not present, there is a trend for BDNF to reduce cell viability. The control condition with amyloid beta is statistically the same as BDNF with amyloid beta ($p=0.812$). Thus, in the presence of amyloid beta, BDNF does not further decrease percent cell viability. More specifically, BDNF does not cause any further harm in the presence of amyloid beta. As these conditions are statistically identical, one cannot claim that BDNF is neuroprotective. Furthermore, there is a trend that BDNF (without amyloid beta) is statistically significantly greater than the control with amyloid beta ($p=0.06$). This can be interpreted as when BDNF is presented alone, it is not as harmful to the cells as amyloid beta. In summation, the control (without amyloid beta) is greater than or equal to BDNF (without amyloid beta), which is greater than or equal to the control (with amyloid beta). These trends, although not statistically significant, are necessary to note since other data presented in this study is consistent with a neurotoxic effect of BDNF. The effect of BDNF is difficult to interpret because by itself it decreases cell viability, while in combination with amyloid beta or LM11A-31, BDNF does not seem to affect the ability of either of those conditions to alter cell viability (Figure 4). BDNF does not make the neurotoxicity of amyloid beta better or worse in this particular experiment. The cytotoxic effects of LM11A-31 are independent of the cytotoxic effects of amyloid beta. BDNF on its own is toxic but does not exacerbate the effects of the other neurotoxins. To illustrate differences between the four groups, a post-hoc test was completed. The Tukey results

showed that the control without amyloid beta was statistically significantly greater than the control with amyloid beta ($p<0.01$). The control without amyloid beta was statistically significantly greater than the BDNF with amyloid beta condition ($p<0.01$).

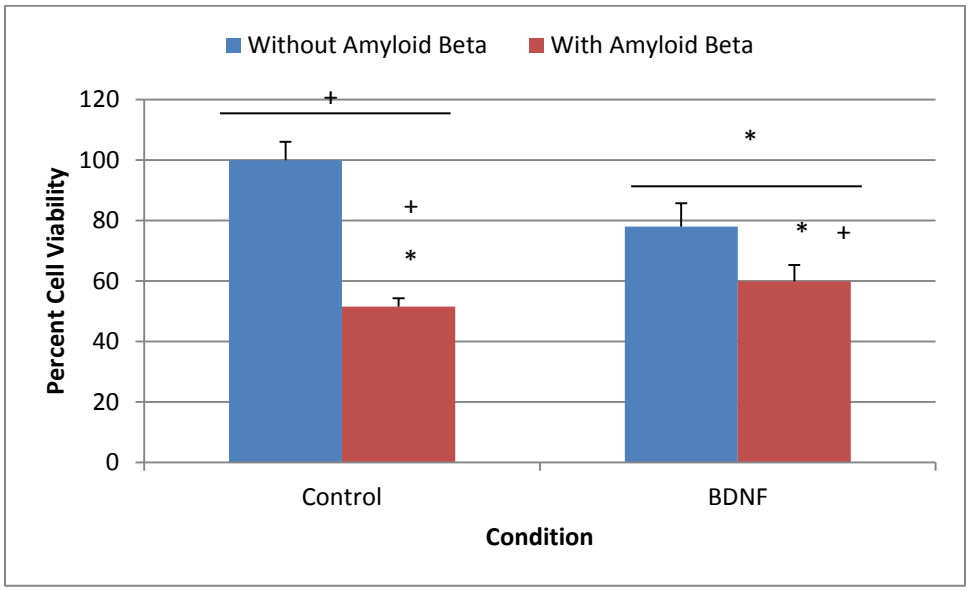

Figure 5: BDNF Stimulation (Same data as Figure 4) 72 hour stimulation of one week aged cortical neurons with 50ng/mL, 100nM of LM11A-31, and 1uM amyloid beta (AB). Data is the mean +/- SEM. (N=12 for the control, N=6 for all other conditions) There was a statistically significant main effect of amyloid beta ($p<0.05$) and an interaction between BDNF and amyloid beta ($p=0.009$). Statistical significance between groups was as follows as indicated by the + symbol: control without amyloid beta and amyloid beta condition ($p<0.01$) as well as the control without amyloid beta and BDNF with amyloid beta condition ($p<0.01$).

Neuronal Morphology and Tubulin Staining

Neuronal morphology was based on tubulin staining (Figure 6). Absolute intensities of the images were obtained for more quantifiable results on cell structure. Based on observations of general cell morphology, glutamate decreased the number of neurons and decreased the intensity of neurons that were still present. BDNF alone seemed to show high intensity tubulin staining. Glutamate + BDNF conditions exhibited a decrease in overall cell count but cytoskeletal structures such as dendrites and axons were more defined when BDNF was present as opposed to glutamate alone (Figure 6 B and D).

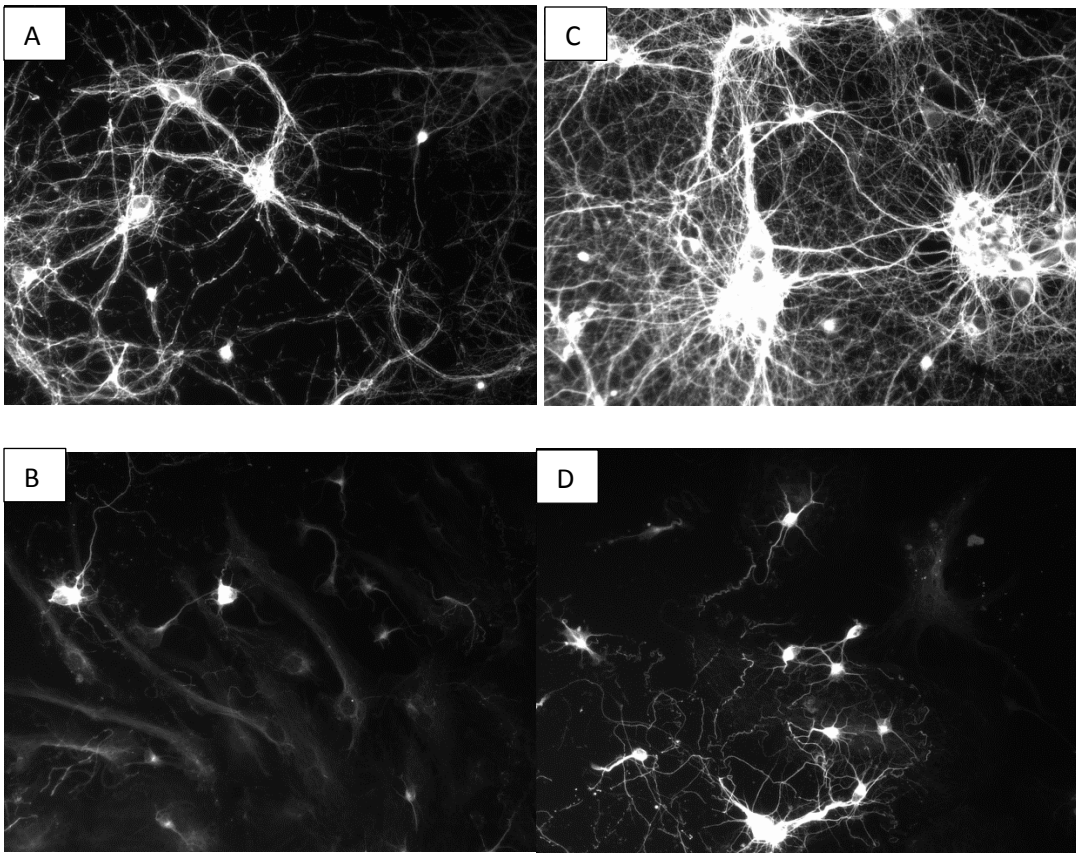

Figure 6: Comparison of Neuronal Morphology Images exhibit tubulin staining via immunocytochemistry to examine number of neurites and total staining per image.
A: control, B: glutamate (0.5mM), C: BDNF (50 ng/mL), D: BDNF + Glutamate

To determine the health of the microtubule structures, relative intensity of tubulin staining was measured in the conditions from Figure 6. Using a two-way ANOVA, there was a significant main effect of glutamate ($p=0.000$) and no main effect of BDNF and no interaction (Figure 7). Glutamate decreased tubulin intensity staining, while BDNF did not show any substantial effects on overall neuronal morphology.

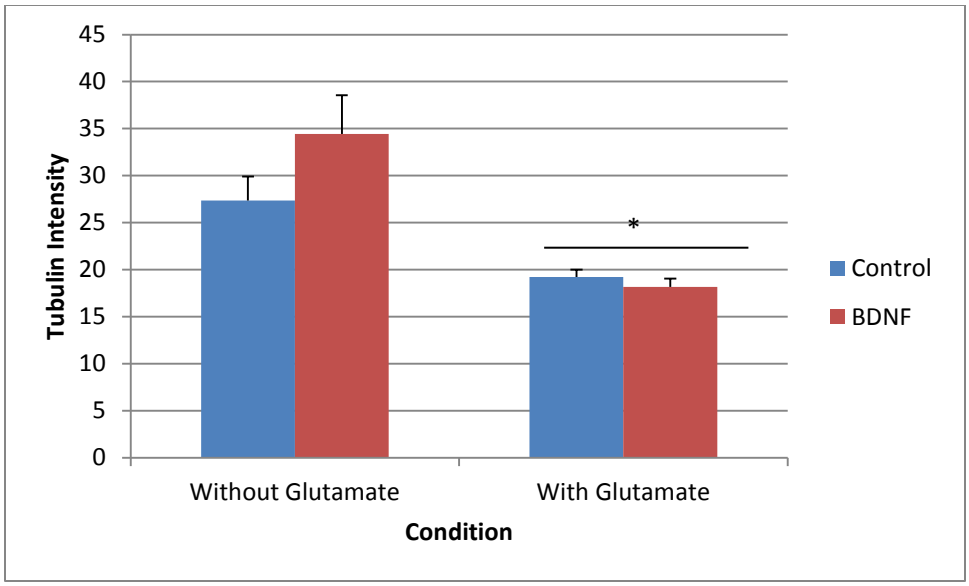

Figure 7: BDNF and the Effects of Glutamate on Tubulin Staining per Image Relative Intensity of 2 week cortical neurons all treated with 0.5mM glutamate and 50ng/mL BDNF. Data is the mean +/- SEM. (N=33 for the control, 32 for glutamate and BDNF, and 30 for BDNF + glutamate) The * symbol indicates a significant main effect of glutamate (p=0.000).

It was hypothesized that varying amounts of BDNF would cause a difference in tubulin intensity. Differing values of BDNF were tested (0, 10, 25, 50, and 100 Ng/mL) both with and without glutamate. It is important to note that each condition also incorporated LM11A-31 including the control (Figure 8). A one-way ANOVA showed that that glutamate significantly decreased tubulin intensity (p=0.000). There was also a significant effect of BDNF whereby the concentration of 10 Ng/mL decreased tubulin intensity in both glutamate and non-glutamate conditions (p=0.001). There were no other effects with any other BDNF concentrations.

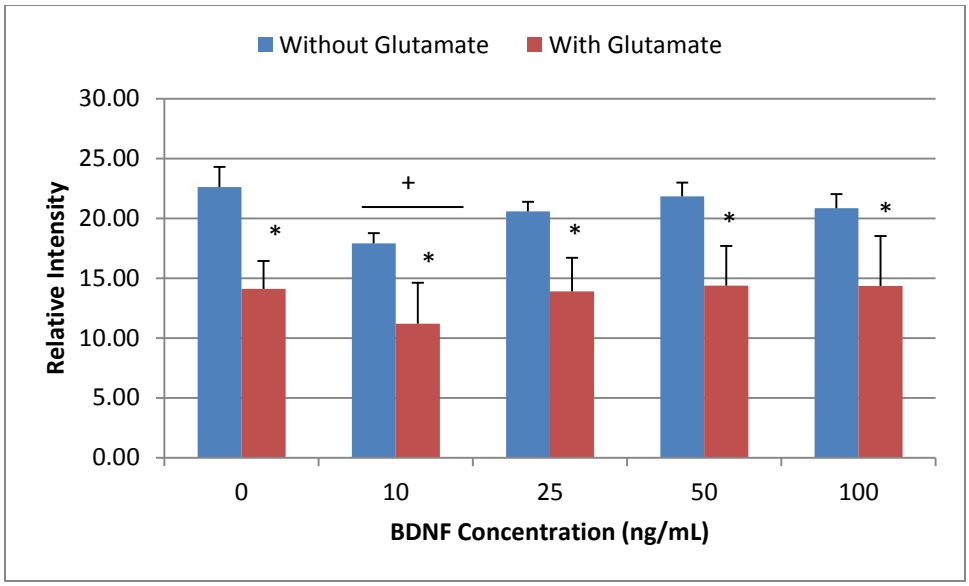

Figure 8: Effect of Glutamate on Tubulin Staining per Image with BDNF and LM11A-31 Compound Relative Intensity of 2 week old cortical neurons all treated with LM11A-31 (100nM) and varying BDNF concentration (with or without 50uM glutamate). Data is mean +/- SEM. (N= 24 for each condition, aside from 100Ng/mL no glutamate and 0Ng/mL with glutamate whereby N=25) The * symbol indicates a significant main effect of glutamate (p=0.000). The + indicates a significant main effect of 10ng/mL BDNF (p=0.001).

Discussion

Research Goals

The main goal of this research was to create an Alzheimer's disease cell culture model using excess glutamate levels and amyloid beta whereby one could test the effects of brain derived neurotrophic factor (BDNF) and LM11A-31 on cell viability and morphology. BDNF has been shown to promote cell survival in culture and can be enhanced *in vivo* by cognitive stimulation such as music (Angelucci et al. 2007b). Scientists have shown that when BDNF and the p75 receptor ligand, LM11A-31, are used in conjunction, neurons *in vitro* tend to have an increased survival rate (Yang et al. 2008).

Model of Neurodegeneration

In order to test the hypotheses, a model of Alzheimer's disease was created using glutamate or amyloid beta in a primary neuronal cell culture. Three main hypotheses were tested. BDNF is protective against glutamate and amyloid beta toxicity. LM11A-31 is protective against glutamate and amyloid beta toxicity. BDNF and LM11A-31 in combination are protective against glutamate and amyloid beta toxicity. In addition to these main hypotheses, other premises that were tested as part of this study were as follows: Glutamate has a main effect on percent cell viability. Amyloid beta has a main effect on percent cell viability. A threshold for toxicity was set to standardize each separate experiment. Percent cell survival rate had to be at or below 80% for the effect of the stressor to be considered neurotoxic. There were a total of 29 cell culture plates that were used for experiments. Seven plates met the desired neurotoxic threshold, six of which had a glutamate stressor and one which contained an amyloid beta stressor. Essentially, seventy-five percent of the experimental data was not included because it did not adequately express glutamate or amyloid beta toxicity. Toxicity was measured using MTS Assays and immunocytochemistry. For each experiment, all conditions were normalized to the control.

Effects of BDNF

One of the main hypotheses tested was whether BDNF was protective against glutamate neurotoxicity. However, when BDNF was present, it decreased percent cell viability (Figure 1). One possible explanation may be that the 50 ng/mL concentration of BDNF was not high enough to produce a protective effect and perhaps at greater concentrations, BDNF would be better able to protect the cells against toxicity. Contrary to this experiment's hypothesis, there have been cases whereby BDNF has inhibited cell growth in rat neuroblastomas (Maki et al. 2014). Although the data measured was based on primary, neuronal cell culture, Maki et al.'s research

can suggest that BDNF is not always beneficial (2014). At low levels of glutamate that were not toxic, BDNF helped to increase cell viability, while at higher glutamate concentrations, BDNF decreased cell viability. Therefore, BDNF alone may not be a method of protection against Alzheimer's disease. Rather, BDNF may only be involved in maintaining regular cell survival rates in healthy individuals.

In this particular experiment, we saw that BDNF does not provide protection against neurotoxicity. We hypothesized that we would see a protective effect of BDNF against glutamate and amyloid beta toxicity based on previous studies by Angelucci et al. (2007 a,b) and Cardenas-Aguayo (2013). The discrepancies between our findings and those of others could be due to varied models. For example, in the Angelucci et al study, a mouse model allowed for the measurement of BDNF levels in the hippocampus following a period of music exposure. Perhaps the beneficial value of elevated levels of BDNF is more visible in an *in-vivo* model (2007a). Yet, the Cardenas-Aguayo study did see an effect of BDNF using a primary neuronal cell culture. We determined our concentration of BDNF for stimulation based on the Cardenas-Aguayo study. Stimulation concentrations we tested ranged from 10 – 100 ng/mL. The Cardenas-Aguayo study stimulated with 20 ng/mL and 100 ng/mL, which was comparable to our own range (2013). However, Cardenas-Aguayo tested specific BDNF peptides representing varying active regions of the neurotrophic factor (2013). Therefore, BDNF in its entirety may not be protective but rather isolating particular groups of BDNF tetra peptides may be more beneficial. In our particular study, active peptide sites were not isolated and this may have been the reason for the toxic effect of BDNF rather than its predicted protective role.

Effects of LM11A-31

The second hypothesis that was tested was whether LM11A-31 would be protective against glutamate toxicity. However, LM11A-31 did not show protective effects on cell survival rates under toxic glutamate conditions, regardless of the concentration of LM11A-31 that was tested (Figure 2). In a study by Massa et al., which used an embryonic neuronal model, all LM11A-31 concentrations above 1nM inhibited cell survival (2006). LM11A-31 is artificial and may not work the same way as other natural receptor ligands (Massa et al. 2006). Some of these differences include variability in binding to particular receptors, amplification of the signal, and interference with other ligands via antagonism. Additionally, LM11A-31 alone may not be enough to aid cell survival. Perhaps enhanced survival signaling may only occur in the presence of neurotrophins such as BDNF (Massa et al. 2006).

Effects of BDNF and LM11A-31

In order to test BDNF and LM11A-31 together, both compounds were combined to examine their joint effect on cell survival. Neurons vulnerable to AD often have neurotrophin receptors, one of which is the p75 receptor. Cells treated with LM11A-31 receptor ligand tend to show enhanced cell survival (Longo and Massa 2013). Yet, rather than treat the neuronal cultures with these compounds once the cells were already induced with neurotoxicity, a period of pretreatment occurred for seven days prior to glutamate stimulation (Figure 3A and B). However, there was no evidence for any effect of pretreatment in our experiment. Although the idea of exposing individuals to BDNF and LM11A-31 before displaying signs of dementia may be relatively new, the idea of pretreatment with BDNF is not a completely new concept. Li et al. explains that neuronal stem cell transplantation may be a potential therapy for AD patients (2015). They found that stem cells pretreated with neurotrophins such as BDNF have a more

successful transplant showing increased numbers of basal forebrain cholinergic neurons (BFCN). The data presented in this research are not conclusive to state that pretreatment is necessary. Variability between Figures 3A and 3B was solely explained by differing effects of glutamate depending on the particular experimental plate. All treated plates in Figure 3A had approximately 60% cell viability, while all treated plates in Figure 3B had close to 40% cell viability.

Both BDNF and LM11A-31 were also tested with amyloid beta toxicity (Figure 4). There was no effect of BDNF alone, but there was an interaction between BDNF and amyloid beta. Additionally, contrary to the initial hypothesis, LM11A-31 decreased cell viability but did not interact with amyloid beta. More specifically, to track the presence of BDNF, an RNAase protection assay can be conducted. The measure of BDNF mRNA correlates to BDNF levels in the hippocampus. When BDNF mRNA is not expressed, neurons may be more susceptible to the effects of amyloid beta (Phillips et al. 1991). Therefore, if BDNF can be elevated by means such as music therapy, this neurotrophic factor may be able to reduce the harmful effects of amyloid beta such as plaque formation and memory loss.

For neuronal morphology quantification, data was based on tubulin staining. As opposed to the MTS assay data, the tubulin staining values represent both the percent cell survival and the amount of axon and dendritic development. In healthy neurons more of the cell volume is taken by the neurites. Therefore, this measure gives greater weight to the intensity from the neurites rather than the soma. Glutamate-induced neurotoxicity was shown by the decreased density and intensity of the neurites in Figure 6. There was also evidence of neurite beading in the axons and dendrites of glutamate-induced cells. By visual examination of the images, neurons treated with BDNF showed increased levels of tubulin staining, and neurons treated with both glutamate and

BDNF appeared to have some minor protection in that there were still a few neurites present (Figure 6). When the data was examined quantitatively, the only statistically significant finding was that there was a decrease in staining due to glutamate and that BDNF did not cause any sustained effects on overall neuronal health nor on relative staining intensity (Figure 7). This is logical because during tubulin staining, antibody binds to stable microtubules present in healthy axons and dendrites. However, in glutamate neuronal cultures, cells are undergoing more stress and will have fewer microtubules causing destabilization and reduced intensity. For example, activation of G protein-coupled metabotropic glutamate receptors (mGluRs) can inhibit the development of processes prompted by the expression of the microtubule-associated protein MAP2c (Huang and Hampson 2000). In varying BDNF concentrations, the relative intensities of cells in glutamate conditions were lower than cells without glutamate (Figure 8). In both glutamate and non-glutamate conditions, 10 Ng/mL BDNF showed a significant effect and decreased tubulin intensity. In the presence of 10 ng/mL BDNF, tubulin staining was lower than in other conditions. Lower relative intensities correlate with unhealthy cells.

One interesting finding when combining the MTS assay with the tubulin staining data is that the BDNF treated neurons showed decreased cell viability (Figure 1) but was able to maintain the relative amount of tubulin staining (Figure 7). The most likely explanation for these two results is that BDNF caused some of the neurons to undergo cell death while the remaining surviving neurons had greater neuritic development (Figure 9).

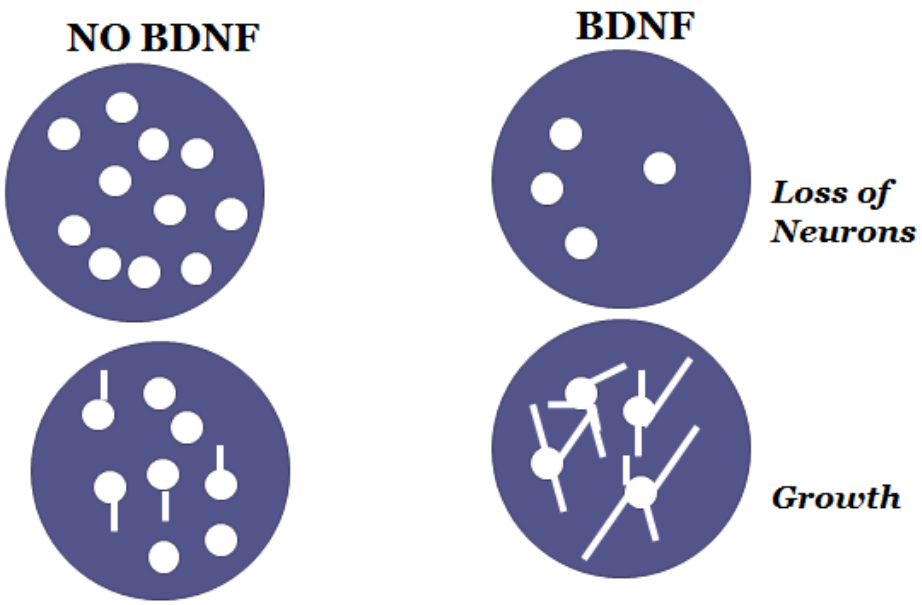

Figure 9: BDNF Survival and Morphology Effect In the absence of BDNF, there is minimal loss of neurons and evidence of growth of axons and dendrites. When BDNF is added to the cell culture, there is a decrease in cell survival rate but an increase in relative tubulin staining and neuritic development.

These findings may indicate that subsets of neurons in the mixed cortical culture were sensitive to the toxic effects of BDNF and another subset of neurons were sensitive to the neurotrophic effects of BDNF. If this is the case, then it would be interesting to try to determine the difference between these two groups of neurons. One possibility may be the differing amounts of expression of p75 and TrkB receptors in these neurons, with those undergoing degeneration expressing more p75 receptors and those having more trophic effects expressing more TrkB receptors. Since these were mixed cortical neuronal cultures, future experiments may aim to isolate one region of the brain for dissection, such as the hippocampus, which may have more pronounced positive responses from BDNF.

In summation, the MTS assay and immunocytochemistry data was conclusive in showing either no effect at all or the presence of neurotoxicity. The data presented did not show protective effects of LM11A-31 and BDNF as originally predicted. The hypotheses were conclusively

rejected. Indeed, other studies by Teng et al. showed similar results to ours in that BDNF did not provide protection towards neurons in toxic AD condition (2005). One explanation may be that proBDNF can be present at the synapse which is not able to participate in TrkB phosphorylation and may lead to greater p75 activity.

There are multiple external factors with regards to the robustness of the model and manipulation that provide further explanations for these data. Inconsistent glutamate toxicity may have been due to the decreased density of glutamate receptors present in the cell culture, not allowing for expected toxicity to be observed. Neurons may have also released endogenous glutamate, which could potentially interact with the exogenous glutamate added to the culture. Glial cells present also release growth factors that could play a role in neuronal health and survival. Moreover, although glutamate toxicity may not have been shown, perhaps there was a loss in synapses and disruption of communication in cells which would not have been measured by the MTS alone or via evaluating cell morphology and intensity levels. Amyloid beta did not prove to be a consistent chemical insult, which may have been due to the concentration chosen or the length of times cells were exposed to this stressor (Figure 4 and 5).

Additionally, perhaps an *in vitro* system may not be the best model to test the hypotheses. Cell cultures lack vasculature present within the brain which could play a role in how neurons respond to BDNF and exogenous factors such as LM11A-31. The blood supply to the brain is necessary for health and protection of neurons. The blood brain barrier prevents cells from being exposed to external contaminants. BDNF cannot cross the blood brain barrier, while LM11A-31 is able to do so (Longo and Massa 2013, Allen at el. 2013). However, since blood flow is not occurring in cell culture as it would in an *in vivo* model, this could play a role in how LM11A-31 and BDNF interact. Further, when viewing the raw data for survival rates within each well for

multiple conditions, there was much variation in the data, even in control conditions. Some wells showed high concentrations of cells beyond 1.000 nm absorbance while other wells were as small as 0.1 nm absorbance. High absorbance values indicate a larger concentration of cells in that particular well and when normalized 1.000 or high is often close to one hundred percent cell viability. This degree of variability may be common to the model. Depending on one's technique in plating cells for primary neuronal cell culture, some wells may have contained different concentrations of cells and thus affected how they responded to AD stressors, BDNF, and LM11A-31. Perhaps cells plated at small concentrations did not survive the initial plating procedure and were not sensitive to compounds such as BDNF and LM11A-31. Also, Alzheimer's disease is a progressive neurodegenerative condition that is prolonged for many years. Hyper-inducing cells with glutamate and amyloid beta and expecting results of toxicity over a period of a few days or weeks may not be representative of the full extent of AD for decades. Alternatively, neurons may have died prematurely even before glutamate was added to the culture and thus no further effect was detectable. However, in other experiments, glutamate toxicity did not work and provided no level of toxicity. Additionally, some plates had to be discarded due to contamination (wells were cloudy/discolored) even prior to stimulations. Contamination may have been caused by numerous factors: non-sterile equipment (i.e. pipet tip touching external surface, conical tube harboring a contaminate from a prior feeding cycle), length of time to plate cells increased possible contaminants entering plates, plates were not sprayed with ethanol prior to placing them back in the incubator, and maintenance of oxygen levels in the incubator. During one particular month in the lab, the Sterile Culture Room was also not accessible due to a water leak. Therefore all stimulations and culture work were completed in an external hood in the laboratory. Replicates of conditions within a particular plate were

consistent. However, any outlier data points were either removed from the data set or, if the plate was highly variable, it was not included at all.

In addition to alterations in the model, the manipulation can also be adjusted. For example, perhaps the concentrations of BDNF and LM11A-31 were not sufficient to protect against neurotoxicity or the levels presented may have been too high whereby they exacerbated neurotoxicity. In this particular model, BDNF and LM11A-31 did not increase cell survival and in several conditions tested, both compounds seemed neurotoxic on their own.

At the cellular level, BDNF and LM11A-31 may be contending for the same binding site on the p75 receptor, while amyloid beta is binding to a different area (Yaar et al. 1997). The p75 receptor ligand can be activated by BDNF. Lee et al. explains that proBDNF can induce cell death by apoptosis (2001). Matured BDNF provides protection by activating the TrkB pathway. It binds to the same site as the p75 receptor ligand and increases percent cell viability. Furthermore, a positive feedback loop exists between amyloid beta and p75 (Zeng et al. 2011). As amyloid beta is activated, p75 receptors are further expressed, leading to further amyloid beta production and ultimately neurodegeneration. One possible protein, TACE, has been shown to play a role in cleaving the p75 receptor, thus breaking this feedback loop (Zeng et al. 2011). The extracellular domain of the p75 receptor is thought to sequester and clear amyloid beta. Additionally, the link between p75 and TrkA plays a role in whether cells are pressed towards the cell survival or death pathway (Zeng et al. 2011). The survival pathway can be enhanced by elements such as the protein TACE as well as more therapeutic options such as cognitive stimulation or being in an enhanced environment with many stimuli.

Future Directions

In future experiments it would be beneficial to continue to test cell viability in Alzheimer's-like conditions to determine if varied concentrations of BDNF and LM11A-31 will have optimal benefits on neuronal health and survival, if any effect at all. Ligands bind to particular proteins or receptors such as the p75 receptor. Synthetic ligands such as LM11A-31 can be produced in the form of a drug and used as agonists or antagonists. Agonists would cause the same response as a natural ligand while antagonists would block the effect of the natural ligand. Determining additional activated receptors (ligand-bound) and their effect on neurons would be beneficial to the development of this project. For instance, Hu et al. suggests that there is a particular death receptor 6 (DR6) which binds to and is a part of the p75 signaling complex (2013). By binding, it increases the rate of neuronal death due to amyloid beta. DR6 antagonists can selectively block the apoptotic function of the DR6/p75 complex while at the same time preserve the pro-survival function of the p75/Trk complex (Hu et al. 2013). This type of antagonist may be converted into a pharmaceutical agent that could potentially decrease the progression of AD. Moreover, another supplement to this research may involve testing if BDNF effects are variable in other brain areas aside from the cortical region. Studies have shown that there is often less BDNF mRNA in the hippocampus of AD patients suggesting that it may directly contribute to the disease progression and loss of cognitive function (Phillips et al. 1991).

Important Elements of Music

The larger idea behind this study is that music can alter substrates within the brain such as elevating levels of BDNF (Angelucci et al. 2007b). Perhaps the cell culture model used in this particular experiment is not appropriate in that there are many other components of music which cannot be directly mimicked in cell culture. Thus the potential benefits of music are not visible in

the data. Neurons in culture grow in a chemical-milieu-based environment. However, cells in culture have no sensory receptors and therefore cannot have an emotional or cognitive interaction with music. Perhaps there is some inherent benefit of becoming involved in music and actually experiencing the various melodies and rhythms. Moreover, ideas to consider beyond the lab include studying whether the effects of prior knowledge of music, such as playing a musical instrument, can enhance benefits of music therapy later in life. Exposure to music at a young age could potentially enhance neurotrophin production in the hippocampus (Angelucci et al. 2007a).

Conclusion

The main focus of this research was to test the potential protective interaction between BDNF and LM11A-31 in the presence of Alzheimer's disease stressors. The cell culture model of Alzheimer's disease involved the use of glutamate or amyloid beta and allowed for measurement of neurodegeneration and cell survival. The main findings of this study fail to support the main three hypotheses. In these particular data, BDNF and LM11A-31 are not protective against cell death in Alzheimer's-like conditions. Whether independently presented or as a co-treatment, the effects of BDNF and LM11A-31 are highly variable. There was no evidence for any effect of pretreatment in our experiment (Figure 3). If pretreatment was beneficial, the glutamate condition in Figure 3 would maintain a lower percent cell viability values than all other groups, since the glutamate condition did not participate in pretreatment. The change seen in the data between Figure 3A and 3B is considered a batch variable, meaning that the difference was likely seen due to variability in the control values between the two experiments. This is because each condition is expressed as a percent of the control and any relative difference in this value will also alter all of the other values. With regard to neuronal morphology, cells with glutamate

conditions had less developed cell structures and decreased tubulin intensity (Figure 6). Additionally, it is important to note that although toxicity from glutamate or amyloid beta was based on the 80% set threshold, the data presented does not include all experiments. Approximately 24% of all plates (7 of a total of 29 plates) used during experimentation showed an appropriate level of toxicity whereby the effects of BDNF and LM11A-31 could actually be tested.

It was hypothesized that BDNF and LM11A-31 would have a joint effect in assisting to maintain neuronal health and cell viability within the AD cell culture model. Certain data exhibited that BDNF and LM11A-31 increased cell viability when presented separately, but again this is highly variable. The results do not show any data whereby either BDNF or the LM11A-31 drug had a beneficial effect separately nor in conjunction with each other. The evidence suggests harm on percent cell survival and health and does not offer any benefit in this particular study. BDNF and LM11A-31 did not enhance cell survival and, in some instances, these reagents were neurotoxic. As some scientists have shown the benefits of BDNF and LM11A-31, this current model would need to be revised before making further conclusions on these compounds' effectiveness. A new model that is more consistent with the results obtained may involve testing the proneuropeptide form of BDNF in addition to the mature BDNF form. Also, rather than simply focus on the p75/TrkB interaction in cell culture, a simultaneous cell culture study of p75/sortilin may provide a better method of comparison between the cell survival and cell enhancement pathways (Lu et al. 2005). An *in vivo* mouse model similar to the Angelucci et al. studies may also be more realistic in determining the effects of music on internal neurotrophic pathways and cell signaling (2007a,b). If the mouse model was successful, it would be valuable to measure BDNF in a human model by monitoring gene expression perhaps through

polymerase chain reaction (PCR) (Buchman et al. 2016). Further experimentation is necessary to gain a more complete understanding of these compounds as a potential therapy for Alzheimer's disease.

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